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Interspecific and sexual shape variation in the filariasis vectors *Mansonia dives* and *Ma. bonneae*

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ABSTRACT

In the South of Thailand, six *Mansonia* species are recorded as filariasis vectors, among which *Ma. bonneae* and *Ma. dives*. These two species are distributed in the same breeding place, mainly the swamp forest, but appear to be of problematic identification using traditional morphological characters. Because of the risk of wrong identification during epidemiological or biological studies, complementary techniques are needed to distinguish the two species.

We used on the same field collected specimens both genetic (DNA barcoding) and phenetic (geometric morphometrics) techniques. Both methods converged to identify two separate entities in accordance with morphological differences and geographic origins. Shape divergence between species was more pronounced in males than in females. Notably, the amount of within species sexual shape dimorphism was much larger than shape divergence as recorded between species.

In spite of these two species of *Mansonia* being evolutionary very close, simple DNA barcoding was resolutive. Geometric morphometrics, because it is a fast and low-cost procedure, appeared as an interesting complement to modern diagnostic techniques applied in medical entomology. It also was able to provide information relevant to the ecology of the two species.

1. Introduction

Filariasis in South East Asia is an important public health problem. Most species in the subgenus Mansonioides of the genus *Mansonia* Blanchard, 1901 (i.e. *Mansonia annulata* Leicester, 1908, *Ma. annulifera* (Theobald, 1901), *Ma. bonneae* Edwards, 1930, *Ma. dives* (Schiner, 1868), *Ma. indiana* Edwards, 1930 and *Ma. uniformis* (Theobald) 1901) are vectors of Brugian filariasis in Thailand, some of which are difficult to distinguish by morphological features. Traditional morphological identification of adult specimens relies on scales and body patterns. Up to now, it has been used to differentiate the *Mansonia* species.

Some pairs of species like *Ma. indiana* and *Ma. uniformis* or *Ma. bonneae* and *Ma. dives*, do not readily separate and may represent a taxonomic problem. Our study focused on the *bonneae – dives* pair. These morphologically very close species exhibit differences in ecology and possibly in their response to control measures. For instance, *Ma. bonneae* is common in peat swamp forest while *Ma. uniformis* is common in open swamp (Apiwathnasorn et al., 2006). Alternative methods for accurate identification are then required to either implement or evaluate control program.

The barcoding technique based on the sequence of the cytochrome oxidase subunit I (COI) has become a popular technique for species identification, although not always satisfactory for very close species (Meier et al., 2006; Elias et al., 2007; Kumar et al., 2007). To evaluate the interest of another potentially diagnostic approach in the case of the two close species *Ma. dives* and *Ma. bonneae*, we submitted the specimens also to geometric morphometry (Dujardin and Slice, 2007; Dujardin, 2008). This technique has not been applied before on species of this genus. Thus, on the field collected specimens from Thailand and Myanmar, we compared on the same individuals the species diagnostic provided by the cytochrome oxidase subunit I (COI) sequence and the one obtained by the morphometric technique.

2. Materials and methods

2.1. Specimen collection

Mosquitoes were collected from the south of Thailand (Surat Thani and Narathiwat) and Myanmar (Dawei) by biting collection and aspirator. From the more than 2000 mosquitoes classified as *Mansonia*, 109 specimens had well preserved diagnostic characters (Rattanarithikul et al., 2005, 2006) and were unequivocally identified as *Ma. bonneae* and *Ma. dives*. These specimens were 28 female

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Table 1
Origin and date of capture of the specimens under study. *n*, number of mosquitoes.

Species	*n* (Gender)	Date	Place
Mansonia bonneae	28 (Female)	April 2005	Surat Thani, Thailand
	22 (Male)	April 2005	Surat Thani, Thailand
Mansonia dives	15 (Female)	February 2005 (3)	Narathiwat, Thailand
		August 2009 (12)	Surat Thani, Thailand
	5 (Male)	February 2005	Narathiwat, Thailand
	16 (Female)	December 2009	Dawei, Myanmar

Ma. bonneae, 24 male *Ma. bonneae*, 52 female *Ma. dives* and 5 male *Ma. dives* (Table 1). Each specimen was analyzed by DNA barcoding and morphometrics.

2.2. DNA extraction

All 109 *Mansonia* specimens were selected for DNA extraction by the QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany) following manufacturers instructions. Only legs of mosquitoes were used. The DNA elution was stored at −20 °C.

2.3. COI amplification

For *Ma. bonneae*, Cytochrome oxidase subunit I (COI) was amplified by PCR using primer pairs, LepF1 forward (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LepR1 reverse (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) primers (Hajibabaei et al., 2006). Each PCR reaction contained 5 μL 10× PCR buffer (20 mM Tris–HCl, pH 8.4, and 500 mM KCl), 2.5 mM $MgCl_2$, 50 μM dNTPs, 0.5 U Platinum® Taq DNA polymerase (Invitrogen, USA), 0.1 μM of each primer, 5 μl of DNA template and the remaining volume of ddH_2O up to 50 μl. The PCR thermal consisted of 1 min at 94 °C followed by 5 cycles of 30 s at 94 °C, 40 s at 45 °C, 1 min at 72 °C, then 35 cycles of 30 s at 95 °C, 40 s at 55 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. For *Ma. dives*, C1J-1718 primer (5′-GGAGGATTTGGAAATTGATTAGTTCC-3′) and C1N-2191 primer (5′-CCCGGTAAAATTAAAATATAAACTTC-3′) (Simon et al., 1994) were used to amplify COI. The PCR thermal consisted of 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final extension step of 10 min at 72 °C. All PCR products were submitted for sequencing.

2.4. DNA sequencing and analysis

COI amplification was sequenced by Fluorescent dye-terminator sequencing (ABI3730xl DNA sequencers). DNA sequencing was performed by using forward and reverse primers as in COI amplification. Nucleotide sequences were multiple aligned using the Clustal X version 2.0.12 (Thompson et al., 1997). Intraspecific and interspecific sequence divergences were calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980). Neighbor-joining (NJ) tree was built using MEGA 4.0 (Tamura et al., 2007) with bootstrapping (1000 replications).

2.5. Morphometrics

Wings of *Ma. bonneae* and *Ma. dives* were digitized by one of us (JK) at 13 anatomical landmarks (Fig. 2). The centroid size (CS) of

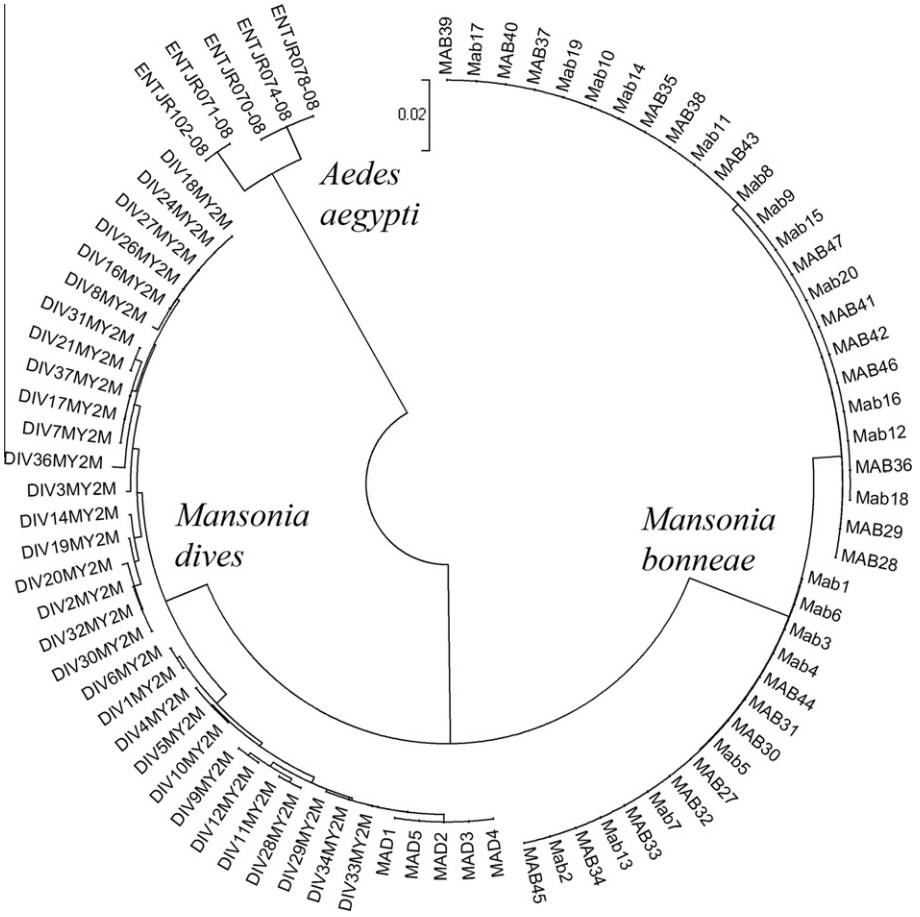

Fig. 1. Neighbor Joining tree built from intraspecific and interspecific sequence divergences using the Kimura 2-parameter (K2P) model. ENT, entomology; JR, Jiraporn Ruangsittichai; MAB, *Mansonia bonneae* from Thailand; MAD, *Mansonia dives* from Thailand; DIVMY2M, *Mansonia dives* (DIV) from Myanmar (MY2M).

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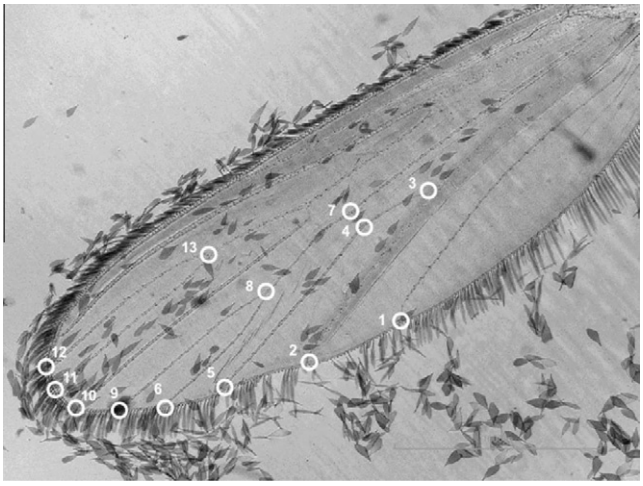

Fig. 2. Wing of *Mansonia* sp. showing the 13 landmarks whose coordinates were used in morphometric analyses. Each landmark is the junction of two different veins, as required by "Type I" landmarks (Bookstein, 1991).

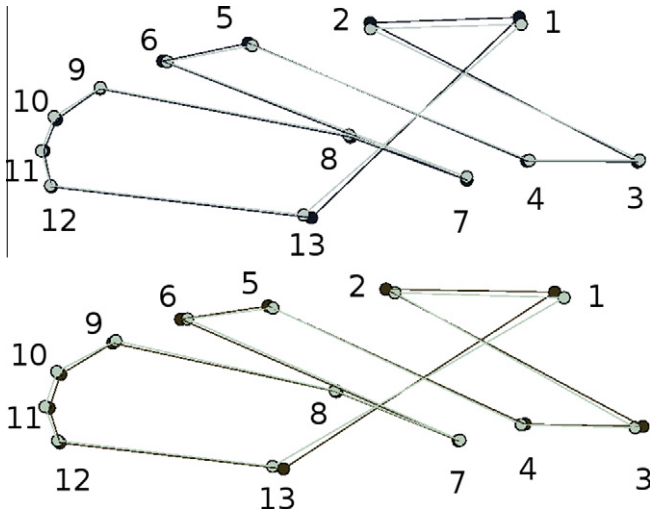

Fig. 3. Polygons are the 13 anatomical landmarks, numbered as in Fig. 2, and connected by a straight line after Procrustes superposition (residual coordinates) of the two species, in females (top) and in males (bottom). Interspecific differences are visually similar in both sexes, slightly more pronounced in males.

each mosquito was computed from the 13 LM configuration: CS is the square root of the summation of the squared distances between the center of the configuration and each landmark (Bookstein, 1991). Size means and variances were analyzed using non-parametric ANOVA (Caro-Riaño et al., 2009).

Each configuration of landmarks was superimposed on the total consensus using the Generalized Procrustes Algorithm (GPA) (Rohlf, 1990). The residual coordinates of the 13 anatomical landmarks provided polygons allowing visual comparisons of mean shapes between species (Fig. 3) and between sexes (Fig. 6). Thus, after size removal through the Procrustes superimposition on the consensus of the total sample (GPA), the residual coordinates were converted into "partial warps" (PW) to allow their processing by standard multivariate analyses.

The principal components of the PW (i.e., the relative warps, or RW) were computed to show the morphospace for both species in females (Fig. 4, left) and in males (Fig. 4, right), separately.

The classification of the wings, or of the individuals (left and right wing averaged), was evaluated using as reference the DNA classification. It was performed through a discriminant analysis (Table 3). For females, the complete set of PW was used as input. For species assignation of male individuals, to take into account some small sample sizes in this sex relative to the number of variables, a reduced set of the RW was used: the nine first RW for the wing classification, representing 81% of the total variation, and the five first RW for the individual classification, representing 93% of the total variation. Classification was based on the shortest Mahalanobis distance, and was validated by a Jack-Knife procedure (VR, see Table 3).

2.5.1. Software

Nucleotide sequences were aligned using the Clustal_X software (Thompson et al., 1997). Neighbor joining (NJ) tree was obtained using MEGA 4.0 software (Tamura et al., 2007) with bootstrapping (1000 runs). All the analyses related to the morphometric study used the CLIC package (http://www.mpl.ird.fr/morphometrics/clic/index.html) (Dujardin, 2008; Dujardin et al., 2010).

3. Results

3.1. DNA

The COI DNA barcodes of *Mansonia* mosquitoes showed 650 basepairs in agarose gel electrophoresis. Thirty-six sequences for 480 basepairs of COI coding regions of *Ma. dives* (GenBank Accession Nos.: from JF811352 to JF811392) were compared with 41 sequences of *Ma. bonneae* (GenBank Accession Nos.: from JF811393 to JF811428). These sequences had high A+T content, the average ranging from 66.5% (*Ma. dives*) to 67.4% (*Ma. bonneae*). The average A+T content at the third position of the codon was 91.3% and 93.4% in *Ma. dives* and *Ma. bonneae*, respectively. This high content of A+T

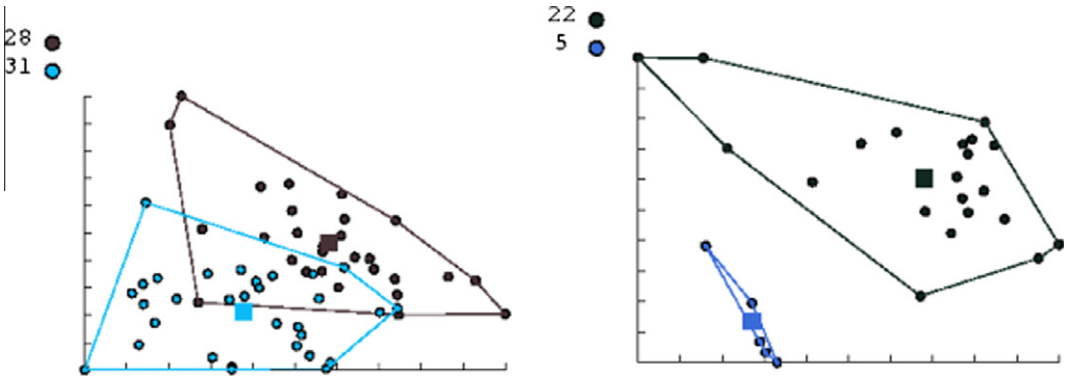

Fig. 4. The two species, *Mansonia dives* and *Ma. bonneae*, are represented by convex hulls in the morphospaces of females (28 *Ma. bonneae* and 31 *Ma. dives*, left hand) and males (22 *Ma. bonneae* and 5 *Ma. dives*, right hand). In each morphospace, the horizontal axis is the first relative warp (RW1), the vertical axis is the second one (RW2). For females, the morphospace represents 51% of the total variation, for males it represents 55%. For species classification using the totality or part of the RW, please see Table 3.

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