Zootaxa 378: 1–14 (2003)
www.mapress.com/zootaxa/

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

378

Molecular confirmation of *Anopheles (Anopheles) lesteri* from the Republic of South Korea and its genetic identity with *An. (Ano.) anthropophagus* from China (Diptera: Culicidae)

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Abstract

Recent malaria transmission in The Republic of Korea led to a search for the identity of the possible vectors. The *Anopheles* fauna of South Korea is presently considered to consist of six species: *Anopheles (Anopheles) sinensis*, *An. (Ano.) lesteri*, *An. (Ano.) pullus*, *An. (Ano.) sineroides*, *An. (Ano.) lindesayi japonicus*, *and An. (Ano.) koreicus*, of which only the former three are considered potential vectors. Based on a combination of published and newly generated rDNA ITS2 sequence we found that *An. lesteri* from South Korea, *An. anthropophagus* from Jiangsu Province, China, and *An. lesteri* from near the type locality in Laguna Province, in the Philippines, are indistinguishable. Also, a species reported in GenBank as *An. lesteri* from Shandong Province, China, is the same as an unnamed species also discovered by us in South Korea. The above are compared to *An. sinensis* from South Korea and the type locality in China. These data indicate that *An. anthropophagus*, an important malaria vector in China, is actually *An. lesteri*. We therefore place *An. anthropophagus* in synonymy with *An. lesteri*. In addition, based on Korean specimens, *An. yatsushiroensis* was recently synonymized under *An. pullus*. We are in agreement with the conclusion that Korean specimens that have morphological attributes previously thought to differentiate these two species are actually just highly variable characters of a single species. However, genetic comparison to specimens from the type locality of *An. yatsushiroensis*, Yatsushiro City, Japan, is still needed to rule out the possibility that this is a valid species.

Key words: malaria, *Anopheles lesteri*, South Korea, taxonomy, Hyrcanus Group

Accepted: 4 November 2003; published: 8 December 2003

Introduction

Because of a recent recrudescence of malaria in South Korea (Cho *et al.* 1994; Feighner *et al.* 1998; Lee *et al.* 1998) we examined the taxonomic status of the possible vectors to verify that the names used in the literature correspond to the biological species that the names represent. This is a serious concern since misidentification of species can cause significant confusion in understanding the epidemiology of malaria transmission and affect control measures.

Most malaria in temperate East Asia, as in Europe, North America, and Central Asia, is caused by *Plasmodium vivax.* This parasite is well adapted to climates with a prolonged cool season. In contrast to *P. falciparum, P. vivax* is capable of establishing chronic liver infections in humans. Untreated, a person may support an infection for years with the liver stage hypnozoites periodically producing blood–stage merozoites (Brunetti *et al.* 1954; Paik *et al.* 1988; Wang 1985). Infected persons may not seek medical care because the symptoms can be relatively mild and self–limited (Shen *et al.* 1998). People who do not clear their infection continue to infect mosquitoes, perpetuating the disease.

The distribution of malaria in East Asia has changed over time, with foci appearing and disappearing. Perhaps the best-described outbreak is the most recent one in the Republic of Korea (South Korea), which began in 1993 with cases in the northeastern part of the country (Chai *et al.* 1994; Cho *et al.* 1994). The number of cases increased exponentially through 1997 (Chai 1999; Lee *et al.* 1998). The outbreak centered on the border area with the People's Democratic Republic of Korea (North Korea), suggesting that a parallel outbreak occurred in that country.

Most species of *Anopheles* in temperate East Asia are part of the Hyrcanus Group of the subgenus *Anopheles*. The Hyrcanus Group of *Anopheles (Anopheles)* is composed of about 29 species (Harbach 1994; Harrison & Scanlon 1975; Manh *et al.* 2000; Ramsdale 2001), some of which are vectors of mosquito-borne diseases including malaria and filarial parasites. Twenty-six species of the group have an Oriental or eastern Palaearctic distribution while 3 species occur in the western Palaearctic (Palaearctic west of China to south of 50°N). In South Korea the known Hyrcanus Group species are: *An. sinensis* Wiedemann, *An. pullus* Yamada, *An. lesteri* Baisas & Hu and *An. sineroides* Yamada. In addition, 2 other *Anopheles* occur there, *An. (Ano.) lindesayi japonicus* Yamada and *An. (Ano.) koreicus* Yamada & Watanabe. Only *An. sinensis, An. pullus*, and *An. lesteri* are considered to be malaria vectors, though one study (Shin *et al.* 2002) showed experimental infection only in *An. sinensis* and *An. lesteri* in Korea. Not discussed in detail here is the close resemblance that *An. sinensis, An. lesteri* and *An. pullus* have to each other, and to the species discussed below. These similarities, in all stages, along with intraspecific variation, have lead to confusion not only in Korea but also regionally.

Anopheles anthropophagus Xu & Feng is generally acknowledged to be the most important vector of malaria in the region, though it has not been found in Korea (but see results below). In parts of China, *An. anthropophagus* also transmits *P. falciparum* (Tang

et al. 1991). *An. sinensis* is also known to be a vector in both China and Korea, though it is considered approximately 20 times less susceptible than *An. anthropophagus* (Liu *et al.* 1990). Natural infection rates of the 2 species in the 1960s consistently showed that *An. anthropophagus* had 1.9 to 14.4 times greater infection rate than *An. sinensis* in the same area (Gu *et al.* 1996). *An. lesteri* and *An. yatsushiroensis* Miyazaki (= *pullus* (Shin & Hong 2001)) are suspected of being vectors because they both bite humans. In addition, sporozoites have been found in *An. pullus* in Korea (Hong 1977).

The published distribution of *An. anthropophagus* extends west to approximately 105°E longitude and north to 33°N latitude (Gu *et al.* 1996). Work completed by one of us (Guan–Hong Song) recently extended the range to 43°N. This species was first split off as a subspecies of *An. lesteri* (Xu & Feng 1975) and then raised to full species status (Ma 1981). Known morphological differences from *An. sinensis* are only reliable in the egg stage with the result that adult identifications are not always reliable (Ma *et al.* 1998). Studies of pupal ultrastructure (Xu *et al.* 1981), chromosomes (Xu & Qu 1991; Zhu *et al.* 1981), a genomic DNA probe (Niu *et al.* 1992), and sequencing of rDNA ITS2 (Ma *et al.* 1998), all showed differences between *An. sinensis* and *An. anthropophagus*. *An. anthropophagus* larvae are usually found in cooler, non–polluted water (Xu *et al.* 1994). Rice fields are a major source of *An. anthropophagus*, particularly late in the season when the rice plants shade the water. *An anthropophagus* is favored by single cropping of rice (i.e., one harvest per year) because water remains deep for a longer period of time and because the rice plants grow to provide a deeper shade. Adult females enter homes readily to seek resting sites and blood meals. There is some evidence that this species can over winter in both the egg and adult stages (Ho *et al.* 1962).

Anopheles sinensis, as currently defined taxonomically, occurs from Pakistan to Japan and from northern China to Indonesia. Given the widespread distribution of this species, there is the possibility that it actually consists of a number of species that are not currently defined by morphological characters (Choochote *et al.* 1998; Ma *et al.* 2001). Larvae of this species are also common in rice fields, tolerating shallower, sunnier water than *An. anthropophagus*. As a result, *An. sinensis* appears two to four weeks earlier than *An. anthropophagus* and is favored by double cropping practices, which can produce thinner shade and shallower water (Xu *et al.* 1994). *An. sinensis* will feed indoors or outdoors, but adults tend to leave a house soon after biting. Although the species is usually considered zoophilic (i.e., preferring to bite animals (Ho *et al.* 1962)), it readily bites humans (Shim *et al.* 1997; Strickman *et al.* 2000). In Shandong Province, China, *An. sinensis* was shown to be the principal vector of malaria and was associated with rice production (Yang *et al.* 1991).

Anopheles lesteri is interpreted as similar morphologically to *An. anthropophagus*, but is more poorly defined taxonomically. *An. lesteri* was described by Baisas & Hu (1936) as *An. hyrcanus var. lesteri* from the Philippines (Santa Mesa, Manila, Luzon). There has been confusion concerning the true identities of the *An. lesteri* populations in Japan, Korea

and China (for a review see Tanaka *et al.* 1979). Taxonomists suspected that some geographic groups of *An. lesteri* were distinct from each other (Xu & Feng 1975). This suspicion culminated in elevation of two forms (*anthropophagus* in China, *paraliae* Sandosham in Southeast Asia (Harrison *et al.* 1990)) to full species status. Northern Asian populations may also differ from the Philippine form, but this question has not been studied. Recent DNA examination of Korean *An. lesteri* showed that it is not the same as *An. anthropophagus* in China (Ma *et al.* 2000). In addition, *An lesteri* is considered a principal vector of malaria in southern China (Beales 1984; Chow 1991; Ho *et al.* 1962; Ma 1981) and was suspected of being a primary vector in Japan and Korea (Kamimura 1968; Otsuru 1949; Tanaka *et al.* 1979). Adults and eggs of Korean *An. lesteri* often exhibit individual morphological variation making them difficult to separate from those of Korean *An. sinensis* using available keys (Lee 1998; Tanaka *et al.* 1979). As presently known, it is not possible to morphologically separate the larvae of Korean *An. lesteri*, *An. sinensis*, *An. pullus* and *An. yatsushiroensis*. Xu & Feng (1975) described *An. lesteri* subspecies *anthropophagus* from central and south China using the characters of the eggs (larger size of the egg and narrower egg deck) and pupae (darker spots at the trumpet bases) to separate it from the Philippine *An. lesteri s. s.* In Japan, these egg and pupal characters are quite variable in *An. lesteri* from various localities and are therefore not reliable.

Here we present evidence based on sequence data from specimens of *An. lesteri* from near its type locality, Luzon, the Philippines, indicating that the identities of *An. lesteri* and *An. anthropophagus* in China, as described above, have been confused and that an additional unnamed or unrecognized similar species is present in the fauna of the region.

Two additional Hyrcanus Group species have been recognized in Korea: *An. pullus* and *An. yatsushiroensis*. A thorough analysis of morphological characters by Shin & Hong (2001) demonstrated that specimens that key to these two species from Korea actually represent a single polymorphic species. Since *An. pullus* is the older name they synonymized *An. yatsushiroensis* under *An. pullus*. We present corroborating molecular evidence and discuss their action.

Materials and methods

The central question concerning the genetic identities of specimens identified as *An. lesteri* and *An. anthropophagus* was explored using rDNA ITS2 sequence from material collected by us in various parts of the ranges of the 2 putative species (Korea, China, Philippines), and also by using ITS2 sequence deposited in GenBank. We also sequenced ITS2 of putative *An. yatsushiroensis* and *An. pullus* from specimens collected by us in Korea. For comparison, we sequenced ITS2 from *An. sinensis*, a related species that at the present time is better characterized than the others (an “outgroup”). For specimens reared by us we first used the adult female characters given in Tanaka *et al.* (1979) for preliminary identifications.

TABLE 1. Summary of collection localities for *Anopheles* species identified using morphological keys (Tanaka *et al.* 1979) and molecular tests (PCR and ITS2 sequence alignment). All collections are males unless otherwise specified as female (F) or larva (L).

Province (Locality)	Coordinates	Date	Collector	Habitat	Collection No.	Morphology	PCR	ITS2 Sequence
KOREA								
Gyeonggi–do (Sokcho)	37°11'N 126°23'E	16-VI-2001	T. Klein A. Schuster	Progeny reared from adults resting in cowshed near cultivated field	KS 4(1)-5, (2)-2, (3)-8, (4)-8, (5)-8, (7)-2	*sinensis*	-	*sinensis*
Gyeonggi–do (Tongilchon, near Munsan)	37°51'N 126°47'E	26-VI-2001	T. Klein A. Schuster Park	Progeny reared from adults collected at dairy farm	KS 7(5)-14, (7)-7, (9)-3, (10)-2, (11)-1, (12)-1, (13)-1, (14)-4, (16)-4,(17)-3, (19)-14, (22)-4, (23)-8, (24)-6, (25)-4,(28)-6, (29)-3, (30)-8, (32)-4 , (33)-3, (34)-11,(35)-5, (36)-12, (37)-3, (38)-4, (39)-4, (40)-4	*sinensis*	-	*sinensis*
					KS 7(1)-4, (6)-4, (8)-7	*yatsushiroensis*	-	*pullus*
					KS 7(27)-2	*pullus*	-	*unknown species*
Gyeonggi–do (Ogeum–ri, Paju)	37°49'N 126°43'E	29-VII-2001	H.C. Kim	Progeny reared from adults collected at cowshed near rice paddy	KS 8(1)-1, (5)-1	*sinensis*	*sinensis*	*sinensis*
					KS 8(10)-1, (16)-1, (39)-1, (76)-1, (96)-1, (111)-1, (42)-1, (51)-1, (57)-2, (113)-1, (124)-1, (130)-1, (143)-1	*yatsushiroensis*	*pullus*	*pullus*

.....continued on the next page

TABLE 1 (continued)

Province (Locality)	Coordinates	Date	Collector	Habitat	Collection No.	Morphology	PCR	ITS2 Sequence
					KS 8(12)-1	*sinensis*	-	unknown species
					KS 8(20)-1, (45)-1, (67)-1, (77)-1, (94)-2	*sinensis*	*sinensis*	-
					KS 8(27)-1, (31)-1, (35)-2	*pullus/yatsushiroensis*	-	*pullus*
					KS 8(59)-1, (62)-2, (88)-2, (104)-2, (107)-1	*lesteri*	-	*lesteri*
Gyeonggi–do (Ganghwa)	37°45'N 126°29'E	1-IX-2001	H.C. Kim	Progeny reared from adults collected at cowshed near rice paddy	KS 9(1)-1, (2)-1, (3)-1,(4)-1, (5)-1, (9)-1, (10)-1, (11)-1, (12)-1	*sinensis*	sinensis	-
					KS 9(6)-1, (7)-1	*pullus*	-	*pullus*
					KS 9(8)-1	*yatsushiroensis*	-	unknown species
Gyeonggi–do (Incheon)	37°28'N 126°38'E	8-IX-2001	H.C. Kim	Progeny reared from adults collected at cowshed in village	KS 10(1)-1, (2)-1, (3)-1, (6)-1	*sinensis*	*sinensis*	-
					KS 10(4)-1, (5)-1	*pullus*	-	*pullus*
CHINA								
Jiangsu (Wuxi)	31°35'N 120°18'N	?-XI-1988	G.H. Song	-	CH 006F, 008F	*anthropophagus*	-	*lesteri*
Guang–dong (Guang–zhou)	23°07'N 113°15'E	23-IV-2002	J. P. Liao	Rice paddy, pH 8.93, turbid water 31.7°C, salinity 0.52 ppt, conductivity 1.06mS	CH 25L, 25-1	*sinensis*	-	*sinensis*

.....continued on the next page

TABLE 1 (continued)

Province (Locality)	Coordinates	Date	Collector	Habitat	Collection No.	Morphology	PCR	ITS2 Sequence
PHILIPPINES								
Luzon: Laguna ('Tanque', Calauan)	14°08'N 121°18'E	29-VII-2002	L.M. Rueda	Hill ditch, pH 6.79, clear water 28.8^{0}C, salinity 0.07 ppt, conductivity 0.07 mS	PH 9-6, 9-11	*lesteri*	-	*lesteri*

Source of Specimens (Table 1). Adults were obtained from individually reared larvae. There were 5 collection sites in South Korea, 1 in the Philippines and 2 in China. Standard methods used by the Walter Reed Biosystematics Unit (Pecor & Gaffigan 1997) were followed for collection and progeny rearing. These collections resulted in 92 progeny broods and an additional 5 adults reared from larvae, and one field–collected larva. A representative from each progeny brood and the 5 individually reared larvae were evaluated. Progeny broods were preserved both for morphological study (paper pinpointed adults with associated larval and pupal exuviae in 80% ethyl alcohol for slide mounting), and for molecular study (frozen at –80 °C). The frozen specimens were later placed in 100% ethyl alcohol before DNA extraction.

We attempted to collect *An. lesteri* specimens from the type locality, Sta. Mesa, Manila, Luzon, but found that it is now an urban area totally lacking typical larval habitats. Baisas & Hu (1936) stated that many cotypes of *An. lesteri* were collected from Calauan, Laguna, Luzon, about 50 km from Santa Mesa, Manila. This locality remains rural and we were able to collect specimens from Calauan for the present study.

Mosquito Identification. Adult specimens were identified using the characters in Tanaka *et al*. (1979). Voucher specimens are deposited in the National Museum of Natural History, Smithsonian Institution, Washington, D.C.

DNA Isolation and Sequencing. DNA was isolated from individual adult mosquitoes by phenol–chloroform extraction as described in Wilkerson *et al*. (1993). Direct sequencing was carried out as described in Wilkerson *et al.* (In press) using their primers. The beginning and end of the rDNA ITS2 was estimated as in Cornel *et al*. (1996).

Results and discussion

Morphological identification of adult female mosquitoes from Korea (Tanaka *et al.* 1979) resulted in identification of *An. sinensis*, *An. pullus*, *An. yatsushiroensis*, *An. lesteri*, and several intermediates between *An. pullus* and *An. yatsushiroensis* (Table 1). Our sequence

of rDNA ITS2 (Fig. 1) revealed four discrete ITS2 sequences: 1) *An. sinensis* Korea, n = 38 (GenBank Acc. No. AY375464) and *An. sinensis* China, n = 4 (GenBank Acc. No. AY375465); 2) unknown species Korea, n = 5 (GenBank Acc. No. AY375466); 3) *An. lesteri* China (locally identified as *An. anthropophagus*), n = 2 (GenBank Acc. No. AY375467), *An. lesteri* Korea, n = 5 (GenBank Acc. No. AY375468), and *An. lesteri* Philippines, n = 2 (GenBank Acc. No. AY375469); and, 4) *An. pullus* (morphologically identified as *An. yatsushiroensis*) Korea, n = 22 (GenBank Acc. No. AY375470) and *An. pullus* Korea, n = 10 (GenBank Acc. No. 375471)). The above, and existing GenBank sequence for *An. sinensis*, *An.* "*lesteri*", and *An.* "*anthropophagus*" are compared and discussed below (summarized in Fig. 1).

Anopheles lesteri. To verify that the name *An. lesteri* in Korea is correctly applied we collected this species from near the type locality (see "Source of Specimens" above) in the Philippines (Laguna, Calauan, "Tanque") (Table 1). The best way to infer conspecifity of populations across large geographic areas is to compare with specimens from the type locality. We were able to do this and found a very close match (a difference of only 2 transitions; 99.3% homology) supporting the assumption that populations in Korea and at the type locality are conspecific. Also, a search of GenBank resulted in matching sequences under the name *An. anthropophagus* (Acc. Nos. AF384172, AJ004941, AF543860). We also obtained dried specimens from Jiangsu, Wuxi, China, identified locally as *An. anthropophagus*, that also match our *An. lesteri* sequence. We conclude that specimens currently identified as *An. anthropophagus*, considered an important malaria vector in China, is actually *An. lesteri*, not a separate species. This same species is also found in Korea. We therefore place *An. anthropophagus* in synonymy with its senior synonym, *An. lesteri*.

Anopheles pullus* and *An. yatsushiroensis. Specimens from Korea morphologically identified as these two species, or with a query, were found to have identical sequence. This supports the conclusion of Shin & Hong (2001) that *An. pullus* and *An. yatsushiroensis* are two names applied to the same variable species in Korea. A search of GenBank resulted in several submissions, all under the name *An. yatsushiroensis*, that also match our sequences (Acc. Nos. AY170923-5, AY186791, AY186792, AF146749). Since the type locality of *An. yatsushiroensis* is in Japan there remains the possibility that it is a separate species not found in Korea. However, we retain the synonymy of *An. yatsushiroensis* under *An. pullus* pending comparison to topotypic specimens.

FIGURE 1. Ribosomal DNA ITS2 sequence for potential malaria vectors belonging to *Anopheles* (*Anopheles*) Hyrcanus Group from Korea, China, Japan and the Philippines. See Table 2 and text for sequence summaries and discussion. The following GenBank accession numbers correspond to the label numbers at the 5' end of the sequence: 1) AY375464; 2) AY375465; 3) AJ004942; 4) AY375466; 5) AF384172, AJ004941 and AF543860; 6) AY375467; 7) AY187728; 8) AY375468; 9) AY375469; 10) AY375470; 11) AY375471. The number of individuals sequenced, of those presented here for the first time, appears in parentheses


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1)  sinensis Korea (38)      1 AATTAGAAG---TGGAAACGTGGACTTACGCAGTGATTGGTGCTGGTCACCACGTCAC-G
2)  sinensis China (4)       1 .........---.............................................-.
3)  sinensis GenBank         1 .........---.............................................-.
4)  Unknown sp. Korea (3)    1 .........--T........CT.........T...................C....G.-A
5)  "lesteri" GenBank        1 .........--T........CT.........T...................C....G.-A
6)  "anthrop." China (2)     1 .........TTTG.C....AGAA.AC.....................................-A
7)  "anthrop." GenBank       1 .........TTTG.C....AGAA.AC.....................................-A
8)  lesteri Korea (5)        1 .........TTTG.C....AGAA.AC.....................................-A
9)  lesteri Philippines(2)   1 .........TTTG.C....AGAA.AC.....................................-.
10) "yatsu." Korea (22)      1 .T..G..T.--T.AT.C..AATA.AC.....C...........C.............TT.
11) pullus Korea (10)        1 .T..G..T.--T.AT.C..AATA.AC.....C...........C.............TT.

sinensis Korea (38)         57 GTCGTGAATAATGATGTAAGATGGGGTCTCGTCGACCCGCTTGCATTTAAAACGT-GTGT
sinensis China (4)          57 ..........................................................-....
sinensis GenBank            57 ..........................................................-....
Unknown sp. Korea (4)       58 ........A.............TT...........T.....................---...
"lesteri" GenBank           58 ........A.............TT...........T.....................---...
"anthrop." China (2)        60 ......C....A.G.......-.A.A.........-T.........C.CGG.AC.--...
"anthrop." GenBank          60 ......C....A.G.......-.A.A.........-T.........C.CGG.AC.--...
lesteri Korea (5)           60 ......C....A.G.......-.A.A.........-T.........C.CGG.AC.--...
lesteri Philippines (2)     60 ......C....A.G.......-.A.A.........-T.........C.CGG.AC.--...
"yatsu." Korea (22)         59 ......C..............-......A..C..G............G..G.AACGCG..
pullus Korea (10)           59 ......C..............-......A..C..G............G..G.AACGCG..

sinensis Korea (38)        116 GTTGGAAAAACCGCTAAG-AAGGCA----GACAAGTAGAAAGGGCTGTGTTCCCGCGGAC
sinensis China (4)         116 ..................-..........-.....................................
sinensis GenBank           116 ..................-..........-.....................................
Unknown sp. Korea (4)      115 ....--.T......CT..---.A.....-.....................................
"lesteri" GenBank          115 ....--.T......CT..---.A.....-.....................................
"anthrop." China (2)       116 ....-...GG....G...AC..A..AGTA.TA..C-..C.GCA.A.............ATT
"anthrop." GenBank         116 ....-...GG....G...AC..A..AGTA.TA..C-..C.GCA.A.............ATT
lesteri Korea (5)          116 ....-...GG....G...AC..A..AGTA.TA..C-..C.GCA.A.............ATT
lesteri Philippines (2)    116 ....-...GG....G...AC..A..AGTA.TA..C-..C.GCA.A.............ATT
"yatsu." Korea (22)        118 ...-----..G..-----------ATGATATCA..C...T.GTA..-........T.....-
pullus Korea (10)          118 ...-----..G..-----------ATGATATCA..C...T.GTA..-........T.....-

sinensis Korea (38)        171 GGCGGAGGAAGTATATTGAGCAGGCGCGTCCTTTGCTATGTGTAGGTATGGAACAGGTGT
sinensis China (4)         171 ..........................................................
sinensis GenBank           171 ..........................................................
Unknown sp. Korea (4)      166 ..............TC.....................CG.....................
"lesteri" GenBank          166 ..............TC.....................CG.....................
"anthrop." China (2)       174 ......A.-----.TC.AG.........C...GA-.GC.....GTAG.....G.......
"anthrop." GenBank         174 ......A.-----.TC.AG.........C...GA-.GC.....GTAG.....G.......
lesteri Korea (5)          174 ......A.-----.TC.AG.........C...GA-.GC.....GTAG.....G.......
lesteri Philippines (2)    174 ......A.-----.TC.AG.........C...GA-.GC.....GTAG.....G.......
"yatsu." Korea (22)        161 --.........A..-CCCAT..........T..G.TGC-......-TAGG....-.......
pullus Korea (10)          161 --.........A..-CCCAT..........T..G.TGC-......-TAGG....-.......

sinensis Korea (38)        231 CTTCCTCTTCTATTTTAATTTTTTTTAAAATTGAGGTAAGGC-ACCAACACGTTTCGATC
sinensis China (4)         231 ..........................................-...................
sinensis GenBank           231 ..........................................-.............T...
Unknown sp. Korea (4)      226 ..............-.......-.....................-.....-.....-.....
"lesteri" GenBank          226 ..............-.......-.....................-.....-.....-.....
"anthrop." China (2)       228 .......A........T.....---A..............ATTT.....---.....-T..
"anthrop." GenBank         228 .......A........T.....---A..............ATTT.....---.....-T..
lesteri Korea (5)          228 .......A........T.....---A..............ATTT.....---.....-T..
lesteri Philippines (2)    228 .......A........T.....---A..............A.TT.....---.....-T..
"yatsu." Korea (22)        215 ..................-.....A.....-..............ATT.....---.....T-...
pullus Korea (10)          215 ..................-.....A.....-..............ATT.....---.....T-...

sinensis Korea (38)        290 GAGATAGCATG-TACGCA--AATAATCATTGTATGGAACCCCTGAACAACGGAACACTTA
sinensis China (4)         290 ..........-.....--.....................................
sinensis GenBank           290 ..........-.....--.....................................
Unknown sp. Korea (4)      282 ....CG.....-.....T--..........A.....................
"lesteri" GenBank          282 ....CG.....-.....T--..........A.....................
"anthrop." China (2)       282 .......TGGAA.GG..TGC..G.GA.TGGAATC....G--T.............CT
"anthrop." GenBank         282 .......TGGAA.GG..TGC..G.GA.TGGAATC....G--T.............CT
lesteri Korea (5)          282 .......TGGAA.GG..TGC..G.GA.TGGAATC....G--T.............CT
lesteri Philippines (2)    282 .......TGGAA.GG..TGC..G.GA.TGGAATC....G--T.............CT
"yatsu." Korea (22)        268 ....C..TGG--.GG.ACGC..CG.ATTGGAAC.ATTGAAA.CAG....G.......-..
pullus Korea (10)          268 ....C..TGG--.GG.ACGC..CG.ATTGGAAC.ATTGAAA.CAG....G.......-..

sinensis Korea (38)        347 TGGCACTAGAG-AACACTACCCAGATTTGTTATGTTAGCGGGCTGGACAACAATAATACA
sinensis China (4)         347 ..........-.....................................................
sinensis GenBank           347 ..........-.....................................................
Unknown sp. Korea (4)      339 ..........-.....................------GCAT....-..T.CT-..CG.....
"lesteri" GenBank          339 ..........-.....................------GCAT....-..T.CT-..CG.....
"anthrop." China (2)       340 ATTAG.A-----.............A.CCG.GCA.A.CGACTGGAAGATG...GTTCTAC
"anthrop." GenBank         340 ATTAG.A-----.............A.CCG.GCA.A.CGACTGGAAGATG...GTTCTAC
lesteri Korea (5)          340 ATTAG.A-----.............A.CCG.GCA.A.CGACTGGAAGATG...GTTCTAC
lesteri Philippines (2)    340 ATTAG.A-----.............A.CCG.GCAGA.CGACTGGAAGATG...GTTCTAC
"yatsu." Korea (22)        325 AA-.G.ACC.TA.............-..GT.G.GAGAGCG.ACGA.C.TCG...GCC.G.G
pullus Korea (10)          325 AA-.G.ACC.TA.............-..GT.G.GAGAGCG.ACGA.C.TCG...GCC.G.G

sinensis Korea (38)        406 GCAAACAA---AGGTCAAA--CAATTATCACTCC--AAGAGTGAGGCCACTCGGTGGTCAG
sinensis China (4)         406 .........----------.--.............-.....................
sinensis GenBank           406 .........----------.--.............-.....................
Unknown sp. Korea (4)      390 ........CAA....------.............---.C.....................T
"lesteri" GenBank          390 ........CAA....------.............---.C.....................T
"anthrop." China (2)       395 CTG.GA.TC--------------.........T---.C.....................A
"anthrop." GenBank         395 CTG.GA.TC--------------.........T---.C.....................A
lesteri Korea (5)          395 CTG.GA.TC--------------.........T---.C.....................A
lesteri Philippines (2)    395 CTG.GA.TC--------------.........T---.C.....................A
"yatsu." Korea (22)        383 AA.GCGTGGCATACAATTCAT..........TACA..C.TA.T..GAC.CGTAC.....A
pullus Korea (10)          383 AA.GCGTGGCATACAATTCAT..........TACA..C.TA.T..GAC.CGTAC.....A
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Anopheles sinensis. To verify the identity of *An. sinensis* in Korea we sequenced specimens collected by us from Korea, from the type locality (Canton, China, n = 4), and compared our sequence to published (Min *et al.* 2002), and GenBank sequence (Acc. No. AJ004942). As can be seen in Fig. 1 there is virtual identity among the sequences. Only a single base pair difference was noted in the GenBank sequence.

***Anopheles* species unknown.** Among the 102 progeny broods used in this study we found 3 different, yet internally consistent, families. The same sequence was reported by Ma *et al*. (2000) and Min *et al*. (2002), and in GenBank Acc. No. AY187728. These authors identified the source species as *An. lesteri*. Morphologically (see below), in our sample there was 1 each identified as *An. pullus*, *An. yatsushiroensis* and *An. sinensis*, of this genetically distinct species. Since some workers consider this species to be *An. lesteri*, while true *An. lesteri* is called *An. anthropophagus*, there is obvious confusion regarding the identity of these species.

Genetic distances and sequence characteristics. Genetic distances (Table 2) between pairs of putative species ranged from 7.6% (*An. sinensis*/"unknown species") to 34.1% (*An. pullus*/*An. sinensis*). *An. sinensis* and the unknown taxon appear to be more related to each other in comparison to the other species, but the difference is still well above the divergence seen in some species complexes such as in the *An. gambiae* complex (0.4–1.6%) (Paskewitz *et al.* 1993). The ITS2 fragment lengths ranged from 438 bp (*An. lesteri*) to 459 bp (*An. sinensis*) (Table 2), about average among *Anopheles* ITS2 lengths (Wilkerson *et al.* In press). GC content was quite uniform, ranging from 45.3% (*An. sinensis*) to 47.3% (*An. lesteri*, Philippines) (Table 2), also usual in genus *Anopheles* (Beebe *et al.* 1999; Fritz 1998; Marinucci *et al.* 1999; Paskewitz *et al.* 1993; Wilkerson *et al.* In press).

Morphological identifications (Table 1). Using the adult female characters in Tanaka *et al*. (1979) it was possible to correctly identify 100% (n = 55) of the molecularly identified *An. sinensis*. However, one of the "unknown species" families also keyed to *An. sinensis*. As mentioned above, females keying to *An. pullus* and *An. pullus/yatsushiroensis* (n = 11) or *An. yatsushiroensis* (n = 16) all had *An. pullus* sequence. However, 3 others, 1 each keying to *An. yatsushiroensis*, *An. pullus* and *An. sinensis* were genetically the "unknown species". All 5 *An. lesteri* families were correctly keyed.

The above findings indicate a need for further study into the actual genetic identities of the species that have been credited with malaria transmission in Korea and in the region. Our conclusions could be crucial for understanding which species are actually responsible for malaria transmission and what literature information, summarized in the introduction, can correctly be applied to these species.

To summarize: 1) Using comparison with topotypic specimens it is known that *An. lesteri* occurs in Korea; 2) *An. anthropophagus* in China is a synonym of *An. lesteri*; 3) As previously reported, *An. yatsushiroensis* and *An. pullus* are conspecific in Korea; 4) *An. yatsushiroensis* is retained in synonymy with *An. pullus* pending comparison with speci-

mens from its type locality in Japan; 5) Species reported to be *An. lesteri* in China represent an unknown species which is also found in Korea; 6) Morphological characters used to identify the species studied here are not 100% reliable.

TABLE 2. Length, GC content and genetic distance based on rDNA ITS2 of some member species of the Hyrcanus Group from China, Philippines and South Korea. See text and Figure 1 for GenBank accession numbers. The sequence numbers match those in Fig. 1.

Species (location)	Length (bp)	GC content (%)	1	2	3	4	5	6	7	8	9	10	11
1 *sinensis* Korea	459	45.3	—										
2 *sinensis* (Guangzhou, China)	459	45.3	0	—									
3 *sinensis* (GenBank)	459	45.1	0.22	0.22	—								
4 Unknown sp. (Korea)	441	46.7	7.57	7.57	7.79	—							
5 *lesteri* (GenBank)	441	46.7	7.57	7.57	7.79	0	—						
6 *anthropophagus* (Jiangsu, China)	438	46.6	28.92	28.92	28.92	28.10	28.10	—					
7 *anthropophagus* (GenBank)	438	46.6	28.92	28.92	28.92	28.10	28.10	0	—				
8 *lesteri* (Korea)	438	46.6	28.92	28.92	28.92	28.10	28.10	0	0	—			
9 *lesteri* (Philippines)	438	47.3	28.68	28.68	28.68	28.12	28.12	0.69	0.69	0.69	—		
10 *yatsushiroensis* (Korea)	443	46.5	34.37	34.37	34.36	33.60	33.60	30.89	30.89	30.89	30.68	—	
11 *pullus* (Korea)	443	46.5	34.37	34.37	34.36	33.60	33.60	30.89	30.89	30.89	30.68	0	—

Acknowledgements

Many thanks to Yvonne Linton and Ralph Harbach for very helpful reviews of the manuscript. This research was performed under a Memorandum of Understanding between the Walter Reed Army Institute of Research and the Smithsonian Institution, with institutional support provided by both organizations. The material presented here reflects the views of the authors and should not be construed to represent those of the Department of the Army or the Department of Defense. We are grateful to Jing-Ping Liao for help in collecting mosquito specimens from Guang-zhou, China.

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