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Research Article

Identification of sibling species in field collected *Anopheles minimus* using ovarian nurse cell chromosome in two malaria endemic areas in Myanmar

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Abstract: Malaria remains a major health problem in Myanmar. *Anopheles dirus* and *Anopheles minimus* are the principal vectors of malaria, but *Anopheles maculatus*, *Anopheles vagus*, *Anopheles annularis*, *Anopheles philippnensis*, *Anopheles stiphensi* and *Anopheles culicifacies* are secondary or suspected vectors of malaria in Myanmar. The aim of the present study was to know the prevalence of sibling species complex of *Anopheles minimus* using ovarian nurse cell polytene chromosome method and determine the potential vector using vector incrimination. Therefore the study was done in Pyin Oo Lwin Township Mandalay Region and Kamamaung Township Kayin State from October 2014 to September 2015. *Anopheles* mosquitoes were collected by animal bait K-net, human bait indoor and outdoor and light trap collection. A total of 1153 *Anopheles* mosquitoes comprising six mosquito species were collected from Pho Ne Dun Village, Pyin Oo Lwin Township. The highest number 553 of *Anopheles minimus* were collected. 953 *Anopheles* mosquitoes (524 from Katine Htit and 429 from Kine Taw) comprising nine species were collected from Kamamaung Township. Of these 93 and 92, *Anopheles minimus* was collected from Katine Htit and Kine Taw villages respectively. After identification blood fat *Anopheles minimus* was kept in paper cups with glucose. When ovary development reached to the semi-gravid stage (Christophe stage), ovary specimens were dissected and preserved in Cornoy's fixative in screw type bottle and ovaries were tapered to vectors polytene chromosome. Head and thorax were homogenized tested for circumsporozoite antigen by ELISA method. Result found that all collected *Anopheles minimus* were observed sibling species A in both areas. Circumsporozoite ELISA test found that only one *Anopheles minimus* A was found sporozoite positive in Kamamaung Township 0.54% (1/185) and not found any sporozoite positivity in *Anopheles minimus* A collected from Pyin Oo Lwin Township (0/553).

INTRODUCTION

In Myanmar Anopheline fauna comprises of 37 *Anopheles* mosquitoes species, only two species are involved in the transmission of malaria. *Anopheles dirus* and *Anopheles minimus* are the primary vectors of malaria, but *Anopheles maculatus*, *Anopheles vagus*, *Anopheles annularis*, *Anopheles philippinensis*, *Anopheles stiphensi* and *Anopheles culicifacies* are secondary or suspected vectors of malaria in Myanmar [1]. In Myanmar main vectors *Anopheles minimus* and *Anopheles dirus* are obtained many parts of the country. *Anopheles minimus* was found foothill and forest fringe, but now it is found in the rice field in plain areas. *Anopheles dirus* species are also bred in deep forest areas but now their larvae were found in men made wells in coastal areas of Mon State and Tanintharyi Region [2, 3], because certain environmental changes like deforestation and vegetation clearance for crop plantations and also rapid growth of population and installation of new rural areas may lead to an increase abundance of mosquito larval habitats [4]. All these primary vectors are species complex each of these morphological species comprises some morphologically indistinguishable. There two kinds of polytene chromosome detection methods (1) salivary gland polytene chromosome and (2) ovarian nurse cell polytene chromosome detection methods. Salivary gland polytene chromosome can be dissected from *Anopheles dirus* only although ovarian nurse cell chromosome can be dissected from other Anophelines species (*Anopheles gambiae*, *Anopheles funastus*, *Anopheles maculipennis*, *Anopheles annularis*, *Anopheles leucosphyrus*, *Anopheles fluviatilis*, *Anopheles maculates*, *Anopheles minimus*, *Anopheles subpictus*, *Anopheles philippnensis*,

Anopheles nivipes and *Anopheles sundaicus* in different areas. About 23 *Anopheles* taxa have been identified so far as the species complex, most notably the *Anopheles gambiae*, *Anopheles funastus* in Africa, *Anopheles quadrimaculatus* in North America [5]. *Anopheles maculipennis* in Europe, *Anopheles culicifacies* in the Indian Subcontinent, *Anopheles dirus*, *Anopheles annularis*, *Anopheles leucosphyrus*, *Anopheles fluviatilis*, *Anopheles maculatus*, *Anopheles minimus*, *Anopheles subpictus*, *Anopheles philippnensis*, *Anopheles nivipes* in South-East Asia and they are important vectors of malaria in different parts of the world [5]. In Myanmar, well breeding *Anopheles dirus* from Mon State was cyto-taxonomically identified as sibling species D [6]. There are five sibling species in *Anopheles culicifacies* as A, B, C, D and E were identified by ovarian nurse cell polytene chromosome method and A, C, D and E are vectors of malaria [7]. A Large number of sibling species B of *Anopheles culicifacies* was found in Paukaung Towship [8]. The primary vector of *Anopheles minimus* is a complex of 5 sibling species designated as species A, B, C, D and E. *Anopheles minimus* E is restricted to the non-malarious Ryukyu Archipelago [9]. Their vectorial capacity varies widely. *Anopheles minimus* is the malaria vector of the Foothill, forest fringe and plain areas in Southeast Asia region [10]. *Anopheles minimus* sibling species A has long been considered to be a primary malaria vector in northeastern state of India [10], and according to ovarian nurse cell polytene chromosome identification of *Anopheles minimus* showed A, B and C were found in China [11], E was discovered in the Ryukyu Archipelago of Japan [9] and A and C was found in Vietnam and Thailand [12,13].

Other researchers revealed that the different sibling species and their biological characteristics were distinguished by molecular methods [14, 15]. Shi Yu-Ming and Ye Yi-Ying [11] mention that in ovarian nurse cell polytene chromosomes of arm 2 can easily be identified the chromosome of the *Anopheles minimus*. Sibling species identification is useful for identifying which species is the potential vector of malaria and which species is abundant in the study area. Vector incrimination is a prerequisite for understanding the role of Anopheline in malaria transmission and has been used to determine which species is the most potential vector and which species is abundant in the study area. It also is used to compare the contribution of individual species to overall malaria transmission. As particularly few literatures were available about sibling species of *Anopheles* in Myanmar. Present study was conducted in Pho Ney Dun village Pyin Oo Lwin Township Mandalay Region, Katine Htit and Kine Taw villages in Kamamaung Township Kayin State. All these villages are situated foothill and forested areas of Myanmar. Malaria transmission is occurred year round. Main malaria vectors of *An. dirus* and *An. minimus* and secondary vectors as *An. maculatus*, *An. annularis*, *An. culicifacies*, *An. vagus and An. aconitus* are abundantly present in all seasons in these areas and need to classify, which sibling species of the main vectors are potentially harmful to villages. Therefore the present study was assessing the prevalence of sibling species complex of *Anopheles minimus* using ovarian nurse cell polytene chromosome method and determined the potential vector using vector incrimination study.

MATERIALS AND METHODS

Study design and period

Study design field and laboratory base cross-sectional descriptive study design were done with field collected ovary of *Anopheles minimus* from August 2014- July 2015.

Study areas

Two malaria endemic areas as were selected according to the abundance of *Anopheles minimus* mosquito by previous literature [1]. Pho Ney Dun Village, Pyin Oo Lwin Township Mandalay Region and Katine Htit and Kine Taw village, Kamamaung Township, Kayin State were selected for *Anopheles minimus* collection.

Pho Ney Dun Village Pyin Oo Lwin Township Mandalay Region: Pho Nay Dun village is situated in Pyin Oo Lwin Township, Infant of the village is Mandalay- Larshio Railway line and Mandalay-Lashio packer car road West of the village is cave of the Pait Chin Myaung which is an ancient Buddha historical place. North of the village is mountains and teak forest of Naung Cho Township Shan State, it is a border Township of Shan State and Mandalay Region. Southern part of the village is foothill areas of Shan mountain range. Paddy fields, bean fields, Sunflower fields are situated in southern parts of the village. About 2000populations are living in 200-250 households in the village. 60% of the populations are found in Myanmar, 20% Shan 10 Napoli and remaining 10% is chin, Hindu, Rakhine and Islam. 90% of the population are Buddha reaming are Christian and Islam only one household family is Hindu. Almost all of the households have one to two long lasting insecticide treaded mosquito nets which are used for the prevention of mosquito bite. 90% of the population are farmers, and remaining 10 % are hunters, teachers, policeman and railway staff. There are 5 monasteries, 1 state high school and 2 primary-school are formed in the village. In the village there are naturally formed many water pools, slowly running gutters and rock pools which are suitable as mosquito breeding places. Each house has one cow shed and in one cow shed has 2-10 cows, and some villagers owned 50-100 cows in the village

Katine Htit and Kine Taw village, Kamamaung Township, Kayin: Katine Htit and Kine Taw village, Kamamaung Township Kayin State was selected for the collection of *Anopheles* mosquitoes and larvae to do sibling species identification by cytogenetic technique.

Katine Htit village: Katine Htit village is situated beside the Yonsalin creek, Kamamaung- Phapun packer car road is situated eastern part of the village which road is crossed to the village and Yunsalin creek is situated in western part of the village. The northern part of the village, one military head quarter is situated which is 3 kilometer away from village. The population is about 1000 in 150 households in the village and households are situated in both site of the Kamamaung – Phapun road. 60% of the whole populations are Buddha and remaining are Christian and Islam. Almost all of the households have one to two long lasting

insecticidal mosquito nets which are used for the prevention of mosquito bite. In this village population, over 85% of the populations are formers remaining are wood cutters, charcoal makers, fisher men, school teachers and government stuff, police man. One RHC, one state high school, one pagoda and tow monastic schools are situated in the village. One police station is situated between Katine Htit and Kine Taw villages.

Kine Taw village: Kine Taw village is situated beside the Yonsalin creek, Kamamaung Phapun packer car road is situated in the eastern part of the village, police station is situated other site of the road it is about one kilo meter away from the village. Yunsalin creek is situated in the west site of the village. Yonsalin bridge is situated in southern part of the village. In the northern part of the village, paddy fields are situated. Kine Taw village is situated about 3 kilo meter far away from the Katine Htit village. The village is 50 kilometer away from Kamamaung city. Kamamaung - Phapun road is across the village. The population is about 1200 and 80% of the population are Kayin remaining are Burma and Muslim. Ninety percent of the households have one to two mosquito nets. In the village population, over 90% of the people are plantation workers, remaining are government workers, hunters, fishers and school teachers. One primary school and one monastery and one midwife house are situated in the village.

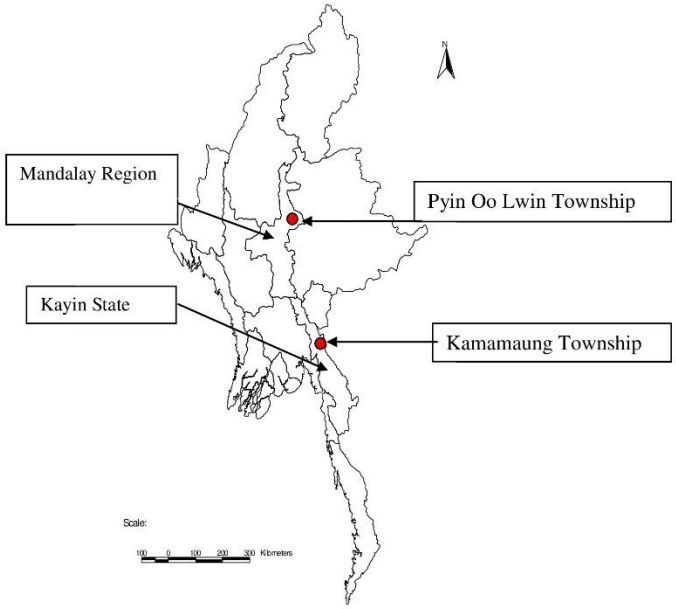

Fig 1. Map of Myanmar and study area

Adult mosquito's collection

Adult mosquitoes were collected by WHO sucking tube with several mosquitoes catching methods using cattle baited big net trap (330x330x180cm) collection, indoor and outdoor human bait gathering, cow sheds gathering and light trap collection in both areas at 18:00 to 06:00 hours for seven days. Mosquitoes were collected according to WHO (16) guideline.

Larvae collection: *Anopheles* larvae were collected in and around the 3 Kilometer radius of the study villages. Water pools, water pockets, sand pools, domestic water wells, footprint, slowly running water in the creek were searched for mosquito's larvae. Larvae were collected according to WHO (16) guideline.

Identification of Mosquitoes

All collected adult mosquitoes and adult mosquitoes emerged from larval surveys were identified by the key of different authors [17-19].

Ovary collection and processing

Blood fed *Anopheles minimus* were collected using WHO sucking tube then put into the paper cup and supplied glucose as food to till the development of ovary. Water soak towel was covered to make constant Humidity and temperature (Temp 27ºC, RH 90%). When ovary development was reached to the semi-gravid stage (Christophe stage) ovary specimens were dissected and preserved in Cornoy's fixative in screw type bottle. Samples were stored at 4ºC in refrigerator till ovarian nurse cell polytene chromosome study.

Identification of sibling species

Preserved ovaries of *Anopheles minimus* were processed in 50% propionic acid and were stained with 2% lacto-aceto-orcein stain according to the method Green and Hunt [20] for making polytene chromosome preparations. All collected samples were stored in a freezer at 4ºC in laboratory till use. The chromosome complement of individual mosquitoes were examined under 40X, and 100X magnification lens of compound light Olympus microscope for species diagnostic inversions were used for identification of the members of *Anopheles minimus* complex.

Salivary gland collection

The head and the thorax of mosquitoes were cut from the body by clean blade and ten each head and thorax of same species were put into individual microfuge tubes containing silica gel as preservative for detection of sporozoites antigen in salivary glands. Head and the thorax of the main vector were kept individually in microfuge tubes. All collected samples were stored in a freezer at 4ºC. This procedure was reducing the probability of detection of CS antigen from other parts of the body [21].

Vector incrimination

After sibling species identification the head and thorax of *Anopheles minimus* specimens were identified for Circumsporozoite antigen by sporozoite ELISA test. Homogenates of individual head and thorax of main vector *Anopheles minimus* and five pools head and thorax of individual species (Secondary vectors) in grinding buffer were put into each well of *P.falciparum, P.vivax210* and *P,vivax*$_{247}$ specific monoclonal antibody coated plates and identified according to Wirtz et al., [22] method.

Statistical analysis

Data entry and analysis was done using Microsoft excel software. Sibling species identification and vector incrimination were determined as percentage population of sibling species and percentage positivity of malaria parasite species.

RESULTS

Table (1) shows that a total of 2106 *Anopheles* mosquitoes were collected out of this 1153 *Anopheles* mosquitoes comprising six mosquito species were collected from Pho Ne Dun Village, Pyin Oo Lwin Township. The highest number 553 of *Anopheles minimus* were obtained in the Pho Ney Dun village followed by *Anopheles maculatus* 285 numbers, the lowest eight numbers of *Anopheles culicisifacies* were observed. Remaining 953 *Anopheles* mosquitoes were collected from Kamamaung Township. Out of this a total of 524 *Anopheles* mosquitoes belonging to 9 *Anopheles* species from Katine Htit and 429 *Anopheles* mosquitoes belonging to same nine species were collected in Kine Taw villages respectively. The highest numbers *of Anopheles culicifacies* 103 and 99 numbers were observed in both Katine Htit and Kine Taw villages followed by 93 and 92 *Anopheles minimus* The lowest numbers 25 (4.77%) of *Anopheles jamsi* from Katine Htit village and 8 (1.87%) of *Anopheles tessalatus* from Kine Taw village were collected. The highest density of *Anopheles minimus* was observed in Pyin Oo Lwin Township. It was 5.95 fold higher than Katine Htit and 6.01 fold greater than Kine Taw village collected *Anopheles minimus*.

Table (2) shows that all collected *Anopheles minimus* from Pho Nay Dun Village, Pyin Oo Lwin and Katine Htit and Kine Taw villages were found species complex A (Fig. 2). Among the collected *Anopheles minimus*, only one (1/93, 1.075%) *Anopheles minimus* from Katine Htit village was found circumsporozoite antigen positive by ELISA method.

Table (3) shows that a total of 738 *Anopheles minimus* were collected from both Townships. Only one1/93(1.075%) *Anopheles minimus* from Katine Htit village was found *Pf* circumsporozoite antigen positive, and only one *Anopheles dirus* from Kine Taw village was found Pv_{210} circumsporozoite antigen positive.

Table 1. Total collection of *Anopheles* mosquitoes from Pyin Oo Lwin and Kamamaung Township

Collected mosquitoes	Pyin Oo Lwin Mandalay Region	Kamamaung Township Kayin State		Total mosquitoes (in Number)
	Pho Ne Dun village (in Number)	Katine Htit village (in Number)	Kine Taw village (in Number)	
Anopheles minimus	553 (47.96%)	93 (17.75%)	92 (21.46%)	738 (35.04%)
Anopheles maculatus	285 (24.72%)	88 (16.79%)	64 (14.92%)	437 (20.75%)
Anopheles vagus	132 (11.49%)	63 (12.03%)	24 (5.59%)	219 (10.40%)
Anopheles culicifacies	8 (0.69)	103 (19.65%)	99 (23.08%)	210 (9.97%)
Anopheles annularis	163(14.14%)	55 (10.50%)	52 (12.12%)	270 (12.82%)
Anopheles tessalatus	0	32 (6.11%)	8 (1.87%)	40 (1.90%)
Anopheles jamsi	0	25 (4.77%)	27 (6.29%)	52 (2.47%)
Anopheles aconitus	12 (1.04%)	34 (6.49%)	36 (8.39%)	82 (3.89%)
Anopheles dirus	0	31 (5.92%)	27 (6.29%)	58 (2.75%)
Total	1153 (100%)	524 (100%)	429 (100%)	2106 (100%)

Table 2. Identification of species complex of *Anopheles minimus* in two different Townships using ovarian nurse cell polytene chromosome

Collected mosquitoes	Pyin Oo Lwin Mandalay Region	Kamamaung Township Kayin State		Total mosquitoes (sporozoite positive rate)
	Pho Ne Dun village	Katine Htit village	Kine Taw village	
	Species complex & sporozoite positive rate	Species complex & sporozoite positive rate	Species complex & sporozoite positive rate	
Anopheles minimus	Species A	Species A	Species A	Species A
Sporozoite	0/553 (0%)	1/93 (1.075%)	0/92 (0%)	738 (0.136%)

Table 3. Circumsporozoite positivity in all collected *Anopheles* mosquitoes in different areas using ELISA technique

Collected mosquitoes	Pyin Oo Lwin Mandalay Region	Kamamaung Township Kayin State		Total mosquitoes (in Number) (% sporozoite +ve)
	Pho Ne Dun village (in Number) (% sporozoite +ve)	Katine Htit village (in Number)(% sporozoite +ve)	Kine Taw village (in Number)(% sporozoite +ve)	
Anopheles minimus	553 (0%)	1/93 (1.075%)	92 (0%)	1/738 (0.135%)
Anopheles maculatus	285 (0%)	88 (0%)	64 (0%)	437 (0%)
Anopheles vagus	132 (0%)	63 (0%)	24 (0%)	219 (0%)
Anopheles culicifacies	8 (0%)	103 (0%)	99 (0%)	210 (0%)
Anopheles annularis	163(0%)	55 (0%)	52 (0%)	270 (0%)
Anopheles tessalatus	0	32 (0%)	8 (0%)	40 (0%)
Anopheles jamsi	0	25 (0%)	27 (0%)	52 (0%)
Anopheles aconitus	12 (0%)	34 (0%)	36 (0%)	82 (0%)
Anopheles dirus	0	31 (0%)	1/27 (3.7%)	1/58 (1.72%)
Total	1153 (0%)	1/524 (0.19%)	429 (0.23%)	2/2106 (0.095%)

DISCUSSION

The present study revealed that the prevalence of sibling species complex of *Anopheles minimus* in different areas of Myanmar using ovarian nurse cell polytene chromosome method and also determined the potential vectors of malaria in the studied areas using circumsporozoite ELISA test. *Anopheles* mosquitoes were collected in Pho Ney Dun village Pyin Oo Lwin Township Mandalay Region and two areas as Katine Htit and Kine Taw villages in Kamamaung Township. of Karin State. Younsalin Creek is situated around the villages are the preferred breeding site of *Anopheles minimus* [23]. A total of 1153 *Anopheles* mosquitoes belonging to 6 *Anopheles* species, *Anopheles minimus* was found the highest number 553 (47.96%) in Pyin Oo Lwin Township. Minimus was observed in high density because paddy fields and plenty of slowly running water places were available in and around the study area. In Kamamaung Township a total of 2106 *Anopheles* mosquitoes belonging to 9 species were collected, of this 524 and 429 *Anopheles* mosquitoes were collected from Katine Htit and Kine Taw villages. *Anopheles culicifacies* was found highest density followed by *Anopheles minimus* in both Katine Htit and Kine Taw villages. It may be because plenty of sand pools and slowly running water areas were available in the band of Yonsalin Creek, and *Anopheles culicifacies* and *Anopheles minimus* larvae were abundantly bred together in these places. The main vector *Anopheles dirus* adult was collected in both Katine Htit, and Kine Taw village by animal and human bait collections and larvae were collected in domestic water wells in both villages of Kamamaung Township.

Anopheles minimus was found in high numbers in forest fringe Foothill and plain areas in the country [24-26]. The pick biting time of both main vector *Anopheles minimus* and *Anopheles dirus* were found 10 to 11: 00 pm in both areas, it was agreed with the other researchers finding who were worked in Bago Yoma, Taikkyi Township Yangon Region, Mon state and Taninthayi Region [3,25-27].

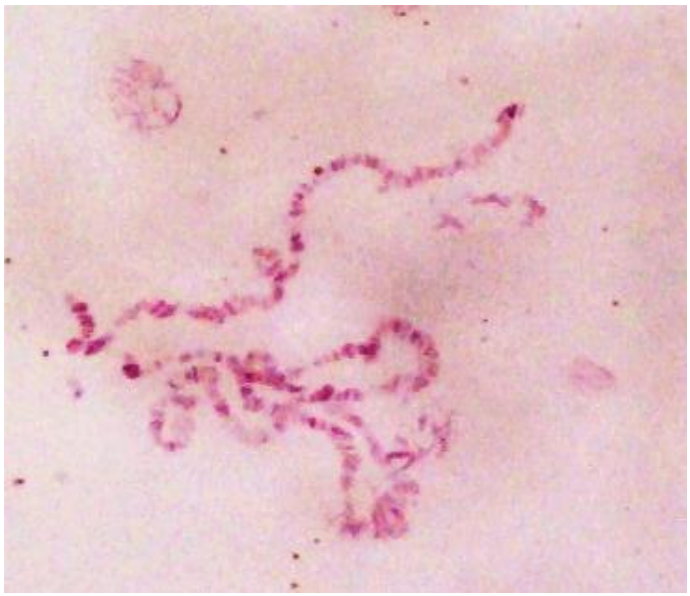

Fig 2. Ovarian nurse cell polytene chromosomes of *Anopheles minimus* A

Present study observed that collected *Anopheles minimus* from both Pyin Oo Lwin and Kamamaung were species complex A. *Anopheles culicifacies* is a co-breeder of *Anopheles minimus* A. *Anopheles minimus* A larvae were collected in high number in sand pools and slowly running

water of Yonsalin creeks, in both villages of Kamamaung. In Pyin Oo Lwin, *Anopheles minimus* A larvae were found in slowly running water and rice field. The result is agreed with the previous researchers; they revealed that *Anopheles minimus* larvae were mostly found in the rice field and slowly running water in Taungoo Township and slowly moving small jungle stem of Yeasitkan Village of Taikkyi Township [28, 26]. *Anopheles dirus* larvae were collected from domestic water wells in raining and cold seasons in both villages. Other researchers revealed that *Anopheles dirus* larvae were abundantly found in rock pools in forested hilly areas of Bago Yoma, Oktwin Township, Bago Region and also larvae were found in domestic water wells in Mon state and Thaninthayi Region, Bamboo stumps in deep forest areas [8, 25-27].

A researcher group in China proposed that the map of chromosome for *Anopheles minimus* from Guangxi [11]. The ovarian nurse cell's polytene chromosomes of *Anopheles minimus* exist as five arms (Fig. 2). They were the telocentric X chromosome, the submetacentric chromosome 2 and the metacentric chromosome 3. The X chromosome is easily recognized by its length and shuttle-shaped zone 6. The studied of ovarian nurse cell polytene chromosome of collected *Anopheles minimus* from Pyin Oo Lwin and Kamamaung Townships were found species complex A. Myat Myat Thu et al., [6] indicated that *Anopheles minimus* A was found in Bago Region by morphologically. High numbers of *Anopheles minimus* were collected in animal bait collection than human bait collection in both Townships. Sungvornyothin et al., [29] revealed that the trophic behavior and host preference of two sibling species, *Anopheles minimus* s.s. High number of *Anopheles minimus* species C, and A were captured on cattle than by outdoor and indoor human landing collection. Other researchers mention that *Anopheles minimus* is most likely to human blood than the animal blood [18].

Other researchers from different countries revealed that two forms of *Anopheles minimus* designated A and B, were described in China [30, 31] based on morphological features in larvae, pupae, and adults [32], and B in Thilandv[13] then confirmed in Vietnam [33], where sympatric homozygotes at the Odh locus (Octanol dehydrogenase) occurred in the absence of heterozygotes. The species were informally named species A and C [13]; the latter designated to distinguish it from B previously described in China [30]. Recently, Chen et al., [34] showed that forms A and B in China are morphological variants of *Anopheles minimus* A. In Japan, Somboon et al. [35] observed species E in Ishigaki Island. Both studies showed hybrid male sterility, which is generally accepted as very clear evidence of specific status. There is now no reason to cast doubt on the specific status of *Anopheles minimus* E [36, 37]. Therefore to date, three species, designated as A, C and E are formally recognized within the Minimus complex [9]. However, the complex may include two other species, species D [38] and specimen no. 157 in Thailand [39]. The specific status of these two entities is uncertain and needs further study. It seems that species D is a chromosomal variant of *Anopheles minimus* A (V. Baimai, personal communication). Van Bortel *et al*., [33] using isozyme electrophoresis detected *Anopheles minimus* A and C species in Northern Vietnam and found the absence of the HP (Humeral pale spot) was common (99%) in *Anopheles minimus* A and common (92%) in *Anopheles minimus* C. The variable nature of the HP in *Anopheles minimus* C precludes it from being an effective marker in separating *Anopheles minimus* A from C. Although other researchers revealed that Polytene chromosomes are often a very powerful means to identify mosquito species [40, 41].

Vector incrimination study of *Anopheles* mosquitoes was tested by ELISA method and found that *Anopheles minimus* A was the potential vector of malaria which mosquito was collected indoor at 11 pm in Katine Htit village. One *Anopheles dirus* was found 1/27 (3.7%) Pv_{210} circumsporozoite antigen was positive, which was collected from outdoor human bait at 10:00 pm in Kine Taw village. Vector incrimination study in the laboratory of Pakistan revealed that *Anopheles culicifacies* B was found sporozoite positive [42]. Although in Myanmar vector incrimination study of *Anopheles culicifacies* B was found sporozoite negative [43]. Other researcher found that vector incrimination study of *Anopheles dirus, Anopheles minimus*, *Anopheles kochi* and *Anopheles maculatus* were circumsporozoite antigen positive in Bokpyin Township in Tanintharyi Region and *Anopheles minimus* and *Anopheles culicifacies* were found circumsporozoite antigen positive in Ann Township, Rakhine State [44]. Other study found that 3.57% of *Anopheles minimus* and 0.54% of *Anopheles maculatus* were sporozoites positive in Taikkyi Township Yangon Region [26].

CONCLUSION

The present study conclusively establishes that the presence of sibling species A of *Anopheles minimus* complexes in studied areas as Pyin Oo Lwin Township, Mandalay Region and Kamamaung Township Kayin State in Myanmar by ovarian nurse cell polytene chrosome method; it is a first time report on the sibling species composition of *Anopheles minimus* in these areas. The sporozoite positivity rate was high in Kayin State. Therefore *Anopheles minimus* is a primary vector of malaria in the study areas. A detailed ovarian nurse cell cytogenetic and PCR study is needed for the identification of the role of *Anopheles minimus* sibling species complexes in other parts of malaria endemic areas of Myanmar, to establish existence and distribution of *Anopheles minimus* sibling species complexes and its vectorial capacity and status of malaria in Myanmar.

COMPETING INTERESTS: The authors have declared that no competing interests exist.

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