Molecular Marker Confirmation for Member of
Anopheles barbirostris Van Der Wulp 1884 in Different Localities

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Abstract

Vector and non-vector forms of *Anopheles barbirostris* have been recognized in Indonesia. However, because of their similarity in morphology, they were considered to be a single species. This information has led to the hypothesis that *Anopheles barbirostris* is a complex of species, which are morphologically indistinguishable from each other by ordinary methods. Objectives of the research was to identify the member of *Anopheles barbirostris* by PCR Assay. Samples were taken from two localities in Java, two in Sulawesi, two in Flores Indonesia, one from Thailand, one from China. The study was to develop a PCR-based technique of rDNA ITS2 region. Results showed that there are at least four species within the *Anopheles barbirostris* population studied, namely *Anopheles barbirostris* species DW, DX, DY and DZ. The length of the sequence amplified for species W, species X, species Y, and species Z were 339bps, 247bps, 165bps. and 157bps, respectively. Verification of the method was carried out with 270 mosquitoes from eight different field-collection sites using various sampling methods. Samples collected from Singaraja-Flores were identified as species W and X. All specimens collected from human bite outdoors were identified as species X; this species showed to be predominant among indoor light trap, indoor human bite and indoor resting collections Samples from Reo-Flores were identified as species W and X. All specimens from Manado and Palopo in Sulawesi were identified as species Z. Similarly only species Y was found in samples from Thailand, while specimens from Salaman and Jambu in Java were identified as species W or species X. These species-specific molecular markers for the *Anopheles barbirostris,* complex appear to be reliable over a wide geographical area. However, larger number of samples is still needed from throughout the range of this species.

Key words: *Anopheles barbirostris*, ITS2, PCR, Specific primer diagnostic

Introduction

The internal transcribed spacer (ITS1 and ITS2) sequences proved useful for resolving the evolutionary affiliation at different taxonomic levels, including diverged taxa, such as cryptic species of mosquitoes (Marrelli *et al.*, 2006). Both ITS1 and ITS2 are flanked by highly conserved regions, which facilitate their examination through construction of primers for use in polymerase chain reaction (PCR). In particular, ITS2 is generally more conserved among species than ITS1, providing an efficient molecular marker for closely related species (Kronefeld *et al.,* 2012; Marelli *et al.*, 2006).

There are at least four species within the *Anopheles barbirostris* population studied (Satoto and Townson, in press). *Anopheles barbirostris* (Van der Wulp, 1884) exhibits confusing variations in morphology, behaviour and vectorial capacity (Choochote *et al.*, 1983; Lien *et al.*, 1975; Partono *et al.*, 1977; Reid, 1962; 1979; Atmosoedjono *et al.*, 1977; Saeung *et al.*, 2008; Paredes-Esquivel *et al.*, 2009; Jariyapan *et al.*, 2010). Some of this evidences suggest that *Anopheles barbirostris* may comprise of cryptic species.

The aim of this study was to develop a PCR-based technique of rDNA ITS2 region

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that could be applied for identification of these species in natural population. The advantage of such techniques is that they are applicable to all live stages from egg to adult, even from museum preserved (Zahler *et al.,* 1995; Rutledge *et al.,* 1999; Townson *et al.,* 1999). DNA prepared from as a little as a single leg of mosquito is more than ample for the PCR allowing the rest of the specimen to be used for other analyses, such as parasite detection (Beebe and Saul, 1995; Walton *et al.,* 1999). These techniques have been applied to a number of anopheline species groups (Paredes-Esquivel *et al.,* 2011; Kronefeld *et al.,* 2012)

Materials and methods
Samples collection
Samples were taken from two localities in Java, two sites in Sulawesi, two sites in Flores, one site from Thailand and two dry samples were sent by colleague from China. All samples were obtained by simple random sampling single catching.

Methods of adult sample collection were using an aspirator, particularly for indoors resting (Ri) and outdoors resting (Ro) collections. Human bite landing collections both indoor (HBi), and outdoor (HBo) were also used. Mosquito CDC light traps, indoor (LTi) and outdoor (Lto). The collections methods were based on Service (1993) and WHO (1975).

Genomic DNA extraction
DNA from Mosquito legs were extracted using livak buffer according to Collins *et al.,* (1987). Livak grinding buffer, consisting of 16 ml 5M NaCl , 5.48 g Sucrose, 1.57 g Tris, 16ml 0.5M EDTA, 2.5 ml 20% SDS in final volume of 100ml, was sterile filtered. Store 5 ml aliquots at –20°C. After thawing keep aliquot at 4°C for no more than 2 weeks; heat in water bath and mix before each use to re-dissolve precipitate. Grind all mosquito legs in 100µl of preheated grinding buffer in 1.5 ml microtube. Or, to maximize total yield, grind in 50 µl, then rinse pestle with a

further 50 µl (100 µl total in tube). Transfer immediately to 65°C. Incubate at 65°C for approximately 30 min. Microsentrifuge briefly to collect condensation. Add 14 µl 8M K-acetate (to final concentration of 1 M). Mix. Incubate on ice for approximately 30 min. Centrifuge for 20 min at 4°C. Transfer supernatant to new 1 ml microtube; be careful not to transfer any debris. If desired re-spin for 20 min and transfer supernatant to new tube. Add 200 µl ethanol and mix. Spin 15 min at 4°C. Remove and discard supernatant, rinse pellet in approx. 100 µl ice-cold 70% ethanol, being careful not to dislodge pellet. Dry pellet: in vacuum centrifuge (5 min) or leave open tubes on bench top for approx. 1 h. Suspend pellet in 100 µl TE; incubate at 65°C for 10 min. Resuspend pellet in 15 µl water.

DNA amplification
The design of specific primers to distinguish members of the *Anopheles barbirostris*, complex has followed generally agreed principles of Ballinger-Crabtree (Ballinger-Crabtree, 1992), Primer have been designed based on sequences difference, whether substitution or indels, which were concentrated at the 3' end of the primer, where they have the greatest effect on inhibiting extension from mismatched primer-DNA templates (Ugozzoli and Wallace, 1991). Where possible, the discriminating mismatch base pair were used. The primers used are shown in Table 1 and 28S primer 5'ATGCTTAAATTTAGGGGGTAGTC 3'

The following amplification product was 1:2000 dilution of DNA template, 2.5 µl of 10x reaction buffer, 400 µM each dNTP, 1 mM $MgCl_2$ 0.5 unit of ampliTaq polymerase, 50 ng of primer DW, 50 ng primer DX, 50 ng primer DY, 25 ng primer DZ and 25 ng primer DU, add sterile water to give total volume of 25 µl. This is overlaid with 50 µl of mineral oil in a 500 µl microfuge tube. The PCR cycling programme was 5 min at 94°C followed by 26 cycles of 1 min 94°C, 1 min 55°C, and 1 min at 72°C with 7 min at 72°C after the last cycle. Amplification was carried out in a

Table 1. Primer used for identification of four species of the *An.barbirostris* complex. D-U is the universal primer that binds in the same position on the ITS2 DNA for all members of the complex. DW to DZ bind at different places to the ITS2 DNA of *An.barbirostris* species W, X, Y,Z

Primer name				Sequence	5' to 3'			Tm (°C)
DU	TGG	TGG	TGA	CTT	TCA	AGT	TC	60.1
DW	GAA	CTT	TTG	GAT	TGT	TCT	CA	56.7
DX	GGA	CTG	TGT	CAA	AGA	GAA	TGT	58.2
DY	GTG	TCG	GTA	GAT	TAC	TAG	TCG	A	57.4
DZ	TTA	CCC	CCG	AAA	AAA	CTG		59.9

Hybaid thermal cycler (HYBAID Omnigene) using DynaZyme or Gibco recombinant thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus*. After the PCR is complete, the entire 25-µ l reaction was electrophoresed on a 1.2% agarose gel containing ethidium bromide to verify product band size. DNA marker (VI) (Supplied by Promega) Mixture of pBR328 DNA, cleaved by *Bgl*I and pBR328DNA, cleaved with *Hif*I to give DNA fragments size: 1500bp - 100 bp. The amplified fragment is visualised by illumination with short wave ultraviolet light. PCR reagent and thermal cycler can be obtained from many different suppliers and this limited experience suggests that reaction conditions need to be optimised for different machines and different reagents, particularly different thermostable polymerase (Ballinger-Crabtree, 1992).

Results

The four consensus sequences for the ITS-2 region of each species were aligned and compared. The universal forward primer (DU) was designed to bind at a conserved region at positions 1256 – 1276 in ITS-2. The reverse primers (DW, DX, DY and DZ) were species-specific and bound to the unique regions . Trial PCR amplifications were performed using all four possible primers pair combination DU with DW, DX, DY and DZ. The PCR condition was optimised with respect to a number of parameters such as concentration of $MgCl_2$, primer, Taq, and DNA template.

The length of the sequence amplified between DU and each of the four species-specific primers is 339 bp for species W, 247 bp for species X, 165 bp for species Y and 157 bp for species Z. It was not possible to design suitable primers that differed in more than two nucleotides for species Y and species Z. Nevertheless primer DY and DZ produce consistent and reliable results. The differences in band size between each species was clearly seen on 1.2 % agarose gel, as shown in Figure 1.

Verification of the method with has been carried out with 270 mosquitoes from seven different field-collection sites using various sampling methods. Figure 2 represents 44 samples collected from Singaraja-Flores, which were identified as 13 species W, and 31 species X. All specimens collected from human bite outdoors were identified as species X; this species was also shown to predominant indoor light trap collections,
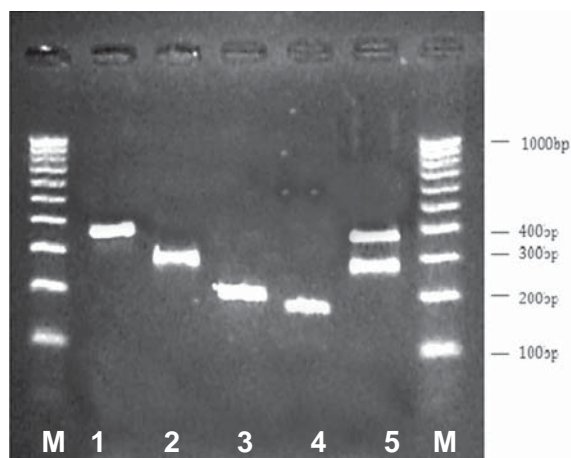

Figure 1. ITS2 DNA bands produced by PCR amplification from different species of *Anopheles barbirostris* complex. Track 1: Species W (339 bp); Track 2: Species X (247 bp); Track 3:Species Y(165 bp); Track 4: Species Z (157 bp) Track 5: Hybrid simulate between W/X. M: DNA ladder (VI) 100 bp size standard
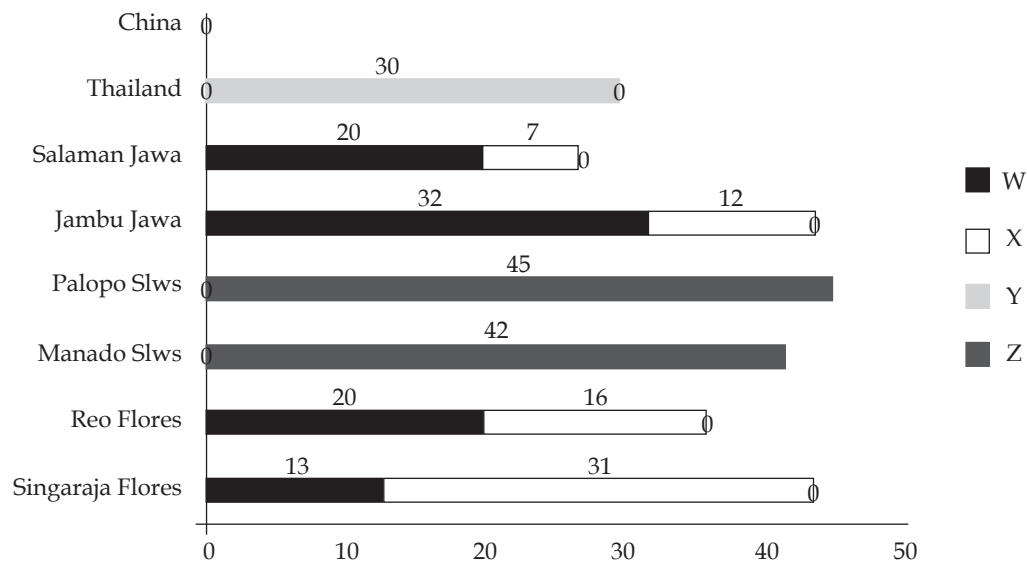
Figure 2. Represent 270 samples collected from seventh different site which were two sites from Jawa and two site from Flores identified species W and X , two sites from Sulawesi identified species Z and one site from Thailand identified species Y. The specimen from China was not appear although specific diagnostic primer (DU) to be able to identify *Anopheles barbirostris* from China

indoor human bait collections and indoor resting samples of samples from Reo-Flores, 20 were identified as species W and 16 as species X. All specimens from Manado and Palopo in Sulawesi were identified as species Z. Similarly only species Y was found in samples from Thailand, while specimens from Salaman and Jambu in Java were identified as species W or species X. Two specimens from China was not detected although specific diagnostic primer (DU) and 28S was able to identify *Anopheles barbirostris* from China

Discussion

The PCR of rDNA-ITS2 region has potential diagnostics tool for members of *Anopheles barbirostris* species complex. This assay has been used by a number of scientists for differentiating a wide variety of closely related mosquito species. Walton *et al.,* (1999) designed primers based on the nucleotide differences in the ITS2 region of five species of the *Anopheles dirus* complex from Thailand.. Similar work was done for the *Anopheles triannulatus* s.l. (Diptera: Culicidae) from Northwestern and Southeastern Colombia

(Rosero *et al.,* 2012), and six *Anopheles maculipennis* sibling species occurring in Europe (Romi *et al.*, 2000). Moreover the method has been used to distinguish 4 of the 5 cryptic sibling species, *Anopheles quadrimaculatus* Say, complex. (Cornel, 1996) as well as *Anopheles hyrcanus* group from Thailand and Indonesia. (Paredes-Esquivel *et al*, 2011). The method has shown that the *Culex vishnui* subgroup includes three important vectors of Japanese encephalitis virus, *Culex tritaeniorhynchus* Giles, *Culex pseudovishnui* Colless, and *Culex vishnui* Theobald (Toma *et al*, 2000).

Using this species-specific PCR reaction, species W and X seems to be sympatric in Java and Flores. No hybrids have been found in those locations where both species W and X occur in sympatry, although the sample sizes are admittedly small. A very small percentage of hybrids in a single locality do not affect the specific status of these closely related species (Colluzi *et al.,* 1979). Meanwhile species Z from both Palopo and Manado in Sulawesi Island occur alopatric. Species Y in this study perhaps one of three species, which is reported present in Thailand (Baimai, 1995). The specimen from China

possibly other species *barbirostris* complexes out of four species have been found.

As conclusion, the number of nucleotide differences in the ITS2 products and species-specific molecular markers for the *Anopheles barbirostris,* complex appear to be reliable over a wide geographical area. However further testing number of samples is needed from throughout the range of this species. We believe that this general approach to the development of species-diagnostic assays on ITS2 region can be extended easily to other complexes of closely related, morphologically indistinguishable species.

Acknowledgment

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