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MOLECULAR EVIDENCE FOR SYNONYMY OF *ANOPHELES YATSUSHIROENSIS* AND *AN. PULLUS*

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ABSTRACT. Identification of species members of the Hyrcanus Group of *Anopheles* is difficult because of intraspecific variation in and interspecific similarity among key characters. Hibernating female *Anopheles pullus* were collected and 6 adults were individually reared. All F_1 progeny of wild-caught *An. pullus* were morphologically identical to *An. yatsushiroensis.* The 5.8S rDNA-ITS2-28S rDNA region from each of 3 *An. pullus* and *An. yatsushiroensis* (wild-caught females) and a portion of the mitochondrial cytochrome oxidase 1 genes from 1 individual of the 2 species were sequenced. The same gene regions from an *An. sinensis* were sequenced to determine the degree of interspecific sequence variation within the Hyrcanus Group. Consensus sequence of the 5.8S rDNA-ITS2-28S rDNA region from 3 individual *An. pullus* was completely identical to that from 3 individual *An. yatsushiroensis.* Examination of molecular data obtained from nuclear DNA and mitochondrial DNA as well as morphological observations in rearing experiments support synonymy of *An. yatsushiroensis* and *An. pullus* in Korea.

KEY WORDS *Anopheles pullus, Anopheles yatsushironesis,* synonymy, molecular systematics

INTRODUCTION

Anopheles (Diptera: Culicidae) contains the most important malaria vector species. Species identification of anophelines, particularly of sibling species, is of vital importance for malaria control. Among 7 species of *Anopheles* found in Korea, *An. sinensis* Wiedemann, *An. lesteri* Baisas and Hu, *An. pullus* Yamada, and *An. yatsushiroensis* Miyazaki are closely related and their differentiation is difficult because of morphological similarity. In addition, large variation occurs in key morphological characters.

Anopheles sinensis is the most widespread and common species, and is the main vector of *Plasmodium vivax* (Ree et al. 1967). In Korea, *Anopheles lesteri* is an extremely rare species and *An. yatsushiroensis* is a secondary vector of malaria (Hong 1977). *Anopheles pullus* and *An. yatsushiroensis* were synonymized based on the morphology of F_1 progeny in rearing experiments (Shin and Hong 2001).

Variable genes, such as protein-coding genes and the control region within mitochondrial DNA, and the intergenic spacer and internal transcribed spacer (ITS) within a nuclear ribosomal DNA (rDNA) unit have been used for solving taxonomic problems. These genes are experimentally convenient because of their short length and the existence of universal polymerase chain reaction (PCR) primers (Kocher et al. 1989, Liu and Bechenbach 1992, Schlotterer et al. 1994, Lunt et al. 1996, Hwang and Kim 1999). In the present study, 5.8S rDNA-ITS2-28S rDNA and partial mitochondrial cytochrome oxidase 1 (mtCOI) gene regions from *An. pullus, An. yatsushiroensis,* and *An. sinensis* were sequenced and compared to test the status of these sibling species.

MATERIALS AND METHODS

Sample collection and rearing: We caught 145 hibernating female *An. pullus* in rice paddies at Samha-Ri, Changheung-Myeon, Yangju-Gun, Gyunggi-Do, Korea (37°65′N, 126°80′E) in January–March 1999. A mobile vinyl tent was used to collect hibernating adults. As the temperature within the tent increased with sunlight, hibernating mosquitoes flew out from the grasses. Female *An. yatsushiroensis* and *An. sinensis* were collected with a light trap at Goyang-Si, Gyunggi-Do, Korea (37°71′N, 126°93′E) in September 1999.

Wild-caught female *An. pullus* and *An. yatsushiroensis* fed upon 1 of the authors. Each of the fed females was kept in a separate cup containing water for oviposition. Each batch of eggs from a female was reared in a separate pan in an insectary (26°C and 14:10 h light : dark). The F_1 progeny were identified based upon morphological characters, and then processed for molecular analysis.

DNA extraction: Three wild-caught female *An. pullus* and 3 female *An. yatsushiroensis* were randomly selected. Wings and palpi were dry mounted. Total DNA was extracted by using a DNeasy tissue kit (Qiagen, Valencia, CA) from the whole body, which was homogenized in a 1.5-ml Eppendorf tube with a sealed blue tip and DNeasy lysis buffer. The concentration and quality of extracted DNA were determined on 1% agarose gels.

PCR amplification and cloning of 5.8S rDNA-ITS2-28S rDNA and partial mtCOI gene regions: The 5.8S rDNA-ITS2-28S rDNA region was amplified with primer 687 (5′-ACC CTG GAC GGT GGA TCA CTY GG-3′) and primer CS250 (5′-GTT RGT TTC TTT TCC TC-3′). Primers 687 and

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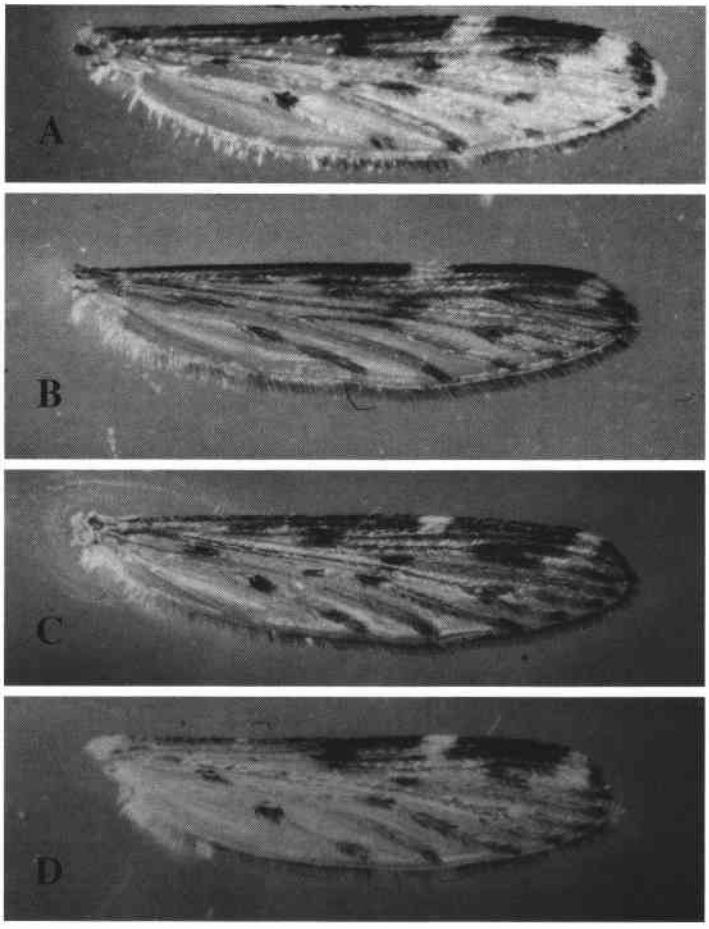

Fig. 1. Wings of female mosquitoes. (A) F_1 progeny (*An. yatsushiroensis* form) of *An. pullus* (B); (B) Wild-caught *Anopheles pullus* (parent of A); (C) F_1 progeny (*An. pullus* form) of *An. yatsushiroensis* (D); (D) wild-caught *An. yatsushiroensis*.

CS250 correspond to conserved sites in the 5.8S rDNA and 28S rDNA domain 1, respectively. A portion of the mtCOI gene was amplified with primers UEA5 (5′-AGT TTT AGC AGG AGC AAT TAC TAT-3′) and UEA8 (5′-AAA AAT GTT GAG GGA AAA ATG TTA-3′). Both genes were amplified under the same reaction conditions. Template DNA (~50 ng) was mixed in a 50-µl amplification reaction with deoxynucleoside triphosphates (0.2 mM), 20 pM of each primer, 2.5 mM $MgCl_2$, 5 µl of 10× PCR reaction buffer (Promega, Madison, WI), and 2.5 U of *Taq* DNA polymerase (Promega). The PCR amplifications were performed for 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The amplified PCR products were purified (Qiagen), ligated into pGEM-T (Promega), and transformed into $CaCl_2$-competent *Escherichia coli* XL1. Recombinant colonies were identified by blue/white screening with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropylthio-β-D-galactoside. Plasmid DNA was isolated by using the Qiaprep spin miniprep purification kit (Qiagen).

DNA sequence analysis: Plasmid DNA sequencing was conducted with a Perkin Elmer 9600 PCR machine (Wellesley, MA) using T7 forward and SP6 reverse vector primers. Sequences were assembled with GeneJockey II (BIOSOFT Co., Cambridge, U.K.). Sequences were aligned with Clustal X (Thompson et al. 1997) and MacClade version 3.0 (available at http://www.macclade.org/old/macclade3.html). A maximum likelihood tree based upon the 5.8S rDNA-ITS2-28S was estimated by using PAUP* (Swofford 2002).

RESULTS

Rearing experiment and morphology observation

Six bloodfed female *An. pullus* were individually reared in separate cages in the laboratory. Some of

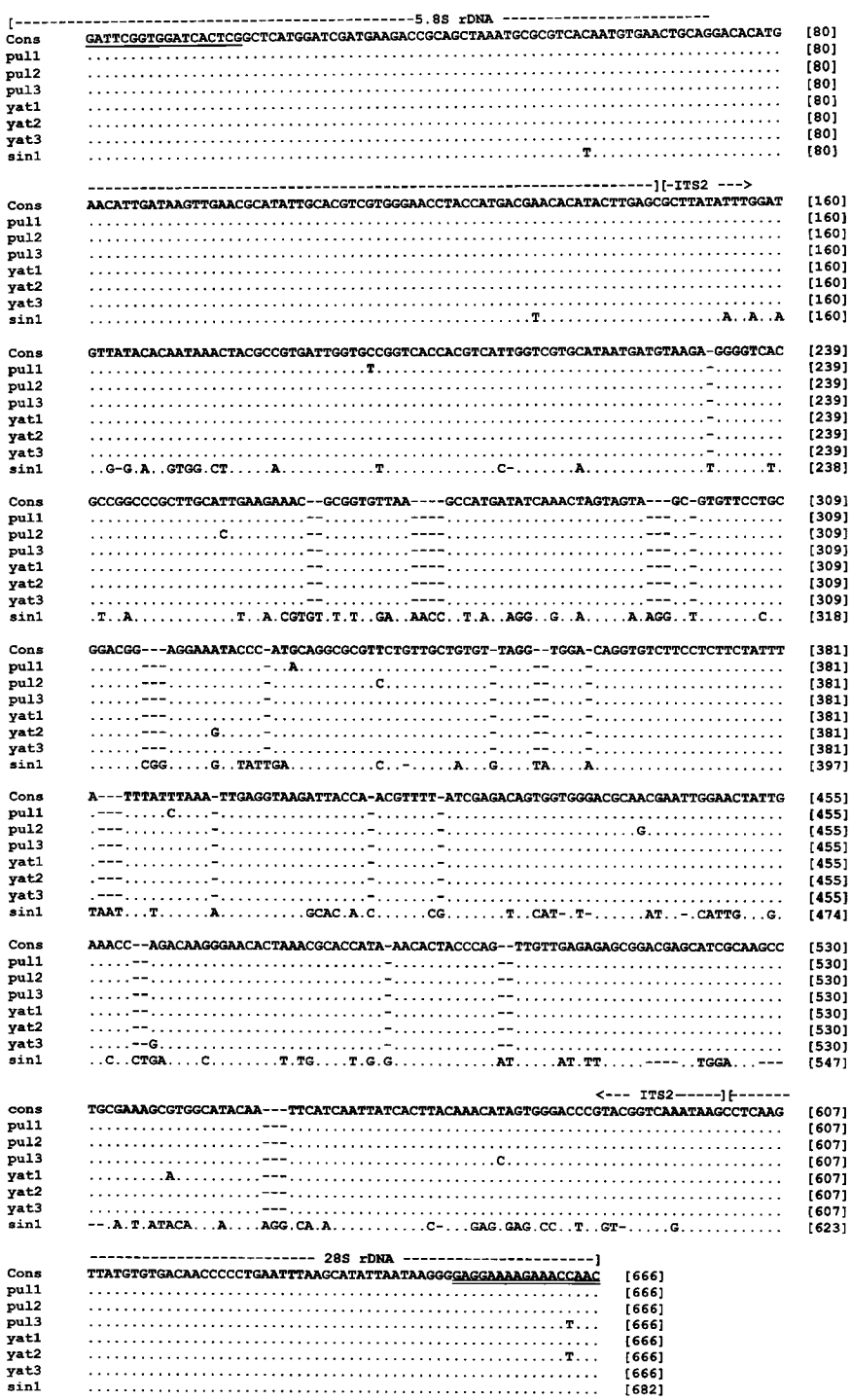

Fig. 2. Nucleotide sequence alignment of 5.8S rDNA-ITS2-28S rDNA regions from 3 individuals of *Anopheles pullus,* 3 individuals of *An. yatsushiroensis,* and 1 individual of *An. sinensis.* Single and double underlines indicate positions of polymerase chain reaction primers, 687 and CS250, respectively. Cons, consensus sequences of 5.8S rDNA-ITS2-28S rDNA of 3 individuals of *An. pullus* or *An. yatsushiroensis*; pul 1–3, *An. pullus* individuals 1–3 (GenBank accession numbers AY339272–AY339274 in order); yat 1–3, *An. yatsushiroensis* individuals 1–3 (AY339275–AY339277 in order); sin 1, *An. sinensis* individual 1 (AY339278); -, alignment gap; ., same nucleotide with that of the 1st line (Cons).

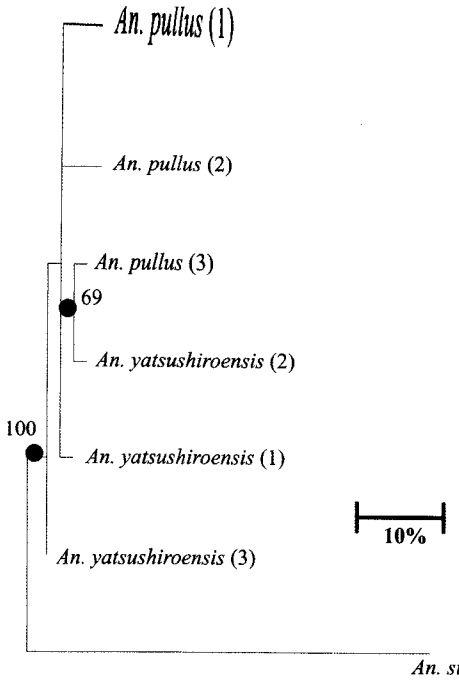

Fig. 3. Maximum likelihood tree inferred from 5.8S rDNA-ITS2-28S rDNA nucleotide sequences of 3 *Anopheles pullus,* 3 *An. yatsushiroensis,* and 1 *An. sinensis.* The branch length of *An. sinensis* is one tenth of its actual length.

the eggs, larvae, and pupae were preserved for morphological observation. All of the F_1 progeny obtained (15 females and 19 males) were morphologically identified as *An. yatsushiroensis.* Two pale fringe spots of the wings, 1 apical (at the termination of veins R_1–M_{1+2}) and the other at the termination of vein Cu_2 were present. In contrast, *An. pullus* had completely dark fringe scales (Fig. 1). We also observed that some of the F_1 progeny of wild-caught *An. yatsushiroensis* were of the *pullus* form (Fig. 1).

Sequence comparison of 5.8S rDNA-ITS2-28S rDNA region

The 5.8S rDNA-ITS2-28S rDNA from *An. pullus* and *An. yatsushiroensis* consisted of complete 5.8S rDNA (142 base pairs [bp]), complete ITS2 (453 bp), and partial 28S rDNA (71 bp) and were 666 bp in length. The length of this region in *An. sinensis* was 682 bp (Fig. 2). Sequences varied at 8 sites among 3 *An. pullus* and 4 sites among 3 *An. yatshshiroensis* but varied at 159 sites between *An. pullus* and *An. sinensis.*

The maximum likelihood tree (Fig. 3) indicates that *An. pullus* and *An. yatsushiroensis* are monophyletic with 100% bootstrap support. In addition, individual 3 of *An. pullus* and individual 2 of *An. yatsushiroensis* were monophyletic with 69% bootstrap support. Both observations are consistent with the hypothesis that *An. yatsushiroensis* is a synonym of *An. pullus.*

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pulCO1  AGTTTTAGCAGGAGCAATTACTATACTTTTAACAGATCGAAATCTAAATACTTCATTTTTTGACCCAGCTGGAGGGGGAG  [80]
yatCO1  ................................................................................  [80]
sinCO1  .........................................T.......C.....C.................A....  [80]

pulCO1  ATCCTATTCTATATCAACATTTATTCTGATTCTTTGGTCATCCAGAAGTTTATATTTTAATTTTACCTGGATTTGGAATA  [160]
yatCO1  ........T.......................................................................  [160]
sinCO1  ........T.......................T.....A.....................................G...  [160]

pulCO1  ATTTCTCATATTATTACACAAGAAAGTGGTAAAAAGGAAACTTTTGGAAATTTAGGAATAATTTATGCTATATTAGCAAT  [240]
yatCO1  ................................................................................  [240]
sinCO1  ..........................................C.....................................  [240]

pulCO1  TGGATTATTAGGATTCATTGTATGAGCTCATCATATATTTACAGTAGGAATAGACGTAGATACACGAGCTTATTTTACAT  [320]
yatCO1  ................................................................................  [320]
sinCO1  ..............T.....T...........C..............T.....................................  [320]

pulCO1  CAGCAACTATAATTATTGCTGTTCCAACTGGAATTAAGATTTTTAGTCGATTAGCTACTATACATGGAACACAATTAACT  [400]
yatCO1  ...........................................T....................................  [400]
sinCO1  ...........................................T.............C.................A  [400]

pulCO1  TATAGCCCTGCTATATTATGATCATTTGGATTTGTATTTTTATTTACAGTTGGAGGATTAACAGGAGTAGTACTAGCTAA  [480]
yatCO1  ........C.......................................................................  [480]
sinCO1  ........A..A...C.....G.....................................G......T.............  [480]

pulCO1  TTCATCAATTGACATTGTATTACATGATACATATTATGTAGTAGCCCATTTCCACTATGTATTATCAATAGGAGCTGTAT  [560]
yatCO1  ................................................................................  [560]
sinCO1  ............T...........A.....................T.....................T.............  [560]

pulCO1  TTACTATTATAGCAGGATTTGTTCATTGATACCCTTTATTAACCGGACTAACAATAAATCCATCTTGATTAAAGTATCAA  [640]
yatCO1  ..G.....................................T.....................C.....................  [640]
sinCO1  ..G.................A...........T..A.........A...T.................A.....................  [640]

pulCO1  TTTGGAATAATATTTATTGGAGTAAATTTAACATTTTCCCTCAACATTTTT  [691]
yatCO1  ...................................................  [691]
sinCO1  ....CT................................................  [691]
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Fig. 4. Nucleotide sequence alignment of partial mtCOI genes from *Anopheles pullus, An. yatsushiroensis,* and *An. sinensis.* Single and double underlines indicate positions of polymerase chain reaction primers, UEA5 and UEA8, respectively. PulCO1, *An. pullus* (AY339279); yatCO1, *An. yatsushiroensis* (AY339280); sinCO1, *An. sinensis* (AY339281); -, alignment gap; ., same nucleotide with that of the 1st line (pulCO1).

Sequence comparison of partial mtCOI gene

Partial mtCOI genes of *An. pullus, An. yatsushiroensis,* and *An. sinensis* were 691 bp. *Anopheles pullus* and *An. yatsushiroensis* differed at 6 sites. In contrast, the mtCOI genes differed at 35 positions between *An. pullus* and *An. sinensis.*

DISCUSSION

Anopheles pullus was described in Korea in 1937 (Yamada 1937) and has so far been found only in the Korean peninsula. *Anopheles yatsushiroensis* was described in Japan in 1951 (Miyazaki 1951), and was 1st collected in Korea in 1967 (Hong and Ree 1968) and in China in 1975 (Xu and Feng 1975). The most important key characters for identification of *An. pullus* and *An. yatsushironesis* are the fringe scales of the wings, with completely dark fringe scales (no pale spot) in *An. pullus,* whereas *An. yatsushiroensis* has 2 pale spots, one apical and the other at the termination of vein Cu_2. The key character to distinguish between *An. yatsushiroensis* and *An. sinensis* is the width of the 2nd pale band of the maxillary palpus, which is wider in the former. The key character to distinguish between *An. pullus* and *An. sinensis* is the presence of 2 pale fringe spots of wings in *An. sinensis* (Yamada 1937, Otsuru and Ohmori 1960, Xu and Feng 1975).

Many different taxonomic opinions or findings have been made on the *An. sinensis* group in Japan and Korea. Nakayama (1942) reared wild-caught female *An. sinensis* from near Tokyo, Japan, in the laboratory and obtained both *sinensis* and *pullus* forms as well as intermediate forms from F_1 progeny. He suggested that *An. pullus* could be a seasonal form of *An. sinensis.* In Korea, Chu (1956) also speculated that *An. pullus* was a variant of *An. sinensis.* A comparative study on morphological characters of male terminalia among *An. sinensis, An. lesteri,* and *An. yatsushironesis* in Japan suggested that these were valid species (Ohmori 1957). Hybridization experiments between members of the *An. sinensis* group from Korea were conducted with artificial copulation. The emergence rates of F_1 and F_2 hybrid progenies between *An. pullus* and *An. sinensis* were 60.2 and 77%, respectively. Those between *An. yatsushiroensis* and *An. sinensis* were 57.0 and 80%, respectively. Chromosomes of the F_1 hybrids were not abnormal and coincided well with those of the parents. Kanda and Oguma (1971) concluded that these were all same species. They also observed a wide range of morphological variation in the appearance of pale fringe spots of *An. sinensis* wings, that is, 0.3–20.5% of wild-caught females had no fringe pale spot at the termination of vein Cu_2 and 20.5% of 78 F_1 progeny of a female *An. sinensis* had no pale fringe spot (Kanda and Oguma 1976).

Recently, Shin and Hong (2001) compared morphological characters between wild-caught females and their F_1 progeny reared under summer temperature conditions (26°C). The F_1 progeny of wild-caught female *An. pullus* included 49.7% of the *pullus* form, 9.6% of the *yatsushiroensis* form, and 40.8% of intermediate forms. The F_1 progeny of wild-caught female *An. yatsushironesis* included only 19.3% of the *yatsushiroensis* form, 55.2% of the *pullus* form, and 25.5% of intermediate forms. They concluded that *An. yatsushironesis* is a junior synonym of *An. pullus.* However, morphological variation is not decisive in the *sinensis* group because of the wide range of overlapping variations.

When identification of certain species on the basis of morphological characters is difficult, molecular markers frequently have been employed to obtain additional evidence (Hwang and Kim 1999). Consensus sequences of the 5.8S rDNA-ITS2-28S rDNA obtained from *An. pullus* and *An. yatsushiroensis* were completely identical. Consensus sequences between *An. pullus/yatsushiroensis* and *An. sinensis* were different in 159 sites. Partial mtCOI gene sequences of *An. pullus* and *An. yatsushiroensis* differed at only 6 base pairs (Fig. 4). Examination of these results suggests that *An. pullus* and *An. yatsushiroensis* are morphological variants of 1 species rather than separate species, confirming that *An. yatsushiroensis* is a synonym of *An. pullus. Anopheles sinensis* is a separate species from *An. pullus.*

Previous studies reported that *An. pullus* was collected in May–June and in September–October but never was found from July to August. In contrast, *An. yatsushiroensis* was found in large numbers with a peak in July (Kim et al. 1997, 1999, 2001; Shim et al. 1997). This result strongly suggests that these are seasonal forms.

Another unusual finding is that all 145 females collected in grasses (a main hibernating place of *An. sinensis*) in winter in Kyonggi-do were *An. pullus* with no *An. sinensis* found. The Medical Entomology Team, National Institute of Health, Korea, also found that the majority of hibernating females collected on grasses in the winter were *An. pullus,* with only a few *An. sinensis* (Shin et al., unpublished data).

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