MORPHOLOGICAL VARIATIONS OF *ANOPHELES MINIMUS* A IN TAK PROVINCE, THAILAND

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Abstract. *Anopheles minimus* Theobald is one of the major vectors of malaria throughout the Oriental Region, and it's complex consists of at least 2 sibling species (A and C) in Thailand. This study aimed to determine the morphological variations of wings of *An. minimus* A and to clarify the specific status of *An. minimus* in Ban Khun Huay, Ban Pa Dae, and Ban Tham Seau, Mae Sot district, Tak Province, Thailand. Anopheline larvae were collected from the fields between October 2002 and September 2003, allowed to emerge into adults in the laboratory and identified by morphological and molecular characterization. About 1,715 of female *An. minimus* A were separated into 8 groups based on their wing scale patterns. Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) assay (ribosomal DNA ITS2) confirmed the identification of *An. minimus* A in all 8 groups.

INTRODUCTION

Anopheles minimus Theobald belongs to the Myzomyia series of subgenus *Cellia,* and its complex consists of 3 sibling species, A, B and C, with the first 2 occurring in Thailand (Green *et al,* 1990; Somboon *et al,* 2001; Harbach, 2004). Other members of the Myzomyia series are found in Thailand including *An. aconitus*, *An. culicifacies, An. jeyporiensis, An. pampanai* and *An. varuna* (Harrison *et al,* 1980). *An. minimus* is one of the most important malarial vectors in hilly-forested regions containing small, cool fresh-water streams. It has been reported in southern and southeastern Asia (Reid, 1968), including southern China, the eastern part of Taiwan and the Ryukyu Archipelago of Japan. It is often been misidentified because of its high morphological variability and similarities to other members of the *Myzomyia* series, such as *aconitus, pampanai* and *varuna.* Since *An. minimus* is a vector of the human malarial parasite, misidentification of this species can be a real problem to public health officials in conducting their vector control activities.

In a study in Thailand, Harrison (1980) found 12 different characters in the wing scales, maxillary palpus and proboscis of *An. minimus,* from a total of 2,264 females collected as adults or reared from 4th stage larvae. Sucharit *et al* (1988) noted 3 different forms of *An. minimus* complex (species A and C) based on wing variations, namely normal *minimus* form M (costa with presector pale spot, PSP), and *varuva* form V (costa without prehumeral pale spot, PHP, humeral pale spot, HP and PSP), *pampanai* form P (costa with HP and PSP). In Chen's study of the molecular and morphological characteristics *An. minimus* in southern Thailand, wings from 150 *An. minimus* A, 144 *An. minimus* C, and 34 *An. aconitus* were examined for the HP and PSP spots, the separation of accessory pale (ASP) and sector pale (SP) spots, and the presence of a median pale spot on the media 1 (M_1) vein (Chen *et al,* 2002). His results showed that an HP spot occurs in low frequencies in wild-caught

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females (7.3-15.6%). A PSP spot is present much more frequently in wild-caught *An. minimus* A (88.4-100%) than in wild-caught specimens of *An. minimus* C (46.9-75.0%) or *An. aconitus* (20.0-28.6%). Concerning the frequency of occurrence of a PSP spot in colony specimens of *An. minimus* A, the range of 81.5-94.1% is within the range for wild-caught mosquitoes, whereas this spot is absent in colony samples of *An. minums* C. One or two median pale spots on the M_1 vein occur much more frequently in wild-caught *An. minimus* A (68.1% of the females, 100% of the males) than in wild-caught *An. minimus C* (28.1% of females, 62.5% of males). However, Chen *et al* (2004) found no significant difference in the occurrence of these spots in colony samples of the two species (75-100%). Because of the variable occurrence of the four wing spot characters in both *An. minimus* A and C they can not be used with any degree of confidence in order to distinguish them. Adults of *An. minimus, An. aconitus, An. culicifacies, An. jeyporiensis,* and *An. varuna* appear very similar to one another, with overlapping ranges of morphological characteristic variations, often resulting in their misidentification (Harrison, 1980). As shown by the results of Chen *et al* (2004), morphological variations of the *An. minimus* complex are multiple and very specific. However, misidentification of morphological variations is very common. The present study is therefore crucial in helping people to understand more about the significant morphological variations of *An. minimus*.

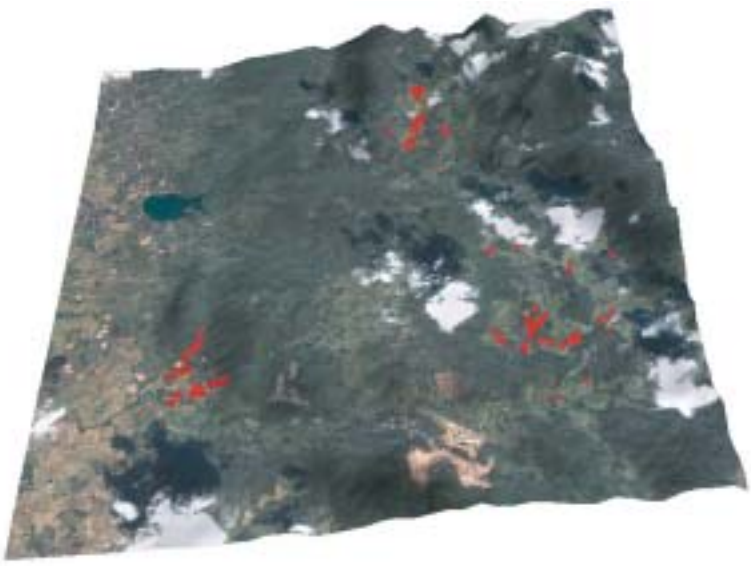

Fig 1–The locations of Ban Khun Huay (in the middle of the red dots at the top), Ban Pa Dae (bottom left) and Ban Tham Seau (bottom right) are displayed on a 100 km^2, true color IKONOS satellite image (resolution 1x1 m) overlaid on a Digital Elevation Model (DEM) to depict 3-dimensions (clouds shown in white, cloud-shadows appear as black areas north of clouds. Red dots represent the breeding habitats of anopheline larvae.

MATERIALS AND METHODS

Anopheline larvae were collected between October 2002 and September 2003 from three villages in Thailand: Ban Khun Huay, Ban Pa Dae, and Ban Tham Seau in Mae Sot district, Tak Province (Fig 1) from diversified habitats, such as stream margins, stream pools, rice fields, rock pools, ground pools, swamps, flood pools, swamp wheel tracks, and animal foot prints. Larvae from each habitat were collected and put into different bags, and labelled with a collection number, depending on the habitat type from which the larvae were obtained. Larvae from each collection were kept alive and sent to a laboratory in Bangkok, then allowed to develop into adults under laboratory conditions. Emerged adults were identified by using the keys of Rattanarithikul and Panthusiri (1994) and Harrison (1980). The terminology and abbreviations of Harbach and Knight (1980, 1982) are used for the morphological wing characters and illustrations, except for wing spot nomenclature (Wilkerson and Peyton, 1990) (Fig 2). *An. minimus* were selected and classified into different groups based on the variation of the wing spots.

We used Montage Explorer and Auto-Montage software programs (Syncroscopy, Frederick, MD) to examine and capture the digital images of mosquito specimens. To study the morphological variations of *An. minimus*, the images from each group of specimens were then compared with each other. The identifications of sampled

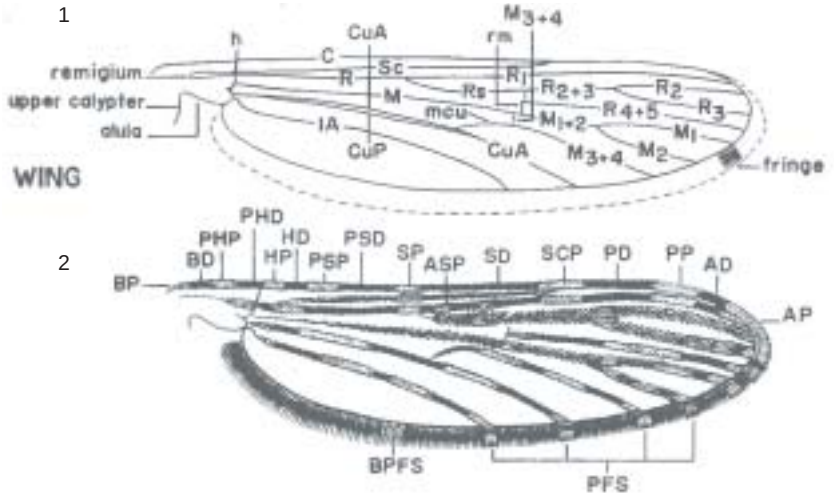

Fig 2–*Anopheles* adult wing, dorsal view, showing veins (1) and wing spots (2) AD, apical dark; AP, apical pale; ASP, accessory sector pale; BD, basal dark; BP, basal pale; BPFS, basal pale fringe spot; HD, humeral dark; HP, humeral pale; PD, preapical dark; PFS, pale fringe spot; PHD, prehumeral dark; PHP, prehumeral pale; PP, preapical pale; PSD, presector dark; PSP, presector pale; SCP, subcostal pale; and SP, sector pale.

specimens from each group of adult *An. minimus* A , *An. minimus* C and *An. aconitus* were confirmed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay (Van Bortel *et al,* 2000). The ribosomal DNA internal transcribed spacer 2 (ITS_2) was amplified using the primers ITS_2A (5′-TGTGAACTGCAGGACACAT-3′) and ITS_2B (TATGCTTAAATTCAGGGGGT-3′). The amplification was carried out in a 25 µl reaction mixture containing 3 mM $MgCl_2$, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 200 µM of each dNTP, 500 nM of each primer, 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 2.5 µl DNA template. The PCR condition was started by denaturing it at 94ºC for 5 minutes, followed by 30 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 1 minute, and a final elongation at 72ºC for 7 minutes. Two restriction enzymes, MspI (5′-CCGG-3′) and Sau96I (5′-GGNCC-3′) (New England Biolabs, Beverly, MA), were used to differentiate the sequences of the various species. Each reaction mixture contained 7 µl of sterile distilled water, 2 µl of buffer provided by the manufacturer, 1 µl of the enzyme and 10 µl of PCR-amplified template. The mixtures were then incubated at 37ºC for two hours. Digested PCR samples were subjected to electrophoresis in a 2% agarose gel to confirm each group of adult specimens. The ITS2 sequence of rDNA for *An. minimus* A is listed in the GenBank (Accession no. AF194504; http://www.ncbi.nlm.nih.gov/entrez/query. fcgi).

RESULTS

About 1,715 adult females of *An. minimus* A that emerged from larvae collected from various habitats in Ban Khun Huay, Ban Pa Dae, and Ban Tham Seau were examined to evaluate the presence of HP and PSP, and a median pale spot on veins R_1, R_2, M_1, M_2, M_{3+4} and 1A. Using the Explorer and Auto-Montage digital images, we separated all females of the *An. minimus* A into 8 distinct groups (Figs 3-10). The identification of sampled specimens from each group was confirmed by PCR-RFLP assay (Van Bortel *et al,* 2000). We concluded that all samples were in fact *An. minimus* A.

DISCUSSION

Morphological variations of characters (*ie*, wings, legs, maxillary palpi, proboscis, thorax and abdomen) have been widely studied in several species of anopheline mosquitoes. Wherever encountered, certain individual adult females may be very difficult to identify due to overlapping variations with other members of the *Anopheles funestus* Group (where *minimus* Subgroup belongs). Molecular technique has been used to study the variations in anopheline mosquitoes. There has been recent study of metaphase karyotypes of the Pyretophorus and Neomyzoyia series subgenus *Cellia* by Baimai *et al* (1996a).

Morphological variations of *An. minimus*

Fig 3–*An. minimus* A, Group 1 showing a typical wing, dorsal view (frequency = 43.10%; found in 738 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa with presector pale (PSP); 2) veins R_1, R_2, M_1 and M_2 without median pale spot; and 3) veins M_{3+4} and 1A with median pale spot.

Fig 4–*An. minimus* A, Group 2, wing, dorsal view (frequency = 21.28%; found in 365 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa without HP and PSP; 2) veins R_1, R_2, M_1, and M_2 without median pale spot; and 3) veins M_{3+4} and 1A with median pale spot.

Fig 5–*An. minimus* A, Group 3, wing, dorsal view (frequency = 4.20%; found in 72 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa with HP and PSP; 2) veins R_1, R_2, M_1 and M_2 , without median pale spot; and 3) veins M_{3+4}, and 1A with median pale spot.

Fig 6–*An. minimus* A, Group 4, wing, dorsal view (frequency = 9.39%; found in 161 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa with PSP; 2) veins R_1, R_2, M_1, M_2 and M_{3+4} without median pale spot; and 3) vein 1A with median pale spot.

Fig 7–*An. minimus* A, Group 5, wing, dorsal view (frequency = 3.90%; found in 67 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa without HP and PSP; 2) veins R_1, R_2, M_1, M_2 and M_{3+4} without median pale spot; and 3) vein 1A with median pale spot.

Fig 8–*An. minimus* A, Group 6, wing, dorsal view (frequency = 3.44%; found in 56 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa with PSP; 2) veins M_1 and M_2 without median pale spot; and 3) veins R_1, R_2, M_{3+4} and 1A with median pale spot.

Fig 9–*An. minimus* A, Group 7, wing, dorsal view (frequency = 11.20%; found in 192 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa with PSP; 2) on veins R_1 and R_2 without median pale spot; and 3) veins M_1, M_2, M_{3+4} and 1A with median pale spot.

Fig 10–*An. minimus* A, Group 8, wing, dorsal view (frequency = 3.58%; found in 61 out of 1,715 adults). Morphological characters (arrows) of this group include: 1) costa with PSP; 2) veins R_1, R_2, M_1 and M_2, and 1A without median pale spot; and 3) vein M_{3+4} with median pale spot.

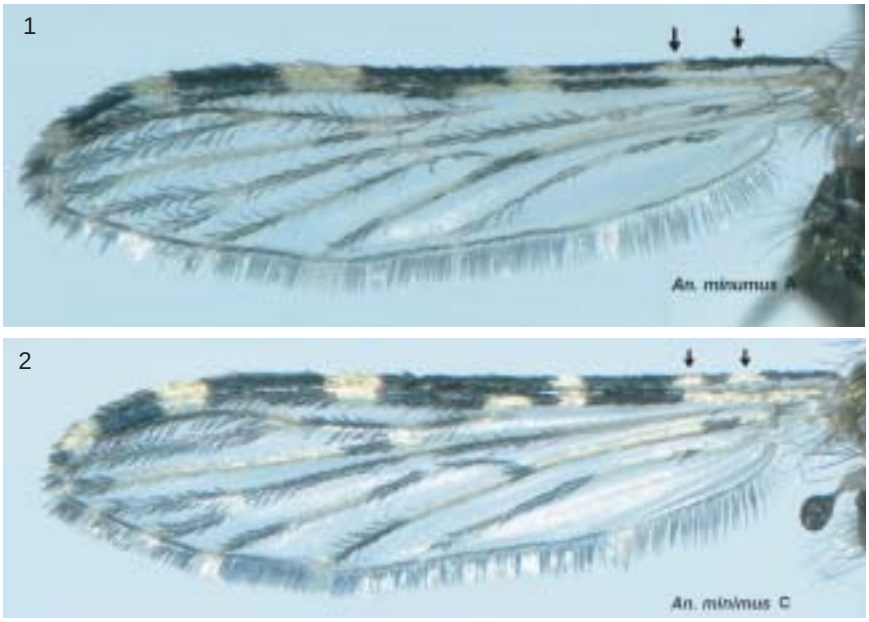

Fig 11–Wing, dorsal view *An. minimus* A (1) and *An. minimus* C (2).

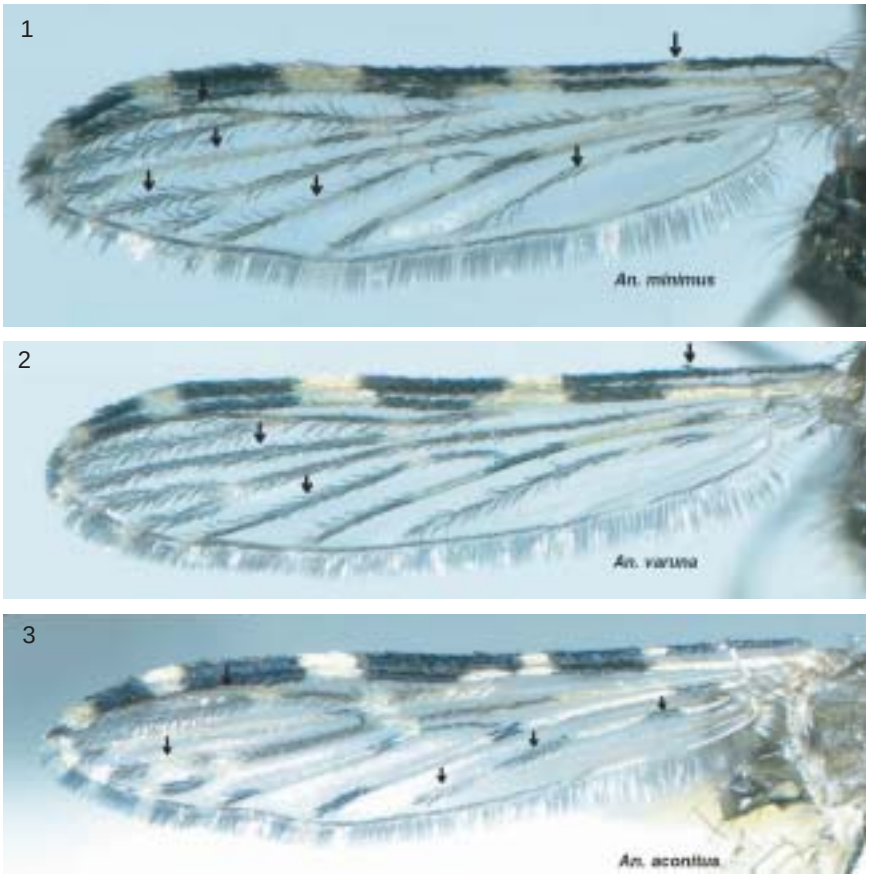

Fig 12–Wing, dorsal view. *An. minimus* A (1), *An. varuna* (2), and *An. aconitus* (3).

were separated into 12 and 3 selected characteristics by Harrison (1980) and Sucharit *et al* (1988), respectively. They were separated into three morphology vaiations. Sucharit *et al* (1988) and Baimai *et al* (1996b) used two characteristics of metaphase karyotypes to distinguish *An. minimus* individual variations. In this study, we found 8 groups of *An. minimus* A from Tak Province showing morphological variations. The first group (Group 1) is a typical *An. minimus* A according to Rattanarithikul and Panthusiri (1994) and Harrison (1980). Separating adult variations of *An. minimus* cannot be accomplished by relying only on the key characters. It also needs properly trained eyes and excellent experience in the one doing the identification. Additional useful characters include the presence of HP and PSP on the costa, and of median pale spot on veins R_1, R_2, M_1, M_2, M_{3+4}, and 1A.

Anopheles minimus A (Fig 11-1) usually has no humeral pale spot (HP) on costa as compared to *An. minimus* C having HP (Fig 11-2).

Some characteristics of *An. minimus* A, such as those found in Groups 2 (Fig 4), 4 (Fig 6) and 5 (Fig 7) are very similar to those of other species, *ie An. varuna* and *An. aconitus* (See Figs 12-1, 2, 3). Being aware of the variable characteristics shared by these species, it will help in preventing the misidentification of target species. Furthermore, it is necessary to conduct molecular analysis (*eg* PCR-RFLP) to confirm morphological identification of those species with highly variable characteristics, such as *An. minimus* A.

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REFERENCES

Baimai V, Kijchalao U, Rattanarithikul R. Metaphase karyotypes of *Anopheles* of Thailand and Southeast Asia. VI. The Pyretophorus and The Neomyzomyia series, subgenus *Cellia* (Diptera: Culicidae). *J Am Mosq Control Assoc* 1996a; 12: 669-75.

Baimai V, Kijchalao U, Rattanarithikul R. Metaphase karyotypes of Anopheles of Thailand and southeast Asia. V. The Myzomyia Series, subgenus *Cellia* (Diptera: Culicidae). *J Am Mosq Control Assoc* 1996b; 12: 97-105.

Chen B, Harbach RE, Butlin RK. Molecular and morphological studies on the *Anopheles minimus* group of mosquitoes in southern China: taxonomic review, distribution and malaria vector status. *Med Vet Entomol* 2002; 16: 253-65.

Green CA, Gass RF, Munstermann LE, Baimai V. Population genetic evidence for two species in *Anopheles minimus* in Thailand. *Med Vet Entomol* 1990; 4: 25-34.

Harbach RE, Kenneth LK. Corections and Additions to Taxonomists' Glossary of Mosquito Anatomy. *Mosquito Systematics* 1982; 13: 201-17.

Harbach RE, Knight KL. Taxonomists' Glossary of Mosquito Anatomy. Marlton, New Jersey: Plexus Publishing, 1980; xi: 415 pp.

Harbach RE. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bull Ent Res* 2004; 94: 537-53.

Harrison BA. Medical entomology studies. XIII. The Myzomyia Series of *Anopheles* (*Cellia*) in Thailand, with emphasis on intra – and interspecific variations (Diptera: Culicidae*) Contrib Am Entomol Inst* (Ann Arbor). 1980; 17: 1-195.

Rattanarithikul R, Panthusiri P. Illustrated keys to the medically important mosquitoes of Thailand. *Southeast Asian J Trop Med Public Health* 1994; 25: 1-66.

Reid JA. Anopheline mosquitoes of Malaya and Borneo. *Stud Inst Med Res Malaya*. 1968; 31: 1-520.

Somboon P, Walton C, Sharpe RG, *et al*. Evidence of new sibling species of *Anopheles minimus* from the Ryukyu Archipelago, Japan. *J Am Mosq Control Assoc* 2001; 17: 98-113.

Sucharit S, Komalairsa N, Leemingsawat S, Apiwathnasorn C, Thongrungkiat S. Population genetic studies on the *Anopheles minimus* complex in Thailand. *Southeast Asian J Trop Med Public Health* 1988; 19: 717-23.

Van Bortel W, Trung HD, Roelants P, Harbach RE, Backeljau T, Coosemans M. Molecular identification of *Anopheles minimus* s.l. beyond distinguishing the members of the species complex. *Insect Mol Biol* 2000; 9: 335-40.

Wilkerson RC, Peyton EL. Standardized nomenclature for the costal wing spots of the genus *Anopheles* and other spotted-wing mosquitoes (Diptera: Culicidae). *J Med Ent* 1990; 27: 207-24.