Anopheles Bionomics in a Malaria Endemic Area of Southern Thailand

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Abstract

Background: Ivermectin mass drug administration (MDA) could accelerate malaria elimination in the Greater Mekong Subregion. This study was performed to characterize the bionomics of *Anopheles* in Surat Thani province, Thailand.

Methods: Mosquitoes were collected via human landing collections, February - October of 2019. *Anopheles* were morphologically identified to species. Primary *Anopheles* malaria vectors were dissected to assess parity status and a subset evaluated for molecular identification and *Plasmodium* detection.

Results: A total of 17,348 mosquitoes were collected. Of 5,777 *Anopheles* mosquitoes, 15 species were identified morphologically. The most abundant *Anopheles* were *Anopheles minimus* s.l. (87.87%, n = 5,035), *Anopheles dirus* s.l. (7.05%, n = 407), and *Anopheles barbirostris* s.l. (2.86%, n = 165). Molecular identification confirmed that of *An. minimus* s.l., 99.80% were *An. minimus* s.s. (n=484) and 0.2% *An. aconitus* (n = 1), of *An. dirus* s.l., 100% were *An. baimaii* (n = 348), and of *An. maculatus* s.l., 93.62% were *An. maculatus* s.s. (n = 44) and 6.38% *An. sawadwongporni* (n = 3). No *Anopheles* were *Plasmodium* positive (n = 879). An average of 11.46 *Anopheles* were captured per collector per night. There were differences between species in hour of collection (Kruskal-Wallis = 80.89, P < 0.0001, n = 5,666), with more *An. barbirostris* s.l. and *An. maculatus* s.l. caught earlier compared to *An. minimus* s.l. (P = 0.0001, P < 0.0001, respectively) and *An. dirus* s.l. (P = 0.0082, P < 0.001, respectively). The proportion of parous *An. minimus* s.l. captured by hour increased throughout the night (Wald Chi-square = 17.31, P=0.000, odds ratio = 1.0535 [1.0279 – 1.0796] 95% CI (n = 3,400). Overall, *An. minimus* s.l. parity was 67.68% (2,375/3,509) with an intra cluster correlation of 0.0378. A power calculation determined that an *An. minimus* s.l. parity reduction treatment effect size = 34%, with four clusters per treatment arm, a minimum of 300 mosquitoes dissected per cluster at an α= 0.05 will provide 82% power to detect a significant difference following ivermectin MDA.

Conclusions: The study area in Surat Thani province is an ideal location to evaluate the impact of ivermectin MDA on *An. minimus* parity.

Background

In 2019, an estimated 229 million cases of malaria occurred worldwide, with approximately 239,000 cases from the Greater Mekong Subregion (GMS). Between 2010 and 2019, the number of malaria cases in the GMS fell by 90%. By 2030, the countries within the GMS have targeted to eliminate malaria [1]. Malaria transmission in the GMS is complex, various *Anopheles* species in the Dirus complex, Minimus complex, and the Maculatus group have been recognized as the primary malaria vectors in the region. From the Minimus complex, *Anopheles minimus* s.s. is the primary malaria vector and it is distributed across the GMS. There are two members of the *An. dirus* complex that are primary malaria vectors, *An. dirus* s.s. occurs east of the Thai-Myanmar border, while An. *baimaii* occurs west of the Thai-Myanmar border [2]. Additionally, members of the *An. maculatus* group are considered primary vectors that can

contribute to malaria transmission, including *An. maculatus* s.s. [3] and to a lesser extent *An. sawadwongporni* which has been incriminated in southern Thailand [4]. Members within a species complex differ in their behavioral characteristics, which in turn drives *Plasmodium* transmission dynamics, therefore, an accurate identification is essential to design and evaluate vector control methods in the GMS.

The diversity of vector species, insecticide resistance, and increasing antimalarial drug resistance are some of the greatest challenges for malaria elimination in the GMS [5]. Moreover, the outdoor-feeding, outdoor-resting and early evening feeding behaviors of GMS malaria vectors [6, 7, 8] also limit the effectiveness of indoor residual spraying (IRS) and long-lasting insecticide-treated nets (LLINs). Therefore, novel vector control measures which target these outdoor-feeding vectors could accelerate malaria elimination in this region. Ivermectin mass drug administration (MDA) to humans has been suggested as a possible malaria parasite transmission control tool as it directly kills *Anopheles* that feed on treated people, regardless of blood feeding time or location. Evidence from West Africa has shown that single ivermectin MDAs can kill wild *An. gambiae* s.l. [9, 10], shift the population age structure [10], reduce the *Plasmodium falciparum* sporozoite rate [10, 11] and repeated ivermectin MDAs can reduce clinical falciparum malaria episodes [12]. In the GMS, at human-relevant concentrations, ivermectin is lethal to *An. dirus, An.minimus*, *An. sawadwongporni*, and *An. campestris*, and inhibits the development (*i.e.* sporogony) of *Plasmodium vivax* in *An. dirus* and *An. minimus* [13]. A clinical trial demonstrated that persons treated with ivermectin (400 μg/kg) effectively reduced *An. dirus* survival by 50% when fed blood from treated persons up to six days post ivermectin, and reduced *An. minimus* survival by 90% when fed blood from treated persons up to ten days post drug administration [14]. This evidence has inspired a large-scale cluster randomized trial in Thailand to assess the effect of ivermectin MDA on entomological and parasitological parameters of malaria transmission.

Evaluating vector control interventions for malaria in the GMS is difficult because transmission occurs primarily in the forest due to *Anopheles* habitat preference, combined with sporadic human entry into the forest for various agricultural and economic pursuits, not all of which are legal [15, 16]. However, most vector control interventions (*e.g.* LLINs and IRS) are applied in the village setting where transmission is less likely to occur, and thus do not directly target the areas of active *Plasmodium* transmission. Thailand has dramatically reduced its malaria burden and has set the goal to eliminate malaria by 2026. Indeed, from 2000 to 2019, there was a 96% reduction in number of malaria cases from 159,120 to 5,832 but the ratio of Thai to non-Thai cases has increased from 57–72% demonstrating that there are still active foci of transmission in Thailand [17]. This reduction in malaria complicates evaluation of *Anopheles* vector control interventions in Thailand due to low rates of *Plasmodium* transmission. For ivermectin MDA evaluation, a site in Thailand needed to be selected where persons live and work with active malaria transmission. Rubber plantations in Thailand offer an ideal location for *Anopheles* vector control intervention evaluation, as mature rubber plantation provides a suitable habitat for vector proliferation, and tend to be located in hilly areas adjacent to natural forests which are ideal habitat for primary malaria vector proliferation. Rubber tappers live and work in the same environment and tappers work throughout the night exposed to wild *Anopheles*, therefore, rubber tappers have higher rates of malaria

than non-rubber tappers [18, 19, 20]. Indeed, a seven-province wide survey of case data from malaria clinics in Thailand from 2013 to 2016 found 60.1% (3,330/5,5541) of *P. falciparum* cases were identified from rubber tappers [20].

Surat Thani province has the greatest amount of rubber plantation area in Thailand, with approximately 3,829 square kilometers [21] and is one of the few provinces afflicted with malaria that is not along an international border. Interestingly, in Surat Thani, the dominant malaria species infecting humans is *P. falciparum*, accounting for 77.58% (519/669) of malaria cases from 2015–2019 [22]. *Plasmodium falciparum* is the ideal parasite to assess during vector control interventions as it is most sensitive to transmission interruption due to its non-relapsing nature. A cross sectional molecular malaria survey conducted in Surat Thani in 2019 demonstrated that persons who stayed outdoors during the night-time were at the highest risk of malaria infection [23]. Three districts were selected for evaluation based on their malaria case incidence comprising 65.13% (338/519) of falciparum cases in Surat Thani from 2015–2019 and high rubber plantation coverage including: Phanom, Vibhavadi, and Khiri Rat Nikhom districts. Historically, IRS with 5% deltamethrin was performed in villages with higher malaria case burden, but this has ceased in Phanom and Vibhavadi in 2015 and Khiri Rat Nikhom in 2016. LLINs are widely distributed throughout all three districts by government and non-governmental organizations. Daytime indoor thermal fogging for *Aedes aegypti* control with 1% deltamethrin or 25% cypermethrin still occurs focally and sporadically in response to local Dengue cases [Personal Communication, Surat Thani Vector-borne Diseases Control Center 11.3].

Little available information regarding vector bionomics in Surat Thani has been published. The largest study conducted in Surat Thani reported a total of 3,778 *Anopheles* mosquitoes collected from February 2015 to December 2016 via human landing collection in Phanom district. Six *Anopheles* species were collected including, *An. dirus* s.l., *An. minimus* s.l., *An. maculatus* s.l., *An. barbirostris* s.l., *An. hycranus* s.l. and *An. tessellatus*. The predominant specie was *An. minimus* with 87.19%. The highest mosquito densities were found between March and May in both years [24]. Molecular identification has verified presence of *An. maculatus* s.s. from Phanom district [25] and *An. minimus* s.s. from Khiri Rat Nikhom district [26]. Due to active *P. falciparum* transmission, asymptomatic malaria observation, presence of primary malaria vectors, and an ideal environment for evaluating a vector control intervention, Surat Thani province was selected as the study area to evaluate the impact of ivermectin MDA on entomological and parasitological parameters.

Baseline entomological surveillance utilizing mosquito human landing collections were performed to evaluate vector abundance, composition, landing activity, and parity rates linked to molecular identification in Surat Thani in 2019. These efforts were done to assess whether the study area was appropriate to assess the impact of ivermectin MDA on *An. minimus* population age structure (*i.e.* parity).

Methods

1) Ethics statement

The study was approved by the Walter Reed Army Institute of Research (WRAIR#2430), the Human Research Protection Office (HRPO Log No.19919.2a/A-19919.2b), and the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Thai MoPH Ref no.25/2560).

2) Field site description and maps

2.1) Field Site Description

Collection sites were located in Khiri Rat Nikhom, Phanom, and Vibhavadi districts, in Surat Thani province (651 km south of Bangkok). Villages with higher malaria case burden as reported by local Vector Borne Disease Units were selected from each district. Populations and house locations were mapped (see next section), and cluster sizes of approximately 300-500 persons were established. In total, 13 clusters were selected for entomological evaluation, 5 clusters in Khiri Rat Nikhom, 4 clusters in Phanom, and 4 clusters in Vibhavadi. Khiri Rat Nikhom is located in the center of Surat Thani (9°1'48"N 98°57'12"E). The western portion of Khiri Rat Nikhom is located in the hills of the Phuket mountain range adjacent to Khao Sok National Park, while the eastern portion is mostly flat. Phanom is in the south west (8°51'18"N 98°48'48"E), covered by mountain and forest. The northwestern portion is protected by the Khao Sok National Park and the southwestern portion protected by the Khlong Phanom National Park. Vibhavadi is a small district situated in the north-central portion of the province (9°14'20"N 98°58'44"E) covered by mountain and forest. The western portion of the district is protected by the Kaeng Krung National Park and Khlong Yan Wildlife Sanctuary. In Surat Thani, the dry season occurs January - February, rainy season lasts March - September, with the heavy monsoon rains occurring October - December.

2.2. Mapping process

Latitude and longitude coordinates of all houses and human landing collection locations were captured using a 60CSx GPS unit (Garmin, Olathe, KS, USA). The open source QGIS software was used to generate maps for each cluster. The terrain data derived from a topographic map with elevator contour line at 25 meters.

3) Mosquito collection, morphological identification, and parity evaluation

3.1) Mosquito collection

Adult mosquitoes were collected from February through October 2019 for two consecutive nights per cluster per month, except Vibhavadi in which collections began in June. Mosquitoes were collected by the human landing collection (HLC) method. Mosquito collector volunteers were local Thai residents, non-pregnant and non-breastfeeding adults (18-62 years old), capable of providing informed consent, and capable of comprehending the HLC method. A canopy was constructed at each outdoor collection site to protect the mosquito collector volunteers from the elements. There were two collection sites per cluster each night. Collection sites were chosen based on proximity to forest or rubber plantation, a house nearby for access to water and electricity, and near potential *An. minimus* s.l. larval habitat. Efforts were made to sample as many areas of each cluster that met the above criteria and were safely accessible. If a

collection site yielded little or no *Anopheles*, then the site was switched to a new location the following night. At each collection site, two mosquito collector volunteers worked together from 18.00 – 24.00 and were replaced by two additional collectors from 24.00 – 06.00. Collections occurred for 50 minutes followed by a 10 minute break every hour. Volunteers were instructed to wear double layer clothing. Each collector exposed only their legs and captured mosquitoes as soon as they landed on them using a plastic collection tube sealed with a cotton ball. The mosquitoes were then transferred to collection cups grouped by hour and separated by each volunteer. The cups were kept in a Styrofoam box and covered with moist towel to keep the mosquitoes humid and alive during transport back to the field station.

3.2 Mosquito morphological identification and Parity dissections

At the field station, mosquitoes were transferred to plastic knockdown chambers and anesthetized with triethylamine (Flynap®, Carolina Biological Supply Company, Burlington, USA) for 2 minutes. Mosquitoes were then identified morphologically with a dissecting stereomicroscope (Stemi 305, ZEISS, Oberkochen, Germany) using a standard key of the adult of *Anopheles* of Thailand [27]. All primary *Anopheles* species such as *An. dirus* complex and *An. maculatus* group, and a subset of 20 *An. minimus* complex per pair from 18.00 – 24.00 and 24.00 – 06.00 were dissected to remove their ovaries. The parous and nulliparous status was assessed visually with a compound microscope (Optika B-190TB, Ponteranica, Italy) and images of the ovaries taken for reference. Then the mosquito sample was bisected between the thorax and the abdomen and stored in labeled 2 ml centrifuge tubes with silica gel desiccant. Processed mosquitoes were shipped back to the Armed Forces Research Institute of Medical Sciences in Bangkok for molecular species identification, and *Plasmodium* infection evaluation of the thorax.

4) Molecular methods for *Anopheles* and *Plasmodium* identification

4.1) DNA Extraction method

The DNA extraction method involved adding 700 µL of phosphate buffer saline (pH7.4) and 4.5 mm steel beads (Bloomfield, USA) into 2 ml tubes containing individual mosquito thorax or abdomen and homogenized by Qiagen tissue lyser II (Qiagen, Hilden, Germany) at 22 Hz for 2 minutes. Mosquito suspension was centrifuged at 12,000 rpm for 5 min. Briefly, a 250 µL aliquot of the *mosquito homogenate* supernatant was used for DNA extraction according to DNA Minikit and Tissue LC 200 DSP protocol by automated extraction machine, a QIAsymphony® SP instrument (Qiagen). The DNA was eluted in 50 µL and stored at -20°C till further use. DNA/RNA free distilled water was included in the extraction process as a negative extraction control.

4.2) Molecular methods for *Anopheles* sibling species identification

To detect anopheline sibling species, Multiplex allele-specific PCR assay (AS-PCR) was used to examine the ITS2 region of DNA and distinguish the members of the Dirus complex (*An. dirus* s.s., *An. scanloni*, *An. cracens*, *An. baimaii*, *An. nemophilous*), Maculatus group (*An. maculatus* s.s., *An. pseudowillmori*, *An. sawadwongporni*, *An. rampae*, *An. dravidicus*) and Funestus group (*An. minimus* s.s., *An. harrisoni*, *An.*

aconitus, *An. varuna*, *An. pampanai*). The following modifications were added to the previously published protocols [28, 29, 30]. The amplification was carried out using total volumes of 25 µL with the final optimized reaction conditions as follows: (i) 1x GoldStar Best Master mix (GoldStar DNA Polymerase, dNTP, PCR stabilizer and enhancer); (ii) 3 specific primers cocktail, each containing 4-5 difference primer pairs to discriminate between *Anopheles* with 400 nM for primer specific for the Funestus group and Maculatus group and 500 nM for the Dirus complex specific primer; (iii) 4% *dimethyl sulfoxide* (*DMSO*) was included only for Dirus complex reactions; (iv) a universal forward primer, located in conserved region of 5.8S gene, and species-specific reverse primers in the ITS2 spacer region were employed to amplify a portion of the mosquito ITS2 region; and (v) 1 µL of genomic DNA was used as template. Positive controls (*i.e. An. minimus* s.s., *An. dirus* s.s., *An. sawadwongporni*) were obtained from mosquito colonies, maintained at the Armed Forces Research Institute of Medical Science Department of Entomology. Negative controls were included in reaction using DNA/RNA free distilled water.

The amplifications were done using a T100 DNA thermal cycler (Bio-Rad, Hercules, CA) under the following PCR conditions. For the Maculatus group, the program was started as initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, primer annealing at 72°C for 30 s and final extension at 72 °C for 10 min. The Dirus complex, was the same as the Maculatus group, except the annealing time was 15 s instead of 30 s. For the Funestus group, which contains the Minimus complex, the PCR cycle was initiated with denaturation at 95°C for 10 min, followed by 35 cycles of amplification at 94°C for 30 s, 45 °C for 30 s; and 72 °C for 40 s, and final extension at 72^{O}C for 10 min. The amplified PCR products were subjected to DNA fragment analysis using *QIAxcel Advanced system* (Qiagen) according to the manufacturer instructions. Briefly, 10 µL of PCR product was used and analysis was performed with QIAxcel *DNA Fast* Analysis *Cartridge* (Qiagen), using the DM190 method and QX 15-bp/1-kb alignment markers. Fragment sizes were calculated using the BioCalculator (Qiagen). Expected amplicons sizes for species discrimination were determined as shown in table 1.

Table 1) Primers designed for *Anopheles* species detection.

Anopheles group	Primer name	Anopheles species	Sequence (5'-3')	Fragment size (bp)	reference
Funestus group (Minimus complex)	ITS2A	Universal forward primer	TGT GAA CTG CAG GAC ACA T		[28]
	MIA	*An. minimus* (sp. A)	CCC GTG CGA CTT GAC GA	310	
	MIC	*An. harrisoni* (sp. C)	GTT CAT TCA GCA ACA TCA GT	180	
	ACO	*An. aconitus*	ACA GCG TGT ACG TCC AGT	200	
	VAR	*An. varuna*	TTG ACC ACT TTC GAC GCA	260	
	PAM	*An. pampanai*	TGT ACA TCG GCC GGG GTA	90	
Maculatus group	5.8F	Universal forward primer	ATC ACT CGG CTC GTG GAT CG		[30]
	MAC	*An. maculatus*	GAC GGT CAG TCT GGT AAA GT	180	
	PSEU	*An. pseudowillmori*	GCC CCC GGG TGT CAA ACA G	203	
	SAW	*An. sawadwongporni*	ACG GTC CCG CAT CAG GTG C	242	
	K	*An. rampae*	TTC ATC GCT CGC CCT TAC AA	301	
	DRAV	*An. dravidicus*	GCC TAC TTT GAG CGA GAC CA	477	
Dirus complex	D-U	Universal forward primer	CGC CGG GGC CGA GGT GG		[29]
	D-AC	*An. dirus* (A), *An. scanloni* (C)	CAC AGC GAC TCC ACA CG	562 (A), 349/353(C)	
	D-B	*An. cracens*	CGG GAT ATG GGT CGG CC	514	
	D-D	*An. baimaii*	GCG CGG GAC CGT CCG TT	306	
	D-F	*An.nemophilous*	AAC GGC GGT CCC CTT TG	223	

Table 1 lists the forward and backward primers used for *Anopheles* species identification. Universal

forward primers; ITS2A, 5.8F, D-U, are for Funestus group, Maculatus group and Dirus complex, respectively. Specific reverse primers of corresponding species and expected amplification sizes for each species are shown.

4.3) DNA sequencing and Data analysis

To confirm the results of multiplex assay a representative of each Anopheline group was selected for confirmation with ITS2 rDNA gene DNA sequencing. Amplification of ITS2 rDNA gene from mosquito DNA extracts was conducted using universal primer ITS2A (5'-TGT GAA CTG CAG GAC ACA T-3') and ITS2B (5'-TAT GCT TAA ATT CAG GGG GT-3') [31, 32]. Briefly, reactions were performed using T100 DNA thermal cycler (Bio-Rad). PCR reaction mixture (25 µL) consisted of 2 µL of mosquito DNA extract, 0.1 U of AmpliTaq® Gold DNA Polymerase (Life Technologies, Carlsbad, CA), 1X Gold buffer, 0.2 mM of dNTP, 2 mM of $MgCl_2$, 0.2 µM of each primer. The PCR condition was programmed as follows: initial denaturation step at 94°C for 10 min, 37 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and final extension step at 72°C for 5 min. The size of the PCR product was determined using *QIAxcel Advanced system* as described above. The PCR product was clean by ExoSAP-IT™ PCR Products Clean up kit, 2 µl of ExoSAP-IT™ was added directly to 5 µl of PCR reaction product, then incubated at 37 ºC for 15 min and then at 80 ºC for 15 min. The PCR product of ITS2 rDNA gene was sequenced using Bigdye® Terminator v3.1 Cycle Sequencing Kit according to Applied Biosystems's protocol with forward and reverse universal primers; ITS2A and ITS2B and run on a SeqStudio Genetic Analyzer (Life Technologies). Forward and reverse nucleotide sequence data for each sample were assembled using Sequencher 5.1 software packaged (Gene Code Corporation, Ann Arbor, Michigan, USA). The *Anopheles* species were identified by phylogenetic analysis. Briefly, the MUSCLE algorithm was used for sequence alignment in Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software [33]. Maximum likelihood trees were constructed with the best fit model of nucleotide substitution with bootstrapping (1000 replicates) using MEGA 6.0 software as described by Tamura et al. [33].

4.4) Real-time PCR for detection of *Plasmodium* spp.

Real-time PCR for *Plasmodium* detection in *Anopheles* thoraxes was performed using 7500 Fast Real-time PCR system (Life Technologies). The primers and probes were modified from previously published protocol of Kimura et al. [34] using 18S subunit of rRNA of *Plasmodium* spp. as a target gene as follows: forward P2F [5'-TAT TCA GAT GTC AGA GGT GAA ATT C-3'], reverse P2R [5'-GAA CCC AAA GAC TTT GAT TTC TCA T-3'], Plasmodium Genus Probe [5'-FAM- ACG ATC AGA TAC CGT CGT AAT CTT-BHQ2-3']. The real-time PCR reaction (25 µL) consisted of 10 µL of *KAPA PROBE FAST qPCR* Master Mix (2X) (Roche, Branford, CT) containing KAPA Taq HotStart DNA Polymerase, dNTPs, $MgCl_2$, and stabilizers, 0.3 µL of each 20 µM primer , 1 µL of 10 µM probe , 1 µL of mosquito DNA extract, and 8.2 µL of nuclease-free water. *Plasmodium falciparum*- and *P. vivax*-infected *An. dirus* s.s. positive controls and uninfected *An. dirus* negative controls were included in every run. The thermocycler conditions included initial steps at 50 ºC for 2 min and 95 ºC for 2 min, followed by 40 amplification cycles at 95 ºC for 15 min and 60 ºC for

30 min and the cut-off values were set automatically at every run. All positive samples were confirmed for *Plasmodium* species by Nested PCR.

4.5) Nested PCR Analysis for *Plasmodium* species differentiation

The Nested PCR was performed to identify 4 *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* using two amplification processes as described by Kimura et al. [34]. The nested PCR was performed using T100 DNA thermal cycler (Bio-Rad). I*nner* and *outer* primers for the *nested PCR are listed in table 2.*

Table 2) Oligonucleotide sequences of primers used in nested PCR analysis for Plasmodium species differentiation

Primer set	Primer name	Sequence (5' to 3')
Primary PCR primer	P1F	ACG ATC AGA TAC CGT AAT CTT
	P2R	GAA CCC AAA GAC TTT GAT TTC TCA T
Secondary PCR primer	FR	CAA TCT AAA AGT CAC CTC GAA AGA TG
	MR	GGA AGC TAT CTA AAA GAA ACA CTC ATA T
	OR	ACT GAA GGA AGC AAT CTA AGA AAT TT
	VR	CAA TCT AAG AAT AAA CTC CGA AGA GAA

Table 2 lists the species-specific primers targeting 18S rRNA gene were used for nested PCR assay. The first-round PCR primers are P1F and P2R. The second-round PCR primers are P1F and species-specific reverse primers; FR, MR, OR and VR for *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*, respectively.

For the first PCR reaction, the reaction mixture (20 µL) consisted of 1X PCR Gold Buffer II (50mM KCl, 15 mM Tris-HCl, pH8.0), 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each specific outer primer set (P1F and P2R), 0.25 units of Amplitaq Gold™ DNA polymerase, 1 µL of DNA template. The condition of the first PCR was as follows: 94 °C for 10 min, 35 cycles of 92°C for 30 s, 60°C for 90 s, and 72°C for 1 min. Nuclease free water was used as negative control. The amplified fragment was analyzed using Qiaxcel advanced system with expected size around 140-160 bp. For the second PCR, the product of the first PCR was diluted (1:50) with water and used it as a template in the second PCR reaction. The second PCR reaction (20 µL) and amplification program were the same as the first PCR, except that the reverse specific primers were used instead of P2R primer. Expected product (about 110 bp) of each *Plasmodium* species was determined by Qiaxel Advanced System as previously described.

5) Statistical analysis

The number of *Anopheles* caught per night was calculated by assessing the total number of *Anopheles* captured at one human landing collection site divided by 2 for the pair of collectors used at each location and this number was then used to calculate the mean number of *Anopheles*. The median mosquito catching time was calculated and the comparison of the distribution of mosquito catching times among groups were assessed using the non-parametric Kruskal-Wallis Test, and pairwise comparisons for significantly different groupings were performed using the post-hoc Dunn's Multiple Comparisons Test to account for the multiple comparisons using Prism version 7.2 (GraphPad Software, San Diego, CA, USA). Parity by cluster and hour was assessed by a logistic regression model and the clustering of outcomes was accounted for by using the robust standard errors. The sample size and power calculations for a cluster randomized trial were performed using the clustersampsi command in STATA version 14 (StataCorp, College Station, TX, USA).

Results

Topographical Maps

Figure 1 depicts a terrain map of Surat Thani with the clusters evaluated from Phanom (PN), Khiri Rat Nikhom (KR), and Vibhavadi (VB). Figure 2 are zoomed in terrain maps depicting the house and mosquito collection locations for each of the 13 clusters evaluated in this study.

Figure 1 depicts the cluster locations that were surveyed for *Anopheles* mosquitoes in 2019 and surrounding terrain features.

Figure 2 depicts enlarged terrain maps for each group of clusters surveyed in 2019. Houses (circles) and mosquito collection sites (triangles) are marked along with cluster boundaries (dashed lines). As much of the cluster that was safely accessible was surveyed for mosquito collections.

Species composition and abundance

In 13 selected clusters from 3 districts, a total of 17,348 adult female mosquitoes were collected representing 6 genera: *Armigeres*, *Anopheles*, *Aedes*, *Culex*, *Mansonia*, and *Coqullitettedia* (Figure 3). A total of 23 Culicine mosquito species were identified from 11,571 collected specimens. The most abundant Culicine species were: 55.59% *Ar. subalbatus*, 30.15% *Ae. albopictus*, 3.21% *Cx. gelidus*, 3.21% *Cx. quinquefasciatus*, and 2.73% *Ma. indiana*, with the remaining 18 Culicine species each comprising less than 1% of the total collection (Figure 4).

Figure 3 represents the proportion of mosquito genera captured in Surat Thani in 2019.

Figure 4 represents the proportion of Culicine species captured from Surat Thani in 2019. *Aedes* (*Ae.*); *Armigeres* (*Ar.*); *Coquilletidia* (*Cq.*); *Culex* (*Cx.*); *Mansonia* (*Ma.*)

Fifteen *Anopheles* species were morphologically identified from a total of 5,777 specimens, of which 10 belonged to the subgenus *Cellia* and 5 to the subgenus *Anopheles*. The predominant *Anopheles* captured

at are study site were primary or suspected vectors and are listed by abundance: 87.17% *Anopheles minimus* s.l. (n = 5,035), 7.05% *An. dirus* s.l. (n = 407), 2.86% *An. barbirostris* s.l. (n = 165), and 1.04% *An. maculatus* s.l. (n = 60). The remaining 11 species comprised 1.79% of the total *Anopheles* captured: *An. donaldi* (n = 32), *An. tessellatus* (n = 27), *An. pollicaris* (n = 23), *An. philippinensis* (n = 11), *An. subpictus* (n = 3), *An. epiroticus* (n = 2), *An. hodgkini* (n = 2), *An. nigerrimus* (n = 2), *An. hyrcanus* s.l. (n = 1), *An. jamesii* (n = 1), *An. kochi* (n = 1). There were only 5 (0.09%) *Anopheles* specimens that could not be morphologically identified to species (Figure 5a).

Overall, *An. minimus* s.l. was the most abundant *Anopheles* species captured across all three districts in Phanom (PN), Khiri Rat Nikhom (KR) and Vibhavadi (VB) (Figure 5a-d). The second and third most abundant Anopheles species by district were: *An. dirus* s.l. and *An. barbirostris* s.l. in PN (Figure 5b), *An. dirus* s.l. and *An. maculatus* s.l. in KR (Figure 5c), and *An. maculatus* s.l. and *An. dirus* s.l. in VB (Figure 5d). Of the total *Anopheles* captured (n = 5,777), primary and secondary malaria vectors comprised 95.26% of the total *Anopheles* collected.

Figure 5 represents the proportion of *Anopheles* species captured identified morphologically from Surat Thani and separated by district.

The average numbers of *Anopheles* specimens collected per volunteer per night are shown in Table 3. Overall, nightly human landing catch did not vary by district for all *Anopheles* with an average of 11.46 *Anopheles* captured per collector per night. There were no differences in mean number of *An.minimus* s.l. captured per collector per night across the three districts. However, more *An. dirus* s.l were captured per person per night from PN, and more *An. maculatus* s.l. per person per night from KR, while fewer *An. barbirostris* s.l. were captured per person per night from KR (Table 3).

Table 3) Mean number of *Anopheles* captured per collector per night from Surat Thani

Study sites	*An.minimus* s.l. Mean (95% CI)	*An. dirus* s.l Mean (95% CI)	*An. barbirostris* s.l. Mean (95% CI)	*An. maculatus* s.l Mean (95% CI)	All *Anopheles* Mean (95% CI)
PN	7.50 (3.50,11.50)	**2.11 (1.02,3.20)**	0.64 (0.34,0.93)	0.01 (0.01,0.02)	10.70 (5.95,15.46)
KR	9.91 (7.88,11.94)	0.35 (0.23,0.47)	**0.12 (0.07,0.17)**	**0.20 (0.10,0.30)**	10.73 (8.64,12.82)
VB	13.47 (7.13,19.81)	0.31 (0.12,0.50)	0.45 (0.18, 0.72)	0.03 (0.01,0.06)	14.46 (8.14,20.78)
All districts	9.99 (8.06,11.92)	0.81 (0.49,1.12)	0.32 (0.22, 0.42)	0.12 (0.06,0.07)	11.46 (9.43,13.50)

Table 3 represents the mean and 95% Confidence Interval (CI) number of *Anopheles* captured per collector per night in Surat Thani in 2019, separated by district. Phanom (PN), Khiri Rat Nikhom (KR), Vibhavadi (VB).

In Phanom, 12 *Anopheles* species were morphologically identified from four clusters. *An. minimus* s.l. was the most abundant *Anopheles* species captured across all four clusters (Figure 6a-d) and the second and third most abundant *Anopheles* species by cluster were: *An. dirus* s.l. and *An. barbirostris* s.l. in PN-01 (Figure 6a), *An. barbirostris* s.l. and *An. dirus* s.l. in PN-02 (Figure 6b), *An. dirus* s.l. and *An. maculatus* s.l. in PN-03 (Figure 6c), and *An. barbirostris* s.l. and *An. dirus* s.l. in PN-04 (Figure 6d).

Figure 6 represents the morphological composition of *Anopheles* identified from 4 clusters in PN district.

In KR, 12 *Anopheles* species were morphologically identified from five clusters. *An. minimus* s.l. was the most abundant *Anopheles* species captured across all five KR clusters (Figure 7a-e) and the second and third most abundant *Anopheles* species by cluster were: *An. barbirostris* s.l. and *An. maculatus* s.l. in KR-05 (Figure 7a), *An. maculatus* s.l. and *An. dirus* s.l. in KR-06 (Figure 7b), *An. dirus* s.l. and *An. tesselatus* in KR-07 (Figure 7c), *An. dirus* s.l. and *An. maculatus* s.l. in KR-08 (Figure 7d), and *An. dirus* s.l. and *An. barbirostris* s.l. in KR-09 (Figure 7e).

Figure 7 represents the morphological composition of *Anopheles* identified from 5 clusters in KR district.

Species diversity was more limited in VB, with only 6 *Anopheles* species captured and morphologically identified. *An. minimus* s.l. was the most abundant *Anopheles* species captured across all four VB clusters (Figure 8a-d) and the second and third most abundant *Anopheles* species by cluster were: *An. barbirostris* s.l. and *An. dirus* s.l. in VB-10, and VB-13 (Figure 8a,d), and *An. dirus* s.l. and *An. barbirostris* s.l. in VB-11 and VB-12 (Figure 8b,c).

Figure 8 represents the morphological composition of *Anopheles* identified from 4 clusters in VB district.

A total of 879 *Anopheles* were identified molecularly to species by PCR or sequencing. Of the Funestus group, which contains the Minimus complex, 99.80% (n=484) were *An. minimus* s.s. and 0.20% (n=1) were *An. aconitus* (Figure 9a). The Dirus complex was 100% *An. baimaii* (n=347) (Figure 9b). Of the Maculatus group, 93.62% were *An. maculatus* s.s. (n=44) and 6.38% were *An. sawadwongporni* (n=3) (Figure 9c). One *An. epiroticus* and one *An. nigerrimus* specimen were identified by sequencing. The Barbirostris group was identified to species based on morphology. Of the Barbirostris group, 69.37% were *An. barbirostris* (n=154), 14.42% were *An. donaldi* (n=32), 10.36% were *An.pollicaris* (n=23), 4.95% were *An. campestris* (n=11), and 0.90% were *An. hodgkini* (n=2) (Figure 9d).

Figure 9 depicts the molecular (a-c) and morphological (d) composition of *Anopheles* species groups and complexes collected from all surveyed clusters in Surat Thani in 2019.

Plasmodium infection status

A total of 879 *Anopheles* mosquitoes were tested to determine *Plasmodium* infection status and none were found to be *Plasmodium* positive.

Anopheles mosquito collection by time

Overall, there were significant differences in hour of collection by species (Kruskal-Wallis = 80.89, P < 0.0001, n = 5,666) with significantly more *An. barbirostris* s.l. and *An. maculatus* s.l. caught earlier in the night compared to *An. minimus* s.l. (P = 0.0001, P < 0.0001, respectively) and *An. dirus* s.l. (P = 0.0082, P < 0.001, respectively). Overall, almost 60% of *An. maculatus* s.l. and 30% of the *An. barbirsostris* s.l. were captured between 18:00 – 20:00 (Figure 10a). In Phanom, there were no significant differences in time of *Anopheles* (n = 1,358) capture between the species (Figure 10b), although too few *An. maculatus* s.l. (n = 1) were captured to be included in the analysis. In Khiri Rat Nikhom, there were significant differences in hour of collection by species (Kruskal-Wallis = 82.19, P < 0.0001, n = 2,884) with significantly more *An. barbirostris* s.l. and *An. maculatus* s.l. caught earlier in the night compared to *An. minimus* s.l. (P < 0.0001, P < 0.0001, respectively) and *An. dirus* s.l. (P = 0.0164, P < 0.001, respectively). In Vibhavadi, there were significant differences in hour of collection by species (Kruskal-Wallis = 21.61, P < 0.0001, n = 1,424) with significantly more *An. minimus* s.l. caught later in the night compared to *An. dirus* s.l. (P = 0.0108) and *An. barbirostris* s.l. (P = 0.0457). Too few *An. maculatus* s.l. (n = 3) were captured in VB to determine significant differences in capture time from the other species. Interestingly, there were significant differences in capture times across all three districts for *An. minimus* s.l. (Kruskal-Wallis = 16.66, P = 0.0002, n = 5,035), *An. dirus* s.l. (Kruskal-Wallis = 17.4, P = 0.0002, n = 407), and *An. barbirostris* s.l. (Kruskal-Wallis = 12.61, P = 0.0018, n = 165). *An. minimus* s.l. were captured later in the night in Vibhavadi compared to Phanom (P = 0.0275) and Khiri Rat Nikhom (P = 0.0002). *An. dirus* s.l. were captured later in the night in Phanom compared to Khiri Rat Nikhom (P = 0.0023) and Vibhavadi (P = 0.0088). *An. barbirostris* s.l. were captured earlier in the night in Khiri Rat Nikhom compared to Phanom (P = 0.0013) and Vibhavadi (P = 0.0273).

Figure 10 depicts the proportion of *Anopheles* species collected by hour from all districts (a), PN (b), KR (c), and VB (d).

Anopheles mosquito parity status

A total of 3,831 *Anopheles* specimens were dissected to determine parity, *An.minimus* s.l. (n = 3,509) and *An.dirus* s.l. (n = 322). Across the three districts the proportion parous of *An.minimus* s.l. ranged from 51.71% to 71.64% and *An. dirus* s.l from 55.32% to 74.71% (Table 4).

Table 4) Parity status of *Anopheles* mosquitoes in Surat Thani

Study sites	*An.minimus* s.l.				*An.dirus* s.l.			
	Parous	Nulliparous	Total	Parity (%)	Parous	Nulliparous	Total	Parity (%)
PN	287	268	555	51.71	130	105	235	55.32
KR	1,437	569	2,006	71.64	65	22	87	74.71
VB	651	297	948	68.67	18	12	30	60.00
Total	2,375	1,134	3,509	67.68	213	139	352	60.51

Table 4 represents the total number of parous and nulliparous dissected *An. minimus* s.l. and *An. dirus* s.l. in 2019, separated by district. Phanom (PN), Khiri Rat Nikhom (KR), Vibhavadi (VB).

Overall, there was a significant increasing trend in the proportion of parous *An. minimus* s.l. captured by hour throughout the night (Wald Chi-square = 17.31, P=0.000, odds ratio = 1.0535 [1.0279 – 1.0796] 95% CI (n = 3,400) (Figure 11a). Only clusters with more than 100 dissected *An. minimus* s.l. were included in the analyses (*i.e.* PN-01, PN-03, KR-05, KR-06, KR-07, KR-08, KR-09, VB-10, VB-11, VB-12). While all clusters showed an increasing trend in the proportion of parous *An. minimus* s.l. throughout the night (Figure 12), only PN-01 and VB-12 were significant (Table 5). For *An. dirus* s.l. there was no significant trend in the proportion of parous mosquitoes by hour throughout the night (Wald Chi-square = 0.46, P=0.497, odds ratio = 0.9702 [0.8891 – 1.0587] 95% CI (n = 229)) (Figure 11b). Only clusters with more than 20 dissected *An. dirus* s.l. (*i.e.* PN-01, PN-03, PN-04) were included in the analysis.

Figure 11 represents proportion of *An. minimus* (a) and *An. dirus* (b) that were parous or nulliparous collected per hour from all clusters with at least 100 dissected *An. minimus* and 20 dissected *An. dirus*.

Table 5) Increasing trend in *An. minimus* s.l. parity by hour for each cluster

Cluster	P-value	Odds Ratio (95% CI)	n
PN-01	**0.002**	**1.1083 (1.0393 - 1.1819)**	**392**
PN-03	0.680	1.0250 (0.9117 - 1.1523)	121
KR-05	0.251	1.0493 (0.9665 - 1.1393)	335
KR-06	0.438	1.0298 (0.9563 - 1.1089)	501
KR-07	0.884	1.0069 (0.9180 - 1.1044)	280
KR-08	0.102	1.0520 (0.9900 - 1.1180)	595
KR-09	0.250	1.0478 (0.9676 - 1.1347)	282
VB-10	0.530	1.0217 (0.9555 - 1.0925)	329
VB-11	0.133	1.1141 (0.9675 - 1.2829)	146
VB-12	**0.034**	**1.0824 (1.0059 - 1.1646)**	**419**

Table 5 depicts the increasing trend in parity by hour and significance by cluster for *An. minimus* s.l. (n = 3,400). CI = confidence interval, n = number of mosquitoes dissected

Figure 12 represents the proportion of *An. minimus* that were parous or nulliparous collected per hour by cluster (n = 3,400).

Power calculation

The purpose of this field study was to assess the potential of Surat Thani to serve as the location for future entomological evaluation of ivermectin MDAs. As *Plasmodium* infection rates in GMS *Anopheles* are extremely low, either survival or parity become the ideal metrics to measure, however, capturing blood-fed *Anopheles* is very difficult rendering assessment of mosquito survival as done previously in Africa impossible. The power calculation to assess the impact of ivermectin MDA on mosquito parity was based on *An. minimus* s.l. as it is a primary malaria vector, the most abundant *Anopheles* species captured in Surat Thani 87.87% (5,035/5,777), and the most ivermectin-sensitive GMS malaria vector evaluated to date [13, 14]. Parity rates (P_0 = 67.68%: 2,375/3,509) from ten clusters wherein more than 100 *An. minimus* s.l. were collected and dissected were used to calculate an intra-cluster correlation (ICC) to be used for sample size calculations. Individual mosquito parity results were assessed with a random effects logistic regression model to obtain an ICC of 0. 0378. To demonstrate an effect, based on a two-sided of alpha = 0.05, a parity rate at baseline and in control villages of P_0 = 67.68%, a treatment effect size = 34% reduction meaning treatment villages with a parity rate of P_1 = 44.67% after MDA, and an ICC = 0. 0378, requires at least four clusters per treatment arm and a minimum of 300 mosquitoes dissected per cluster to provide 82% power. A conservative treatment effect size of 34% was predicted based on a previously developed model for ivermectin MDA [35] parameterized with *An. minimus* s.s. ivermectin susceptibility data [14]. This treatment effect size is reasonable and supported by the following:

ivermectin MDAs in West Africa reduced the parity rate in *An. gambiae* by 30% [10], *An. minimus* (7-day-LC_{50} = 14.7 ng/ml) [14] is more susceptible to ivermectin than *An. gambiae* (7-day-LC_{50} = 15.9 ng/ml) [36], and we plan to administer 400 µg/kg ivermectin at our field site whereas 150 – 200 µg/kg was used in West Africa MDA trials [10].

Discussion

This study represents the most intensive and detailed analysis of *Anopheles* bionomics from Surat Thani, Thailand. These surveys were conducted to determine which clusters in Surat Thani were most appropriate for evaluation of the entomological impacts of ivermectin MDA on *Anopheles*. Most (95.26%) of the *Anopheles* species collected from PN, KR, and VB districts were primary or secondary malaria vectors. In addition to detailed hourly collection data, this study presents the parity status throughout the night for the primary malaria vectors, *An. dirus* s.l. and *An. minimus* s.l. Finally, a power calculation was performed to determine the number of clusters that should be utilized in entomological evaluation to assess the impact of ivermectin MDA on *An. minimus* parity.

Surat Thani is unique in that it is a malarious province in Thailand that is not located along an international border. It has some of the highest rainforest cover in the GMS and the highest amount of rubber plantation in Thailand, making it an ideal habitat for GMS malaria vectors. Indeed, of the fifteen species captured in these surveys, eleven of them were either primary malaria vectors (*An. minimus* s.l., *An. dirus* s.l., *An. maculatus* s.l.), secondary malaria vectors (*An. epiroticus*), or suspected malaria vectors (*An. barbirostris* s.l., *An. hodgkini*, *An. hyrcanus* s.l., *An. nigerrimus*, *An. philippinensis*, *An. kochi*, *An. tessellatus*,). *An. minimus* s.l. and *An. dirus* s.l. accounted for 87.17% (5,035/5,777) and 7.05% (407/5,777) of the total collection, respectively. Molecular identification confirmed that of *An. minimus* s.l. 99.80% were *An. minimus* s.s. (n = 484) and 0.2% *An. aconitus* (n = 1), and of *An. dirus* s.l. 100% were *An. baimaii* (n = 348). Since the secondary and suspected vectors were captured in such low numbers, it is likely that the primary malaria vectors, *An. minimus* s.s. and *An. baimaii* are largely responsible for *Plasmodium* transmission in the study area. However, as no specimens were *Plasmodium* positive (n = 879) it cannot be confirmed that these vectors are solely responsible for transmission. A previous study in Ubon Ratchathani province, in northeastern Thailand, reported that suspected malaria vectors *An. barbirostris* s.l., *An. philippinensis*, and *An. hyrcanus* s.l. are highly zoophagic, feeding mostly on cattle [37]. Since we only used the HLC method this may explain the low abundance of these species in the study area, making it difficult to rule them out as possible contributors to *Plasmodium* transmission in Surat Thani.

Primary malaria vectors in the GMS tend to be collected more frequently outdoors than indoors [7, 8]. Since the aim of this study is collect as many human host-seeking *Anopheles* as possible, all HLCs were performed outdoors. Results from a cross-sectional survey in Surat Thani in the same study area where HLCs performed indicated that staying outdoors is a primary risk factor for asymptomatic *Plasmodium* carriage, suggesting that most of the transmission occurs outside the home [23]. This finding reinforces

the potential usefulness of ivermectin MDA in the GMS as it can target the *Anopheles* malaria vector regardless of location or time.

Vibhavadi had the lowest *Anopheles* species diversity, with only six species collected. However, this reduced diversity could be due in part to seasonality and the limited sampling duration from July to October, while Khiri Rat Nikhom and Phanom had mosquitoes collected from February to October. Another interesting point about mosquito collections in Vibhavadi was that *An. minimus* s.l. were captured later in the night compared to Phanom (P = 0.0275) and Khiri Rat Nikhom (P = 0.0002). This could be due production of durian in Vibhavadi as thermal fogging with malathion occurs at nighttime for the control of several arthropod pests, in particular *Scirtothrips dorsalis* (Order Thysanoptera; Family Thripidae) and *Allocaridara malayensis* (Order Homoptera; Family Psyllidae), and this fogging was observed at some of the mosquito collection sites during HLCs in Vibhavadi clusters. Vibhavadi has the fourth highest coverage (1.33%) of durian plantation of the 19 districts in Surat Thani with Khiri Rat Nikhom the eighth highest coverage (0.33%) and Phanom twelfth highest coverage (0.18%) [38].

The abundance of *An. dirus* s.l. varied among districts, with the greatest numbers captured in PN. This is likely due geographical (Figs. 1 and 2) and biological characteristics as the PN study site is surrounded by national parks comprised of primary old growth rain forest and steep hillsides. Furthermore, rubber plantations adjacent to the forest create a suitable habitat for *An. dirus* s.l. proliferation [2, 19]. *An. dirus* s.l. were captured later in the night in Phanom compared to Khiri Rat Nikhom (P = 0.0023) and Vibhavadi (P = 0.0088) but this may have been artifact due to the fewer number of *An. dirus* s.l. captured in Khiri Rat Nikhom and Vibhavadi districts.

These surveys report new records for Surat Thani for the Barbirostris group including: *An. hodgkini*, *An. donaldi*, and *An.* pollicaris. However, it is possible these species may have just been counted as *An. barbirostris* s.l. previously as they are difficult to distinguish morphologically [24]. Future work in this study area should identify the members of the Barbirostris group by molecular methods as not all species in this group are malaria vectors [39]. Another new record for Surat Thani was *An. sawadwongporni*, but again this may have not been identified in previous surveys due to lack of molecular analysis [24]. Additionally, *An. baimaii* is a new record for Surat Thani, likely missed due to lack of molecular analysis. It is somewhat surprising that more members of the Dirus complex were not identified as *An. dirus* s.s., *An. cracens*, *An. nemophilous*, and *An. scanloni* have been observed in adjacent provinces of Phang Nga, Krabi, Nakhon Si Thammarat, and Ranong [2].

There were no *Plasmodium* positive *Anopheles* specimens (n = 879) detected. However, this is not very surprising as the likelihood of finding sporozoite-infected *Anopheles* in the GMS is very low, typically lower than 1:1,000 [8]. This is why the population age structure (*i.e.* parity rate) was selected as the primary entomological outcome indicator to assess impact of ivermectin MDA on *Anopheles* populations in Surat Thani. This study found an overall *An. minimus* s.l. parity of 67.68% (2,375/3,509) which is comparable with other surveys in Thailand [40, 41, 42, 43]. Similar to Sithiprasana et al. [41], little fluctuation in *An. minimus* s.l. parity was observed from cluster to cluster, with an ICC value of 0.0378.

There was a significant trend for increasing *An. minimus* s.l. parity by hour of collection suggesting that older *An. minimus* tend to feed later at night (Fig. 11a) but no trend was observed for *An. dirus* s.l. (Fig. 11b). A power calculation determined that an *An. minimus* s.l. parity reduction treatment effect size = 34%, with four clusters per treatment arm, a minimum of 300 mosquitoes dissected per cluster at an $\alpha = 0.05$ will provide 82% power to detect a significant difference in the population age structure (*i.e.* parity).

Conclusion

An abundance of *Anopheles* primary malaria vectors were captured in Surat Thani. This study illustrates that Surat Thani will be an ideal field site for evaluating the impacts of ivermectin MDA on local *Anopheles* population age structure.

Abbreviations

Aedes (*Ae.*); *Anopheles* (*An.*); *Armigeres* (*Ar.*); *Coquilletidia* (*Cq.*); *Culex* (*Cx.*); Greater Mekong Subregion (GMS); Human landing collection (HLC); Indoor residual spraying (IRS); Long-lasting insecticide-treated nets (LLINs); *Mansonia* (*Ma.*); Mass drug administration (MDA); *Plasmodium* (*P*); Polymerase chain reaction (PCR); sensu lato (s.l.); sensu stricto (s.s.)

Declarations

Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70–25.

Ethics approval and consent to participate

The study was approved by the Walter Reed Army Institute of Research (WRAIR#2430), the Human Research Protection Office (HRPO Log No.19919.2a/A-19919.2b), and the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Thai MoPH Ref no.25/2560). All mosquito collectors were enrolled by appointed Vector-Borne Disease Control center staff.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NW, KCK designed the study; NW, OK, KCK, PT, UN, performed mosquito collections and field processing; PS, KK, AK, WN performed mapping efforts; JS, RT performed molecular analyses; SC, MM, KCK performed statistical analyses; ST, VS, SAD, PWM, WN, JSP, JS provided administrative oversight; NW, KCK wrote the first draft; all authors read and approved the final manuscript.

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References

1. World Health Organization. World malaria report 2020: 20 years of global progress and challenges. Geneva2020. p. Licence: CC BY-NC-SA 3.0 IGO.
2. Obsomer V, Defourny P, Coosemans M. The *Anopheles dirus* complex: spatial distribution and environmental drivers. Malaria Journal. 2007;6:e26.
3. Manguin S, Garros C, Dusfour I, Harbach R, Coosemans M. Bionomics, taxonomy, and distribution of the major malaria vector taxa of *Anopheles* subgenus Cellia in Southeast Asia: an updated review. Infection Genetics Evolution. 2008;8(4):489–503.

4. Rattanarithikul R, Konishi E, Linthicum K. Detection of *Plasmodium vivax* and *Plasmodium falciparum* circumsporozoite antigen in anopheline mosquitoes collected in southern Thailand. The American Journal of Tropical Medicine Hygiene. 1996;54(2):114–21.
5. Cui L, Yan G, Sattabongkot J, Chen B, Cao Y, Fan Q, et al. Challenges and prospects for malaria elimination in the Greater Mekong Subregion. Acta Trop. 2012;121(3):240–5.
6. Van Bortel W, Trung H, Hoi L, Ham N, Chut N, Luu N, et al. Malaria transmission and vector behaviour in a forested malaria focus in central Vietnam and the implications for vector control. Malaria Journal. 2010;9:e373.
7. Trung H, Van Bortel W, Sochantha T, Keokenchanh K, Briët O, Coosemans M. Behavioural heterogeneity of *Anopheles* species in ecologically different localities in Southeast Asia: a challenge for vector control. Tropical Medicine International Health. 2005;10(3):251–62.
8. Sinka M, Bangs M, Manguin S, Chareonviriyaphap T, Patil A, Temperley W, et al. The dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. Parasites Vectors. 2011;4:e89.
9. Sylla M, Kobylinski K, Gray M, Chapman P, Sarr M, Rasgon J, et al. Mass drug administration of ivermectin in south-eastern Senegal reduces the survivorship of wild-caught, blood fed malaria vectors. Malaria Journal. 2010;9:e365.
10. Alout H, Krajacich B, Meyers J, Grubaugh N, Brackney D, Kobylinski K, et al. Evaluation of ivermectin mass drug administration for malaria transmission control across different West African environments. Malaria Journal. 2014;13:e417.
11. Kobylinski K, Sylla M, Chapman P, Sarr M, Foy B. Short Report: Ivermectin Mass Drug Administration to Humans Disrupts Malaria Parasite Transmission in Senegalese Villages. The American Journal of Tropical Medicine Hygiene. 2011;85(1):3–5.
12. Foy B, Alout H, Seaman J, Rao S, Magalhaes T, Wade M, et al. Efficacy and risk of harms of repeat ivermectin mass drug administrations for control of malaria (RIMDAMAL): a cluster-randomised trial. Lancet. 2019;393(10180):1517–26.
13. Kobylinski K, Ubalee R, Ponlawat A, Nitatsukprasert C, Phasomkulsolsil S, Wattanakul T, et al. Ivermectin susceptibility and sporontocidal effect in Greater Mekong Subregion. *Anopheles* Malaria Journal. 2017;16:e280.
14. Kobylinski K, Jittamala P, Hanboonkunupakarn B, Pukrittayakamee S, Pantuwattana K, Phasomkulsolsil S, et al. Safety, pharmacokinetics, and mosquito-lethal effects of ivermectin in combination with dihydroartemisinin-piperaquine and primaquine in healthy adult Thai subjects. Clin Pharmacol Ther. 2020;107(5):1221–30.
15. Erhart A, Ngo D, Phan V, Ta T, Van Overmeir C, Speybroeck N, et al. Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. Malaria Journal. 2005;4:e58.
16. Prothero R. Malaria, forests and people in Southeast Asia. Singap J Trop Geogr. 1999;20(1):76–85.
17. Division of Vector Borne Diseases. Malaria Online: The Digital Surveillance System for Thailand Malaria Elimination. Thailand: Department of Disease Control Ministry of Public Health Thailand;

2019. pp. 1–46.

18. Singhasivanon P, Thimasarn K, Yimsamran S, Linthicum K, Nualchawee K, Dawreang D, et al. Malaria in tree crop plantations in south-eastern and western provinces of Thailand. Southeast Asian J Trop Med Public Health. 1999;30(3):399–404.
19. Bhumiratana A, Sorosjinda-Nunthawarasilp P, Kaewwaen W, Maneekan P, Pimnon S. Malaria-associated rubber plantations in Thailand. Travel Medicine Infectious Diseases. 2013;11(1):37–50.
20. Chujun S, Chaivisit P, Chutinantakul A. Epidemiological characteristics and factors related to malarial disease in Thai and migrant patients in the upper part of southern Thailand. Disease Control Journal. 2019;45(4):380–91.
21. Rubber. A total planting area, total harvesting area, total yield, yield per rai, for country level, regions level, and provincial level [Internet]. Office of Agricultural Economics. 2018 [cited 19 May 2020]. Available from: http://www.oae.go.th/view/1//TH-TH.
22. Thailand Malaria Elimination Program [Internet]. Thailand Ministry of Health. 2019 [cited 21 April 2020]. Available from: http://203.157.41.215/malariaR10/index_v2.php.
23. Shimizu S, Chotirat S, Dokkulab N, Hongchad I, Khowsroy K, Kiattibutr K, et al. Malaria cross-sectional surveys identified asymptomatic infections of *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium knowlesi* in Surat Thani, a southern province of Thailand. International Journal of Infectious Diseases. 2020;96:445–51.
24. Aumaung B, Sevana J, Sinakom B, Yongchaitrakol S, Muernrat Y. Study on long lasting insecticidal nano jacket (LLINJ) for malaria vector control. Disease Control Journal. 2019;44(4):448–58.
25. Muenworn V, Sungvornyothin S, Kongmee M, Polsomboon S, Bangs M, Akrathanakul P, et al. Biting activity and host preference of the malaria vectors *Anopheles maculatus* and *Anopheles sawadwongporni* (Diptera: Culicidae) in Thailand. Journal of Vector Ecology. 2009;34(1):62–9.
26. Tainchum K, Kongmee M, Manguin S, Bangs M, Chareonviriyaphap T. *Anopheles* species diversity and distribution of the malaria vectors of Thailand. Trends in Parasitology. 2015;31(3):109–19.
27. Rattanarithikul R, Harrison B, Harbach R, Panthusiri P, Coleman R, Panthusiri P. Illustrated keys to the mosquitoes of Thailand. IV. *Anopheles*. Southeast Asian J Trop Med Public Health. 2006;37:1–128.
28. Walton C, Handley J, Kuvangkadilok C, Collins F, Harbach R, Baimai V, et al. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. Med Vet Entomol. 1999;13(1):24–32.
29. Garros C, Koekemoer L, Coetzee M, Coosemans M, Manguin S. A single multiplex assay to identify major malaria vectors within the African *Anopheles funestus* and the Oriental *An. minimus* groups. The American Journal of Tropical Medicine Hygiene. 2004;70(6):583–90.
30. Walton C, Somboon P, O'Loughlin S, Zhang S, Harbach R, Linton Y, et al. Genetic diversity and molecular identification of mosquito species in the *Anopheles maculatus* group using the ITS2 region of rDNA. Infection Genetics Evolution. 2007;7(1):93–102.
31. Beebe N, Saul A. Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction–restriction fragment length polymorphism analysis. The American

Journal of Tropical Medicine Hygiene. 1995;53(5):478–81.

32. Saeung A, Baimai V, Otsuka Y, Rattanarithikul R, Somboon P, Junkum A, et al. Molecular and cytogenetic evidence of three sibling species of the *Anopheles barbirostris* Form A (Diptera:Culicidae) in Thailand. Parasitol Res. 2008;102(3):499–507.
33. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.
34. Kimura M, Kaneko O, Liu Q, Zhou M, Kawamoto F, Wataya Y, et al. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitol Int. 1997;46(2):91–5.
35. Slater H, Walker P, Bousema T, Okell L, Ghani A. The potential impact of adding ivermectin to a mass treatment intervention to reduce malaria transmission: a modelling study. J Infect Dis. 2014;210(12):1972–80.
36. Kobylinski K, Foy B, Richardson J. Ivermectin inhibits the sporogony of *Plasmodium falciparum* in *Anopheles gambiae*. Malaria Journal. 2012;11:e381.
37. Marasri N, Overgaard H, Sumarnrote A, Thanispong K, Corbel V, Chareonviriyaphap T. Abundance and distribution of *Anopheles* mosquitoes in a malaria endemic area along the Thai-Lao border. Journal of Vector Ecology. 2017;42(2):325–34.
38. Surat Thani provincial. product information: durian [Internet]. Office of the Permanent Secretary for Ministry of Agriculture and Cooperatives. 2015 [cited 19 May 2020]. Available from: https://www.opsmoac.go.th/suratthani-dwl-files-401091791958.
39. Brosseau L, Udom C, Sukkanon C, Chareonviriyaphap T, Bangs M, Saeung A, et al. A multiplex PCR assay for the identification of five species of the *Anopheles barbirostris* complex in Thailand. Parasites Vectors. 2019;12(1):e223.
40. Gingrich J, Weatherhead A, Sattabongkot J, Pilakasiri C, Wirtz R. Hyperendemic malaria in a Thai village: dependence of year-round transmission on focal and seasonally circumscribed mosquito (Diptera: Culicidae) habitats. J Med Entomol. 1990;27(6):1016–26.
41. Sithiprasasna R, Linthicum K, Liu G, Jones J, Singhasivanon P. Some entomological observations on temporal and spatial distribution of malaria vectors in three villages in northwestern Thailand using a geographic information system. Southeast Asian J Trop Med Public Health. 2003;34(3):505–16.
42. Rattanarithikul R, Linthicum K, Konishi E. Seasonal abundance and parity rates of *Anopheles* species in southern Thailand. J Am Mosq Control Assoc. 1996;12(1):75–83.
43. Zollner G, Sattabongkot J, Vaughan J, Kankaew P, Robert L, Thimasarn K, et al. Longitudinal Evaluation of malaria epidemiology in an isolated village in Western Thailand: I. Study site and adult Anophelene bionomics. Southeast Asian J Trop Med Public Health. 2016;47(3):341–65.

Figures

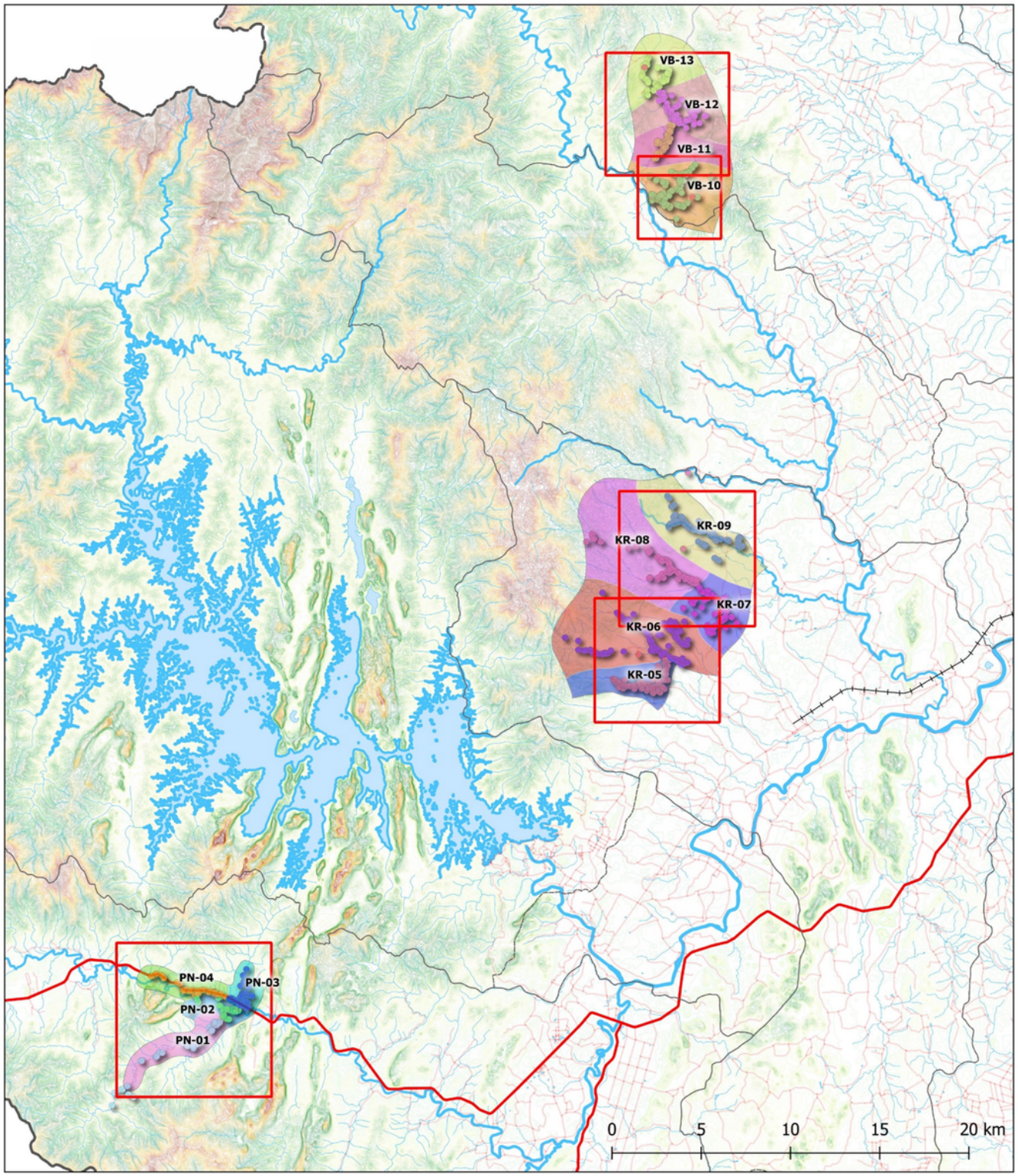

Figure 1

Surat Thani study site terrain map Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

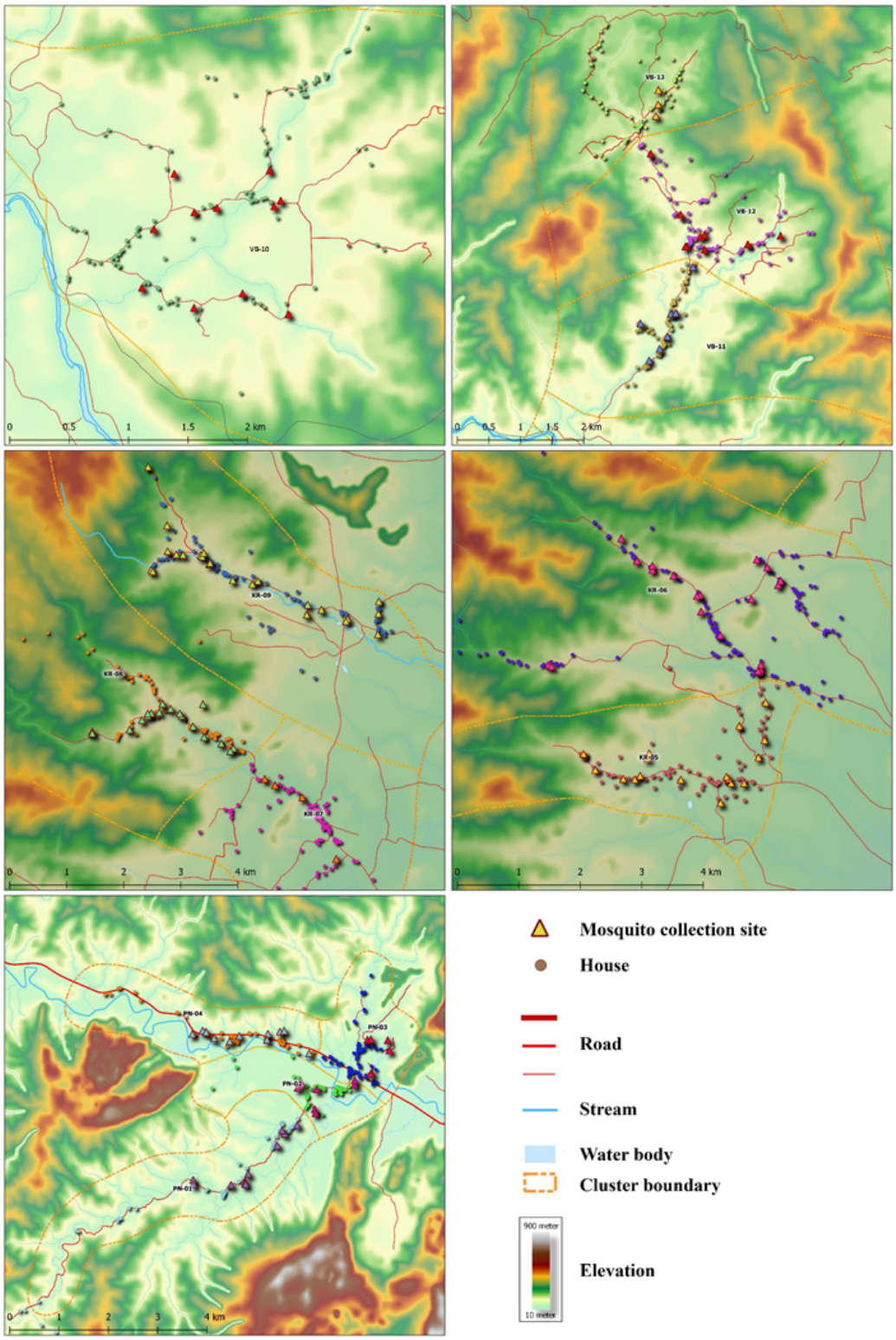

Figure 2

Enlarged terrain maps of surveyed clusters Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

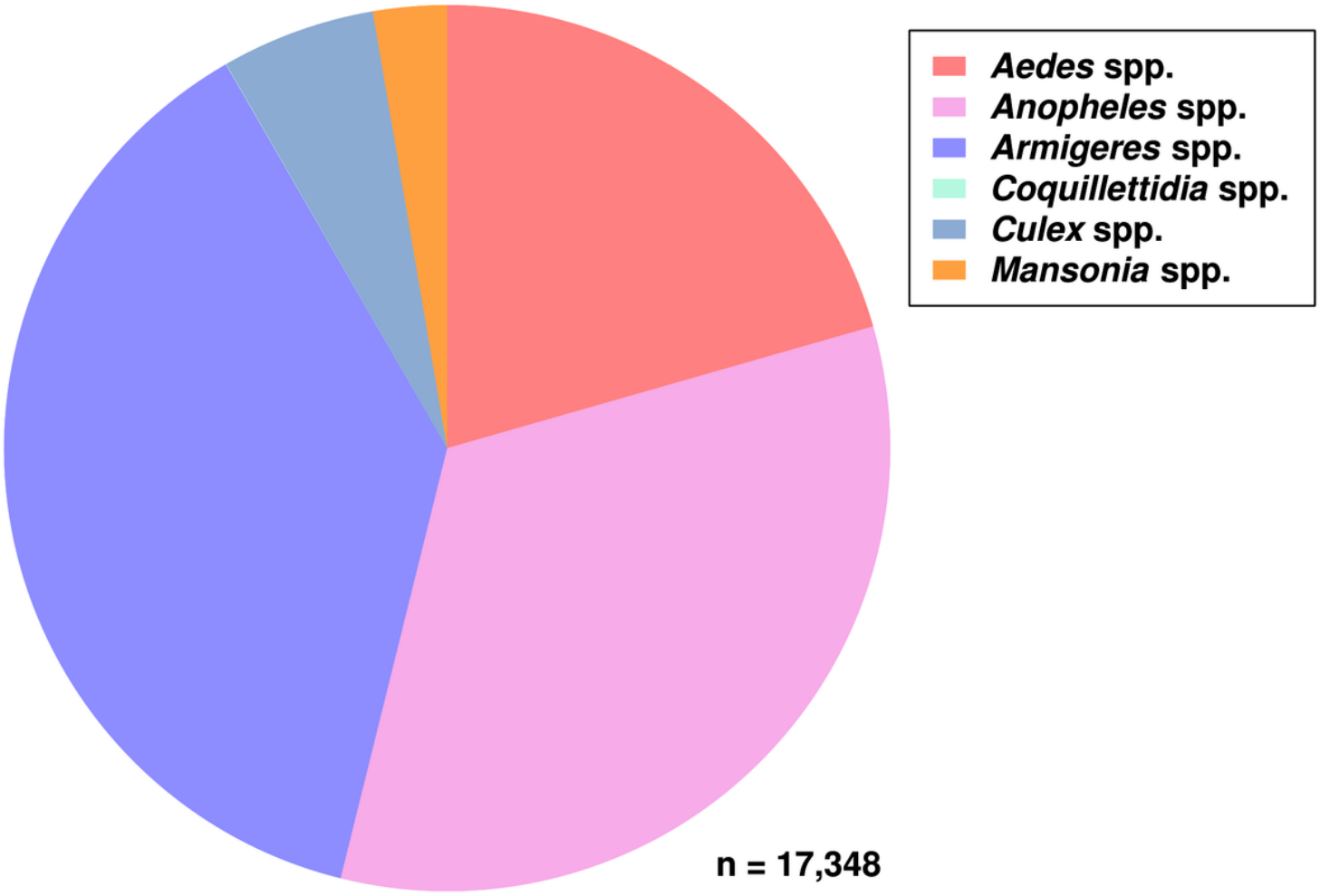

Figure 3

Proportion of mosquito genera captured

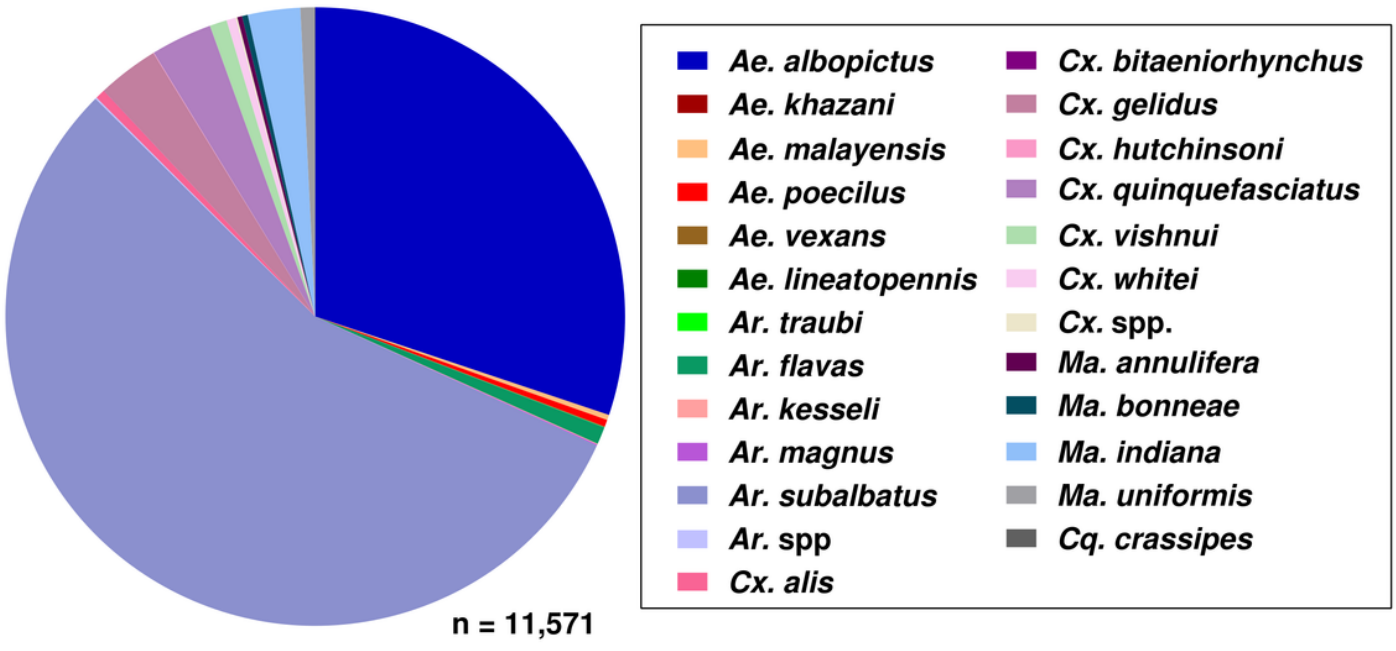

Figure 4

Proportion of Culicine species captured

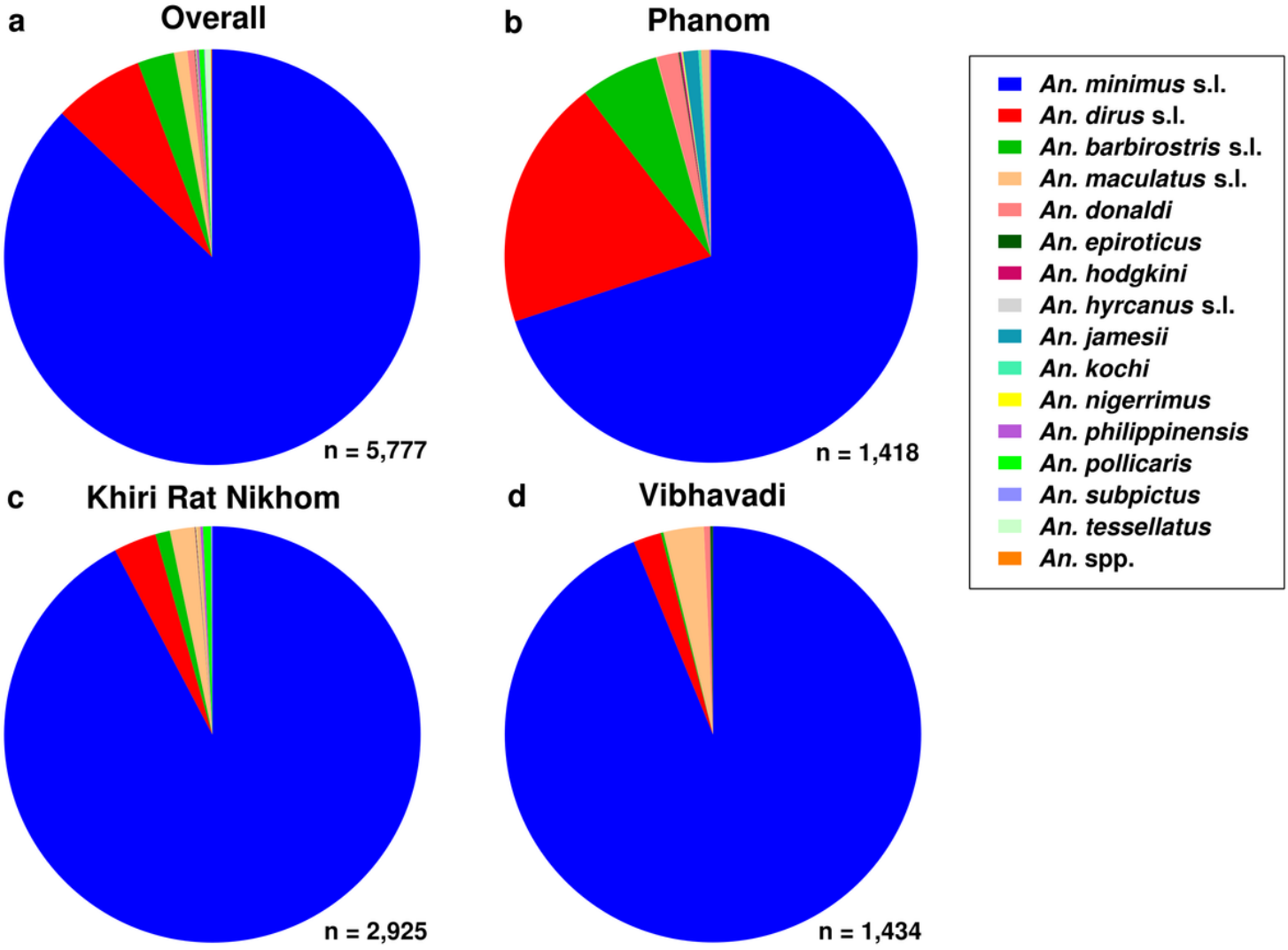

Figure 5

Proportion of Anopheles species captured

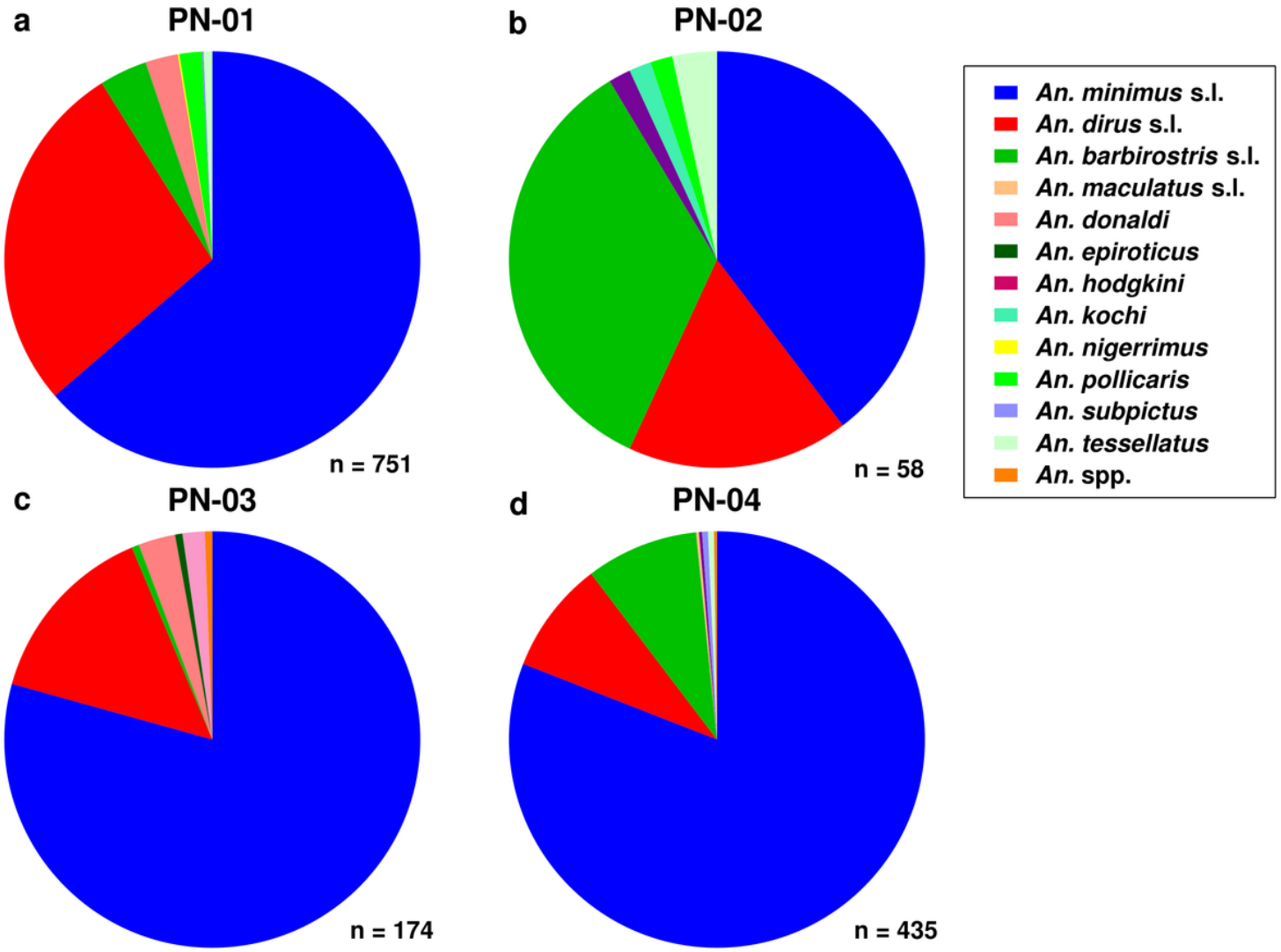

Figure 6

Anopheles species morphologically identified from Phanom (PN) district

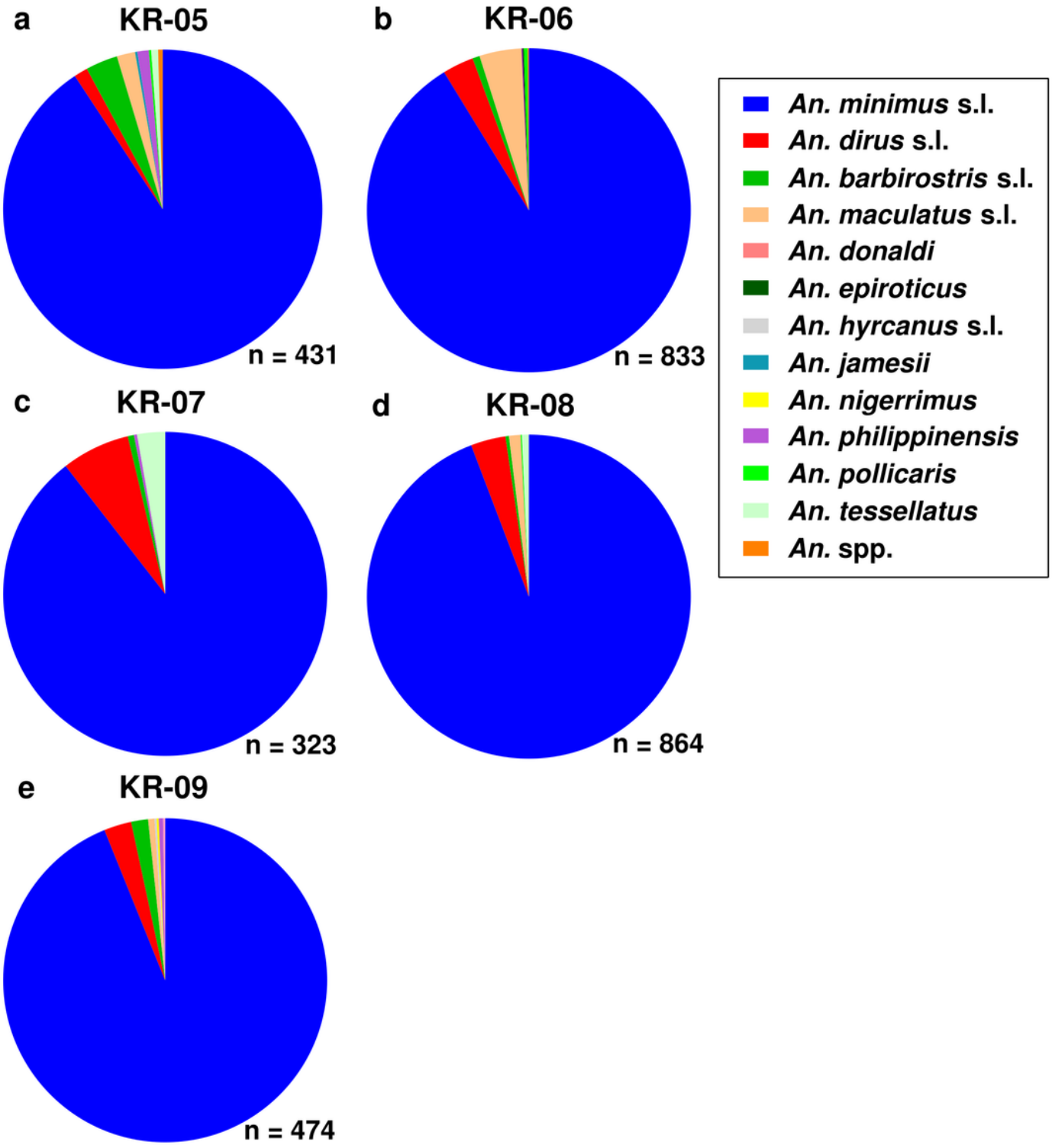

Figure 7

Anopheles species morphologically identified from Khiri Rat Nikhom (KR) district

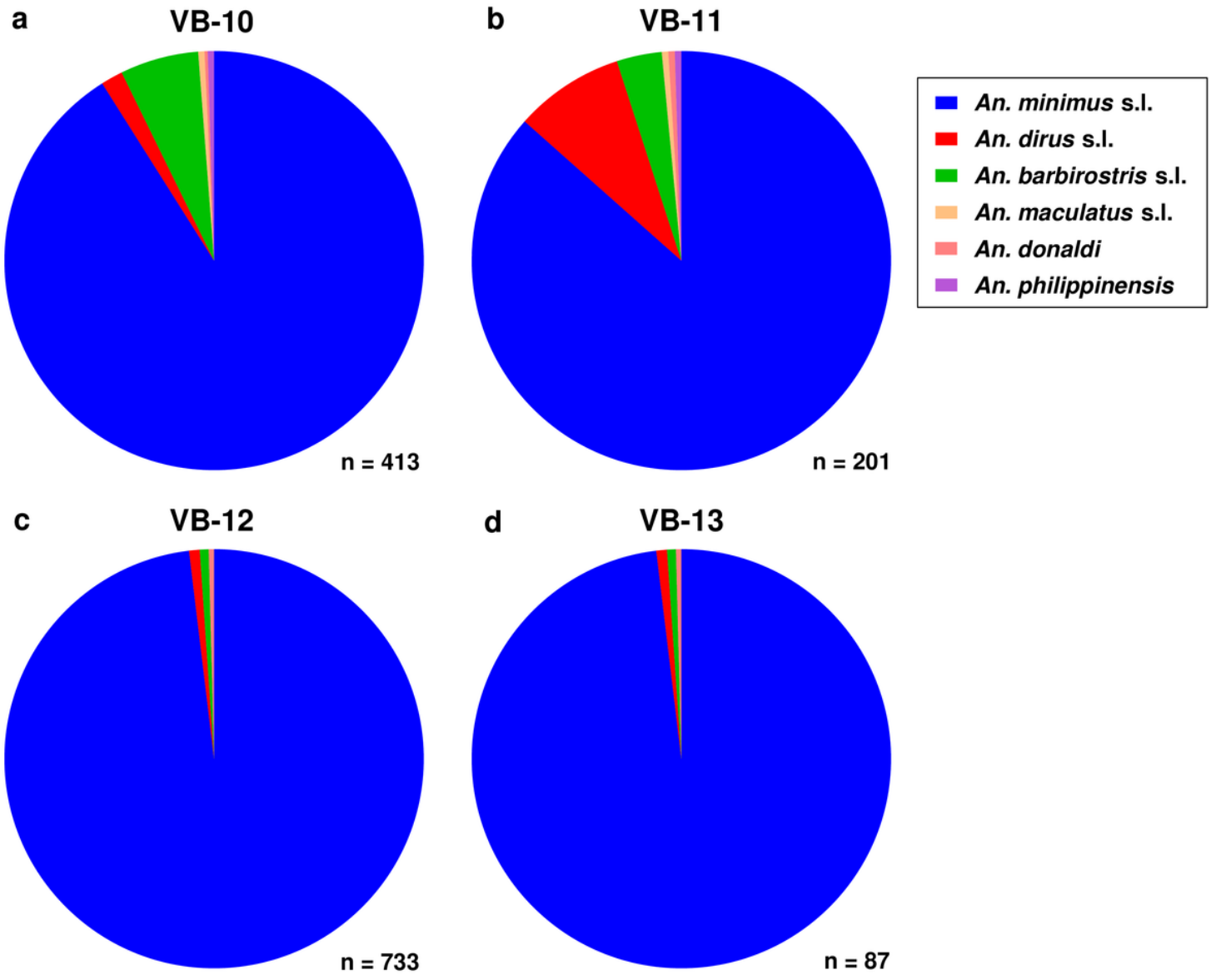

Figure 8

Anopheles species morphologically identified from Vibhavadi (VB) district

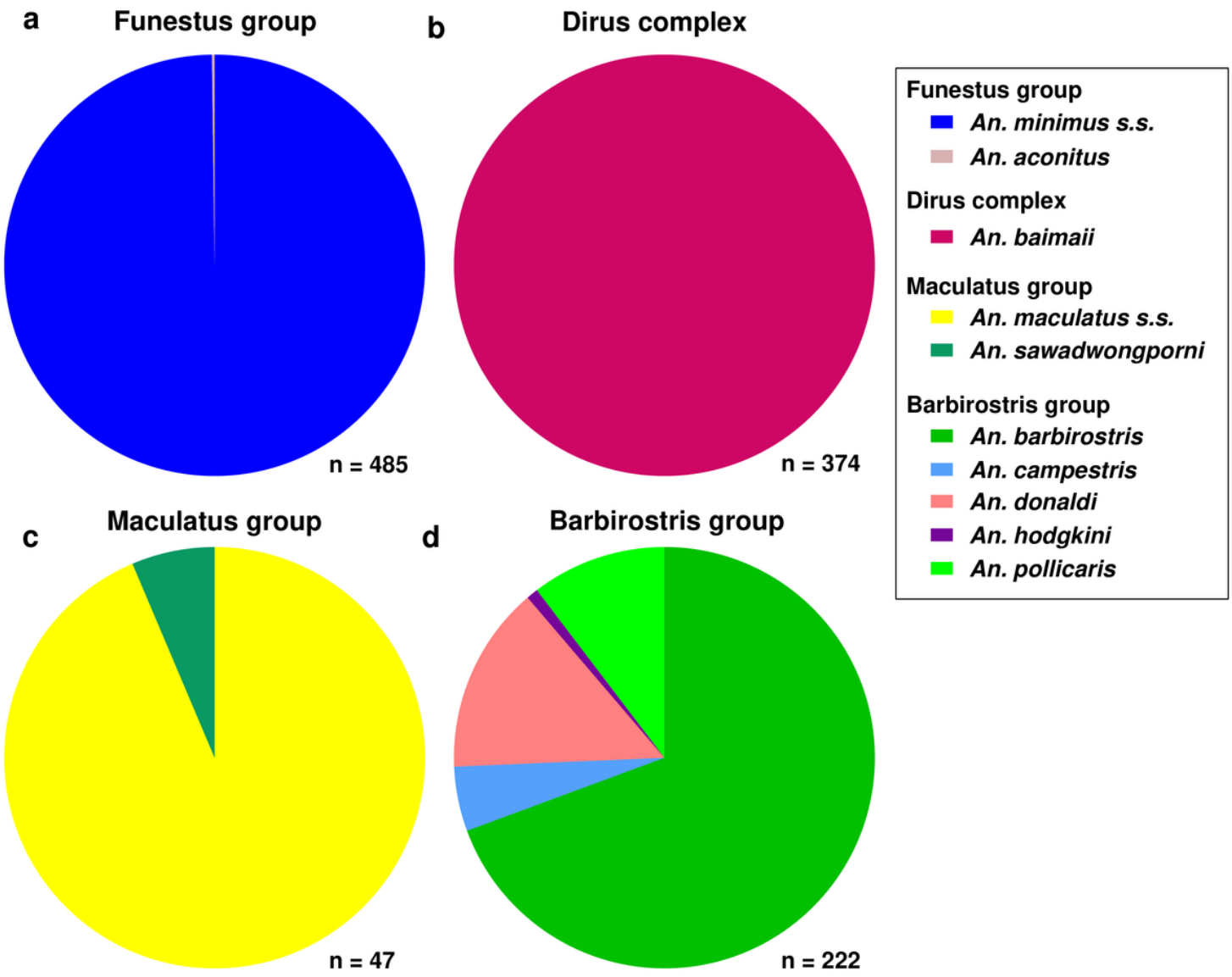

Figure 9

Anopheles Species groups and complexes identified molecularly (a-c) and morphologically (d) from Surat Thani

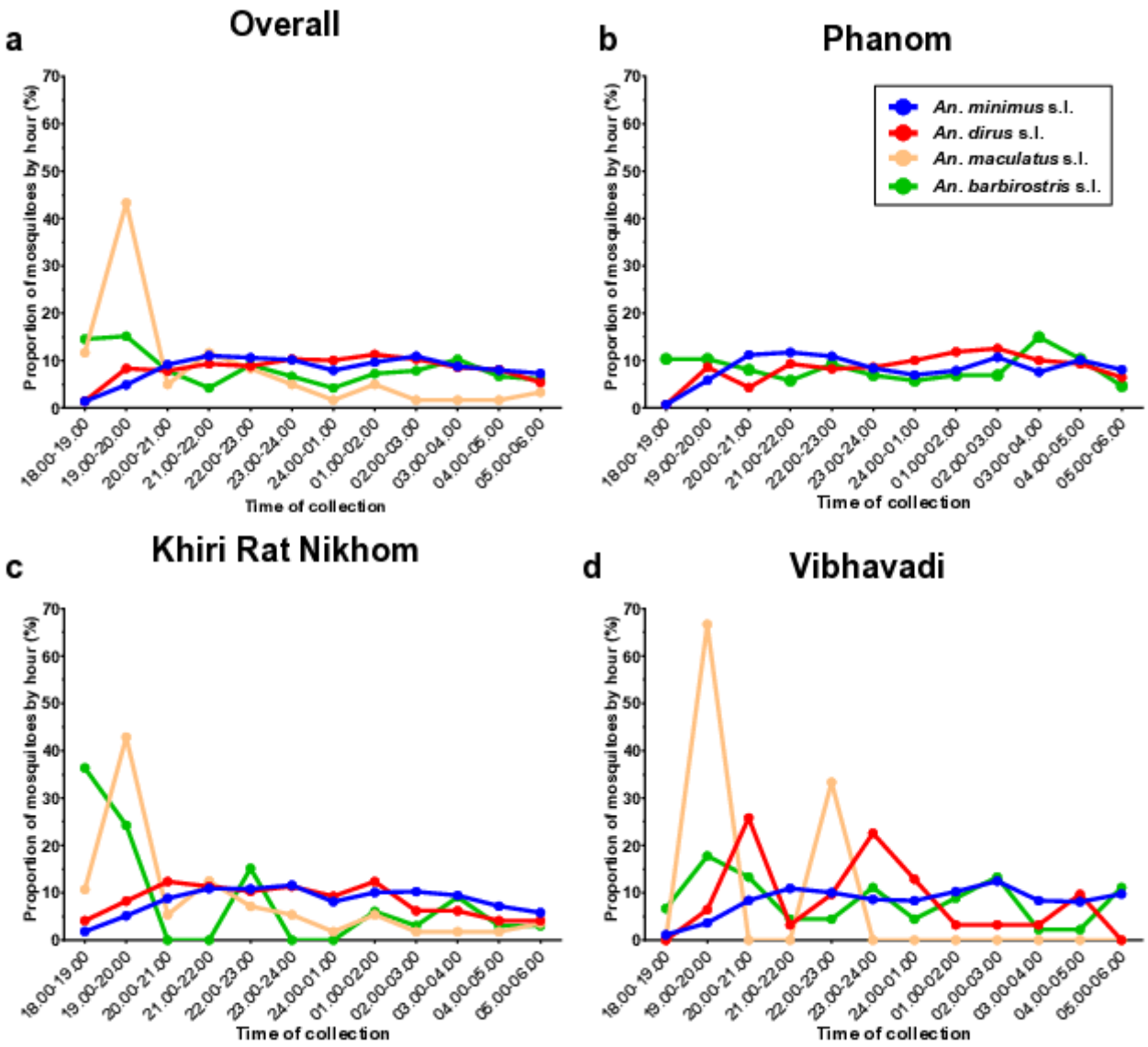

Figure 10

Proportion of Anopheles species collected by hour from all districts

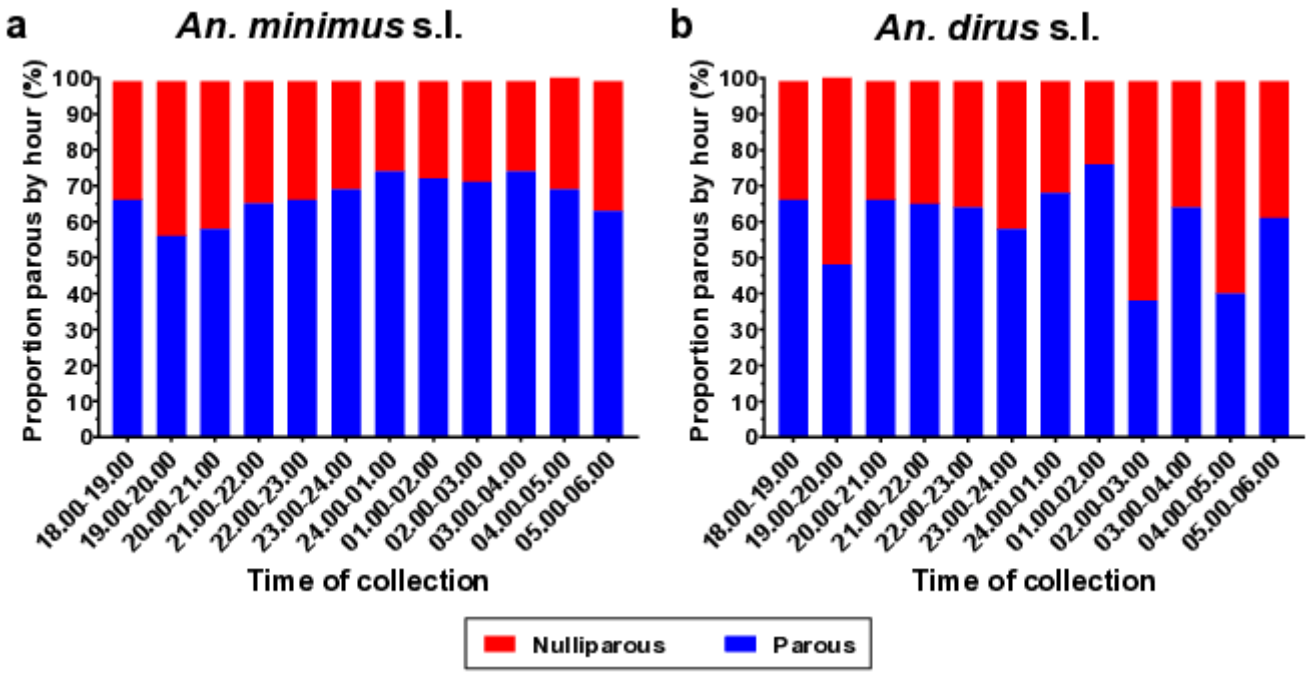

Figure 11

Proportion parous by hour for Anopheles minimus (a) and An. dirus (b) from all three districts

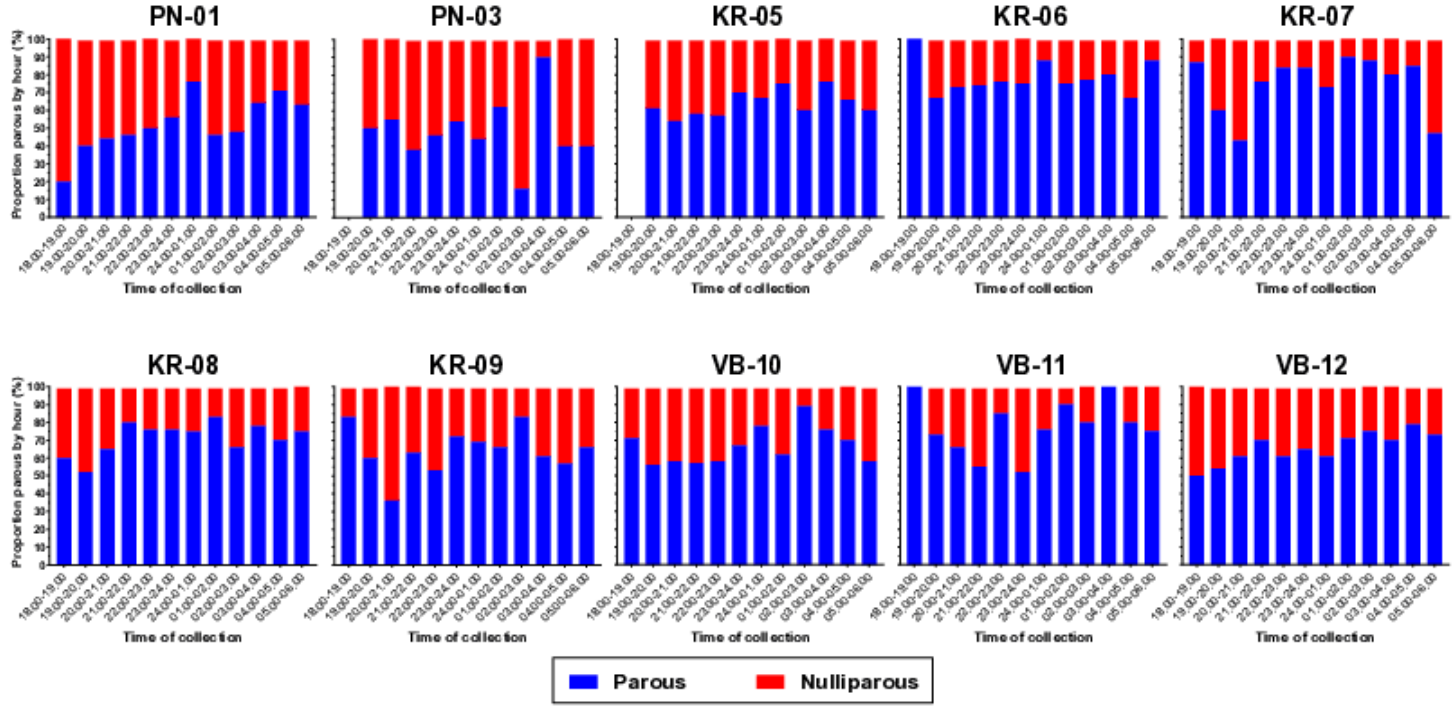

Figure 12

Proportion parous by hour and cluster for Anopheles minimus