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Species-specific primers for the detection of lymphatic filariasis vectors: *Mansonia bonneae* and *Mansonia dives*

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Abstract. Lymphatic filariasis remains a public health problem in some areas of Thailand and Southeast Asia. The nocturnally subperiodic and periodic form of brugian filariasis is transmitted by the *Mansonia* species. It is difficult to distinguish especially wild caught specimens between certain species due to morphological similarities. In the present study, species-specific primers were designed for amplification of *cytochrome oxidase subunit I (COI)* markers to simplify the identification of two morphologically similar species: *Ma. bonneae* and *Ma. dives* by PCR detection method. The primer specificity was checked against associated *Mansonia* species as well as other species of mosquitoes. It was found that the developed species-specific primer sets have proved to be effective for reliable identifying the two morphologically similar species of *Mansonia.* We therefore recommend to use these primer sets as a tool for epidemiological investigations on lymphatic filariasis.

INTRODUCTION

Lymphatic filariasis is considered to be among the most important public health problems in South East Asia (Sudomo *et al*., 2010; Taylor *et al*., 2010; Triteeraprapab *et al*., 2001; Yokmek *et al*., 2013). *Brugia malayi* filariasis is caused by mosquito vectors releasing larvae (microfilaria) which periodically circulate in human and animal blood systems until the host develops symptoms (Taylor *et al*., 2010; Triteeraprapab *et al*., 2001). In Thailand, the disease is mostly found in the Southern region, and the endemic areas are particularly located in Nakhon Si Thammarat, Narathiwat Surat Thani, Pattani, Phattalung, and Yala province (Kanjanopas *et al*., 2001). Identification and detection of infective vector mosquitoes are necessary to monitor and follow up large-scale control programs. Morphological characterization is conventional, simple and inexpensive method used in the identification of most mosquito species. However, morphological features of certain members of the genus *Mansonia* are very similar in appearance leading to misidentification when the field specimens are not in good condition. This is true for the *Ma. bonneae* and *Ma. dives* whose features resemble in structure. The identification merely relies on few scales on supraalar area (Rattanarithikul *et al*., 2006). Moreover, they both breed in the swamp forests (Ruangsittichai *et al*., 2011).

The genomic approach to taxon analysis is generally used for the family Culicidae (Garros *et al*., 2004; Manonmani *et al*., 2003; Smith and Fonseca, 2004). Molecular methods are more effective than the conventionally morphological identification. DNA-based identification does not require the specimens to be intact or undamaged

(Dhananjeyan *et al*., 2010; Iranpour *et al*., 2010). In particular, immature, partial or whole specimens can be easily amplified by molecular markers (Marquardt & Kondratieff, 2005; Patsoula *et al*., 2007). Also the mitochondrial cytochrome oxidase subunit I has been recognised as a potential molecular marker for insect identification (Cywinska *et al*., 2006; Hebert *et al*., 2003a; Hebert *et al*., 2003b; Kumar *et al*., 2007; Rueanghiran *et al*., 2011).

At present, genomic identification through DNA sequencing approach has been extensively applied in entomological research (Cywinska *et al*., 2006; Kumar *et al*., 2007; Reibe *et al*., 2009; Wang *et al*., 2012). However, some laboratories may have limited access to DNA sequencers or a lack of expertise to conduct DNA sequence analysis for the species identification. As a result, diagnostic PCR using specific primers offers a cost-effective approach for the purpose. Unfortunately, as yet, there are no specific primers available for *Mansonia* spp. In this study, attempt has been made in developing species-specific DNA markers using the *COI* gene for identifying *Ma. bonneae* and *Ma. dives*.

MATERIALS AND METHODS

Mosquito samples for the development of species-specific primers

Six species reported as natural vectors of brugian filariasis in Southeast Asia namely *Ma. annulata*, *Ma. annulifera*, *Ma. bonneae*, *Ma. dives*, *Ma. indiana* and *Ma. uniformis* (Rukkeartskul, 1981) were collected from several locations of Thailand during 2005 and 2009 and identified using morphological taxonomic keys (Rattanarithikul *et al*., 2005; Rattanarithikul *et al*., 2006). The collection included 9 samples of *Ma. annulata* from Narathiwat province; 9 samples of *Ma. annulifera* and 11 samples of *Ma. bonneae* from Surat Thani province; 10 samples of *Ma. dives* from Tak province (2) and Surat Thani province (8), 7 samples of *Ma. indiana* from Narathiwat province (2) and Phra Nakhon Si Ayutthaya (5); and 10 samples of *Ma. uniformis* from Phatthalung province. To design specific primers for *Ma. annulata*, we used the partial *COI* sequences from GenBank (HQ341634-42) from a previous study (Rueanghiran *et al*., 2011).

Extraction, amplification and sequencing

The identified samples were individually extracted from a single leg using a QIAmp DNA Mini kit. To develop the specific primers, seven to ten samples for each species of *Mansonia* were amplified and sequenced at the *COI* region using the previously described primer sets C1J-1718/C1N-2191 (Simon *et al*., 1994) and C1-J-2090/TL2-N-3014 (Zhang and Hewitt, 1996). PCR assay was performed in a volume of 50 µl containing 10x PCR buffer, 2.5 mM $MgCl_2$, 5 µM dNTP mix, 0.1 µM of each primer, 1U Platinum *Taq* polymerase (Invitrogen™, Carlsbad, CA). The PCR programming was done according to Rueanghiran *et al.* (2011) with an annealing temperature of 50ºC for both primer sets. The resulting DNA product was sequenced in both directions. For each sample, two fragments of DNA sequences were assembled to constitute approximately 1240 bp of nucleotide and the sequence results were submitted to GenBank with accession numbers KX816465-KX816511.

Species-specific primers for *Ma. bonneae* and *Ma. dives*

COI sequences of each species and available online GenBank sequences were aligned using BioEdit (Hall, 1999). We used fifty-six sequence data to perform multiple alignment; subsequently, species-specific primers for *Ma. bonneae* and *Ma. dives* (Table 1) were designed based on the variable region of the aligned sequences.

PCR reactions for the detection of *Ma. bonneae* (Bonneae set) were performed in 25 µl of the final volume PCR solution containing 2 µl 10x DreamTaq buffer, 160 µM dNTPs, 0.1 µM of each primer (MBF/MBR), 0.5 U DreamTaq DNA polymerase (Thermo Scientific) and 1 µl of DNA template. Amplification steps included initial denaturation at 95ºC for 2 minutes, followed by 35 cycles of denaturation at 95ºC for 1

Table 1. The species-specific primers for the detection of *Mansonia bonneae* and *Ma. dives;* primer sequences, annealing temperatures (Ta °C) and amplicon size (bp)

Species	Forward primer sequence	Reverse primer sequence	Ta (°C)	Size (bp)
Ma. bonneae	MBF (5'-GGA TTA ACC GGA GTC ATTT-3')	MBR (5'-TAT TCC AGC AAG GCC TAAA-3')	55	300
Ma. dives	D120 (TGG AAC TGG ATG AAC CGT TTA T)	DR608 (5'-AAT CCT AAT AAT CCA ATT GCT AG-3')	55	550

minute, annealing at 55°C for 1 minute, and 1 minute extension at 72°C, plus a final elongation step at 72°C for 10 minutes.

For the detection of *Ma. dives*, PCR (Dives set) was conducted in 25 µl of reaction containing 5 µl 10x PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP mix, 0.1 µM of each primer (D120 and DR608), 1U Platinum Taq polymerase (Invitrogen) and 1 µl of DNA template. PCR amplification steps included initial denaturation at 94°C for 2 minutes; 29 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final heating at 72°C for 10 minutes. Finally, PCR-products were visualised with GelRed on 1.5% agarose gel. A positive *Mansonia* control (sequencing sample) and one negative control (H_2O) were always included in each PCR batch.

The efficacy of these specific primer sets was then tested on individuals from different populations of the same species, and their specificity was examined on mosquito species of different genera. For the target validation of specific-primers, one to two amplified PCR products from reference samples were randomly selected for purification using FavorPrep™ PCR Clean-UP Kit, and were sequenced by First BASE, Malaysia. Then, the amplified reference sequences were compared with our *Mansonia* sequence and the NCBI nucleotide to confirm the species identity.

RESULT

Mosquito primer evaluation

In confirming species identity, the results of the target test showed sequence similarities between a tested PCR-amplicon and its corresponding species. One microliter of DNA template extracted from one leg of an individual mosquito was sufficient to produce PCR product. Regarding the specificity of each primer pair with the described methods, the species-specific primer pairs developed in this study showed no non-specific annealing and amplified the intended target region. There was no cross-amplification with other species with which the primer pair was tested. The PCR of MBF/MBR and D120/DR608 were tested with *Ma. bonneae* and *Ma. dives*. Furthermore, they were also determined on four member species of the genus *Mansonia* (*Ma. annulifera*, *Ma. annulata*, *Ma. indiana* and *Ma. uniformis*) and other mosquitoes found in Thailand (*Aedes albopictus*, *Ae. togoi*, *Anopheles dirus*, *An. minimus*, *Culex quinquefasciatus* and *Toxorhynchites*), with negative outcomes.

Practical use of the species-specific primers

To examine the specificity of the primers for practical use, ninety-five mosquito samples including *Ma. annulifera* (10), *Ma. annulata* (10), *Ma. bonneae* (13), *Ma. dives* (15), *Ma. indiana* (10), *Ma. uniformis* (10), *Ae. albopictus* (5), *Ae. togoi* (5), *An. dirus* (5), *An. minimus* (5), *Cx. quinquefasciatus* (5) and *Toxorhynchites* sp. (2) were analysed with the specific primer sets developed in the present study: the Bonneae and Dives set. A single electrophoresis band was noticed in the electrophoresis. The two sets of primer pairs gave sharp amplicons of the expected size, which confirmed the successful and accurate amplification of the target regions. The primers (MBF/MBR) successfully amplified the *COI* fragment of *Ma. bonneae*, with a product size of 300 base

pairs (Figure 1); meanwhile, the D120/ DR608 pair only amplified a 550 bp fragment from *Ma. dives* (Figure 2).

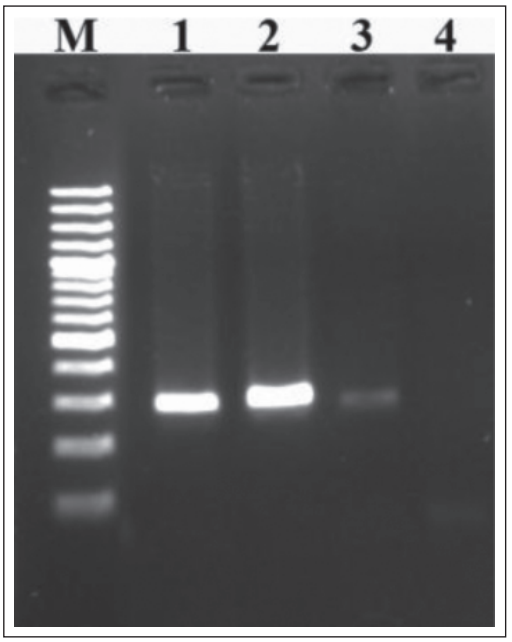

Figure 1. Amplifation efficacy for *Mansonia bonneae COI*-specific primer set against *Mansonia* species on agarose gel. Lane M, 100 bp ladder; lane 1-3, MBF/MBR primer against *Ma. bonneae*; and lane 4, blank control.

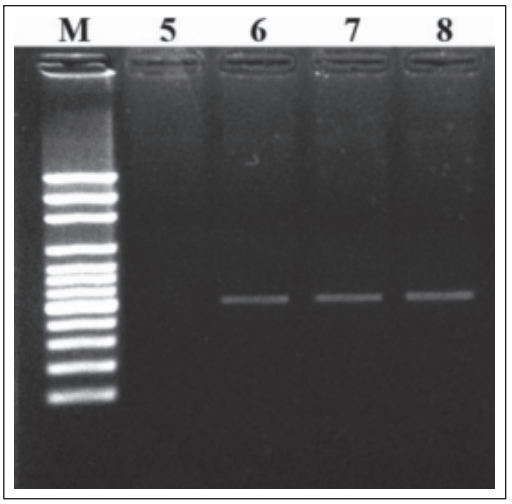

Figure 2. Electrophoretic analysis of *Mansonia dives* COI-specific primer using PCR. Lane 5, *Aedes albopictus*; lane 6-8, *Mansonia dives* against D120/ DR608 primer (550 bp); and lane M, molecular weight marker (100 bp).

DISCUSSION

Vector identification is a primary step in the surveillance system and control of vector-borne diseases (Wang *et al*., 2012). The southernmost region of Thailand is still a foci of brugian filariasis with multiple reservoirs and diverse environments for *Mansonia* mosquitoes (Apiwathnasorn *et al*., 2006; Sudomo *et al*., 2010; Triteeraprapab *et al*., 2001). Traditional morphology-based taxonomy to distinguish species is still commonly used for identification. Geometric morphometrics, quantitative analysis of size and shape of wing venation, has been successfully used to characterise the two species namely *Ma. dives* and *Ma. bonneae*, and to distinguish sexual dimorphism within its species (Ruangsittichai *et al*., 2011). However, the complete structure of specimens is necessary. The precise identification of species, is a relatively simple task, but can become extremely difficult when the condition of submitted specimens are poor. The physical structure of mosquitoes are generally very delicate, and they can lose taxonomically important characters during prolonged examination process or during specimen collection, transportation, preservation and categorisation. Whenever researchers encounter ambiguous cases, the molecular method is one of the accepted methods of identification. Mosquito identification using molecular techniques typically targets the ribosomal DNA (rDNA) region (Brengues *et al*., 2014; Eamkum *et al*., 2014; Fahmy *et al*., 2015). The mitochondrial *COI* gene from this study effectively identified two similar *Mansonia* spp. using the developed species-specific primers. The success of the developed species-specific primers (MBF/ MBR and D120/DR608), which do not require sequencing, allowed the reasonable cost and time for the identification of indistinct species. In addition, we had the opportunity to test the primers MBF/MBR and D120/ DR608 on five samples of *Ma. dives* and the results were consistent.

In conclusion, our study demonstrated the usefulness and characteristics of the *COI* species-specific primers for the

detection of *Ma. bonneae* and *Ma. dives*. According to the results, the species-specific PCR assay described here can be used as a fast-testing, reliable platform that is a cost-effective diagnostic tool to differentiate *Ma. bonneae* and *Ma. dives*.

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