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 F1 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 yatsushiroensis, 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 F1 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. Batch of eggs from a female was reared in a separate pan in an insectary (26°C and 14:10 h light: dark). The F1 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 TTC TTT TCC TC-3'). Primers 687 and
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 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 MgCl₂, 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 CaCl₂-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. 1. Wings of female mosquitoes. (A) F₁ progeny (*An. yatsushiroensis* form) of *An. pullus* (B); (B) Wild-caught *Anopheles pullus* (parent of A); (C) F₁ progeny (*An. pullus* form) of *An. yatsushiroensis* (D); (D) wild-caught *An. yatsushiroensis*. 

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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).
**An. pullus (1)**

**An. pullus (2)**

**An. pullus (3)**

**...**

**An. yatsushiroensis (2)**

**...**

**An. yatsushiroensis (3)**

**An. sinensis (3)**

![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.](image)

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**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. yatsushiroensis 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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**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. yatsushiroensis 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 Cu2. 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 F1 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. yatsushiroensis 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 F1, and F2 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 F1 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 Cu2 and 20.5% of 78 F1 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 F1 progeny reared under summer temperature conditions (26°C). The F1 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 F1 progeny of wild-caught female An. yatsushiroensis included only 19.3% of the yatsushiroensis form, 55.2% of the pullus form, and 25.5% of intermediate forms. They concluded that An. yatsushiroensis 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 yatsushiroensis 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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