SHORT COMMUNICATION

A Polymerase Chain Reaction-Based Diagnostic to Identify Larvae and Eggs of Container Mosquito Species from the Australian Region

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J. Med. Entomol. 44(2): 376–380 (2007)

ABSTRACT Dengue outbreaks occur regularly in parts of northern Queensland, Australia, and there is concern that these outbreaks may spread with the introduction and range expansion of the two main vectors *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse). Problems encountered in separating larvae of endemic and exotic container mosquito species resulted in the development of a polymerase chain reaction diagnostic procedure that uses a restriction enzyme to cut the internal transcribed spacer region 1 of the ribosomal DNA to separate *Ae. aegypti* and *Ae. albopictus* from a number of common local container mosquito species which can be used at any stage of the life cycle, including eggs up to 8 wk of age. Identification was possible using desiccated or alcohol-preserved specimens with a response time of <24 h after receipt of the specimens.

KEY WORDS polymerase chain reaction diagnostic, container mosquito species, internal transcribed spacer region 1, *Aedes* mosquitoes

Regular outbreaks of dengue occur in Australia's Torres Strait Island communities, and the early 1980s saw this disease reappear in northern Queensland (Russell and Kay 2004). Australia's main dengue vector is *Aedes* (*Stegomyia*) *aegypti* (L.) whose distribution is mostly limited to greater Queensland and Tennant Creek in the Northern Territory (Sinclair 1992; Whelan et al. 2004; P. Mottram, personal communication); however, temperate Australia remains receptive to this mosquito. *Aedes* (*Stegomyia*) *scutellaris* (Walker), an endemic species occurring on the tip of Cape York Peninsula, the Torres Strait Islands, and Papua New Guinea (PNG) also has been incriminated as a vector of the dengue virus and is thought to be responsible for outbreaks in rural areas of PNG (Mackerras 1946).

Aedes (*Stegomyia*) *albopictus* (Skuse) is an important dengue vector first found in the Alexishafen region on the north coast of PNG in 1972 (Schoenig 1972). Since this time, it has spread throughout PNG and into the southwest Pacific where it was recorded in 1992 on Daru Island (Is.) (PNG) in the Torres Strait (Cooper et al. 1994). In 2004, it was identified on Yorke Is. in the Torres Strait, where it seems to be outcompeting *Ae. scutellaris* (Ritchie et al. 2006). In 2005, it was found on 10 of the 17 inhabited islands of the Torres Strait, although established populations have not as yet been discovered on the Australian mainland.

There have been at least 28 interceptions of *Ae. albopictus* between 1997 and 2005; all interceptions came from seaport entries or illegal foreign fishing vessels (Russell et al. 2005). A difficulty with this monitoring is accurate and timely species identification of eggs, larvae, and pupae because problems exist in separating these species from common endemic container mosquito species, including: *Ae. scutellaris*, *Aedes katherinensis* (Woodhill), *Aedes notoscriptus* (Skuse), and *Aedes tremulus* (Theobald).

Rearing early instars to adults is not feasible, and there are difficulties in separating correctly mounted fourth instars of *Ae. albopictus*, *Ae. scutellaris*, and *Ae. katherinensis* due to a lack of local keys for these species. Indeed, Lamche and Whelan (2003) have had difficulties identifying fourth instars of *Ae. albopictus* intercepted from a foreign vessel in Darwin. Consequently, there is a requirement for a simple DNA-based diagnostic tool to identify dengue vectors and closely related nonvector species at all stages of their life cycle. This tool could deliver accurate and reliable identification, enhance mosquito surveillance, and reduce interception response times, all of which may be vital to the successful control of an incursion. Here, we describe a simple polymerase chain reaction (PCR)-

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Table 1. Species used in this study, their collection location, the method of collection, and the number of specimens processed

Species	Collection location[a] (method)[b]	*n* (sites)[c]
Ae. aegypti	Australia, QLD, Cairns (L)	5 (2)
	Australia, QLD, Townsville (L)	3 (1)
	Australia, QLD, Camooweal (L)	2 (1)
	Australia, NT, Tennant Creek (L)	9 (3)
	Australia, NT, Nhulunbuy, Indonesian boat (L)	5 (1)
	Australia, Torres Strait, Thursday Is. (L)	3 (1)
	PNG, Port Moresby (HB, L)	3 (1)
	Vanuatu, Port Vila (L)	1 (1)
	Timor Leste (L, HB)	7 (3)
	Thailand, Bangkok (AC)	2 (1)
	Vietnam, Hanoi (AC)	3 (1)
	Burma, Yangon (AC)	3 (1)
Ae. albopictus	Australia, Torres Strait, Yorke Is. (L)	4 (3)
	PNG, Daru, Kiunga (L)	19 (3)
	PNG, Port Moresby (LT, HB)	19 (6)
	PNG, Lae (LT)	7 (2)
	PNG, Buka and Bougainville Is. (L, HB, LT)	16 (5)
	Timor Leste, Dili, Balibo, Maliana (L, HB)	21 (5)
	Vietnam, Quang Binh Province (L)	3 (1)
	Thailand, Bangkok (AC)	3 (1)
Ae. notoscriptus	Australia, QLD, Ipswich (L)	4 (1)
	Australia, QLD, Brisbane (LT)	9 (1)
	Australia, QLD, Wide Bay (LT)	4 (1)
	Australia, NT, Darwin (L)	8 (2)
	Australia, NT, Humpty Doo (L)	4 (1)
	Australia, NT, Howard Springs (L)	4 (1)
	Australia NT, Melville Is. (L)	8 (2)
Ae. scutellaris	Australia, Torres Strait, Yorke Is. (L)	65 (16)
	PNG, Daru, Kiunga (L)	12 (5)
	PNG, Port Moresby (LT)	8 (2)
Ae. katherinensis	Australia, NT, Kakadu (L)	12 (3)
Ae. tremulus	Australia, NT, Darwin (L)	2 (2)
	Australia, NT, Katherine (L)	1 (1)
	Australia, NT, Tennant Creek (L)	15 (2)
	Australia, NT, Melville Island (L)	8 (2)

[a] NT, Northern Territory; QLD, Queensland.
[b] AC, adults from established colony; HB, human biting collection; L, larval collection; LT, CO_2-baited light trap.
[c] *n* is number of specimens processed; sites is the number of locations from which collections were made.

based procedure that will differentiate the eggs, larvae, and adults of the main (common) endemic Australian container *Aedes* and *Culex* species from the exotic dengue vectors *Ae. albopictus* and *Ae. aegypti.*

Materials and Methods

Mosquito Material. The material used in this study and the sites and method of collection are shown in Table 1 and Fig. 1. In addition, *Culex* (*Culex*) *quinquefasciatus* (Say) and *Culex* (*Culiciomyia*) *pullus* (Theobald) also were examined because they commonly cohabit with the container mosquito *Aedes* species, and as early instars their larvae could be confused with *Aedes* larvae (Lee et al. 1989). *Ae. aegypti* eggs were obtained from a colony maintained at the Australian Army Malaria Institute (Brisbane, Queensland, Australia).

DNA Extraction. DNA was extracted from adults, larvae, and eggs using the procedure described in Beebe et al. (2005); additionally, a rapid DNA isolation procedure was tested that involved grinding a small part of the mosquito in 100 μl of TE and immediately boiling it for 5 min. One microliter of either extraction was used in the PCR reaction. This rapid procedure is simple but should not be used if further evaluation of the DNA is required.

Time-Scale Analysis of Desiccated Eggs. Eggs of *Ae. aegypti*, laid on filter paper, were desiccated and stored at 26°C and 75% RH for 1, 2, 8, 16, 120, and 160 wk. Ten replicate individual eggs were processed (DNA extracted and amplified as described here) for each of these time periods. Additionally, using the rapid TE method described above, DNA was isolated from 10 individual eggs that had been desiccated for 1 wk.

Specimen Preservation for Shipment. To avoid shipping specimens in alcohol, we examined whether usable DNA could be obtained from specimens immersed in 70% alcohol for 1 h and then dried. Forty adult and fourth instars were treated in this manner, and then 10 specimens from each stage were stored for 1, 2, 5, and 10 d at ambient temperature. Untreated specimens were used as controls. DNA was then extracted from each specimen as described above and processed by PCR analysis for species identification.

PCR Amplification and Analysis. All PCR amplifications were carried out in a 48-well, 0.2-ml PCR microtiter plate (Astral Scientific Pty Ltd., Gymea, New South Wales, Australia) or in 0.2-ml microfuge

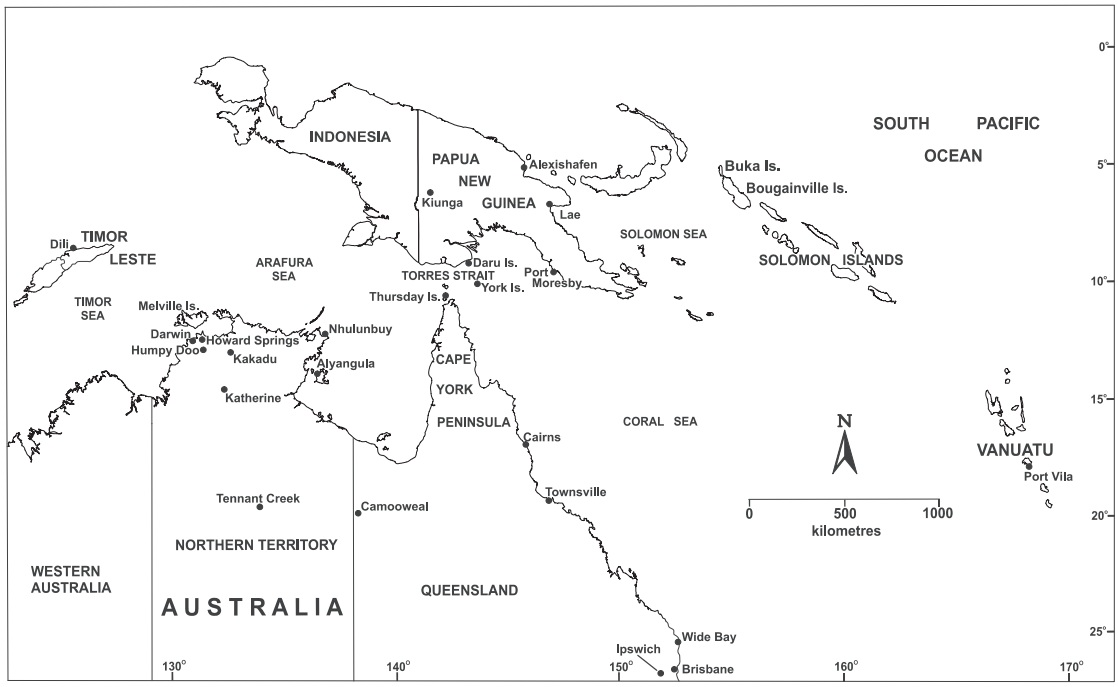

Fig. 1. Map of the Australian region indicating collection locations and place names mentioned in the text.

tubes on either a MJ-PTC 200 Thermo-cycler (MJ Research, Waltham, MA) or an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The final 25-μl PCR mixture contained 1 μl of the template DNA, 0.4 μM each primer, 1.25 mM $MgCl_2$, 1.5 mM each dNTP, 1X *Taq* reaction buffer (Biotech, Perth, WA), and 1 U of *Taq*DNA polymerase. Primers used for amplification of the internal transcribed spacer region 1 (ITS1) were those used in Beebe et al. (2000): forward primer ITS1A, 5′-CCT TTG TAC ACA CCG CCC GTC G, and reverse primer ITS1B, 5′-ATG TGT CCT GCA GTT CAC A. The cycling regime was 94°C for 4 min and then 35 cycles of 94°C for 30 s, 51°C for 40 s, and 72°C for 30 s. PCR products were size separated on a 1.2% agarose gel to confirm product size (5 μl). Restriction analysis was carried out in a 0.5-ml microfuge tube containing 5 μl of PCR product and 5 μl of 2X RsaI buffer (premade stock) containing 1–2 U of RsaI enzyme per reaction (NEB II, New England Biolabs, Natick, MA). The mixture was incubated at 37°C for 2 h and then size separated on a 3.0% agarose gel (Low Melt, Progen Biosciences, Brisbane, Australia) at 100 V for 30 min. Finally, the gel was stained with 5 μg/ml ethidium bromide for 15 min and viewed at 312 nm.

Results

DNA Extraction. Both the DNA extraction and rapid isolation in TE provided sufficient DNA from larvae and eggs to produce PCR products by using 35 cycles. Two different ITS1-size PCR products were observed on a 1.2% agarose gel. *Ae. albopictus*, *Ae. aegypti*, *Ae. tremulus*, and *Ae. notoscriptus* produced a band of ≈750 bp, whereas *Ae. scutellaris* and *Ae. katherinensis* produced products at ≈600 bp (Fig. 2A).

The resulting restriction fragment length polymorphisms delivered diagnostic banding profiles for *Ae.*

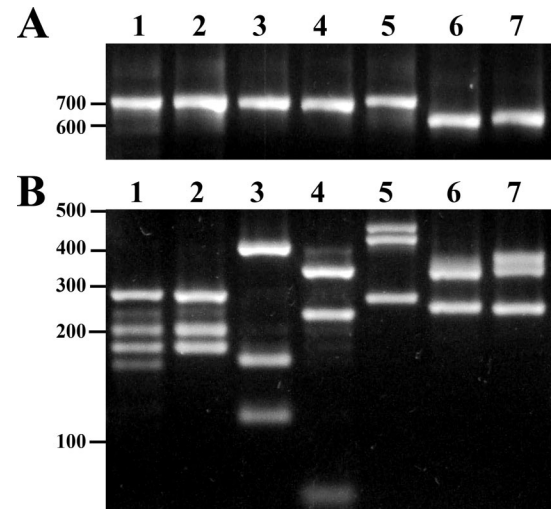

Fig. 2. The PCR amplified ITS1 product size separated in a 1.2% agarose gel (A) and digested with RsaI and separated in 3.0% agarose gel (B). Lanes 1–2 contain the two restriction fragment length polymorphism variants of *Ae. albopictus* (York Island), lane 3 is *Ae. aegypti* (Cairns), lane 4 is *Ae. tremulus* (Darwin), lane 5 is *Ae. notoscriptus* (Darwin), lane 6 is *Ae. katherinensis* (Kakadu) and one restriction fragment length polymorphism variant of *Ae. scutellaris*, and lane 7 is the second restriction fragment length polymorphism variant of *Ae. scutelaris.*

albopictus, *Ae. aegypti*, and *Ae. notoscriptus*. For *Ae. albopictus* there were two ITS1 restriction fragment length polymorphism variant profiles distinct from all other species with major bands at ≈170, 190, 200, and 290 bp in the first profile and 190, 200, and 290 bp in the second (Fig. 2B, lanes 1 and 2). Both variant profiles showed no population or geographic restriction and were identified from the same collection sites in Timor Leste, Torres Strait, and PNG. The faint bands at ≈170 and 250 bp are due to the presence of intragenomic ITS1 sequence variant in the rDNA array. *Ae. aegypti* (lane 3) produced bands at ≈120, 180, and 400 bp and showed no restriction fragment length polymorphism variants. *Ae. tremulus* produced bands at ≈240 and 330 bp (lane 4) and showed no restriction fragment length polymorphism variants. *Ae. notoscriptus* (lane 5) produced bands at ≈280 and 420 bp with a third band at ≈460 bp occurring with varying intensity in different individuals. *Ae. scutellaris* produced two banding profile variants with the first showing bands at 250 and 320 bp (Fig. 2B, lane 6), while the second variant showed bands at 250, 320, and 380 bp (Fig. 2B, lane 7). The first variant profile from *Ae. scutellaris* was also the same as that identified for *Ae. katherinensis* (Fig. 2B, lane 6), and although several different restriction enzymes were tested, none could be found to separate these two mosquito species.

Cx. quinquefasciatus and *Cx. pullus* (from Darwin) also were assessed for ITS1-amplified products and restriction fragment length polymorphism profiles. *Cx. quinquefasciatus* mosquitoes produced a larger ITS1 PCR product (≈850 bp) than all container mosquito species tested and delivered two distinct restriction fragment length polymorphism variants with major bands for variant 1 at 120, 140, 180, 190, and 240 bp and variant 2 at 80, 120, 170, 190, and 230 bp; *Cx. pullus* produced a 750-bp PCR product and a distinct restriction fragment length polymorphism profile with major bands at ≈100, 140, 160, and 180 bp (N.W.B., unpublished data). The ITS1 region for these *Culex* mosquitoes seemed highly polymorphic, and the observed variants occurred in individuals taken from the same collection site.

Time-Scale Analysis of Desiccated Eggs. All eggs for weeks 1, 2, and 8 produced DNA that could be PCR amplified. After 8 wk, the PCR amplification success decreased with only 4/10, 2/10, and 1/10 of the eggs processed for weeks 16, 120, and 160, respectively. All 10 eggs processed using the rapid DNA isolation method produced suitable DNA for species identification.

Specimen Preservation for Shipment. Viable DNA was extracted from all alcohol-treated specimens up to day 5 posttreatment and in 10/10 adults and 9/10 larvae on day 10 posttreatment. Controls not treated were 3/5 adults and 2/5 larvae on day 5 and 0/5 adults and 2/5 larvae on day 10. Because most shipments should be achieved within 5 d, this simple preserving method should be sufficient to allow specimens to be sent without constant immersion in alcohol.

Discussion

Timely and accurate identification of possible incursion species in border surveillance programs is important in terms of providing a rapid control response at the detection site to ensure containment. The limiting factor in identifying exotic container-inhabiting mosquito incursions has been the stage they are discovered (i.e., eggs, immature larvae, and pupae), which can prevent the immediate identification of specimens.

We describe a PCR-based mosquito species diagnostic procedure for the Australian region that will discriminate the two exotic species *Ae. aegypti* and *Ae. albopictus* from a number of common endemic container mosquito species: *Ae. notoscriptus*, *Ae. scutellaris*, *Ae. katherinensis*, and *Ae. tremulus*. The method can be used on all stages of the life cycle, including individual eggs <8 wk old. Desiccated eggs or larvae and adults fixed in 70% alcohol for 1 h can be sent overnight through the normal postal service with no apparent loss in usable DNA. Because the number of intercepted specimens is usually small (<20), specimens could be processed within 24 h, which is considerably faster and more reliable than rearing eggs and instars through to adults for morphological identification.

The rDNA ITS1 marker was selected because it is a highly polymorphic gene spacer flanked by conserved gene sequences (SSU/18S and 5.8S); thus, all mosquitoes should produce a PCR product. This should permit any anomalous results encountered, such as unexpected restriction fragment length polymorphism profiles from other exotic *Aedes* mosquitoes, to be further investigated and permit the diagnostic capability of this procedure to develop beyond the species described here. The rDNA-transcribed spacers contain nucleotide polymorphisms that account for the presence or absence of restriction sites, and sequence repeats that undergo insertion/deletion events that can augment the diagnostic capacity of the restriction sites by enhancing the restriction fragment length polymorphism band size variation. This method should be applicable for separating distantly related species, provided restriction fragment length polymorphism profiles do not overlap and obscure identification.

Of note is the limit of resolution for closely related species such as *Ae. scutellaris* and *Ae. katherinensis*. The overlapping PCR-restriction fragment length polymorphism profiles from *Ae. scutellaris* and *Ae. katherinensis* were interesting (Fig. 2B, lanes 6), because past cross-mating studies on these mosquitoes by Woodhill (1949, 1950) suggested reproductive compatibility, although not bidirectional and consistent with *Wolbachia*-mediated cytoplasmic incompatibility (Yen and Bar 1973, Sinkins et al. 1997).

The rDNA gene family is organized as a multicopy tandem array of genes and spacers showing sequence similarity within a species and sequence diversity between species, although the evolutionary process is not resolved (Dover 2002, Nei and Rooney 2005).

Nonetheless, the DNA turnover process can spread (or remove) sequence variants within the rDNA array (homogenization) and also can operate to spread variants through an interbreeding population (fixation). When the rate of mutation exceeds the rate of homogenization, sequence copy variants will be observed within the genome, such as the weaker restriction fragment length polymorphism bands at 170 and 250 bp observed in *Ae. albopictus.* The restriction enzyme RsaI was specifically selected for this procedure so as to minimize the number of restriction fragment length polymorphism variants observed in *Ae. albopictus,* i.e., four different restriction fragment length polymorphism variants were produced with MspI.

This diagnostic tool for container-mosquito species was specifically developed to differentiate larva and eggs of *Ae. albopictus* and *Ae. aegypti* from common endemic *Aedes* species in southern New Guinea and northern Australia and thus assist incursion monitoring of these species. The recent global expansions of *Ae. aegypti* and *Ae. albopictus* suggest that they would be unlikely to produce different restriction fragment length polymorphism profiles to those shown here, however, subtle variation may be found in the endemic species. This procedure may have applications beyond the initial intended goal discussed above (i.e., discriminating mosquito cell lines); however, we would recommend the procedure be evaluated specifically for the region where it may be used.

Acknowledgments

We thank Geoff Kumjew of AQIS for specimens from an Indonesian fishing vessel; Bill Pettit, Matthew Shortus, and Jeffery Kennedy for specimens of *Ae. aegypti* from northern Australia; and Donna Mackenzie for technical support. We also acknowledge the following for providing specimens used in this study: Armed Forces Research Institute for Medical Sciences, Bangkok, Thailand; Military Institute of Hygiene and Epidemiology, Hanoi, Vietnam; and Department of Medical Research, Yangon, Myanmar.

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Received 23 May 2006; accepted 4 December 2006.