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Intraspecific Hybridization of *Anopheles sinensis* (Diptera: Culicidae) Strains from Thailand and Korea

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***Anopheles* (*Anopheles*) *sinensis* [Wiedemann (1828)] is a member of the *hyrcanus* species group, and it has been incriminated as the natural or experimental malaria vectors in the Republic of Korea, Japan, China, and Indonesia. In Thailand, however, *An. sinensis* seems to be of little medical importance. Hybridization tests among the three iso-female lines (isolines) of *An. sinensis* [i.e., Form A (X, Y_1) and Form B (X, Y_2) (Thailand strain), and Form B (X, Y_2) (Korean strain)] were established based on two distinct types of metaphase chromosomes and geographical differences. The chromosomal form of the Korean strain was first identified from this study. Results of reciprocal and back crosses indicated that both karyotypic forms of the *An. sinensis* Thailand and Korean strains were genetically compatible, and provided viable progenies and completely synaptic polytene chromosomes. The sequences of the rDNA internal-transcribed spacer 2 (ITS2) and mitochondrial cytochrome c oxidase subunit II (COII) among the *An. sinensis* strains were nearly identical to each other, and the intraspecific sequence variability was very low (0.0–0.6%). Sequence comparisons among the cryptic inter-species (i.e., *An. sinensis*, *An. lesteri*, and *An. yatsushiroensis*), however, revealed extensive divergence, and the intraspecific variability ranged from 12.2 to 34.6%. Therefore, it is concluded from these results and previous vector ability studies that the *An. sinensis* Forms A and B exhibit cytological polymorphic races that have different vector abilities in their transmission of malaria, depending on their geographical locations.**

Keywords: *Anopheles sinensis*; Cytochrome c Oxidase Subunit II; Internal Transcribed Spacer 2; Intraspecific Hybridization; Karyotypic Forms.

Introduction

Anopheles (*Anopheles*) *sinensis* (Wiedemann, 1828) is a member of the *hyrcanus* species group that belongs to the *Myzorhynchus* series (Harrison and Scanlon, 1975). Throughout the Republic of Korea, it has for a long time been incriminated as the most dominant natural vector of *Plasmodium vivax* in (Chow, 1970; Ree, 2000). Recently, it was considered as an important vector that is involved in the re-emerging of *P. vivax* in the border between South Korea and North Korea since 1993 (Chai, 1999; Ree *et al*., 2001; Whang *et al.*, 2002). Lee *et al.* (2001) reported an experiment that was highly susceptible to the wild-caught *An. sinensis* that are indigenous to *P. vivax* (sporozoite rate = 33.4%; 4/12). Recently, 28,286 Korean anopheline mosquitoes were tested by an enzyme-linked immunosorbent assay for identifying the presence of *P.vivax* 210 and 247 circumsporozoite (CS) proteins by Coleman *et al.* (2002). They reported that two pools (i.e., 9 and 10 *An. sinensis/An. lesteri*) were positive for the *P. vivax* 247 CS protein.

It was also incriminated as the natural and experimental vectors of *P. vivax* in other countries - i.e., Japan (Otsuru and Ohmori, 1960), China (Ho *et al.*, 1962), and Indonesia (O'Connor, 1980). In Thailand, *An. sinensis* seems to have only a small medical importance (Harrison and

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Abbreviations: COII, cytochrome c oxidase subunit II; ITS2, internal-transcribed spacer 2.

Scanlon, 1975). Additionally, the laboratory feeding of two karyotypic forms of Thailand *An. sinensis* (Forms A and B) on *P. vivax* revealed that they were poor vectors (sporozoite rates = 0.00–5.88%) (Rongsriyam *et al.*, 1998). Many morphologically-undistinguishable anopheline mosquitoes have composed complexes that consist of several reproductively-isolated sibling species. Cytogenetic analyses, including chromosomal forms, have proved to be one of the most powerful tools for identifying the existence of sibling species in the anopheline species complexes. Based on metaphase karyotype studies, at least two karyotypic forms of *An. sinensis* have been reported from Thailand and Taiwan (Baimai *et al.*, 1993). They are as follows: 1) The *An. sinensis* Form A (X, Y_1). Its Y_1 chromosome is subtelocentric or acrocentric, and it has only a small portion of the short arm. 2) Form B (X, Y_2). Its Y_2 chromosome is clearly submetacentric with the short arm approximately one-half the length of the long arm. Recently, crossing studies of sympatric *An. sinensis* Forms A and B from the Mae Hong Son Province, Northern Thailand revealed that they were genetically compatible, providing viable progenies and completely synaptic polytene chromosomes (Choochote *et al.*, 1998).

The question of whether or not it is just a single cosmopolitan species or a complex, which consists of at least two reproductively-isolated taxa, needed to be answered. Therefore, to identify the species status of *An. sinensis*, we performed a hybridization experiment among *An. sinensis* iso-female lines (isolines), which are geographically isolated and have two different chromosomal forms, and sequence comparisons of the rDNA internal-transcribed spacer 2 (ITS2) and mitochondrial cytochrome c oxidase subunit II (COII) sequences among *An. sinensis* strains, and among cryptic inter-species, i.e., *An. sinensis, An. lesteri* and *An. yatsushiroensis.* The latter two species were selected as references because they (with *An. Pullus)* are known as the closest relatives of *An. sinensis* among *hyrcanus* group mosquitoes. They are also sometimes grouped as members of the *An. sinensis* complex (Ree, 2002).

Materials and Methods

Isolines of *An. sinensis* Forms The five isolines (denoted as Sinen i1SACM, Sinen i2SACM, Sinen i1SBCM, Sinen i1SBKR, and Sinen i3SBKR) of the *An. sinensis* Forms A and B were established, based on the two distinct types of metaphase chromosomes and geographical differences. For the *An. sinensis* Forms A (Sinen i1SACM and Sinen i2SACM) and B (Sinen i1SBCM) of the Thailand strain, they were established by using single wild-caught females from the San Sai District, Chiang Mai Province. For Form B (Sinen i1SBKR, and Sinen i3SBKR) of the Korean strain, they were established by using selected females from laboratory-raised, mixed colony of the *An. sinensis* strain from the So-Rea District (Korea). These five isolines were successfully colonized for more than 5 consecutive generations and were used for the experiments. Three isolines (Sinen i2SACM, Sinen i1SBCM and Sinen i3SBKR) were used for the hybridization and karyotype studies. Figure 1 shows pictures of metaphase chromosomes of the testes and ovaries of the three isolines that were prepared from the newly-emerged adult males and females using the method of Choochote *et al.* (2001).

Hybridization study Intraspecific crossing experiments among the three isolines of the *An. sinensis* Forms A and B (Sinen i2SACM, Sinen i1SBCM and Sinen i3SBKR) were done by following the method of Choochote *et al.* (1998). Briefly, the reciprocal and back crosses were carried out by using virgin females and males, then their viability was compared (i.e., hatching rates, survival rates, pupation rates, emergence rates, adult sex-ratios) with the parental crosses. The F_2-progeny that failed to survive was the criterion for the reproductive isolation. The salivary gland polytene chromosomes of the 4th larvae from the crosses were investigated using the technique that was described by Kanda (1979).

DNA extraction, amplification, and sequencing Genomic DNA was extracted from individual mosquitoes of 5 isolines and a wild-caught mosquito from the Chongup Province, Chonllabukdo, South Korea using the protocol for animal tissue samples in the DNeasy Tissue kit (Qiagen, Co.). Primers to amplify the ITS2 region of rDNA were designed, based on the conserved sequences of various eukaryotes. The primers are 18S+1600 (5′-GCG TTG ATT ACG TCC CTG CCC TTT G-3′) and 28S-60 (5′-GTT GGT TTC TTT TCC TCC-3′). Primers to amplify the COII region of mtDNA were designed based on the comparisons of the *An. gambiae* and *Drosophila melanogaster* sequences. The spanning position of the AnoCO2+1 (5′-GAT TAG TGC AAT GAA TTT AAG C-3′) and AnoCO2END (5′-GAG ATC ATT ACT TGC TTT CAG TC-3′) primers are 2973–2995 and 3725–3748, respectively, in the complete *An. gambiae* sequence (Beard *et al*., 1993). PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min and extension at 72°C for 2 min, then a final extension step at 72°C for 5 min. The PCR fragments were gel purified by glass milk extraction (GeneClean III kit, BIO 101) and directly cycle-sequenced using a Big-Dye Terminator sequencing kit (PE Applied Biosystems). The reaction products were electrophoresed on an ABI 310 automated DNA sequencer (PE Applied Biosystems). Both of the strands were sequenced and aligned using the Clustal X multiple alignment program (Thompson *et al.*, 1997). The following sequences were retrieved from the GenBank database and included in the alignments for analyzing the sequence variability: ITS2 (accession No. AJ004942) and COII (AF325715) sequences of *An. sinensis* strain of China (Sinen WCCNA); ITS2 sequences of *An. lesteri* (Leste WCCNA; AF145464) and *An. yatsushiroensis* (Yatsu WCNKR; AF325715), the closely related species of *An. sinensis.* The sequence data

that are presented in this article have been submitted to GenBank under accession numbers AY130463−AY130474.

Results

Hybridization study Table 1 shows the details of the embryonation, hatchability, pupation, and emergence of the parental, reciprocal, and back crosses among the three laboratory-raised isolines of the *An. sinensis* Forms A and B.

Observation on the hatchability, pupation, emergence, and adult sex-ratio of the parental, reciprocal, and back crosses among the three isolines of the laboratory-raised *An. sinensis* Forms A and B revealed that all of the crosses yielded viable progenies. No evidence of genetic incompatibility was observed. The hatchability, pupation, emergence rates, and ratio adult female/male of parental, reciprocal, and back crosses were as follows: 70.88–80.56%, 84.46–89.56%, 91.89–100%, and 0.72–0.89; 75.23–91.90%, 82.98–94.93%, 100% and 0.71–1.09; 72.35–95.13%, 81.23–98.10%, 96.02–100% and 0.78–1.18, respectively. The salivary gland polytene chromosomes of the 4th larvae from all of the reciprocal crosses showed complete synapsis along the whole length of all of the autosomes and X-chromosome (Figs. 2A and 2B). Nonetheless, the floating, heterozygous inversion on 3 L was found in one preparation from the crosses of the Form A female (Thailand strain) with the Form B male (Korea strain), but at a very low frequency (Fig. 2C).

Sequence analysis of ITS2 and COII regions Individual mosquitoes of five isolines and a wild-caught mosquito from Chongup (denoted as Sinen WCSKR; the chromosomal form was not determined) were used for sequencing the ITS2 and COII regions. All of the sequences for both regions were the same in length without indels (insertion or deletion sequences). The alignments for ITS2 and COII are given in Figs. 3 and 4, respectively.

All 6 of the newly-obtained ITS2 sequences of the *An. sinensis* strains were 469 bp in length; one base longer than that of the China strain that was retrieved from GenBank. The level of intraspecific variability in the ITS2 sequences among the *An. sinensis* strains that was examined in this study was very low with 0.0 to 0.6% differences (Table 2). The sequences of the three Korean strains (two Form B isolines and one wild-caught mosquitoes) were completely identical. However, there was the exception of the A/G mixture sequence (denoted as R) at position 465. The sequence of the Thailand Form B strain was

Table 1. Cross mating among isolines of *An. sinensis* Form A and B.

Cross	Total eggs* (No.)	Embryonation rate**	No. hatched (%)	No. pupation (%)	No. emergence (%)	No. females and males from total emergence (%)	
Female x Male						Female	Male
Parental crosses							
ATxAT	421 (191, 230)	84	332 (78.86)	296 (89.16)	272 (91.89)	117 (43.01)	155 (56.99)
BTxBT	355 (172, 183)	88	286 (80.56)	253 (88.46)	253 (100)	119(47.04)	134 (52.96)
BKxBK	419 (135, 284)	71	297 (70.88)	266 (89.56)	261 (98.12)	109 (41.76)	152 (58.24)
Reciprocal crosses							
ATtxBK	382 (159, 223)	96	348 (91.10)	299 (85.92)	299 (100)	156 (52.17)	143 (47.83)
BKxAT	274 (123, 151)	87	217 (79.20)	206 (94.93)	206 (100)	94 (45.63)	112 (54.37)
BTxBK	327 (154, 173)	82	246 (75.23)	221 (89.84)	221 (100)	92 (41.63)	129 (58.37)
BKxBT	358 (172, 186)	97	329 (91.90)	273 (82.98)	273 (100)	127 (46.52)	146 (53.48)
Back crosses							
ATx(ATxBK)F_1	472 (176, 296)	86	368 (77.97)	339 (92.12)	335 (98.82)	154 (45.97)	181(54.03)
(ATxBK)F_1xBK	269 (101, 168)	85	213 (79.18)	201 (94.37)	193 (96.02)	96 (49.74)	97(50.26)
BKx(BKxAT)F_1	347 (152, 195)	74	253 (72.91)	227 (89.72)	224 (98.68)	108 (48.21)	116 (51.79)
(BKxAT)F_1xAT	428 (175, 253)	89	368 (85.98)	361 (98.10)	361 (100)	159 (44.04)	202 (55.96)
BTx(BTxBK)F_1	405 (186, 219)	76	293 (72.35)	238 (81.23)	229 (96.22)	114 (49.78)	115 (50.22)
(BTxBK)F_1xBK	390 (123, 267)	97	371 (95.13)	337(90.84)	337(100)	148 (43.92)	189 (56.08)
BKx(BKxBT)F_1	291 (103,188)	90	218 (74.91)	192 (88.07)	192 (100)	104 (54.17)	88 (45.83)
(BKxBT)F_1xBT	376 (122, 254)	96	346 (92.02)	329 (95.09)	325 (98.78)	146 (44.92)	179 (55.08)

AT, *sinensis* Form A (Sinen i2SACM, Thailand strain); BT, *sinensis* Form B (Sinen i1SBCM, Thailand strain), BK, *sinensis* Form B (Sinen i1SB, Korea strain).

* Two selective egg-batches of inseminated females from each cross.

** Dissection from one hundred eggs.

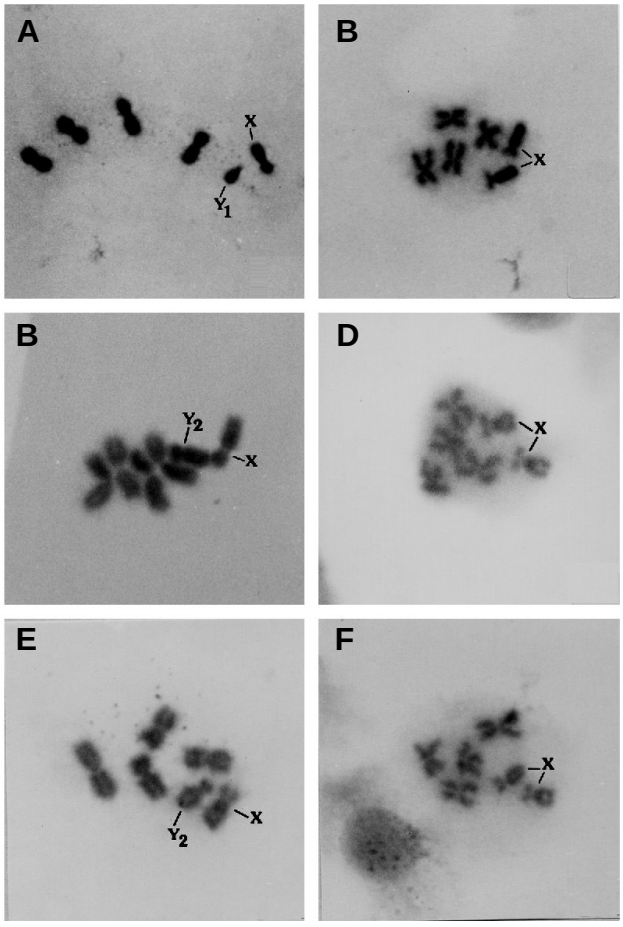

Fig. 1. Metaphase karyotype of isolines of *Anopheles sinensis* (Giemsa staining). **A.** Testis chromosomes of Form A (Thailand strain) showing X, Y_1-chromosomes. **B.** Ovary chromosomes of Form A (Thailand strain) showing X-chromosomes. **C.** Testis chromosomes of Form B (Thailand strain) showing X, Y_2-chromosomes. **D.** Ovary chromosomes of Form B (Thailand strain) showing X-chromosomes, which are similar to Form A. **E.** Testis chromosomes of Form B (Korean strain) showing X, Y_2-chromosomes, which are similar to Form B (Thailand strain). **F.** Ovary chromosomes of Form B (Korean strain) showing X-chromosomes, which are similar to Forms A and B (Thailand strain). Isolines of Sinen i2SACM, Sinen i1SBCM, and Sinen i1SBKR are denoted as strains of Thailand Form A, Thailand Form B, and Korean Form B.

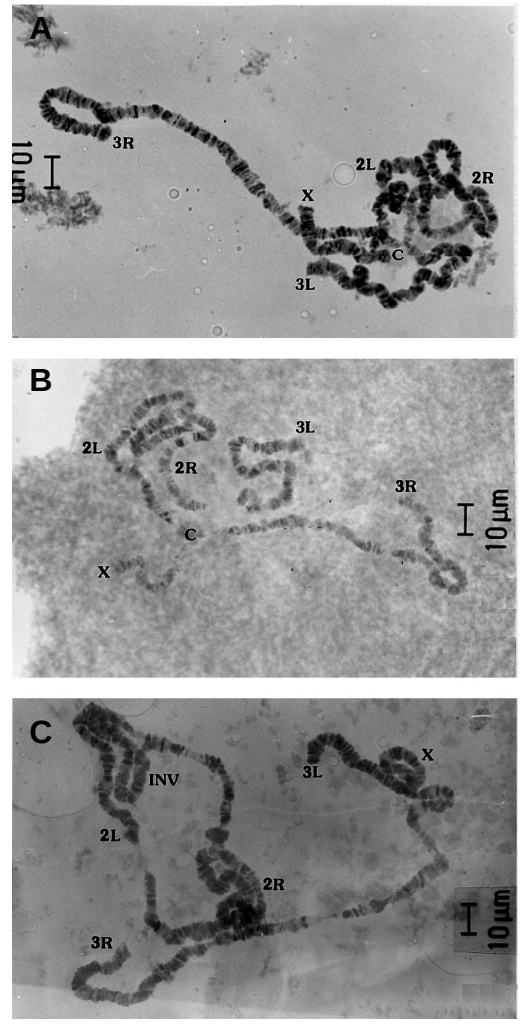

Fig. 2. Salivary gland polytene chromosomes of F_1-hybrid 4th larvae of *Anopheles sinensis*. **A.** Form A male (Thailand strain) × Form B female (Korean strain) showing complete synapsis in all arms. **B.** Form B female (Thailand strain) × Form B male (Korean strain) showing complete synapsis in all arms. **C.** Form A female (Thailand strain) × Form B male (Korean strain) showing complete synapsis in all arms, except floating, heterozygous inversion (INV) on 2 L, which was found in only one preparation. Isolines of Sinen i2SACM, Sinen i1SBCM, and Sinen i1SBKR are denoted as strains of Thailand Form A, Thailand Form B, and Korean Form B.

the same as those of the Korean strains. The China strain showed an identical sequence with the Koreans, except the one indel sequence at position 466. The sequences of the two Thailand Form A strains were identical to each other, but they differed from the Korean strain by three nucleotides. These were positioned at 94, 143, and 337.

On the other hand, in case of comparing the sequences of *An. sinensis* with those of two closely-related species (*An. lesteri* and *An. yatsushiroensis*), the ITS2 sequence divergences among these cryptic species ranged quite highly from 12.2 to 34.6% (Table 2).

All of the COII genomic sequences of the *An. sinensis* strains (including the China strain) contained 663 nucleotides without length variations, coding 221 amino acids, including an ATG initiation codon, but no putative termination codons were found. Within the strains, there were two amino acid substitutions; one was in the China strain at position 53, and the other was in the Thailand B strain

Table 2. Summary of sequence variations of ITS2 region among *Anopheles sinensis*, *An. lesteri*, and *An. yatsushiroensis.*

Species	Length (bp) difference	Variation (%)[1]			
		Total	Substitution	Deletion	Insertion
Sinensis FormB/FormA	0	3 (0.6)	3 (0.6)	0(0.0)	0(0.0)
sinensis[2]*/lesteri*	18	59 (12.2)	39 (8.1)	1 (0.2)	19 (3.9)
sinensis[2]*/lesteri*	16	163 (33.7)	117 (24.2)	15 (3.1)	31 (6.4)
lesteri/yatsushiroensis	2	167 (34.6)	121 (25.1)	24 (5.0)	22 (4.6)

[1] Number of variable sites (with percentage in parentheses).

[2] Sequence of Sinen WCSKR (a wild-caught mosquito from Chongup province, South Korea).

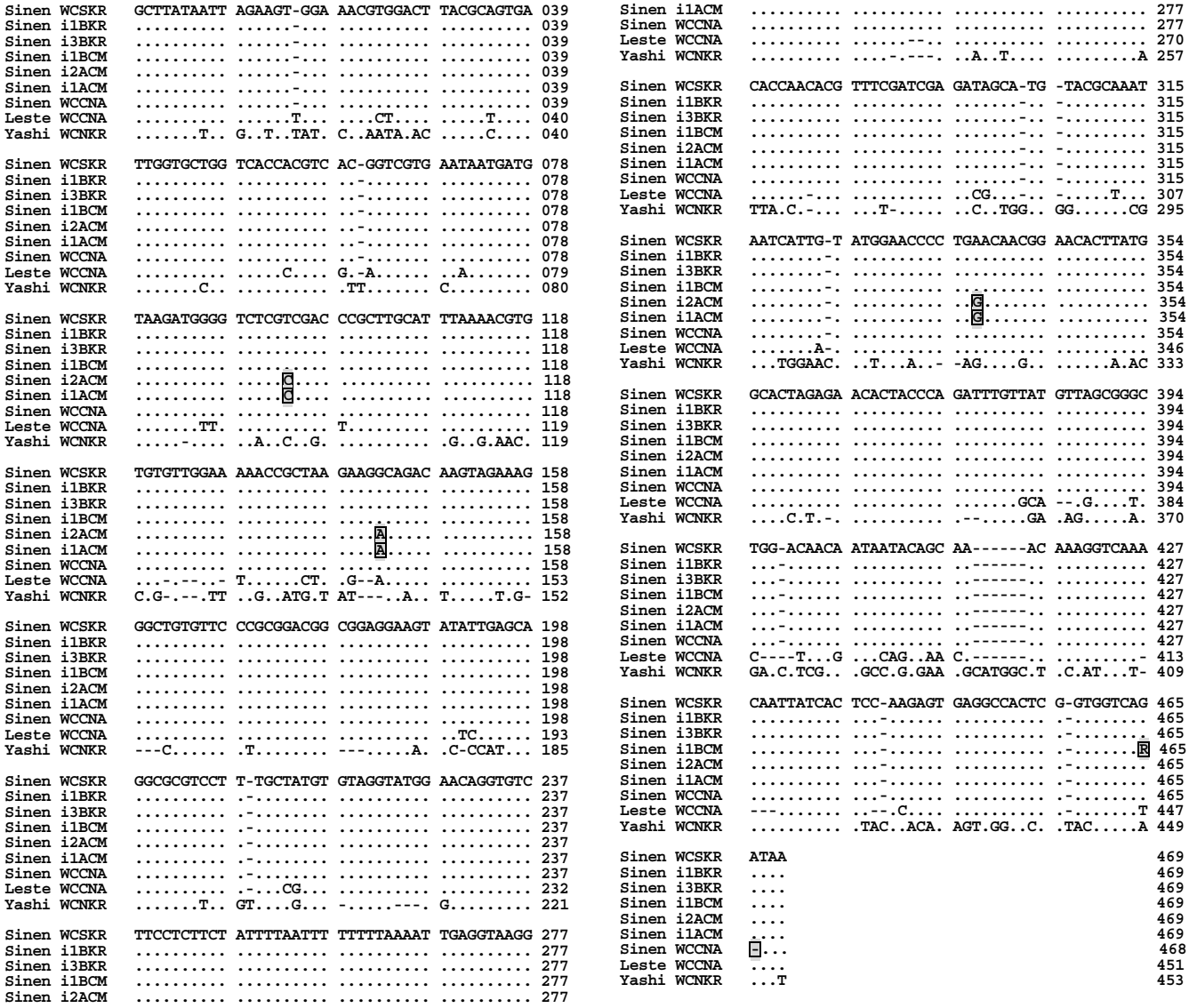

Fig. 3. Aligned rDNA ITS2 sequences for 7 *Anopheles sinensis* strains and two *hyrcanus* anopheline species, *An. lesteri* and *An. yatsushiroensis*. Dots indicate the sequence identity in the other sequences that were compared with Sinen WCSKR (an *An. sinensis* strain); dashes represent gaps introduced to maximize the overall sequence similarity. Variable nucleotides among *An. sinensis* strains are in rectangular. The GenBank accession numbers are as follows: Sinen WCKR (AY130474); Sinen i1BKR (AY130469); Sinen i3BKR (AY130470); Sinen i1BCM (AY130471); Sineni 1ACM (AY130472); and Sinen i2ACM (AY130473). Name sequences are defined in the text.

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Sinen WCSKR   MATWANLGLQ DSSSPLMEQL NFFHDHTLLI LTMITILVGY 040
Sinen i1BKR   .......... .......... .......... .......... 040
Sinen i3BKR   .......... .......... .......... .......... 040
Sinen i1BCM   .......... .......... .......... .......... 040
Sinen i1ACM   .......... .......... .......... .......... 040
Sinen i2ACM   .......... .......... .......... .......... 040
Sinen WCCNA   .......... .......... .......... .......... 040

Sinen WCSKR   IMGMLMFNQF TNRYLLHGQT IEIIWTVLPA IILMFIAFPS 080
Sinen i1BKR   .......... .......... .......... .......... 080
Sinen i3BKR   .......... .......... .......... .......... 080
Sinen i1BCM   .......... .......... .......... .......... 080
Sinen i1ACM   .......... .......... .......... .......... 080
Sinen i2ACM   .......... .......... .......... .......... 080
Sinen WCCNA   ..S....... .......... .......... .......... 080

Sinen WCSKR   LRLLYLMDEI NTPSITLKSV GHQWYWSYEY SDFLNLEFDS 120
Sinen i1BKR   .......... .......... .......... .......... 120
Sinen i3BKR   .......... .......... .......... .......... 120
Sinen i1BCM   .......... .......... .......... ...M...... 120
Sinen i1ACM   .......... .......... .......... .......... 120
Sinen i2ACM   .......... .......... .......... .......... 120
Sinen WCCNA   .......... .......... .......... .......... 120

Sinen WCSKR   YMIPTNELET NGFRLLDVDN RIVLPMNNQI RILVTATDVL 160
Sinen i1BKR   .......... .......... .......... .......... 160
Sinen i3BKR   .......... .......... .......... .......... 160
Sinen i1BCM   .......... .......... .......... .......... 160
Sinen i1ACM   .......... .......... .......... .......... 160
Sinen i2ACM   .......... .......... .......... .......... 160
Sinen WCCNA   .......... .......... .......... .......... 160

Sinen WCSKR   HSWTVPSLGV KVDATPGRLN QINFLINRPG LFFGQCSEIC 200
Sinen i1BKR   .......... .......... .......... .......... 200
Sinen i3BKR   .......... .......... .......... .......... 200
Sinen i1BCM   .......... .......... .......... .......... 200
Sinen i1ACM   .......... .......... .......... .......... 200
Sinen i2ACM   .......... .......... .......... .......... 200
Sinen WCCNA   .......... .......... .......... .......... 200

Sinen WCSKR   GANHSFMPIV IESIPMNYFI K                     221
Sinen i1BKR   .......... .......... .                     221
Sinen i3BKR   .......... .......... .                     221
Sinen i1BCM   .......... .......... .                     221
Sinen i1ACM   .......... .......... .                     221
Sinen i2ACM   .......... .......... .                     221
Sinen WCCNA   .......... .......... .                     221
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Fig. 4. Aligned mitochondrial COII amino acid sequences for 7 *Anopheles sinensis* strains. Dots indicate the sequence identity in the other sequences that were compared with Sinen WCSKR. Variable amino acids among the *An. sinensis* strains are in rectangular. The GenBank accession numbers are as follows: Sinen WCKR (AY130463); Sinen i1BKR (AY130464); Sinen i3BKR (AY130465); Sinen i1BCM (AY130468); Sinen i1ACM (AY130467); and Sinen i2ACM (AY130466). Name sequences are defined in the text.

at position 114 (Fig. 4).

Discussion

A hybridization experiment and/or testing of reproductive isolation at the postmating barrier is still one of the most efficient and reliable diagnostic tools to differentiate intra-taxon of anopheline species to a sibling species and/or subspecies. Hybrid inviability, sterility, or breakdown are criteria of genetic incompatibilities. These include insemination, embryonation, hatchability, larva survival, pupation, emergence, adult sex-ratio, abnormal morphology, and reproductive system, as well as the degree of asynaptic polytene chromosomes (Kanda *et al.*, 1981; Kitzmiller, 1976). Nonetheless, the genetically-compatible one does not entirely rule out its sibling species status, since the investigation of assortative mating and/or premating barrier (Paterson, 1980) by using paracentric inversions of polytene chromosomes, biochemical, and molecular genetics should be done intensively prior to a definite conclusion (Subbarao, 1988). However, a genetically-incompatible one could be entirely differentiated in its sibling species status.

Several intra-taxa of the Asian anopheline species, which were primarily detected of morphological, biological, and cytological differences and/or variations, led to the doubtful status of sibling species and/or subspecies. Subsequently, it was clearly confirmed by hybridization experiments. These include the *An. barbirostris* complex (Choochote *et al.*, 1983), *An. maculatus* complex (Chabpunnarat, 1988; Takai *et al.*, 1987), *An. culicifacies* complex (Subbarao *et al.*, 1988), and *An. dirus* complex (Baimai *et al.*, 1987; Sawadipanich *et al.*, 1990). Nevertheless, a point to be remembered is that colonies that are established from species-specific diagnostic characteristics of progeny from isolines have to be used. A laboratory colony that is established from a mixed, natural population may be a mixture of two or three species (Subbarao, 1988).

The *An. sinensis* strains from Thailand and Korea were selected for the hybridization studies, because these two allopatric strains consisted of geographically-reproductive isolation (approximately a 2,300-mile distance from each other). In addition, the former or tropical strain is not a vector of malaria in Thailand (Harrison and Scanlon, 1975; Rongsriyam *et al.*, 1998); whereas, the latter or temperate strain has been incriminated as the natural vector of *P. vivax* in the Republic of Korea since 1967 (Chow, 1970). It was recently considered as an important vector that is involved in the reemergence of *P. vivax* in the border between South Korea and North Korea (Chai, 1999). Therefore, some degree of genetic incompatibility is expected, even though they have similar karyotypic forms (Forms A and B in the Thailand strain, and Form B in the Korean strain). However, the results of reciprocal and back crosses - among the three laboratory-raised isolines, which were representative of the karyotypic forms - indicated that the *An. sinensis* Thailand and Korean strains were genetically compatible, providing they had viable progenies and completely synaptic polytene chromosomes.

Comparative studies of the nucleotide sequences of the ITS2 and COII regions among 7 *An. sinensis* strains (including 5 isolines) revealed nearly identical and/or very low intraspecific variations (variation rate ~0.6%). However, the interspecific sequence variations of the ITS2 region, among the three cryptic species members of the *hyrcanus* species group, were extremely high (12.2 to 34.6%).

Based on this evidence, as well as previous studies (Coleman *et al.*, 2002; Lee *et al.*, 2001; Rongsriyam *et al.*, 1998), it can be confidently concluded that the *An. sinensis*

Forms A and B exhibit two cytological-polymorphic races. These have different vector abilities in the transmission of malaria, depending on their geographical locations.

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References

Baimai, V., Andre, R. G., Harrison, B. A., Kijchalao, U., and Panthusiri, L. (1987) Crossing and chromosomal evidence for two additional sibling species within the taxon *Anopheles dirus* Peyton and Harrison (Diptera: Culicidae) in Thailand. *Proc. Entomol. Soc. Wash.* **89**, 157–166.

Baimai, V., Rattanarithikul, R., and Kijchalao, U. (1993) Metaphase karyotypes of *Anopheles* of Thailand and Southeast Asia: I. The *hyrcanus* group. *J. Am. Mosq. Control. Assoc.* **9**, 59–67.

Beard, C. B., Hamm, D. M., and Collins, F. H. (1993) The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. *Insect Mol. Biol.* **2**, 103–124.

Chabpunnarat, S. (1988) Cytogenetic study of the *Anopheles maculatus* complex. Bangkok, Mahidol University, MSc Thesis.

Chai, J. Y. (1999) Re-emerging *Plasmodium vivax* malaria in the Republic of Korea. *Korean J. Parasitol.* **37**, 129–143.

Choochote, W., Sucharit, S., and Abeyewickreme, W. (1983) Experiments in crossing two strains of *Anopheles barbirostris* Van Der Wulp 1884 (Diptera: Culicidae) in Thailand. *Southeast Asian J. Trop. Med. Public Health* **14**, 204–209.

Choochote, W., Jitpakdi, A., Rongsriyam, Y., Komalamisra, N., Pitasawat, B., and Palakul, K. (1998) Isoenzyme study and hybridization of two forms of *Anopheles sinensis* (Diptera: Culicidae) in Northern Thailand. *Southeast Asian J. Trop. Med. Public Health* **29**, 841–847.

Choochote, W., Pitasawat, B., Jitpakdi, A., Rattanachanpichai, E., Riyong, D., Leemingsawat, S., and Wongkamchai, S. (2001) The application of ethanol-extracted *Gloriosa superba* for metaphase chromosome preparation in mosquitoes. *Southeast Asian J. Trop. Med. Public Health* **32**, 76–82.

Chow, C. Y. (1970) Bionomics of malaria vectors in the Western Pacific region. *Southeast Asian J. Trop. Med. Public. Health* **1**, 40–57.

Coleman, R. E., Kiattibut, C., Sattabongkot, J., Ryan, J., Burkett, D. A., Kim, H. C., Lee, W. J., and Klien, T. A. (2002) Evaluation of anopheline mosquitoes (Diptera: Culicidae) from the republic of Korea for *Plasmodium vivax* circumsporozoite protein. *J. Med. Entomol.* **39**, 244–247.

Harrison, B. A. and Scanlon, J. E. (1975) The subgenus *Anopheles* in Thailand. *Cont. Am. Ent. Inst.* **12**, 1–307.

Ho, C., Chou, T. C., Chen, T. H., and Hsueh, A. T. (1962) The *Anopheles hyrcanus* group and its relation to malaria in East China. *Chin. Med. J.* **81**, 71–78.

Kanda, T. (1979) Improved techniques for the preparation of polytene chromosomes for some anopheline mosquitoes. *Mosq. News* **39**, 568–574.

Kanda, T., Takai, K., Oguma, Y., Chiang, G. L., Cheong, W. H., Sucharit, S., Joesoef, A. M., and Imajo, S. (1981) Evolutionary genetics of the *Anopheles hyrcanus* group, the *leucosphyrus* group and the *Pyretophorus* group in East Asia and Pacific area; in *Cytogenetics and Genetics of Vectors*, Proceedings of a symposium of the XVIIth International Congress of Entomology, Pal, R., Kitzmiller, J. B., and Kanda, T. (eds.), pp. 31–60, Tokyo, Kohdansha Ltd., Elsevier Amsterdam, New York, Oxford Biomedical.

Kitzmiller, J. B. (1976) Genetics, cytogenetic and evaluation of mosquitoes. *Adv. Genet.* **18**, 316.

Lee, H. W., Cho, S. H., Shin, E. H., Lee, J. S., Chai, J. Y., Lee, S. H., and Kim, T. S. (2001) Experimental infection of *Anopheles sinensis* with Korean isolates of *Plasmodium vivax*. *Korean J. Parasitol.* **39**, 177–183.

O'Connor, C. T. (1980) The *Anopheles hyrcanus* group in Indonesia. *Mosq. Syst.* **12,** 293–305.

Otsuru, M. and Ohmori, Y. (1960) Malaria studies in Japan after World War II. Part II. The research for *Anopheles sinensis* sibling species group. *Jpn. J. Exp. Med.* **30**, 33–65.

Paterson, H. E. (1980) A comment on "mate recognition systems". *Evolution* **34**, 330–331.

Ree, H. I. (2000) Unstable vivax malaria in Korea. *Korean J. Parasitol.* **38**, 119–138.

Ree, H. I. (2002) Studies on *Anopheles sinensis*, the vector species of vivax malaria in Korea; in *Today's Malaria Epidemiology and Vector Control*, Proceedings of the 2nd Seminar of Tropical Medicine, Yong, T. S. (ed.), pp. 33–52, Yonsei Medical Center.

Ree, H. I., Hwang, U. W., Lee, I. Y., and Kim, T. E. (2001) Daily survival and human blood index of *Anopheles sinensis*, the vector species of malaria in Korea. *J. Am. Mosq. Control. Assoc.* **17**, 67–72.

Rongsriyam, Y., Jitpakdi, A., Choochote, W., Somboon, P., Tookyang, B., and Suwonkerd, W. (1998) Comparative susceptibility of two forms of *Anopheles sinensis* Wiedemann 1828 (Diptera: Culicidae) to infection with *Plasmodium falciparum, P. vivax, P. yoelii* and the determination of misleading factor for sporozoite identification. *Southeast Asian J. Trop. Med. Public Health* **29**, 159–167.

Sawadipanich, Y., Baimai, V., and Harrison, B. A. (1990) *Anopheles dirus* species E: chromosomal and crossing evidence for another member of the *dirus* complex. *J. Am. Mosq. Control. Assoc.* **6**, 477–481.

Subbarao, S. K., Vasantha, K., and Sharma, V. P. (1988) Studies on the crosses between the sibling species of the *Anopheles culicifacies* complex. *J. Hered.* **79**, 300–303.

Takai, K., Kanda, T., Oguma, K., and Sucharit, S. (1987) Morphological differentiation in *Anopheles maculatus* of Thailand accompanied with genetical divergence assessed by hybridization. *J. Am. Mosq. Control. Assoc.* **3**, 148–153.

Thompson, J. D., Gibson, T. J., Plewniak, D. E., Jeanmougin, F., and Higgins, D. G. (1997) The CLUSTAL X-windows interface: flexible strategies for multiple sequence alignment aided by quality analysis. *Nucleic Acids Res.* **25**, 4876–4882.

Whang, I.-J., Jung, J., Park, J.-K., Min, G.-S., and Kim, W. (2002) Intragenomic length variation of the ribosomal DNA intergenic spacer in a malaria vector, *Anopheles sinensis*. *Mol. Cells* **14**, 158–162.