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Identification of four species of the *Anopheles hyrcanus* complex (Diptera: Culicidae) found in Korea using species-specific primers for Polymerase Chain Reaction Assay

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Abstract: For identification of four sibling species of the *Anopheles hyrcanus* complex found in Korea, the 5.8 rDNA-ITS2-28S rDNA region of each species was sequenced and the species-specific primers were designed. The amplified PCR products obtained from each species were analyzed by electrophoresis on 3% agarose gel. The result showed a single species-specific band, i.e. 559 bp, 432 bp, 322 bp and 192 bp for *An. sinensis*, *An.* sp., *An. lesteri* and *An. pullus*, respectively. In conclusion, the species-specific PCR primers designed from ITS2 variable regions functioned successfully and specifically, and can be applied as a useful tool for identifying species of the Hyrcanus complex found in Korea.

Key words: identification, *Anopheles hyrcanus* complex, PCR, Korea

INTRODUCTION

In the Republic of Korea (South Korea) vivax malaria had been eradicated in 1979. However, malaria re-emerged in 1993 and an outbreak occurred in northern parts of Kyonggi-do and Kangwon-do, with confirmed cases of 1 in 1993, 20 in 1994, 107 in 1995, 356 in 1996, 1,724 in 1997, 3,932 in 1998, 3,621 in 1999, 4,142 in 2000, 2,556 in 2001, 1,799 in 2002 and 1,170 in 2003 (Ree, 2000; Lee et al., 2002; Ministry of Health and Welfare, 2004). Malaria cases reported in DPR Korea (North Korea) were 204,428 in 2000, 300,000 in 2001, 241,190 in 2002 and 46,251 in 2003 (unpublished data). For understanding the epidemiological features of vivax malaria in the Korean peninsula taxonomical studies on the *Anopheles hyrcanus* complex are urgently required, as several vector species are included in the complex.

The Hyrcanus group of the genus *Anopheles* (Diptera: Culicidae) consists of 18 related species (Harrison, 1972; Xu and Feng, 1975), of which five species, *An. sinensis*, *An. lesteri*, *An. pullus*, *An. yatsushiroensis* and *An. sineroides* are found in Korea (Kim et al., 2000; Ree, 2003). *An. sinensis* was confirmed as the main vector species in Korea (Ree et al., 1967; Lee et al., 2000; Strickman et al., 2001), and natural infection of *An. yatsushiroensis* to the malaria parasite was also reported (Hong, 1977). Recent works revealed that *An. yatsushiroensis* Miyazaki, 1951 is a synonym of *An. pullus* Yamada, 1937 (Shin and Hong, 2001; Hwang et al., 2004), and that *An. anthropophagus* from China and *An. lesteri* from Japan are the same species (Hwang et al., unpublished data). Based on a combination of published and newly generated rDNA ITS2 sequence, Wilkerson et al. (2003) found that *An. lesteri*

from Philippines, *An. lesteri* from South Korea and *An. anthropophagus* from China are all identical; so *anthropophagus* is a synonym of *lesteri*. It is well known that *An. anthropophagus* in central and southern parts of China is the primary vector species of vivax malaria. Therefore, *An. lesteri* in the Korean peninsula would play an important role in malaria transmission. Tanaka et al. (1979) considered that *An. lesteri* would be the probable primary vector of malaria in Japan rather than *An. sinensis*. Little work has been done on *An. lesteri* of the Korean peninsula and Japan, mainly because of morphological similarity between *An. sinensis* and *An. lesteri*. These species of the Hyrcanus group are so morphologically similar that they are extremely difficult or impossible to identify at the species level. Therefore, species identification and confirmation are of vital importance for determination of the vector species of malaria and their vector efficiency.

We designed the species-specific primers of four species of the Hyrcanus complex found in Korea for accurate identification of these species in field collections, by using a Polymerase chain reaction (PCR) assay. The primers for *An. sineroides* were not made, because this species can be easily identified with morphological characteristics, such as the presence of a humeral pale spot and three black spots on the anal vein (Ree, 2003).

Materials and Methods

Total DNA was extracted using a DNeasy tissue kit (Qiagen Co.) from the whole body of each of five species. The 5.8S rDNA-ITS2-28S rDNA region which is the most variable in amino acid sequences among the Hyrcanus group was amplified with the primer 687 (5′-ACCCTGGACGGTGGATCACTYGG-3′) and the primer CS250 (5′-GTTAGTTTCTTTTCCTC-3′). The amplified PCR products were purified by a PCR product purification system (Qiagen Co.), and ligated into the pGEM-T easy vector system (Promega Co.). The ligation mixture was used to transform $CaCl_2$-competent *Escherichia coli* XL1. The selection of clones was executed by the blue/white screening method with X-gal and IPTG. Plasmid DNA was isolated using the Qiaprep spin miniprep purification kit (Qiagen Co.), and the sequencing was conducted with a Perkin Elmer 9600 PCR machine. Final DNA sequence construction, alignment and comparison were facilitated using Gene Jockey II for sequence editing (BIOSOFT Co.). Clustal X^8 for multiple sequence alignment, MacClade ver 3.0 were used for sequence alignment and manipulation (Thompson et al., 1997). For the comparison of the Hyrcanus complex, the species-specific PCR primers were designed based on interspecies nucleic acid sequence variation in the second internal transcribed spacers (ITS2) of the nuclear ribosomal DNA gene array.

Results and Discussion

The DNA sequence of the 5.8S rDNA-ITS2-28S rDNA region was constructed, aligned and compared in Fig. 1. The species-specific PCR primers for identifying four species of the Hyrcanus group found in Korea designed from the most variable regions are 687 and Sin-S for *An. sinensis*, Ant-S and CS250 for *An. lesteri*, 689 and Pul-S for *An. pullus* (including form *yatsushiroensis*), and 688 and Asp-S for *Anopheles* sp. (Table 1). The primer positions and orientation are given in Fig. 1.

The PCR products differed in length: 559 bp for *An. sinensis*, 322 bp for *An. lesteri*, 192 bp for *An. pullus*/form *yatsushiroensis*, and 432 bp for *An.* sp., and they are separated on 2% agarose gels as shown in Fig. 2. This figure also shows that the PCR product of each species produced a specific band of its own and did not respond to the other species primers.

The sequence comparison of the 5.8S rDNA-ITS2-28S rDNA revealed that there is an unknown discrete species (*Anopheles*

Table 1. Species-specific PCR primers for identifying 4 species of the Hyrcanus complex found in Korea.

Primer code		Primer nucleotide sequences (5′→3′)
687	(+)	ACC CTG GAC GGT GGA TCA CTY GG
CS250	(−)	GTT AGT TTC TTT TCC TC
688	(+)	GGG AAC CTA CCA TGA CGW A
689	(+)	CAG GTG TCT TCC TCW TCY A
Ant-S	(+)	AAG TAG TAA ACA GCA GCA G
Sin-S	(−)	GCT GTA TTA TTG TTG TCC A
Pul-S	(−)	ATT GTA TGC CAC GCT TTC G
Asp-S	(−)	TTT GCT GTA TCG TTA GGA CC

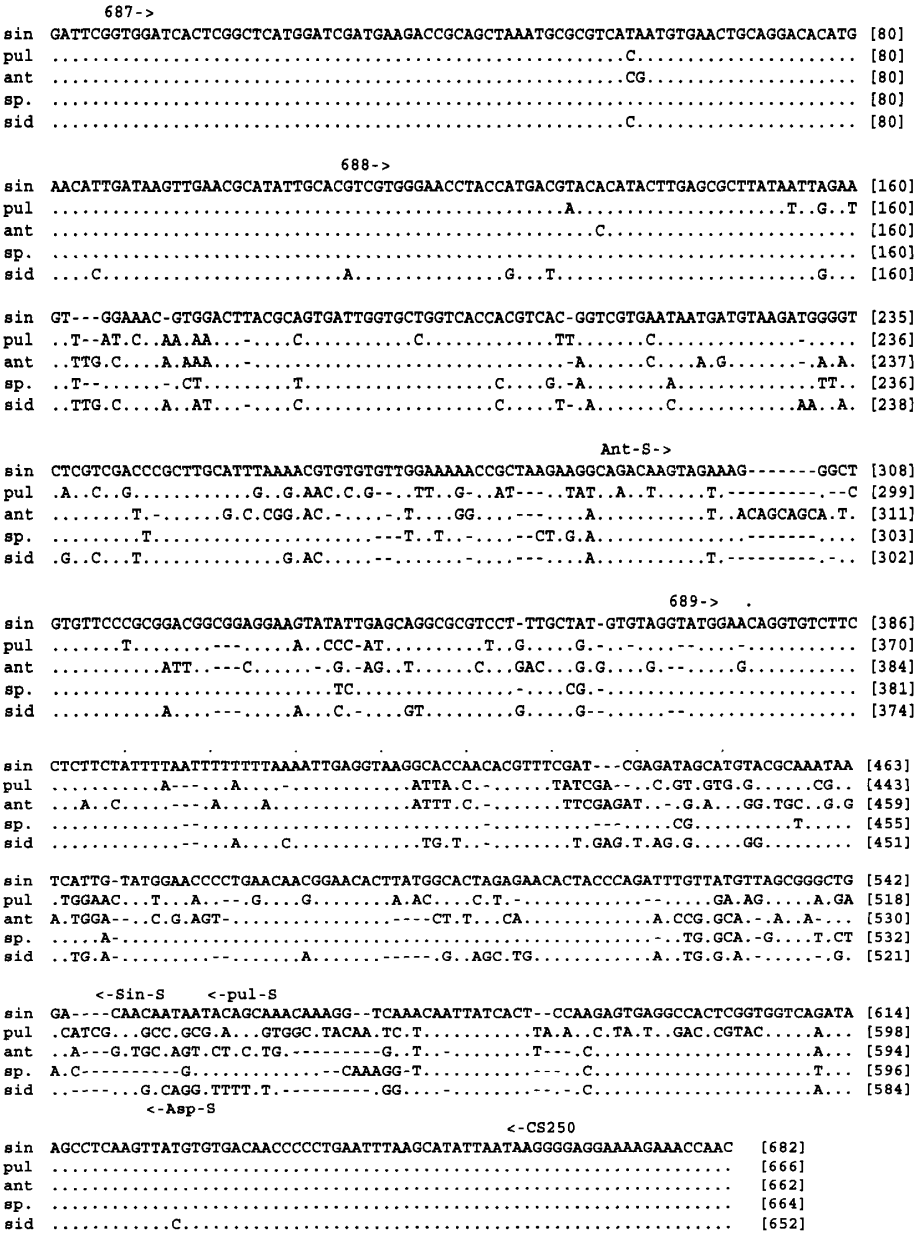

Fig. 1. Nucleotide sequence alignment of 5.8S rDNA-ITS2-28S rDNA from *An. sinensis* (sin), *An. pullus* (pul), *An. anthropophagus/lesteri* (ant), *An.* sp. (sp.) and *An. sineroides* (sid). The primer positions are given. Gen Bank accession numbers AY339278, AY339272, AJ620897, AJ620896 and AJ620895, in order. -, alignment gap; ., same nucleotide with that of the 1st line.

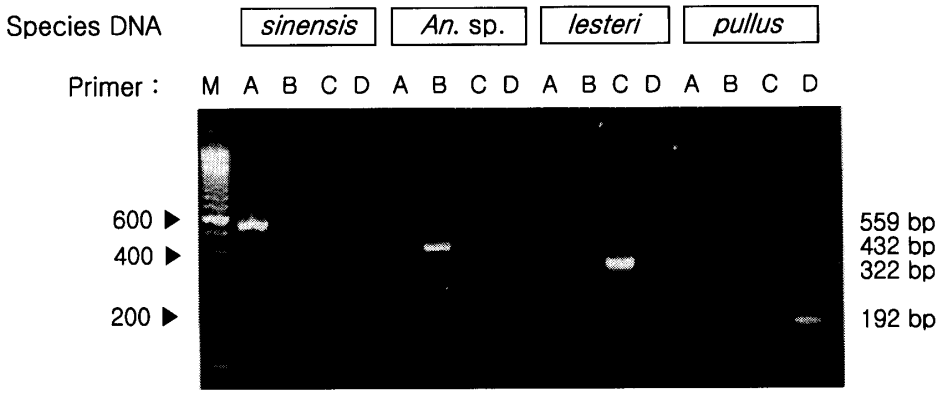

Fig. 2. Specificity of four *Anopheles* species-specific primers. M, 100 bp ladder. A, *sinensis*-specific primers; B, *An.* sp.-specific primers; C, *lesteri*-specific primers; D, *pullus*-specific primers.

sp.) among *An. sinensis* population. The gene differences appeared at 67 bp between *An. sinensis* and *Anopheles* sp. (an unknown species), at 132 bp between *An. lesteri* and *An.* sp., and at 143 bp between *An. pullus* and *An.* sp. The species-specific primers for *An.* sp. produced a 432 bp band on the agarose gels. This unknown species was morphologically identical to *An. sinensis*. We found that considerable numbers of *An.* sp. co-exist with *An. sinensis* population (unpublished). Further studies are required for this unknown species, not only in taxonomical but also in behavioral and malaria vector efficiency aspects.

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