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Development of cytogenetic method for identification of *Anopheles culicifacies* species complex and its applications at malaria endemic areas of Myanmar

**Maung Maung Mya, **Pe Than Htun, **Sein Min & **Sein Thaung*

*Parasitology Research Division
**Medical Entomology Research Division
Department of Medical Research (Lower Myanmar)

Cytogenetic method for detailed identification of *An. culicifacies* was developed and tested in the field and laboratory. The objective of the study was to develop polytene chromosome cytogenetic method for *An. culicifacies* and to identify its sibling species complex by ovarian nurse cell polytene chromosome technique in malaria endemic areas of Myanmar. The study was conducted in two endemic areas, Paukkaung Township, Bago Division and Madaya Township, Mandalay Division. Adult mosquitoes were collected by animal baited big net trap and WHO recommended sucking tube in the field. Blood fed *An. culicifacies* were kept for 12 hours, 24 hours and 36 hours and ovaries were dissected and fixed in 10% methyl alcohol and ethyl alcohol fixatives in the field. In main laboratory, they were kept at 4˚C in refrigerator. Ovarian nurse cell chromosome bands were examined by compound microscope under 400x magnifications. Blood fed *An. culicifacies* accounting 613 specimens from Bago Division and 35 specimens from Mandalay Division were examined and the result revealed that both methyl alcohol and ethyl alcohol were found to be suitable fixatives and preservatives for prolong period. Up to 70% to 80% of clear and accurate polytene chromosome bands were detected from the 36 hours aged ovary group. Furthermore, sibling species of *An. culicifacies* were studied in detail by using this cytogenetic method and found to be type "B" of *An. culicifacies* complex. It is assumed that in Myanmar type B species are abundant in Bago and Mandalay Divisions. However, further study is needed to find out the presence of other type of species complexes in endemic areas. Although *An. culicifacies* is not a primary vector in Myanmar, it has been abundantly present in association with primary vectors such as *An. dirus* and *An. minimus* in endemic areas. Better understanding of species complex distribution and the role in malaria transmission will be beneficial in prevention and control of malaria.

INTRODUCTION

In Myanmar, 37 *Anopheles* mosquito species were recorded and *An. dirus* and *An. minimus* are major vectors of malaria. Secondary vectors, namely *An. culicifacies, An. annularis. An. maculatus* are abundant in central and lower Myanmar. Khin Maung Kyi [1] stated that *An. culicifacies* is not a vector of malaria in hyperendemic foot-hill areas of Myanmar. *An. culicifacies* was the earliest to be identified amongst the Myzomyia series of Anopheles (Cellia) in Thailand [2]. *An. culicifacies* is regarded as the malaria vector in Sri Lanka [3, 4] and cytotaxonomical identification of *An. culicifacies* from Southern Sri Lanka identified it as species B [5]. In South East Asia, there are 4 sibling species of *An. culicifacies* comprising species A and B [5], species C [6], and species D [7, 8]. Species A, C and D are main vectors of malaria and B was a poor vector of malaria in India and Pakistan [9, 10]. Vector potential of these species varies depending on the region. Sibling species of *An. dirus*

were identified by salivary gland polytene chromosome method at the Medical Entomology Research Division (MERD), Department of Medical Research (Lower Myanmar) (DMR-LM). Morphological identification techniques are unable to distinguish among sibling species. Each sibling species may have its own distri-bution, biology, and may vary in response to insecticides and capacity to transmit malaria. It is important to develop a reliable cytogenetic method for identification of *An. culicifacies* species complex which can be performed in field areas. Therefore it was attempted to identify the *An. culicifacies* sibling species complex by polytene chromosome method in the wild caught mosquito population at malaria endemic areas in Myanmar.

MATERIALS AND METHODS

Selection of study areas

The selection was based on the importance and representativeness of malaria endemic areas.

Field study areas

Gon-min-soe Village, Paukkaung Township, Bago Division and Se-daw-gyi Village, Madaya Township, Mandalay Division were selected for the collection of blood fed wild adult *An. culicifacies* mosquitoes to identify its sibling species complex by determining the ovarian nurse cell chromosome. Gon-min-soe Village, with the population of about 1500, lies in a valley on the Pyae-Paukkaung-Oktwin road (Mahar-buhar road). Most of the villagers are farmers, government timber staff and some are bamboo cutters. Se-daw-gyi Village is situated beside the Se-daw-gyi Canal and it is 3 miles away from Se-daw-gyi Dam, Madaya Township, Mandalay Division. About 2000 population are residing in the village and one primary school and one Rural Health Center (RHC) are situated in the village. Majority of the population are government staffs and dam workers, minority are farmers and fishermen.

Laboratory study

Cytotaxonomical study of all collected *An. culicifacies* samples from both Gon-min-soe and Se-daw-gyi villages was done in MERD, DMR (LM).

Study design

The combination of a field and laboratory-based study.

Mosquito sampling methods and samples

Adult mosquito collection

Adult mosquitoes were collected by several methods using cattle baited big net trap and hut collection, indoor and outdoor resting night collection, outdoor resting morning collection and morning indoor resting collection in the field.

Larval collection

Anopheles mosquitoes larvae were collected from sand pools in stream-bed, water pools in rocky place, canal bed, water pools in creeks which were within 3 kilometer radius around the study villages. Larvae were kept in standard chamber for emergence to adult.

Ovaries specimen collection

Ovaries of all semi-gravid *An. culicifacies* were dissected and separated out and kept in Cornoy's fixative (methanol and acetic acid mixture) in screw-capped bottles in field station. At the laboratory, the Cornoy's fixative fixed specimens were stored in refrigerator at 4ºC for ovarian nurse cell identification within six months. Field collected other Anopheles mosquitoes were not dissected for ovarian nurse cell chromosome study but they were taken alive to the laboratory of MERD, DMR (LM) for colonization and taxonomical research works.

Cytogenetic method

Procedures

1. Wild caught adult mosquitoes and adult mosquitoes emerged from pupae collected during larval surveys were identified by the keys of Barraud [11],

Peyton and Scanlon, [12], Delfinado [13]. Reid [14], Zavortink [15], Harrison and Scanlon [16], Payton and Harrison [17] and Harrison [18].

2. Blood fed *An. culicifacies* were kept in paper cups for ovarian development (Christopher stage III) for 12 hours, 24 hours and 36 hours, and then killed by chloroform vapours.
3. The ovaries were dissected and fixed in 1 part glacial acetic acid and 3 parts methanol or ethanol (Cornoy's fluid). After fixation, ovaries were kept for 36 hours at room temperature. (After this stage, specimens can be kept up to 6 months in 4ºC).
4. Ovaries were softened in a drop of 50% propionic acid for a minute on a glass slide.
5. Propionic acid was removed and a few drops of diluted 2% lacto-aceto-orcein stain were added and kept for 10 minutes.
6. Tapping on the cover slip with a blunt ended tapper to obtain evenly spread chromosome.
7. After 3 minutes, slides were examined with Leica imaging system using IM50 software.
8. Polytene chromosomes were identified by chromosomal maps of sibling species A, B, C and D [9] and data were analyzed by Microsoft Excel.

RESULTS

Detail description of Anopheline mosquitoes collection is shown in Table 1. The table shows that a total of 4 anopheles species from Gon-min-soe Village and 10 anopheles species from Se-daw-gyi Village were caught in survey periods. Maximum number of *An. culicifacies* adult and larvae were collected in Gon-min-soe Village, followed by *An. maculatus* and *An. stephensi*. In Se-daw-gyi Village, maximum number of *An. vagus* and *An. annularis* were collected, followed by *An. phillipinensis* and *An. culicifacies.* Twenty-four *An. minimus* adults were collected in cattle bait but *An. dirus* adults could not be collected from human and cattle baits and *An. dirus* larvae could not be collected in both areas. High numbers of *An. culicifacies* larvae were found in sand pool on the bed of Gon-min-soe Creek and Se-daw-gyi Canal.

Ovarian nurse cell development

After blood meal the fertilized eggs were developed in the ovary and at the same time different stages of ovarian nurse cell and chromosome were also developed. Figure 1 shows that 12 hours and 36 hours aged ovarian nurse cell and chromosome development were present in upper parts of the follicle in ovarian cell.

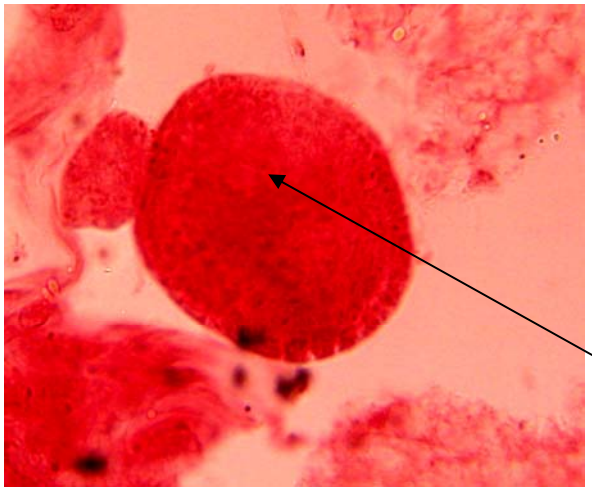
A total of 648 *An. culicifacies* from Bago and Mandalay Divisions were collected and examined for the ovarian nurse cell polytene chromosome analysis. Readable chromosomes were obtained from 216 specimens of 36 hours after blood meal groups. Up to 70-80% of specimens fixed in both ethanol and methanol fixatives showed clear chromosome bands (X and arm 2). Percent detection of chromosome bands were not significantly different between 12 hours, 24 hours and 36 hours aged ovaries and the outcome of methanol fixative and ethanol fixative was not statistically different. The chromosome detection rates are also not different between Bago and Mandalay Divisions. All 648 specimens were identified as *An. culicifacies* sibling species "B" when compared with the reference bands of X and arms 2 chromosome figures (Fig. 2 & 3). The chromosome of 36 hours aged ovaries was found to be highest and clearest chromosome bands in both areas (Fig. 4).

DISCUSSION

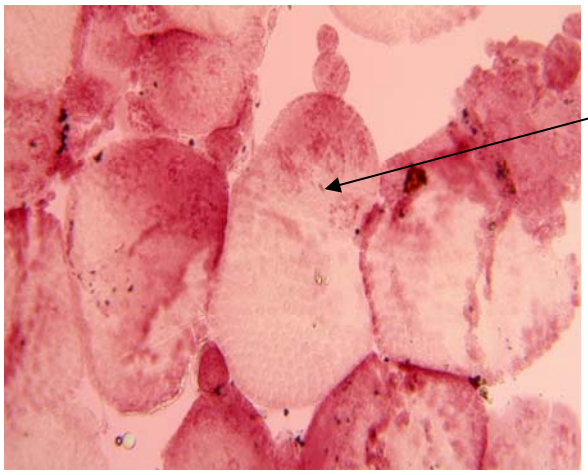
From the entomological aspect, *An. culicifacies* was found in high numbers during the study periods in both Gon-min-soe Village and Se-daw-gyi Village. *An. culicifacies* could not be assumed as the main vector of malaria since *An. minimus* may play a main vector of transmitting malaria in the region.

Table 1. Anopheline mosquitoes collection from two malaria endemic areas during survey

Anopheles species	Gonminsoe Village, Paukkaung Township, Bago Division				Saedawgyi Village, Madaya Township, Mandalay Division			
	Cattle bate	Morning collection	Night collection	Total number of collection	Cattle bate	Morning collection	Night collection	Total number of collection
An. minimus	-	-	-	-	24	-	-	24
An. maculatus	-	-	2	2	52	-	6	58
An annularis	-	-	-	-	188	-	-	188
An. phillipinensis	-	-	-	-	120	-	-	120
An. culicifacies	21	136	456	613	30	5	-	35
An. vagus	3	4	14	21	151	23	15	189
An. aconitus	-	-	-	-	22	-	-	22
An. tesslatus	-	-	-	-	12	-	-	12
An. stephensi	8	2	28	38	8	-	-	8
An. jamasii	-	-	-	-	10	-	-	10
Total no. of Anopheline mosquitoes collection	32	142	500	674	617	28	21	666



12 hours aged ovarian nurse cell and chromosome development in follicle



36 hours aged ovarian nurse cell and chromosome development in follicle

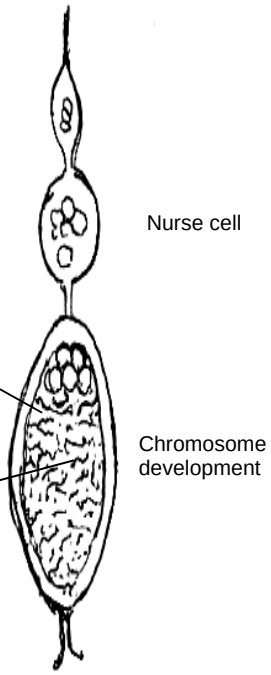


Fig. 1. Developmental stages of ovarian nurse cell and follicle in ovary

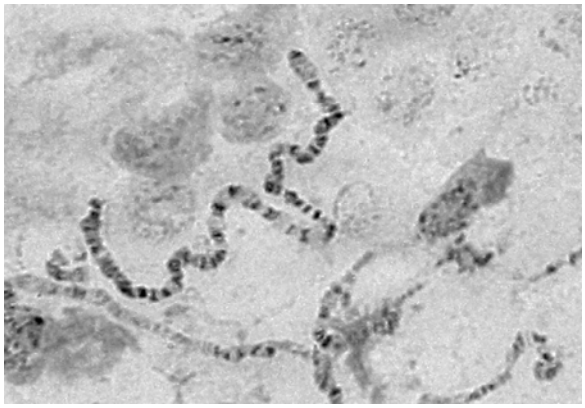


Fig. 2. Arm 2 chromosome after preparation of polytene chromosomes method of *An. culicifacies* sibling species B

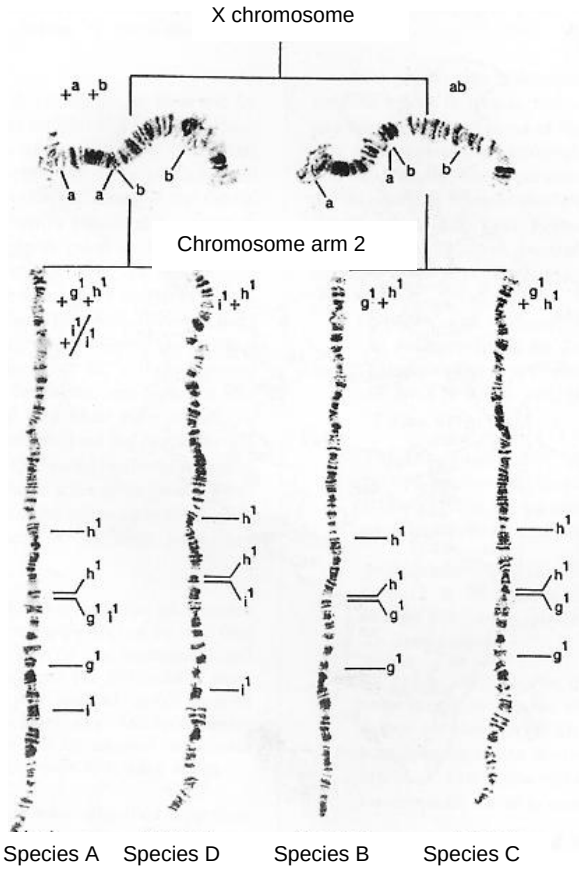


Fig. 3. Schematic representation of polytene chromosomes of *An. culicifacies* sibling species (Subbarao *et al*., 1988)

Anopheles dirus played secondary role due to its absence during the study period. Dissection reports in the past on *An. culicifacies* indicated that it was not a major vector of malaria in Union of Myanmar [1, 2] but laboratory feeding experiment

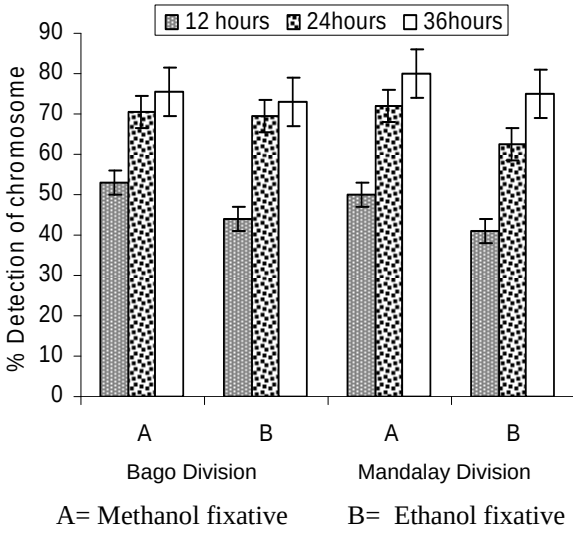


Fig. 4. Comparison of detectable chromosome with age specific ovaries fixed in methanol and ethanol fixatives

using *An. culicifacies* sibling species A and B mosquitoes in India and Pakistan showed that both species supported sporogony of *P. falciparum* and *P. vivax* [10, 19]. *An. culicifacies* type "B" was reported to be a main vector in Sri Lanka [20] but poor vector in India [9]. *An. culicifacies* is the major vector of malaria in rural areas of northern India [19] and also indicated that in northern India where *An. culicifacies* sibling species A and B are prevalent, salivary gland dissections of mosquitoes collected in a few villages around Delhi indicated that species A, with a sporozoite rate of 1.65%, is definitely a vector of malaria. However, the role of species B could not be established. *An. culicifacies* collection from Tha-bye-wa Village, Oktwin Township, Bago Division revealed that they are propable secondary vector but vector incrimination study confirmed *An. dirus* and *An. minimus* to be primary vectors of malaria, and positive for *P. falciparum* and *P. vivax* sporozoite antigen in their salivary gland by ELISA method [21, 22, 23].

Diluted 2% lacto-aceto-orcein stain is a very good stain for polytene chromosome staining and chromosome bands are clearly observed by microscopy. In cytotaxonomical study, 12 hours aged ovarian nurse

cell and chromosome were found weak and faint bands when stained with diluted 2% lacto-aceto-orcein stain but 24 and 36 hours aged had strong resolution and dark bands were observed but readable. Sharp and clear chromosomes were obtained from the specimens of more than 36 hours after blood meal group and it was good enough for ovarian nurse cell polytene chromosome study with clear identification of sibling species complex (Fig. 2). Other researchers revealed that ovaries up to Christopher stage III (after blood meals) were good for polytene chromosome study for *An. culicifacies* complex [5, 24]. Some 70-80% of specimens showed clear chromosome bands (X and arm 2) with fixed in both ethanol and methanol fixatives (Fig. 4). Both ethanol and methanol fixatives were suitable but methanol fixative have slight advantage over ethanol fixative for ovarian nurse cell polytene chromosome cytogenetic study.

Present study showed that type "B" species are abundant in Bago and Mandalay Divisions. The banding pattern was that of Xab which includes sibling species B and C. On the basis of the banding pattern on chromosome arm 2, all dissected samples of *An. culicifacies* were identified as sibling species B. Therefore *An. culicifacies* B is abundantly present in Paukkaung Township, Bago Division and Madaya Township, Mandalay Division. Ovarian nurse cell cytogenetic method was found to be accurate, simple and can be applied in the field situation. Although *An. culicifacies* is not a primary vector in Myanmar, they are found abundantly in malaria endemic area of foothill areas together with the primary vectors of *An. dirus* and *An. minimus.* Better understanding of species complex distribution and their role in malaria transmission will be beneficial for malaria prevention and control.

Conclusion

Ovaries of *An. culicifacies* developed after 36 hours blood meal provided best chromosome band in both ethanol and methanol fixatives. Present cytogenetic method for identification of *An. culicifacies* complex was able to identify species complex "B". This information will be valuable in prevention and control of malaria. In malaria endemic areas, where *An. culicifacies* is present with other main vectors of malaria, polytene chromosome cytogenetic method could be used to identify sibling species complex. It is applicable for field based identification of anopheles vector sibling species. Application of polytene chromosome method should be encouraged and used as a tool for malaria prevention and control. Further studies are required to find out the presence of other type of species complexes in malaria endemic areas. Vectorial capacity of *An. culicifacies* type "B" should be studied in detail.

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