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

Yukiko Higa*, Takako Toma[1], Yoshio Tsuda[2], and Ichiro Miyagi[1]

Department of Vector Ecology and Environment, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523;
[1]Laboratory of Environmental Health, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Okinawa 903-0215; and
[2]Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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, Ae. albopictus, Ae. riversi, Ae. flavopictus*, and *Ae. 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 riversi* Bohart and Ingram, *Aedes flavopictus* Yamada, and *Aedes daitensis* Miyagi and Toma, have been described to date in the Ryukyu Archipelago, Japan, although *Ae. 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 *Ae. aegypti*, the most domesticated species (3,4), and *Ae. albopictus* prefer vegetation in the vicinity of domestic environments, *Ae. riversi* and *Ae. 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).

Ae. aegypti and *Ae. 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 epidemics 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 *Ae. aegypti* and *Ae. 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 *Ae. aegypti* and *Ae.*

*Corresponding author: Mailing address: Department of Vector Ecology and Environment, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Nagasaki, Nagasaki 852-8523, Japan. Tel: +81-95-819-7811, Fax: +81-95-819-7812, E-mail: yukko@nagasaki-u.ac.jp

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 into 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 Ryukyus 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. riversi* 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′-GCG 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 MgCl$_2$ (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 1. Origin of female mosquitoes examined

Mosquito species	Origin	Date of collection
Aedes (*Stegomyia*) *aegypti*	Chiang Mai, Thailand (F[1])	Laboratory strain (>F15)
Aedes (*Stegomyia*) *albopictus*	Akita Prefecture, Japan (F)	July, 2000
	Saitama Prefecture, Japan (F)	July, 2000
	Nagasaki Prefecture, Japan (F)	September, 2000
	Okinawa Prefecture, Japan (F)	June, 2000
	Ishigaki Island, Japan (F)	August, 2000
Aedes (*Stegomyia*) *flavopictus*[2]	Miyagi Prefecture, Japan	April to June, 1996
	Saga Prefecture, Japan	April to June, 1996
	Okinawa Prefecture, Japan	April to June, 1996
	Ishigaki Island, Japan	April to June, 1996
	Iriomote Island, Japan	April to June, 1996
Aedes (*Stegomyia*) *riversi*	Nagasaki Prefecture, Japan (F)	May, 2005
	Okinawa Prefecture, Japan (F)	January, 2003
	Ishigaki Island, Japan (F)	June, 2000
	Iriomote Island, Japan (F)	January, 2003
Aedes (*Stegomyia*) *daitensis*	Minami-Daito Island, Japan (F)	November, 2001

[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*) mosquitoes in the Ryukyu Archipelago, Japan

| Mosquito species	Reverse primer	Sequence of primer (5′ to 3′)
Aedes (*Stegomyia*) *aegypti*	aeg.r1	TAACGGACAC CGTTCTAGGC CCT
Aedes (*Stegomyia*) *albopictus*	alb.r1	GTACTAGGCT CACTGCCACT GA
Aedes (*Stegomyia*) *flavopictus*	fla.r3	ACCRCAAGCA AGCCTCRTCG TA
Aedes (*Stegomyia*) *riversi*	riv.r1	GTGTCGTCCG GGGTKAMCGT
Aedes (*Stegomyia*) *daitensis*	dai.r1	ACGGGTTGGT TGGCAAAAGC CGT

R = A/G; K = T/G; M = A/C.
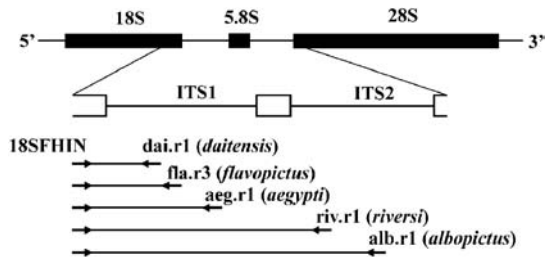
Fig. 1. rDNA gene group, the region amplified and sequenced, and the location and direction of primers used in the species identification assay.

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× PCR buffer (Promega); 0.04 mM each of dATP, dCTP, dGTP, and dTTP (Promega); 3.0 mM MgCl$_2$ (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, AB 548796–AB548801), *Ae. albopictus* (ITS1, AB548761–AB548768; ITS2, AB548788–AB548795), *Ae. riversi* (ITS1, AB548775–AB548782; ITS2, AB548802–AB548809), *Ae. flavopictus* (22); and *Ae. daitensis* (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. daitensis* 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. daitensis* and 410 bp for *Ae. flavopictus*; Table 3 and Fig. 2). This extensive electrophoresis may not, however, be a practical issue as *Ae. daitensis* is endemic to the Daito islands, which are isolated from the other regions inhabited by *Ae. flavopictus* (7). Although *Ae. daitensis* 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
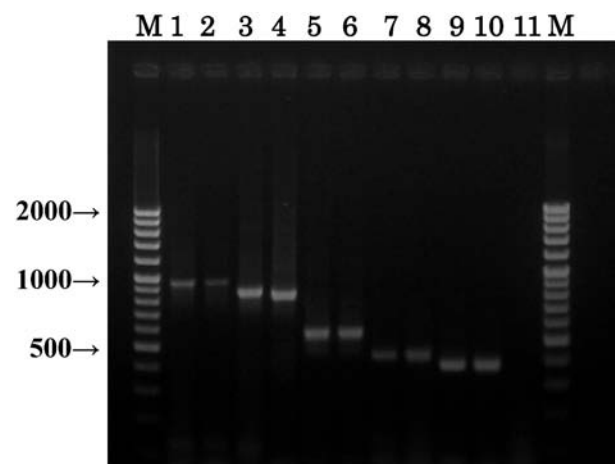

Fig. 2. Species-diagnostic PCR for five species of the subgenus *Stegomyia* in the Ryukyu Archipelago and mainland Japan. Lane 1, *Ae. albopictus* (Nagasaki); 2, *Ae. albopictus* (Okinawa); 3, *Ae. riversi* (Nagasaki); 4, *Ae. riversi* (Ishigaki Island); 5, *Ae. aegypti* (Thailand); 6, *Ae. aegypti*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

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

Mosquito species	Length of ITS1	Length of ITS2	Approximate amplified size[1]
Aedes (*Stegomyia*) *aegypti*	425–435	206–217	550
Aedes (*Stegomyia*) *albopictus*	426–434	394–408	950
Aedes (*Stegomyia*) *flavopictus*	347–409	331–404	410
Aedes (*Stegomyia*) *riversi*	298–332	411–446	800
Aedes (*Stegomyia*) *daitensis*	328–330	384–401	390

[1]: Including partial sequences of 18S (190 bp) for 5 species and 5.8S (155 bp) for *Ae. albopictus* and *Ae. riversi*.

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. daitensis* (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 Tsushima 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.

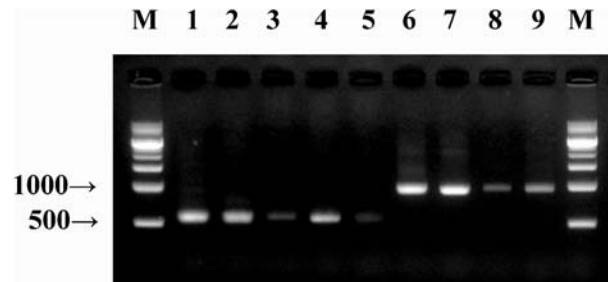The distribution of two major dengue vectors, *Ae.*

Fig. 3. Species-diagnostic PCR for *Ae. aegypti* and *Ae. albopictus* from various countries. Lanes 1–5, *Ae. aegypti*; 1, Vietnam (Larva, F6); 2, El Salvador (Larva, F2); 3, Thailand (Female, >F50); 4, Indonesia (Female, 2008 field collected); 5, Kenya (Larva, 2009 field collected). Lanes 6–9, *Ae. albopictus*; 6, Singapore (Larva, F3); 7, Nagasaki, Japan (Larva, >30F); 8, Tokyo, Japan (Female, 2005 field collected); 9, Vietnam (Larva, F12). M, size marker. 2% agarose gel was used.

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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