Multiplex PCR assay for the identification of eight Anopheles species belonging to the Hyrcanus, Barbirostris and Lindesayi groups

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Abstract

Background: Genus Anopheles mosquitoes are the primary vectors of human malaria, which is a serious threat to public health worldwide. To reduce the spread of malaria and identify the malaria infection rates in mosquitoes, accurate species identification is needed. Malaria re-emerged in 1993 in the Republic of Korea (ROK), with numbers peaking in 2004 before decreasing to current levels. Eight Anopheles species (Anopheles sinensis, Anopheles pullus, Anopheles belenrae, Anopheles Lesteri, Anopheles kleini, Anopheles sineroides, Anopheles koreicus, Anopheles lindesayi) are distributed throughout Korea. Members of the Anopheles Hyrcanus group currently cannot be identified morphologically. The other species of Anopheles can be identified morphologically, except when specimens are damaged in traps. The purpose of this study was to develop a rapid and accurate method for simultaneous molecular identification of the eight Anopheles species present in the ROK.

Methods: Anopheles spp. used in this study were collected near/in the demilitarized zone in ROK, where most malaria cases are reported. DNA from 165 of the Anopheles specimens was used to develop a multiplex PCR assay. The internal transcribed spacer 2 (ITS2) region of each species was sequenced and analyzed for molecular identification.

Results: DNA from a total of 165 Anopheles specimens was identified to species using a multiplex diagnostic system. These included: 20 An. sinensis, 21 An. koreicus, 17 An. lindesayi, 25 An. kleini, 11 An. Lesteri, 22 An. sineroides, 23 An. belenrae, and 26 An. pullus. Each species was clearly distinguished by electrophoresis as follows: 1,112 bp for An. sinensis; 925 bp for An. koreicus; 650 bp for An. lindesayi; 527 bp for An. kleini; 436 bp for An. Lesteri; 315 bp for An. sineroides; 260 bp for An. belenrae; and, 157 bp for An. pullus.

Conclusion: A multiplex PCR assay was developed to identify Anopheles spp. distributed in ROK. This method can be used to accurately identify Anopheles species that are difficult to identify morphologically to determine species distributions and malaria infection rates.

Keywords: Anopheles, Multiplex PCR assay, Malaria, Korea

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Asia, the Mediterranean, and South America (7%) [2]. Climate change and the expansion of cross-border trading may have contributed to recent increases in malaria risks worldwide [3, 4].

Members of the genus Anopheles are vectors of Plasmodium spp., the causative agent of malaria. Plasmodium spp. that are considered human pathogens include: Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium knowlesi, the latter previously considered a monkey malaria [5]. In the Republic of Korea (ROK), P. vivax, P. falciparum and P. malariae were eradicated in 1979 by the National Malaria Eradication Service (NMES) of the Korean Government [6], and the World Health Organization (WHO) declared the country malaria free [7]. However, malaria reappeared in 1993 near the demilitarized zone (DMZ) in northern Gyeonggi Province [8]. Except for imported malaria cases, only P. vivax is present in ROK and, following its peak of >4000 cases in 2010, continues to be responsible for 300–500 cases annually [9–11].

In ROK there are eight Anopheles species (Anopheles sinensis, Anopheles lesteri, Anopheles pullus, Anopheles kleini, Anopheles sineroides, and Anopheles belenrae belonging to the Hyrcanus group; Anopheles koreicus belonging to the Barbirostris group; and, Anopheles lindesayi belonging to the Lindesayi group) [12–15]. Recently, two species, An. lesteri and An. kleini, were proposed to be the primary vectors of malaria in ROK, while An. sinensis is considered a poor vector. Anopheles lesteri showed a large number of P. vivax sporozoites (up to 2105) in the salivary glands when compared to An. sinensis (0–14) in a single microscope field (750 × 560 μM). Also, An. kleini had higher oocyst rates of P. vivax (8.8%) in the midgut than An. sinensis (4.2%) [15–18]. In another study, while An. kleini and An. sinensis demonstrated similar numbers of oocysts, An. kleini demonstrated +1 (1–10 sporozoites) to +4 (>1000 sporozoites) salivary gland infections, while An. sinensis only had +1 salivary glands [19]. Recent evidence indicates that An. pullus and An. belenrae are poor to moderate vectors of malaria in ROK (Ubalee, R., pers. comm.). While An. sineroides has been implicated as a malaria vector, its status is unknown. Although there are no records of malaria infections in An. koreicus, several members of the Barbirostris group are primary vectors of malaria in Southeast Asia [20, 21]. While An. lindesayi has not been found positive for malaria in ROK, it has been implicated as a vector of P. malariae in Southeast Asia [22]. Accurate identification of Anopheles species to determine their distribution and malaria infection rates in order to develop vector control measures is needed in ROK.

Accurate species identification and subsequent monitoring of Anopheles spp. is necessary to identify their geographic distributions, larval habitats and population dynamics to manage or conduct epidemiological investigations that identify the most likely sites where infections occurred. Although scales on wings (wing patterns) and spots on legs are used as the primary key characters for species identification, it is extremely difficult if the characters are lost during collections [12, 23]. In addition, An. sinensis, An. lesteri, An. kleini, An. belenrae, and An. pullus are morphologically very similar and species cannot be identified using current morphological characters [13, 24–26]. Although a multiplex PCR assay to identify six species of the Hyrcanus group was developed [27], molecular diagnostics for all eight Anopheles species in ROK had not yet been developed. In this study, a new multiplex PCR assay was developed to identify all Anopheles species simultaneously that are present in ROK.

Methods
Sample collection
Eight species of Anopheles mosquitoes used in the study were collected at six sites in/near the DMZ where most malaria infections are contracted: 1) Neutral Nations Supervisory Commission (NNSC) camp adjacent to the Panmunjom (37°57′17.19″N; 126°40′47.91″E); 2) Daegeondong (village of approximately 200 residents inside the DMZ (37°56′28.31″N; 126°40′37.38″E)); 3) South Gate (South gate entrance to the DMZ) (37°56′03.53″N; 126°43′15.46″E)); 4) Camp Bonifas (US Army installation (37°55′55.25″N; 126°43′21.73″E)); 5) Warrior Base (US Army training sites approximately 3 km from the south gate of DMZ), (37°55′03.96″N; 126°44′29.74″E); and, 6) Dagmar North training area (37°58′29.85″N; 126°50′40.88″E). Mosquitoes were collected using Mosquito Magnets® (Woodstream Corp., Lancaster, PA, USA) (Fig. 1). The distance between the two farthest collection points: (2: Daeseongdong and 6: Dagmar North training area) was about 15.2 km. Other points were approximately 3.9 km distant from 3: South gate. Collected mosquitoes were identified morphologically to Anopheles spp. [23, 28] and then stored at −70 °C until used.

Identification and primer design
Genomic DNA used in this study was extracted using the Chelex protocol [29]. Identification of six species (An. sinensis, An. pullus, An. belenrae, An. lesteri, An. kleini, An. sineroides) was performed using a multiplex PCR assay diagnostic method [27]. The universal primers for the mitochondrial gene cytochrome c oxidase subunit 1 (COI) region (LCO1490: 5′-GGA TCA ATC ATA AAG ATA TTT G-3′/HCO2198: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) were used as species identifiers for An. koreicus and An. lindesayi [30]. Each sample
was sequenced by Macrogen (Macrogen Daejeon, Korea) and analysed using BLAST.

Two pairs of universal primers (An-ITS2-U1; forward primer: 5′-ATC GAT GAA GAC CGC AGC TA-3′/ reverse primer: 5′-CAA CAC GAC TCC ATG GTA CG-3′; An-ITS2-U2; forward primer: 5′-AAC GGG AGA AGC TCA GCA C-3′/reverse primer: 5′-GAC TTC TTG GTC CGT GTT TCA-3′) were designed between the 5.8 S and 28 S regions of the ribosomal DNA (rDNA) to analyse the entire internal transcribed spacer 2 (ITS2) sequences for the eight Anopheles species.

PCR amplification of the whole ITS2 region was conducted as follows. Each individual reaction mixture (25 μl) included: 0.2 mM each dNTP, 0.4 μM each primer, 1X PCR buffer, 1.5 mM MgCl2, and 0.5 units Taq DNA polymerase (Ro01AM; TaKaRa, Shiga, Japan) with 1.0 μl genomic DNA extracted from an individual specimen. The PCR cycling conditions were as follows: denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 60 s; and final extension at 72 °C for 5 min. Each product was visualized on 1.5% (wt/vol) agarose gels stained with ethidium bromide (VWR Life Science), and then sequenced in both directions by Macrogen. Sequence data were analysed using Bioedit v7.2.6.1 [31] and deposited in the National Center for Biotechnology Information (NCBI) under the following accession numbers:

An. sinensis—MW546412, MW546421; An. pullus—MW546424, MW546423; An. lesteri—MW546426; An. sineroides—MW546417, MW546414; An. kleini—MW546419, MW546415; An. belenrae—MW546422, MW546418; An. koreicus—MW546413, MW546416; An. lindesayi—MW546425, MW546420.

Multiplex PCR assay for eight Anopheles species
Universal forward and species-specific reverse primers were designed for the eight species of Anopheles present in ROK. Reverse primers for the three species (An. sinensis, An. koreicus, An. lindesayi) were designed using the 28 S rDNA region, while primers for the remaining species were designed using the ITS2 region (Table 1). The multiplex PCR assay was conducted in a 25-μl reaction mixture containing 0.4 μM each primer, 1X PCR buffer, 0.2 mM each dNTP, 0.5 units Taq Hotstart DNA polymerase (R007A, TaKaRa), and 1.0 μl genomic DNA from an individual specimen. PCR amplification was performed under conditions of denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min; and final extension at 72 °C for 5 min. The PCR products were visualized on ethidium-bromide-stained 2.0% (wt/vol) agarose gels (VWR Life Science). The whole aligned sequences showing positions for the universal primers and the specific reverse primers are described in Fig. 2.

Results
Molecular species diagnosis
A total of 165 DNA samples extracted from individual Anopheles species were used: An. sinensis (20), An.
An. koreicus (21), An. lindesayi (17), An. kleini (25), An. lesteri (11), An. sineroides (22), An. belenrae (23), and An. pullus (26). A gel showing the products of multiplex PCR assay separated by agarose gel electrophoresis for the eight species is shown in Fig. 3 (1112 bp for An. sinensis; 925 bp for An. koreicus; 650 bp for An. lindesayi; 527 bp for An. kleini; 436 bp for An. lesteri, 315 bp for An. sineroides; 260 bp for An. belenrae; 157 bp for An. pullus). This method allowed identification of all eight Anopheles spp., including An. koreicus and An. lindesayi, and is comparable to the current molecular diagnosis method applied to identify six Anopheles species belonging to members of the Anopheles Hynycanus group present in ROK [27]. All samples used in this study were identified using the multiplex assay. The results of species identification for An. koreicus and An. lindesayi, which were not included in the previous

| Species                  | Universal forward primer (5′→3′) | Reverse primer (5′→3′) | Product length (bp) |
|--------------------------|----------------------------------|------------------------|---------------------|
| Anopheles sinensis       | ATC GAT GAA GAC CGC AGC TA        | TAG GGT CAA GGC ATA CAG AAG G | 1112 bp |
| Anopheles koreicus       | TAT CGT GGC CCT CGA CAG           |                        | 925 bp |
| Anopheles lindesayi      | ACC ATC TAC TGCC CGA CAG          |                        | 650 bp |
| Anopheles kleini         | TTT GTT GAT AAC TTG TAT CGT CCA TC|                        | 527 bp |
| Anopheles lesteri        | CAG TCT TTT GCA GCC CAT TC        |                        | 436 bp |
| Anopheles sineroides     | CGC GCA CGC TCA GAT ATT           |                        | 315 bp |
| Anopheles belenrae       | TGT CCT AGG CGG TTA TCA ACA       |                        | 260 bp |
| Anopheles pullus         | CGG CGT AGT TTA TTG TGT ATA ACA TC|                        | 157 bp |
method [27] using this molecular assay, were also consistent with morphological identification results.

**Accurate species identification for the vector control**

In Africa and Southeast Asia where malaria is widespread, multiplex PCR assays have been developed and used to identify species accurately and to investigate malaria vector distributions and infection rates [32–40]. In addition, the ITS2-based multiplex PCR assay was used to detect two unknown species (after named as *An. belenrae* and *An. kleini* by Rueda [13]) in ROK [26]. Accurate species identification, using both morphological and molecular methods is important to confirm species identification and monitoring vector populations [41]. Several studies have described accurate species identification as a part of vector surveillance programmes. In India, *Anoph eles minimus*, a primary malaria vector, was morphologically misidentified as *Anopheles fluvialitis*, while each species was identified correctly using PCR of the ITS2 regions [42]. In South Africa, *Anopheles vaneedeni* also was reported as a new malaria vector during a malaria surveillance programme using the ITS2 region for specific identification [43]. Molecular diagnostic methods have been used to monitor invasive species, e.g., *Aedes albopictus* and *Aedes aegypti*, to verify morphological identification of specimens, as well as screening for potential new invasive species in Europe [44]. These studies support the importance of accurate species identification for monitoring vector populations and distributions, as well as supporting pathogen surveillance programmes.

**Application of new diagnostic method**

The eight *Anopheles* species present in ROK included in three groups (Hyrcanus Barbirostris, Lindesayi) can be identified based on a new multiplex molecular-based method. Morphological identification of these species is challenging, particularly in cases when legs or wing scales used as the primary identification characters are missing or damaged during collections. The method described here enables simple and accurate identification requiring only PCR of individual specimens followed by electrophoresis. It would also be useful to acquire geographic, habitat and population distributions of *An. koreicus* and *An. lindesayi* that are less frequently collected than the other species. Since the re-emergence of vivax malaria in ROK in 1993, most malaria cases have been attributed to exposure near the DMZ. Although the reason for the concentrated outbreak of malaria in/near the DMZ is uncertain, one of the primary vectors, *An. kleini*, is more prevalent near the DMZ than south of Seoul [45]. Additionally, there are reports of higher numbers of malaria cases in the Democratic People’s Republic of Korea (DPRK, North Korea) that provide a source of malaria-infected blood meals for mosquitoes that subsequently migrate south across the DMZ [46–49]. Identification of species distributions and malaria infection rates would assist in understanding the malaria distribution pattern in ROK, in addition to developing vector and malaria mitigation strategies. Recently, two species (*An. lesteri* and *An. kleini*) showed higher infection rates in artificial experiments than *An. sinensis* that was previously considered to be the primary vivax malaria vector in ROK [18, 19]. In China, *An. sinensis* and *An. lesteri* were considered the primary malaria vectors [50]. However, *An. lesteri* demonstrated more anthropophilic behaviour and 20 times higher sporozoite rates (0.58%) than *An. sinensis* (0.02%) [51, 52]. In addition, the annual distribution of *P. vivax* cases varies with environment factors that impact on mosquito population densities, which may be further impacted by climate change [53, 54]. Thus, continuous monitoring of malaria vectors is needed. The new multiplex ITS2-28S rDNA-based method eliminates the requirement for multiple PCR analyses.

![Fig. 3 Representative results of agarose gel electrophoretic separation of multiplex PCR products. M: 100 bp molecular marker; lane 1, *An. pullus*; lane 2, *An. belenrae*; lane 3, *An. sineroides*; lane 4, *An. lesteri*; lane 5, *An. kleini*; lane 6, *An. lindesayi*; lane 7, *An. koreicus*; lane 8, *An. sinensis*; lane 9, negative control.](image-url)
and is useful for monitoring Anopheles spp. distributions and population densities in ROK.

Conclusion
In this study, a new molecular diagnostic method was developed for the identification of eight Anopheles spp. present in ROK. This multiplex PCR assay is a simple and accurate method to identify Anopheles spp. and can be used as a surveillance tool for monitoring malaria vector population distributions in ROK.

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Authors’ contributions
WJB developed the new diagnostics for the eight species of Anopheles spp. and drafted the manuscript. KSC and HCK designed the study. HCK, JR, HSL, SYL, MSK, STC and TAK collected the mosquitoes from the DMZ area and helped draft the manuscript. KSC helped draft the manuscript with analysis of the data. All authors have read and approved the final manuscript.

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Availability of data and materials
All data generated during this study are included in the article. Sequences used in this study are deposited in NCBI database (https://www.ncbi.nlm.nih.gov/genbank/) as follow accession numbers: MW546412, MW546421 MW546424 MW546423 MW546426 MW546417 MW546414 MW546419 MW546415 MW546422 MW546418 MW546413 MW546416 MW546423, MW546420.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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