UTILITY OF A SET OF CONSERVED MITOCHONDRIAL *CYTOCHROME OXIDASE SUBUNIT I* GENE PRIMERS FOR *MANSONIA ANNULATA* IDENTIFICATION

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Abstract. DNA-based identification system using the mitochondrial *cytochrome oxidase subunit I (COI)* gene has enabled validation of many species in certain taxonomic groups. These primer combinations were able to work universally across Insecta. Here, a set of three primer pairs were successful in amplifying *COI* of *Mansonia annulata* mosquito, a potential vector of *Brugia malayi*. By merging all three amplicons the whole *COI* was obtained. Primer pair TY-J-1460/C1N2087 amplified 5′ region of *COI*, LepF1/LepR1 the central and C1J2090/TL2N3014 the 3′ region, generating *COI* amplicons of 650, 700 and 950 base pairs, respectively. When *Ma. annulata* sequences were compared with those from online sources, they formed a cluster group that is clearly distinct from other allied species.

Keywords: *Mansonia annulata*, mitochondrial DNA, *COI*, primer

INTRODUCTION

Mansonia mosquitoes have been described as primary vectors of Malayan filariasis (rural forest parasite) in many countries of Asia, including Thailand, Malaysia, Indonesia, The Philippines, India and Sri Lanka (Sasa, 1976; Denhan and Mc Greevy, 1977; WHO, 1979). Six species of Mansonoides have been found in Southeast Asia with vector capability to transmit *Brugia malayi*. Generally, *Mansonia* breed in swamp while the immature stages are commonly found attached to roots of aquatic plants. In the southern part of Thailand where elephantiasis occurs, not only *Ma. annulata* was found to be an abundant species, but also was described as a vector of subperiodic *B. malayi* (Apiwathnasorn *et al*, 2006b) due to its biting pattern, and thereby playing a significant role in transmission and parasite maintenance. Thus, it has been considered that *Ma. annulata* is certainly suitable for molecular study as a model of its group.

One of the promising molecular markers used to study biodiversity in mosquitoes is mitochondrial *cytochrome oxidase subunit I (COI)* gene, which has been used for taxonomy, phylogenetic and species identification (Fairley *et al*, 2000; Linton *et al*, 2001; Dusfour *et al*, 2004;

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Cywinska *et al*, 2006; Patsoula *et al*, 2006; Kumar *et al*, 2007). COI is the largest of the three mitochondria genome-encoded cytochrome oxidase subunits (Clary and Wolstenholme, 1985) and one of the largest proteins encoded in metazoan mitochondrial genome. In addition, *COI* also has a number of superior characteristic: haploid, uniparentally inherited and resistance to degradation, making it suitable to be an ideal marker. According to mtDNA data in NCBI database, *COI* is one of the most intensively studied of the 13 protein-coding genes in mitochondrial genome. Analysis of short genomic regions shows a promising trend in rapid species identification and a suitable target gene for a bioidentification system has been *COI* (Hebert *et al*, 2003a, b).

Among genes used for examining different taxa, *COI* has been used to identify mosquitoes and butterflies in many taxa (Hebert *et al*, 2003a, 2004; Cywinska *et al*, 2006; Kumar *et al*, 2007). Many studies of various mosquito vectors (*Anopheles*, *Aedes* and *Culex*) have applied molecular markers in order to identify species (Fairley *et al*, 2000; Linton *et al*, 2001; Dusfour *et al*, 2004; Patsoula *et al*, 2006). However, the group of *Mansonia,* which is a major vector of lymphatic filariasis, has never been studied in detail before; in contrast, studies of its biology and morphology are constantly expanding (Apiwathnasorn *et al*, 1991, 2006a, b; Rattanarithikul *et al*, 2006; Samung *et al*, 2006). Although morphology identification is easy and wildly used in the field, but during surveillance specimens can be damaged (losing important identification characteristic), which make them impossible to be identified. Hence, molecular identification that could identify mosquito species even from a small piece of tissue from any developmental stage would be advantageous and useful.

Several universal primers of mitochondrial *COI* have been designed for different purposes (Lunt *et al*, 1996). Recently, some primers have been designed that might be suitable for identifying insect species (Simon *et al*, 1994; Zhang and Hewitt, 1997; Hebert *et al*, 2004). Here, we report on four sets of *COI* primers used for molecular identification of *Ma. annulata.*

MATERIALS AND METHODS

Specimens and DNA preparation

Specimens representing potential vectors in filariasis endemic area were collected from Ban Toh Daeng and Sirindhorn Research and Nature Study Center (Apiwathnasorn *et al*, 2006b) in Narathiwat Province, Thailand (6º 4′N, 101º 58′E) from 2008 to 2009. Adult mosquito species were identified based on morphology using taxonomic keys (Rattanarithikul *et al*, 2005, 2006). Specimens were stored at -80ºC until used for DNA extraction. DNA was extracted from a leg of an individual mosquito using QIAmp® DNA Mini kit according to the manufacturer's protocol.

PCR and sequencing

Primer LepF1 and LepR1 (Table 1) were used to amplify *COI* region (Hebert *et al*, 2004). PCR solution contained 5 l 10x PCR buffer, 2.5 mM $MgCl_2$, 5 M dNTP mix, 0.1 M of each primer, 1U Platinum *Taq* polymerase (Invitrogen™, Carlsbad, CA) and 5 l of DNA template. PCR thermocycling conditions were as follows: 94ºC for 1 minute; five cycles of 94ºC for 30 seconds, 45ºC for 40 seconds, 72ºC for 1 minute; 35 cycles of 94ºC for 30 seconds, 55ºC for 40 seconds, 72ºC for 1 minute; with a final heating at 72ºC for 10 minutes. Similar conditions were employed with the other three pairs of primers (TYN1438/C1N2087, TYN1460/

Table 1
Primers used to amplify regions of *Ma. annulata COI.*

Primer name	Sequence (5′ to 3′)
LepF1	ATTCAACCAATCATAAAGATATTGG
LepR1	TAAACTTCTGGATGTCCAAAAAATCA
TY-N-1438	GAATAATTCCCATAAATAGATTTACA
C1-N-2087	AATTTCGGTCAGTTAATAATATAG
C1-J-2090	AGTTTTAGCAGGAGCAATTACTAT
TL2-N-3014	TCCAATGCACTAATCTGCCATATTA
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC

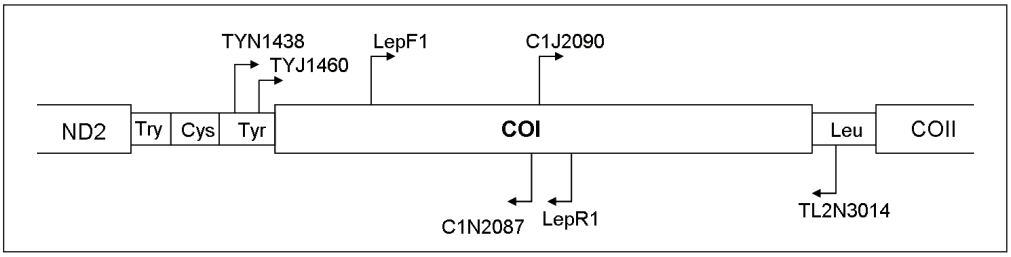

Fig 1–Relative location and directionality of the seven mitochondrial *COI* primers. The sequences of the primers are listed in Table 1.

C1N2087 and C1J2090/TL2N3014) (Table 1) (Zhang and Hewitt, 1997) but with annealing temperature at 41ºC, 44ºC and 50ºC, respectively, and the PCR solution contained 5 l 10x PCR buffer, 1.5-2.0 mM $MgCl_2$, 200 M dNTP mix, 0.2 M of each primer, 1U Platinum *Taq* polymerase (Invitrogen™) and 5 l of DNA template. Amplicons were analysed by electrophoresis in 1.5% agarose gel. DNA sequences were determined by fluorescent dye-terminator sequencing using ABI 3730XL sequencer. The location of the primers and directionality are shown in Fig 1. Both sense and antisense DNA sequences were assembled and the bases were verified manually. All sequences were aligned using Bioedit (Hall, 1999). Pairwise nucleotide sequence divergences were calculated using Kimura 2-parameter (K2P) model and neighbor-joining (NJ) analysis using MEGA 4 (Tamura *et al*, 2007) was employed to examine relationships among taxa.

RESULTS

In total, nine *Ma. annulata* sequences were obtained in the study and were deposited in GenBank (accession numbers: HQ341634-42). PCR amplification resulted in a single band from each primer pair, but TYN1438 / C1N2087 did not generate a distinct band (Fig 2). The approximate amplicon sizes from TYJ1460/C1N2087, LepF1/LepR1 and C1J2090/TL2N3014 primer pair was 650, 700, and 950 bp, respectively.

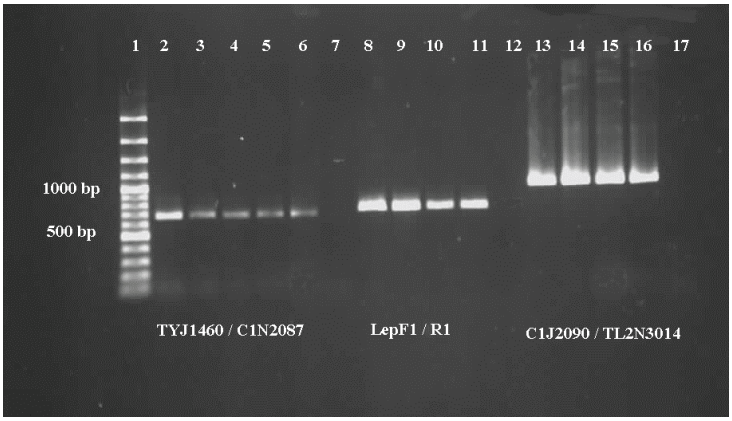

Fig 2–Agarose gel electrophoresis of PCR amplicons from DNA of individual *Ma. annulata* specimen. Amplification conditions are described in Materials and Methods. Lane 1, 100 bp marker; lanes 2-6, 8-11 and 13-16, amplicons from primer pairs TYJ1460/C1N2087, LepF1/LepR1 and C1J2090/TL2N3014, respectively; lanes 7, 12 and 17, PCR negative controls.

The *COI* sequences were AT rich, and when all three fragments were assembled they created a full length of *Ma. annulata COI* (1,516 bp). There were 1484 monomorphic sites, 32 polymorphic sites and 8 singleton variable sites (site positions: 129, 163, 404, 474, 675, 780, 783, 1074) and 24 parsimony-informative sites (site positions: 222, 231, 255, 258, 261, 388, 408, 417, 498, 531, 642, 750, 828, 849, 861, 900, 903, 969, 1050, 1051, 1104, 1107, 1212, 1404). In order to analyze patterns of *COI* nucleotide divergence we used a total of 46 mtDNA sequences, divided into 9 genera and 36 species of mosquitoes. Profiles that were used to compare with our *Ma. annulata COI* sequences were derived from online database (GenBank and www.boldsystem.org). The overall mean genetic distance (K2P) computed for the different species of Culicidae was 16%. Neighbor-joining analysis showed individuals of a single species always grouped closely together (Fig 3). There was generally a high bootstrap support (100%) for the terminal branches at the species level of *Ma. annulata*. The average intraspecific divergence of *Ma. annulata* was 1% and sequence divergences were even more increased among species in different genera.

DISCUSSION

This study establishes the utility of mitochondrial *COI* primers in *Ma. annulata* identification and conducted a phylogenetic analysis of selected taxa. The selection of *Mansonia* was based on sample availability and importance as transmitter of lymphatic filaria. As seen clearly from agarose gel analysis, these primers can amplify satisfactorily *COI* fragments of *Ma. annulata* samples.

TYN1438/C1N2087 primer set could not produce distinct bands in *Ma. annulata* samples. This might be caused by TYN1438 located in $tRNA^{Tyr}$ gene, forming a stable secondary structure, affecting amplification. Alternatively, transposition of tRNA might have happened, or that the nucleotide sequence of this primer is not conserved (Zhang and Hewitt, 1997). For these reasons TY-J-1460 was selected to replace TYN1438. C1J2090/TL2N3014 primer set has been shown to work well among different taxa. The downstream region of the amplicon (≈400 bp) is the most conserved part of insect *COI* gene (Zhang and Hewitt, 1997), accounting for the success in amplification. Furthermore, this PCR amplicon contains more than 60% of *COI* gene, comprising of different variable regions. Primer pair, LepF1/LepR1, had good capability in amplification. There

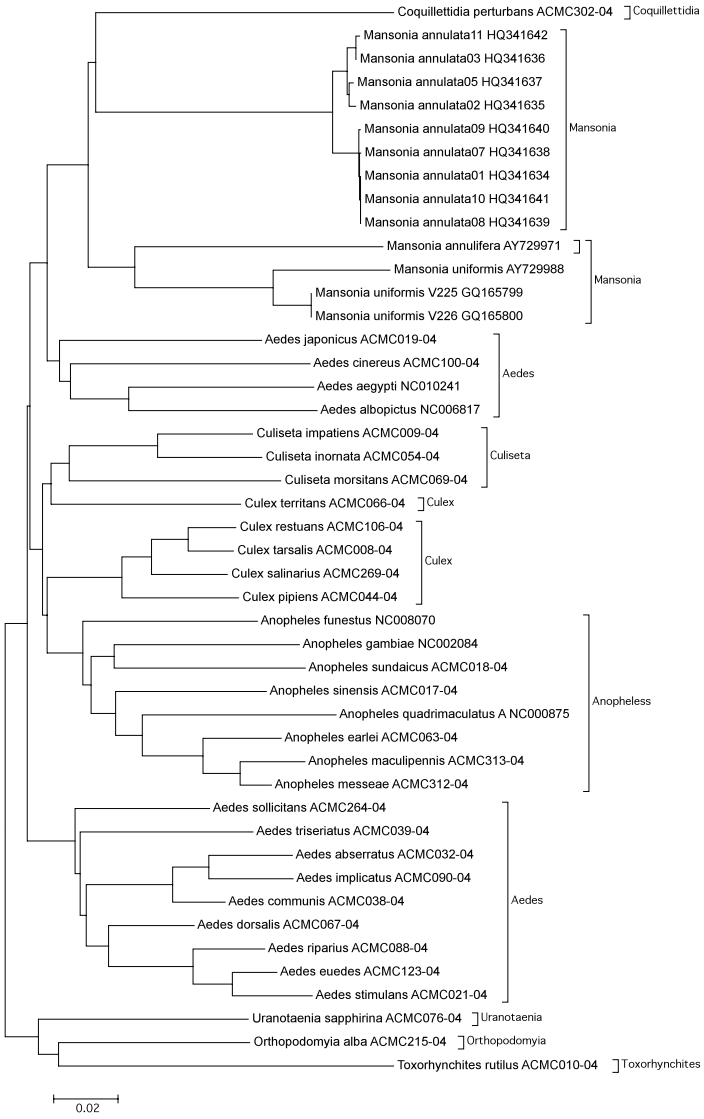

Fig 3–Phylogenetic tree of Kimura 2-parameter distances of *COI* from *Ma. annulata* and online mosquito sequences (GenBank and www.boldsystem.org).

have been reports that it was suitable for old specimens (10-20 years old) than new samples (Hebert *et al*, 2004), but from our experiments it new specimens also worked as well.

Ma. annulata COI sequences had a strong A + T bias similar to Canadian mosquito sequences (Cywinska *et al*, 2006). Our genetic distance results (intraspecific divergences) of *Ma. annulata* conformed the work of Avise (2000) with intraspecific divergence rarely >2% and mostly <1%. Likewise, Hebert *et al* (2003b) suggested that the K2P divergences between different species of mosquitoes is >2%. From the phylogenetic tree, *Coquillettidia*

pertuban was grouped near *Mansonia*, and this might be associated with their related morphology characteristics, because *Coquillettidia* is listed in tribe *Mansoniini* as is *Mansonia*. Consequently, our phylogeographic studies established that conspecific divergences are typically lower than congeneric values, and sequences from the same species present similar cluster patterns.

Molecular systematics delimiting insect species have tended to rely on a specific fragment (~600 bp) from the 5´ end of *COI* as DNA barcode region (Hebert *et al*, 2003a, b). Usually *COI* from an insect contains 1,536 bp (*Drosophila yakuba*) (Clary and Wolstenholme, 1985). Instead of focusing on the 5′ region of *COI*, other 900 base pairs at 3′ end are as good as the upstream region (Roe and Sperling, 2007). Parts of *COI* contain conserved regions and others have high variability. Therefore if DNA sequence increase in length, the probability of obtaining many diverse regions will increase. Hence, our sequence study expanded to other 900 bp regions of the *COI* different from the barcode region.

In summary, the current study demonstrated that an achievement of using universal primers to amplify parts of *COI* of *Ma. annulata*. Not only have we obtained almost the whole *COI* sequence but also established the effectiveness in differentiating species of mosquitoes. The result from NJ tree is in general agreement with prior taxonomy based on morphology, and clusters of uniform species form tight cohesion clearly distinct from their allied species. The strategy employed using *Ma. annulata* can be employed with other species of *Mansonia*.

ACKNOWLEDGEMENTS

This research work is supported by the Thailand Research Fund, grant number MRG5280206, and partially by Research Assistant (RA) scholarship from the Faculty of Graduate Studies, Mahidol University, for the academic year 2010.

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