

MOSQUITO CYTOGENETIC, ELECTROPHORETIC AND
CROSS MATING STUDIES

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OBJECTIVES : To use the latest cytogenetic, cross mating and electrophoretic techniques to : (a) delineate the vector species and vector strains of mosquito species in Thailand and Southeast Asia as a check against current morphological species concepts; (b) identify rapid and accurate techniques and discriminating characters for differentiating sibling species in vector species complexes; and (c) accurately determine genetic variation in natural populations of vector species and correlate this variation with the susceptibility of the vector(s) to infection with human pathogens.

BACKGROUND : These studies are a continuation of projects initiated in 1978 and outlined in the 1977-78 annual report (1). The recognition of sibling or cryptic species in important vector species groups of mosquitoes is steadily increasing with each year, and is essential for developing effective control programs and understanding the epidemiology of vector-borne diseases (2). Cytogenetic and electrophoretic techniques have been shown to be effective in differentiating such cryptic species and in determining the genetic variability in natural populations of mosquitoes (3). These techniques, when coupled with morphological studies and hybridization experiments, provide the best basis for species and vector strain differentiation.

METHODS : Cytogenetic techniques employed include a modification of the standard chromosome squash technique (4) for salivary polytene chromosomes, the Coluzzi technique (5) for polytene ovarian nurse cell chromosomes, a modified technique (6) for larval brain metaphase preparations, and the standard technique (7) for pupal testes meiotic preparations. The electrophoretic techniques and the enzyme terminology and abbreviations are those of Steiner and Joslyn (8).

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Chromosome maps and electrophoretic starch-gel esterase patterns are being made for selected laboratory colony strains or species. These maps or patterns will serve as "standards" for later comparisons with other strains and/or species. Once "standard" maps or esterase patterns have been established, wild collected populations will be sampled to survey the variations occurring in natural populations. Wild and/or colony strains of currently recognized morphological species that exhibit sufficient cytogenetic and/or electrophoretic differences will be studied further by hybridization experiments to determine if they are conspecific.

RESULTS : Colonization efforts continued to receive major emphasis this year. Two Thailand strains of *Anopheles nivipes* were colonized after considerable effort. Both strains are reluctant to feed on small mammals, thus they are currently maintained on a human blood source. In addition, both strains would not oviposit on moist filter paper or in open bowls of water. Thus, oviposition is obtained by confining individual females over water in vials. A manuscript (9) describing the materials and methods used in colonizing *An. nivipes* and *An. philippinensis* is near completion. Colonization of *philippinensis* was fairly simple, as this species quickly adapted to feeding on hamsters and to ovipositing either on moist filter paper or in open bowls of water.

Three colonies of members of the Leucosphyrus Complex also were initiated. Two colonies of the Fraser's Hill form were firmly established, with one derived from a number of females, while the second is an isoline from a single female. The third colony was started from eggs kindly provided by Dr. H.R. Bhat, Deputy Director, National Institute of Virology, Pune, India. Dr. Bhat obtained these eggs from females of a *dirus*-like member of the complex collected in Karnataka State, India. The 3 colonies of the Leucosphyrus Complex, unlike the *nivipes* colonies, were easily initiated and have adapted well to feeding on hamsters and ovipositing on damp filter paper. All 5 colonies discussed above are maintained by artificial copulation.

With the addition of the above 5 colonies, the Department of Medical Entomology now houses and maintains colonies of 13 species/strains of anophelines: *An. dirus* (Bangkok colony strain), Fraser's Hill form (Thailand strain - from several females), Fraser's Hill form (Thailand strain - isoline from single female), *An. sp. near dirus* (Indian, Karnataka strain), *An. balabacensis* Perlis form (Malaysia, IMR strain), *An. takasagoensis* (Taiwan, Peiyuan-Tunggho strain), *An. maculatus* (Malaysia, IMR strain), *An. maculatus* (Thailand, Nakhon Nayok strain), *An. maculatus* (Thailand, Cholburi (Huai Kuum) strain), *An. minimus* (Thailand, Saraburi (Tap Quang) strain), *An. nivipes* (Thailand, Phrae strain), and *An. philippinensis* (Thailand, Rayong (Klaeng) strain). These colonies are currently being used in taxonomic, cytogenetic, cross mating and malaria susceptibility studies. Only one colony, *An. balabacensis* Perlis form, is self mating. The rest are maintained by the artificial copulation technique.

Larval salivary polytene chromosome preparations of the Fraser's Hill form revealed that zone 6 on the X chromosome is considerably shorter than that seen on the X chromosome of *An. dirus* (10). Work is continuing on a comparison of the banding patterns on zone 6 for *An. dirus*, Fraser's Hill form, *An. balabacensis* Perlis form and *An. takasagoensis*.

At the beginning of this FY collaborative efforts were initiated with Mr. Chris A. Green, South African Institute for Medical Research, on a survey of the ovarian nurse cell polytene chromosomes of a number of known or potential vectors of malaria in Southeast Asia. Mr. Green spent 1 1/2 months in Thailand training personnel in the Department of Medical Entomology, AFRIMS, and Department of Biology, Faculty of Science, Mahidol University, in the techniques for harvesting and making high quality ovarian chromosome preparations. In addition, he also made a large number of chromosome preparations for several species in Thailand for comparison with other preparations he previously made in the Philippines and Taiwan. Since his departure from Thailand over 400 additional preparations have been made and sent to Green for analysis. Based on the ovarian chromosome preparations analyzed to date, *Anopheles annularis*, *minimus*, *jamesii*, *splendidus* and *philippinensis* all exhibit specific differences, without evidence of any cytogenetic sibling species. On the other hand, *An. maculatus* and *An. nivipes* preparations from Thailand revealed several distinct chromosomal types having complicated fixed inversions for each species, without evidence of heterozygous individuals. Since these distinct arrangements are sympatric, this is considered presumptive evidence for sibling speciation. Recently (11), *An. culicifacies*, the primary vector of malaria in large parts of the Middle East and India, was discovered to consist of 2 cytogenetic species. Cross mating experiments have confirmed these 2 species, however, no morphological differences have been found to separate them. Like the *culicifacies* problem, additional evidence, e.g., crossing experiments, is needed to verify the *An. maculatus* and *nivipes* sibling species. As noted below, metaphase karyotype data also suggest different species in *maculatus* and *nivipes*, however, intra-specific variations are clouding the karyotype analysis. The 3 colonies of *An. maculatus* currently maintained at AFRIMS all belong to one chromosomal species of *maculatus* and are completely compatible in crossing studies. As soon as known colonies of the *maculatus* sibling species have been started morphological studies will be initiated to identify potential characters to use for field identification. These studies on *maculatus* and *nivipes* are continuing as rapidly as possible. The malaria vector status of both species in the different regions of Thailand urgently needs resolution.

Our karyotype studies were enhanced considerably by the use of a technique which produces slides that do not require refrigeration and are preserved with a permanent mounting media. This technique was taught by Dr. Baimai, and involves the use of Giemsa stain and Colcemid. Karyotype slides prepared by this method are superior to those used previously, and will allow a much more rapid analysis of heterochromatin and karyotype polymorphism.

Recently, intraspecific polymorphism of sex chromosome heterochromatin has been described in 2 species in the Gambiae Complex of *Anopheles* (12). These workers suggest the possible role of sex chromosome heterochromatin in controlling fertility and mating behavior of *Anopheles* mosquitoes. We have also found considerable sex chromosome polymorphism in several of the species under study. In the Leucosphyrus Complex at least 3 types of Y and 2 types of X chromosomes have been observed in the Fraser's Hill form. No variations have been observed in karyotypes of the *An. dirus*, *An. takasagoensis* and *An. balabacensis* Perlis form colonies, however, all 3 of these are old colonies that have survived considerable selection pressure and are probably

homozygous for most characters. On the other hand, the Fraser's Hill form colony is only in the 9th generation and the karyotype polymorphs were detected in the 2nd through the 5th generations. One polymorph of the Y chromosome already was becoming very infrequent by the 5th colony generation.

The metaphase mitotic and meiotic chromosomes of the Fraser's Hill form are nearly telocentric like those of *An. dirus* and *An. takasagoensis*, and short like those of *takasagoensis*. The karyotype chromosomes of *An. balabacensis* Perlis form, however, are acrocentric with a very distinct short arm beyond the centromere, and approximately the same length as those of *An. dirus* (13). To date, the karyotype chromosomes of *An. balabacensis* Perlis form are very distinct and the only ones in the Leucosphyrus Complex that are distinctly acrocentric.

In the Maculatus Complex at least 3 types of Y and 3 types of X chromosomes have been seen. Some of these differences in *maculatus* are obviously intra-specific as crossing experiments and ovarian chromosome preparations for the 3 colonies indicate a single species. However, one colony (Huai Kuum) having a very short Y chromosome, also was significantly less susceptible to *Plasmodium cynomolgi* infections than the other 2 colonies having much longer Y chromosomes (14). The X chromosomes for the Huai Kuum colony were polymorphic (2 types), but identical to those in the other 2 colonies. We currently consider this susceptibility difference in the Huai Kuum colony to be an intraspecific strain difference. On the other hand, *maculatus* from other areas of Thailand have very distinct fixed chromosomal arrangements on certain arms of the ovarian polytene chromosomes. At least 3 distinct cytogenetic types of *maculatus* have been identified in Thailand to date. Specimens presumed to be identical to these types all have a very distinct 3rd type of X chromosome in karyotype preparations, that was not seen in the 3 colonies of *maculatus* currently maintained at AFRIMS. These recently found cytogenetic types of *maculatus* have not been tested in crossing or susceptibility studies. Although polymorphism occurred in the Gambiae Complex species discussed above (12), the intraspecific ranges of variation still did not compromise the diagnostic karyotype differences for the 2 species. Obviously, the current karyotype studies on *maculatus* must proceed very cautiously to properly identify and separate the intraspecific polymorphs from the interspecific karyotype differences.

Karyotype differences have also been detected among the X and Y chromosomes of the 2 *An. nivipes* colonies and those of the *An. philippinensis* colony at AFRIMS. Since *nivipes* possibly exists as 2 distinct cytogenetic species in Thailand (unpublished data) we are not certain whether the karyotype differences we have observed in our 2 *nivipes* colonies represent species or strain differences. The few crosses between the 2 *nivipes* colonies performed to date, have been inconclusive and additional crosses are needed. The karyotype of *An. philippinensis* is polymorphic (2 types) for the X chromosome, however, only one type of Y chromosome has been identified. The Y of *philippinensis* is very short and quite distinct from the Y chromosomes from the 2 *nivipes* colonies.

Reciprocal cross mating studies between the IMR and Nakhon Nayok strains, and the Nakhon Nayok and Huai Kuum strains of *An. maculatus* have revealed complete compatibility, with no evidence of non-viability or sterility even in

F₂ crosses. These data agree well with ovarian polytene chromosome studies which show no asynapsis for F₁ hybrids from these crosses, and no discernable differences in chromosome banding patterns. Accordingly, we are concluding that the Nakhon Nayok and Huai Kuum strains from Thailand are conspecific with the IMR strain from Kuala Lumpur, Malaysia. The IMR strain is recognized as a very good vector strain for human malaria parasites.

Crossing studies were also conducted with the *An. nivipes* (Khon-buri strain) and *An. philippinensis*. These reciprocal crosses resulted in reduced viability and gross morphological evidence of sterility in F₁ males and backcross males. Small non-functional testes, fragile vasa efferentia and non-active big headed sperm were found in these males. These data clearly confirm *An. nivipes* as a distinct species from *An. philippinensis*.

Crossing studies are underway between *An. dirus* and the Fraser's Hill form, and strong healthy F₁ hybrids have been produced in both directions. However, some male F₁ hybrids have abnormal testes without sperm, or sperm that have big heads and are non-motile. Backcrosses are currently in progress and to date, parental females mated to these F₁ hybrid males have either failed to oviposit, or eggs that were oviposited have failed to hatch.

These studies are continuing, and electrophoretic studies are planned for the coming year.

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