



Identification of Five Sibling Species of the *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand by a Polymerase Chain Reaction Assay

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Abstract

Anopheles barbirostris is a complex of six sibling species that are almost identical in adult morphology. The lack of accurate identification method, diagnosis based on only adult morphological characters has led to misidentification. This complex has been incriminated, as malaria and filarial vector in Indonesia and Thailand but knowledge of the epidemiology and vectorial roles of this species group are poorly known. For this purpose, a diagnostic PCR system to differentiate among five sibling species of the *An. barbirostris* species complex in Thailand was developed by using two PCR reactions. Based on nucleotide differences in the ITS2 sequences, novel degenerate primer was constructed for amplification in combination with a universal reverse primer to generate amplification products of specific length for each of species. The first PCR reaction can identify *An. barbirostris* species complex in four species, i.e., *An. dissidens*, *An. saeungae*, *An. barbirostris* species A3, and *An. barbirostris* s.s. by using universal primer pairs and the second PCR reaction can distinguish species *An. barbirostris* s.s. from *An. wejchoochotei* by using new primer and universal reverse primer. Additionally, *An. saeungae* can be clearly differentiated on PCR product from *An. barbirostris* s.s. and *An. wejchoochotei* by using new primer.

Keywords: *Anopheles barbirostris* complex, PCR identification, ITS2, mosquitoes

Introduction

Mosquitoes of genus *Anopheles* are responsible for the transmission of malaria disease, one of the most serious health problems in developing countries. Many of the primary anopheline vectors of malaria parasites in Southeast Asia belong to member of sibling species complexes (Baimai, Green, Andre, Harrison, & Peyton, 1984; Subbarao, 1998). Additionally, sibling species complex often contain both vector and non-vector species and two or more member species are often found sympatrically. Accurate species identifications are necessary for the study of *Anopheles* mosquitoes, i.e., diversity, behaviour, epidemiology, malaria transmission, relationships of *Anopheles* evolution particularly, malaria vector control programs. Correct identification of *Anopheles* species enables estimations of vector competence and important behavioral characteristics by species, leading to design the effective control method. Unfortunately, members of these species complexes are often share similar morphological characters, creating difficulties in identifying mosquitoes responsible for disease transmission and potentially leading to misidentification (Lounibos & Conn, 2000; Van Bortel et al., 2001).

In the past, mosquito taxonomists have achieved the accurate identification of members of the sibling species complexes by using several traditional methods, i.e., morphologic characteristics, cytogenetic, crossing experiment and isoenzyme markers (Norris, 2002). Nevertheless, terms of time and expertise required for the identification of large numbers of field-collected specimens are still limitations of these techniques (Norris, 2002). Moreover, these techniques are not practicable for the routine identification and also not applicable for all developmental stages of the mosquito (Norris, 2002). Since there are several limitations of the traditional methods, molecular methods have been developed and received great attention in a decade ago. The methods



have been applied to many group of mosquito species complex not only to sibling species, but also to members of closely related groups with overlapping morphologic characters (Scott, Brogdon, & Collins, 1993; Cornel, Porter, & Collins, 1996; Proft, Maier, & Kampen, 1999; Manonmani et al., 2001;).

The Barbirostris Subgroup of the Myzorhynchus Series includes six formally described species of almost identical morphology (Reid, 1962; Taai & Harbach, 2015) and at least one additional species, i.e., *An. barbirostris* species A3 (Saeung et al., 2008). Zoophagic *Anopheles barbirostris* is similar with the anthropophagic *Anopheles campestris* in external morphology, therefore it is often confused in the field identification. The pattern of wing scales used to distinguish these species (Reid, 1962) is not valid in Thailand (Harrison & Scanlon, 1975) and in a recent pictorial key for *Anopheles* mosquitoes (Rattanaarithikul, Harrison, Harbach, Panthusiri, & Coleman, 2006) does not use this character.

An. barbirostris has been incriminated as a natural vector of *Plasmodium vivax* and *Brugia malayi* in Indonesia (Syafuruddin et al., 2007). In Thailand, it was formerly considered a suspected vector of malaria and/or filariasis (Iyengar, 1953). Recently, mosquitoes *An. barbirostris* complex has been incriminated as potential natural vectors of *P. vivax* in the Aranyaprathet district, Sa Kaeo province (Limrat, Rojruthai, Apiwathanasorn, Samung, & Prommongkol, 2001; Apiwathanasorn, Prommongkol, Samung, Limrat, & Rojruthai, 2002) and has been implicated in the increase in malaria cases in Thailand (Sattabongkot, Tsuboi, Zollner, Sirichaisinthop, & Cui, 2004). The morphological, cytological, hybridization and molecular analysis have recently revealed that *An. barbirostris* complex in the Thai population consisted of at least six sibling species, namely, *An. wejchoochotei* (formerly *An. campestris*-like), *An. dissidens* (formerly *An. barbirostris* species A1), *An. saeungae* (formerly *An. barbirostris* species A2), *An. barbirostris* species A3 (Saeung et al., 2007; 2008), *An. barbirostris s.s.* (formerly *An. barbirostris* species A4) (Suwannamit et al., 2009) and *An. campestris* (Reid, 1962). According to our previous studies, we could not distinguish *An. barbirostris s.s.* from *An. wejchoochotei* by using PCR method based on ITS2 (Suwannamit et al., 2009).

In this study, we described the development and use of a method, based on two PCR reactions for identifying *An. barbirostris s.s.* and *An. wejchoochotei* and distinguish the four cryptic species of Barbirostris complex, *An. dissidens*, *An. saeungae*, *An. barbirostris* species A3 and *An. barbirostris s.s.*, except *An. campestris* due to the lack of samples.

Methods and Materials

Mosquito

The five isoline colonies of mosquito from the previous collection, i.e., *An. wejchoochotei* (Chiang Mai, iHCE6: F48), *An. dissidens* (Chiang Mai, iACA6: F46), *An. saeungae* (Phetchaburi, iAPA13: F46), *An. barbirostris* species A3 (Kanchanaburi, iAKA5: F46), and *An. barbirostris s.s.* (Chiang Mai, iACA18: F36) samples were examined in this study. They were routinely maintained in the insectariums at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand for many consecutive generations by the techniques described by Choochote, Sucharit, and Abeywickreme (1983).

Extraction of DNA

Genomic DNA was extracted from a single individual adult mosquito using a DNeasy[®] blood and tissue kit (Qiagen) according to the manufacturer's instructions.



PCR identification assay

Method for identification was arrived by conducting two PCR reactions. The first PCR reaction, the ITS2 region of the rDNA was entirely amplified by PCR using primers described by Beebe and Saul 1995. The sequence of the forward primer was complementary to a conserved region of the 5.8S rDNA (ITS2A; 5'-TGTGAACTGCAGGACACATG-3') whereas the reverse primer annealed to a conserved 28S rDNA region (ITS2B; 5'-ATGCTTAAATTTAGGGGGTA-3'). PCR was carried out in a 20 µl reaction containing 0.5 U of Ex Taq (Takara), 1× Ex Taq buffer, 2 mM of MgCl₂, 0.2 mM of each dNTP, 0.25 µM of each primer, and 1 µl of the extracted DNA. The amplification profile comprised initial denaturation at 95°C for 1 minute, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplified products were electrophoresed through 0.6% agarose gel. After staining with ethidium bromide, the gel was visualized under a UV light.

For the second reaction consisted of the ITS2B primer along with new species-specific forward primer (ITS2FA4Cm; 5'-ATCCAATTT(C/T)(G/T)TT(T/G)GGTG-3'). The condition and compositions of the PCR mixtures were the same as for the amplification of the ITS2 region, except that the ITS2A forward primer was replaced with the ITS2FA4Cm primers. The amplified products were electrophoresed through 2.0% agarose gel.

Results

Our strategy for the specific amplification to identified members of the *An. barbirostris* species complex was two PCR reactions by using new and universal primers (Table 1). The first PCR reaction was done by using primer pairs ITS2A and ITS2B for identified *An. barbirostris* mosquitoes to *An. dissidens*, *An. saeungae*, *An. barbirostris* species A3 and *An. barbirostris s.s.* This could be verified experimentally as depicted in Fig. 1. The ITS2 PCR product of the *An. barbirostris* species A3 was clearly different in length from *An. dissidens*, *An. saeungae*, *An. barbirostris s.s.* and *An. wejchoochote* while PCR product of *An. dissidens* was slightly different from *An. saeungae*, *An. barbirostris s.s.* and *An. wejchoochote* (Table 2). For *An. saeungae*, *An. barbirostris s.s.* and *An. wejchoochote*, ITS2 PCR product of three species was quite similar in length (Fig. 1, lane 2, 4 and 5, Table 2). The second PCR reaction was used to distinguish *An. barbirostris s.s.* from *An. wejchoochote* by using primer ITS2FA4Cm and ITS2B. As shown in Fig. 2, amplification with these primers resulted in the product in the expected size (420 and 486 bp) and yielded products of different sizes for each species; *An. barbirostris s.s.* and *An. wejchoochote* (lane 4 and 6).

Additionally, PCR product of 1:10000 dilution PCR product of *An. barbirostris s.s.* and *An. wejchoochote* by using ITS2A and ITS2B primer was similar pattern with extracted DNA and several unexpected bands were obtained for *An. barbirostris s.s.* (Fig. 2, lane 3). Nevertheless, the both of PCR products of *An. saeungae* was not obtained by using these primer pair (Fig. 2, lane 1 and 2). The success of the PCR demonstrated that these primers could distinguish *An. barbirostris s.s.* from *An. wejchoochote*.

Table 1 Details of the three primers designed for the two PCR reactions for the identification of the five species of the Barbirostris complex

Primer name	Sequence (5'-3')	CG%	Tm (°C)
ITS2A	5'-TGTGAAGTGCAGGACACATG-3'	50	52
ITS2B	5'-ATGCTTAAATTTAGGGGGTA-3'	35	46
ITS2FA4Cm	5'-ATCCAATTT(C/T)(G/T)TT(T/G)GGTG-3'	38	49

Table 2 PCR product sizes and primers used for identification of the five species of the Barbirostris complex

Primers	Species	PCR product size (bp)
ITS2A, ITS2B	<i>An. dissidens</i>	1861
	<i>An. saeungae</i>	1717
	<i>An. barbirostris</i> species A3	1070
	<i>An. barbirostris</i> s.s.	1676
	<i>An. wejchoochotei</i>	1651
ITS2FA4Cm, ITS2B	<i>An. saeungae</i>	-*
	<i>An. barbirostris</i> s.s.	420
	<i>An. wejchoochotei</i>	486

Note: * not amplified

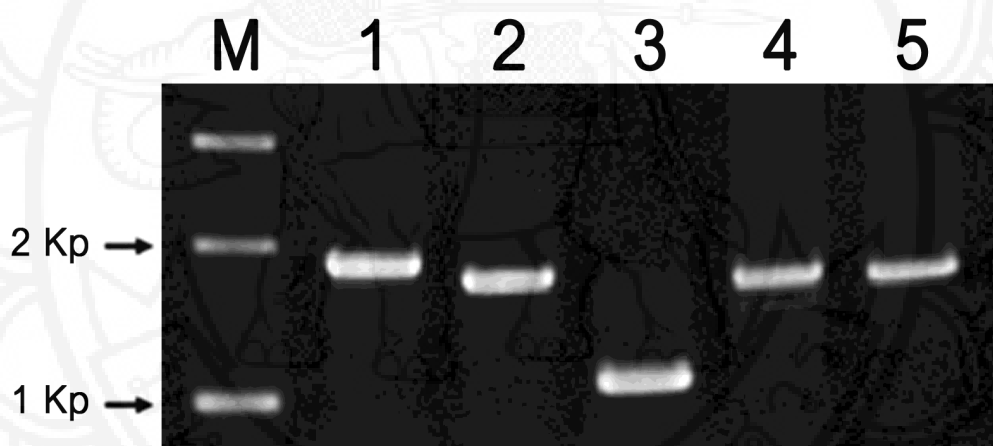


Figure 1 PCR products of ITS2 amplification for *An. barbirostris* species complex by using ITS2 and ITS2B primer on a 0.6% agarose gel

Note: Lane M, 1 Kb molecular weight ladder; Lane 1-5, *An. dissidens*, *An. saeungae*, *An. barbirostris* species A3, *An. barbirostris* s.s., and *An. wejchoochotei*, respectively

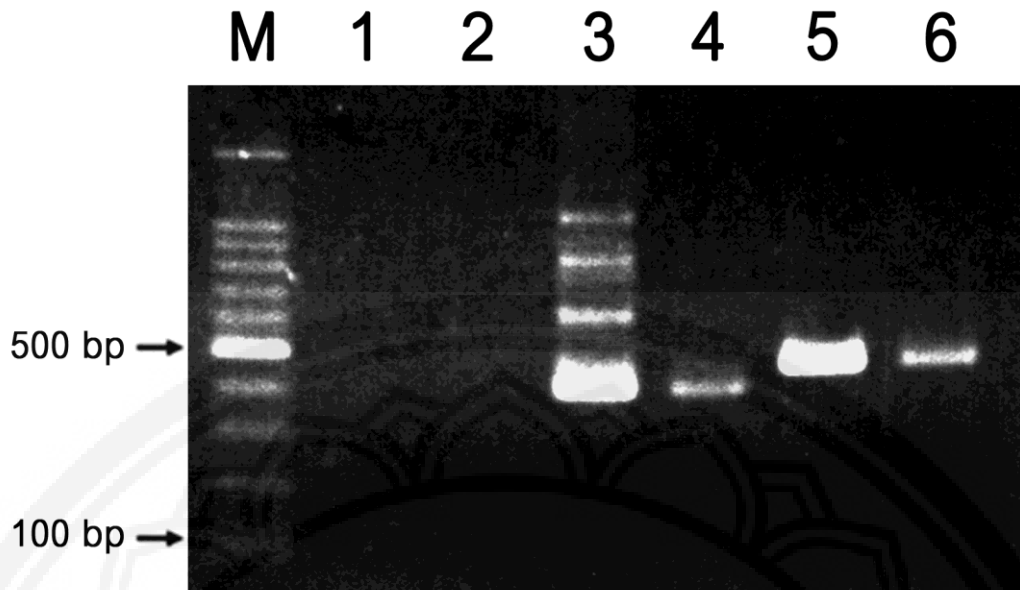


Figure 2 Specific PCR products for *An. saeungae* and *An. barbirostris s.s.* and *An. wejchoochotei* by using ITS2FA4Cm and ITS2B primers on a 2% agarose gel. 10000 fold serial dilutions of the first PCR reaction product were amplified by PCR for reducing unexpected bands

Note: Lane M, 100 bp molecular weight ladder; lane 1 and 2, 1:10000 dilution PCR product and gDNA of *An. saeungae*; lane 3 and 4, 1:10000 dilution PCR product and gDNA of *An. barbirostris s.s.*; lane 5 and 6, 1:10000 dilution PCR product and gDNA of *An. wejchoochotei*

Discussion

Comprehensive understanding of the epidemiology and transmission dynamics of malaria in any given area is obtained from precise knowledge of the biology and distribution of vector species. However, precise knowledge has been limited by inexistence reliable and accurate identification methods. Morphologic characteristics and other traditional methods are often difficult to apply because of a number of specimens or technical sections. Therefore, the limitations of these methods have been circumvented with DNA-based methods because all developmental stages of the both sexes can be identified. PCR assay based on nuclear ribosomal ITS2 sequence has already provided a powerful diagnostic tool for the study of anopheline species complexes, such as *Anopheles gambiae* (Scott et al., 1993), *Anopheles maculipennis* (Proft et al., 1999), *Anopheles quadrimaculatus* (Cornel et al., 1996), *Anopheles fluviatilis* (Manonmani et al., 2001), and *Anopheles dirus* (Xu, Xu, & Qu, 1998).

In the study of *An. barbirostris* species complex, our previous study failed to distinguish *An. barbirostris s.s.* from *An. wejchoochotei* by using primers followed the previous studies of Saeung et al. (2007, 2008). Since it appeared that the primers were not specific enough to distinguish them. Because PCR product band of *An. barbirostris s.s.* was the same size as the PCR product of *An. wejchoochotei* (Fig. 1, lane 4 and 5). Consequently, ITS2 region of *An. barbirostris s.s.* contains similar sequence and length as *An. wejchoochotei*. The length of the ITS2 region in the two species of *An. barbirostris* species complex, *An. barbirostris s.s.* and *An. wejchoochotei* was found to be 1676 and 1651 bp, respectively. Therefore, in this study we describe the development of PCR identifications assay of five of six sibling species of *An. barbirostris* species complex in Thailand.



Two PCR reactions were performed for this purpose. Primer pairs followed the previous studies successfully identify *An. barbirostris* complex to *An. dissidens*, *An. saeungae*, *An. barbirostris* species A3 and *An. barbirostris* s.s. in the first PCR reaction. And the second PCR reaction successfully distinguish *An. barbirostris* s.s. from *An. wejchoochotei* by using new primer in combination with ITS2B primer. Interestingly, PCR product of *An. saeungae* by ITS2A & ITS2B in the first reaction was quite similar in length with *An. barbirostris* s.s. and *An. wejchoochotei*. Although we can distinguish *An. saeungae* from *An. barbirostris* s.s. and *An. wejchoochotei* in the first reaction but it is difficult to do. However, as shown in Fig. 2., PCR product of *An. saeungae* was not obtained whereas *An. barbirostris* s.s. and *An. wejchoochotei* was obtained in different size in the second reaction. Hence, we can use these primers in order to identify and/or confirm *An. saeungae*.

Recently, a multiplex PCR based on *COI* sequences was developed for the identification of five sibling species in this complex (Wilai et al., 2020). However, the assay requires six primers for PCR reactions. The other methods available till date for the identification of sibling species in this complex were the karyotyping of the ovarian polytene and metaphase chromosomes. Although cytogenetic is reliable method to distinguishes any cryptic species (e.g., Coluzzi, Petrarca, & Deco, 1985), it is not practicable for the routine identification and also not applicable for both sex and all developmental stages of the mosquito (Norris, 2002). Since this method requires the larval or adult stage but specimen rearing is tedious, time consuming and may be not successful for some species. Additionally, the result interpretation needs much experience and training. For this reason, it is not widely used in field studies. Moreover, recent study (Saeung et al., 2008) suggested that metaphase karyotype couldn't use to differentiate the sibling species of *An. barbirostris* Form A in Thai populations.

Unfortunately, in this study we did not include *An. vanderwulpi*, which found in Indonesia and *An. campestris* due to lack of samples. Thus, the further study, primers to identify two this species have been designed and analyzed with field samples.

Conclusion and Suggestion

In conclusion, the results obtained from this study showed that this identification method provides a reliable tool for the differentiation of five Thai *An. barbirostris*. The method is likely to be a powerful tool in survey of this complex over the large geographic area. Although PCR method needs sophisticated equipment and sensitive reagent, it is easy to apply in practice and many specimens can be examined by a single person in time unit. Moreover, it is highly sensitive, requiring only a small part of a mosquito and DNA also could be recovered from all specimen of extensive preserved and preservation by a wide variety of simple conservation methods, be it fixation in alcohol or drying with silica gel. Further study, we need to validate this method with field samples for screening *An. barbirostris* species complex for large- scale of geographic distribution and epidemiological studies of malaria in Southeast Asia.

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