

# A Multiplex PCR Assay for Six *Aedini* Species, Including *Aedes albopictus*

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## Methodology

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# Abstract

## Background

Mosquitoes, as vectors of various human pathogens, are significant drivers of serious human illness. In particular, those species in the *Aedini* tribe, which typically transmit dengue virus, Chikungunya fever virus, and Zika virus, are increasing their range because of climate change and international commerce. In order to prevent mosquito-borne disease, accurate mosquito species identification and monitoring are needed. The goal of this work was to develop a rapid and simple molecular diagnostic method for six morphologically similar *Aedini* species (*Aedes flavopictus*, *Aedes albopictus*, *Ochlerotatus koreicus*, *Ochlerotatus japonicus*, *Ochlerotatus togoi*, and *Ochlerotatus hatorii*) in Korea.

## Methods

In total, 109 samples were used in this study. The internal transcribed spacer 2 (ITS2) regions from all six species were amplified, sequenced and analyzed using Mega 6. Once regions that were consistently different in sequence between all six species were identified, multiplex primers were designed to amplify them to generate species-specific fragments distinguishable by their size.

## Results

Uniquely sized fragments were generated in *Aedes flavopictus* (495bp), *Aedes albopictus* (438bp), *Ochlerotatus koreicus* (361bp), *Ochlerotatus togoi* (283bp), *Ochlerotatus hatorii* (220bp), and *Ochlerotatus japonicus* (160bp). Pairwise distance analysis showed that the difference was  $35.0 \pm 1.5\%$  between *Aedes* spp. and *Ochlerotatus* spp.,  $17.4 \pm 0.2\%$  between *Aedes albopictus* and *Aedes flavopictus*, and  $11.1 \pm 0.3\%$  between *Ochlerotatus koreicus* and *Ochlerotatus japonicus*.

## Conclusions

In this study, a multiplex PCR assay for six species of the *Aedini* tribe was developed. This assay is more accurate than morphological identification and will be useful for monitoring and controlling these vector mosquitoes.

# Background

Mosquitoes are insects which are vectors for diseases such as malaria, Japanese encephalitis, and dengue virus. The threat of mosquito-borne diseases is increasing in parallel with expansion of their mosquito vector ranges. Mosquito ranges are expanding because of climate change and increased introductions into novel territory facilitated by international travel and commerce (Reiter 2001; Tatem et al., 2012). More than 250 million people are exposed to mosquito-borne dengue viruses, and more than 400,000 peoples die from malaria each year (WHO, 2020).

Among the *Aedini* tribe, *Aedes* sp. and *Ochlerotatus* sp. are known to transmit dengue virus, Chikungunya virus, and yellow fever virus, and have been recently shown to be the main vector of Zika virus (Bhatt et al., 2013; Attaway et al., 2016). The main species that serve as vectors for these diseases are *Aedes aegypti* and *Aedes albopictus*. Notably, the range of *Ae. albopictus*, which is a potential vector for dengue and Zika virus, has been expanding worldwide since the 20th century as climate change and international transport accelerate. It is currently identified as a major invasive species (Benedict et al., 2007; ISSG, 2009). *Aedes albopictus* is more heat- and stress-tolerant and is more widely distributed in Asia than *Ae. aegypti* (Lwande et al., 2019). In addition, this species can carry 22 more strains of arboviruses than *Ae. aegypti*, and is likely to be a major vector in areas where *Ae. aegypti* is absent (Mitchell et al., 1995; Gratz, 2004; Medlock et al., 2015).

In Korea, 12 species of mosquitoes, including *Ae. albopictus*, are known to transmit vector-borne diseases (Lee, 2017). Every mosquito-borne disease is managed by the Korea Disease Control and Prevention Agency (KDCA) as 'a legal infectious disease' necessitating extra surveillance because of the possibility of being introduced from abroad and exacerbated by climate change (KDCA, 2020). Although there have been no reported cases of these viruses as indigenous to Korea, a mosquito infected with dengue virus was found near the airport in Incheon, Korea in 2019 suggesting that the spread of vector-borne diseases due to introductions and climate change is a real threat (KDCA, 2019).

There are six species of the *Aedini* tribe (*Aedes albopictus*, *Aedes flavopictus*, *Ochlerotatus japonicus*, *Ochlerotatus koreicus*, *Ochlerotatus togoi*, and *Ochlerotatus hatorii*) living in Korea which are difficult to identify and distinguish morphologically. *Aedes flavopictus*, a potential vector of the dengue virus, was thought to be restricted to Korea and Japan but has recently been found in Europe (Srisawat et al., 2015; Ibáñez-Justicia et al., 2019). *Ochlerotatus japonicus* has recently spread to North America and Europe and is known to transmit West Nile virus, Chikungunya virus and dengue virus (Turell et al., 2001; Schaffner et al., 2011; Koban et al., 2019). *Ochlerotatus koreicus* is a common species that lives in urban areas, and it is a vector of Japanese encephalitis and Chikungunya virus (Shestakov and Mikheeva, 1966; Ciocchetta et al., 2018). *Ochlerotatus togoi*, which is the main vector of *lymphatic filariasis*, lives in coastal areas of east Asia and also exists along the coast of North America and South America (Cheun et al., 2011; Wada et al., 2011; Peach et al., 2019).

Successful mitigation of these mosquito-borne diseases requires regular monitoring of the range and population density of their mosquito vectors. This monitoring requires rapid and accurate methods to identify mosquito species. However, morphological identification is difficult, especially if the scales or legs which are used in identification are lost (Tanaka et al., 1979; Ree et al., 2003), and the development of a rapid and accurate identification method would be extremely useful. In this study, we describe the development of a new molecular diagnostic method for these six mosquito species using the internal transcribed spacer 2 (ITS2) of the ribosomal RNA (rRNA) region.

# Methods

## 1. Mosquito sample collection and information

From August to September 2019, adults and larval mosquitoes were collected from mosquito habitats in five regions (Chiak-mountain, Yongmun-mountain, Daedeok-mountain, Bibong-mountain, and Bomokpo port) in Korea (Fig. 1). Adult specimens were collected using BG-sentinel™ (BGS) traps (Biogents, Regensburg, Germany) with lactic acid and dry ice followed by identification using morphological criteria (Tanaka et al., 1979; Ree *et al.*, 2003). Identified samples were stored at -70°C. Larvae were collected from water pools around streams and reared in the laboratory. Hatched larvae were used for the study after identification.

## 2. Sequence determination of the ITS2 region of six Aedini species

Each DNA was extracted using the AccuPrep® DNA Extraction Kit (Bioneer, K3032). Universal primers (Forward primer: 5'-AGG ACA CAT GAA CAC CCA CA-3')/Reverse primer: 5'-CTC GCA GCT ACT CAG GGA AT-3') were designed from sequences registered in the National Center for Biotechnology Information (NCBI) with the following accession numbers: *Ae. flavopictus* - AF353524; *Ae. albopictus* - MN062758; *Oc. koreicus* - KF471622; *Oc. togoi* - EU980394; and *Oc. japonicus* - KF471614). The sequence of the ITS2 region of *Oc. hatorii* was not available as of this study, and so the sequence of the phylogenetically close *Oc. togoi* was used instead (Soghigian et al., 2017). The analyzed sequence data were deposited in NCBI (accessions *Ae. albopictus* - MT992619; *Ae. flavopictus* - MW040082; *Oc. koreicus* - MW046043; *Oc. japonicus* - MW046046; *Oc. hatorii* - MW046045; *Oc. togoi* - MW046044).

Individual reaction mixtures (25 µl) contained: 0.4 µM of each primer; 1X PCR buffer; 0.2 mM of each dNTP; 1.0 mM MgCl<sub>2</sub> and; 0.5 units of Taq DNA polymerase (TaKaRa, R001AM) with 1.0 µl of the genomic DNA extracted from an individual specimen. Amplification was conducted on a Thermal Cycler Dice (TaKaRa, TP350) as follows: one cycle of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, and final extension at 72°C for 10 min. PCR products were visualized on 1.5% (wt/vol) agarose gels stained with Ethidium Bromide (VWR life science), and then sequenced in both directions by Macrogen (Macrogen Daejeon, Korea). Sequences were aligned and analyzed using BLAST and Bioedit v7.2.6.1 (Johnson et al., 2008; Hall 1999)

## 3. Multiplex PCR assay for six Aedini species

Table 1  
Universal forward primer sequences and specific reverse primer sequences for six species.

Species	Forward primer (5'↯3')	Reverse primer (5'↯3')	Product length (bp)
<i>Ae. flavopictus</i>	AGG ACA CAT GAA CAC CCA CA	TGA GGC CTA CTG ACT TGA CTT G	495
<i>Ae. albopictus</i>		GGA GCA CAC TGA GAG TTC CA	438
<i>Oc. koreicus</i>		GCC TAC TGA TTG ACG GGG TA	361
<i>Oc. togoi</i>		AGG CGG TGG AGT GTA TGG	283
<i>Oc. hatorii</i>		CAA TGT TTT ACC GCT GTT TGC	220
<i>Oc. japonicus</i>		TAT ACT ACG CTG CCG AGA GG	160

Specific multiplex primer sets for the six species were designed using the same universal forward primer used for sequencing and reverse primers designed from the sequence to amplify fragments within the ITS2 region of different lengths for each species. The multiplex PCR was conducted in a 25 µl reaction with 0.4 µM of each primer (Table 1), 1X PCR buffer, 0.2 mM of each dNTP, 1.0 mM MgCl<sub>2</sub>, 0.5 units of Taq DNA polymerase (TaKaRa, R001AM) and 1.0 µl of genomic DNA of an individual specimen. The PCR amplification was conducted as follows: one cycle of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, and final extension step at 72°C for 5 min. The products were visualized on 2.0% (wt/vol) agarose gels with Ethidium Bromide (VWR life science) and sequenced as described in Sect. 2.

## 4. Phylogenetic analysis for six Aedini species

For phylogenetic analysis, Neighbor-joining method under the Kimura 2-parameter model was conducted using MEGA software version 6 (Tamura et al., 2013) to verify phylogenetic relationships and compare with the results of morphological identification. Bootstrapping based on the ITS2 sequence data was conducted with 1,000 replicates and genetic diversity between the species was compared using pairwise distances.

# Results And Discussion

## 1. Comparison of ITS2 sequence and multiplex PCR results

In total, 109 samples of DNA extracted from individual mosquitoes were used for the study (20 of *Ae. flavopictus*, 24 of *Ae. albopictus*, 5 of *Oc. japonicus*, 20 of *Oc. koreicus*, 20 of *Oc. hatorii*, and 20 of *Oc. togoi*)

The lengths of the sequenced fragments of the ITS2 regions of the six *Aedini* species were: *Ae. flavopictus* 580 bp, *Ae. albopictus* 576 bp, *Oc. koreicus* 450 bp, *Oc. togoi* 451 bp, *Oc. hatorii* 406 bp, and *Oc. japonicus* 456 bp. The sequence of each fragment was aligned (using the Basic Local Alignment Search Tool – BLAST) with the existing sequence registered with NCBI (accessions KF471630, MK765859, JF430391, FJ641870, KF471619, GU121103, EU980394, MN062758, KU497619, MF623839, AF353524, AF353553, and AF353551)

Six multiplex primer sets consisting of the aforementioned universal forward primer paired with a species-specific reverse primer from within the ITS2 region were designed to amplify fragments of different species-specific lengths (Fig. 2). A gel showing the results of multiplex analysis of DNA from each species is shown in Fig. 3 (*Ae. flavopictus* 495 bp, *Ae. albopictus* 438 bp, *Oc. koreicus* 361 bp, *Oc. togoi* 283 bp, *Oc. hatorii* 220 bp, *Oc. japonicus* 160 bp).

## 2. Results of phylogenetic analysis using Mega 6

The ITS2 sequences of the six *Aedini* species used in this study and other ITS2 sequences registered in NCBI from other species in the *Aedini* tribe (accessions KF471630, MK765859, JF430391, FJ641870, KF471619, GU121103, EU980394, MN062758, KU497619, MF623839, AF353524, AF353553, and AF353551) were analyzed to determine phylogenetic relatedness (Fig. 4). *Aedes albopictus* and *Ae. flavopictus* which are morphologically similar were closely related as were *Oc. koreicus* and *Oc. japonicus*. The phylogenetic tree was clearly divided into *Aedes* sp. and *Ochlerotatus* sp., but *Oc. hatorii* and *Oc. togoi* were only distantly related to the other *Ochlerotatus* sp. A pairwise analysis of genetic distances using the Kimura 2-parameter calculation and the interspecies ITS2 region showed  $35.0 \pm 1.5\%$  difference between *Aedes* spp. and *Ochlerotatus* spp.,  $17.4 \pm 0.2\%$  difference between *Ae. albopictus* and *Ae. flavopictus*, and  $11.1 \pm 0.3\%$  difference between *Oc. koreicus* and *Oc. japonicus* (Table 2). Although the species were morphologically indistinguishable, the phylogenetic relationship between six species of *Aedini* tribe as well as between the genera was confirmed through this progress.

Table 2  
An analysis of pairwise distances using the Kimura 2-parameter calculation for six *Aedin*

Accession number	DQ 341113	MT 992619	MN 062758	MF 623839	KU 497619	AF 353524	AF 353551	AF 353553	MW 040082	MW 046043	MK 765859	JF 430391	KF 471630
DQ341113	-												
MT992619	36.44%	-											
MN062758	36.23%	0.17%	-										
MF623839	36.11%	0.35%	0.17%	-									
KU497619	36.31%	0.70%	0.52%	0.70%	-								
AF353524	36.67%	17.59%	17.41%	17.27%	17.63%	-							
AF353551	36.67%	17.83%	17.65%	17.32%	17.86%	0.00%	-						
AF353553	36.47%	17.41%	17.24%	17.09%	17.45%	0.69%	0.69%	-					
MW040082	36.75%	17.27%	17.45%	17.30%	17.66%	0.17%	0.17%	0.70%	-				
MW046043	35.56%	34.77%	35.00%	35.00%	35.45%	34.75%	34.75%	34.75%	34.61%	-			
MK765859	36.12%	36.26%	36.04%	36.04%	36.26%	35.41%	35.41%	35.41%	35.71%	1.56%	-		
JF430391	36.12%	36.26%	36.04%	36.04%	36.26%	35.41%	35.41%	35.41%	35.71%	1.56%	0.00%	-	
KF471630	35.56%	35.23%	35.00%	35.00%	35.45%	34.75%	34.75%	34.75%	35.06%	0.44%	1.56%	1.56%	-
MW046046	35.75%	34.38%	34.60%	34.68%	35.12%	35.48%	35.48%	35.48%	35.33%	10.63%	10.99%	10.99%	11.09%
KF471619	35.67%	34.74%	34.52%	34.60%	34.82%	35.18%	35.18%	35.18%	35.48%	11.06%	10.51%	10.51%	11.06%
FJ641870	35.96%	34.82%	34.60%	34.68%	35.12%	35.48%	35.48%	35.48%	35.78%	11.54%	11.43%	11.43%	11.54%
GU121103	36.17%	34.81%	34.59%	34.67%	34.89%	35.24%	35.24%	35.24%	35.54%	11.49%	10.94%	10.94%	11.49%
MW046045	34.24%	31.17%	31.42%	31.42%	31.92%	30.42%	30.42%	30.42%	30.25%	11.28%	11.25%	11.25%	11.78%
MW046044	40.31%	34.54%	34.76%	34.62%	34.84%	37.67%	37.67%	37.44%	37.53%	19.16%	19.71%	19.71%	19.66%
EU980394	40.00%	34.68%	34.46%	34.31%	34.54%	37.36%	37.36%	37.14%	37.67%	19.12%	19.17%	19.17%	19.12%
Out group for <i>Culex pipiens pallens</i> : DQ341113. <i>Ae. albopictus</i> : MT992619, MN062758, MF623839, KU497619. <i>Ae. flavopictus</i> : MW040082, AF353524, AF353551, KF471630. <i>Oc. japonicus</i> : MW046046, KF471619, FJ641870, GU121103. <i>Oc. togoi</i> : MW046044, EU980394. <i>Oc. hatorii</i> : MW046045.													

## 3. Application of multiplex PCR molecular diagnostic method

All of the species in this study were very similar morphologically, and were identified by fine differences in the leg, scales, scutum and scutellum (Tanaka et al., 1979; Ree et al., 2003).

The tediousness of accurate morphological identification and the fact that legs and scales may be lost during collection or storage results in a substantial mis-identification rate. The multiplex PCR assay we developed can reduce this mis-identification rate and is simple – requiring only PCR followed by electrophoresis. A multiplex PCR assay for *Anopheles* sp. which transmit malaria has also been developed and is being used to monitor certain vector species of *Anopheles* sp. (Scott et al., 1993, Joshi et al., 2010).

The multiplex PCR assay we developed for *Aedini* species enables simple and accurate identification and monitoring of species of mosquitoes that carry *flaviviruses* such as dengue virus, yellow fever virus, and Zika virus. The data provided by consistent and accurate monitoring of mosquito populations through this method can potentially be used to guide national public health measures, such as quarantining and thus help prevent the spread of infectious mosquito-borne diseases.

## Conclusions

In this study, a multiplex PCR assay was developed to identify six *Aedini* species which can transmit various diseases in Korea. The resulting assay provides a simple and accurate molecular identification tool for these six species, which are difficult to identify morphologically. These species are expected to spread globally due to climate change and increasing international trade. This tool will, therefore, be useful for control of the vectors for several infectious diseases.

## Declarations

### Availability of data and materials

The datasets generated during the current study are included within the article. Sequences used in this study are deposited in NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) under the accession numbers MT992619 MW040082 MW046043 MW046046 MW046045 MW046044.

### Author's contributions

WJB designed the study, developed the new multiplex PCR assay method for six *Aedini* species and drafted the manuscript. MHW and STC assisted with collecting mosquitoes and identifying mosquito species. JR designed the study with WJB, and assisted with analysis of the data and helped draft the manuscript. KSC revised and improved the manuscript. All authors approved the final manuscript.

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### Ethics 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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## Figures



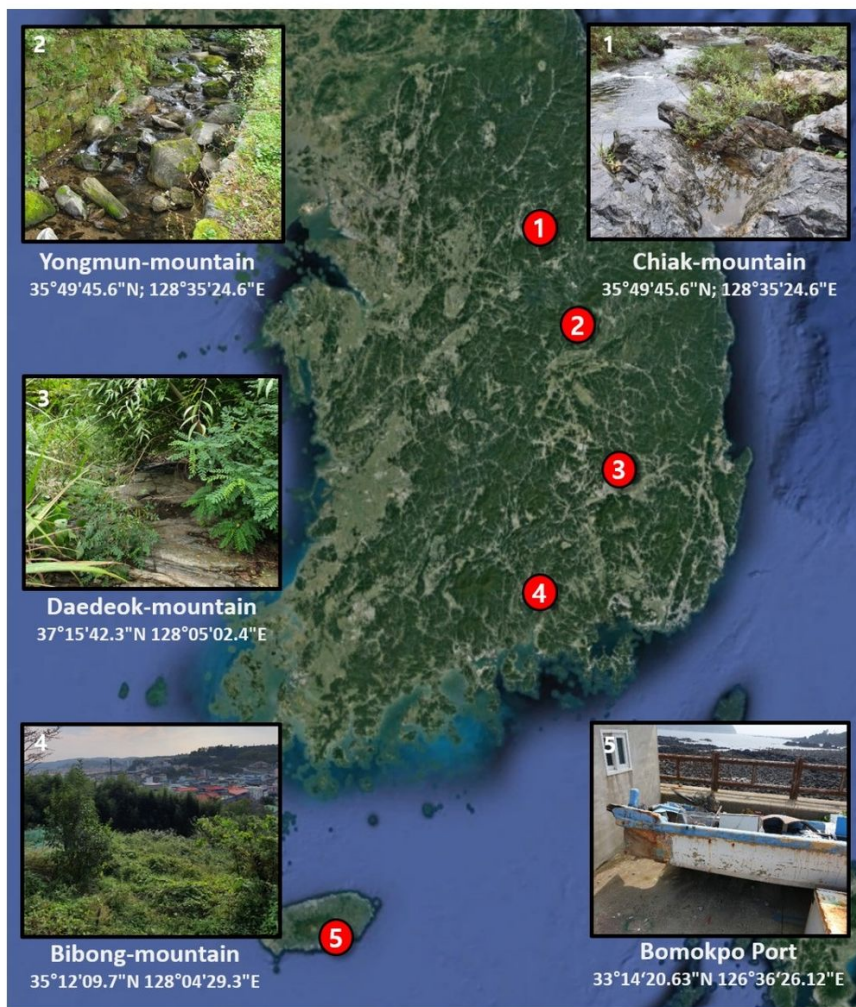


Figure 1

Collection sites of the six Aedini species in Korea. 1: Chiak mountain. 2: Yongmun-mountain. 3: Daedeok-mountain. 4: Bibong-mountain. 5: Bomokpo port. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

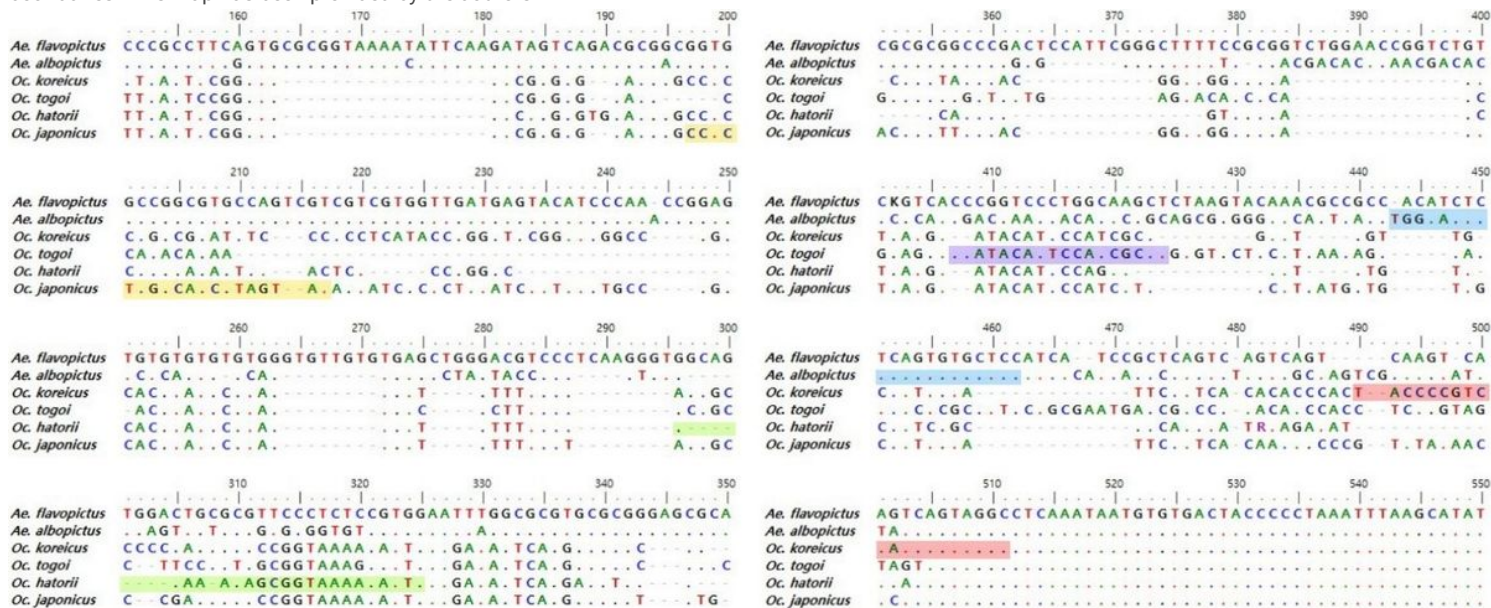
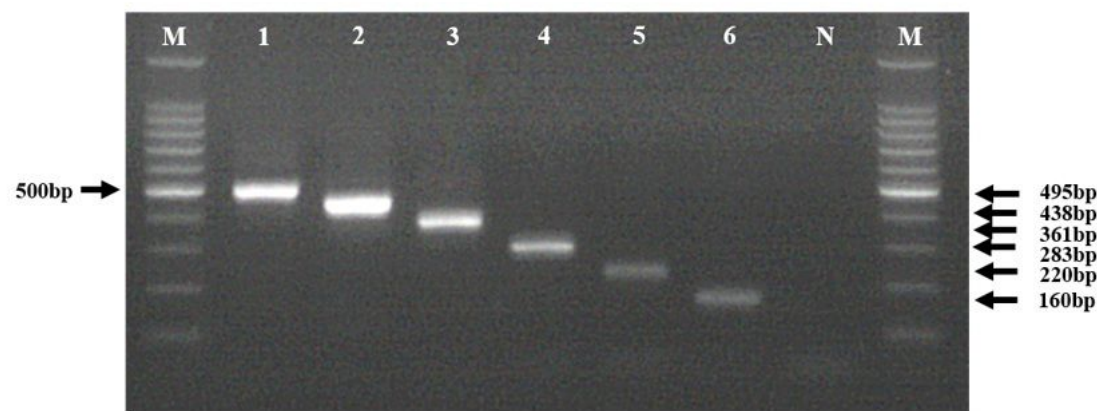
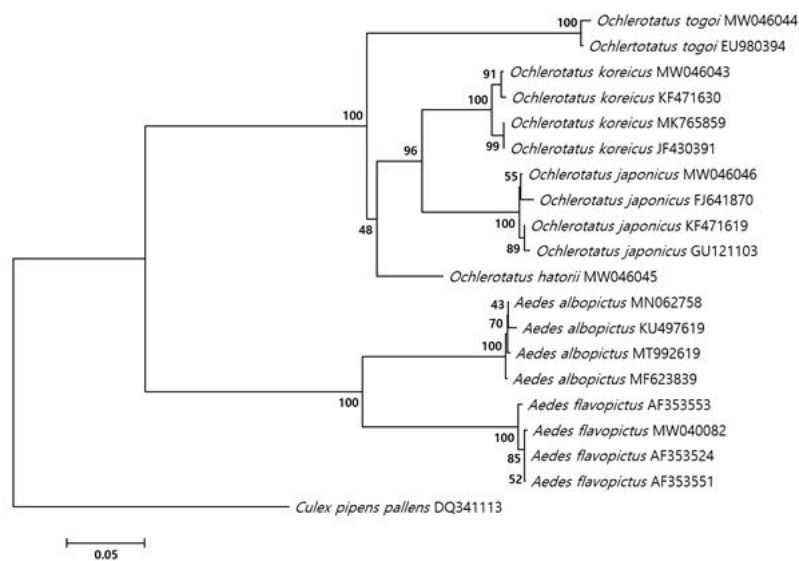


Figure 2

Position of six specific reverse primers within the ITS2 region.



**Figure 3**  
A result of multiplex PCR assay for six Aedini species. M: 100bp molecular marker; lane 1: *Ae. flavopictus*; lane 2: *Ae. albopictus*; land 3: *Oc. koreicus*; lane 4: *Oc. togoi*; lane 5: *Oc. hatorii*; lane 6: *Oc. japonicus*.



**Figure 4**  
Neighbor joining phylogenetic tree (Kimura 2-parameter genetic distance calculating method) using ITS2 sequences of six species.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [WooJunBanggraphicalabstractsize.jpg](#)