A RAPID POLYMERASE CHAIN REACTION BASED METHOD FOR IDENTIFICATION OF THE *ANOPHELES DIRUS* SIBLING SPECIES

Ngo Thi Huong[1], Piengchan Sonthayanon[2], Albert J Ketterman[2] and Sakol Panyim[2]

[1]Institute of Malariology, Parasitology and Entomology, Quinhon, Vietnam;
[2]Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand

Abstract. A simple polymerase chain reaction (PCR) based method was developed to differentiate the *Anopheles dirus*, species A, B, C and D in Thailand using specific primers designed from species specific sequences. The PCR protocol was optimized to obtain products of 120 bp, 75 bp, 60 bp and 172 bp for species A, B, C and D, respectively. This method used a cocktail of four primer sets to identify these *An. dirus* sibling species. The method is very sensitive as only a small portion of mosquito was required allowing the rest of the mosquito to be used for other analyses. Specimens also kept for up to 14 years could be analyzed unambiguously from either larvae or adult. This method is advantageous over other PCR-based methods for identification of malaria vectors because it does not require any specific DNA extraction. A mosquito specimen was homogenized in 1x PCR buffer, then the supernatant directly used for PCR identification, allowing a large number of samples to be processed at the same time. It provides a simple and rapid practical method for screening *An. dirus* species, which is essential in malaria vector epidemiological studies in Southeast Asia.

INTRODUCTION

Anopheles dirus one of the major malaria vectors in Southeast Asian countries including Thailand, Vietnam, Lao PDR and Myanmar. *An. dirus*, according to Peyton and Harrison (1979), belongs to a species complex (Rosenberg *et al*, 1990). The complex consists of seven morphologically similar species. Five species of which have been recognized in Thailand as *An. dirus* A, B, C, D and F (Baimai *et al*, 1988a; b). Sibling species complexes are reproductively distinct but can not be distinguished by morphological features alone and require alternative methods for identification. It is important to distinguish these species in disease vector control programs as the different species may exhibit differences in ecology, vectorial capacity and response to control measures (White, 1972).

The definitive method for species identification of mosquito malaria vectors is to perform cross-breeding experiments against laboratory colonies of known species (Hill and Crampton, 1994). Enzyme electromorphs also have been used as a means of vector identification for members of *An. dirus* from Thailand (Green *et al*, 1992). The limitation of this technique is that not all sibling species have isoenzyme patterns (Yong *et al*, 1983). Moreover, specimens must be stored frozen in liquid nitrogen prior to identification (Hill and Crampton, 1994). The most reliable and widely used methods for species identification are chromosomal techniques. *An. dirus* can be separated on the basis of metaphase chromosomes using the Giemsa and Hoechst 33258 staining technique (Baimai *et al*, 1984). However, this method is not always successful because readable polytene chromosomes can only be obtained from fourth-instar larvae but field collected females sometimes die before laying eggs. Another limitation is the skill necessary to perform the chromosome spread technique.

To improve the ability for sibling species

Correspondence: Piengchan Sonthayanon,
Fax: (+66) 2441-9906
Email: stprj@mahidol.ac.th

identification, DNA based methods have been investigated. DNA based technologies differ from most other identification techniques in that they detect the DNA itself. DNA remains constant irrespective of life cycle stage or environment, allowing identification techniques to work on both sexes and all developmental stages. DNA based methods of identification ultimately rely upon the use of either DNA probe hybridization or polymerase chain reaction (PCR).

The polymerase chain reaction has been used for identification of several species complexes. Ribosomal DNA intergenic regions have been developed for species identification of the *Anopheles gambiae* complex (Scott *et al,* 1993) and the *Anopheles maculipenis* complex (Porter and Collins, 1991). Recently, ribosomal DNA internal transcribed spacer 2 has been used in allele specific amplification to differentiate five species of the *An. dirus* complex in Thailand (Walton *et al,* 1999). However, the method still requires genomic DNA extraction from single mosquitos.

DNA probe for the species identification of the *An. dirus* complex in Thailand were successfully identified by both radiolabeled and non-radiolabeled probes (Panyim *et al,* 1988a; b). With species-specific sequence information available for *An. dirus*, we have developed a simple and rapid practical method for species identification of *An. dirus* using PCR based method

MATERIAL AND METHODS

Mosquito specimens of *An. dirus* species A, B, C and D were chromosomal identified by Dr Visut Baimai of the Department of Biology, Faculty of Science, Mahidol University. Some samples of mosquito species A, B and D were obtained from AFRIMS and Malaria Division, Department of Communicable Disease Control, Bangkok Thailand. This study also included 73 wild-caught female *An. dirus* from several provinces in Central Vietnam.

Extracted genomic DNA of *An. dirus* A, B and C were obtained from the Institute of Molecular Biology and Genetics, Mahidol University.

Species specific sequences of *An. dirus* A, B, C and D contained in plasmids, namely pMU-A40.1#5 (128 bp), pMU-B5 (124 bp), pMU-C19.2 (278 bp) and pMU-D10 (295 bp) respectively were obtained from the Institute of Molecular Biology and Genetics.

Crude DNA from a single mosquito was prepared for PCR amplification. A single mosquito was homogenized with 50 µl of 1x PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.01 % gelatin, pH 9) in an eppendorf tube for 3-5 minutes. The solution was boiled at 100ºC for 10 minutes, then quickly placed on ice for 5 minutes. The cell debris was removed by centrifugation at 14,000 rpm for 30 seconds. Then, 5 µl of the supernatant, which contained genomic DNA, was used directly for PCR amplification.

The PCR amplification method was performed in a total of 50 µl containing 200 µM dNTPs each, 5 µl of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin; pH 9.5), primers of species A and D 0.4 µM each, primers of species B 0.6 µM each and primers of species C 0.8 µM each and 5 µl template. The mixture was preheated 2 minutes at 94ºC, then 1 µl of *Taq* DNA polymerase was added at 72ºC followed by 25 cycles of amplification at 94ºC for 15 seconds, 60ºC for 15 seconds, 72ºC for 15 seconds and a final extension step at 72ºC for 5 minutes.

After amplification, the PCR product was analysed by 3% agarose gel electrophoresis. The gel was stained with ethidium bromide and the DNA was visualized and photographed under ultraviolet light.

RESULTS

Primer design

Four primer sets for identification of *An. dirus* species A, B, C and D were selected as shown in Table 1. The melting temperatures of the primers were selected to be similar to

Table 1
Primers used for identification of *An. dirus* sibling species A, B, C and D.
T_m (melting temperature) was calculated by the formula $T_m = 4(G+C) + 2(A+T)$.

Species	Specific sequence (bp)	Primer's name	Primer sequences (5′-3′)	Position (5′-3′)	Length (bp)	T_m	G+C (%)	Product size (bp)
A	128	FA2	TCG GGT TCT ATA ATA TTC GCT	9	21	58	38	120
		RA2	GAC CTA GTG TTT GGG AAG GT	109	20	60	50	
B	124	FB2	GCT TCA AGA CCA AAA CCA TCA	15	21	60	42	75
		RB2	GAA TTT ACA ACT TTT GAC CTG G	68	22	60	36	
C	278	FC3	ATT CTG TGC CAA AAT TGT ACC T	1	22	60	36	60
		RC3	TTG TCC GAA ACT GGC TTC T	42	19	56	47	
D	295	FD1	AGG GCA CAA AAG TTA TTA ACT T	11	22	58	34	172
		RD1	GTG AAG AGC GAA TAT TGT AGC	162	21	60	42	

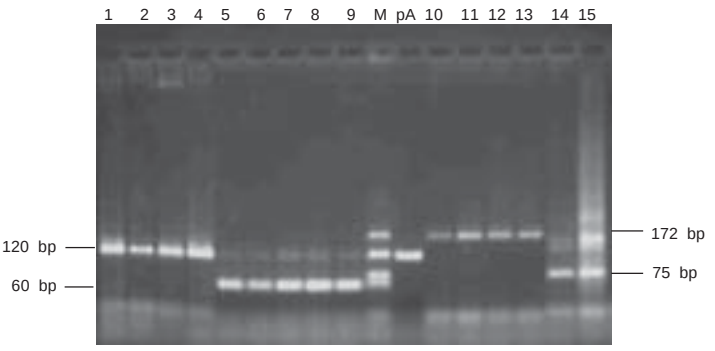

Fig 1–PCR amplification from chromosomal identified mosquito species A, B, C and D. M: DNA marker (172, 129, 75, and 60 bp) pA: pMU-A40.1#5 DNA. Lanes 1-4: individual mosquitos, species A. Lanes 5-9: individual mosquitos species C. Lanes 10-13: individual mosquitos, species D. Lanes 14-15: individual mosquitos species B.

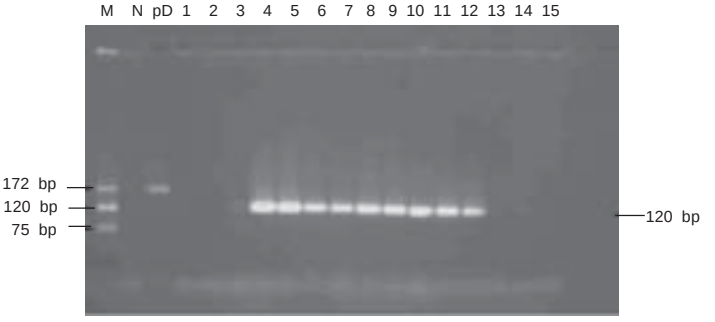

Fig 2–PCR amplification from body parts of *An. dirus* species A M: DNA marker. N : no DNA template. pD: pMU-D10 DNA Lanes 1-3: From heads of individual mosquitos. Lanes 4-6: From thorax of individual mosquitos. Lanes 7-9: From abdomen of individual mosquitos. Lanes 10-12: From legs of individual mosquitos. Lanes 13-15: From wings of individual mosquitos.

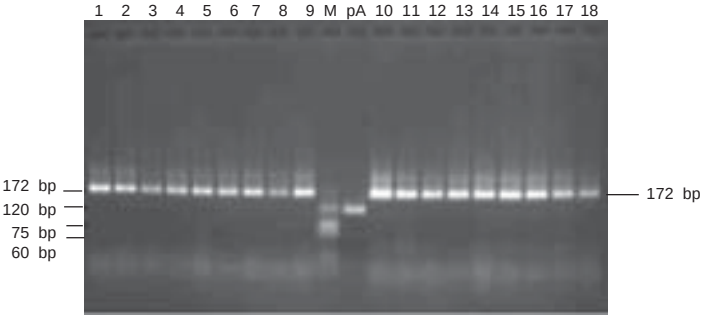

Fig 3–PCR amplification from identified *An. dirus* species D kept in isopropanol since 1986. M: DNA marker. pA : pMU-A40.1#5 DNA. Lanes 1-8: individual adult mosquitos. Lanes 9-18: individual larva.

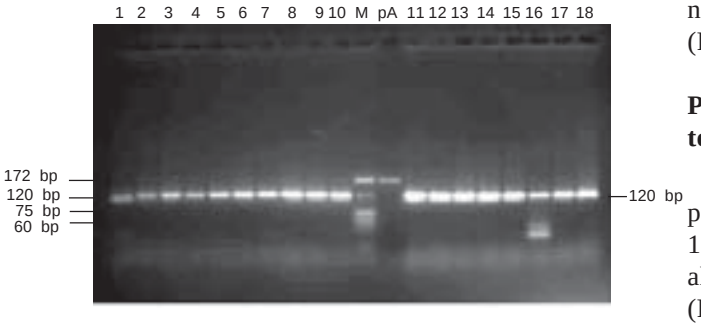

Fig 4–PCR amplification from unknown *An. dirus* collected from Central Vietnam. M: DNA marker. pA: pMU-D10 DNA (172 bp). Lanes 1-18: individual mosquitos collected from Khanh Phu-Khanh Hoa.

enable combining all the primers in one PCR reaction for species identification. However, G-C content of all primers was not similar. It varied from 34% to 50% because of the size limitation but selected primers yielded PCR products that could be differentiated on agarose gel.

PCR amplification from single identified mosquitos

PCR amplification was performed using 5 μl of supernatant containing crude genomic DNA from single mosquitos of species A, B, C and D. The expected PCR products of 120 bp, 75 bp, 60 bp and 172 bp were obtained from the single mosquitos of species A, B, C and D, respectively (Fig 1). The specificity of this method was performed by blind test with 109 chromosomal identified mosquitos of species A, B, C and D including 20 A, 45 B, 39 C and 5 D. One hundred percent of the samples were correctly identified by the PCR method.

PCR amplification from isolated body parts of a single mosquito

Body parts of single mosquitos of species A including head, thorax, abdomen, wing and leg were used to prepare 5 μl of crude DNA used for PCR amplification. The results demonstrated that a thorax, abdomen or leg of a mosquito could be used for species identification. The head gave a very weak signal and no PCR product was obtained from the wings (Fig 2).

PCR amplification from specimens after long term storage

The method could be used on samples preserved in 95 % ethanol or isopropanol since 1986. Either larva or adult could be used, although larva yielded a slightly higher signal (Fig 3).

PCR amplification from unknown *An. dirus* from Vietnam

A total of 73 field-collected specimens from several provinces in Central Vietnam were examined by this method. 67 specimens were identified as species A. Four specimens gave negative results and two specimens yielded two PCR products. (Table 2 and Fig 4).

DISCUSSION

Although the primers selected for identification of species A were highly specific; they also amplified genomic DNA of species C. This band was the same size as the PCR product of species A, that is, 120 bp. It is possible that genomic DNA of species C might contain similar sequence as species A. Several studies support this possibility. A genetic study of the relationships of 5 species of *An. dirus* suggested that species C has recently diverged from species A and has retained the chromosomal banding sequence identical to the standard ancestor (Poopittayasataporn and Baimai, 1995). Recently, Walton *et al* (1999) found that the ITS2 (Internal Transcribed Spacer 2) sequence in the ribosomal DNA of species A and C are almost identical. In the central hypervariable region of ITS2 sequence of species A and C there is a micro-satellite composed of CA and GT repeat units. It is only in this area that *An. dirus* A and C differ from each other by a single base change and a GT repeat unit. In addition, DNA probe of species A crossed hybridized with genomic DNA of species C (Chanama, 1994), but not vice versa (Sonthayanon, unpublished data). Primers for

Table 2
List of unknown *An. dirus* collected from the Central Vietnam.

No.	Location	Time of collection	PCR identification			Total
			Species A	(-)	2 PCR products	
1	Khanh Phu-Khanh Hoa	1995, 1999, 2000	29	0	1	30
2	Iakor-Gialai	1996-1999	16	0	0	16
3	Van Canh-Binh Dinh	1986	10	2	1	13
4	An Truong-Binh Dinh	1989	2	1	0	3
5	Eale-Daklak	1996	1	0	0	1
6	Son Hoa-Phu Yen	1990-1991	9	1	0	10
	Total		67	4	2	73

species B could be used for identification of single mosquitos of this species. Although these primers did not generate a single PCR product, the pattern of PCR products did not interfere with the identification of other PCR products. Primers for species C and D were highly specific to the target sequence. Using a mixture of all four primer sets it was possible to identify 109 specimens of *An. dirus* A, B, C and D. The results obtained were in agreement with the chromosomal identification. This method provided a rapid and simple method to identify unknown *An. dirus* species found in Central Vietnam. Most of the specimens were identified as species A. This result indicates that *An. dirus* species A found in Thailand and Central Vietnam are similar. Several specimens gave negative results. It is possible that the DNA was degraded during the long period of preservation or the specimens might represent new members in the *An. dirus* complex. Another two specimens gave two distinct PCR products. Therefore, overall the data suggests that these specimens might belong to one or more unidentified groups within the *An. dirus* complex. However, more samples need to be analyzed to confirm this possibility.

In conclusion, the results obtained from this study demonstrated that this method is highly sensitive, needing only a small part of a mosquito for species identification. Specimens of either larvae or adult stage that had been preserved for 14 years could be identified unambiguously. These primer sets are very specific to the *An. dirus* species, generating no PCR products with other anopheline mosquitos tested including *An. balabacensis, An. leucosphyrus, An. minimus, An. aconitus and An. jeyporiensis*. Even a blood meal in the mosquito did not give a problem for species identification. The method is very simple compared to other PCR based identification methods of malaria vectors because it does not require any specific DNA extraction. It allows processing large numbers of samples at the same time, providing a simple and rapid practical method for screening *An. dirus* species. This will be a valuable tool for malaria vector epidemiological studies in Southeast Asia.

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