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Characterization Of Anopheline Species Composition Along The Bhutan-India Border Region

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MPH Thesis

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Abstract:

Bhutan is aggressively embarking on a path towards malaria elimination. Despite substantial progress, Bhutan remains vulnerable to imported malaria. The majority of cases are in Sarpang district, which shares a border with the state of Assam in India. However, the anopheline species responsible for autochthonous malaria transmission have not been well characterized. Therefore, a comparison of the *Anopheles* species in Sarpang was made with published records of anopheline mosquitoes in neighboring Assam. An assessment of *Anopheles* species composition was undertaken from June to July 2014 in four Sarpang villages adjacent to the Sarpang-Assam border. Five sampling methods were employed: (1) human landing catches, (2) cattle-baited catches, (3) CDC light traps, (4) indoor resting catches and (5) resting boxes. Female anopheline mosquitoes were identified to species using a morphological key. These results were compared to published literature on anopheline ecology and vectorial roles in Assam. The two suspected malaria vectors in Bhutan, *Anopheles culicifacies* (n=189) and *An. pseudowillmori* (n=205), were abundant in the Sarpang villages. However, in Assam, only *An. culicifacies* species B, a relatively incompetent vector, has been documented. In contrast, the primary malaria vectors of Assam, *An. minimus* and *An. baimaii*, were absent in the Sarpang collections. If *An. culicifacies* is not a competent vector in Sarpang, the other recovered species – *An. pseudowillmori* and *An. maculatus* – may be the responsible vectors for malaria transmission in Sarpang. Nonetheless, molecular methods are required to identify members of several sibling species complex in this region; however, adequate equipment and additional training of personnel will be necessary to address this difficulty.

Background:

Since the inception of its malaria elimination efforts in 1964, Bhutan has experienced a few outbreaks, most notably one that involved 39,852 indigenous cases in 1994[1]. Nonetheless, it has achieved substantial decline in malaria incidence, with only 82 indigenous cases and 24 imported cases in 2012[2,3]. Although it has already reached the elimination stage since 2011, Bhutan remains vulnerable to malaria, particularly in the malaria-endemic and malaria epidemic-prone *dzongkhags* or districts (Figure 1)[1]. The epidemic-prone districts, in which 32% of total population reside, usually have fewer cases, but are prone to cases occurring during summer months[4]. In contrast, the low-lying malaria-endemic districts, in which 31% of total population reside, are more likely to have the highest malaria incidence in Bhutan, usually peaking between March and July[4]. Located in this region and sharing a border with a malaria-endemic Indian state, Sarpang, Samdrup Jongkhar and Samtse districts account for at least 70% of the country's reported malaria cases[5]. Given the increasing economic development and job opportunities in Bhutan, a more dynamic cross-border movement is occurring and anticipated in the future. Located strategically near the Bhutan-India border, the Ministry of Health's Vector-borne Diseases Control Program (VDCP) is primarily responsible for malaria surveillance, case management and vector control throughout Bhutan. Current surveillance methods, including active case detection, are successfully capturing incident cases, but more aggressive surveillance approaches are needed to achieve successful elimination and avoid reintroduction of cases in the future. Importantly, such measures must be combined with an increased understanding of vector ecology and diversity, so that Bhutan can not only achieve its goal of "no indigenous malaria by 2016", but also sustain that goal with a tailored long-term vector control strategy.

In Bhutan, two important malaria parasite species are present, *Plasmodium falciparum* and *P. vivax*, which in 2012 encompassed 43% and 57% of confirmed cases, respectively[4]. Transmission occurs when a competent anopheline mosquito bites an infected human and subsequently transmits the parasite to an uninfected host. Although about 25 *Anopheles* species were observed between 1962 and 2013, the relative contribution of these species to malaria transmission in Bhutan is not well-understood[4,6]. In the past, *An. minimus, An. fluviatilis* and *An. dirus* were suspected to transmit malaria but none were identified in 2013[1,6]. Two competent vectors, *An. pseudowillmori* and *An. culicifacies*, are currently suspected as primary vectors due to strong preference for blood feeding on humans in indoor and outdoor settings, their abundance, and higher field infection rate[1]. Effort to incriminate these and other species have been hampered by the lack of trained vector biologists and advanced equipment[6]. The conventional method, which involves dissecting mosquitoes and examining salivary glands for *Plasmodium* sporozoites, has been challenging and time-consuming for inexperienced workers. Studies in other malaria endemic regions with low parasitemia have addressed some of the eco-epidemiological challenges[7,8,9,10,11].

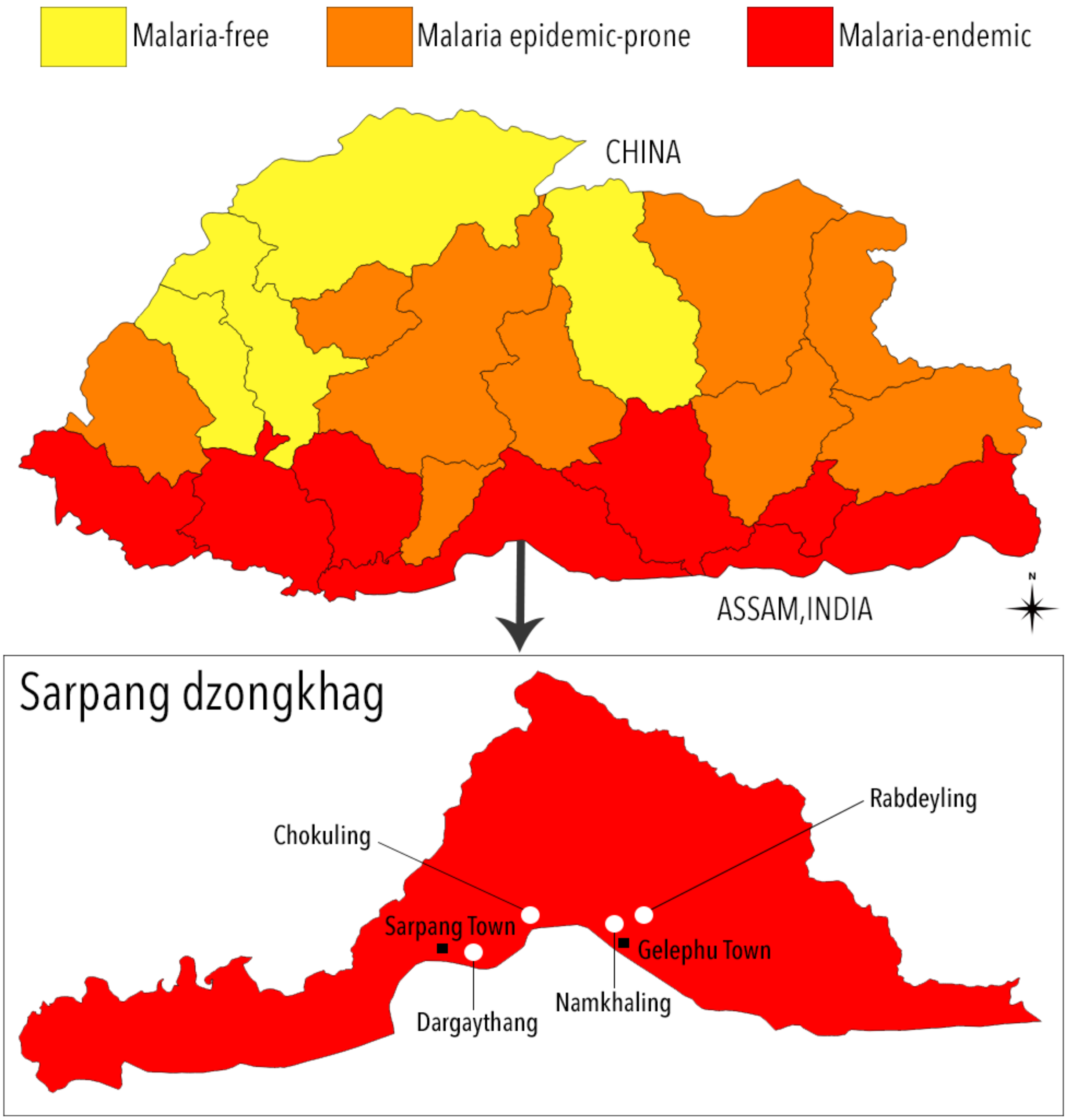

Figure 1. Top: Malaria risk in Bhutan, stratified by *dzongkhag* or district. Bottom: Four study sites selected in Sarpang, a malaria-endemic district which shares its border with India.

Understanding population dynamics and ecology of mosquito vectors is a pre-requisite to long-term success in malaria elimination and prevention, especially in resource-limited settings[12]. Some mosquitoes belonging to a sibling species complex are morphologically indistinguishable yet exhibit ecological, behavioral, or physiological differences, resulting in different vectorial capacities[13]. *Anopheles culicifacies*, for example, is species complex that includes five sibling species informally named species A, B, C, D and E. Four species of the complex (A, C, D, E) are reportedly malaria vectors in neighboring India, where they are estimated to be responsible for the transmission of 60-65% of all cases of malaria in peri-urban and urban environments[59]. Therefore, thorough examination of factors for incriminating mosquito vectors is essential for developing cost-effective and selective vector control strategy. Currently, Bhutan depends on biannual indoor residual spraying (IRS) and triennial long lasting insecticide-treated nets (LLIN)[4], which were developed to reduce daily survival rates of indoor mosquitoes only. However, majority of malaria cases were those who spent more time outdoor[4]. Because mosquitoes can adopt new behavior in response to insecticide-based intervention, information on vector feeding and resting preferences will be useful in assessing the effectiveness of current strategies[14,15]. Such behavioral plasticity may also alter vector population dynamics and provide opportunity for secondary vectors to take on primary vectorial roles, requiring vigilance for all possible vectors[12,16]. Despite lacking signs of insecticide resistance, Bhutan has employed robust use of insecticides for a long time and is within close proximity to areas with insecticide-resistant mosquitoes[4]. Loss of efficacy in insecticide-based vector control can possibly lead to a catastrophic outbreak, such as that in 2009[4]. An improved knowledge database on mosquito vectors is therefore required to prepare for the emergence of insecticide-resistance and enable development of novel malaria vector control[17,18,19].

To assist Bhutan in eliminating indigenous malaria transmission by 2016, the current study aims to improve the current understanding of *Anopheles* species in malaria-endemic Sarpang district via an entomological investigation and a literature review-based comparison of species found in the neighboring Assam state of India. Sarpang district was selected because it has consistently reported the highest number of malaria cases annually[2]. The timeline of the entomological investigation overlapped with annual malaria transmission trends since 2011, which are influenced by climatic conditions, cross-border human movement and the characteristics of available vectors[4]. At the selected study sites (Figure 1), a comprehensive entomological data collection for each *Anopheles* species was undertaken, including 1) abundance, 2) landing rates, and 3) blood-feeding behavior[20]. Mosquito collection and identification as well as geographical information systems (GIS) mapping of study sites were conducted in conjunction with the VDCP in Sarpang district, Bhutan. The Sarpang-Assam border becomes a major concern to controlling malaria in Bhutan because the most populous Indian state accounts for 42% of malaria cases in the northeastern region[21]. Additionally, more than 60% of these cases are caused by *P. falciparum*, which, unlike *P. vivax*, can cause severe disease or death[22]. Aside from social factors (such as civic unrest, poverty, and migration), environmental factors (such as heavy rainfall, recurrent floods, and inaccessible terrain) provide an optimal setting for rich anopheline fauna and thus, a perennial malaria transmission[22]. While cross-border movement continues to challenge efforts in halting malaria introduction into Bhutan, *Anopheles* species composition is likely to be changing in response to ecological changes caused by climate change, deforestation and urbanization. Because Sarpang and Assam have similar biomes, the literature review complements the findings from the entomological study. Subsequently, results of the current study will be used assist in designing future entomological research aimed at incrimination of vectors and the further development of strategies to control and eliminate malaria in Bhutan.

Hypotheses:

1. *Anopheles* species composition in the study sites of Sarpang district is similar to that established in several localities across Assam.
2. The same *Anopheles* species are responsible for local malaria transmission in both Sarpang and Assam.

Specific Aims:

To compare species composition of anopheline mosquitoes in malaria-endemic Sarpang district of Bhutan to that in Assam state of India.

- To select four study sites in Sarpang based on malaria incidence history and proximity to towns and Sarpang-Assam border
- To estimate the relative abundance of *Anopheles* species captured indoor and outdoor using various trapping methods
- To compare the landing rate of anopheline mosquitoes using data from human and animal bait captures
- To carry out a literature review on *Anopheles* species composition in several locations in Assam

Methods:

A) Entomological investigation in Sarpang district

Study Sites Selection

Data on malaria incidence from 2009 to 2013, including count and coordinates, were mapped using ArcGIS, version 10.1 (Esri, Redlands, CA). The spatial distribution of cases was assessed in terms of their proximity to two areas with the largest economic activities and human movement, namely, Sarpang (26°51′51.70″N, 90°16′08.57″E) and Gelephu (26°52′14.51″N, 90°29′08.00″E) towns. The latter is situated closer to a border checkpoint between Bhutan and India. Based on consultation with the VDCP, two study sites from each area were selected for entomological field data collection.

Mosquito Collections

Mosquitoes were collected between June 2 and July 27, 2014 in each study site. Five sampling methods were deployed: 1) human landing catch (HLC), 2) cattle-baited catch, 3) CDC light traps, 4) indoor resting catch and 5) resting boxes.

Aimed at sampling anthropophilic blood-seeking *Anopheles*, 12-hour indoor and outdoor HLCs were carried out in conjunction with trained mosquito collectors from VDCP about twice a week. HLCs were executed in such a way each mosquito collector will collect any mosquito that lands on his/her bare leg. Cattle-baited catches have been successful in collecting a large number of zoophilic anopheline mosquitoes in Bhutan[6] and were therefore used with outdoor HLC simultaneously. For sampling blood-seeking mosquitoes, CDC light traps were set up outdoors in dark outdoor areas with minimal wind exposure.

These three methods were done to understand the blood-seeking behavior of anopheline mosquitoes from 6:00 pm to 6:00 am, whereas indoor resting catches were aimed at sampling blood-fed mosquitoes that rest indoors. Common resting sites are dark and cool areas, such as underneath a table and behind a cabinet. Blood-fed mosquitoes that rest outdoor were sampled using resting boxes method during the day. Guided by a recommended protocol, twenty resting boxes were positioned outdoor and checked twice a day for resting anopheline mosquitoes[23].

Mosquitoes were collected using aspirators and transferred into collection cups, which were labeled by sampling method, site and date. All mosquito collectors were asked for written informed consent. Only mosquitoes from this study were used in subsequent identification and analysis.

Mosquito Species Identification

Mosquito collection cups were transported to the facilities at VDCP. Mosquitoes were identified to species using the key by Rattanarithikul et al and examined for evidence of blood using a dissecting microscope[24,25]. Female (non-blood-fed, blood-fed or gravid) anopheline mosquitoes were recorded by species, abundance, collection date, and study site.

B) Literature Review of *Anopheles species* in Assam state of India

Relevant papers were searched for via PubMed. The keywords used in the search are "*Anopheles*", "malaria vector", "Assam", and "northeastern India". Papers that were not available through PubMed were acquired from other web platforms, such as ResearchGate. To compile a list of species in Assam, papers that included information on species name, location and methods of trapping were given high priorities. Other papers that provided supplementary information on the bionomics and epidemiology of each species were also considered.

Results:

A) Entomological investigation in Sarpang district

Study Sites Selection

Four study sites were selected along the Sarpang-Assam border (Figure 1). Two of these were located in close proximity to Sarpang town, namely the villages of Dargaythang (26°51'20.50"N, 90°18'28.10"E) and Chokuling (26°54'46.45"N, 90°21'29.94"E). Local malaria cases, involving *P. vivax* and *P. falciparum* infections, were reported from these villages during the period of this study. Located close to Gelephu Town, two other study sites were selected in the villages of Rabdeyling (26°52'33.50"N, 90°29'45.30"E) and Namkhaling (26°51'50.30"N, 90°29'10.50"E). Although these two villages had local malaria cases in the past five years, none was reported in 2014.

Dargaythang reported the most recent local malaria incidence during the study period. The study site also has a stretch of rice paddy fields, in which more *Culex* *spp.* larvae were observed compared to that of *Anopheles spp.* Most households maintain the paddy fields and own livestock, predominantly cows, goats, and chickens. Concrete and wooden houses were located relatively far from one another but they were situated along the major highway. Houses in Chokuling, however, were mostly located in areas without road and transportation system. Similarly, households in this village were farmers that were responsible over paddy fields and maize plantation. They also had two communal fish ponds, one of which had *Culex spp.* larvae. Not far from the study site was a large cattle shed that held approximately twenty cows.

Namkhaling has the highest density of human settlement compared to the other three sites. Therefore, the tillable area is limited. In the study site, cattle sheds sheltered approximately eight cows. During the second half of the study period, an irrigation channel that goes through this village dried up due to construction work. Namkhaling is also located near massive areas of low-lying paddy fields shared between Bhutan and Assam. Anopheline larvae were observed in one of the fields, suggesting the availability of suitable larval habitats. Rabdeyling, located north of Namkhaling, primarily consists of fields ranging from maize to betel nut, and paddy fields. Permanent wooden or concrete houses were situated far apart from one another. Make-shift temporary settlements for Indian workers were also within the locale. Larval sampling was attempted but larval habitats appeared to be suitable for non-anopheline species. Additionally, large, government-owned fish ponds were situated 700 meters away from the study site. However, these farm fishes were reported to feed on mosquito larvae.

Mosquito Collection and Species Identification

1,698 female anopheline mosquitoes were collected throughout the study, specifically from two 12-hour night collections in each of four study sites. Coupled with Rattanarithikul et al. key, morphological characteristics of wings, hindlegs, and palpi revealed that eight species were collected: *Anopheles vagus, An. pseudowillmori, An. culicifacies, An. maculatus, An. peditaeniatus, An umbrosus, An. nigerrimus* and *An. jamesii* (Table 1)[24]. Four of these species are pictured in Appendix A.

Mosquitoes were primarily obtained from 12-hour cattle-baited catches. A large number of *An. vagus* were collected from cattle-baited catch in the four study sites (n=1,226). The relative abundance of each *Anopheles* species, stratified by study site, can be found in Appendix B. Based on the general hourly pattern in *Anopheles* species composition, *An. vagus* predominantly seeks blood during first six hours of the evening, whereas *An. culicifacies* during the second six hours. The temporal dynamics of *Anopheles* species composition in each study site are depicted in eight individual graphs (Appendix C).

A disproportionate number of *Culex* species was collected from 12-hour human landing catch (HLC). Light traps and resting boxes yielded few *Anopheles* and tended to attract other insects. Several blood-fed and gravid resting female *Anopheles* were found indoors.

Anopheles spp.	#
vagus	1226
pseudowillmori	205
culicifacies	189
maculatus	36
peditaeniatus	13
umbrosus	13
nigerrimus	12
jamesii	4
TOTAL	**1698**

Table 1. Eight Anopheles species were collected from four study sites in Sarpang. See Appendix B for relative abundance in each study site.

B) Literature Review of *Anopheles* species in Assam state of India

Overview

A list of *Anopheles* species in Assam was generated based on information collected from eleven papers published between 2004 and 2015 (Table 2). Although a total of 61 *Anopheles* species have been observed in India[26], at least 23 species were observed in several locations across Assam: *An. aconitus, An. annularis, An. baimaii, An. barbirostris, An. crawfordi, An. culicifacies, An. dirus, An. dravidicus, An. fluviatilis, An. jamesii, An. jeyporiensis, An. kochi, An. maculatus, An. minimus, An. pallidus, An. philippinensis/nivipes, An. pseudowillmori, An. splendidus, An. subpictus, An. tessellatus, An. vagus, An. varuna* and *An. karwari*. Additionally, two authors reported the presence of mosquitoes of *Anopheles hyrcanus, An. barbirostris, and An. maculatus* group[27,28]. GPS coordinates of species sightings, if available, are shown in Appendix D.

The most important malaria vectors in Assam are *An. minimus, An. fluviatilis* and *An. baimaii* (previously *An. dirus* species D)[29,30,31]. *Anopheles minimus* was reported to be present almost throughout the year whereas *An. baimaii* was observed only during the summer/ monsoon months (between May and September)[30,31]. *Anopheles fluviatilis* was reported to contribute to malaria during winter months[30]. In other parts of India, *An. culicifacies* (species A, B, C, D, and E), *An. stephensi, An. subpictus* (species A, B, C and D) and the *An. sundaicus* (cytotype D) complexes have been considered medically important, including *An. minimus, An. fluviatilis* (species S, T, U, and V) and *An. baimaii* [26,31,32]. *Anopheles* *annularis* (species A and B), *An. philippinensis/nivipes* and *An. varuna* were reported to be minor malaria vectors[26,32]. Other *Anopheles* species may also be opportunistic malaria vectors[27].

An. minimus

Anopheles minimus has been incriminated as a primary malaria vector in several locations, including the Kamrup, Nalbari, and Karbi Anglong districts of Assam[33,34,35]. Although this species varied in abundance from one location to another, *An. minimus* was present almost all year round and specimens were found to contain *Plasmodium* sporozoites (except in Lakhimpur) in all districts of Assam[30]. In areas where they have displayed high sporozoite rates, the anopheline mosquitoes were strongly associated with *P. falciparum*[30]. However, a study in forest-fringes villages of Sonitpur district reported absence of sporozoites in *An. minimus*[36]. *Anopheles minimus* was also outnumbered by *An. philippinesis/nivipes, An. annularis*, and sometimes, *An. culicifacies*[36]. Nonetheless, *An. minimus* displayed the highest anthropophilic index (AI), making it an efficient malaria vector along the forested areas of Himalayan foothills in northeastern India[37,38,39,40,] It displayed the highest human biting rates in summer/ monsoon months, in Morigaon and Darrang and between 1:00 am and 4:00 am[22,37]. *Anopheles minimus* also favored an altitude ranging from sea level to 1600 meters[38]. The larvae of this species were found in low to moderate saline water in drains, water canals, humid swampy area, and unused water tanks[39]. Based on the well-established bionomics of this species, a GIS-based model has shown that northeastern India is conducive for *An. minimus* population[38]. A study suggested that sibling species of *An. minimus* may exist in Assam, as in Thailand[28,41].

An. fluviatilis

As a morphological and seasonal form of *An. minimus*, *An. fluviatilis* has been incriminated as a malaria vector in the Boko area of Kamrup district, Assam[29,42,43]. It was observed in Sonitpur, Lakhimpur, and Dhubri districts[44,45]. The species was abundant during winter months, when other malaria vectors were relatively scarce and IRS spraying was absent[22]. Thus, it became the most efficient vector during this period, leading to an interrupted malaria transmission in northeastern India[29,46]. However, in India, *An. fluviatilis* was composed of three reproductively isolated species, namely, species S, T, and U[47]. Species S was predominantly anthropophagic whereas species T and U were zoophagic[48]. Thus, *An. fluviatilis* reported by Das et al were likely to be of the latter species[36]. Additionally, the same sub-species might represent specimens from Medeluajan and Panirara that lacked the *Plasmodium falciparum* and/or *P. vivax* circumsporozoite protein[27].

Only species U has been documented in Kamrup district of Assam[49]. *Anopheles fluviatilis* fed on human between 8:00 pm and 4:00 am during all seasons but was most active between 11:00 pm and 02:00 am both indoors and outdoors[50]. However, another study observed *An. fluviatilis* feeding exclusively before midnight[51]. Aside from the genetic variability between sibling species, the capacity of mosquitoes to adapt to focal extrinsic events might explain the markedly different feeding patterns[32,52]. *Anopheles fluviatilis* preferred slow running streams and stream channels near villages as their larval habitat[53].

An. baimaii (previously *An. dirus* species D)

Following the revision in the taxonomy of Leucosphyrus Group by Sallum et al (2005), *An. dirus* species D has been renamed *An. baimaii*[54]. Therefore, papers on *An. dirus* that were published prior to 2005 must be interpreted carefully. Stating that the ecology of *An. baimaii* and its role in malaria transmission in Assam have been widely studied, Prakash et al (2005) referred to several papers on *An. dirus,* including one by Dutta et al (1996)[55,56]. In an updated report on malaria vectors in India, *An. dirus* was limited to Southeast Asia[31]. Although *An. dirus* may exist in northeastern India, *An. baimaii* is more likely to be a primary malaria vector in Assam, given its abundance and vector competence in the region[31]. *Anopheles baimaii* causes at least 50% of reported human malaria cases in northeastern India, and is a highly anthropophilic, endophagic and exophagic mosquito[31,56,57,58,59]. Unlike *An. fluviatilis*, *An. baimaii* was a malaria vector during warm and wet monsoon months[31,60]. As an efficient vector of forest malaria in northeastern India, a small number of *An. baimaii* was adequate for sustaining malaria transmission, involving either *Plasmodium falciparum* and *P. vivax* [30,61]. *Anopheles baimaii* fed mostly around midnight[55]. Their flight range is about 1.5 kilometers[38]. During the day, they rest in the forest mostly on tree trunks, avoiding direct sun light[57]. The larvae of *An. baimaii* were regularly found in small, transient pools of hard water during wet season and in streams of tropical wet evergreen forests during dry season[57,62].

An. culicifacies

Prior to 1960s and during 1970s, malaria control in India mostly targeted *An. culicifacies.* This species has been recorded in all parts of India, including in the Himalayas[31]. It was incriminated as a malaria vector in Garubandha area in Sonitpur district of Assam[63]. *Anopheles culicifacies* has contributed to 60 to 70% of malaria cases occurring in plains of rural India annually[31,64]. Sibling species of the *An. culicifacies* complex (species A, B, C, D, and E) varied in terms of vectorial capacity as well as relative anthropophagy and zoophagy, which may be influenced by season and availability of different bloodmeal types[31]. Sibling species A, C, D, and E were vectors of both *Plasmodium vivax* and *P. falciparum* but only species E was anthropophagic[31]. Species B, a relatively ineffective vector, was prevalent throughout India, including in the northeastern region[31,47]. The *An. culicifacies* complex has exhibited a wide range of anthropophagic indices (2%-80%) in India[32]. However, it displayed strong anthropophagy in the absence of cattle bloodmeal[65,66]. Species A, B, and C fed throughout the night, peaking around midnight whereas species D fed only until midnight[31]. All sibling species preferred to rest indoors in human dwellings and cattle sheds[31]. Their larval habitat preferences included rainwater, clean irrigation water, and riverine ecology[31]. Species A was abundant in villages with wells, whereas species B in villages with streams[67]. As a fast-invading species, *An. culicifacies* required targeted control, especially in deforested areas[31]. Additionally, most of the population have developed resistance toward most insecticides, posing threat to neighboring regions[31].

<u>An. pseudowillmori, An. maculatus, and An. dravidicus</u>

As one of the nine members in the Maculatus Group of the Neocellia Series, *An. pseudowillmori* is related to *An. maculatus* and *An. dravidicus* Christophers, both of which were recorded in Assam, India[59,68]. Allele-specific PCR assays have been used to distinguish between these species[69]. Due to previous misidentifications based on morphological characters as well as influence of geographical location, the vectorial roles of the three species are still ambiguous[59]. They were generally zoophilic but might feed on humans indoor and outdoor[59]. The larvae of *An. maculatus* were found in pools of water near rivers and waterfalls[59]. Adults preferred early evening feeding and open to partially shaded habitats that are within 100-400 meters from human dwellings[59]. Although *An. maculatus* is the least zoophilic and the most prevalent species in India, it might only be important in hilly and deforested areas of eastern India[59,68]. However, a study in Thailand reported that *An. pseudowillmori* had a higher man-biting rate compared to *An. maculatus* in Thailand[49]. Capable of maintaining a low grade transmission in the absence of more efficient vectors, *An. pseudowillmori* was a primary malaria vector in Tibet and a suspected vector in Bhutan[1,4,49,70]. This species made up 60% of collected Maculatus group specimens in a study across northeastern India, which was at an altitude of 40 – 2000 m[68]. In fact, 97% of specimens in the neighboring Arunachal Pradesh state were *An. pseudowillmori.* Internal transcribed spacer 2 (ITS2) of *An. pseudowillmori* from northeast India was found to be similar to that in Thailand but different from that in China[71]. The larval habitat preferences of this species in Thailand included rice fields, stream margins, ponds, pits and wells[72]. Despite being closely related to *An. maculatus*, *An. dravidicus* is not considered important in malaria transmission[59].

Other *Anopheles* species in Assam

Hyrcanus Group is one of the most complex anopheline groups, comprising of members that are important vectors of mosquito-borne diseases[26]. Differences in morphological characteristics are not apparent unless immature skins of the specimens are available for analysis[73]. Although seven members of *Anopheles hyrcanus* group have been recorded in India, only *An. crawfordi* was found in Assam[45,74]. Several other studies found unidentified members of this group in Assam[27,28]. *Anopheles nigerrimus* was reported to be predominant in India but was relatively rare in northeastern India compared to *An. peditaeniatus*, a member of the Hyrcanus Group[75]. ITS2 sequencing has confirmed the presence of *An. crawfordi, An. peditaeniatus* and *An. sinensis* in northeastern India[26]. To date, none of these species has been incriminated as a malaria vector. Despite not being considered medically important species, *An. aconitus, An. annularis, An. jeyporiensis, An. kochi,* and the *An. philippinensis/nivipes* complex were found infected with sporozoites in Assam[27,32]. In 1969, *An. philippinensis/nivipes*, which are two separate species that are difficult to differentiate morphologically, contributed to malaria transmission in the state[76]. This species complex also displayed some anthropophilic feeding preference[61]. Based on ITS2 sequencing, *An. nivipes* was reported to be more prevalent in Assam compared to *An. philippinensis*[77]. *Anopheles vagus* is zoophilic but can bite humans occasionally[32]. This species was highly abundant in Assam but has only been incriminated in Bangladesh, supporting the importance of some relatively ineffective vectors as opportunistic contributors[37,78]. Therefore, other *Anopheles* species that are listed in Appendix D but are not well-studied and elaborated in the current study cannot not be entirely dismissed in the study of malaria ecoepidemiology.

Figure 2. Assam state of India shares its border with four other Indian states, Bhutan and Bangladesh. Dark green-colored areas represent Assamese districts that were included in the literature review. Light green-colored areas represent Assamese districts that were not included in the literature review due to absence of information.

Author	List of *Anopheles* species recorded	Trapping Method
Das NG, et al (2004)	*An. annularis, An. barbirostris, An. crawfordi, An. culicifacies, An. kochi, An. minimus, An. philippinensis, An. tesselatus, An. vagus, An. varuna*	NBC (indoor), LT (indoor), LT (cattle shed)
Das NG, et al (2011)	*An. culicifacies s.l., An. annularis, An. dirus s.l., An. fluviatilis s.l., An. philippinensis/nivipes, An. varuna*	LT (indoor), LT (cattle shed)
Das NG, et al (2015)	*An. philippinensis/nivipes, An. annularis, An. minimus, An. culicifacies s.l., An. fluviatilis s.l., An. dirus s.l., An varuna*	LT (indoor)
Dev et al (2004)	*An. maculatus, An. culicifacies s.l., An. annularis, An. dirus s.l., An. philippinensis/nivipes, An. varuna, An. aconitus, An. barbirostris, An. hyrcanus group, An. jeyporiensis, An. kochi, An. minimus, An. pallidus, An. splendidus, An. subpictus, An. tessellatus, An. vagus*	DRC (indoor), NBC (indoor)
Dhiman S, et al (2012)	*An. philippinensis/nivipes, An. culicifacies, An. annularis, An. minimus*	LT (indoor, near cattle sheds)
Gopalakrishnan R, et al (2014)	*An. annularis, An. barbirostris, An. crawfordi, An. culicifacies, An. philippinensis/nivipes, An. vagus, An. aconitus, An. jamesii, An. karwari, An. subpictus, An. minimus, An. fluviatilis, An. kochi*	LT (indoor), DRC (indoor)
Kalita JC, et al (2014)	*An. minimus*	-
Prakash A, et al (2004)	*An. aconitus, An. annularis, An. barbirostris group, An. culicifacies s.l., An. dirus s.l., An. fluviatilis s.l., An. hyrcanus group, An. jeyporiencis, An. kochi, An. maculatus group, An. minimus s.l., An. philippinensis-nivipes complex, An. splendidus, An. tesellatus, An. varuna, An. vagus*	LT (indoor)
Sarma DK, et al (2012)	*An. baimaii*	LT (indoor)
Sarma NP, et al (2012)	*An. philippinensis-nivipes complex*	LT (indoor)
Saxena R, et al (2014)	*An. annularis, An. culicifacies, An. minimus, An. philippinensis/nivipes, An. varuna*	DRC (outdoor), LT (outdoor), NBC (indoor), NBC (cattle shed)
Singh et al (2011)	*An. maculatus, An. pseudowillmori, An. dravidicus*	LT (indoor)

Table 2. List of papers selected for *Anopheles* checklist review in Assam. See Appendix D for species-stratified list and study locations. (NBC= night baited catch (human/cattle), LT = light trap, DRC = day resting catch)

Discussion:

The current study is the first to use 12-hour anopheline mosquito collections in Bhutan and to comprehensively review literature on malaria vectors in the neighboring Assam state of India. Therefore, it offers up-to-date evidence on the potential malaria vectors in southern Bhutan, particularly in areas along Sarpang-Assam border. Minimal immigration control and no health screening were observed at the border between May and July 2014. Majority of the border-crossing population were Assamese carrying out their daytime routine in Gelephu town of Sarpang district. They were bringing in goods from India, including fresh vegetable, fruits and meat, and provided services as tailors, barbers, construction workers, waiters/waitresses and so on. Although most return to their homes in Assam, some employees are settled in Bhutan until their work contracts end. At present, a large number of Indians work at the large-scale hydropower construction projects in other districts[4]. Active case detection usually targets these areas but these mountainous locations are relatively less susceptible to malaria introduction compared to plains in southern Sarpang, where competent vectors are highly abundant. Most malaria cases still occur in Sarpang district in the past three years but the trend may change in response to climate change and ecological changes, supporting the immediate need to understand current eco-epidemiology of malaria in southern region.

Mountain species	*An. maculatus, An. willmori, An. lindesayii, An. baileyi, An. aitkenii, An. bengalensis, An. splendidus, An. fluviatilis, An. dirus*
Plain species	*An. pseudowillmori, An. vagus, An. subpictus, An. culicifacies, An. jamesii, An. pseudojamesii, An. annularis, An. philippinensis/nivipes, An. kochi, An. peditaeniatus, An. aconitus, An. barbirostris, An. barbumbrosus, An.umbrosus, An. minimus*

Table 3. List of mountain and plain *Anopheles* species. See Appendix E for complete checklist from 1962 till 2014.

According to the Ministry of Health and published papers, more than half of the 25 *Anopheles* species observed in Bhutan since 1962 are common in plains (Table 3)[3,79]. In 2013, 16 species were documented: *An. maculatus, An. willmori, An. pseudowillmori, An. vagus, An. philippinensis, An. splendidus, An. nivipes, An. jamesii, An. pseudojamesii, An. balabacensis, An. kochi, An. tessellatus, An. peditaeniatus, An. lindesayii, An. baileyi,* and *An. bengalensis* (Appendix E). However, during the entomological investigation conducted between June and June 2014, only *An.*

maculatus, An. pseudowillmori, An. vagus, An. jamesii, An. culicifacies, and *An. peditaeniatus* were caught (Table 1; Appendix E). The three previously incriminated species (*An. minimus, An. fluviatilis,* and *An. dirus*) continued to be absent from Bhutan for many years. However, as observed in Assam, *An. fluviatilis* is expected to be prevalent only in winter months. Therefore, future entomological investigations are recommended to be consistently conducted throughout the year to understand the seasonal variation in anopheline species composition and vectorial roles.

Data from cattle-baited catches provided a great insight into the types and relative abundance of *Anopheles* in southern Sarpang. Consistent with its high zoophilly and previous studies conducted in Assam, a large number of *An. vagus* were obtained from cattle-baited catch (Appendix E)[27,28,74]. Although this species has been largely overlooked in Bhutan, *An. vagus* was incriminated as a human malaria vector in Bangladesh[78]. However, the two suspected malaria vectors were abundant across the four study sites. Third to *An. vagus* in total abundance, *An. culicifacies* was more dominant in both Namkhaling (n=76) and Rabdeyling (n=72) compared to *An. pseudowillmori* (Table 1; Appendix B). Meanwhile, *An. pseudowillmori* was more dominant in Dargaythang (n=137) compared to *An. culicifacies* (Table 1; Appendix B). A consistent pattern in the blood-seeking preference between *An. vagus* and *An. culicifacies* was observed: when *An. vagus* declined in number toward the end of the first six hours, *An. culicifacies* grew in number (Appendix C). Whether this temporal blood-seeking pattern is merely coincidental or resulted from interspecies competition, further research is needed.

Anopheles culicifacies has been present in Bhutan almost every year since 1989. Due to the lack of resources to carry out molecular identifications in Bhutan, little is still known about the distribution of five sibling species from the *An. culicifacies* complex (species A, B, C, D and E). Although species A, C, D, and E are vectors in India, only species B has been documented in the northeastern region of India, including Assam[30,46]. Thus, *An. culicifacies* collected from the study sites may be of species B. However, a polymerase chain reaction (PCR)-based identification is strongly recommended to confirm this assumption. If this is true, the incompetent malaria vector is unlikely to contribute significantly to malaria transmission in southern Bhutan, rendering efforts to control *An. culicifacies* less useful. Moreover, this species has displayed resistance against multiple insecticides[30]. *Anopheles pseudowillmori,* another suspected malaria vector of Bhutan, was relatively abundant during the study and present in the country since 1998 (Table 1; Appendix B; Appendix E). However, misidentification of *An. pseudowillmori* can easily occur since it shares similar morphological characteristics with *An. maculatus* and *An. dravidicus*[69]. Albeit scarce, *An. maculatus* was also collected from the study sites and has been present in Bhutan since 1989 (Table 1; Appendix B; Appendix E). In fact, *An. maculatus* was once a malaria vector in Bhutan[48]. Although *An. maculatus* is slightly more anthropophilic than *An. pseudowillmori*, the latter has been incriminated in nearby Himalayan region and is a good secondary malaria vector[48,70]. *Anopheles pseudowillmori* has also been reported to be prevalent in Arunachal Pradesh state of India, suggesting that an ecological comparison between southern Bhutan and the Indian state may yield useful information about the bionomics of species[67]. Similarly, PCR-based identification will be helpful in accurately differentiating the closely related species, *An. pseudowillmori* and *An. maculatus*, both of which exist and display varying vectorial roles in Sarpang *dzongkha*.

From the entomological study, only *Culex spp*. were successfully caught via human landing catch (HLC). This suggested that female anopheline mosquitoes, using these methods and timing of collection, preferred non-human bloodmeals. Additionally, the study was largely limited by the extremely low yield from light traps and outdoor day resting boxes. Surprisingly, almost all studies conducted in Assam successfully collected vectors and non-vectors using light traps indoors and outdoors (Table 2). Reasons for the lack of effectiveness with these methods are not known. Environmental factors specific to the region are likely to have contributed to the observed dynamics of *Anopheles* in the four study sites. Aside from availability of suitable larval habitats as well as the fluctuations in climatic factors (temperature, humidity and wind), the relative density of humans compared to animals within each study site may influence the blood-seeking pattern of *Anopheles spp*. Additionally, across all four sites, a large number of livestock, such as cows, goats, sheep, and chickens, were within close proximity to human settlements. In fact, most households in the rural region of Sarpang district are farmers. It may be possible that their role as dead-end hosts have contributed to the decline in malaria incidence in Bhutan. However, attempts to understand the causal relationship between livestock abundance and number of reported cases is greatly challenged by the limited amount of information as well as environmental heterogeneity.

Despite not being identified as vectors in India nor Bhutan, *An. peditaeniatus and An. nigerrimus* were collected from the entomological study. These species have been reported to contribute to malaria transmission in other parts of Asia. As members of the Hyrcanus Group, *An. peditaeniatus* and An. nigerrimus are difficult to differentiate but the former is more prevalent in India[75]. More importantly, the two primary malaria vectors in Assam, *An. minimus* and *An. baimaii* (previously *An. dirus* species D), have been absent for many years as well as during the entomological investigation. However, with the rise of global temperature, insecticide spraying, and changes in ecology, the spatial and temporal distribution of these important malaria vectors may be different within the next decade. Lack of funding and trained technicians poses a significant challenge to understanding vector diversity in Bhutan, whether from the aspect of research or surveillance activities. For example, because of the lack of data on the relative abundance of *Anopheles* species, limited inference can be made about their influence on the declining rate of malaria incidence. As a result, incriminating a malaria vector remains challenging. Another issue lies in the lack of molecular technologies in Bhutan, though these are becoming increasingly affordable in developing nations. Without molecular identification of sibling species members from the *An. culicifacies* complex – which vary in larval habitat, resting, and feeding preferences – optimal and cost-effective vector control strategies cannot be designed. PCR-based bloodmeal analyses, which involves quantifying the extent of anthropophagy or/and zoophagy of the collected anopheline mosquitoes, will assist in incriminating malaria vector, supporting the need for additional research funds, and the continued development of evidence-based parasite and vector control strategies in Bhutan.

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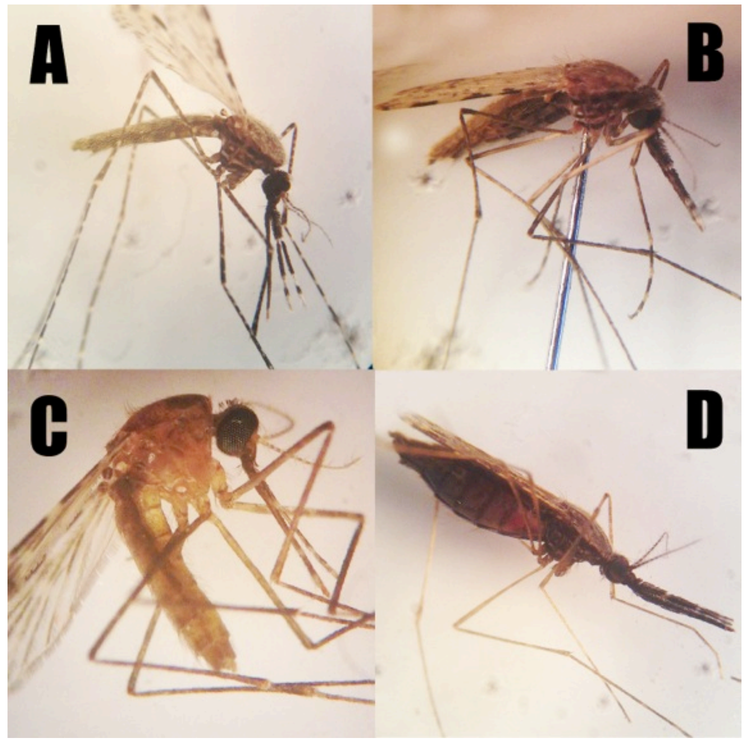
Human Subjects Review

Yale research ethics board has exempted this study due the absence of human subjects in the study. However, ethical clearance has been obtained from the Research Ethics Board of Health (REBH), Ministry of Health, Bhutan for involving VDCP's trained mosquito collectors in human-landing catch. As detailed in written informed consent, the trained personnel were exposed to very minimal disease or injury risk as they were undertaking regularly practiced methods within regular surveillance study sites.

Appendix A

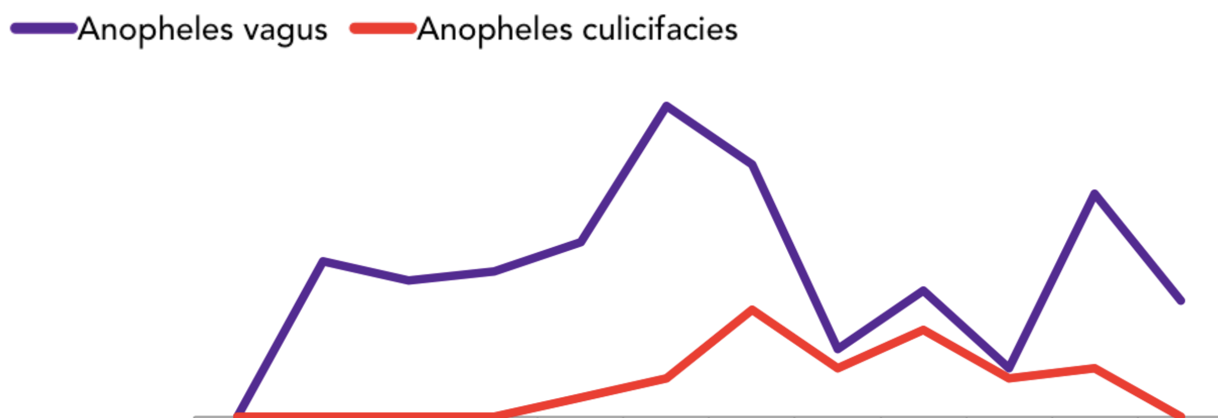
A) *An. pseudowillmori*
B) *An. vagus*
C) *An. culicifacies*
D) *An. nigerrimus*



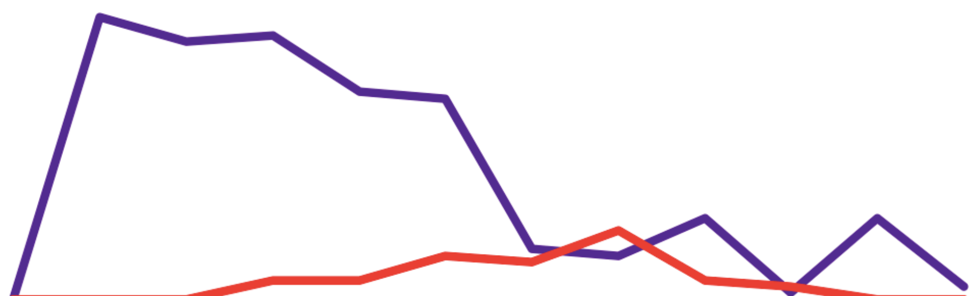
Appendix B

Anopheles spp.	Namkhaling	Rabdeyling	Chokuling	Dargaythang	**Total**
vagus	418	522	32	254	**1226**
pseudowillmori	5	52	11	137	**205**
culicifacies	76	72	13	28	**189**
maculatus	4	5	26	1	**36**
peditaeniatus	1	0	0	12	**13**
umbrosus	0	8	0	5	**13**
nigerrimus	3	9	0	0	**12**
jamesii	0	3	0	1	**4**
Total	**507**	**671**	**82**	**438**	**1698**

Namkhaling

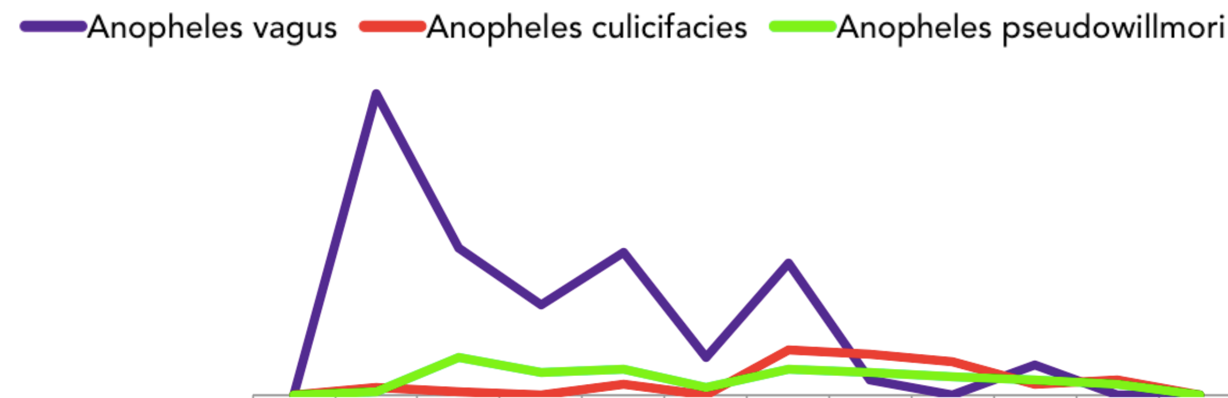


	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	16	14	15	18	32	26	7	13	5	23	12
Anopheles culicifacies	0	0	0	0	2	4	11	5	9	4	5	0

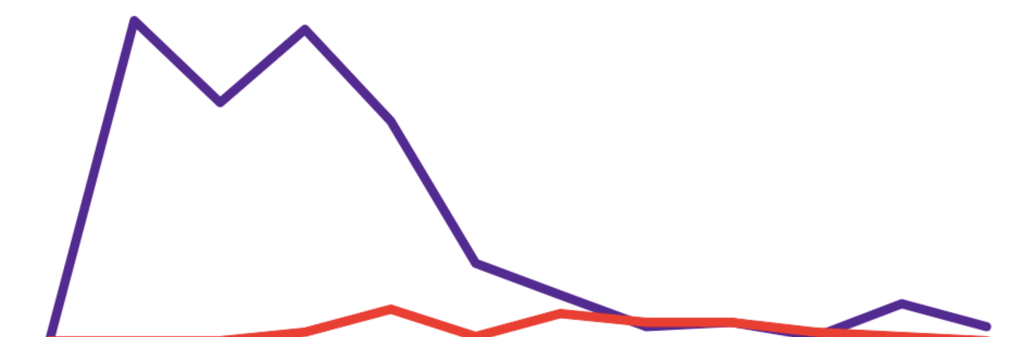


	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	45	41	42	33	32	8	7	13	1	13	2
Anopheles culicifacies	0	0	0	3	3	7	6	11	3	2	0	0

Rabdeyling

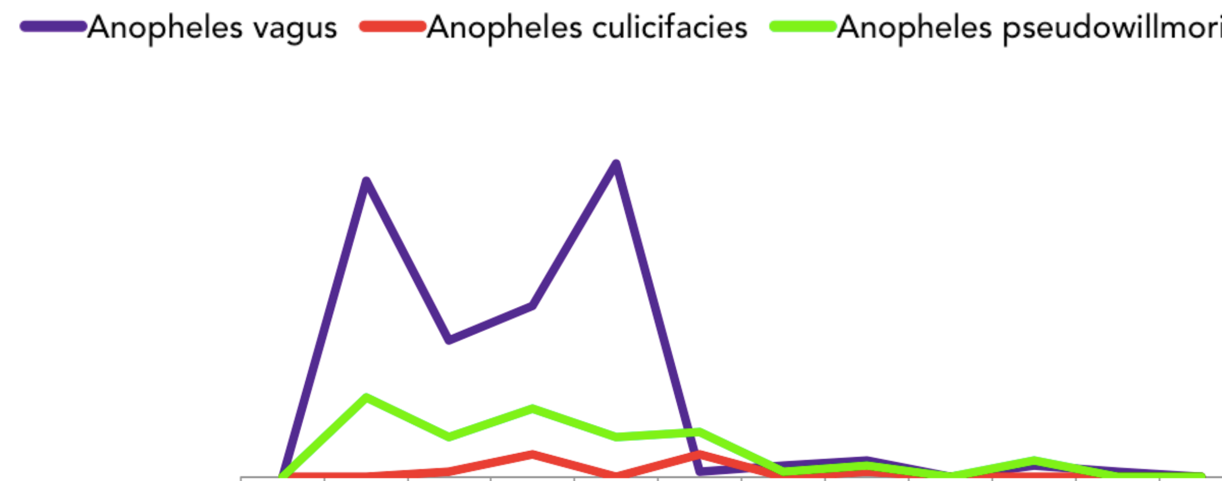


	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	80	39	24	38	10	35	4	0	8	0	0
Anopheles culicifacies	0	2	1	0	3	0	12	11	9	3	4	0
Anopheles pseudowillmori	0	1	10	6	7	2	7	6	5	4	3	0

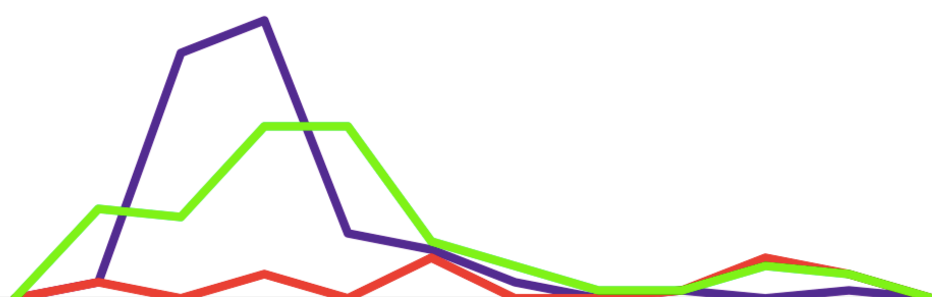


	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	70	52	68	48	17	10	3	4	1	8	3
Anopheles culicifacies	0	0	0	2	7	1	6	4	4	2	1	0

Dargaythang

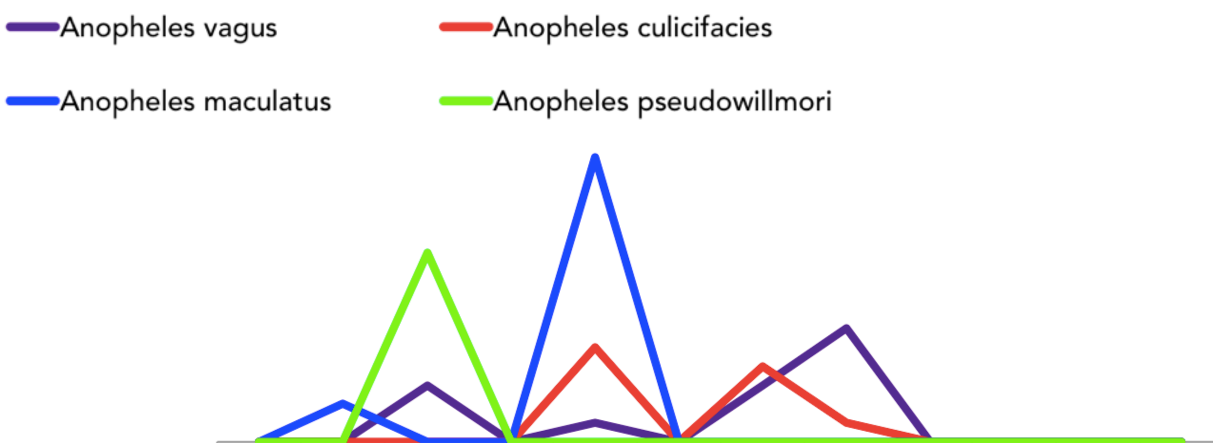


	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	52	24	30	55	1	2	3	0	2	1	0
Anopheles culicifacies	0	0	1	4	0	4	0	1	0	0	0	0
Anopheles pseudowillmori	0	14	7	12	7	8	1	2	0	3	0	0

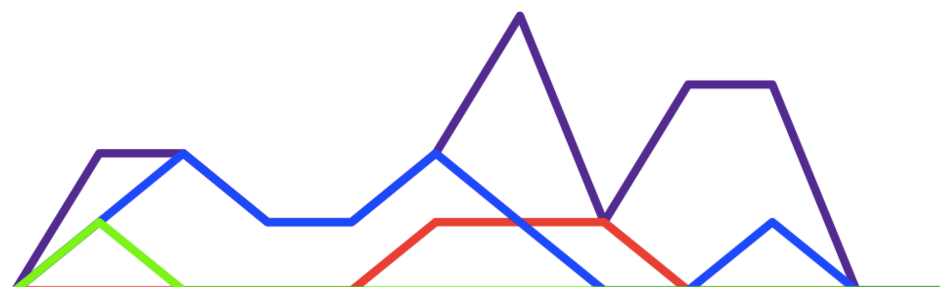


	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	2	30	34	8	6	2	0	1	0	1	0
Anopheles culicifacies	0	2	0	3	0	5	0	0	1	5	3	0
Anopheles pseudowillmori	0	11	10	21	21	7	4	1	1	4	3	0

Chokuling



	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	0	3	0	1	0	3	6	0	0	0	0
Anopheles culicifacies	0	0	0	0	5	0	4	1	0	0	0	0
Anopheles maculatus	0	2	0	0	15	0	0	0	0	0	0	0
Anopheles pseudowillmori	0	0	10	0	0	0	0	0	0	0	0	0



	6 to 7	7 to 8	8 to 9	9 to 10	10 to 11	11 to 12	12 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Anopheles vagus	0	2	2	1	1	2	4	1	3	3	0	0
Anopheles culicifacies	0	0	0	0	0	1	1	1	0	0	0	0
Anopheles maculatus	0	1	2	1	1	2	1	0	0	1	0	0
Anopheles pseudowillmori	0	1	0	0	0	0	0	0	0	0	0	0

Appendix D

A) Group Maculatus:

Anopheles maculatus, An. dravidicus Christophers, *An. notanandai* Rattanarithikul & Green, *An. rampae* Harbach &

Anopheles spp.	Author	District	GPS Coordinates
An. maculatus group*	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (6)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
An. maculatus	Singh S, et al (2012)	Tinsukia (2) • Margherita (1) • Mullock gaon (1) Karbi Anglong (8) • Budong (4) • Rong Hong Robong (3) • Hatidandi forest camp (1) Dima Hasao (11) • Longma 2 (11) Golaghat (17) • Bokakhat (17)	Tinsukia • N-27◦ 17′ E-95◦ 39′ • N-27◦ 21′ E-95◦ 37′ Karbi Anglong • N-25◦ 47′ E-92◦ 33′ • N-25◦ 48′ E-92◦ 32′ • N-26◦ 33′ E-93◦ 10′ Dima Hasao • N-25◦ 08′ E-93◦ 03′ Golaghat • N-26◦ 37′ E-93◦ 36′
	Dev V, et al (2004)	Kamrup	-

Somboon, *An. sawadwongporni* Rattanarithikul & Green, *An. dispar* Rattanarithikul & Harbach, *An. greeni* Rattanarithikul & Harbach, *An. pseudowillmori* (Theobald) *and An. willmori* (James)

Anopheles spp.	Author	District	GPS Coordinates
An. pseudowillmori	Singh S, et al (2012)	Tinsukia (1) • Mullock gaon (1) Karbi Anglong (1) • Umbasor (1) Dima Hasao (4) • Longma 2 (1) • Jatinga (3)	Tinsukia • N-27◦ 21′ E-95◦ 37′ Karbi Anglong • N-25◦44′ E-92◦ 29′ Dima Hasao • N-25◦ 08′ E-93◦ 03′ • N-25◦07′ E-93◦ 01′
An. dravidicus **Christophers**	Singh S, et al (2012)	Nalbari (1) • Goybari, Nagrijuli (1) Dima Hasao (3) • Jatinga (3)	Nalbari • N-26◦ 43′ E-91◦ 44′ Dima Hasao • N-25◦07′ E-93◦ 01′

B) Group Funestus:

Anopheles spp.	Author	District	GPS Coordinates
***An. culicifacies* Giles 1901**	Das NG, et al (2011)	Sonitpur (887) • Bengenajuli (218) • Sapairaumari Pathar (429) • Nigam (240)	2°200 E–93°450 E and 26°200 N–27°050 N
	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (30)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Dhiman S, et al (2012)	Sonitpur (514) • 4 villages	26°40′44.9″N to 92°47′42.5″E 26°41′09.4″N to 92°46′46.6″E 26°41′56.9″N to 92°48′09.9″E 26°42′01.2″N to 92°46′50.1″E
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-

Anopheles spp.	Author	District	GPS Coordinates
***An. culicifacies* Giles 1901 (continued)**	Das NG, et al (2004)	Sonitpur (47) • Kekurijan (15) • Balijanbanua (21) • Duflagurh Tea Estate (11)	92° 20′E–93°45′E and 26° 20′N–27° 05′N
	Saxena R, et al (2014)	Sonitpur (656)	92° 20'-93°45' E and 26° 20' - 27° 05' N
***An. fluviatilis* James 1902**	Das NG, et al (2011)	Sonitpur (304) • Bengenajuli (88) • Sapairaumari Pathar (116) • Nigam (100)	2°200 E–93°450 E and 26°200 N–27°050 N
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
***An. varuna* Iyengar 1924**	Das NG, et al (2011)	Sonitpur (244) • Bengenajuli (92) • Sapairaumari Pathar (43) • Nigam (109)	2°200 E–93°450 E and 26°200 N–27°050 N

Anopheles spp.	Author	District	GPS Coordinates
***An. varuna* Iyengar 1924 (continued)**	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. (2004)	Jorhat • Medeluajan and Panirara (31)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N
	Das NG, et al (2004)	Sonitpur (118) • Kekurijan (41) • Balijanbanua (69) • Duflagurh Tea Estate (8)	92° 20′E – 93°45′E and 26° 20′N – 27° 05′N
	Saxena R, et al (2014)	Sonitpur (8)	92° 20' – 93°45' E and 26° 20' – 27° 05' N
***An. aconitus* Donitz 1902**	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. (2004)	Jorhat • Medeluajan and Panirara (76)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
***An. jeyporiensis* James 1902**	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. (2004)	Jorhat • Medeluajan and Panirara (16)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′

Anopheles spp.	Author	District	GPS Coordinates
An. minimus **Theobald 1901**	Dev V, et al (2004)	Kamrup • Sonapur (332) Karbi Anglong • Manja (282) Kokrajhar • Gossaigaon (89) Sonitpur • Rangapara (142) Darrang • Tanglang (382) Nagaon • Kathiatoli Golaghat • Bokakhat (303) Lakhimpur • Lakhimpur (5) Goalpara • Agia (105) Morigaon • Nellie (130) Nalbari • Kumarikata (46)	-
	Prakash A, et al. (2004)	Jorhat • Medeluajan and Panirara (702)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′

Anopheles spp.	Author	District	GPS Coordinates
An. minimus **Theobald 1901 (continued)**	Kalita JC, et al. (2014)	Kamrup Metropolitan • Maligaon rail colony (40) • Maligaon Pandughat (24) • Garchowk (28) • Khanapara (66)	-
	Dhiman S, et al (2012)	Sonitpur (90) • 4 villages	26°40′44.9″N to 92°47′42.5″E 26°41′09.4″N to 92°46′46.6″E 26°41′56.9″N to 92°48′09.9″E 26°42′01.2″N to 92°46′50.1″E
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
	Das NG, et al (2004)	Sonitpur (157) • Kekurijan (50) • Balijanbanua (81) • Duflagurh Tea Estate (26)	92° 20′E–93°45′E and 26° 20′N–27° 05′N
	Saxena R, et al (2014)	Sonitpur (42)	92° 20'-93°45' E and 26° 20' - 27° 05' N

C) Group Annularis:

Anopheles spp.	Author	District	GPS Coordinates
An. annularis **van der Wulp 1884**	Das NG, et al (2011)	Sonitpur (2018) • Bengenajuli (667) • Sapairaumari Pathar (761) • Nigam (590)	2°200 E–93°450 E and 26°200 N–27°050 N
	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (69)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Dhiman S, et al (2012)	Sonitpur (526) 4 villages	26°40′44.9″N to 92°47′42.5″E 26°41′09.4″N to 92°46′46.6″E 26°41′56.9″N to 92°48′09.9″E 26°42′01.2″N to 92°46′50.1″E
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-

Anopheles spp.	Author	District	GPS Coordinates
***An. annularis* van der Wulp 1884 (continued)**	Das NG, et al (2004)	Sonitpur (136) • Kekurijan (61) • Balijanbanua (56) • Duflagurh Tea Estate (19)	92° 20′E – 93°45′E and 26° 20′N –27° 05′N
	Saxena R, et al (2014)	Sonitpur (192)	92° 20' – 93°45' E and 26° 20' – 27° 05' N
***An. philippinensis/ nivipes* complex**	Das NG, et al (2011)	Sonitpur (2902) • Bengenajuli (773) • Sapairaumari Pathar (1455) • Nigam (674)	2°200 E – 93°450 E and 26°200 N – 27°050 N
	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (1377)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Dhiman S, et al (2012)	Sonitpur (744) 4 villages	26°40′44.9″N to 92°47′42.5″E 26°41′09.4″N to 92°46′46.6″E 26°41′56.9″N to 92°48′09.9″E 26°42′01.2″N to 92°46′50.1″E
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N

Anopheles spp.	Author	District	GPS Coordinates
***An. philippinensis/ nivipes* complex (continued)**	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
	Das NG, et al (2004)	Sonitpur (310) • Kekurijan (101) • Balijanbanua (183) • Duflagurh Tea Estate (26)	92° 20′E–93°45′E and 26° 20′N–27° 05′N
	Saxena R, et al (2014)	Sonitpur (156)	92° 20'-93°45' E and 26° 20' - 27° 05' N
	Sarma NP, et al (2012) * Ratio of *An.nivipes*: *An. philippinensis* is based on molecular identification	Karbi Anglong (50) • Longnit (46:4)* Golaghat (2) • Bagori (2:0)* Nagaon (5) • Kaziranga (2:0)* • Kanchanjuri (3:0)* Dibrugarh (1) • Saraipung (0:1)* Jorhat (30) • Titabor (29:1)* Nalbari (131) • Kumarikata (101:0)* • Tamulpur (30:0)*	Karbianglong • N-25°33′ E-93°50′ Golaghat • N-26°21′ E-92°40′ Nagaon • N-26°39′ E-93°20′ • N-26°42′ E-93°27′ Dibrugarh • N-27°23′ E-95°34′ Jorhat • N-26°46′ E-81°24′ Nalbari • N-26°25′ E-91°25′ • N-26°17′ E-91°21′
***An. pallidus* Theobald 1901**	Dev V, et al (2004)	Kamrup	-

D) Group Tessellatus

Anopheles spp.	Author	District	GPS Coordinates
***An. tessellatus* Theobald**	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (15)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Das NG, et al (2004)	Sonitpur (29) • Kekurijan (2) • Balijanbanua (27)	92° 20′E–93°45′E and 26° 20′N–27° 05′N

E) Group Jamesii:

Anopheles spp.	Author	District	GPS Coordinates
***An. jamesii* Theobald 1901**	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
***An. splendidus* Koidzumi 1920**	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (12)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′

F) Complex Subpictus

Anopheles spp.	Author	District	GPS Coordinates
***An. subpictus* Grassi 1899**	Dev V, et al (2004)	Kamrup	-
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-

G) Group Leucosphyrus:

Anopheles spp.	Author	District	GPS Coordinates
An. baimaii **Sallum & Peyton 2005** (previously An. dirus Species D)	Sarma et al (2012)	Dibrugarh • Soraipung forest (9) Jorhat • Titabor (1) Golaghat and Nagaon • Kaziranga National Park (10) Dima Hasao • Jatinga (4) Kamrup • Kamrup (19)	Dibrugarh • N-27◦ 23′ E-95◦ 34′ Jorhat • N-26◦ 36′ E-94◦ 17′ Golaghat and Nagaon • N-26◦ 34′ E-93◦ 10′ Dima Hasao • N-25◦ 11′ E-93◦ 02′ Kamrup • N-26◦ 17′ E-91◦ 92′
An. dirus **Peyton & Harrison**	Das NG, et al (2011)	Sonitpur (285) • Bengenajuli (213) • Sapairaumari Pathar (0) • Nigam (72)	2°200 E–93°450 E and 26°200 N–27°050 N
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (41)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Dev V, et al (2004)	Kamrup	-
	Das NG, et al (2015)	Sonitpur • Bengenajuli • Sapairaumari Pathar • Nigam	92° 20′ E – 93° 45′ E and 26° 20′ N – 27° 05′ N

H) Group Barbirostris:

Anopheles spp.	Author	District	GPS Coordinates
An. barbirostris group*	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (45)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
An. barbirostris **van der Wulp 1884**	Dev V, et al (2004)	Kamrup	-
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
	Das NG, et al (2004)	Sonitpur (782) • Kekurijan (461) • Balijanbanua (294) • Duflagurh Tea Estate (27)	92° 20′E–93°45′E and 26° 20′N–27° 05′N

* *An. freyi, An. koreicus, An. barbirostris, An. campestris, An. donaldi, An. franciscoi, An. hodgkini, An. pollicaris, An. ahomi, An. barbumbrosus, An. manalangi, An. reidi, An. vanus*

I) Group Hyrcanus:

Anopheles spp.	Author	District	GPS Coordinates
An. hyrcanus group*	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. (2004)	Jorhat • Medeluajan and Panirara (832)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
***An. crawfordi* Reid 1953**	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
	Das NG, et al (2004)	Sonitpur (347) • Kekurijan (123) • Balijanbanua (132) • Duflagurh Tea Estate (92)	92° 20′E–93°45′E and 26° 20′N–27° 05′N

* *An. anthropophagus, An. argyropus, An. belenrae, An. changfus, An. chodukini, An. dazhaius, An. engarensis, An. hailarensis, An. heiheensis, An. hyrcanus, An. junlianensis, An. kiangsuensis, An. kleini, An. kummingensis, An. kweiyangensis, An. liangshanensis, An. nimpe, An. pseudopictus, An. pullus, An. sinensis, An. sineroides, An. xiaokuanus, An. xui, An yatsushiroensis, An. crawfordi, An. kiangsuensis, An. lesteri, An. paraliae, An. peditaeniatus, An. vietnamensis, An. nigerrimus, An. nitidus, An. pseudosinensis, An. pursati*

J) Group Kochi

Anopheles spp.	Author	District	GPS Coordinates
An. kochi	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. (2004)	Jorhat • Medeluajan and Panirara (233)	Catchment area of Titabor primary health center N-26◦ 35′ E-94◦ 12′
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
	Das NG, et al (2004)	Sonitpur (449) • Kekurijan (155) • Balijanbanua (257) • Duflagurh Tea Estate (37)	92° 20′E–93°45′E and 26° 20′N–27° 05′N

K) Others

Anopheles spp.	Author	District	GPS Coordinates
***An. vagus* Donitz 1902**	Dev V, et al (2004)	Kamrup	-
	Prakash A, et al. 2004.	Jorhat • Medeluajan and Panirara (775)	Catchment area of Titabor primary health center • N-26◦ 35′ E-94◦ 12′
	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-
	Das NG, et al (2004)	Sonitpur (342) • Kekurijan (71) • Balijanbanua (175) • Duflagurh Tea Estate (96)	92° 20′E–93°45′E and 26° 20′N–27° 05′N
***An. karwari* James 1903**	Gopalakrishnan R, et al. (2014)	Lakhimpur • Balitika • Paruwa • Rupkurua • Udmari	-

Appendix E

Species	1962	1976	1986	1989	1998-2007	2008	2009	2010	2011	2012	2013	2014
MACULATUS GROUP												
An. (Cel.) maculatus (Theobald,1901) M	+	+	-	+	+	+	+	+	+	+	+	+
An. (Cel.) willmori (James,1903) M					+	+	+	+	+	+	+	-
An. (Cel.) pseudowillmori (Theobald, 1910) P					+	+	+	+	+	+	+	+
SUBPICTUS GROUP												
An. (Cel.) subpictus Grassi, 1899 P					+	+	-	-	-	-	-	-
An. (Cel.) vagus Doenitz,1902 P	+	+	+	+	+	+	+	+	+	+	+	+
ANNULARIS GROUP												
An. (Cel.) annularis Van der Wulp, 1884 P	+	+	+	+	+	+	+	+	+	-	-	-
An. (Cel.) philippinensis Ludlow,1902 P					+	+	+	+	+	+	+	-
An. (Cel.) splendidus Koidzum, 1920 M		+	+	+	+	+	+	+	+	+	+	-

An. (Cel.) nivipes Theobald, 1903					+	-	+	+	+	+	+	-
JAMESII GROUP												
An. (Cel.) jamesii Theobald, 1901 P			+	+	+	-	+	+	+	+	+	+
An. (cel.) pseudojamesii Theobald 1901 P			+	+	+	+	+	+	+	+	+	-
MINIMUS GROUP												
An. (Cel.) minimus Theobald, 1901	+	+	-	+	-	-	-	-	-	-	-	-
An. (Cel.) aconitus P			+	+	+	-	-	-	-	-	-	-
CULICIFACIES SUB-GROUP												
An. (Cel.) culicifacies Gales,1901 P	+	+	-	+	+	+	+	+	+	+	-	+
An. (Cel.) fluviatilis James,,1902			+	+	+	-	-	-	-	+	-	-
An. (Cel.) jeyporiensis James,1902		+	+	-	-	-	+	+	-	+	-	-
An. (Cel.) balabacensis Baisas, 1936		+	+	+	+	-	+	+	+	+	+	-
An. (Cel.) kochi Doenitz, 1901 P			+	+	+	+	+	+	+	+	+	-

An. (Ano.) barbirostris Van der Wulp,1884 <u>P</u>			+	-	-	-	-	+	-	-	-	-
An. (Cel.) tessellatus Theobald 1901.			+	-	-	-	-	-	-	+	+	-
An.(Ano.) aitkenii (Reid and Knight, 1961) <u>M</u>				+	-	-	-	-	-	-	-	-
An. (Ano.) peditaeniatus (Leicester,1908) <u>P</u>					+	+	+	+	+	+	+	+
An. (Ano.) lindesayii cameronensis Edward, 1929 <u>M</u>					+	+	+	+	-	-	+	-
An. (Ano.) baileyi Edwards, 1929 <u>M</u>					+	-	-	-	-	-	+	-
An. (Ano.) bengalensis Puri,1930 <u>M</u>					+	-	-	-	-	+	+	-

+ indicates the presence of the *Anopheles* species in the specified year in Bhutan
- indicates the absence of the *Anopheles* species in the specified year in Bhutan
<u>P</u> indicates plain species
<u>M</u> indicates mountain species

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