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A newly recognized species in the *Anopheles* Hyrcanus Group and molecular identification of related species from the Republic of South Korea (Diptera: Culicidae)

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Abstract

We report here a previously unrecognized mosquito species from the Republic of South Korea that is closely related to *Anopheles* (*Anopheles*) *sinensis*. We also present an rDNA ITS2-based method for identification of this and the other morphologically similar *Anopheles* from the country. The PCR assay is a multiplex of seven primers in a single reaction that unambiguously identifies all included species. The other species include *An. sinensis*, *An. lesteri*, *An. pullus*, and a previously reported unknown *Anopheles*. Based on the ITS2 sequence, the two unnamed species have genetic distances from *An. sinensis* of 9.1% (unknown 1) and 10.7% (unknown 2), and are 10.6% different from each other. Adult females of *An. sinensis* and unknown 2 are morphologically indistinguishable, while unknown 1, *An. lesteri* and *An. pullus* can usually be separated from each other and from *An. sinensis* using available keys and descriptions. This PCR identification tool offers vector biologists and malaria epidemiologists a means to identify the potential vectors of malaria parasites in South Korea.

Key words: malaria, *Anopheles*, PCR identification, Hyrcanus Group, South Korea

Introduction

Recent malaria transmission in the Republic of South Korea has resulted in an increased effort to incriminate the vector or vectors of this disease (reviewed in Wilkerson et al. 2003). Reported in the above study was the existence of an unknown species ("unknown

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1”) related to *Anopheles (Anopheles) sinensis* Wiedemann in South Korea, and a description of the rDNA Internal Transcribed Spacer 2 (ITS2) sequence for those two species plus two other members of the Hyrcanus Group (Harbach, 2004) in South Korea that could be confused with *An. sinensis*; *An. pullus* Yamada and *An. lesteri* Baisas & Hu. Wilkerson et al. (2003) also reconfirmed that *An. pullus* (the senior synonym) and *An. yatsushiroensis* Xu & Feng were genetically identical in Korea and showed that *An. anthropophagus* Xu & Feng is a junior synonym of *An. lesteri*. Continued collection of specimens, rearing of progeny broods, and sequencing of representative specimens resulted in the recognition of another unknown species (“unknown 2”) related to *An. sinensis*. The two “unknown” species have been formally described and named (Rueda, 2005). Rueda (2005) provides a discussion of distinguishing characters for the species which usually will serve to separate all but *An. sinensis* and unknown 2. Below, we provide the ITS2 sequence for *An*. unknown 2 and give a PCR method for identification of five of the Hyrcanus Group species found in South Korea. *An. sineroides* Yamada was not included since it is easily distinguishable morphologically.

Materials and Methods

Source of specimens. PCR identifications were carried out on the same specimens reported in Wilkerson et al. (2003). An additional 50 specimens identified morphologically as belonging to the Hyrcanus Group (Tanaka et al. 1979, Lee 1998) were also tested. These include examples from three progeny broods of the previously unrecognized *An.* “unknown number 2” (Table 1). Morphological and DNA vouchers of the above are deposited in the Smithsonian Institution, National Museum of Natural History. A synoptic collection is kept by one of us (Hueng-Chul Kim) in his laboratory.

Morphological identification. Adult female specimens included in this study were first identified morphologically using either Tanaka et al. (1979) or Lee (1998).

DNA isolation and sequencing. DNA was isolated from individual adult mosquitoes by phenol-chloroform extraction as described in Wilkerson et al. (1993). The rDNA ITS2 was amplified using conserved sequence found in the 5.8S subunit, ITS2 forward (5’-TGTGAACTGCAGGACACATGAA–3’) and in the 28S subunit, ITS2 reverse (5’-ATGCTTAAATTTAGGGGGTAGTC–3’) (Cornel et al. 1996). PCR products were directly sequenced using Big Dye 3.0 (Applied Biosystems Inc. –ABI) with an ABI 3100 sequencer (ABI). The sequence was then edited and analyzed using Sequencher (v4.2, AB). Sequence of *An. sinensis*, *An. lesteri*, *An. pullus*, and *An*. unknown number 1 are those of Wilkerson et al. (2003). GenBank accession numbers for the above are in Wilkerson et al. (2003). The accession number for the *An*. unknown 2 reported here is AY753740. Alignment was carried out with Clustal W (v. 1.82), with final alignments done manually. The boundaries of the rDNA ITS2 were estimated following Cornel et al. (1996). Sequences shown in Fig. 1 are of the ITS2 only. Complete amplicons have an additional

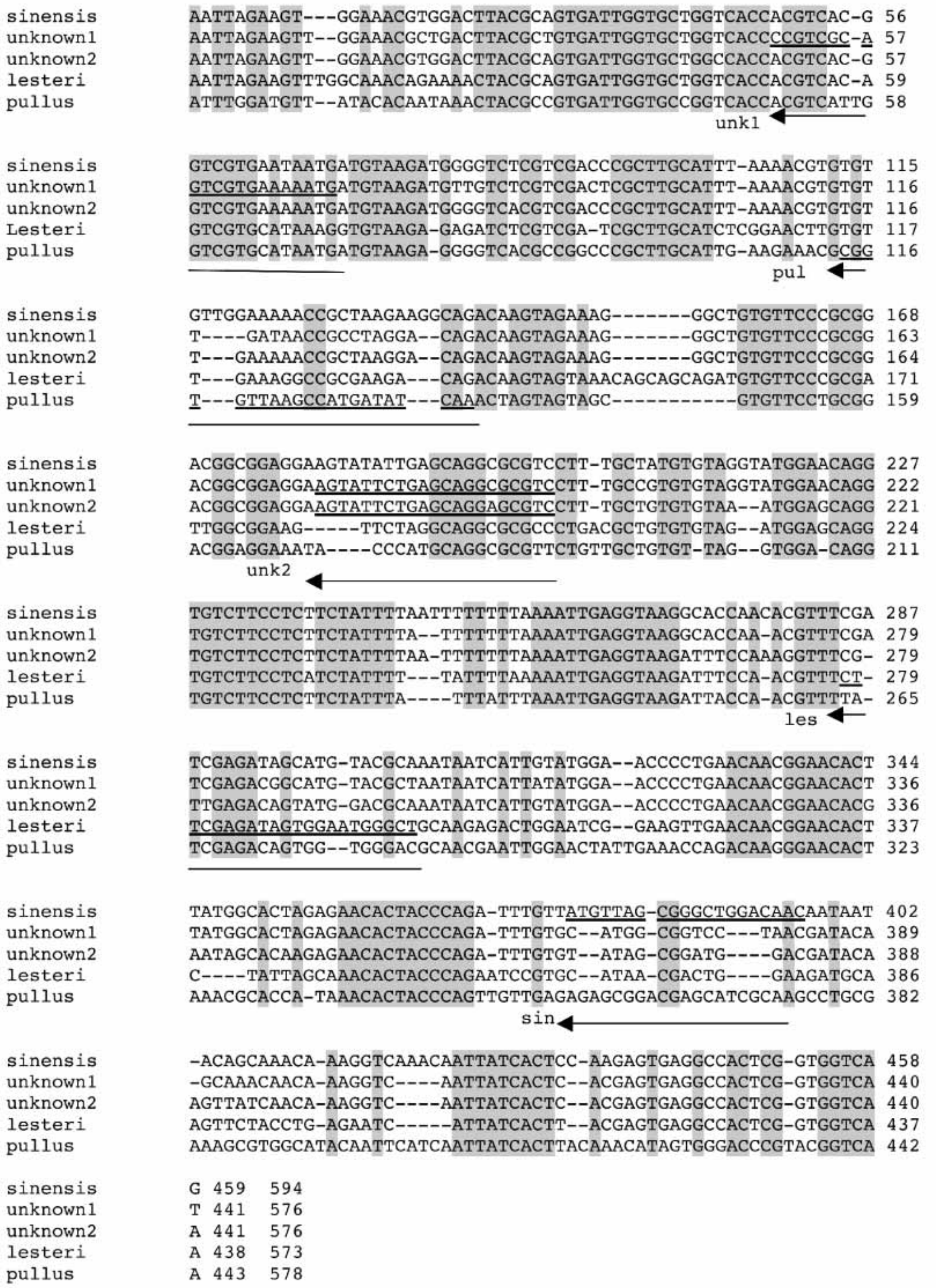
92 bases from the 5.8S subunit and 43 bases from the 28S subunit (total ITS2 and flanking sequence given in Fig. 1).

TABLE 1. Summary of collection localities for species belonging to the *Anopheles* Hyrcanus Group reported here for the first time.

Province (Locality)	**Coordinates**	**Date**	**Collector**	**Habitat**	**Country prefix, Collection No., (progeny brood no.)**	**Species identified by PCR and/or sequence**
Korea						
Ogeum-ri, Paju-Paju	37º 40’ 00” N 126º 54’ 00” E	July 22, 2002	W.J. Lee	cow shed	KS2-3(1,5)	*sinensis*
					KS2-3(2-4,6,7)	*pullus*
Jeonju	35º 49’ 19” N 127º 8’ 56” E	July 26, 2002	W.J. Lee	cow shed	KS2-4(1-5)	*sinensis*
Camp Casey	37º 54’ 15” N 127º 05’ 23” E	July 26, 2002	T.A. Klein	cow shed	KS2-5(1)	*lesteri*
Cheongpyeong	36º 40’N 128º 22’E	August 2, 2002	H.-C. Kim	cow shed	KS2-6(1,3,5)	*sinensis*
					KS2-6(2)	Unknown 2
Kunsan	35º 58’ 43” N 126º 47’ 41” E	August 2, 2002	T.A. Klein	cow shed	KS2-7(1-6)	*sinensis*
Majeong-ri, Paju	37º 50’N 126º 49’E	June 5, 2004	W.J. Lee	cow shed	KS4-2(1), KS4-2(17)	Unknown 2
					KS4-2(3,7,9,11,16,19-21,24-27,31,34-35,37-38,42-43,45-48,50,52)	*sinensis*

Primer design and multiplex PCR mixture (Table 2). Species-specific reverse primers were designed based on species-specific sequence in the ITS2. When these primers are combined with universal ITS2 forward primer, they produce PCR products of different lengths for each species. This makes it possible to identify the five very similar species of the Hyrcanus Group in South Korea in a single reaction. The multiplex reaction also contains the universal ITS2 reverse primer so that if the DNA of a non-target species is tested, or no species-specific primer is included, a band representing the entire ITS2 will result (Fig. 2, lane 11). This serves as an internal control for the presence of amplifiable DNA.

Distance analysis. A Neighbor Joining (NJ) analysis as implemented in PAUP* v.4 (Swofford 1998) was carried out using uncorrected “p” setting, with gaps treated as missing data. A distance matrix is presented in Table 3.



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sinensis     AATTAGAAGT---GGAAACGTGGACTTACGCAGTGATTGGTGCTGGTCACCACGTCAC-G 56
unknown1     AATTAGAAGTT--GGAAACGCTGACTTACGCTGTGATTGGTGCTGGTCACCCCGTCGC-A 57
unknown2     AATTAGAAGTT--GGAAACGTGGACTTACGCAGTGATTGGTGCTGGCCACCACGTCAC-G 57
lesteri      AATTAGAAGTTTGGCAAACAGAAAACTACGCAGTGATTGGTGCTGGTCACCACGTCAC-A 59
pullus       ATTTGGATGTT--ATACACAATAAACTACGCCGTGATTGGTGCCGGTCACCACGTCATTG 58
                                                            unk1

sinensis     GTCGTGAATAATGATGTAAGATGGGGTCTCGTCGACCCGCTTGCATTT-AAAACGTGTGT 115
unknown1     GTCGTGAAAAATGATGTAAGATGTTGTCTCGTCGACTCGCTTGCATTT-AAAACGTGTGT 116
unknown2     GTCGTGAAAAATGATGTAAGATGGGGTCACGTCGACCCGCTTGCATTT-AAAACGTGTGT 116
Lesteri      GTCGTGCATAAAGGTGTAAGA-GAGATCTCGTCGA-TCGCTTGCATCTCGGAACTTGTGT 117
pullus       GTCGTGCATAATGATGTAAGA-GGGGTCACGCCGGCCCGCTTGCATTG-AAGAAACGCGG 116
                                                                  pul

sinensis     GTTGGAAAAACCGCTAAGAAGGCAGACAAGTAGAAAG-------GGCTGTGTTCCCGCGG 168
unknown1     T----GATAACCGCCTAGGA--CAGACAAGTAGAAAG-------GGCTGTGTTCCCGCGG 163
unknown2     T---GAAAAACCGCTAAGGA--CAGACAAGTAGAAAG-------GGCTGTGTTCCCGCGG 164
lesteri      T---GAAAGGCCGCGAAGA---CAGACAAGTAGTAAACAGCAGCAGATGTGTTCCCGCGA 171
pullus       T---GTTAAGCCATGATAT---CAAACTAGTAGTAGC-----------GTGTTCCTGCGG 159

sinensis     ACGGCGGAGGAAGTATATTGAGCAGGCGCGTCCTT-TGCTATGTGTAGGTATGGAACAGG 227
unknown1     ACGGCGGAGGAAGTATTCTGAGCAGGCGCGTCCTT-TGCCGTGTGTAGGTATGGAACAGG 222
unknown2     ACGGCGGAGGAAGTATTCTGAGCAGGAGCGTCCTT-TGCTGTGTGTAA--ATGGAGCAGG 221
lesteri      TTGGCGGAAG-----TTCTAGGCAGGCGCGCCCTGACGCTGTGTGTAG--ATGGAGCAGG 224
pullus       ACGGAGGAAATA----CCCATGCAGGCGCGTTCTGTTGCTGTGT-TAG--GTGGA-CAGG 211
                 unk2

sinensis     TGTCTTCCTCTTCTATTTTAATTTTTTTAAAATTGAGGTAAGGCACCAACACGTTTCGA 287
unknown1     TGTCTTCCTCTTCTATTTTA--TTTTTTAAAATTGAGGTAAGGCACCAA-ACGTTTCGA 279
unknown2     TGTCTTCCTCTTCTATTTTAA-TTTTTTAAAATTGAGGTAAGATTTCCAAAGGTTTCG- 279
lesteri      TGTCTTCCTCATCTATTTT---TATTTTAAAAATTGAGGTAAGATTTCCA-ACGTTTCT- 279
pullus       TGTCTTCCTCTTCTATTTA----TTTATTTAAATTGAGGTAAGATTACCA-ACGTTTTA- 265
                                                                 les

sinensis     TCGAGATAGCATG-TACGCAAATAATCATTGTATGGA--ACCCCTGAACAACGGAACACT 344
unknown1     TCGAGACGGCATG-TACGCTAATAATCATTATATGGA--ACCCCTGAACAACGGAACACT 336
unknown2     TTGAGACAGTATG-GACGCAAATAATCATTGTATGGA--ACCCCTGAACAACGGAACACG 336
lesteri      TCGAGATAGTGGAATGGGCTGCAAGAGACTGGAATCG--GAAGTTGAACAACGGAACACT 337
pullus       TCGAGACAGTGG--TGGGACGCAACGAATTGGAACTATTGAAACCAGACAAGGAACACT 323

sinensis     TATGGCACTAGAGAACACTACCCAGA-TTTGTTATGTTAG-CGGGCTGGACAACAATAAT 402
unknown1     TATGGCACTAGAGAACACTACCCAGA-TTTGTGC--ATGG-CGGTCC---TAACGATACA 389
unknown2     AATAGCACAAGAGAACACTACCCAGA-TTTGTGT--ATAG-CGGATG----GACGATACA 388
lesteri      C----TATTAGCAAACACTACCCAGAATCCGTGC--ATAA-CGACTG----GAAGATGCA 386
pullus       AAACGCACCA-TAAACACTACCCAGTTGTTGAGAGAGCGGACGAGCATCGCAAGCCTGCG 382
                                       sin

sinensis     -ACAGCAAACA-AAGGTCAAACAATTATCACTCC-AAGAGTGAGGCCACTCG-GTGGTCA 458
unknown1     -GCAAACAACA-AAGGTC----AATTATCACTC--ACGAGTGAGGCCACTCG-GTGGTCA 440
unknown2     AGTTATCAACA-AAGGTC----AATTATCACTC--ACGAGTGAGGCCACTCG-GTGGTCA 440
lesteri      AGTTCTACCTG-AGAATC-----ATTATCACTT--ACGAGTGAGGCCACTCG-GTGGTCA 437
pullus       AAAGCGTGGCATACAATTCATCAATTATCACTTACAAACATAGTGGGACCCGTACGGTCA 442

sinensis     G 459  594
unknown1     T 441  576
unknown2     A 441  576
lesteri      A 438  573
pullus       A 443  578
```

FIGURE 1. Ribosomal DNA ITS2 sequence for five *Anopheles* Hyrcanus Group species from the Republic of South Korea. Bases in the gray areas are common to all species. Species-specific primers and direction of amplification are indicated by arrows: sin = *An. sinensis*, unk1 = *An*. unknown 1, pul = *An. pullus*, unk2 = *An*. unknown 2, les = *An. lesteri*. Two numbers are given at the end of the figure: 1) ITS2 length, and 2) total length of amplified fragment, which includes 135 bases from the flanking regions.

TABLE 2. Species-specific primers used to identify five species in Hyrcanus Group from South Korea. Reactions were a multiplex of these five primers and the universal ITS2F and ITS2R primers. Diagnostic band size(s) are on the right. *The *An. sinensis* primer was first reported by Ma et al. (1998).

Species	Species-specific primers (name: sequence)	Diagnostic bands	
An. sinensis	sin: 5'– GTTGTCCAGCCCGCTAACAT –3'*	488bp	
An. lesteri	les: 5'– AGCCCATTCCACTATCTCGAAG –3'	391bp	
An. pullus	pul: 5'– TTGATATCATGGCTTAACACCG –3'	227bp	
An. unknown 1	unk1: 5'– CATTTTTCACGACTGCGACGG –3'	287bp	162bp
An. unknown 2	unk2: 5' – GACGCTCCTGCTCAGAATACT – 3'	288bp	

TABLE 3. Neighbor Joining distance matrix, using uncorrected "p" with gaps treated as missing data.

		1	2	3	4	5
1	*sinensis*	-				
2	unknown 1	0.09106	-			
3	unknown 2	0.10698	0.10560	-		
4	*lesteri*	0.26778	0.27309	0.23632	-	
5	*pullus*	0.33152	0.33957	0.31407	0.29896	-

Mosquito PCR identification. Adult mosquitoes used here were preserved either in 100% ethanol or in some cases originated from dry pin-pointed museum specimens. DNA was extracted by boiling a single mosquito leg in 20 µl of TE for 10 minutes and then the DNA was spun at high speed (10,000g) for 10 minutes. One µl of template supernatant was added to a 25 µl PCR reaction containing 1X buffer (10mM Tris-HCl, pH8.3, 50mM KCl); 2.5mM MgCl2; 200µM dNTP; 6pmol of each ITS2 forward, reverse and unknown 1 (unk1) primers, and 4pmol each of *An. sinensis* (sin), *An. lesteri* (les), *An. pullus* (pul), and unknown 2 (unk2) primers; and 0.5 U Taq polymerase (ABI). The primer sequences are listed in Table 2 and their locations in the ITS2 are shown in Fig. 1. PCR reactions were performed using the following parameters: 95°C for 2 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, final extension at 72°C for 7 min. PCR products were visualized on a 2% agarose gel containing ethidium bromide.

Results and Discussion

We present here discovery of a second unknown *Anopheles* Hyrcanus Group species from South Korea that is closely related to *An. sinensis* and is also related to a previously discovered unknown *Anopheles* from the same region. We also provide an rDNA ITS2-based method for identification of all five morphologically similar species belonging to the Hyrcanus Group in South Korea.

We raised three progeny broods of the *An.* unknown 2 and sequenced the ITS2 from at least one individual of each. The ITS2 sequences were identical in all individuals sequenced, showing no indels or muations. The ITS2 of the *An*. unknown 2 is 441 bases long with a total amplicon, including flanking sequence, of 576, the same as *An*. unknown 1. It has a GC content of 45%, similar to *An*. unknown 1 (47%), and the other related species: *An. sinensis* 45%, *An. lesteri* 47%, *An. pullus* 47% (Wilkerson et al. 2003). Note that in the figure caption in Wilkerson et al. (2003) numbers 5 and 7 are reversed. The table itself and the description in the text are correct.

A pairwise comparison of the five species treated here (Table 3) shows that the two unknown species and *An. sinensis* are much more similar to each other, about a 10% difference between them, than to *An. lesteri* and *An. pullus*, which are approximately 24–34% different from each other and the *sinensis*/unknowns. These differences are sufficient for design of species-specific primers.

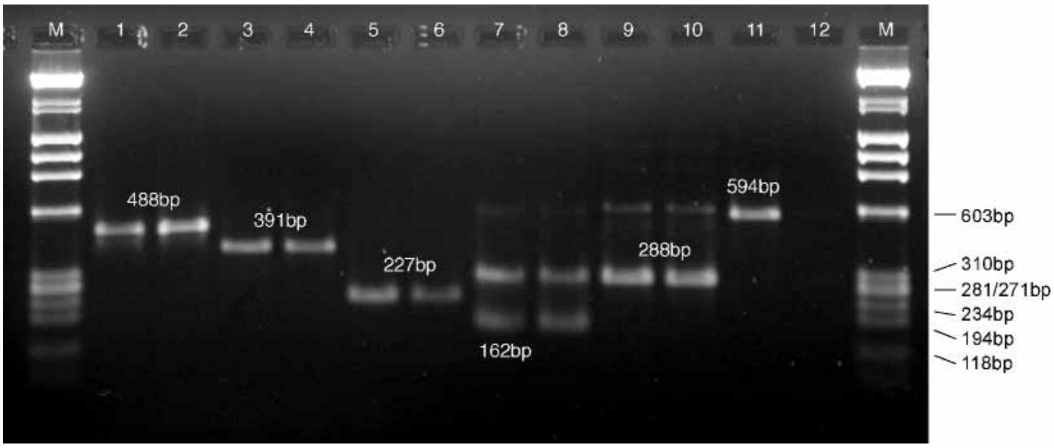

FIGURE 2. Results of amplification of rDNA ITS2 of *An. sinensis* (lane, progeny brood number): (1) KS8(67), (2) KS8(94); *An. lesteri*: (3) KS8(59), (4) KS8(88); *An. pullus*: (5) KS8(76), (6) KS8(86); *An.* unknown 1: (7) KS8(12), (8) KS7(27); *An.* unknown 2: (9) KS2-6(2) and (10) KS4-2(1); (11) positive control, complete ITS2 amplicon of KS8(67), *An. sinensis*; (12) negative control, no template. M: DNA ladder consisting of lambda DNA digested by *Hind* III, and phiX174 DNA digested with *Hae* III (Sigma, St. Louis, MO).

As stated in the introduction, *An. sinensis* and unknown 2 are not separable morphologically, and key characters to separate *An. sinensis* from unknown 1, *An. lesteri* and *An. pullus* are not completely reliable (see Rueda (2005) for a discussion of available characters). Use of a multiplex PCR assay with five species-specific primers and the universal ITS2F and ITS2R primers resulted in unambiguous species identifications of the five species. The addition of the ITS2R primer to the reaction mix served as a positive control for detection of *Anopheles* DNA that is not from one of the five species, or to confirm the presence of amplifiable DNA in a sample. In other words, the species-specific band was observed with and without the complete ITS2 amplicon (ITS2 plus flanking regions) due to direct primer competition that depends on the starting concentration of the mosquito DNA. Examples of this can be seen in lanes 7–10 of Fig. 2.

We successfully tested the multiplex PCR reaction mix described above against at least one individual from progeny broods of 96 *An. sinensis*, 29 *An. pullus*, 10 *An. lesteri*, 3 *An*. unknown 1, and 3 *An*. unknown 2, all from South Korea. Molecular-based diagnostic methods such as the one reported here, will enable entomologists to correctly identify and correlate potential vectors with the diseases they carry.

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