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Mosquitoes (Diptera: Culicidae) from Villages and Forest Areas of Rural Communes in Khanh Hoa and Binh Phuoc Provinces, Vietnam

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Abstract

This study presents the diversity of mosquitoes collected from communes, endemic with malaria and dengue, located in Khanh Hoa and Binh Phuoc Provinces, Vietnam. A total of 10,288 mosquitoes were collected in the village and forested sites using standard larval dippers, cow-baited traps, ultra-violet light traps, and mechanical aspirators. Mosquito taxa were identified morphologically and species complexes/groups were further characterized molecularly. Five genera of mosquitoes were morphologically identified: *Anopheles Meigen* (21 species), *Aedes Meigen* (2 species), *Culex Linnaeus* (5 species), *Mansonia Blanchard* sp., and *Armigeres Theobald* sp. The PCR-based identification methods allowed the distinction of members of Maculatus Group, Funestus Group, and Dirus Complex; and DNA barcodes enabled the further identification of the Barbirostris Complex. Data reported here include the first report of *An. saeungae* Taai & Harbach and *An. wejchoochotei* Taai & Harbach from Vietnam, and re-emphasizes the significance of using molecular data in an integrated systematic approach to identify cryptic species and better understand their role in disease transmission.

Key words: mosquito diversity, PCR-based identification method, DNA barcodes, Vietnam

Vector-borne diseases have a significant impact on morbidity in the Greater Mekong Sub-region (GMS) (Jones et al. 2008; Hii and Rueda 2013), where malaria, dengue, and Japanese encephalitis (JE) are major public health threats (Solomon et al. 2003; Hewitt et al. 2013; Undurraga et al. 2013). In Vietnam, *Anopheles dirus* Peyton and Harrison and *An. minimus* Theobald have a significant role in malaria transmission (Sanh et al. 2008; Do Manh et al. 2010; Hii and Rueda 2013; Edwards et al. 2019), while *An. maculatus* Theobald, *An. aconitus* Dönitz, *An. philippinensis* Ludlow, *An. sawadwongporni* Rattanarithikul and Green, *An. sinensis* Wiedemann and members of the *An. hyrcanus* group have been identified as secondary malaria vectors (WHO 2007; Do Manh et al. 2010; Hii and Rueda 2013). Nevertheless, a full understanding of the role of these species in malaria transmission in Vietnam is unclear due to the presence of cryptic species within implicated vector taxa. Further, few entomological investigations properly differentiate collected mosquitoes beyond genus, which may not account for differences in behavior, feeding habits, and geographic range (Hii and Rueda 2013).

Morphological misidentification of sibling members in an *Anopheles* species complex or group is common due to shared morphological characteristics. In conjunction with morphological analysis, molecular techniques, such as PCR-based identification methods and DNA barcoding, have been used as complementary approaches to accurately identify many complex and group members. This approach has enabled researchers to better understand mosquito species distribution, ecology, behavior, and role in malaria transmission (Walton et al. 1999, 2007; Van Bortel et al. 2000; Huong et al. 2001; Manonmani et al. 2001; Garros et al. 2004; Dusfour et al. 2007; Hempolchom et al. 2013; Phunngam et al. 2017; Brosseau et al. 2019; Wilai et al. 2020).

Aedes aegypti (Linnaeus) and *Ae. albopictus* (Skuse) are primary and/or secondary vectors of dengue worldwide (Higa 2011). All four serotypes of dengue flaviviruses occur in Vietnam (Ha et al. 2003; Tuan et al. 2017; Quyen et al. 2018), but the incidence rate and transmission patterns differ among various regions in the country (Vu et al. 2014; Nguyen et al. 2019). Japanese encephalitis, caused by another flavivirus (Pearce et al. 2018), is transmitted by *Culex gelidus* Theobald, *Cx. quinquefasciatus* Say, *Cx. tritaeniorhynchus* Giles, and *Cx. vishnui* Theobald throughout Vietnam (Ohba et al. 2011; Kuwata et al. 2013).

Although rarely reported, the incidence of concurrent malaria and dengue infections appears to be increasing in co-endemic areas throughout Asia (Sahu et al. 2016). Recent epidemiologic reports of dengue and malaria transmission in two Vietnamese provinces follow this concerning pattern. To improve the understanding of Anopheline and Culicine mosquito diversity in Vietnam, we used morphological and molecular methods to identify mosquitoes collected from rural communes in Khanh Hoa (central-eastern) and Binh Phuoc (southwestern) Provinces, Vietnam.

Materials and Methods

Study Areas

Mosquito collections were carried out in October 2018 (rainy season) and March 2019 (dry season) in Khanh Thanh and Cau Ba communes within the Khanh Vinh district, located in Khanh Hoa Province, and in Bu Gia Map and Dac O communes, within the Bu Gia Map district, located in Binh Phuoc Province (Fig. 1). Khanh Hoa Province is mountainous, with over half of it covered by forest areas. The average monthly temperatures are between 23°C (December and January) and 27°C (April and August). The yearly precipitation varies between 1,400 and 2,800 mm. The rainy season is between September and December while the dry season is between January and April (Van Bortel et al. 2004). Binh Phuoc Province is mostly surrounded by primary forests and has an equatorial monsoon climate with two distinct seasons: rainy (May to October) and dry (November to April). The temperature averages around 25°C during the day and falls between 7 and 9°C at night (Ngo et al. 2014).

Specimen Collections

Sample collection was conducted in two different environments: in villages (indoor and around houses) and forest fringe areas. Mosquito larvae were collected from artificial habitats such as water-holding containers (e.g., discarded containers, tanks, jars, flower vases, tires, and other similar objects), from inside and/or around houses (peridomiciliary), from natural habitats such as streams around villages and in the forested areas using a standard larval dipper (350 ml, 13 cm diameter; BioQuip, Rancho Dominguez, CA, USA). The larvae were transferred by pipette into plastic bags (WhirlPak, BioQuip, CA, USA) and transported to the entomological laboratory at the National Institute of Hygiene and Epidemiology (NIHE) for identification.

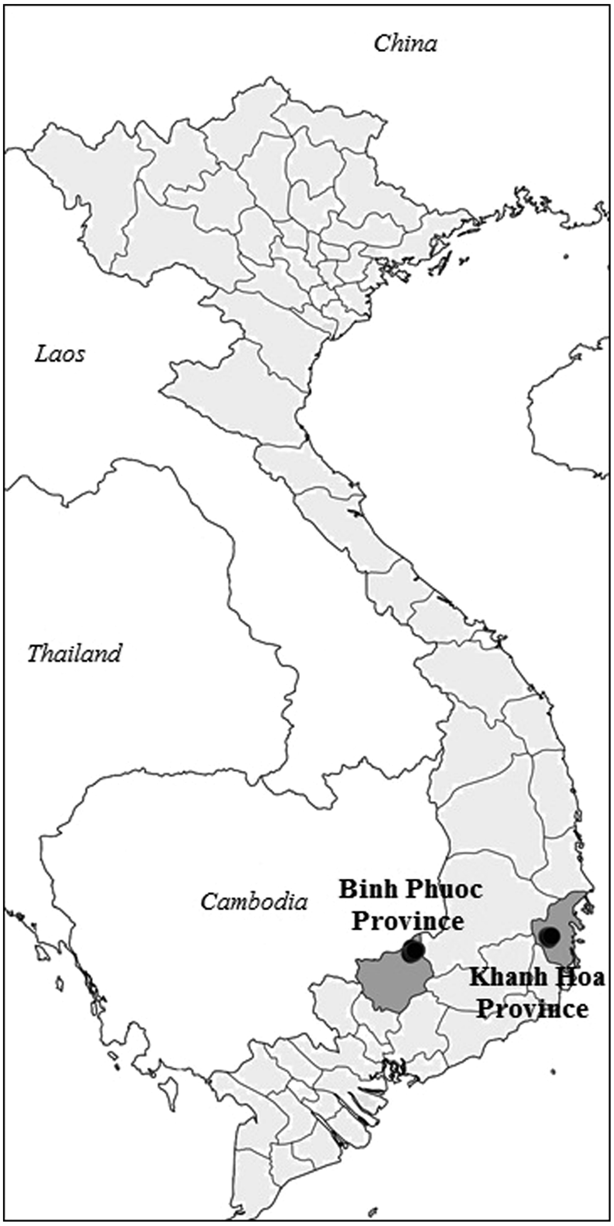

Fig. 1. Sampling locations in Khanh Hoa and Binh Phuoc Provinces, Vietnam.

Adult mosquitoes were collected using ultra-violet light traps (LT), cow-baited traps (CBT), and peridomiciliary with mechanical aspirators. Three to six LTs were set up from 6 pm to 6 am in each commune (30% in villages and 70% in forest areas) for three nights in October 2018 and four nights in March 2019. Two CBT per commune were performed, one in a forest area and one in a village. During CBT, 4–5 people collected mosquitoes off a tethered cow for 10–15 min every hour using a manual aspirator from 6 pm to 6 am for three nights in October 2018 and for four nights in March 2019. Resting adult mosquitoes were collected with manual aspirators indoors and outdoors in the villages (from 7 am to 11 pm and 1 pm to 5 pm; for 4 d in each commune).

The use of cows for CBT during this study was reviewed by a veterinarian in accordance with U.S. Department of Defense and applicable U.S. federal laws and determined to be a field study that did not require review by an Institutional Animal Care and Use Committee.

Species Identification

Adult specimens were pinned on paper points, labeled with a unique collection number and identified under a stereoscope following Stojanovich and Scott (1966) and an identification key developed by the Institute of Malariology, Parasitology, and Entomology (IMPE 2008). Voucher specimens were deposited in the national mosquito collections of the Smithsonian Institution, National Museum of Natural History, Washington DC.

Species belonging to Dirus Complex, Funestus Group, and Maculatus Group were further characterized using PCR-based identification methods in accordance with Walton et al. (1999) for Dirus Complex, Garros et al. (2004) for Funestus Group, and Walton et al. (2007) for Maculatus Group. In addition, DNA barcodes (658-bp of the *COI*) were obtained and analyzed following Motoki et al. (2019a). DNA was extracted from the removal of a single leg from pinned adult specimens or the whole ethanol-preserved adults using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany, http://www.qiagen.com) in accordance with the manufacturer's instructions. Molecular identification was obtained using primers listed in Supp Table S1 (online only).

The PCR protocol for Dirus Complex (Walton et al. 1999) consisted of a 5-min denaturation at 94°C and 32 cycles at 94°C for 15 s, 45°C for 15 s and 72°C for 30 min, followed by 5-min extension at 72°C; for Funestus Group (Garros et al. 2004), 2-min denaturation at 94°C and 40 cycles at 94°C for 30 s, 45°C for 30 s and 72°C for 40 s, followed by 5-min extension at 72°C; and for Maculatus Group (Walton et al. 2007) 5-min denaturation at 94°C and 35 cycles at 94°C for 1 min, 55°C for 30 s and 72°C for 30 s, followed by 5-min extension at 72°C. PCR amplicons were electrophoresed in 1.5% TBE agarose gels stained with GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, USA) at 100V for 15–20 min, before ultraviolet visualization. Specimens were identified according to the length of the product (Supp Table S1 [online only]).

The PCR protocol for *COI* was followed by Motoki et al. (2019a), using primers developed by Folmer et al. (1994) (Supp Table S1 [online only]). The PCR cycle was 94°C for 1 min, 5 cycles of 94°C for 40 s, 45°C for 40 s and 72°C for 1 min, followed by 30 cycles at 94°C for 40 s, 49°C for 40 s and 72°C for 1 min, and a 5-min extension at 72°C. Amplicons were electrophoresed and visualized using the same methods detailed in the above PCR protocol. Positive samples were purified by adding 1 µl of diluted ExoSAP-IT (1:10) in 10 µl of PCR product. The PCR product was incubated using a thermal cycler at 37°C for 30 min, then at 80°C for 15 min.

Sequencing reactions were carried out in both directions using an ABI Big Dye Terminator kit v.3.1 (Applied Biosystems, Warrington, UK) and analyzed in an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences of *COI* were edited in Sequencher^{TM} v.5.4.6 (Genes Codes Co., Ann Arbor, MI, USA). Similarities with publicly available sequences were assessed using the Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi) and through the Identification System of the Barcode of Life Database (BOLD). Alignments were made using Geneious 9.1.6. (Kearse et al. 2012). The best evolutionary model (GTR+G) was selected using jModelTest v.2.1.10 (Darriba et al. 2012). Maximum likelihood analysis was performed with MEGA v.7 (Kumar et al. 2016), with bootstrap supports (Felsenstein 1985) based on 1000 replicates. Intra and interspecific variation was calculated using the Kimura-2 parameter method (Kimura 1980) conducted in MEGA v.7 (Kumar et al. 2016).

Results

Mosquito Identification

Overall, 10,288 specimens were collected in Khanh Hoa and Binh Phuoc Provinces. According to morphological identifications, mosquitoes represented 26 taxa from five genera: *Aedes* Meigen, *Anopheles* Meigen, *Armigeres* Theobald, *Culex* Linnaeus, and *Mansonia* Blanchard (Table 1). *Anopheles* and *Culex* mosquitoes with missing or damaged body parts were assigned to *Anopheles* spp. and *Culex*. spp., respectively. *Culex* and *Aedes* were morphologically identified to species, while *Armigeres* and *Mansonia* were morphologically identified to genus (Table 1).

Based on the PCR-based identification methods (Table 2), all *An. dirus* s.l. (79 specimens) were identified as *An. dirus* s.s. Specimens of Maculatus Group (668 specimens) were identified molecularly as *An. maculatus* sensu stricto (s.s.) (70%, 468/668), and *An. sawadwongporni* (26%, 175/668). The remaining 4% (*n* = 25) could not be identified by the molecular method used. Despite the few samples of Minimus Complex (*n* = 4) identified by morphological diagnostic characters, the PCR-based identification method detected one *An. minimus* s.s., two *An. harrisoni* Harbach and Manguin, and one specimen could not be identified molecularly. Lastly, all but one of the specimens morphologically identified as *An. aconitus* (75 specimens) were molecularly confirmed as *An. aconitus*; one specimen was molecularly identified as *An. varuna* Iyengar. Specimens that could not be identified by molecular methods were assigned to *Anopheles* spp. (Table 2).

In addition, DNA barcodes of 40 specimens were generated (GenBank numbers: MT380479-MT380518) and were compared to sequences already published in the GenBank. Comparison of sequences matched between 98.5 and 100% of similarities with the closest publicly available sequences (Supp Table S2 [online only]), allowing the verification of 16 *Anopheles* species (Fig. 2). The *COI* sequences of *An. kochi* Dönitz, *An. minimus*, *An. philippinensis*, *An. vagus* Dönitz and *An. varuna* were not generated. *Anopheles barbirostris* van der Wulp, *An. dissidens* Taai and Harbach, *An. saeungae* Taai and Harbach and *An. wejchoochotei* Taai and Harbach were identified initially by morphological characters as *An. barbirostris* s.l., but were not identified molecularly using the multiplex PCR method (Brosseau et al. 2019; Wilai et al. 2020). However, the *COI* sequences allowed for further discrimination of the sibling members and correctly identified these four species (Fig. 2). The intraspecific variation in the K2P between the *COI* sequences ranges from 0.002 to 0.017, and the interspecific variation was ≥0.027 (Supp Table S3 [online only]).

Diversity of *Anopheles* in Village and Forest Areas

A total of 1,798 adult *Anopheles* species were collected using CBT (96.3%, 1,731/1,798) and LT (3.7%, 67/1,798) (Supp Table S4 [online only]). Based on the morphological identification, 11 species of *Anopheles* were identified and four species complexes/groups (Dirus Complex, Maculatus Group, Funestus Group, and Barbirostris Complex) (Table 1). Twenty-one *Anophele*s species were identified after using molecular identification techniques (Table 2). Only resting *Ae. aegypti* and *Ae. albopictus* were collected using manual aspirators in villages (Supp Table S5 [online only]).

Of 1,198 *Anopheles* adults collected in Khanh Hoa Province, 705 (59%) specimens were found in Khanh Thanh and 493 (41%)

Table 1. Mosquitoes identified based on morphological diagnostic characters, collected in Khanh Hoa and Binh Phuoc Provinces, Vietnam

Species	Khanh Hoa Province		Binh Phuoc Province		Total
	KT*	CB	BGM	DO	
An. aconitus	133(107)**	66(45)		29	228
An. barbirostris s.l.	12(1)	8(4)	3	24	47
An. crawfordi	25	1			26
An. dirus s.l.	74	1	1	3	79
An. jamesii	1	6			7
An. karwari	1		17	4	22
An. kochi	97	31	3		131
An. maculatus s.l.	36	193	364	75	668
An. minimus s.l.		1	2	1	4
An. peditaeniatus	94	75		1	170
An. philippinensis	68	21	10	46	145
An. sinensis	67	12	2	3	84
An. splendidus	9	31	2		42
An. tessellatus		9			9
An. vagus	151	79	4		234
Anopheles spp.	45	8	1	5	59
Ae. aegypti	109(107)	609(583)	346(338)	268(253)	1,332
Ae. albopictus	964(956)	218(216)	181(169)	(88)	1,451
Cx. fuscochephala	98	130	5		233
Cx. gelidus	59	57	1	2	119
Cx. quinquefasciatus	12	75	28	163	278
Cx. tritaeniorhynchus	91	450	61	72	674
Cx. vishnui	2,421	940	97	235	3,693
Culex spp.			49	1	50
Armigeres sp.	135	7	176	117	435
Mansonia sp.	55	11		2	68
TOTAL	4,757	3,039	1,353	1,139	10,288

Abbreviations*: **KT= Khanh Thanh Commune; **CB** = Cau Ba Commune; **BGM** = Bu Gia Map Commune; **DO** = Dac O Commune.

**In parenthesis represents the larval samples included in the total sample size of each species.

in Cau Ba. In Khanh Thanh, *An. vagus* (21.4%,), *An. kochi* (13.8%), *An. peditaeniatus* (Leicester) (13.3%), *An. dirus* (10.5%), *An. philippinensis* (9.6%), and *An. sinensis* (9.5%) represented 78.1% of the collected species (Table 2). In Cau Ba, *An. sawadwongporni* (26%), *An. vagus* (16%), *An. peditaeniatus* (15.2%), *An. maculatus* (10.8%), *An. splendidus* Koidzumi (6.3%) and *An. kochi* (6.3%) represented 80.6% of the collected species (Table 2).

In Binh Phuoc Province, a total of 600 *Anopheles* specimens were collected, 409 specimens in Bu Gia Map and 191 specimens in Dac O. In Bu Gia Map, *An. maculatus* (81.9%) was the dominant species followed by *An. sawadwongporni* (5.9%) (Table 2). In Dac O, *An. maculatus* (32.5%), *An. philippinensis* (24.1%), and *An. aconitus* (14.1%) were the most abundant species and represented 70.7% of all species collected (Table 2).

Throughout this study, adult *Anopheles* species were collected around villages (62.1%; 1,117/1,798) and forest environments (37.9%; 681/1,798) (Table 2). *Anopheles dirus* was principally collected from forest environments (98.7%, 78/79), whereas, *An. maculatus* and *An. sawadwongporni* were collected in all locations and environments (Table 2). *Anopheles maculatus* was the most abundant species collected in both communes in Binh Phuoc Province and was the dominant species in Bu Gia Map (Table 2).

Observation on *Anopheles*' Host-Seeking Activity in Response to Cattle-Baited Traps

The host-seeking activity in response to CBT for *An. kochi*, *An. peditaeniatus*, and *An. vagus* occurred before 7 pm, while *An. dirus* peaked between 8 and 9 pm in Khanh Thanh (Fig. 3A); *An. sawadwongporni*, peak activity was between 7 and 8 pm in Cau Ba (Fig. 3B). *Anopheles maculatus* peak activity was between 8 and 9 pm, rising higher between 11 pm and 12 am in Bu Gia Map (Fig. 4A), and occurred between 9 and 10 pm, with lower peak activity observed between 1 and 2 am in Dac O (Fig. 4B).

Larval Species Collected from Natural and Artificial Habitats

Larvae of *An. aconitus* (*n* = 152) and *An. barbirostris* s.l. (*n* = 5) were collected in streams and small rivers (natural breeding habitats) (Table 1). Larvae of *Ae. aegypti* (*n* = 1,281) and *Ae. albopictus* (*n* = 1,429) were collected in artificial habitats found indoor or around houses in villages within Khanh Hoa and Binh Phuoc Provinces (Table 1). In Khanh Thanh, *Ae. albopictus* corresponded to 90% (956/1,063) of all collected *Aedes* larvae; while *Ae. aegypti* (73%, 583/799) was more commonly collected in Cau Ba (Table 1; Supp Table S5 [online only]). *Aedes aegypti* larvae were more common in Bu Gia Map (67%, 338/507) and Dac O (74%, 253/341) (Table 1; Supp Table S5 [online only]).

Larvae of *Ae. aegypti* from Khanh Thanh were mostly collected from plastic drums (34%), plastic jars (16%), and discarded containers (15%) while larvae of *Ae. albopictus* were mostly collected in discarded containers (38%), plastic drums (23%), and ceramic jars (20%) (Table 3). In Cau Ba, *Ae. aegypti* were predominately found in plastic drums and discarded containers (31% each) with *Ae. albopictus* larvae were found in discarded containers (37%) and plastic jars (33%) (Table 3). In Bu Gia Map, *Ae. aegypti* larvae were mostly found in plastic jars (35%) and ceramic jars (26%), and *Ae. albopictus* were found in plastic

Table 2. Adults of *Anopheles* species collected in villages and forest areas in Khanh Hoa and Binh Phuoc Provinces, Vietnam.

Species	Khanh Hoa Province				Binh Phuoc Province				Total
	KT**		CB		BGM		DO		
	V	F	V	F	V	F	V	F	
*An. aconitus**	26	–	10	11	–	–	1	26	74
*An. barbirostris**	10	–	–	2	–	–	5	1	18
*An. crawfordi**	12	13	–	1	–	–	–	–	26
*An. dirus**	–	74	–	1	1	–	–	3	79
*An. dissidens**	–	–	–	-	1	–	8	–	9
*An. harrisoni**	–	–	1	–	1	–	–	–	2
*An. jamesii**	1	–	1	5	–	–	–	–	7
An. karwari	1	–	–	–	13	4	1	3	22
An. kochi	51	46	23	8	–	3	–	–	131
*An. maculatus**	3	15	51	2	300	35	17	45	468
*An. minimus**	–	–	–	–	1	–	–	–	1
*An. peditaeniatus**	36	58	29	46	–	–	–	1	170
An. philippinensis	67	1	21	–	6	4	20	26	145
*An. saeungae**	1	–	1	1	2	–	–	–	5
*An. sawadwongporni**	5	8	109	19	18	6	1	9	175
*An. sinensis**	34	33	5	7	2	–	–	3	84
*An. splendidus**	8	1	10	21	1	1	–	–	42
An. tessellatus	–	–	–	9	–	–	–	–	9
An. vagus	125	26	30	49	3	1	–	–	234
*An. varuna**	–	–	–	–	–	–	1	-	1
*An. wejchoochotei**	–	–	–	–	–	–	9	1	10
Anopheles spp.	10	40	15	5	4	2	5	5	86
Total	390	315	306	187	353	56	68	123	1,798

*Species identified based on PCR-based identification methods and/or sequence of *COI* generated for Maximum Likelihood tree; other species (unmarked) identified using the morphological method.

***Abbreviations*: **KT**= Khanh Thanh commune; **CB** = Cau Ba commune; **BGM** = Bu Gia Map commune; **DO** = Dac O commune; **V** = village, **F** = forest.

drums (31%) and tires (30%) (Table 3). In Dac O, *Ae. aegypti* were mostly collected most from plastic jars (81%) and *Ae. albopictus* in flower vases (83%) (Table 3). No larvae of other species were collected in association with *Aedes* larvae from artificial habitats in the villages.

Discussion

The GMS includes six countries, each with a high diversity of mosquito vector species, including many *Anopheles* complexes/groups, with high heterogeneity in geographic distribution patterns and behavioral plasticity between species (Trung et al. 2005; Sinka et al. 2011; Cui et al. 2012). This heterogeneity and behavioral plasticity require periodic reevaluation of mosquito distributions to target vector control efforts and obtain a better understanding of disease transmission cycles, particularly in co-endemic areas with concurrent disease transmission patterns.

This study utilized multiple methodologies to better sample and identify the mosquitoes at field sites in two Vietnamese provinces with reports of overlapping malaria and dengue transmission. Previous entomological surveys carried out in a transect from the northern to the southeastern regions of Vietnam, demonstrated that CBT was a suitable collection method for primary and secondary malaria vectors throughout the country (Garros et al. 2008). This method, along with the use of light traps, resulted in the collection of 21 *Anopheles* species during this study. Additionally, the other larval and adult collection methods used in indoor and outdoor locations within villages resulted in the identification of *Ae. aegypti*, *Ae. albopictus*, *Cx. fuscochephala* Theobald, *Cx. gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, *Cx. vishnui*, *Armigeres* sp., and *Mansonia* sp. The biodiversity of known malaria vectors, arbovirus vectors, and other mosquitoes in this area represents a challenge that public health and vector control professionals will have to work together to address.

The PCR-based identification methods employed in this study were crucial to discerning the cryptic species of Maculatus Group and Minimus Complex. The cryptic species, *An. maculatus* s.s./*An. sawadwongporni* of the Maculatus Group and *An. minimus* s.s./*An. harrisoni* of the Minimus Complex, have been found in sympatry and implicated in malaria transmission in Vietnam (Do Manh et al. 2010; Van Bortel et al. 2010). These data re-emphasize the need for accurate mosquito identification to ensure cryptic vectors are considered when investigating malaria transmission cycles and to successfully direct vector control operations (Manguin et al. 2008; Do Manh et al. 2010; Sinka et al. 2011).

Recently, Taai and Harbach (2015) revised *An. barbirostris* s.l. in Thailand based on molecularly identified progeny broods and described three new members of this complex: *An. dissidens*, *An. saeungae*, and *An. wejchoochotei*. *Anopheles barbirostris* s.l. has been collected in the northern and central regions of Vietnam (Garros et al. 2008; Van Bortel et al. 2010), thus it is plausible that these reports may include more than one species of the Barbirostris Complex. In this study, the sequences of the *COI* confirmed, for the first time, that *An. saeungae* and *An. wejchoochotei* are present in Vietnam. Our findings expand the known species and regional distribution of the Barbirostris Complex in Vietnam. These species should be further investigated to provide a better understanding of how the Barbirostris Complex influences malaria maintenance and transmission in Vietnam.

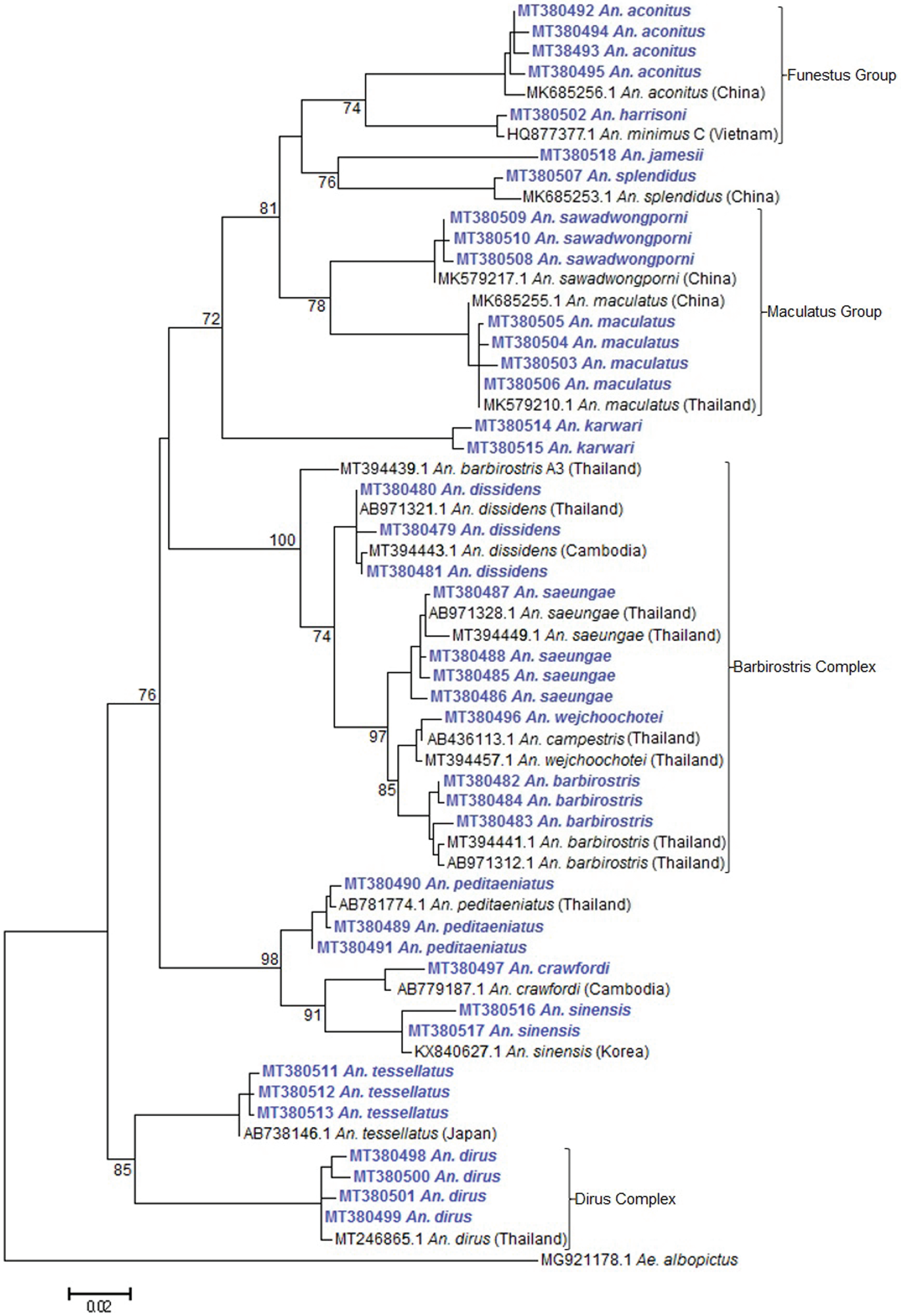

Fig. 2. Maximum likelihood tree of *COI* of *Anopheles* species. Sequences generated during this study have initials MT38. GenBank sequences included specimens from Cambodia, China, Korea, Japan, Thailand, and Vietnam. Bootstrap values ≥ 70%

The primary malaria vector, *An. dirus*, has been collected in high densities from forested areas in the southern and central provinces of Vietnam and contributes to the maintenance of malaria transmission in these regions (Obsomer et al. 2007; Sinka et al. 2011; Marchand et al. 1997, 2011; Ngo et al. 2014). Seventy-four specimens of *An. dirus* were found in Khanh Thanh commune, Khanh Hoa Province; however, very few specimens were collected in Cau Ba commune from the same province or Bu Gia Map and Dac O communes in Binh Phuoc Province. This suggests that province-level vector mapping may not be

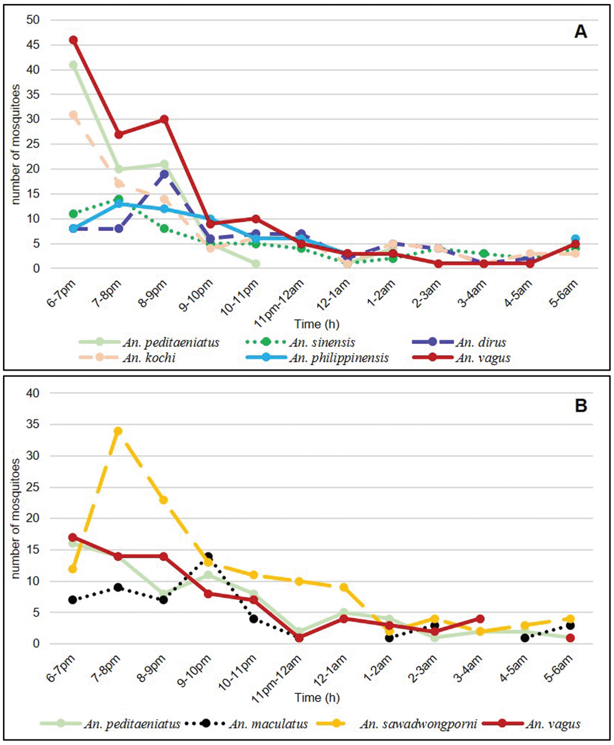

Fig. 3. Host-seeking activity in response to cattle-baited traps in Khanh Hoa Province, Vietnam. **A**. Khanh Thanh commune. **B**. Cau Ba commune

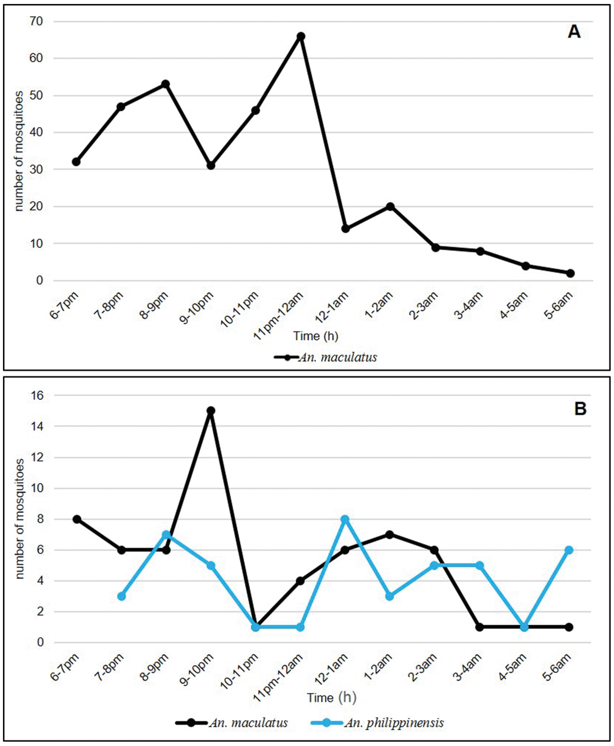

Fig. 4. Host-seeking activity in response to cattle-baited traps in Binh Phuoc Province, Vietnam. **A**. Bu Gia Map commune. **B**. Dac O commune

sufficiently accurate to understand the geographic distribution of *An. dirus* in Vietnam.

Ngo et al (2014) collected 17 *Anopheles* species in Bu Gia Map commune, Binh Phuoc Province, including predominately the primary vectors *An. dirus* and *An. minimus,* and low densities of other *Anopheles* species. Interestingly, low densities of *An. dirus* (*n* = 1), *An. minimus* (*n* =1), other *Anopheles* species (n varied from 1 to 24), and a high density of *An. maculatus* (*n* = 335) were collected during this study from the same commune. A possible explanation for the varying density of malaria vectors is the use of insecticide (e.g., indoor residual spray and insecticide-treated nets and long-lasting insecticidal nets), which can reduce regional mosquito populations (Parajuli et al. 1981; Garros et al. 2005; Dev and Manguin 2016; Nguyen 2020). However, this should not work for *An. maculatus.* which has been found to be resistant, or suspected to be resistant to alpha-cypermethrin, lambda-cyhalothrin (Hieu and Duyen 2015), permethrin, and deltamethrin (NIMPE 2012) in the central and central highland regions of Vietnam. While *An. dirus* and *An. minimus* are still sensitive for alpha-cypermethrin and lambda-cyhalothrin in the central region of the country (Nguyen 2020). Nevertheless, additional insecticide resistance studies are needed to clarify the susceptibility of malaria vectors to insecticide in the studied locations.

A number of secondary vectors (*An. harrisoni*, *An. kochi*, *An. maculatus*, *An. peditaeniatus*, *An. philippinensis*, *An. sawadwongporni*, and *An. vagus*) were collected herein. In Hanh Chuon village, Truong Xuan Commune, Quang Binh Province of Vietnam, *An. harrisoni, An. maculatus*, and *An. sawadwongporni* were involved in malaria transmission (Do Manh et al. 2010). *Anopheles maculatus* is considered a primary malaria vector in eastern India, southern Thailand, peninsular Malaysia, and Java (Green et al. 1991; Rahman et al. 1993; Rattanarithikul et al. 1996; Barcus et al. 2002; Coleman et al. 2002); *An. sawadwongporni* is an important malaria vector in Thailand (Somboon et al. 1998; Coleman et al. 2002); while *An. harrisoni* was incriminated for the first time as a vector of malaria in Hanh Chuon village, Truong Xuan Commune, Quang Binh Province of Vietnam (Do Manh et al. 2010). As mentioned before, *Anopheles maculatus* was the most abundant species in Binh Phuoc Province, and *An. sawadwongporni* in Khanh Hoa Province. Despite those species being involved in malaria transmission in Hanh Chuon village, the vector status of *An. maculatus* and *An. sawadwongporni* in Khanh Hoa and Binh Phuoc Provinces is still unclear. Therefore, due to the high density of these species collected in our study, and their importance in malaria transmission in neighboring countries, further large-scale studies are essential to elucidate the role of these species in malaria transmission in Vietnam.

Economic development, such as high rates of urbanization, and a favorable climate have been linked to changes in the range of dengue vectors (Motoki et al. 2019b). These factors may explain the expansion of the range for flavivirus and alphavirus vectors within Vietnam. Vector control remains the primary measure to prevent dengue transmission, so understanding the changes in the range of these vectors is critical to targeting control measures. *Aedes aegypti* has been found commonly in urban areas (Tsuda et al. 2006; Jansen and Beebe 2010), while *Ae. albopictus* has been generally associated with peri-urban and rural environments (Tsuda et al. 2006; Braks et al. 2003). However, recently *Ae. albopictus* has been reported in urban areas (Lambrecht et al. 2010; Li et al. 2014). Only larvae of *Ae. aegypti* and *Ae. albopictus* were collected from indoor and outdoor artificial habitats in villages. Shared larval habitats and reports of distribution and abundance shifts of resident *Ae. albopictus* or *Ae. aegypti* after the establishment of the other species suggest that competitive displacement may have occurred (Braks et al. 2003). Interestingly, in Khanh Thanh and Dac O communes both species where collected in the same habitat; however, *Ae. albopictus* was dominant in Khanh Thanh and *Ae. aegypti* was dominant in Dac

Table 3. Artificial habitats of *Ae. aegypti* and *Ae. albopictus* in villages located in Khanh Hoa and Binh Phuoc Provinces, Vietnam

Artificial breeding habitats	Khanh Hoa Province				Binh Phuoc Province			
	KT*		CB		BGM		DO	
	aeg	*alb*	*aeg*	*alb*	*aeg*	*alb*	*aeg*	*alb*
Flower vase (1–5 L)[1,2]	6	9	5	15		21	1	73
Discarded container[2,3]	16	363	182	79	33	45	7	9
Plastic jar (5–20 L)[1,2]	17	18	61	71	119		204	3
Plastic drum (>100 L)[1,2]	36	220	180	22	72	52	20	
Tank (>500 L)[2]	8	63	26	2	25		6	2
Aquarium[2]	5	32	19	5			5	1
Ceramic jar (5–80 L)[1,2]	14	189	81	5	89		6	
Tire[2]		36	28	16		51	4	
Bamboo node[2]	5	26		1				
Total	107	956	583	216	338	169	253	88

[1] Indoor breeding habitats.

[2] Outdoor breeding habitats.

[3] Anything which can store rainwater <10 l, for example, coconut shell.

Abbreviation*: **KT= Khanh Thanh commune; **CB** = Cau Ba commune; **BGM** = Bu Gia Map commune; **DO** = Dac O commune; ***aeg*** = *Aedes aegypti*; ***alb*** = *Ae. albopictus*; L = liter.

O. Additional research is needed to better understand the distribution of these two dengue vectors.

Many *Anopheles* samples were collected during the first hour of collection (between 6 pm and 7 pm), indicating that peak host-seeking behavior may occur earlier than 6 pm, possibly during the day time, especially in dark environments, such as in forest areas in Khanh Hoa and Binh Phuoc. This may not be an isolated observation as biting activity during the day was observed in *Anopheles* species in northern Cambodia (Vantaux et al. 2021), and in Laos (Marcombe et al. 2020). Herein, only CBT was conducted, thus, we were not able to infer the anthrophophily of *Anopheles* species in the malaria transmission context. However, sample collection that includes daytime hours, before 6 pm, should be considered in future studies to determine the frequency and occurrence of daytime activity for these vectors.

There are some limitations to this study. Collection methods were not related to human-baited trap, and parasites infections were not examined, limiting our ability to confirm which species were involved in malaria transmission in the studied areas. Therefore, the use of human-baited trap methods and determination of parasites infection in *Anopheles* species should be considered for future studies to better understand the dynamics of malaria transmission in these areas. Larval surveys were conducted near homes (indoors and outdoors) in villages; future larval surveys should be conducted in sylvatic habitats to check for the presence of *Ae. albopictus* which are usually reported in rural and forested areas.

This study presented preliminary results; continuous research must be conducted in these areas to better understand the diversity and range of mosquitos in these areas. Further studies that address the limitations noted here will improve the knowledge of the biology, ecology, and behavior of mosquito vectors to better understand human disease transmission and implement targeted strategies to prevent disease transmission in these areas of Vietnam.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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