Original Article

A Multiplex PCR-Based Molecular Identification of Five Morphologically Related, Medically Important Subgenus Stegomyia Mosquitoes from the Genus Aedes (Diptera: Culicidae) Found in the Ryukyu Archipelago, Japan

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SUMMARY: Internal transcribed spacer regions of ribosomal DNA were sequenced, and new species-specific primers were designed to simplify the molecular identification of five morphologically related subgenus Stegomyia mosquito species—Aedes aegypti, Aedes albopictus, Aedes rivieri, Aedes flavopictus, and Aedes daitensis—found in the Ryukyu Archipelago, Japan. Each newly designed primer was able to amplify a species-specific fragment with a different size. Conditions for multiplex PCR were optimized to identify all five species in a single PCR. This method is a convenient tool for entomological field surveys, particularly in arbovirus endemic/epidemic areas where some of these species coexist.

INTRODUCTION

The subgenus of Stegomyia of the genus Aedes (Diptera: Culicidae) is a diverse species group as regards morphology and distribution (1, 2). Indeed, many Aedes (Stegomyia) spp. have evolved as endemic species on isolated islands, especially in the South Pacific (2). Some mosquito species of this subgenus cause serious infectious diseases, such as dengue fever (DF)/dengue hemorrhagic fever (DHF), yellow fever, chikungunya fever, and other arbovirus-related diseases, in humans (3–5).

Five Aedes (Stegomyia) spp., namely Aedes aegypti (L.), Aedes albopictus (Skuse), Aedes rivieri Bohart and Ingram, Aedes flavopictus Yamada, and Aedes daitensis Miyagi and Toma, have been described to date in the Ryukyu Archipelago, Japan, although Aedes aegypti has not been collected since the 1970s (6, 7). The remaining four species continue to be collected in the Ryukyu Archipelago and/or mainland Japan, and their geographic distributions are known to overlap (6). However, although their larvae exhibit similar habitat requirements (6, 7), the ecological niches of adult Stegomyia mosquitoes differ to some extent. Thus, whereas Aedes aegypti, the most domesticated species (3, 4), and Aedes albopictus prefer vegetation in the vicinity of domestic environments, Aedes rivieri and Aedes flavopictus are adapted to a forest environment (8). Aedes galloisi and Aedes wadai, which also belong to the subgenus Stegomyia, are rare species which are confined to the northern part of Japan, mountains of the Kyushu island and isolated islands (6).

Aedes aegypti and Aedes albopictus are distributed worldwide and are known to transmit dengue virus in Asia, the South Pacific, and the Americas. These species were first identified as dengue vectors of the epemics in the early 20th century in the Ryukyu Archipelago and mainland Japan (3–5, 9), although other mosquito species of this subgenus are also regarded as potential vectors in regions where they are found (10). As the DF/DHF vaccine is unavailable for practical use, transmission of the disease can only be prevented by reducing human-vector contact. The recent involvement of Aedes aegypti and Aedes albopictus in DF/DHF and chikungunya fever pandemics in many countries (11–16) has highlighted the need for correct identification of these mosquito species for vector control, as such vectors are expected to play different roles in transmission and often co-inhabit domestic environments (17).

Although Stegomyia spp. have similar white scale patterns, adult morphological characteristics, such as white scales on the scutum and white bands on the legs, are very useful in distinguishing the individual species (1). However, as larval characteristics overlap, damage to adult specimens during collection can often complicate the species identification process, therefore it is necessary to establish new identification methods that can be applied to morphologically damaged specimens. Owing to the importance of accurate identification, medical entomologists have recently paid attention to molecular techniques. Beebe et al. have developed a molecular identification technique for container-breeding mosquito species, including Aedes aegypti and Aedes...
*albopictus* in Australia, which allowed them to differentiate mosquito species using PCR (18). However, this method requires the use of restriction enzymes following PCR. In 2009, a combination of forward universal 5.8S and reverse primers specific to *Ae. albopictus* from Corsica was developed, although it was not subsequently confirmed that this primer was applicable to specimens from other regions (19).

In the present study, to simplify the molecular identification technique for *Stegomyia* spp., including the important dengue and chikungunya vectors, *Ae. aegypti* and *Ae. albopictus*, internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA), which can be used as genetic markers to differentiate mosquito species (20–22), were sequenced and new species-specific primers were designed. The PCR conditions were optimized for multiplex PCR. Furthermore, these primers were applied to species found in mainland Japan and other countries worldwide as well as the Ryukyu Archipelago. This new PCR method is expected to support rapid epidemiological surveillance to reduce the risks arising from introduction of viruses in Japan from neighboring countries, such as Taiwan and Southeast Asia.

**MATERIALS AND METHODS**

**Mosquito samples:** The females of five species of *Aedes*, subgenus *Stegomyia*, found in the Ryukyu Archipelago were used for the present study. As *Ae. aegypti* has not been collected in the last 30 years in either the Ryukyu or mainland Japan, a sample taken from a laboratory strain collected in Chiang Mai, Thailand was used. All samples collected in the field or obtained from laboratory strains were preserved in absolute ethanol prior to DNA extraction. *Ae. albopictus*, *Ae. flavopictus*, and *Ae. rivensi* from mainland Japan were also included in the present analysis in order to apply the newly designed primers to specimens from all over Japan (Table 1). Generally, 1–3 female mosquitoes from each location were examined individually. The sequences available in GenBank were used for *Ae. flavopictus* (22). The taxonomy of the genus *Aedes* followed the Walter Reed Biosystematics Unit (WRBU) (23).

**Cloning and sequencing:** DNA extraction, PCR, electrophoresis, cloning, and sequencing were performed according to Toma et al. (21). Total DNA was extracted using the DNeasy tissue kit (Qiagen, Valencia, Calif., USA) and ITS regions of rDNA, including 18S, 5.8S, and 16S, were amplified by PCR using the forward primer: 18SFHIN, 5'-GTA AGC TTC CTT TGT ACA CAC CGC CCG T-3', and the reverse primer: CP16, 5'-GCC GGT ACC ATG CTT AAA TTT AGG GGG TA-3' (20,24). Each 50 μl reaction solution contained 30 ng of template DNA, 1 × PCR buffer (Promega, Madison, Wis., USA), 0.1 mM each of dATP, dCTP, dGTP, and dTTP (Promega), 1.5 mM MgCl₂ (Promega), 2.5 U of Taq DNA polymerase (Promega), and 6 pmol of the primers described above. Amplification was performed using a program of 1 cycle at 97°C for 4 min, 30 cycles at 96°C for 30 s, 48°C for 30 s, 72°C for 2 min, and 1 cycle at 72°C for 4 min. Amplified PCR products (approximately 1,300 bp) were verified by 1.5% agarose gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen). The purified fragments were cloned with pGEM-T Easy Vector (Promega) and *Escherichia coli* (Stratagene, La Jolla, Calif., USA). Two clones derived from a single female were processed in most experiments. All clones were purified using the QIAprep spin miniprep kit (Qiagen) and sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The sequencing reactions were extended in both directions using BigDye Terminator ver. 3.0 (Applied Biosystems) with forward primers T7, SP6, and CP17 complementary to 5.8S gene (24) and a reverse primer.

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Origin</th>
<th>Date of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes (Stegomyia) aegypti</em></td>
<td>Chiang Mai, Thailand (F¹)</td>
<td>Laboratory strain (&gt;F15)</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) albopictus</em></td>
<td>Akita Prefecture, Japan (F)</td>
<td>July, 2000</td>
</tr>
<tr>
<td></td>
<td>Saitama Prefecture, Japan (F)</td>
<td>July, 2000</td>
</tr>
<tr>
<td></td>
<td>Nagasaki Prefecture, Japan (F)</td>
<td>September, 2000</td>
</tr>
<tr>
<td></td>
<td>Okinawa Prefecture, Japan (F)</td>
<td>June, 2000</td>
</tr>
<tr>
<td></td>
<td>Ishigaki Island, Japan (F)</td>
<td>August, 2000</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) flavopictus</em>²</td>
<td>Miyagi Prefecture, Japan</td>
<td>April to June, 1996</td>
</tr>
<tr>
<td></td>
<td>Saga Prefecture, Japan</td>
<td>April to June, 1996</td>
</tr>
<tr>
<td></td>
<td>Okinawa Prefecture, Japan</td>
<td>April to June, 1996</td>
</tr>
<tr>
<td></td>
<td>Ishigaki Island, Japan</td>
<td>April to June, 1996</td>
</tr>
<tr>
<td></td>
<td>Irionote Island, Japan</td>
<td>April to June, 1996</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) rivensi</em></td>
<td>Nagasaki Prefecture, Japan (F)</td>
<td>May, 2005</td>
</tr>
<tr>
<td></td>
<td>Okinawa Prefecture, Japan (F)</td>
<td>January, 2003</td>
</tr>
<tr>
<td></td>
<td>Ishigaki Island, Japan (F)</td>
<td>June, 2000</td>
</tr>
<tr>
<td></td>
<td>Irionote Island, Japan (F)</td>
<td>January, 2003</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) daitensis</em></td>
<td>Minami-Daito Island, Japan (F)</td>
<td>November, 2001</td>
</tr>
</tbody>
</table>

¹: Fresh female samples collected in fields or obtained from laboratory strains were preserved in absolute ethanol prior to DNA extraction.
²: Data from GenBank (22).
Table 2. Newly designed reverse primers specific to five morphologically related *Aedes* (Stegomyia) mosquitos in the Ryukyu Archipelago, Japan

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Reverse primer</th>
<th>Sequence of primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes (Stegomyia) aegypti</em></td>
<td>aeg.r1</td>
<td>TAACCCGACAC CGTCTAGGC CCT</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) albopictus</em></td>
<td>alb.r1</td>
<td>GTACTAGGCT CACTGCA CACT GA</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) flavopictus</em></td>
<td>fla.r3</td>
<td>ACCRCAAGCA AGCCTRTGC TA</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) rivensi</em></td>
<td>riv.r1</td>
<td>GTGTCGTCGC GGGTKMCGT</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) daintensis</em></td>
<td>dai.r1</td>
<td>ACGGTTTGTT TGCGAAAAC CGT</td>
</tr>
</tbody>
</table>

R = A/G; K = T/G; M = A/C.

Fig. 1. rDNA gene group, the region amplified and sequenced, and the location and direction of primers used in the species identification assay.

58Sr complimentary to 5.8S gene (20).

**Species-diagnostic PCR**: The rDNA sequences were aligned using GENETYX ver. 7 (Genetyx, Tokyo, Japan) and Mega 3.1 software (Center for Evolutionary Medicine and Informatics, Phoenix, Ariz., USA). Variable sequence sites in the ITS1 and ITS2 regions for the five species were used to design species-diagnostic reverse primers with the forward primer 18SFHIN (Table 2, Fig. 1). The newly designed primers were examined individually to confirm that each one specifically amplified the expected size of fragments. To perform a species diagnosis in a single reaction, the PCR conditions were optimized as follows: 30 ng/µl template DNA; 1 x PCR buffer (Promega); 0.04 mM each of dATP, dCTP, dGTP, and dTTP (Promega); 3.0 mM MgCl2 (Promega); 1.0 U of Taq DNA polymerase (Promega); 2.4 pmol of each primer in 20 µl of reaction mixture; and 1 cycle at 96°C for 12 min, 40 cycles at 96°C for 30 s, 52°C for 30 s, 72°C for 90 s, and 1 cycle at 72°C for 4 min.

**Identification of *Ae. aegypti* and *Ae. albopictus* from various countries**: *Ae. aegypti* from Vietnam (Ho Chi Minh), El Salvador, Thailand (Chiang Mai), Indonesia (Bali) and Kenya (Kisumu), and *Ae. albopictus* from Japan (Nagasaki and Tokyo), Singapore, and Vietnam (Ho Chi Minh) were identified using our multiplex PCR method. For a region where *Ae. aegypti* and *Ae. albopictus* were expected to coexist, primers for these two species only were used for PCR identification.

**Accession numbers for DNA sequences in GenBank database**: The following accession numbers were used: *Ae. aegypti* (ITS1, AB548769–AB548774; ITS2, AB548766–AB548801), *Ae. albopictus* (ITS1, AB548761–AB548768; ITS2, AB548788–AB548795), *Ae. rivensi* (ITS1, AB548775–AB548782; ITS2, AB548802–AB548809), *Ae. flavopictus* (22); and *Ae. daintensis* (ITS1, AB548783–AB548787; ITS2, AB548810–AB548814).

**RESULTS AND DISCUSSION**

The primers designed on the basis of DNA sequences from ITS regions were found to amplify a specific fragment with a different size for each of the *Aedes (Stegomyia)* spp. inhabiting the Ryukyu Archipelago and mainland Japan. Fig. 2 shows that the primers used were able to effectively distinguish specific differences as different species had different bands, with the same band being visualized in the same species irrespective of its collection locality. Electrophoresis was performed for 1.5 h with 2.0% Nusieve agarose gel to differentiate *Ae. daintensis* from *Ae. flavopictus*. This process was necessary because of the only small difference in length of the PCR products for these two mosquito species (390 bp for *Ae. daintensis* and 410 bp for *Ae. flavopictus*; Table 3 and Fig. 2). This extensive electrophoresis may not, however, be a practical issue as *Ae. daintensis* is endemic to the Daito islands, which are isolated from the other regions inhabited by *Ae. flavopictus* (7). Although *Ae. daintensis* coexists with *Ae. albopictus* in the Daito islands, it is easy to distinguish one from the other on the basis of the much greater difference in length of their PCR products (Table 3, Fig. 2). Combinations of morphology, distribution records, and molecular information are useful for the identification.

Fig. 2. Species-diagnostic PCR for five species of the subgenus Stegomyia in the Ryukyu Archipelago and mainland Japan. Lane 1, *Ae. albopictus* (Nagasaki); 2, *Ae. albopictus* (Okinawa); 3, *Ae. rivensi* (Nagasaki); 4, *Ae. rivensi* (Ishigaki Island); 5, *Ae. aegypti* (Thailand); 6, *Ae. aegypti* (Thailand); 7, *Ae. flavopictus* (Saga); 8, *Ae. flavopictus* (Okinawa); 9, *Ae. daintensis* (Minami-Daito Island); 10, *Ae. daintensis* (Minami-Daito Island); 11, Negative control. M, size marker. Females were used.
of taxonomically related mosquito species.

* Ae. flavopictus * forms a morphologically diverse species complex (7), and genetic variations are also exhibited among the three subspecies of * Ae. flavopictus * in Japan (22). The species complex also exhibits differences in the length of the ITS regions in the present study. Thus, the variation in ITS length was larger in * Ae. flavopictus * (62 bp in ITS1, 73 bp in ITS2) than in * Ae. aegypti * (10 bp in ITS1, 11 bp in ITS2), * Ae. albopictus * (8 bp in ITS1, 14 bp in ITS2), * Ae. riversi * (34 bp in ITS1, 35 bp in ITS2), and * Ae. daintensis * (2 bp in ITS1, 17 bp in ITS2) (Table 3). As the conserved sequences in ITS1 were chosen for a reverse primer, an equivalent size of PCR products was obtained for the * Ae. flavopictus * spp. complex (Fig. 2).

It was worth demonstrating that the difference in length of the ITS region among * Ae. riversi * populations is relatively large despite the minimal difference suggested by electrophoresis (Table 3, Fig. 2). * Ae. riversi * is common and widely distributed among the islands of the Ryukyu Archipelago and the Kyushu and Shikoku islands, although its known habitats in the latter islands are confined to coastal areas in lowland-type natural forests of evergreen broad-leaved trees (lowland-type lucidophyllous forests) (6,7,25,26). In light of its unique distribution in the Kyushu and Shikoku islands, two hypotheses were suggested to explain the present distribution of this species. One of these hypotheses proposed that the species could expand as pioneers originating from the Ryukyu Archipelago, whereas the other proposed that this species had distributed continuously when the Kyushu islands formed part of the Asian continent (26). The common occurrence of * Ae. riversi * on several continental islands ranging from Danjo to Tushima in Nagasaki, Kyushu strongly supports the latter hypothesis and suggests that the present distribution is a relic remaining after environmental changes on the Kyushu and Shikoku islands depleted the original populations (26). The lengths of ITS1 and ITS2 were 298–320 bp and 411–426 bp, respectively, for the population from the Ryukyu Archipelago population, and 328–332 and 413–446 bp, respectively, for specimens from the Kyushu island population (Nagasaki). Although there was no reproductive isolation between the Ryukyu Archipelago and Kyusyu island populations (27), the fact that the ITS is longer in * Ae. riversi * in Kyushu than in the Ryukyu Archipelago suggests some habitat-related speciation of this species. Further study is, however, required to determine the geographical origin of this species.

The distribution of two major dengue vectors, * Ae. aegypti * and * Ae. albopictus *, has changed dramatically over the last two decades. * Ae. albopictus * has expanded into new regions outside its original habitat (3,28), and the relative abundance of * Ae. aegypti * and * Ae. albopictus * has varied in many regions due to environmental changes resulting from human activities that favor either * Ae. aegypti * or * Ae. albopictus * (3). Dynamic differences in the infestation of both species are therefore likely to influence the epidemiological features of DF/DHF or other arbovirus-linked diseases in these regions, thus meaning that the accurate identification of dengue vectors is critical. The newly designed primers reported herein were found to amplify a specific DNA fragment with a different size for each species from various countries (Fig. 3). These species-specific primers can therefore be used to identify specimens, especially those from dengue and chikungunya epidemic/endemic areas, where * Ae. aegypti * and * Ae. albopictus * coexist. At the same time, they should be useful for rapid surveillance for a new introduction of either * Ae. aegypti * or * Ae. albopictus *. In such an event, it would be relatively straightforward to collect the eggs of the suspected invader, identify the species and link the result to an appropriate control program. Indeed, we have confirmed the utility of the species-specific fragments described in this study using the DNA from a single egg (Higa et al., unpublished).

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**Table 3.** The lengths of ITS1 and ITS2, and amplified sizes of PCR products for five morphologically related *Aedes (Stegomyia)* mosquitoes in the Ryukyu Archipelago, Japan

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Length of ITS1</th>
<th>Length of ITS2</th>
<th>Approximate amplified size[^1]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes (Stegomyia) aegypti</em></td>
<td>425–435</td>
<td>206–217</td>
<td>550</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) albopictus</em></td>
<td>426–434</td>
<td>394–408</td>
<td>950</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) flavopictus</em></td>
<td>347–409</td>
<td>331–404</td>
<td>410</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) riversi</em></td>
<td>298–332</td>
<td>411–446</td>
<td>800</td>
</tr>
<tr>
<td><em>Aedes (Stegomyia) daintensis</em></td>
<td>328–330</td>
<td>384–401</td>
<td>390</td>
</tr>
</tbody>
</table>

[^1]: Including partial sequences of 18S (190 bp) for 5 species and 5.8S (155 bp) for *Ae. albopictus* and *Ae. riversi*.
present study. We would also thank Dr. Osamu Komagata of the National Institute of Infectious Diseases, Tokyo, for assisting with data management and for critically reviewing the manuscript.

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