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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 analysed 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

Background

Malaria has a major impact on global public health with more than 200 million people infected and about 4,00,000 deaths annually [1]. Most malaria is reported in Africa (93%), with the remainder reported in Southeast

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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 Panmunjeom (37°57′17.19″N; 126°40′47.91″E); 2) Daeseongdong (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′-GGT CAA ATC ATA AAG ATA TTG 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

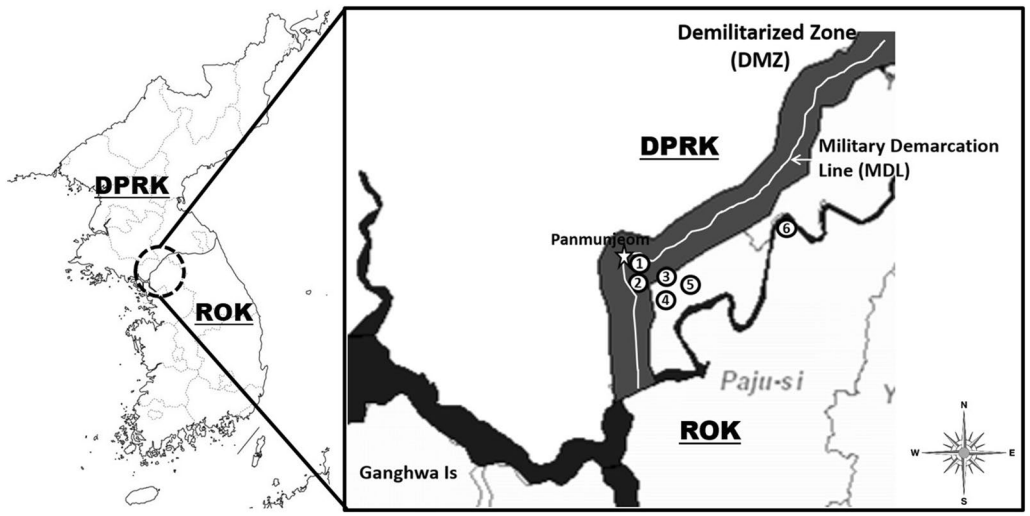

Fig. 1 Map of the northern part of Gyeonggi province denoting *Anopheles* collection sites at (**1**) NNSC (Neutral Nations Supervisory Commission camp adjacent to the Panmunjeom); (**2**) Daeseongdong (village of approximately 200 residents inside the DMZ), (**3**) South Gate (South gate entrance to the DMZ) (**4**) Camp Bonifas (US Army installation), (**5**) Warrior Base (US Army training area approximately 3 km from the south gate of DMZ), and (**6**) Dagmar Noth (US Army training area) denoting the six *Anopheles* collection sites in/near the demilitarized zone (DMZ)

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 $MgCl_2$, and 0.5 units *Taq* DNA polymerase (R001AM; 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, Radnor, PA, USA), 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.*

Table 1 PCR primers for the eight *Anopheles* species

Species	Universal forward primer (5′→3′)	Reverse primer (5′→3′)	Product length (bp)
	ATC GAT GAA GAC CGC AGC TA		
Anopheles sinensis		TAG GGT CAA GGC ATA CAG AAG G	1112
Anopheles koreicus		TAT CGT GGC CCT CGA CAG	925
Anopheles lindesayi		ACC ATC TAC TGC CTG AAC GTG	650
Anopheles kleini		TTT GTT GAT AAC TTG TAT CGT CCA TC	527
Anopheles lesteri		CAG TCT CTT GCA GCC CAT TC	436
Anopheles sineroides		CGC GCA CGC TCA GAT ATT	315
Anopheles belenrae		TGT CCT AGG CGG TTA TCA ACA	260
Anopheles pullus		CGG CGT AGT TTA TTG TGT ATA ACA TC	157

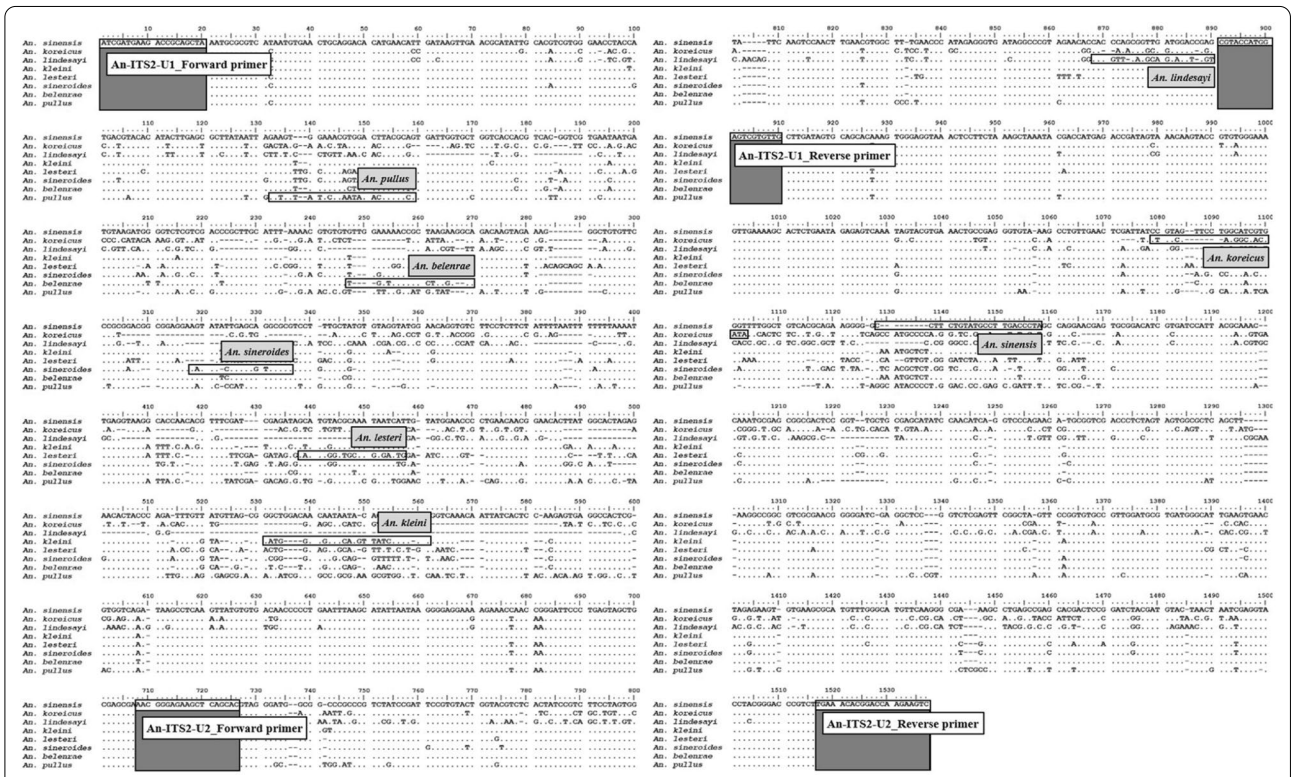

Fig. 2 The whole aligned sequences showing positions for two pairs of the universal primers (An-ITS2-U1 and An-ITS2-U2) and the specific reverse primers between 5.8 S and 28 S ribosomal DNA

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* Hyrcanus 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

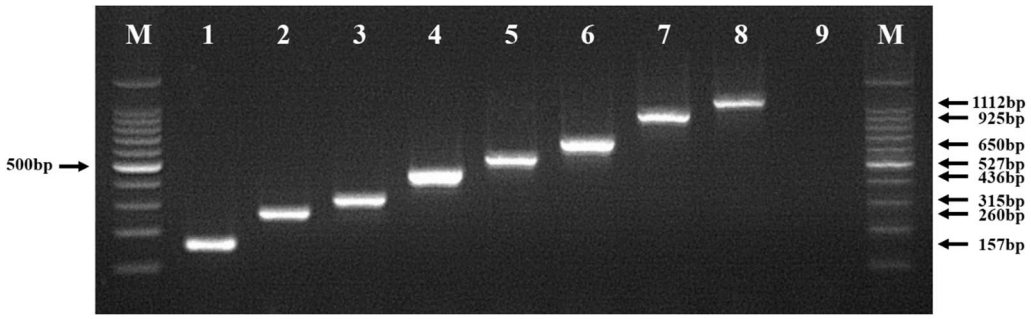

Fig. 3 Representative results of agarose gel electrophoretic separation of multiplex PCR products. M: 100 bp molecular marker; lane 1, *An. pullus*; lane 2, *An. belenrae*; lane 3, *An. sineroides*; lane 4, *An. lesteri*; lane 5, *An. kleini*; lane 6, *An. lindesayi*; lane 7, *An. koreicus*; lane 8, *An. sinensis*; lane 9, negative control

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, *Anopheles minimus*, a primary malaria vector, was morphologically misidentified as *Anopheles fluviatilis*, 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

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 MW546425, 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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