WORKING MATERIAL

THE POTENTIAL FOR GENETIC CONTROL OF MALARIA-TRANSMITTING MOSQUITOES

REPORT OF A CONSULTANTS GROUP MEETING
ORGANIZED BY THE
JOINT FAO/IAEA DIVISION OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE
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INTRODUCTION

Since the beginning of the Joint FAO/IAEA Division Programme on the research and development of insect pest control methodology, emphasis has been placed on the basic and applied aspects of implementing the Sterile Insect Technique (SIT). Special emphasis has always been directed at the assembly of technological progress into workable systems that can be implemented in developing countries. The general intention is to solve problems associated with insect pests that have an adverse impact on public health and the production of food and fibre.

For certain insects, SIT has proven to be a powerful method for control, but for a variety of reasons this technology has not been tried on an operational scale for most of the pest species of insects that exact a toll on the endeavors of humans. The Joint FAO/IAEA Division convened a Consultants Group Meeting to examine "The Potential for Genetic Control of Malaria-Transmitting Mosquitoes", with emphasis to be placed on the SIT. A group of five scientists met, 26-30 April 1993, to examine the current status and the future potential of genetic control for malaria mosquitoes. In most of the tropical, developing countries, and to some extent in temperate regions of the world, *Anopheles* mosquitoes cause havoc by transmitting malaria, a dreaded disease that causes high mortality amongst children and diminishes productivity of adults. The importance of malaria as a deterrent to further economic growth in a large part of the world cannot be over-emphasized. Malaria is a severe problem because there are inadequacies in the technology available for control.

As a result of the deliberations at the meeting, the consultants prepared a list of recommendations concerning the consensus opinions about the development of genetic control for malaria vector control. The following report presents the findings and recommendations of the Consultants Group Meeting.

In order to provide continuity and avoid duplication of effort, information on meetings and copies of reports, sponsored by the Joint FAO/IAEA Division, that have direct bearing on genetic control, were provided to the group. Just over 10 years ago, a Consultants Meeting on "Investigations in East Africa on the Feasibility of the Sterile Insect Technique (SIT) to Control the Anopheline Mosquito" took place during September 1982. In 1984 the Joint FAO/IAEA Division sponsored a Consultants Group on "Research and Development of the Sterile Insect Technique (SIT) for Use Against the Malaria Vector, *Anopheles arabiensis* in Mauritius". Neither of these proposed projects were pursued beyond the planning phase done by the consultants. There have been some major developments in insect genetics and genetic control since those two meetings were held. More recently, the Joint FAO/IAEA Division convened an Advisory Group Meeting on "Genetic Methods of Insect Control" during November 1987. This group of advisors examined the status of genetic control technology and made recommendations that included a strong component of modern genetic engineering for the future development of genetic control. On pages 15 and 16 of their report, the Advisory Group examined the prospects for genetic control of mosquitoes and recommended an effort for *An. arabiensis*. Generally, the recommendations by that Group included most of the current thinking on applying molecular techniques. Although good progress has been made, scientists still cannot offer a genetic control system based on genetic engineering.

This situation should be taken as a challenge to increase our efforts toward that end. The reader is referred to those reports for background material, which was inclusive of most genetic control approaches.

At the beginning of the present Consultants Group Meeting, the group was asked to:

(a) evaluate whether the use of genetic control within the context of an integrated mosquito management programme is appropriate as a strategy for the control/eradication of malaria mosquitoes;

(b) determine which genetic control option should be the method of choice;

(c) evaluate candidate species for genetic control and determine possible locations for pilot studies; and

(d) generate ideas for research that would be relevant in solving problems that are directly pertinent to genetic control.

The members of the group reviewed progress in their specific areas of expertise and discussed strategies for meaningful pilot studies. In the remainder of this report, several topics are summarized and recommendations made for what the group decided would be an appropriate role for the Joint FAO/IAEA Division.

1. THE MALARIA SITUATION

Malaria is one of the most important tropical diseases. It has been estimated that more than 110 million clinical cases occur per year with more than 270 million people infected. Other figures range from 300-500 million cases per year world-wide. Mortality ranges from 1-2 million cases per year. More than 2 billion people are at risk, including not only local populations, but travellers, tourists and immigrants. Imported cases into Europe and North America have the potential to start local outbreaks in areas where the vectors exist, but the parasite is not present. This has occurred several times in numerous countries, and the frequency and magnitude of such outbreaks are only likely to increase in the future because of the movement of people out of areas where malaria exists.

One approach to the malaria problem is to increase local awareness and introduce community-based methods of control into primary health care systems. Conceptual and practical strategies have been developed. At the same time, there is great effort to look at new technologies which can be used to control the parasite and vector. Malaria control programmes vary from region to region and from country to country. Most programmes rely on epidemiological data, drug therapy and vector control. At present there is a general lack of sound systems for rapidly collecting epidemiological data that can be analyzed at regional and local levels for decision-making. Malaria cases are usually detected passively, although in a few countries active case detection is attempted. Active case detection is usually not done on a comprehensive basis because of the lack of human resources. Treatment regimes and malarial drugs used depend on the parasite species, resistance to drugs and availability of drugs. Much concern, at present, is related to drug

resistance and the lack of new drugs on the shelf which can be used as resistance has increased throughout the world.

Almost all preventive measures against malaria vectors involve the use of adulticides, either sprayed inside houses or by space spraying. There are exceptions where small-scale programmes use environmental management, bed nets, or biological control. Few of these programmes actually practise Integrated Pest Management (IPM) in the sense that multiple strategies are used at an appropriate time and space scale. A good example of an IPM approach is that used in rice fields in China. A biocide, *Bacillus thuringiensis israeliensis* (BTI) and fish are used together. The IPM concept is that when rice fields are initially flooded, fish are introduced. These fish need time to establish and increase in population size in order to be effective mosquito predators. Therefore, BTI is used during the initial flooding period to control anophelines. The plant azolla is also helpful in certain rice growing areas by preventing anopheline breeding. IPM is a key concept which needs to be adhered to in future malaria control programmes. Vector management will need to go beyond adulticiding and look more closely at larval ecology and natural regulating systems. Adult biology, such as host seeking, mating behaviour and male ecology needs to be addressed for the future use of genetic control strategies.

In summary, there is a world-wide increase in drug resistance of the parasite, insecticide resistance in malaria vectors, and local health programmes are stretched to their limits; not only because of the increase in malaria, but also due to the enormous increase in other diseases in tropical countries. The situation emphasizes the urgency of research, development and implementation of new technologies for malaria control at regional and local levels.

2. GENETIC CONTROL METHODS

There are several major approaches to the genetic control of anopheline mosquitoes. These include:

(a) the SIT;

(b) chromosomal aberrations;

(c) hybrid sterility;

(d) cytoplasmic incompatibility; and

(e) manipulation of genes (e.g. refractoriness) for genome alteration through dilution, replacement or displacement.

Other somewhat marginal genetic schemes can be envisioned, but the candidate approaches listed above have been studied in the past. There are certain advantages inherent to the use of SIT and the other currently theoretical genetic control systems. These include:

(a) the possibility of eradication;

(b) the effectiveness for quarantine;

(c) less expensive in the long run than IPM programmes for some pests;

(d) the lack of pollution of the environment; and

(e) species-specificity and compatibility with other biological control methods.

Therefore, the value and need for development of SIT and other genetic control systems are unquestioned.

2.1 Sterile Insect Technique

Implementation of an operational SIT programme is a complex affair, in which huge numbers (hundreds of millions) of a pest species are produced in a factory, sterilized by exposure to ionizing radiation (or with chemosterilants), and released into the native habitat. The initial expense of constructing facilities combined with the expense of conducting the campaign have been a deterrent to more widespread use of this method of control/eradication. The costs of control must be looked at over the long run, not just the present, and if this is done, favourable cost-benefit ratios are usually projected.

The rationale for SIT is simple. The target species is first subjected to adulticides (to eliminate most of the fertile, inseminated females) and larvicides (to eliminate most of the population), and then this important part of the process is followed by inundative releases of competitive, sterile males. The intent is to induce enough sterility in the native population to overcome the natural biotic potential of the target species.

A successful sterile male release experiment resulted in the elimination of a population of *Anopheles albimanus* at Lake Apastepeque (a small isolated site east of San Salvador, El Salvador) in the early 1970s. In a subsequent, larger experiment on the Pacific coastal plain of El Salvador, a moderate amount of success was achieved through the release of sterile males in a 20 km^2 area in the late 1970s. High levels of sterility were achieved in the release zone, but total population control was not possible because of immigration of fertile females from the surrounding areas. For the last year of that latter experiment, a genetic sexing strain was used to produce one million sterile males per day.

There are also precedents for using sterile insect releases to control *Culex quinquefasciatus* in experiments conducted on a small island, Seahorse Key, located off the Gulf coast of Florida. In these experiments, releases of either chemosterilized or radiosterilized males resulted in the virtual elimination of *Cx. quinquefasciatus* from the island. Experiments in India also indicated that significant sterility could be achieved by releasing sterile males of *Cx. quinquefasciatus*.

The experiments cited above prove the potential usefulness of SIT for mosquito control. However, there are needed refinements in at least three important aspects, viz. automated rearing, sterilization, and distribution of insects. For practical purposes, improvements in these areas will also be of importance in the use of future types of genetic control. Since the basic principle of SIT involves overflooding the native population with sterile or genetically-altered insects, the means must be available to mass-produce, sterilize, and distribute sterile males that are vigorous and competitive. This must be done economically, otherwise, the methodology is not likely to receive favourable consideration, even if it is highly effective. In the past, the approach to mass-production procedures used in all of the SIT evaluations have simply been an extension of the methods used for laboratory colonies. Instead of rearing one pan of larvae, virtually the same methods were used to produce one hundred pans. Research should be aimed at:

(a) minimizing the detrimental effects of colonization on the gene pool;

(b) standardized diets;

(c) automation; and

(d) quality control.

Sterilization of the males is a tricky step in the procedure, because male mosquitoes are fragile insects that are subject to debilitating damage with excessive handling. The use of radiation exacts a toll on competitiveness, but this can be minimized by irradiating young adults. An effort should be made to minimize the effects of sterilization and handling. Pupae are easily sterilized by treatment with chemosterilants, but the tiny quantities of residues in the young adults might have a detrimental effect on predators. Distribution of the sterile males at the point where they can mate with native females is an area of research that has hardly been touched. It will certainly depend on the particular target species, but for most SIT programmes the distribution of sterile insects by means of aircraft is the most feasible alternative. Past efforts have been done by a combination of vehicle and manual, and while the release from a vehicle might work for urban situations, the use of aircraft should be studied for most species.

In 1989 and 1992, the Joint FAO/IAEA Division convened Research Co-ordination Meetings on "Genetic Engineering Technology for the Improvement of the Sterile Insect Technique". Groups of scientists met on these two occasions to examine the current status and future potential for improvement of SIT. For the most part, their recommendations are still appropriate at this time. In the report from 1989, the consultants concentrated most of their recommendations on:

(a) genetic sexing, which can provide enormous savings of 40-50% of the cost of rearing;

(b) genetic sterilization, which would eliminate the need for using ionizing radiation;

(c) methods for the development of biochemical assays and probes for use in monitoring factory strains, with the aim of avoiding genetic deterioration during mass-production;

(d) monitoring the mating behaviour of released males through the use of biochemical probes; and

(e) biochemical assays for pesticide resistance in target populations.

In the 1992 report, the recommendations of the participants were aimed more at specific goals within genetic engineering technology. The Advisory Group strongly urged the Joint FAO/IAEA Division to:

(a) increase its support for research into the development of transformation systems;

(b) encourage the development of systems for the application of genetically transformed insects;

(c) support the expansion of molecular technology into genome mapping; and

(d) encourage research in the area of symbiont-insect interaction.

For the most part, the recommendations of both Advisory Groups are still appropriate at this time.

2.2 *Genetic Sexing and Chromosomal Aberrations*

Most control strategies developed for anophelines based on the release of large numbers of mosquitoes will be restricted to the release of males only. This necessitates the development of genetic sexing strains either based on conventional male-linked translocations or genetically transformed mosquitoes carrying some form of inducible sex-specific gene-construct. Genetic sexing lines using conventional technology can be routinely produced in anopheline mosquitoes and for many of the major vectors such lines have already been constructed. Unfortunately, many of these lines have been lost, but the selectable mutations necessary for their construction can still be used. Recent developments in the molecular analysis of sex determination in many insect species should provide alternative strategies to produce all-male progeny in anophelines. Anophelines cannot be sexed by mechanical methods; therefore, the requirement of genetic sexing for released mosquitoes should be an integral part in the development of small-scale, mass-rearing facilities.

There is an extensive body of literature on the theoretical use of chromosomal aberrations (viz. translocations, inversions and compound chromosomes) for genetic control. As a result of the promising theoretical models, chromosomal translocations and other aberrations have been extensively studied in many

anopheline mosquitoes, e.g. *An. gambiae, An. albimanus, An. stephensi* and *An. culicifacies*. These aberrations have been studied from two different points of view:

(a) As tools for the genetic analysis of the species being investigated.

(b) Their use for release in genetic control strategies.

With regard to the first approach, work in this area is essential in developing strains for the identification and maintenance of valuable mutations, e.g. vector incompetence. The development of these chromosomal aberration stocks should be a part of genetic control strategies being adopted. These studies also lead to a deeper understanding of the genome organization of anophelines. Except for a few competitive mating trials (limited in both size and scope) in field tests and population replacement trials in laboratory tests, the use of aberrations for direct genetic control has not been tried for any species of mosquito. With some exceptions, the experiments with anopheline and culicine mosquitoes were generally positive in terms of mating competitiveness. For *An. albimanus*, homozygous translocation and inversion stocks were synthesized, but no field tests were attempted because of a lack of strains refractory to malaria and also a lack of financial support. Maintenance of strains in particular combinations was difficult because of genetic "leakage", caused by recombination, under the conditions of mass-production. It is not evident how the development of such aberrations could be improved, and further research in this area for genetic control is not encouraged.

2.3 *Hybrid Sterility*

Naturally occurring hybrid sterility, presumably caused by genetic differences between members of the various *Anopheles* species complexes, has been well documented and new instances of cryptic speciation are still being discovered. Crosses between members of a species complex usually produce sterile hybrid males, most of which are morphologically normal with partially developed reproductive organs. Development of the reproductive organs varies from reduced size to total atrophy. These sterile hybrid males are comparable to radiosterilized or chemosterilized males and therefore potentially can be used in suppression programmes. There has been a single, unsuccessful attempt to use sterile hybrid males from a particular cross to control a field population of *An. gambiae*. This experiment, in which two species of the complex were crossed to produce sterile males to attack a third species, should not be used as conclusive proof that the general approach of harnessing hybrid sterility is of no avail. The design was suspect and improvement could be made. If hybrid sterility is tried, there is a need for genetic sexing for mass-production; in this case one stock would be needed to produce females. In species where procedures have been standardized to colonize sibling species, F_1 hybrid males should be produced to study their mating behaviour. As in many of the areas, where two or more members of a species complex are sympatric, studies should be undertaken to examine the choice of the hybrid males in choosing their female partners.

2.4 *Cytoplasmic Incompatibility*

Cytoplasmic incompatibility occurs naturally, and for the most part, work on this phenomenon in mosquitoes has been done with the *Culex pipiens* group and *Aedes scutellaris* group. The *Cx. pipiens* group has a world-wide distribution and consists basically of 6 species. The *Ae. scutellaris* group is found in S.E. Asia and the Pacific islands and has 36 species; the island distribution probably contributes to the differentiation of so many species and incompatible types. This same type of incompatibility also occurs in other insects, e.g. in *Drosophila simulans* rickettsia-like organism (RLO) endosymbionts were found and incompatibility between conspecific populations was observed. When populations from different geographical regions in the *Cx. pipiens* complex are crossed, the result is either compatibility in one of the reciprocal crosses (unidirectional compatibility), incompatibility in both reciprocals (bidirectional incompatibility), or compatibility in both reciprocals (bidirectional compatibility). Similar compatibility/incompatibility relationships were observed between members of the *Ae. scutellaris* group, but the incompatibility is observed only in interspecific crosses. In *Cx. pipiens*, incompatibility is observed both in inter- and intra-specific crosses. Rickettsia-like organisms, *Wolbachia pipientis*, observed in the gonads of these mosquitoes are considered responsible for the incompatibility relationship observed between the strains. Because of strict maternal inheritance without any involvement of nuclear genes, incompatibility was considered as a genetic control tool. In field experiments (in India during the early 1970s) where incompatible males were released, a reasonable degree of sterility was observed. This phenomenon was also used to replace populations in laboratory cages with the intention of seeing the value of this phenomenon in replacing the target population with a population carrying desirable genes. However, the presence of natural polymorphisms for compatibility (incompatibility) types complicated the usefulness of this system for genetic control, and further development was suspended. With the availability of molecular techniques, it is now genetically possible to transform endosymbionts with desirable genes and introduce them into a target population. This is also considered as an attractive alternative for the transformation of mosquitoes directly. Such an exercise, i.e. transforming endosymbionts, has been done recently with a symbiotic bacterium (*Rhodocorcus rhodnii*) of tsetse flies, which are vectors of African trypanosomiasis.

Until now, cytoplasmic incompatibility in anophelines in intraspecific crosses has not been reported. However, recent work with interspecific crosses between some members of the *An. culicifacies* complex has provided the first evidence, in that in certain crosses, there is either total sterility or 1-2% hatch, and the resulting hybrid males are sterile, while the reciprocal crosses are fertile and sterile hybrid males and fertile hybrid females are produced. The interspecific crosses which show sterility are of interest as these appear to be similar to those observed in *Cx. pipiens* and *Ae. scutellaris* groups. Tetracycline treatment of male larvae in these crosses resulted in 80-90% egg hatch. These results suggest for the first time the possibility of the presence of endosymbionts in anophelines. As noted recently by C.F. Curtis (in a short note in Nature), the incompatibility phenomenon itself could be used to replace the natural population with that of a desirable one and the endosymbionts responsible for the incompatibility could be genetically transformed and introduced into insects. Because of the recent

interest in transforming mosquitoes through genetically transformed endosymbionts, this preliminary observation needs to be pursued. This line of approach is still in the developmental stage.

2.5 *Genetic Engineering for Genome Modification*

Recent developments in the technology of molecular genetics have led to serious re-evaluation of the prospect for genetic-based strategies in the control of malaria vectors. While a number of genetic modifications are envisioned, the currently favoured approach is to reduce or eliminate the mosquito's capacity to support development of the malaria parasite. The key biological consideration that underlies this choice is the enormous reproductive capacity of mosquitoes which enables populations to rebound rapidly when any population-reduction based strategy is relaxed. This goal of population replacement distinguishes this form of genetic control from the population-reduction strategies such as SIT. Three key problems need to be solved before this strategy can be tested:

(a) The tools for mosquito genetic engineering need to be developed.

(b) A parasite-inhibiting gene and its relevant controlling sequences need to be identified.

(c) Techniques for efficiently driving that transgenic construct into the natural population must be developed.

Progress has been made in all three areas.

2.5.1 *Genetic Engineering Tools*

The most important tool is a system of stable and efficient germ-line transformation. This will require a method for introducing DNA into mosquito embryos, a vector or other directed process that will result in integration of genes into the nuclear genome of germ-line progenitor cells, and reporter genes that can be used to detect transformation events. Microinjection has been used effectively to introduce DNA into preblastoderm mosquito embryos, and in three published cases the DNA was integrated into germ-line cells in rare random recombination events. More emphasis is now being placed on the use of biolistics. Because of the enormous success with mobile elements in developing *Drosophila* transformation systems, considerable effort has been directed toward comparable transformation systems for mosquitoes, especially for the malaria vector, *An. gambiae*. The P-element vectors are clearly not useful as mosquito transformation vectors, but other elements, e.g. hobo, mariner and minos that have been identified from both *Drosophila* and other insects, may prove to be effective in mosquitoes. Investigations on those three elements are currently in progress.

Genetic transformation constructs will also require reporter genes as indicators of successful transformation. Several reporters have been developed. The most widely used thus far is the bacterial neomycin phosphotransferase gene (*neo*), which is routinely used to confer antibiotic resistance to transformed organisms

The major vector of malaria in Sri Lanka is *Anopheles culicifacies B.* It has been colonized in India and some genetic studies have been carried out on this species. Sri Lanka has an adequate malaria control programme and infrastructure. Some national scientific capabilities exist and there appears to be semi-isolated valleys where pilot studies could be conducted.

Other anopheline species, such as the neotropical malaria vector, *An. darlingi*, and the *An. dirus* complex from Asia, were considered to need further studies on colonization and genetics before being considered candidates.

More information needs to be gathered on the local conditions where the above candidate species exists; in particular, the presence of more than one vector, malaria epidemiology, ecological setting and national scientific capabilities.

4. CRYPTIC SPECIES OF ANOPHELES AND THEIR IMPACT ON GENETIC METHODS OF CONTROL

Of the more than 450 species of *Anopheles* mosquitoes that have been identified and named, perhaps a dozen are major vectors of malaria, and 3 to 4 dozen are vectors of lesser significance. Most of the vector species are members of closely related sibling species. During the past decade, population genetic analysis has identified complexes for all the major *Anopheles* subgenera. Because members of such species complexes are usually genetically isolated by premating barriers, this form of genetic structure will clearly have important implications for all types of genetic control strategies. It will be essential to understand the relative contribution to malaria transmission made by the particular species in such a complex. Additional information on identifying complexes and the species transmitting malaria in the field is essential. The preliminary stages of any genetic control programme should include a detailed analysis of the ecology, population genetics and transmission biology of the target mosquito. Where a species complex is involved, this will require techniques for rapidly identifying single individuals of the different member species. Currently, the most widely accepted and applied methods include polytene chromosomes, allozymes, and DNA restriction enzyme polymorphisms. The DNA techniques for cryptic species identification have been used for analysis of a wide diversity of insects and are quite appropriate for anopheline mosquitoes. Among the most promising of recently developed population genetic techniques in this area are random amplified polymorphic DNA (RAPDs) and microsatellite short tandem repeats (STR loci). Both techniques depend on the polymerase chain reaction (PCR) and thus require only very small amounts of genomic DNA to identify polymorphic genetic loci. These loci can be mapped genetically and cytogenetically (*in situ* hybridization). Microsatellite markers, the alleles of which are co-dominant, are preferable to RAPDs, which are in general dominant markers, but RAPDs have the advantage that they are relatively easier to accumulate. Such markers are currently available only for *An. gambiae* and its sibling species, but a set sufficient for population genetic studies could probably be developed for other vector species within a year.

5. ROLE OF THE JOINT FAO/IAEA DIVISION IN RESEARCH, DEVELOPMENT AND IMPLEMENTATION OF GENETIC CONTROL PROGRAMMES FOR MALARIA MOSQUITOES

In the preceding sections of this report the consultants summarized the considerable potential for genetic control in suppression of malaria vectors. As pointed out in Section 2.1, there are some notable weaknesses in our current knowledge on automated mass-rearing, dispersal of genetically altered mosquitoes, and quality control of factory-reared anopheline mosquitoes. The Joint FAO/IAEA Division has played a key role in tactical research, development, implementation, and training over the past 20 years, for the most part with agricultural and veterinary insect pests. The Seibersdorf Laboratory is a recognized centre of excellence with a superior record in bridging the gap between academic research and practical application in genetic control techniques. This role of technology transfer is a very important function. Therefore, the consultants agreed that the Joint FAO/IAEA Division is in a unique position to provide a research and development programme and training that would be complementary to the basic academic research being pursued by scientists at various universities, public health groups, and national and international institutes scattered around the world. Regardless of the final genetic approach, there will be a need for mass-rearing and/or genetic sexing technology. For SIT, there is a necessity for this technology. For the successful implementation of any genetic control technique for anopheline mosquitoes in the field, the active participation and co-operation of national and international organizations is essential. The consultants are convinced that the research and development programme proposed in the recommendations of this document is complementary and can be fully integrated with related work being carried out by other organizations. An attempt should be made to include participants from such organizations at all stages.

6. RECOMMENDATIONS

6.1 The Joint FAO/IAEA Division should organize a Co-ordinated Research Programme and host a meeting of interested scientists at some point during the next 12 months. For the purposes of population genetics and colonization of anophelines, it is essential to include scientists from developing countries that have serious malaria problems.

6.2 The Joint FAO/IAEA Division should initiate a 3-5 year research and development project at its Seibersdorf facility for the development of automated procedures for the mass-production, sterilization, and dispersal of anopheline mosquitoes. This effort should be provided with enough laboratory space for producing one-half million mosquitoes per week. This project will require a staff of at least two scientists. One scientist should be a geneticist so as to pursue the practical aspects of implementing genetic sexing, genetic sterilization, biochemical probes, and other aspects of genetic control schemes. The second scientist should concentrate on automated mass-rearing and quality control methods for anopheline mosquitoes.

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Annex 2

WORKING PAPERS

GENETIC METHODS FOR CONTROL OF MOSQUITOES

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This summary of past experiments and pilot projects on the genetic control of mosquitoes is not intended to be an exhaustive review. The bulk (shown in quotes) of this paper was presented at a symposium sponsored by the agency in 1987. "Emphasis has been placed on: (1) successful research efforts on use of the sterile insect technique (SIT) and the synthesis of useful heritable chromosomal aberrations, (2) the technical limitations in our present ability to use SIT, (3) the need for genetic evaluation of the structure of natural populations, and (4) a look at the future impact of recombinant DNA methods on genetic control. A generally optimistic point of view of the current and future situations from the author's perspective will be expressed, with emphasis on successful research efforts. Past failures will not be ignored, but neither will they be presented as irrefutable evidence of doom for genetic control.

PAST SIT PROGRAMS

The earliest research efforts in the form of extensive laboratory and field evaluation on genetic methods for the control of mosquitoes concentrated on the evaluation of the sterile insect technique (SIT). In applying SIT, the rationale for control lies in the use of inundative releases of sterile males which produce gametes bearing one of more dominant lethals caused by a treatment with ionizing radiation or mutagenic chemicals. In practice then, the degree of control is directly related to the genetic lethal load (=sterility) that is imposed on a population by matings between the sterilized and native insects. The lethal load does not persist in the population when releases of sterile insects are terminated. In general, the first experimental trials with mosquitoes employed an approach similar to the successful effort with the screwworm fly, Cochliomyia hominovorax. Radiation was used to sterilize laboratory-reared mosquitoes, which were released into the natural habitat. The expected results, i.e., an increased genetic lethal load that would cause either population decline or control, were not achieved, and the negative results of these experiments led to the general conclusion that the use of radiation was not suitable because somatic damage was excessive (see Patterson et al. [1] for references). There were also obvious problems, fully documented in one case, with the release

of laboratory-adapted mosquitoes, in that the males did not mate with the wild females [2].

Dame [3] presented a comprehensive discussion of the basic requirements that are necessary for making SIT a workable solution to mosquito control, a reality, rather than just a fanciful idea. As he pointed out, it is very important to identify and solve the practical problems that are inherent to SIT, the goal of which is to use an insect factory and automated production and distribution systems to rear, sterilize, and distribute a sufficient number of sterile males that are competitive in mating with wild females. Success is also contingent upon the availability and understanding of basic knowledge on the biology and ecology of a species. If one examines the earlier experiments with SIT for mosquito control, it is very easy to detect violations of several of the basic requirements outlined by Dame [3]. Of course, this was to be expected, for in retrospect, there were no clear guidelines for being successful in this type of research, because of the relative newness at that time." In the experiments conducted with [2] Anopheles quadrimaculatus, the work was done at Lake Panasoffkee in Central Florida. We now know that there are three sibling species (A, B, and C) present in the natural population at that site, and furthermore at certain times of the year, species C is the major type. In the original work, the behavior of the sterile males of the laboratory strain was shown to be ineffective in mating with wild females. However, the impact of the sibling species complex was not evaluated because the researchers were completely unaware of the presence of more than one species.

"Patterson et al. [4] demonstrated that it was possible to use radiation-sterilized males for the induction of high genetic load into natural populations of Culex quinquefasciatus in two separate experiments, one in India and the other on Seahorse Key in Florida. Generally, if the males were irradiated at the end of the pupal stage or as young adults, the males were competitive enough (25-50 %) to have a significant effect on the reproductive ability of native mosquitoes. These experiments seemingly refuted some of the earlier results and definitely indicated that releases of irradiated males was a viable means to obtain mosquito control or suppression.

Chemosterilants were tested in the earlier experimental phase of genetic control, but for the most part these highly mutagenic compounds were difficult to use for the sterilization of male mosquitoes, because in general they were effective sterilants only when fed to larvae or adults. Treatments of larvae usually caused excessive somatic damage, and feeding the adults was impractical. The situation with using chemosterilants changed drastically when White [5] discovered that thiotepa could be used to sterilize pupae of Aedes aegypti, and subsequent work

provided structurally related sterilants that were even better [6].

The first successful small-scale SIT experiment with a species of mosquito was conducted on the island of Seahorse Key, off the west coast of Florida by Patterson et al. [7]. They controlled a population of Culex quinquefasciatus by overflooding a natural population with chemosterilized (treated with thiotepa) males.

In 1970, a large research effort designed to develop and test genetic control for mosquitoes was initiated in New Delhi, India, under the joint sponsorship of the World Health Organization and the Indian Council of Medical Research. This group of scientists did some outstanding research on radiosterilization, chemosterilization, rearing, ecology, cytoplasmic incompatibility, and chromosomal aberrations over a period of about five years (see Rao, 1984 [8] and the other papers in vol. 6 of The Journal of Communicable Diseases for many of the significant references)." Dr. Subbarao, one of the consultants at our meeting was on the staff. This work demonstrated that a significant level of sterility could be caused in field populations of Culex quinquefasciatus by the release of either chemosterilized or radiosterilized males in a typical SIT approach. Good results were also obtained with the release of males of a cytoplasmically incompatible strain, but subsequent work revealed that the Indian field strains were polymorphic for the compatibility types.

"In the early 1970's, a successful experimental trial, resulting in the control of a relatively small, isolated population of Anopheles albimanus, was conducted at Lake Apastepeque in El Salvador by a team of USDA/ARS scientists [9]. This effort was followed by a much larger experimental trial [10], in a 150 km^2 area on the coastal plain of El Salvador in which releases of sterile males peaked at approximately one million sterile males per day for a period of one year. This pilot study was the largest on record and was deemed successful in terms of inducing sterility within the release zone, but migration of fertile females into the study area was a significant factor that mitigated population control. The release rate was not sufficient to have an effect over the entire 150 km^2 area, but a four month experiment in an area of ca. 20 km^2 effectively controlled the breeding of A. albimanus. This large-scale test was the first SIT experiment to make use of a genetic sexing strain, designated MACHO. Radiation was used to induce a translocation-inversion complex that linked propoxur resistance to the Y chromosome. The females of this strain could be selectively killed by treatment with propoxur in the egg stage; thus, it was possible to rear only males, which cut the costs of production of sterile males in half."

During the past year, I have initiated an effort to find an alternative for using radiation or chemosterilants to sterilize mosquitoes. Radiation usually causes some loss in competitiveness and the residue problem is a part of using chemosterilants. In order to circumvent these problems, I have used either radiation or ethyl methane sulphonate to induce non-biting mutants. Mosquitoes can feed on defibrinated blood soaked on a cotton (or other absorbent material) pad. The idea is to isolate dominant mutant types that cannot penetrate the skin with their mouthparts, but can develop eggs by feeding on blood pads. A computer model, written by D. E. Weidhaas, tested the idea of releasing males homozygous for a dominant, non-biting, mutant gene. Instead of sterile eggs in the F_1 progeny, the resulting F_1 females cannot bite. The model showed this strategy would be one generation slower in eradication of a population than SIT. So far, about 10,000 females of Anopheles albimanus have been screened, and 30 non-biting females were detected by feeding blood in a membrane. Most of these non-biters can survive on sugar water, but although they tried to feed, only four have been able to take blood from a cotton pad. Of those four, only one laid eggs that hatched and the progeny could feed through a membrane. In our control groups, all of the females can feed, which means that we are making some genetic change that confers non-biting. The computer model was also extended to examine the hypothetical release of males that were homozygous for a dominant, non-biting mutant, either a dominant or recessive autogeny trait, and an incompatibility trait. At a ratio exceeding the equilibrium point, the wild type population was replaced by the non-biting, autogenous type. The hypothetical models look very promising, but making the non-biting mutants, manipulating autogeny, and finding incompatibility could well be projects that will require significant progress in molecular biology.

"Beginning in the 1960's a small number of geneticists and entomologists with dual training began work in the area of heritable chromosomal aberrations (translocations, inversions,compound chromosomes,etc.) and lethal traits for the genetic control of mosquitoes. The basic ideas (and the original references) underlying the use of heritable genetic mechanisms and much of the progress in this area was reviewed by Seawright [11]. The most important advance that resulted from this type of genetic manipulation has been the production of genetic sexing strains [12], containing visible or conditional lethal mutants, which are constructed so that the females can be killed selectively. A full discussion of the advantages and usefulness of genetic sexing techniques can be found in a review by LaChance [13]. For mosquitoes, this has been relatively easy because of the availability of dominant insecticide resistance traits, which serve as selectable markers. The resistance gene is made holandric via the induction of a Y-linked reciprocal translocation; therefore, a strain can be made that is composed

of resistant males and susceptible females. If there is a problem with leakage of resistant females due to genetic recombination, this problem can be averted by the induction of an inversion, as demonstrated by Seawright et al. [12] in the assembly of the MACHO strain of A. albimanus. As is the case with all research, luck will occasionally give the researcher a boost, as was the case for a genetic sexing strain of Anopheles quadrimaculatus species A, for which there was a malathion resistance gene that was located inside a naturally-occurring paracentric inversion [14]. There was also a report by Sakai and Baker [15] on the use of a sex-linked temperature sensitive lethal trait for the synthesis of a genetic sexing strain for Culex tritaeniorhynchus.

Except for the use of the MACHO strain in the large-scale SIT test in El Salvador, there never have been any attempts to conduct large pilot or operational programs based on the release of mosquitoes bearing chromosomal aberrations constructed for the genetic control of a species. Several male-linked translocations have been tested in limited competitive mating field trials with promising results (see review by Seawright [12])."

My research group (P. E. Kaiser, M. Q. Benedict, S. E. Mitchell, S. G. Suguna and I) made a concerted effort to synthesize compound chromosomes during a two-year period, 1979-1980. We made homozygous autosomal translocations and mutant markers to use in a capture scheme to detect compounds amongst progeny from irradiated mosquitoes; we screened over 250,000 mosquitoes and recovered the proper phenotypes, but the putative compound types produced no progeny.

TECHNICAL LIMITATIONS

The principal technical limitations involve three of the most important aspects of SIT, and for practical purposes these will also be of importance in the use of future types of genetic control. Since the basic principle of SIT involves overflooding the native population with sterile or genetically-altered insects, the means must be available to mass produce, sterilize, and distribute sterile males that are vigorous and competitive. This must be done economically; otherwise, the methodology is not likely to receive favorable consideration, even if it is highly effective. This is a very demanding task, but as was demonstrated in the large-scale experiment in El Salvador, there is some reason for optimism. In a conversation with David A. Dame, it was pointed out that by the time most of the problems had been solved with the technical limitations mentioned above, the funds for the project had been exhausted and civil unrest had started in the country, so the project was abandoned at just the time when real progress was in the offing. From personal experience and interviews with scientists who have been involved in SIT research on a practical scale (i.e. pilot studies or

larger such as the screwworm and mediterranean fruit fly programs), it takes time and considerable effort to discover and correct all of the seemingly minor problems that are in reality very important to the success of such an endeavor. The only way to detect all of the flaws in a system is to attempt the actual conduct of large-scale experiments that are very expensive and could end in failure, because of unanticipated complications and/or the lack of innovative personnel who can recognize and cope with technical details. There is no guarantee that a suitable system can be evolved that will meet all of the economic criteria, but unless a large-scale effort is attempted, it is impossible to predict the outcome. It takes a great deal of fortitude and considerable political skill to organize and conduct expensive ventures that are regarded by many administrators and scientists as high risk technology, and if failure is the final outcome there can be serious consequences for the reputations and careers of the scientists.

In the past, the approach to mass production procedures used in all of the SIT evaluations have simply been an extension of the methods used for laboratory colonies. Instead of rearing one pan of larvae, virtually the same methods were used to produce one hundred pans. Of course, it is a logical manner by which mosquitoes can be reared for limited testing, but in the long run research on mass production will be required. The research should be aimed at (1) minimizing the detrimental effects of colonization on the gene pool, (2) standardized diets, (3) automation, and (4) quality control.

Sterilization of the males has been and will continue to be a tricky step in the procedure, because male mosquitoes are fragile insects that are subject to debilitating damage with excessive handling. The use of radiation exacts a toll on competitiveness, but this is a minimal effect when young adults are irradiated. However, the adult stage is subject to more damage through handling. Pupae are easily sterilized by treatment with chemosterilants, but there are tiny quantities of residues in the very young adults and this might have a detrimental effect on predators [19]. The residues persist for a short time (ca. 24 hours), should not be hazardous to humans or domestic animals, and should not accumulate in the food chain. Seawright et al. [20] found that the higher residues were related to the general health of the pupae, with vigorous insects having much smaller amounts in them, so superior rearing and handling would minimize this problem.

Distribution of the sterile males at the point where they can mate with native females is an area of research that has hardly been touched. Certainly, it will depend on the particular target species, but for most SIT programs the distribution of insects by means of aircraft is the most feasible alternative. Past efforts have been done by a combination of vehicle and on foot, and while the release from vehicle might work for urban

situations, the use of aircraft should be studied for most species.

POPULATION GENETICS

The success of any genetic control system (past, present, and future) depends on a sufficient knowledge of the target species and its distribution. One of the most significant mistakes that could be made would be to release sterile males of a single species into a complex of sympatric sibling species, because even if the SIT worked, only one sibling form would be affected. Sibling species complexes are common in the Culicidae, and new descriptions of cryptic species appear in the literature on a regular basis. The available taxonomic keys are usually of no value in discovering sibling species. The only useful way to study whether a complex exists is through genetic and biochemical analyses involving the use of hybridization crosses, polytene chromosomes, allozymes, diagnostic DNA probes, mitochondrial DNA, and cuticular hydrocarbons. We have used all of these methods in our work with the A. quadrimaculatus complex to uncover and develop simple diagnostic tests for identifying the five morphologically indistinguishable species that have so far been found in natural populations. After working with these techniques, we have determined that, as with other organisms, allozyme and mtDNA analysis is most appropriate for the initial screen of a species of anopheline mosquito." In my laboratory, I now have an experienced team working on the population genetics of anophelines. After our past experience and setting up "assembly line" processes for our research in this area, we can now assess the presence of sibling species in a short period of time. Dr. Collins will present a summary of the status of molecular techniques for population analysis.

MOLECULAR BIOLOGY - THE FUTURE

Cockburn et al. [21] reviewed the status of molecular biology and the potential for applying these techniques in the genetic control of economically important insects. An update of this review was recently finished and should appear soon [22]. Some of the problems encountered in designing operational systems should be more easily solved by a molecular approach than by conventional genetic and cytogenetic methods. The use of classical genetic approaches involving the use of radiation and chemical mutagens to induce useful mutants and chromosomal aberrations followed by breeding program to construct useful strains is a laborious, expensive process that is not always successful. Although there is some opposition [23] to the stampede in the direction of recombinant DNA techniques for the development of future genetic control systems, it is time to accept the reality that molecular biology is going to revolutionize our concepts in most biological and agricultural areas. As mentioned above, there are some perfectly good

indications that we could control some species of mosquitoes with practical improvements of our present knowledge of SIT. However, I think that anyone interested in the possibility of improving tremendously in our present situation should read Cockburn et al. [21] and Cockburn and Seawright [22] and then proceed to become familiar with the literature that describes how to actually manipulate genes. Although it should be obvious, perhaps it is prudent to point out that the basic and applied research required to use recombinant DNA technology for developing novel genetic control systems will be demanding, expensive, and sophisticated. The real benefits are in the future, and this fact must be remembered before embarking on research in this area. There are so many different ways to approach using molecular techniques in the development of genetic control technology that the only limitations are the imagination of the scientists and the funding available for this work. In my own personal view, I do not think that this approach to pest control is a high risk venture. Instead, I think that adequate funding of directed efforts will be successful.

Dr. Collins will present a summary of the current status of genetic engineering of anopheline mosquitoes.

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WORKING PAPER:

NEOTROPICAL ANOPHELINES AND THE MALARIA PROBLEM ON A REGIONAL BASIS

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This presentation will review the malaria situation in the Americas, discuss the present status of regional vectors and update the knowledge on these vectors as it relates to genetic control.

A. The Malaria Problem in the Americas - In the Americas 21 countries and territories combined have more than 1 million cases of malaria per year (PAHO, 1992). This is an alarming increase from the 270,000 reported cases in 1974 and 600,000 in 1980. The mortality is appproximately 156/100,000 inhabitants. The population at risk is estimated to be over 280 million people which represents 40% of the human population in malaria areas and potential areas (Table 1).

In Mexico, Central America, Belize and Panama over 190,000 cases of malaria were registered in 1991. By country the percentage of Plasmodium falciparum ranged from 0.1% in Mexico to 27% in Panama. In the Caribbean malaria is restricted to the Island of Hispaniola (small outbreak in Trinidad, 11 cases). Haiti reported 25,511 cases and the Dominican Republic 377 cases. Almost all of the cases were P. falciparum. In the Andean region of South America (Bolivia, Peru, Colombia and Venezuela), over 260,000 cases were reported with over 50% being P. falciparum. In the Southern Cone countries of Argentina and Paraguay, 4,900 cases were registered with 8,464,000 being exposed.

The majority of the malaria in the Americas is in the Amazon Basin, including Brasil, the Guyanas, Colombia and Venezuela south of the Orinoco River. A total of 627,894 cases were registered in 1991 with over 80% being P. falciparum. Brasil registered 10% of the cases in the world outside of Africa.

B. Important Neotropical Vectors - The major vectors of malaria in the Americas have been summarized in various publications (Forattini, 1962; Faran, 1980; Linthicum, 1988, Fleming, 1986; Deane, 1986; Zimmerman, 1992). Recent studies on vector implication are presented in Table 2. If we start from the southern United States of America we can note that there has been several small outbreaks in recent years due to immigration of people from malarious countries into Southern California. The vector was a new species An. hermsi.

In Mexico and Central America, An. albimanus and An. pseudopunctipennis continue to be the major malaria vectors. Anopheles vestitipennis is a vector in southern Mexico. Anopheles darlingi is present and may be involved in transmission in Mexico, Guatemala and Honduras.

In the Caribbean on the Island of Hispaniola, An. albimanus is the principal vector; although the recent finding of An. pseudopunctipennis in Haiti may change this situation. In the Lesser Antilles, An. aquasalis is the principal potential vector.

In Colombia the major vectors are An. darlingi, An. nuneztovari, and An. albimanus. Other local vectors have been reported (Table 2). Along the Pacific Coast of South America there have been few recent studies on vector implication. The major vectors are An. albimanus and An. pseudopunctipennis. In addition, An. calderoni has been implicated as a secondary vector as well as several members of the Nyssorhynchus group (Table 2). In Bolivia, An. darlingi and An. pseudopunctipennis are considered the major malaria vectors.

In Chile malaria has not been detected for more than 40 years, but the vector An. pseudopunctipennis is still abundant in areas where immigation from malarious areas of other countries occurs.

In Venezuela, north of the Orinoco river the major vectors are An. nuneztovari and An. aquasalis. Potential secondary vectors are An. albitarsis, An. pseudopunctipennis, and An. neivai. South of the Orinoco river An. darlingi is the major vector. In the Guyanas the main vectors are An. darlingi and An. aquasalis.

Anopheles darlingi is the most important vector of malaria in Brasil. Recent studies have confirmed this (Table 2). Along the coast An. aquasalis has been a major vector over the years, but at present the parasite is not present. Many other species have been implicated as vectors in Brasil by dissection, and ELISA. Their actual contribution to malaria transmission needs further study.

Finally, in Paraguay, An. darlingi is the suspected vector and in Argentina An. pseudopunctipennis is the major vector.

C. Genetic Studies and Topics related to Genetic Control in the Neotopics - Research on genetics of neotropical anophelines has been mostly related to solving taxonomic confusion. Most of this work has been done on the subgenus Nyssorhynchus (Fig. 1). Anopheles albimanus, for which sterile male release has been done on a pilot study level, is by far the most genetically studied anopheline in the neotropics. Besides there being the presence of photomaps various studies have revealed at least 37 genetic markers (Frederickson, 1992). It is considered a single species and recent

work using rDNA showed no genetic evidence to suspect the existence of sibling species (Beach et al., 1989).

Conn (1990) present an updated photomap of larval polytene chromosomes of An. nuneztovari from western Venezuela and compared her results with those of Kitzmiller (1973). She found a significant increase in polymorphic frequency of the 2LA position and proposed that it was due to environmental modifications that have taken place over the last 16 years. Isoenzyme studies confirm this (Ranjel, per. comm.). Continuation of this genetic research using chromosomes, isoenzymes and rDNA restriction analysis demonstrated significant regional variation to propose more than one species (Conn, pers. comm.; Ranjel, pers. comm.). In particular, the difference between western Venezuela and Brasil is apparent. The genetic results correlate with differences in biting behavior.

Anopheles aquasalis had been resurrected from the tarsimaculatus group and later separated into several species and then made one (Faran, 1980). Kitzmiller & Chow (1971) presented the first map of the salivary gland chromosomes. Stiener et al. (1981 and Narang et al. (1979) studied isoemzymes of An. aquasalis. Recent work on photomaps of larval salivary glands (Moncada, 1992) and isoenzymes (Vele, 1993) have confirmed that in Venezuela it is the same species. There appears to be a slight genetic difference at the extreme of its range near Rio de Janeiro (Conn, pers. comm.). Presently there is more isoenzyme work being conducted in Venezuela and Trinidad & Tobago.

There always has been the suspicion that An. albitarsis is a complex of species (Forattini, 1962) Chromosome maps have been made by Kreutzer et al. (1976). Recently, Linthicum (1988) resurrected An. marajoara from An. albitarsis and concluded that it was not conspecific. Anopheles allopha was sunk under An. albitarsis. Rosa-Frieta (1989) described a new species, An. deaneorum from this complex in Brasil; on morphological grounds. In 1990, Rosa-Frieta et al. examined the An. albitarsis complex from 9 locations in Brasil and the type locality in Argentina. They examined behavioral differences, morphological characteristics and used multienzyme electrophoresis to determine population differences. Morhologically only An. deaneorum was distinct. Phenetic and isoenzymes showed 2 population clusters and 4 probable distribution groups. Results were compared with the cytogenetic work of Kreutzer et al. (1976) and the electrophoretic study of Stiener et al. (1982). They concluded that there were only 2 species. Obviously more research with this complex is need before the taxonomic situation is clear.

Anopheles darlingi, the most important present day vector of malaria in the Neotropics, is believed to be a complex of cryptic species based on behavioral and genetic studies. Chromosome studies by Kreutzer et al. (1972) and Tadei (1982a, 1982b) produced

larval chromosome photomaps for Brasil. In general they agreed that there was a high amount of chromosomal polymorphism and that this polymorphism changed over seasons. Rosa-Frieta et al. (1992) compared isoenzymes, behavior and morphology of 3 populations of An. darlingi in Brasil and concluded that none of the 3 populations could be considered a separate species. Their results were supported by preliminary results for 8 endonucleases which showed no major polymorphism among populations studied (Rosa-Frieta et al., 1991).

The taxonomic status of An. triannulatus has been questioned by Fernandes (1993) on morphological grounds. She considers there to be 2 species in Brasil. Anopheles trinkae, a vector in Brasil has been sunk under An. dunhami (Peyton, pers. comm.). In addition, preliminary genetic and morphological studies show that An. oswaldoi, An. strodei, and An. rangeli need taxonomic revison.

In the subgenus Anopheles, the secondary vector An. punctimacula has been split into 2 species, An. calderoni and An. punctimacula (Wilkerson, 1991).

The important vector An. pseudopunctipennis was thought to be a complex of species (or subspecies) for quite some time (Forattini, 1962). A chromosomal map was produced by Baker et al. (1965). Recently Manguin et al. (pers comm.) evaluated the taxonomic status of this species from 8 countries using isoenzyme electrophoresis and could find no evidence for more than one species. To the contrary Estrada Franco et al. (1992) concluded in a preliminary report that there were two allopatric species, one species from Mexico and the other from Peru and Bolivia. They compared isoenzymes, restrictive DNA and did hybridization experiments.

In conclusion it can be seen that there has been a renewed interest in the genetics of the neotropical anophelines, but it is presently concentrated on solving taxonomic problems.

D. **Vector Biology as it Relates to Genetic Control** - In the above discussion on taxonomy there is a lack of research mentioned on experimental crossing of species. After all the true test of speciation is if there is interbreeding. The only neotropical anopheline that is self-reproducing in the laboratory is An. albimanus. Galvão et al. (1944) mentioned that they had colonies of An. albitarsis, An. argyritarsis, and An. tarsimaculatus (An. aquasalis). Since then colonies of An. darlingi, An. deaneorum, An. aquasalis, An. psuedopunctipennis and An. albitarsis have been maintained by introducing wild females or inducing copulation (Bates, 1947; Warren et al., 1980; Arruda et al., 1982; Buralli & Bergo, 1988; Klien et al. 1990). Forced copulation can maintain a population, but operationally it is costly and time consuming. For large genetic studies including sterile male releases it is an

important criteria to have laboratory colonies that are self-reproducing.

Knowledge of natural mating behavior may help in the design of laboratory facilities to rear anophelines. Unfortunately it is a neglected field of study. Only 5 species have been reported swarming and 2 mating in nature. Natural occurring swarms and mating has been observed for An. aquasalis in Trinidad (Senior White et al., 1953). They also observed swarming of An. neomaculipalpus and collected An. albitarsis and An. oswaldoi in the same swarm as An. aquasalis. Anopheles pseudopunctipennis has been observed swarming and found mating in exit traps (Borda & Downs, 1956).

Multiple mating would have a significant effect on the ability of sterile males to compete with natural males. For the neotropical anophelines An. nuneztovari was shown to be 15% poliandric (Scarpassa et al., 1992) and An. darlingi to be completely monandric in Manaus, Brasil (Santos et al., 1981). Another Anopheles species which extends into Mexico, An. quadrimaculatus showed some multiple mating but was almost exclusively monogamic (French & Kitzmiller, 1965).

An important consideration for the introduction or alteration of a natural population by genetic means is the interchange of genetic material between natural populations. This is directly related to the ability of a species to disperse. Most information is known about An. albimanus which has been known to fly great distances aided by winds (Frederickson, 1992). In a designed experiment to observe dispersal this species was shown to fly 3 km in 6.5 days. Anopheles darlingi may be the only other neotropical anopheline for which concrete data on dispersal is known. In a mark-release experiment done in the Amazon, marked individuals were collected up to 7km from the release point (Charlwoood & Alecrim, 1989). They also found survivorship to be 65% between ovipositions.

There is no doubt that more and better estimates of parameters involved in population biology are needed for neotropical anophelines. More laboratory and field studies on ecological genetics are essential and these studies should run parallel to molecular genetic studies.

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Table 1. Malaria in the Americas, 1991*

Country	Registered cases	Population at risk	Parasite (%)
Mexico, Central America, Belize, & Panama	188,938	variable 0.2- 16.6/ 1000	variable, P. falciparum 0.1% - 27%
Haiti and Rep. Dominicana	25,888	8 million	almost all P. falciparum
Andean Subregion Bol, Peru, Ecu, Col, & Ven	259,375	53.5 million	P. falciparum > 50%
Southern Cone Arg & Paraguay	4,900	8.5 million	P. vivax >98%
Amazon Basin Brasil**, Guyanas, Col & Ven	627,894	- - -	P. falciparum 80%
21 Countries & Territories	> 1 million	> 280 million	Depends on Eco-region

* From "Informe de la reunion international sobre el paludismo en las Americas (CTD/MCM/IM/92.3).

** Brasil alone represents 10% of the world cases outside of Africa.

Table 2. Anopheles species recently found positive for Plasmodium in the Americas**

Location	Anopheles sp.	Method used	Author
Chiapas, Mexico	An. vestitipennis [1]	ELISA	Loyola et al. (1991)
Chiapas, Mexico	An. albimanus [3]	filtration	Ramsey et al. (1986)
Nicaragua	An. albimanus [1]	ELISA	PAHO (1991)
Guatamala	" "	"	
Junin, Dept., Peru	An. trinkae, An. rangeli [3] An. oswaldoi, An. nuneztovari An. pseudopunctipennis An. sp. nr. fluminensis	salivary glands	Hayes et al. (1987)
Anadoas, Peru	Nyssorhynchus [1]	ELISA	Need (pers. comm
Charambirá, Colombia	An. neivai [1,2]	IRMA	Carvajal et al. (1989)
Colombia	An. albimanus [1]	IRMA	Herrera et al. (1987)
"	An. albimanus, An. allopha [2] An. darlingi, An. neomaculipalpus	IRMA	" "
Bajo Calima, Colombia	An. nuneztovari [3]	salivary glands	Fajarado & Alzat (1987)
Caqueta - Putumayo, Colombia	An. rangeli [1]	ELISA glands neg.	Suarez et al. (1991)
La Lengueta, Barinas, Venezuela	An. nuneztovari, An. oswaldoi [1] An. albitarsis	ELISA	Rubio (1991)
Sucre State, Venezuela	An. aquasalis [1]	ELISA 1 gland pos.	Caseres (pers. comm.)
Payapal, Bolivar, Venezuela	An. darlingi [3]	salivary glands	Berti (pers. comm.)
Para State, Brazil	An. darlingi [2]	IRMA & ELISA	Arruda et al.* (1986)
"	An. darlingi, An. albitarsis [1] An. nuneztovari, An. triannulatus An. oswaldoi		"

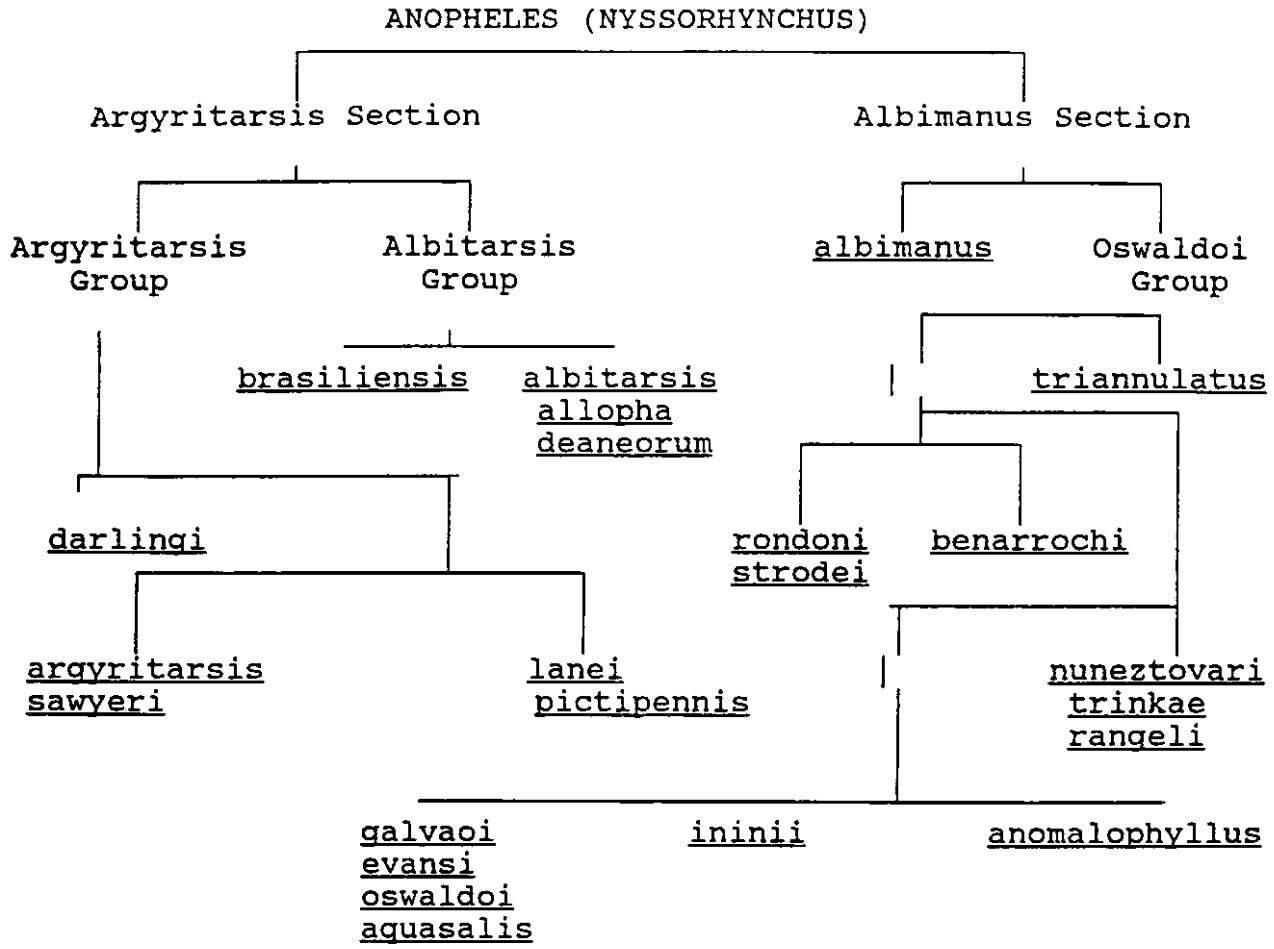
1- Plasmodium vivax, 2- P. falciparum, 3- Plasmodium sp.
* salivary glands were found positive for sporozoites, except for An. oswaldoi.
** modified from Zimmerman (1992).

Table 2 (cont) Anopheles species recently found positive for Plasmodium in the Americas*

Location	Anopheles sp.		Method used
Rondonia State, Brazil	An. darlingi, An. braziliensis[2] An. albitarsis, An. oswaldoi	IRMA	Oliveira-Ferreira et al. (1990)
"	An. darlingi, An. triannulatus[1] An. albitarsis, An. strodei, An. braziliensis	IRMA	" "

1- Plasmodium vivax, 2- P. falciparum, 3- Plasmodium sp.
* modified from Zimmerman (1992).

Figure 1. Neotropical Anopheles subgenus Nyssorhynchus*



* modified from Stiener et al., 1982.

WORKING PAPER:

Vector Control of Malaria in the American Region (WHO/PAHO)

R.H. Zimmerman, PAHO/WHO

Giglioli (1956) stated that "The control of malaria by modern insecticide techniques rests fundamentally on the biting and resting habits of the anopheline species responsible for transmission". His statement from 1956 is still applicable today in the American region. There have been few changes in this approach. Adulticides are usually the only form of control and most use takes place inside houses.

However, certain neotropical anophelines, because of natural or evolved behavioral characteristics or resistance to insecticides, do not always rest inside sprayed houses. Gabaldon (1978) classified malaria transmitted by these vectors as refractory malaria. The five principal vectors of malaria in the Americas are to varying degrees responsible for refractory malaria (Table 1). It can be seen that most biting takes place outside and a larger portion of the population rests outside rather than inside.

Table 1. Biting and Resting Behavior of the major Anopheline Vectors in the Neotropics

Species	Biting Behavior	Location of Biting	Resting Behavior
An. darlingi	Crepuscular/ Midnight	Exophagy > Endophagy	Exophily > Endophily
An. albimanus	Unimodal Before Midnight	Exophagy >> Endophagy	Exophily >> Endophily
An. nuneztovari	Midnight/ Before 10pm	Exophagy >> Endophagy	Exophily >>> Endophily
An. aquasalis	Crepuscular	Exophagy >> Endophagy	Exophily >>> Endophily
An. pseudo-punctipennis	Crepuscular	Exophagy >> Endophagy	Exophily ? Endophily

Because of refractory malaria there is the need to reorientate national control programs. A few examples exist in the Americas. In Colombia, where both An. albimanus and An. neivai are vectors a pilot project was developed along the Pacific Coast . Anopheles neivai was controlled by removing bromeliad from trees near the village which were their larval habitats. Control of An. albimanus was achieved by draining breeding sites and using Bacillus thuringiensis israelensis (BTI) at sites that could not be drained. Results were excellent (Rojas et al., 1992).

In Venezuela, along the norhteast coast where An. aquasalis is the principal vector various vector characteristics were taken into consideration in order to lower malaria. This species has a crepuscular biting activity, bites more outside than inside, rests on vegetation around houses before and after hostseeking and has larval breeding sites which are accessible for treatment. Indoor house spraying was discontinued, space spraying was done around houses using portable sprayers between 6-10pm weekly, and BTI was applied to the major breeding sites, by hand, biweekly. This program was run at the State level. Results showed a decrease in malaria of over 90% in less than a year (Zimmerman & Berti, 1993). Another advantage of this approach was that it may be possible to reduce the use of insecticides even further when more persistant BTI formulations are available. Also, this site was part of an national marine park and this approach reduced a potential conflict between human health and environmental health because no draining was done nor was chemical insecticide used in the park.

In El Salvador malaria has decreased considerably over the last 10 years due to the implementation of an integrated program. This program combined malaria stratification, decentralization, training and environmental management (PAHO, 1992).

It should be mentioned that PAHO malaria personnel were directly involved in the promotion and design of all three projects and participated in the operational phase of the projects in El Salvador and Venezuela.

Historically the Ministries of Health of each country in the Americas have been in charge of all aspects of malaria control. Over the last several years there has been a conceptual change in malaria programs from erradication to control of the disease. Also, there is a move to decentralize the health services and place the responsibility for health at the district or municipality level. Vector control at the local level is a component of primary health care and has been a topic of considerable discussion. In 1987, a scientific working group designed a strategy to strengthen local health services in the area of vector-borne disease (WHO, 1987). Major recommendations were 1) to train medical entomologists at the professional and local level, 2) to introduce aspects of vector control into staff education programs, 3) to form a core group of

professionals which has the ability to provide technical support for the local programs, 4) to give medical entomologist a favorable career structure in national health systems, 5) to design and monitor programs, and 6) to apply biological control agents when the situation dictates.

Research needs involved statification, socioeconomic and education projects, and operational research. Operational research consisted of studying behavior, productivity and workloads of workers under varying epi-sociological situations, optimizing the delivery of community-based vector control measures, and cost effective studies on different operational schemes. There was only scant mention of vector research on biology and control.

The new control technology that will be used in primary health care will have to come from national and international research teams. As mentioned in my previous talk there is a lot we still need to know about the dynamics of vector-borne disease transmission. This knowledge eventually will be transfer to local health programs.

One of PAHO major strategies is to support research and development which benefits primary health care. In Table 2, I have listed some areas of vector related research which should be considered.

Table 2. Research and Development which Benefits Primary Health Care

1. Knowledge engineering	- Intelligent Geographical Information Systems (GIS)
2. Ecological classification of Malaria	- Landscape Ecology (Pattern and Process) - Remote Sensing
3. New Control Methods	- Insecticide Delivery Systems - Barrier Sprays - Biocide Development
4. Population Biology	- Life History Strategies
5. Molecular Biology & Genetics	- Ecological Genetics - Transgenetic Technology - Sterile Insect Technology

All of these areas are essential components in our search for new ways to control malaria none is mutually exclusive. Perhaps the one area which may benefit local health systems most because it readily feeds back to the local level is knowledge engineering. It can be envisioned that local vector control specialists will be able to use a menu driven program which aids them in routine decision making and problem solving. Such an approach is being

discussed in agriculture and natural resource management (Coulson et al., 1990).

In order for information systems to function knowledge from other fields of science is essential. We are presntly classifying vectors and malaria by ecoregions in the neotropics using vector distribution, epidemiology, human activities, and ecological charateristics of the ecoregions (Rubio & Zimmerman, unpub. manuscript).

The field of landscape ecology provides the opportunity along with remote sensing to observe and predict effects of human activities on vector populations and malaria transmission. At present there is a NASA (USA)/Mexico project using remote sensing and GIS to predict vector and malaria distribution in Chiapas State, Mexico. We anxiously await the results.

New integrated control methods are needed which are cost effective and can be managed at the local level. This will involve a considerable input into design and testing control technology.

To better understand the population dynamics of vector species much more empirical data is necessary. Life history strategies need to be defined. Behavior characteristics such as mating and host seeking need to be better understood.

Molecular biology and genetics are fields which may provide new technology that can be transferred to regional and local programs. Generation of molecular cytological and genetic maps are a first step, but much more is needed. Also, in the field of ecological genetics, a better understanding of the mechanism by which vectors respond to the environment on a genetic level coupled with the potential to modify these responses is required.

These research areas combine to form the bases by which genetic control such as sterile insect technology can be implemeted for the control of malaria in regional and local programs.

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INDIAN SUB-CONTINENT ANOPHELINES AND THE MALARIA PROBLEM ON A REGIONAL BASIS

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I Malaria Vectors in the Indian Sub-continent :

The countries in the Indian sub-continent come under the Oriental region. Of the 450 anopheline species reported so far 286 species are from this region.

Table 1 gives the list of important vectors prevalent in each country of the sub-continent (Rao, 1984). Species which may have been found positive with sporozoites but not considered important in the transmission of malaria are not included in the list.

The Indian anopheline fauna consists of about 56 species. Seven of these species viz., A. culicifacies, A. dirus, A. fluviatilis, A. minimus, A. philippinensis/A.nivipes, A. stephensi and A. sundaicus are of primary importance and A. annularis and A. varuna are of secondary importance in the transmission of malaria. A. culicifacies, which has a wide distribution in the Indian sub-continent, is a major vector of rural malaria in India. In Sri Lanka in addition to A. culicifacies, A. subpictus and A. annularis have recently been identified as major vectors. A. culicifacies is a vector of importance in Pakistan along with A. stephensi and A. superpictus. A. dirus, A. minimus, A. philippinensis and A. annularis are vectors in

Bangladesh and these are the species which are vectors too in the north-eastern states of India. In Nepal, A. minimus, A. fluviatilis, A. annularis and A. maculatus have been reported. However, A. minimus seems to have disappeared from Nepal. Though A. culicifacies is present in Nepal, its role as a vector has not been established. It is worth mentioning here that in the Terai region on the Indian side of the Nepal border, A. culicifacies is an established vector.

Following is a brief description of the vector species in the region :

ANOPHELES CULICIFACIES COMPLEX

A. culicifacies has now been identified as a complex of 4 sibling species, provisionally desginated as species A,B,C and D. The four species were recognized on the basis of assortative mating observed between populations having different inversion arrangements in their chromosomes i.e., absence of inversion heterozygotes in the natural populations (Green and Miles, 1980; Subbarao et al., 1983, 1988a). The four species which have specific mate recognition systems in wild, have post-mating barrier only betwen species A and B and A and C (Subbarao et al. 1988b). Genetic relationship of species D with other sibling species has not yet been established with certainty.

Methods available for the identification of sibling species:

i) Fixed paracentric inversions : The four species are distinguishable by the diagnostic paracentric inversions

observable on polytene chromosomes (fig. 1) (Subbarao et al., 1988a). However, Species D can only be recognized at the population level in areas where species A and D are sympatric and i^4 inversion is polymorphic in species A (i^1 is diagnostic for species D) (Vasantha et al., 1992).

ii) Structural variation at metaphase stage : Species A and C have sub-metacentric Y-chromosome while species B has acrocentric Y-chromosome (Vasantha et al., 1982, 1983).

In Rameshwaram island where only species B was identified based on diagnostic inversions, two types of male mitotic karyotypes were found : i) Y-chromosome acrocentric and ii) Y-chromosome sub-metacentric (Subbarao et al., 1993).

iii) Electrophoretic variations : Two variants of LDH enzyme system distinguish species A and D from B and C (Adak et al., 1993); Fast allele is fixed with a high frequency in species A and D and Slow allele in B and C.

iv) DNA probes : A DNA probe which recognizes species B is available with Dr. Gunasekaran in Sri Lanka and one with Dr. Colin Malcolm in U.K.

Thus, polytene chromosomes are the only diagnostics available for distinguishing all the members of the complex.

Laboratory colonies : Anopheles culicifacies was colonized for the fist time in Pakistan in 1976 (Ainsley, 1976) and in 1977 in India (Ansari et al., 1977). Colonies of species A,B and C

established from progeny of single females collected from field and identified for sibling species are being maintained at Malaria Research Centre (MRC), Delhi (Subbarao et al., 1983). A colony with $X+^{a}+^{b}$ $2i^{1}+h^{1}$ chromosome arrangements has been established, but can not be said with certainty whether it is polymorphic form of species A or species D. Procedures have been standardized to mass rear A. culicifacies. However, it may be pointed out that even though the procedures have been standardized to maintain colonies, for reasons not known, egg laying suddenly goes down and many a times, lines are lost. Initial establishment of lines still continues to be problematic.

Genetic maps and mutant stocks in sibling species :

Several induced and spontaneous morphological mutants and biochemical markers were isolated and their inheritance pattern was estalished at the Pakistan Medical Research Centre, Lahore, Pakistan. These markers have been assigned to their respective linkage groups (Parvez, et al., 1985). Later, the colonies from which these mutants were isolated were identified as species A. A few markers were isolated at MRC and two of the autosomal markers in species B are being maintained (Subbarao et al., 1982). Pure lines for enzyme allelomorphs are also being maintained (Adak et al., 1988). List of mutants isolated in species A and B are given in Table 2 and an updated linkage map in fig. 2.

Chromosomal aberrations were also induced in species A (Baker,et al., 1978). A genetic sexing strain using male linked translocation and dieldrin resistant gene has been developed in

species A (Baker et al., 1981). Males sexed by this system and treated with a chemosterilant (bisazir) were slightly less competitive in field but were equally competetive in laboratory experiments (Reisen et al., 1981).

There are no reports on A. culicifacies after 1985 from Pakistan and it is not known whether the strains isolated there are still being maintained or not.

Biological variations among the members of the complex :

Distribution of sibling species in India is given in Fig. 3. In India, species B is found almost throughout the country wherever A. culicifacies is encountered. Species B alone is found in some areas whereas in other areas it is found sympatric with A or with C or with D in combination with A and C. The proportions of occurrence vary in an area with changes in seasons (Subbarao et al., 1987a).

Species composition observed in the countries of the sub-continent is given in table 3. In Pakistan both species A and B were found sympatric in several localities (Mahmood et al.,1984); in Iran, and Yemen only species A, while in Nepal, Sri Lanka and Thialand only species B (Subbarao, 1988) have been reported.

All four species are predominantly zoophagic (Joshi et al., 1988), with only 3-7% of species A and less than 1% of species B,C and D feeding on human blood. However, in Orissa 3% of species C had human blood.

Both P. falciparum and P. vivax sporozoites have been detected by IRMA in species A,C and D (Subbarao et al., 1988c, 1992). One species B out of about 2500 examined had P. vivax circumsporozoite antigen.Epidemiological studies also established that species B had no role in the transmission of malaria (Subbarao et al., 1988d).

In Pakistan, while species A and B are sympatric, species A was incriminated. However, in laboratory feeding experiments, both species A and B developed sporozoites (Mahmood et al., 1984). In Sri Lanka and Rameshwaram island, only species B is found and several A. culicifacies sensu lato were found with sporozoites. As two mitotic karyotypes are observed in Rameshwaram island, the possibility of the new population i.e., species B with sub-metacentric Y-chromosome as a vector should be examined. The presence of the new karyotype in Sri Lanka should also be examined.

Species A is more susceptible to DDT and remains susceptible to malathion for longer periods than B (Subbarao et al., 1988; Raghavendra et al., 1992). Of species B and C, C developed resistance faster to malathion than B (Raghavendra et al., 1991). All three species are highly resistant to HCH.

ANOPHELES STEPHENSI

A. stephensi is an important vector of malaria in urban areas of India and Pakistan. In some rural areas it transmits malaria along with A. culicifacies. This species has been the subject of research for the past 30-40 years in many laboratories

all over the world. Two races, the type form A. stephensi sensu strictu and variety mysorensis, were described on the basis of differences of the egg width, length and the number of ridges on the egg float (Sweet and Rao, 1937 and Rao et al., 1938). In recent surveys in India, a new egg float category with ridge numbers intermediate between type form and mysorensis was found. This was designated as 'intermediate' (Subbarao et al. 1987b). The three egg ridge categories respectively have 14-22, 12-17 and 9-15 ridges on the egg floats. In urban areas only type form with an occassional occurrence of the intermediate category is observed. All three categories - type form, intermediate and var. mysorensis are observed in semi-urban areas while intermediate and mysorensis are seen in rural areas. Hence, the three categories were refered to as 'ecological variants'. An analysis of results from crosses between the three categories indicated the genetic basis for the ridge number to be polygenic (Subbarao et al., 1987b).

In urban A. stephensi, a large number of polymorphic inversions are found, while in rural areas only 2b inversion and that too, with a low frequency is found. Similar observations were made in Pakistan (Mahmood and Sakai, 1984). Though rural and urban A. stephensi populations were found to be genetically distinct, neither pre- nor post-copulatory barriers have been observed so far.

Mutant stocks and linkage maps :

Several morphological mutants and biochemical markers were

isolated in A. stephensi. These markers were analyzed for their inheritance pattern and a linkage relationship between the markers was also established for many of the markers isolated. list of mutants isolated so far is given in Table 4. Parvez et al., (1985) have prepared a linkage map for the available mutants. Since then a few more markers have been isolated and studied (Adak et al., 1990, 1991, 1992 and 1993). A revised map is presented in fig 4. Discrepancies observed in the map of Parvez et al. (1985) have been corrected in the present map.

Refractory strain :

Two lines refractory to P. falciparum and one highly susceptible were isolated in this species (Fieldman and Ponnudurai, 1989). In the refractory lines, median number of oocysts developed were less than 4% of the oocysts developed in an unselected susceptible line. In the highly susceptible selected line, oocyst number was double than that found in the unselected line. Lines refractory to P. falciparum were not refractory to P. vivax.

Genetic sexing :

A line in which dieldrin gene located on linkage group 3 translocated to Y-chromosome was isolated (Robinson, 1986). The line T(Y-D1) shows extremely close linkage between male determining chromosome and dieldrin resistant gene. Treatment of 1st instar larvae with a diagnostic dose of dieldrin (0.1ppm) to kill females affected the emergence of males. Subsequently, a simple technique has been developed exposing newly emerged adults

to dieldrin coated papers for the production of males (Robinson, 1987). A cytological analysis of this line revealed the presence of a 3 chromosome multiple translocation in which part of the Y-chromosome formed the differential segment.

Malcolm and Mali (1986) isolated two Y-linked translocations T(Y-3)20 and T(Y-3)24, which had breakpoints almost completely linked to Black-larva, a morphological mutant on chromosome 3. T(Y-3)20 showed 0.05% recombination frequency and T (Y-3)24 showed 0.9%. Because heterozygotes for Bl can be differentiated from homozygous Bl phenotype at larval stage, full black females can be separated from half black males. Another Y-linked translocation, T(Y-3)39 in which the breakpoint showed 0.7% recombination with an adult morphological mutant short palpi (sp) was also isolated. Mating competitiveness of males produced by these genetic sexing lines needs to be tested.

ANOPHELES FLUVIATILIS COMPLEX :

This is an important malaria vector in India and Nepal and may be in some parts of Pakistan. It is found in hills upto an altitude of 2500 meters above the sea level, foot hills and plains. This taxon has now been identified as a complex of 3 sibling species (Subbarao et al., 1993). Absence of inversion heterozygotes between the populations differing in fixed paracentric inversions was the basis of recognizing the three sibling species provisionally designated as species S,T and U. The species composition observed in the areas surveyed is species S with T, and T with U. Species S is found predominant in hilly

forested areas where malaria incidence is very high. This species has been found to be highly anthropophagic (80-90%) while the other two species are highly zoophagic. Efforts are in progress to establish pure colonies of these species. A detailed study on the bionomics of the three species to establish the biological variations with reference to malaria transmission is in progress at Malaria Research Centre, Delhi.

ANOPHELES MINIMUS COMPLEX :

This taxon has been identified as a species complex based on morphotaxonomy and isozyme studies (Sucharit et al., 1988). Species A and C found in Thialand were distingsuished by Est-2 alleles, alleles 100 and 102 are predominant in species A and 98 allele in species B. Green et al. (1990) studied several enzyme systems and identified ODH as the diagnostic enzyme to distinguish between A and C. Species B is found in China. A small sample of A. minimus from Assam, a north eastern state in India, was found to be species C. A. minimus from Bangladesh has not been examined for sibling species.

ANOPHELES DIRUS COMPLEX :

7 species have been identified in this complex. These are species A,B,C,D,E,F and takasagoensis (Baimai, 1988). The isomorphic species can be distinguished on the basis of distinct patterns of major blocks of constitutive heterochromatin in sex-chromsomes as they appear in the mitotic karyotype and banding sequences in the polytene chromsome preparations. In southern

part of India in the state of Karnataka species E is found (Sawadipanich et al., 1990) and A. dirus from north eastern states of India has not yet been identified. A. dirus from Bangladesh has been identified as species D.

ANOPHELES SUNDAICUS :

This is an important vector in the coastal regions as it prefers to breed in brackish water. This species has now disappeared from coastal regions of West Bengal, Visakapatnam and Orissa state in the mainland of India and now it is found only in Andaman and Nicobar islands in the Bay of Bengal. This species is also reported from Bangladesh, Myanmar, Malaysia, Thialand and Indonesia. It is an important vector in Java island of Indonesia.

Attempts to colonize this species have not been successful. Species exhibits variations with regards to breeding places - fresh water and brackish water; resting places - indoor and outdoor resting and host feeding preferences - highly zoophagic in some areas and highly anthropophagic in other areas. These biological differences suggest the possibility of existence of sibling species which appears to be a common feature in anopheline species.

ANOPHELES PHILIPPINENSIS/NIVIPES COMPLEX :

A. philippinensis is found in north-eastern, central, and southern states except in Tamil Nadu and Kerala in India. But it is important as a malaria vector only in the north-eastern states where it is found in high densities.

A. nivipes and A. philippinensis were reported to differ by two fixed paracentric inversions 't' and '1' in chromosome arms 2 and 5 respectively (Green et al., 1985). Using these diagnostic inversions, the so-called A. philippinensis populations from the two north-eastern states of Assam and Meghalaya were identified as A. nivipes.

A. philippinensis is a vector in the plain areas of Bangladesh. In light of our observation in Assam, populations from Bangladesh have to be cytotaxonomically examined.

A. nivipes is a complex of two species differing by a paracentric inversion in the X-chromosome (loc cit.).A. nivipes from Assam were identified as species A.

ANOPHELES ANNULARIS COMPLEX :

In some eastern parts of India, this species is considered as a vector of some importance. In other parts, it is found in abundance during colder months but has not been implicated in malaria transmission. In Nepal, it is of considerable importance.

In one of the WHO reports(1984),it was mentioned that two sibling species were identified differing in an inversion in the X-chromosome. But no published report has been available to examine the Indian populations. Only one type of X-chromosome (as reported in Green, 1985) was found in all the specimens examined so far. However, two different chromsome 2s were observed differing by a paracentric inversion with a total absence of heterozygotes in several areas. These have been

provisionally designated as species A and B (MRC, Annual Report, 1992).

ANOPHELES MACULATUS COMPLEX :

This taxon has been identified as a complex of 8 sibling species. 16 fixed inversions are used to distinguish 8 species (Green et al., 1992). In Nepal A. willimori transmits malaria. In India A. pseudowillimori has been found (Rattanarathikul and Green, 1986) but A. maculatus s.l. throughout the range of distribution has not been examined cytotaxonomically.

ANOPHELES SUBPICTUS COMPLEX:

A. subpictus has been identified as a complex of two sibling species, A and B (Suguna, 1982). The two species differ by a fixed paracentric inversion in the X-chromosome and also differ in their egg morphology, chaetotaxy and larval salinity tolerance (Reuben and Suguna, 1983). Species A was found in all localities surveyed in Tamil Nadu, while species B was found exclusively in coastal villages where breeding of A. subpictus is observed in brackish water. Populations from villages around Delhi were cytologically identified as spcies A found in Tamil Nadu, but the number of ridges on the egg floats did not correspond (Subbarao et al., 1988a). In Sri Lanka, a fresh water breeding A. subpictus which differs cytologically from species A was observed (Abheyvardhana, AMC, Sri Lanka, personal communication).

A. subpictus sensu lato was found with natural plasmodial infections in coastal villages of Tamil Nadu, India (Panicker et

al., 1981) and in areas from Madhya Pradesh state in India where only fresh water breeding A.subpictus is found (Kulkarni, 1983). In Mahavalley irrigation project areas in Sri Lanka, several A. subpictus s.l. were found with P. vivax and P. falciparum sporozoites (Amerasinghi et al., 1992). The sporozoite rates in A. subpictus were almost equal to those of A. culicifacies, which earlier was considered to be the only major vector in Sri Lanka. A. subpictus in these areas was found breeding in fresh water.

These observations suggest for further detailed cytotaxonomic, morphometric and entomological studies both in India and Sri Lanka.

II Malaria situation in the region :

Malaria has been a serious problem in this region. Malaria cases reported from the countries in the sub-continent are given in Table (WHO Report, 1992). In this report, it is stated that the reduction in number of reported cases from 9 million to 5 million (excluding cases from Africa and China) between 1976 and 1984 was largely due to the control of malaria resurgence in India. This shows the significant number of malaria cases in India compared to the other areas of the region. In Bangladesh and Bhutan about 90% of the cases are contributed from forest areas while in Sri Lanka forest malaria is negligible, and it is confined to a few pockets in gem-mining areas (Sharma et al., 1991). Vectors in forest areas are A. minimus, A. dirus and in some areas A. fluviatilis. In India about 30% are contributed from forests and the remaining are from rural and urban areas.

In Nepal about 40% of the cases are from forest areas. In Pakistan, malaria cases are mostly from rural and urban areas.

III The R & D activites in India :

The National Program to control malaria has been in operation under the National Malaria Eradication Program, Ministry of Health and Family Welfare, Govt. of India. Though the main emphasis of the program is on the control of malaria, this program does carry out research on certain aspects of vectors. The program has 72 zonal entomology units in different parts of the country. At the zonal units, mosquito collections are made and the susceptibility of vectors to different insecticides is tested. Major research on malaria in the country is being carried out at Malaria Research Centre (MRC). under the Indian Council of Medical Research Centre, Delhi, Ministry of Health and Family Welfare, Govt. of India. At the Centre, research on all aspects of malaria vectors is being pursued. A well-established insectary at the Centre caters to the needs of basic research. For the past 20 years, scientists at the Centre have been working an various genetic aspects of malaria vectors.

MRC has 13 field stations in different parts of the country. These field station were established to test the feasibility of bio-environmental strategy to control malaria in different ecological and epidemiological situations. In most of these field stations in addition to the field studies, basic research on vectors is also being carried out. The other insititutes and

universities where research on genetic aspects of malaria vectors is being pursued are : Vector Control Research Centre, Pondicherry; Bangalore University, Bangalore, Karnataka state; Punjab University, Chandigarh and Zoology Dept., University of Pune, Pune, Maharashtra state. There may be many other institutes/universities where minor studies on malaria vectors are being studied.

IV Possible location and a species that could be attacked in a Pilot Project to study the use of genetic control :

A. culicifacies is the major vector in Pakistan, India and Sri Lanka and malaria incidence is high in this region. In India 60-70% malaria cases are annually transmitted by this species. Any method developed to control this species and consequently malaria would be a welcome proposition. However, because of the wide distribution of the species (Fig.), presence of 4 sibling species with specific mate recognition systems and distinct distribution pattern of sibling species, this species may not be a suitable target species for genetic control.

A. stephensi is an important vector in Iran, Iraq, Pakistan and India. In India and Pakistan, the main vector in urban areas is Anopheles stephensi and in peri-urban areas A. culicifacies transmits malaria. Distribution of this species is given in fig . Urban areas are increasing in number and subsequently, population growth. A. stephensi would therefore find new urban areas and foci of malaria transmission would increase with urban growth. 45-50% of all malaria cases in Tamil Nadu state in

southern India are contributed by Madras city alone. In 1990, 51,312 cases were reported from Madras and 12,092 were reported from Delhi. Because of accute shortage of water and irregular supply of water, overhead tanks are extensively constructed in urban areas. In many towns and cities in addition to overhead tanks, wells are dug sometimes. Further, in construction areas temporary water storing tanks are constructed at the ground level. All these are favourite breeding sites for A. stephensi. A. stephensi transmits malaria in rural areas too.

This species has been the subject of extensive genetic studies in many laboratories world over. So far no biological species has been identified i.e., it is one interbreeding population throughout its range of distribution.

For a pilot project to test the potential of genetic control, A. stephensi appears to be a suitable target spcies. It would be ideal if an urban area is selected making sure that A. stephensi is absent in surrounding rural areas.

Keeping in view the past experiences of genetic control experiments in India, the target species and the area have to be carefully selected. And the concerned authorities have to be fully briefed and consulted before a final decision is made for the possible use of genetic control operations against malaria vectors.

Table 1 MALARIA VECTORS IN THE INDIAN SUB-CONTINENT

Country	Bangladesh	India	Nepal	Pakistan	SriLanka
Anophelines	29	55	36	25	23
Vectors					
A. annularis	+	+	+	-	+
A. culicifacies	-	+	-	+	+
A. dirus	+	+	-	-	-
A. fluviatilis	-	+	+	*	-
A. minimus	+	+	-	-	-
A. philippinensis/ A. nivipes	+	+	-	-	-
A. stephensi	-	+	-	+	-
A. sundaicus	-	+	-	-	-
A. superpictus	-	-	-	+	-
A. subpictus	-	?	-	-	+
A. maculatus	-	-	+	-	-

* May be in some parts
+ Vector of malaria
- Absent, even if present not considered a vector
? Role as a vector is doubtful

Table 2 : List of morphological and biochemical markers in A. culicifacies complex

Marker	Genetic nature	Reference
Linkage Group I		
Species A		
Rose-eye (re)	rec.	Sakai et al., 1977
White-eye (rew)	rec.	Sakai and Baker, 1980
Golden-body (go)	rec.	Sakai et al., 1981b
Ventrally spaced (Vs)	indom.	Sakai et al., 1981c
Red spotted eye (rs)	rec.	Akhtar and Sakai,1984
Species B		
White-eye (w)	rec.	Subbarao et al., 1982
Me 2 alleles	cod.	Adak et al., 1988
Linkage Group II		
Species A		
ODH 3 allles	cod.	Ahmed et al., 1978
GOT 2 alleles	cod.	Sakai et al., 1979a
Maroon-eye (ma)	rec.	Sakai et al., 1979a
Black (Blk)	dom.	Dubash et al., 1982a
Green (Blkgn)	indom.	Dubash et al., 1982b
Yellow (Blky)	rec.	Dubash et al., 1982b
Abnormal eye (ae)	rec.	Sakai et al., 1985
Brown eye (be)	rec.	Sakai et al., 1985
Colorless eye (c)	rec.	Sakai et al., 1985
Scarlet eye (sca)	rec.	Sakai et al., 1985
Vermilion-eye (v)	rec.	Adak et al., 1983
Species B		
Creamish-larva	rec.	Subbarao et al., 1982
Linkage Group III		
Species A		
ACPH 2 alleles	cod.	Ahmed et al., 1978
PGM 2 alleles	cod.	Dubash et al., 1981
EST 4 alleles	cod.	Dubash et al., 1982c
Est 2 alleles	cod.	Dubash et al., 1982c
Species B		
Red-thorax	dom.	Subbarao et al., 1982

rec. - recessive, cod. - codominant and indom. - incompletely dominant

Table 3 SPECIES COMPLEXES IDENTIFIED IN VECTORS IN THE INDIAN SUB-CONTINENT

Complexes	Sibling species composition in Bangladesh	India	Nepal	Pakistan	SriLanka
A. annularis (2)	?	A,B	?	-	?
A. culicifacies (4)	?	A,B,C,D	B	A,B	B
A. dirus (7)	D	E,D (?)	-	-	-
A. fluviatilis (3)	?	S,T,U	-	-	-
A. maculatus (8)	?	?	-	-	-
A. minimus (3)	?	C	-	-	-
A. subpictus (2)	-	A,B	-	-	?

( ) No. of sibling species identified
? Sibling species composition not known
- Complex absent

Table 4 : List of morphological and biochemical markers in A. stephensi

Marker	Genetic nature	Reference
Linkage Group I		
White-eye (w)	rec.	Aslamkhan, 1973
Rose-eye (wro)	rec.	Aslamkhan and Gul, 1979
Chestnut-eye (wt)	rec.	Rathor et al., 1983b
Red-eye (r)	rec.	Sharma et al., 1979
Scarlet eye (wsea)	rec	Akhtar and Sakai, 1985
Pigmentless (p)	rec.	Akhtar and Sakai, 1985
Red-spotted eye (prs)	rec.	Akhtar and Sakai, 1985
Me 2 alleles	cod.	Adak et al., 1993
Linkage Group II		
ADH 3 alleles,dimer	cod.	Iqbal et al., 1973a
ACPH 2 alleles	cod.	Iqbal et al., 1973b
Stripe larva (stp)	indom.	Sakai and Baker,1974
Colorless-eye (c)	rec.	Sharma et al., 1977
Green larva (gl)	rec.	Suguna, 1981
Spot larva (spl)	rec.	Suguna, 1981
Maroon-eye (mar)	rec.	Mahmood and Sakai,1982
EST-3 2 alleles	cod.	Adak et al., 1984
EST-4 3 alleles	cod.	Adak et al., 1984
EST-5 3 alleles	cod.	Adak et al., 1984
2nd-3rd coastal Spots fused (2-3f)	rec.	Akhtar and Sakai, 1985
Spotless wing (sl)	rec.	Akhtar and Sakai, 1985
Green larva (gn)	rec.	Akhtar and Sakai, 1985
IDH-2 2 alleles	rec.	Adak et al.
Linkage Group III		
PGM 5 alleles	cod.	Bullini et al., 1971
EST-2 2 alleles	cod.	Iqbal et al., 1973b
Green-larva (g)	rec.	Subbarao and Adak, 1978
Greenish brown larva (gb)	rec.	Sharma et al., 1979
Diamond palpus (dp)	rec.	Sakai et al., 1981a
Black scale (bs) Curved proboscis	rec.	Rathor and Rashid, 1983
Short palpus (sp2)	rec.	Rathor et al., 1983a
Black larva (Bl)	indom.	Akhtar et al., 1982
Short curved probiscis/ Palpus (Scp)	rec.	Akhtar and Sakai, 1985
Golden-yellow (gy)	rec.	Adak et al., 1990
6-PGD 2 alleles	cod.	Adak et al., 1992
MDH 2 alleles	cod.	Adak et al., 1992

rec. - recessive, cod. - codominant and indom. - incompletely dominant

Table 5

Malaria situation in the Indian sub-continent

Country	No. of malaria cases in millions	Pf %
Bangladesh	.054	63.2%
India	1.78	35-37%
Nepal	.023	7.5%
Pakistan	.08	50%
Sri Lanka	.287	20%

Figure 1 DIAGNOSTIC INVERSION GENOTYPES AND MALE KARYOTYPES

A. culicifacies COMPLEX

Species A - $X^{+}\ ^{a\ b}_{+}$, $2^{+}\ ^{1\ 1}_{+}$  Y-sub-metacentric

Species B - Xab, $2g^{+}\ ^{1\ h}_{+}$  Y-acrocentric

Species C - Xab, $2^{+}\ ^{g\ 1}_{h}$  Y-sub-metacentric

Species D - $X^{+}\ ^{a\ b}_{+}$, $2i^{+}\ ^{1\ h}_{+}$  Y-sub-metacentric

Fig.2 Linkage map of A.culicifacies

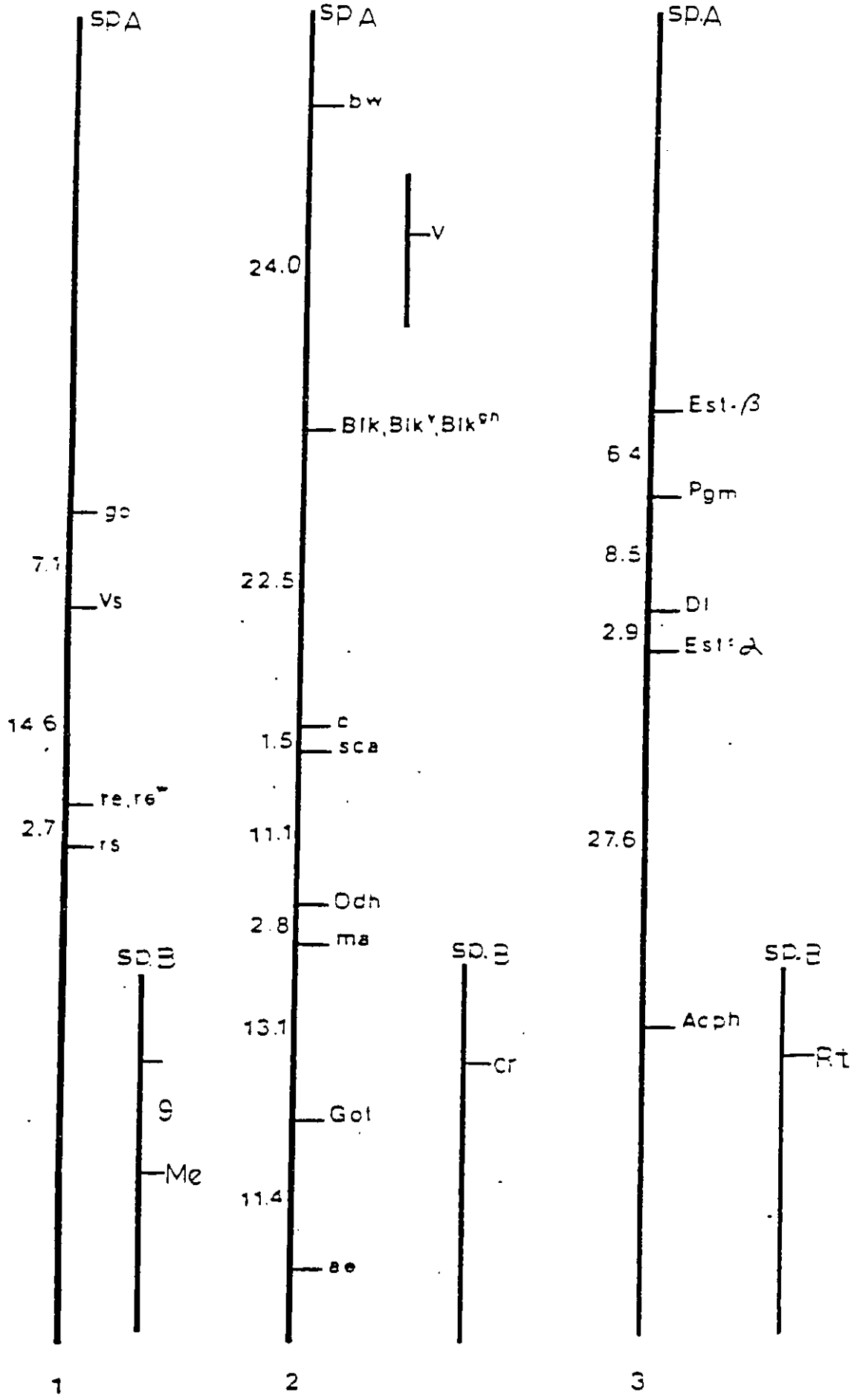


Fig.3

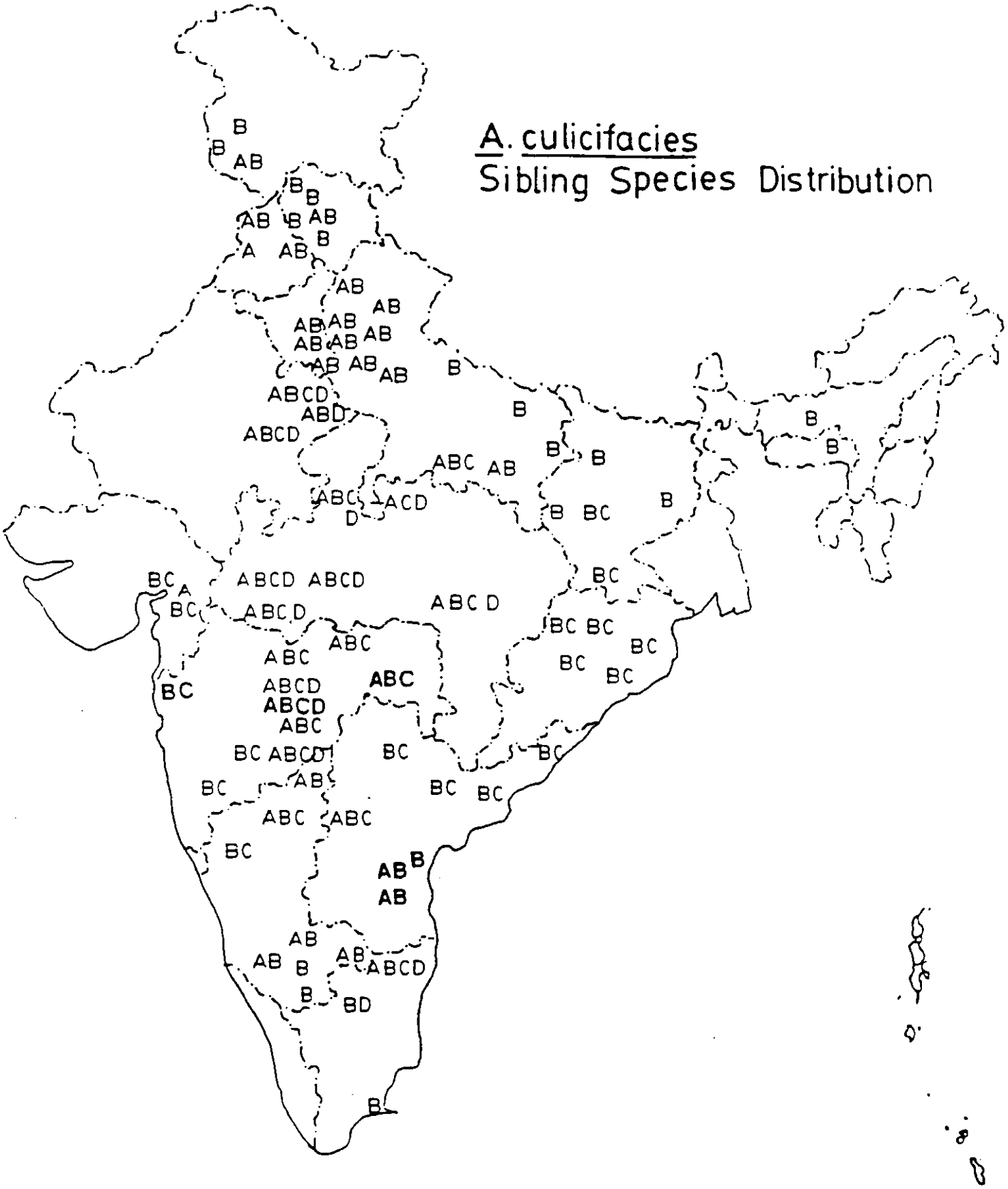
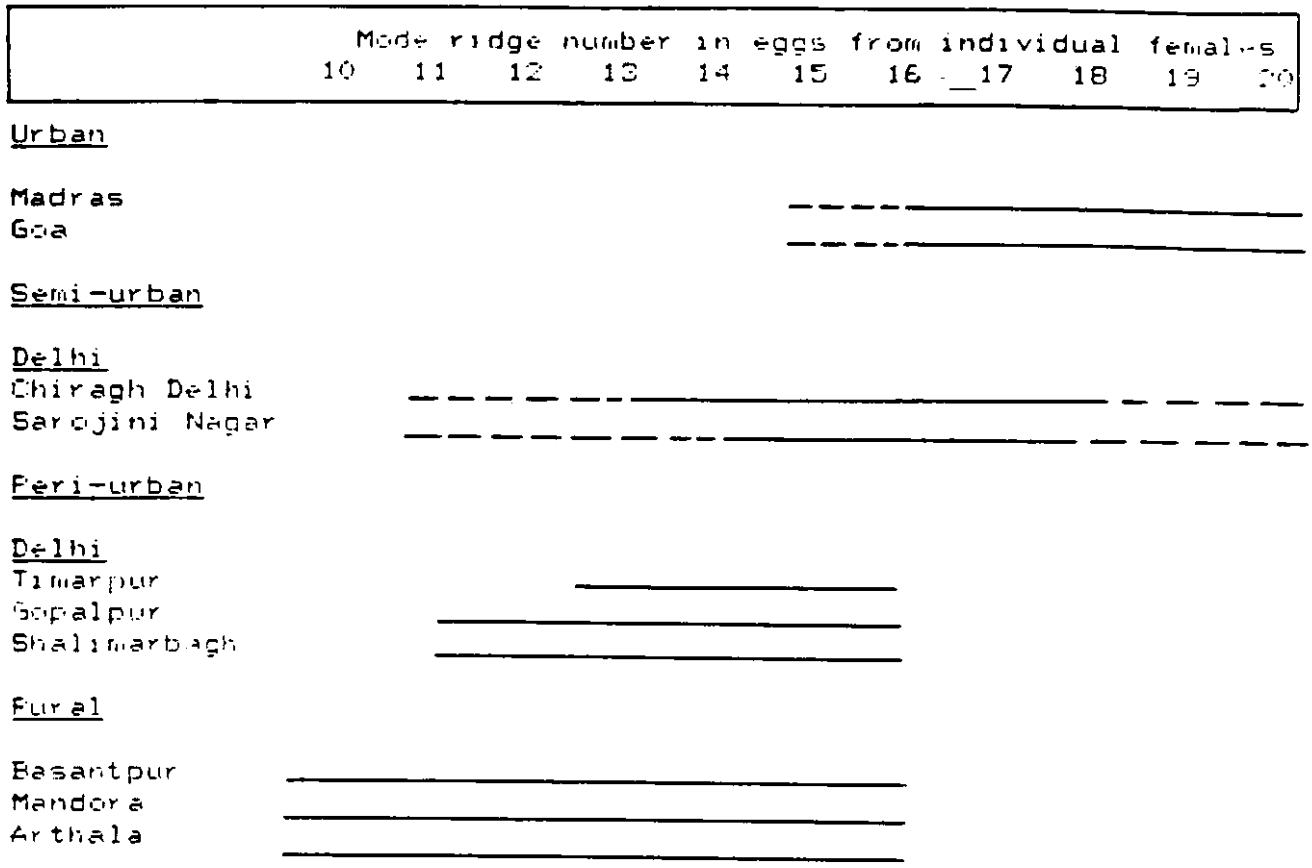


Figure 4 **A. STEPHENSI EGG-FLOAT RIDGE NUMBER**



Type form 16-19 Intermediate 13-16 Var. mysorensis 10-14

Figure 5 Linkage map of A.stephensi

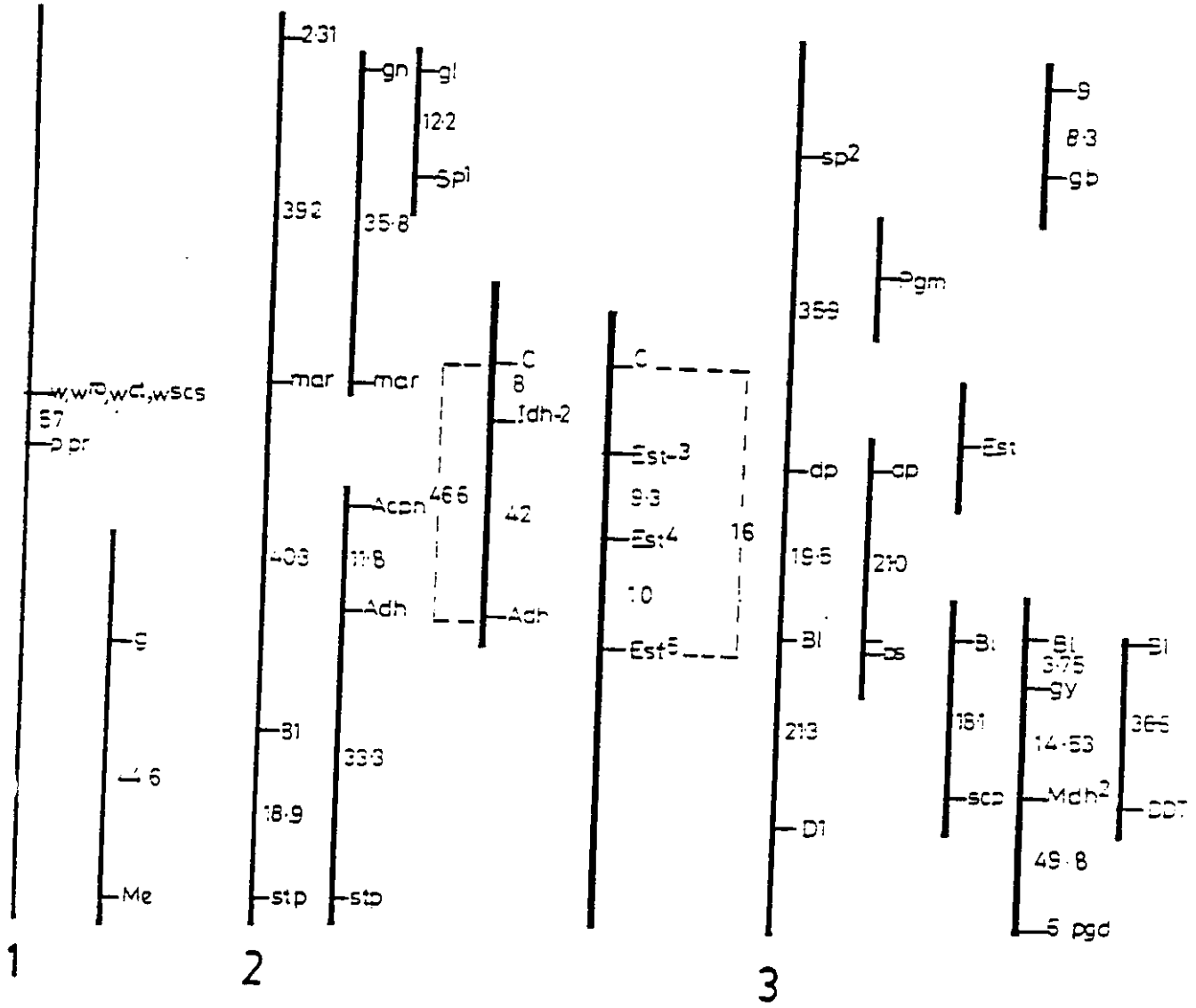


Fig. 6 Distribution of A.culicifacies in the Indian Sub-continent

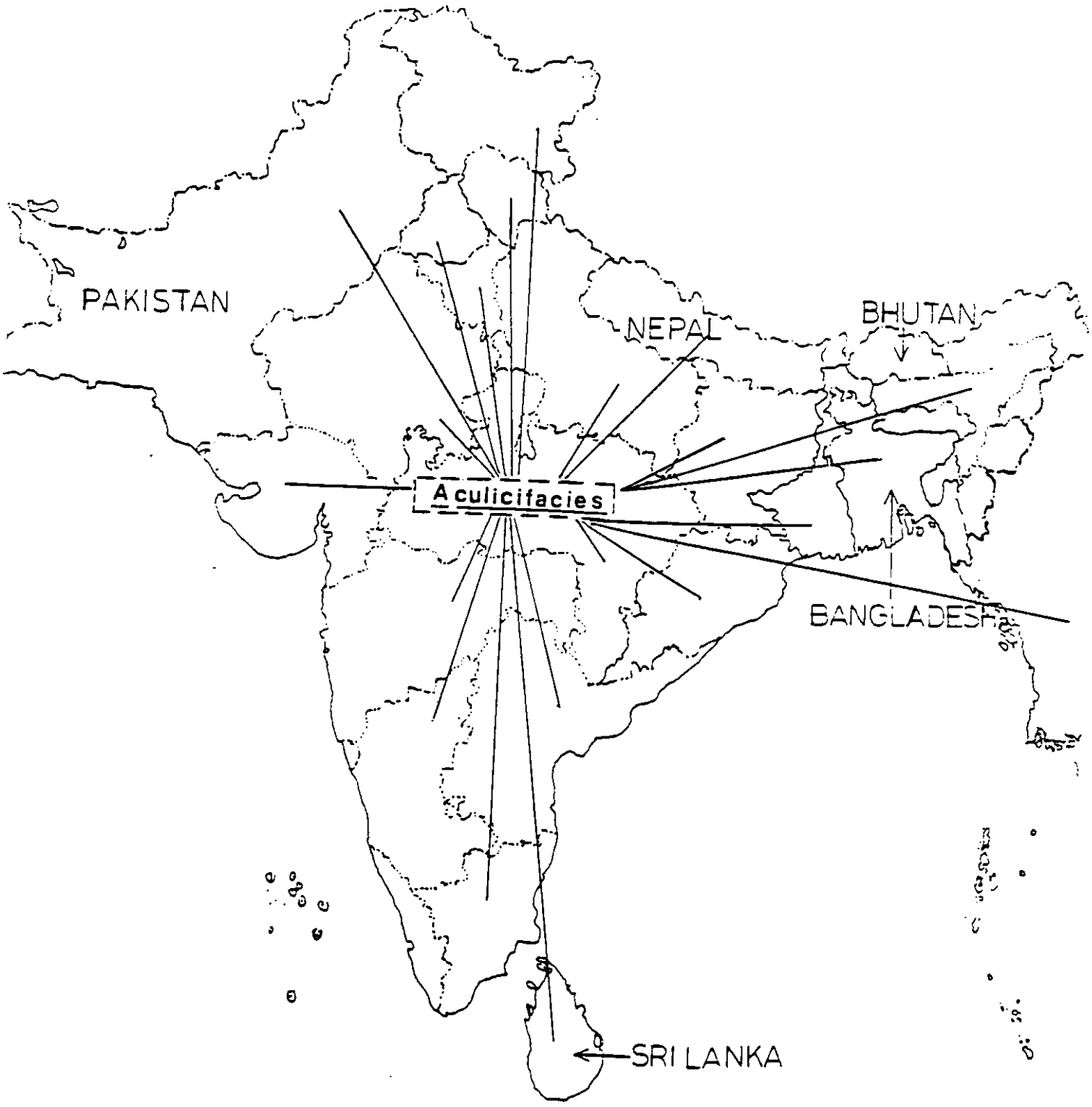
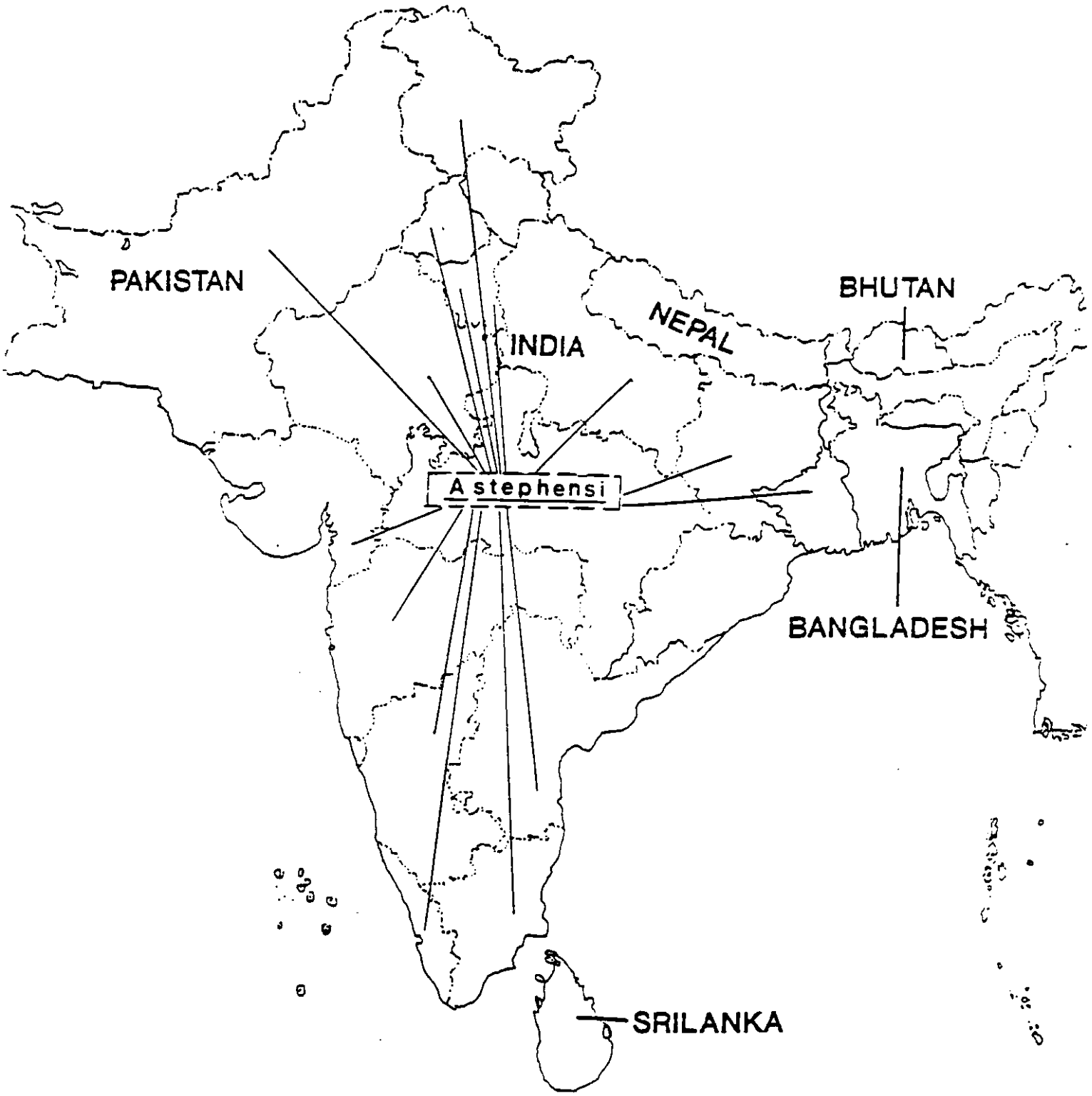


Fig. 7 Distribution of A.stephensi in the Indian Sub-continent



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MOLECULAR PERSPECTIVES ON THE GENETICS OF MOSQUITOES

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I. Introduction

Mosquitoes have been a focus of scientific study since the turn of the century, when they were first linked with human diseases. This review concentrates on the three most intensely studied genera, *Anopheles*, *Culex*, and *Aedes*. These genera include the principal vectors of three major groups of human pathogens: malaria parasites of the genus *Plasmodium*, filarial worms of the genera *Wuchereria* and *Brugia*, and numerous arboviruses. Anophelines are the only mosquitoes known to

transmit human malaria parasites, a group of organisms that may be responsible for more morbidity and mortality worldwide than any other human pathogen. Anophelines also transmit filarial worms, as do *Culex* and *Aedes* species. Among the 14 or more different mosquito genera known to harbor arboviruses (Mattingly, 1973), the most important are *Culex* and *Aedes,* which include the principal vectors of yellow fever, dengue, and most encephalitis-causing arboviruses.

Although the connection between mosquitoes and human diseases has been recognized for more than a century (see Harrison, 1978), instances of successful control of either vector populations or the disease agents they transmit are limited in both number and scope in non-Western countries. Most notable has been the failure to eliminate malaria from much of the developing world, especially tropical Africa, in spite of an almost 20-year worldwide malaria eradication campaign directed by the World Health Organization. Attempts to control mosquitoes have been frustrated by both their enormous reproductive capacity and their genetic flexibility. The latter is evidenced not only by rapid development of insecticide resistance but also by the multitude of closely related, cryptic species complexes, some of which appear to be undergoing incipient speciation in the process of adapting to human environments (Coluzzi, 1984; Coluzzi *et al.,* 1985). Further impediments to the control of vector-borne diseases include the rapid spread of drug resistance throughout parasite populations, the increasing movement of people into and out of disease-endemic regions, and the limited funds and public health infrastructure in most developing countries. Thus the widespread use of residual insecticides or antiparasitic drugs has not been and will not be a sustainable solution to the problem of vector-borne disease control. New approaches are needed. The enormous impact of recent developments in molecular genetics on questions of basic biology and human disease has stimulated a reexamination of the prospects for genetic manipulation of vector populations as a means for reducing or eliminating vector-borne diseases, especially malaria (World Health Organization, 1991a,b). Though the specific disease control scenarios currently being considered, such as the replacement of competent vector populations with pathogen-refractory genetic strains, may not be realized, the increase in knowledge of basic mosquito biology on which these control scenarios depend will inevitably stimulate novel approaches to the control of mosquito-borne diseases.

The identification of new vector control strategies depends on deepening our basic understanding of all aspects of mosquito biology from

molecular to population levels, including the relationship between the mosquito and the pathogens it transmits. Molecular genetic research over the past two decades has led to the development of recombinant DNA technology, providing the tools for detailed study of individual genes, genome organization, population genetics, and evolution. The application of this technology in mosquito research, now underway in a growing number of laboratories, has been greatly accelerated by adapting molecular techniques and drawing on the already extensive molecular data base from *Drosophila melanogaster,* one of the best genetically understood higher organisms (Ashburner, 1989). Many regulatory and structural DNA sequences are sufficiently conserved between *Drosophila* and mosquitoes such that some *Drosophila* gene constructs and transformation tools may function in mosquitoes with little modification. Cloned *Drosophila* DNA will be valuable in isolating relatively conserved homologous coding and regulatory sequences from mosquito genomic DNA and cDNA libraries and directly from polymerase chain reaction (PCR)-amplified genomic DNA and RNA. However, molecular genetic studies are also confirming the perspective of classical taxonomists, who would warn that mosquitoes and *Drosophila* are on two deeply diverged branches of their order. Thus we believe it is appropriate to caution that many homologous genes in *Drosophila* and mosquitoes may be so diverged that cloned *Drosophila* sequences will not hybridize specifically under any conditions to their mosquito homologues.

The formal genetics and cytogenetics of mosquitoes have been reviewed elsewhere (Wright and Pal, 1967; Kitzmiller, 1976; Steiner *et al.,* 1982). The present review attempts to integrate the variety of recent molecular studies performed on mosquitoes of the genera *Anopheles, Culex,* and *Aedes.* Because such studies have been initiated only recently, this review will rely heavily on unpublished information generously contributed by many colleagues and will inevitably highlight the need for more work on all fronts. The first section deals with how molecular techniques have been used to reveal genetic differentiation, both for the identification of cryptic species and for the understanding of population structure and evolution. Next is a discussion of genome organization as revealed by reassociation kinetics, molecular cloning, and genome mapping. Following this, a section covers the molecular biology of specific physiological systems, including insecticide resistance, immune mechanisms, oogenesis, and salivation. Finally, identification of endogenous mobile elements and potential transformation systems for both cell lines and embryos are considered.

II. Genetic Differentiation

A. Classification

Mosquitoes are classified as lower flies within the insect order Diptera. The branching of lineages leading to lower and higher flies is ancient, probably dating from the Triassic (McAlpine and Wood, 1989). Thus, comparison of the coding regions of the α-amylase gene from three Lepidoptera and three Diptera (the lower fly *Anopheles merus* and two higher flies, *D. melanogaster* and *Drosophila virilis*), shows the *Anopheles* to be as diverged from the *Drosophila* as it is from the Lepidoptera (D. Hickey, unpublished). The family Culicidae, to which mosquitoes belong, is considered monophyletic, probably originating from a Chaoborid-like ancestor (White, 1980). Three subfamilies of the Culicidae are generally recognized: Anophelinae, Toxorhinchitinae, and Culicinae. With the single known exception of *Chagasia bathana* (which, like the chaoborids, has a diploid chromosome number of eight), all mosquitoes have a diploid chromosome number of six (White, 1980).

To determine evolutionary relationships among taxa, Rao and Rai (1987a, 1990) analyzed chromosomal rearrangements, differences in amount and position of C-banded heterochromatin, and nuclear DNA amounts in mosquitoes and their close relatives. Although they state that Toxorhinchitinae is closer to Chaoboridae and is primitive within Culicidae, they present a phylogenetic tree in which the anopheline branch is the first to diverge from the culicid line followed later by the Toxorhinchitinae and finally reaching the Culicinae branches. The phylogenetic relationship among representative species from most of the major genera in the family Culicidae and several related lower flies has also been examined by comparison of DNA sequences from a hypervariable domain of the large ribosomal subunit (28S) coding region (C. Porter, D. Wesson, and F. Collins, unpublished). Both phenetic and cladistic analyses of this ribosomal DNA (rDNA) support a monophyletic origin of the family Culicidae but suggest a very early evolutionary divergence between the Anophelinae and the subfamilies Toxorhynchitinae and Culicinae (Fig. 1). Indeed, differences in this rDNA region are greater among the members of the three different *Anopheles* subgenera represented in Fig. 1 than between the subfamilies Culicinae and Toxorhynchitinae. Other evidence from anophelines supports their extensive divergence from other mosquitoes, including significantly smaller chromosomes and lower nuclear DNA content (Rao and Rai, 1990), and their unique possession of dimorphic sex chro-

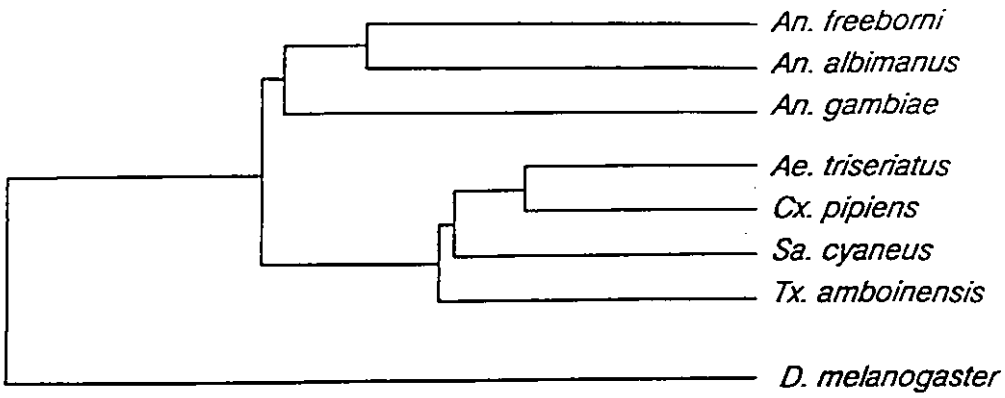

FIG. 1. Similarity dendrogram inferred from comparison of D2 expansion domains of 28S rDNA coding regions.

mosomes and long-period interspersion of repetitive sequences in the genome (Black and Rai, 1988). Taken together, the data suggest that Anophelinae is primitive and Toxorhynchitinae intermediate with Culicidae. Interestingly, only Anophelinae and Culicinae contain vectors of human pathogens, because of obligate blood feeding by the adult females. The reproductive strategy of the Toxorhynchitinae does not involve blood feeding, an ability that seems to have been lost.

B. PATTERNS OF GENETIC VARIATION

The ribosomal RNA genes (rDNA) are frequent targets for evolutionary comparisons over a broad range of taxonomic hierarchies because of contrasting levels of variation among their architectural components (reviewed in Beckingham, 1982; Gerbi, 1985). In 20 mosquito species of the subfamilies Anophelinae and Culicinae, 40–1000 rDNA repeat units are tandemly arrayed at a single locus on the sex-determining chromosomes, except for *Aedes triseriatus* and one of two strains of *Aedes albopictus* examined, each of which have an additional rDNA locus on an autosome (Kumar and Rai, 1990a; A. Kumar and K. S. Rai, unpublished). As in other Diptera, each mosquito rDNA repeat unit consists of a transcribed region containing highly conserved 18S, 5.8S, and 28S coding sequences separated by spacers that evolve extremely rapidly (Faria and Leoncini, 1988; McLain and Collins, 1989; McLain *et al.*, 1989; Collins *et al.*, 1989; Beach *et al.*, 1989; Black *et al.*, 1989; Gale and Crampton, 1989). Adjacent to the transcribed region is the nontranscribed intergenic spacer (IGS), which is usually highly variable in length within and between individuals due to different numbers of small internal subrepeats.

An understanding of the underlying architecture of the rDNA IGS

allows the use of IGS probes to measure population subdivision. Whereas very little difference in the set of IGS length variants is detected among individuals from laboratory colonies, examination of specimens from field populations has revealed variation that increases with genetic and geographic distance (Collins *et al.*, 1989). In a study of gene flow among *Anopheles gambiae* populations from seven different villages in western Kenya, seven different rDNA spacer genotypes based on length variants were identified (McLain *et al.*, 1989). The frequency distribution of these genotypes revealed restricted gene flow between populations separated by less than 10 km and complete isolation of populations at distances no greater than a few hundred kilometers. Preliminary results from a study of field-collected *Anopheles albimanus* specimens from Panama similarly showed intra- and inter-mosquito differences in spacer genotypes, with more variation between than within populations. However, no particular variant was characteristic of populations from a given site (Beach *et al.*, 1989; A. M. de Merida, C. Porter, and F. Collins, unpublished). Unlike the situation in *Anopheles,* however, most of the IGS variation in *Aedes* was accounted for by diversity within populations rather than between populations, with no correlation between genetic and geographic distances based on total IGS length (Black *et al.*, 1989; Kambhampati and Rai, 1991c). Among the newly established United States populations of this mosquito, variation at the IGS has occurred rapidly and independently in adjacent populations.

Other rDNA sequences have also been used in evolutionary studies of several species groups within the family Culicidae. The most extensive work to date has involved anopheline mosquitoes in the subgenus *Anopheles,* with a focus on species in the North American *Anopheles maculipennis* group (C. Porter and F. Collins, unpublished). Two regions of the rDNA were analyzed in this work: the rDNA internal transcribed spacer (ITS2) separating the 5.8S and 28S coding regions and a hypervariable domain (D2 expansion domain) near the 5′ end of the 28S gene. Relationships among members of closely related species complexes (such as the *Anopheles quadrimaculatus* or *Anopheles punctipennis* complexes) were determined primarily by ITS2 sequence analysis, whereas comparison of the D2 region of the 28S gene proved more useful for more diverged taxa. For example, whereas the ITS2 sequences of the morphologically identical species *Anopheles freeborni* and *Anopheles hermsi* differ by approximately 3.7%, no differences were observed in the D2 region. Among the four species in the *An. quadrimaculatus* complex, differences in the ITS2 regions ranged from

4.5 to 10.7%, whereas D2 region sequence differences ranged from 0.5 to 2.2%. In sequence comparisons among different species complexes in the *An. maculipennis* group, large domains of the ITS2 region could not be aligned. In contrast, most of the D2 region remained taxonomically informative in all comparisons within the subgenus *Anopheles*. Very little within-species variation in the ITS2 sequences has been observed in anopheline mosquitoes, possibly because their rDNA is tandemly arranged at a single locus on either the X or both the X and Y chromosomes and is subject to relatively high levels of within-locus homogenizing processes.

Just as more IGS length variation was found in *Aedes* than *Anopheles* populations, analysis of the ITS2 regions from various *Aedes* species and allied genera revealed more intraspecific sequence variation than was observed in the genus *Anopheles* (D. Wesson, C. Porter, and F. Collins, unpublished). Again, almost all variation in *Aedes* ITS2 sequences was observed in single individuals, and most was due to the presence or absence of short insertions/deletions of between 1 and 6 bp. These differences were not randomly distributed among ITS2 clones but occurred as correlated sets, suggesting the presence of several distinct ITS2 types within one individual. Though the basis of this typology in *Aedes* ITS2 sequences has not yet been established, it is consistent with independent rDNA spacer sequence divergence that could occur at multiple rDNA loci. Indeed, rDNA has been localized by *in situ* hybridization to two different chromosomes in 2 of 13 *Aedes* species examined (Kumar and Rai, 1990a; A. Kumar and K. S. Rai, unpublished). This level of rDNA spacer variation may be characteristic of all mosquitoes on the culicine–toxorhynchitine branches of the Culicidae with homomorphic rather than heteromorphic sex chromosomes. In all mosquitoes examined thus far, the position of all or at least some of the rRNA genes is conserved on the sex-determining chromosomes (Kumar and Rai, 1990a).

Comparison of the 28S D2 regions and ITS2 sequences indicates that the subfamilies Culicinae and Toxorhynchitinae are much more closely related than the *Anopheles* subgenera *Cellia*, *Anopheles*, and *Nyssorhynchus* (Fig. 1). Though within-species polymorphisms in the ITS2 region complicate its use for phylogenetic analysis of *Aedes* and related genera, overall conservation of ITS2 sequences, even between different genera such as *Aedes* and *Hemagogus*, allows much of this region to be aligned (D. Wesson, C. Porter, and F. Collins, unpublished). By contrast, levels of ITS2 sequence divergence among members of anopheline cryptic species complexes are equivalent to those

observed among different *Aedes* subgenera. This may reflect the subjectivity of higher taxonomic groupings of mosquitoes as much as differences in their biology.

Mitochondrial DNA (mtDNA) is another useful tool for genetic analyses. It is commonly used to investigate population structure because it is maternally inherited and different mtDNA genomes recombine only rarely, if at all. The complete mitochondrial genomes of two anophelines, *An. quadrimaculatus* sp. A (Cockburn *et al.*, 1990) and *An. gambiae* (D. Mills Hamm and F. Collins, unpublished), and portions of the *Ae. albopictus* (HsuChen *et al.*, 1984; Dubin *et al.*, 1986) mitochondrial genome have been cloned and sequenced. In sequence, gene order, and orientation of coding regions, these mosquito mtDNAs are similar to those of *Drosophila yakuba* and *D. melanogaster*. As has been observed within all other insect and vertebrate mtDNAs sequenced, the base composition is highly A + T rich and very little noncoding intergenic sequence is present. However, the mtDNA of *Ae. albopictus* and *An. gambiae* (and thus probably all mosquitoes) do differ from *Drosophila* with respect to the order of three tRNA genes (Dubin *et al.*, 1986; D. Mills Hamm and F. Collins, unpublished). Based on sequence data, the *An. quadrimaculatus* and *D. yakuba* mtDNA origins of replication, both extremely A + T rich, have no significant homology (Cockburn *et al.*, 1990). Variation in the size of the mtDNA between the two species has been attributed to length variation in the origin of replication (Cockburn *et al.*, 1990). Restriction analysis of several anophelines also shows some intraspecies length polymorphism in this region (Collins *et al.*, 1990; F. Collins and C. Porter, unpublished; S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished). The phylogenetic usefulness of sequence-level changes in A + T-rich mtDNA is limited to closely related species because of the rapid saturation of changes at silent codon positions, and the concomitant difficulty in distinguishing identity by descent from identity due to reversion (e.g., DeSalle *et al.*, 1987). On the other hand, structural changes such as the tRNA differences noted above may be useful phylogenetic characters for investigating higher level taxonomic questions outside of Culicidae.

Surveys of mtDNA restriction fragment length polymorphisms (RFLPs) have been conducted among species in the *Aedes scutellaris* and *Ae. albopictus* subgroups (Kambhampati and Rai, 1991a) and among populations of *Ae. albopictus* (Kambhampati and Rai, 1991b) and *Aedes aegypti* (W. J. Tabachnick and D. Zuhn, unpublished). Intraspecies mtDNA RFLPs have also been assessed for sibling species within the *Anopheles albitarsis* (S. K. Narang, T. A. Klein, J. B. Lima,

and A. T. Tang, unpublished), *An. gambiae* (F. Collins and C. Porter, unpublished), *An. freeborni* (Collins *et al.*, 1990), and *An. quadrimaculatus* (S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished) complexes. Although extensive interspecific divergence was revealed in the *Ae. scutellaris* and *Ae. albopictus* subgroups, there was surprisingly little within- or among-population variation in mtDNA haplotypes, even among geographically very distant populations in the case of *Ae. albopictus*. Among 17 worldwide populations of *Ae. albopictus* surveyed, only 3 revealed unique mtDNA RFLPs with a battery of 12 restriction enzymes, and no RFLPs were detected within populations using 4 restriction enzymes (Kambhampati and Rai, 1991b). The result of a survey of mtDNA RFLPs among several populations of *Aedes aegypti* was consistent with low-level intraspecies polymorphism (W. J. Tabachnick and D. Zuhn, unpublished). Where intraspecies polymorphism was detected in the *An. quadrimaculatus* complex, one haplotype usually predominated in all populations surveyed (S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished). Species-specific haplotypes were detected in the case of two sympatric species in the *An. albitarsis* complex (S. K. Narang, T. A. Klein, J. B. Lima, and A. T. Tang, unpublished) and the *An. quadrimaculatus* complex (S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished). Only the *An. gambiae* complex showed high levels of intraspecific mtDNA polymorphism (F. Collins and C. Porter, unpublished). For example, analysis of polymorphism among 30 field-collected *Anopheles arabiensis* from Kenya using restriction enzymes that surveyed roughly 7% of the mtDNA genome revealed five distinct haplotypes, four of which were present at frequencies of 0.13 or higher. Similar levels of polymorphism were observed in four other species in this complex, but none of the restriction profiles was unique to a single species. Comparison of within- and between-species mtDNA sequence polymorphism over a 1400-bp region encoding the ND4 and ND5 subunits and several tRNA genes confirmed this relatively high level of intraspecies polymorphism (0.55% difference between three different specimens of *An. gambiae*) relative to interspecies differences (1.3% consistently different bases between *An. gambiae* and *An. merus* and only 0.13% difference between *An. gambiae* and *An. arabiensis*). In fact, the number of shared mtDNA haplotypes and specific nucleotide site polymorphisms suggests recent and perhaps continuing mtDNA introgression between these latter two species.

Though many of these population and species complex studies of mtDNA are preliminary, the widely reported low intraspecific levels of

restriction site variation suggest that mtDNA may not be as useful a tool for population genetic analysis of mosquitoes as it has proved to be for other organisms. The only obvious exceptions appear to be the members of the *An. gambiae* complex, which are species with very large and temporally stable geographic distributions in tropical and subtropical Africa. The distributions of both of the *Aedes* species studied have become widespread only relatively recently through association with human commerce (Kambhampati and Rai, 1991a), and the *An. quadrimaculatus* and *An. freeborni* complex mosquitoes are North American species that probably experience substantial fluctuations in overall population size and distribution during winter months.

More recently, a technique known as RAPD-PCR (Williams *et al.*, 1991) has been used to analyze population structure and for species identification. It is based on the use of individual random sequence tenmers as PCR primers. RAPD-PCR amplification of insect genomes results in a complex pattern of bands, visualized by electrophoresis and ethidium bromide staining of agarose gels. This technique revealed large numbers of polymorphisms between individuals and populations of *Ae. aegypti* (Ballinger-Crabtree *et al.*, 1992), but demands meticulous care in its application and in interpretation of the results (Kambhampati *et al.*, 1992).

C. Identification of Cryptic Species

Until fairly recently, only small numbers of morphologically indistinguishable mosquito species were recognized, most notably the *An. maculipennis* complex discovered in the 1930s followed by the *An. gambiae* complex in the 1960s. The idea that many species cannot be easily identified in the adult stage was difficult for many "cabinet" taxonomists (see Bates, 1949) and for many nontaxonomists reluctant to see their malaria control studies impeded (B. Harrison, personal communication). The existence of large numbers of mosquito species that are nearly identical or that are morphologically indistinguishable as adults is finally gaining wide recognition, and *Anopheles* vector identification in particular is now recognized as a major problem in many areas of the world. Of the 66 *Anopheles* species currently believed to be important vectors of human malaria parasites, at least 55% belong to closely related groups of nearly identical species, or to "cryptic species" complexes (B. Harrison, unpublished). As methods for identifying mosquito species move closer to direct genome analysis, from comparisons of morphological characters through mitotic and

polytene chromosome banding patterns to isozymes and, ultimately, DNA sequence, the rate of discovery of new cryptic species has increased. Though interesting from an evolutionary perspective, the existence of cryptic sibling species complexes also poses practical problems in vector control because different complex members may differ in their involvement in disease transmission and their response to various control measures (Coluzzi, 1992). It is therefore important to assess differences between species as they relate to disease transmission, such as biting and resting preferences of adult females, seasonal distribution and abundance, competitive ability, and microhabitat preferences. These data are relevant to the choice and monitoring of vector control strategies and the ability to predict the impact of human activities such as deforestation, irrigation, and desalination on disease transmission (Coluzzi, 1984; 1992).

1. Random Repetitive Probes

Several DNA-based methods have been developed to distinguish among the members of cryptic species complexes. The most widely used approach has been to identify species-specific differences in the undefined highly repeated component of the genome by differential screening of small-fragment genomic libraries with labeled homologous and heterologous genomic DNA. The highly repeated sequences isolated by this strategy typically consist of tandem repeats of 50- to 100-bp monomers present in numbers between 10,000 and 100,000 or more per genome. The high copy numbers make these probes extremely sensitive, enabling species identification in some cases from less than 1 ng of target DNA (or small portions of a specimen). Several laboratories have explored the use of dried or isopropanol-fixed mosquitoes in a squash–blot format, nonisotopic detection systems, and synthetic oligonucleotides based on the probe repeat sequence, all with favorable results. Such modifications should facilitate the use of these assays in studies designed to address population-level questions. Ideally, species diagnosis should be based on probes that are absolutely species specific over the entire species range. However, assays have been devised using a combination of probes that hybridize to different subsets of species in a complex. The species specificity of such probes depends on copy number or sequence variation. These possibilities can be distinguished by varying the stringency of the hybridization conditions, though this step has generally not been taken in published reports. The validity of the probes for species diagnosis should be assessed by careful field testing, especially in areas where they are to be

used, because highly repetitive sequences can diverge rapidly in both copy number and sequence between and even within populations (see Section III,B).

DNA dot–blot or mosquito squash–blot assays have been developed for at least four different anopheline complexes, the *An. quadrimaculatus, Anopheles dirus, An. gambiae,* and *Anopheles farauti* complexes. A squash–blot assay to separate species A, B, C, and D of the *An. quadrimaculatus* complex is based on probes that hybridize intensely to one species and sometimes hybridize slightly to one or more other members of the complex (Cockburn, 1990). In the *An. dirus* complex, cloned probes have been developed that uniquely hybridize with sibling species D (Yasothornsrikul *et al.*, 1986; Panyim *et al.*, 1988) or species B (Kertbundit *et al.*, 1986). The probes are sensitive enough to allow positive identifications from as little as 5 ng of DNA, estimated to be about 1/150th the amount of DNA available from a single mosquito. Panyim *et al.* (1988) tested species D probe pMU-D76 against all four species using progeny of females collected from a single geographic area (eight nearby sites). It is unclear whether this probe is valid throughout the range of the species or whether the other probes have been field tested. Another assay for distinguishing the members of this complex has recently been reported (Yasothornsrikul *et al.*, 1988). Preliminary data from the progeny of single field-caught specimens of each species revealed unique restriction fragment patterns of highly repeated DNA superimposed on a background smear of genomic DNA visualized from ethidium bromide-stained gels. Although presented as an alternative method for species diagnosis with the advantages of speed and simplicity, it is unlikely to replace sensitive dot–blot or squash–blot approaches.

DNA dot–blot and mosquito squash–blot protocols have also been developed for the identification of five members of the *An. gambiae* complex, based on the use of cloned probes containing highly repetitive DNA sequences (Gale and Crampton, 1987a,b, 1988). Each probe is very sensitive due to high copy number, but when used alone cannot distinguish all species and must be used in combination with other probes for positive species identification. One probe, pAna1, is male specific and separates female *An. arabiensis* from the other freshwater species only by probing inseminated spermathecae. In spite of these limitations, the technique has been successfully applied to wild-caught female mosquitoes, which were stored in isopropanol and later squashed onto nitrocellulose (G. Yemane, S. Hill, R. Urwin, A. T. Haimanot, and J. Crampton, unpublished). Three probes were used to differentiate mixed populations of *An. gambiae, An. arabiensis,* and

Anopheles quadriannulatus from four locations in Ethiopia. Although 149 of the 153 specimens collected were identified with these probes, none of these identifications were confirmed by an alternative method such as analysis of polytene chromosomes. As is the case in other laboratories, the technique is being improved for field use by incorporating several modifications that increase its speed, simplicity, safety, and economy. These include the substitution of cloned probes by synthetic oligonucleotides 21–26 bases long (Hill *et al.*, 1991a) and the use of nonradioactive labeling and detection protocols (Hill *et al.*, 1991b, 1992).

Similar work with the *An. farauti* complex led to the development of two sets of probes to distinguish three known members, *Anopheles farauti* Nos. 1, 2, and 3 (Booth *et al.*, 1991; Cooper *et al.*, 1991). Identifications using the probes for mosquitoes captured over a broad geographic area were verified by established identification methods. Although the use of these probes resulted in correct identifications most of the time, there were problems with specificity (Booth *et al.*, 1991) and sensitivity (Cooper *et al.*, 1991).

2. *mtDNA and rDNA Probes*

Species-specific RFLPs in mtDNA genomes or the rDNA multigene family have been described for at least three species complexes, the *An. quadrimaculatus, An. gambiae,* and *An. freeborni* complexes. In the *An. quadrimaculatus* complex, species-specific differences were found in the restriction enzyme map for both mtDNA (S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished) and rDNA (S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished). One enzyme, *Cla*I, was found to produce diagnostic mtDNA restriction patterns for each species (Mitchell, 1990); all others tested required separate digestions with two different enzymes to discriminate between all four sibling species (S. Mitchell, S. K. Narang, A. F. Cockburn, J. A. Seawright, and M. Goldenthal, unpublished). Species-specific differences were found in the rDNA IGS or ITS regions of members of the *An. freeborni* (Collins *et al.*, 1990) and *An. gambiae* (Collins *et al.*, 1987, 1989; Finnerty and Collins, 1988) complexes. Diagnostic methods for the *An. gambiae* species complex based on rDNA RFLPs have been validated from field collections by comparison with both cytogenetic (Collins *et al.*, 1988a) and allozyme (Collins *et al.*, 1988b) methods. Interestingly, crossing studies combined with rDNA RFLP analyses of field-collected *An. freeborni* and its sibling species *An. hermsi* not only confirmed the DNA probe method developed to distinguish this cryptic species pair

but also invalidated a character, an X chromosome inversion, previously considered to be diagnostic for *An. hermsi* (Fritz *et al.*, 1991).

Detection of species-specific differences in the rDNA spacers has been extended by using the polymerase chain reaction in various *Anopheles* complexes, including *An. gambiae* (Paskewitz, and Collins, 1990; J. Scott, W. Brogdon, and F. Collins, unpublished). *An. freeborni* (Porter and Collins, 1991), and *An. maculatus* (L. G. Chiang, C. Porter, and F. Collins, unpublished). Though this format is relatively expensive in its requirement of a thermal cycler and thermostable polymerase, it is very rapid, relatively simple, and has virtually 100% sensitivity and specificity. Furthermore, the amount of DNA required is so small that living specimens can be identified by using DNA from a single segment of a mosquito leg. The actual identification is accomplished by visualizing the PCR-amplified DNA in an ethidium bromide-stained gel, without the steps of transferring DNA to a membrane and probing. In the *An. gambiae* complex, the method exploits differences in the 5′ end of the IGS. In the published work, three primers were used to distinguish *An. gambiae* from *An. arabiensis,* one universal plus-strand primer and two species-specific minus-strand primers. When combined in the same PCR reaction, fragments of unique lengths are produced based on the source of template DNA. This has now been extended to permit identification of five members of the complex using various combinations of one universal plus-strand primer with five species-specific minus-strand primers, each of which amplifies a single fragment of unique length diagnostic of a single species (J. Scott, W. Brogdon, and F. Collins, unpublished). Successful species identification can be achieved from a PCR reaction that incorporates all six primers, but in practice usually no more than three members of the *An. gambiae* complex are sympatric in any part of Africa.

Extensive comparison of the approximately 450-bp ITS2 sequences from 48 different field and colony specimens of five members of the *An. gambiae* complex revealed very few (one to five) species-specific differences from the consensus. By contrast, differences among ITS2 sequences in many other *Anopheles* cryptic species complexes, including the *An. freeborni*, *An. maculatus*, *An. quadrimaculatus*, and *An. punctipennis* complexes, are considerably higher and more readily exploited in a PCR-based species diagnostic assay. For example, an assay used to distinguish *An. freeborni* and *An. hermsi* uses four primers—two universal primers from conserved coding regions that flank the ITS2, and two species-specific primers from within the ITS2, which produce a fragment of characteristic size for each species (Porter and Collins, 1991). Amplification of DNA from other sympatric anopheline species

produced much larger fragments that represented extension between the two universal primers across the entire ITS2.

III. Genome Characterization

A. Genome Organization

Genome organization can be described by the haploid DNA content and the amount, types, and dispersion patterns of repetitive DNA sequences. These parameters need to be considered before isolating repeated sequence components from the genome or screening genomic libraries for single-copy sequences. They also form the basis for comparative studies of genome evolution. In general, the biological significance of each of these features and their relationship to one another is far from understood. However, what is clear is the remarkable rate of divergence of these genome characteristics among closely related species and even among populations of the same species.

1. Genome Size

The genome sizes of various *Anopheles, Culex,* and *Aedes* species, including several strains of *Ae. albopictus,* have been measured by various methods (Table 1). Most genome size determinations were made by relative Feulgen cytophotometry, in which the level of Feulgen staining in the test sample is compared with that of a standard of known DNA content (measured by direct chemical analysis). Genome size has also been estimated by reassociation kinetics (see below). Among the many organisms for which haploid DNA content has been measured, there is no simple correlation between genome size and organismal complexity. There does, however, seem to be an overall trend in the family Culicidae, with the most primitive genus, *Anopheles,* possessing an average genome size at least two times smaller than the more recently evolved *Culex* or *Aedes* (Rao and Rai, 1990). Overall, there is about an eightfold variation in haploid DNA content among the mosquito species that have been examined, from 0.23 pg in *Anopheles labranchiae* (Jost and Mameli, 1972) to 1.9 pg in *Aedes zoosophus* (Rao and Rai, 1987a,b). Perhaps more striking is the enormous variation in genome size among different populations of the same species, even those collected from the same geographic location. Thus the threefold variation in DNA content among 23 species of *Aedes* (Rao and Rai, 1987b) is matched by a threefold variation in DNA content among *Ae. albopictus* sampled throughout its range and a 1.7-fold variation between two populations from Singapore (Kumar and Rai,

TABLE 1
Genome Size of Various Anophelines, Culicines, and Aedines[a]

Species strain	Genome size (pg) ± standard error	Ref.
Anopheles ishbranchiae	0.23 ± 0.04	Jost and Mameli (1972)
Anopheles micoparvus	0.24 ± 0.04	Jost and Mameli (1972)
Anopheles stephensi	0.24 ± 0.03	Jost and Mameli (1972)
Anopheles freeborni	0.29 ± 0.03	Jost and Mameli (1972)
Anopheles quadrimaculatus (sp. A)	0.24 ± ?; 0.24[b] ± ?	Rao (1985); Black and Rai (1988)
Anopheles gambiae	0.27[b] ± ?	Besansky and Powell (1992)
Culex pipiens	1.02 ± 0.19; 0.54 ± ?; 0.53[b] ± ?	Jost and Mameli (1972); Rao (1985); Black and Rai (1988)
Culex quinquefasciatus	0.54 ± 0.01	Rao and Rai (1990)
Culex restuans	1.02 ± 0.04	Rao and Rai (1990)
Aedes aegypti	0.81 ± 0.03; 0.83[b] ± ?	Rao and Rai (1987b); Warren and Crampton (1991)
Aedes pseudoscutellaris	0.59 ± 0.01	Rao and Rai (1987b)
Aedes triseriatus	1.52 ± 0.06	Rao and Rai (1987b)
Aedes zoosophus	1.90 ± 0.06	Rao and Rai (1987b)
Aedes albopictus		
Kent Ridge (Singapore)	0.75 ± 0.02	Kumar and Rai (1990b)
Amoy (Singapore)	1.29 ± 0.06	Kumar and Rai (1990b)
Koh Samui (Thailand)	0.62 ± 0.02	Kumar and Rai (1990b)
Houston-61 TX (United States)	1.66 ± 0.08	Kumar and Rai (1990b)

[a] All measurements by Feulgen cytophotometry unless otherwise indicated.
[b] Measurement by DNA reassociation kinetics.

1990b). The wide range of intraspecific differences in genome size emphasizes the speed with which the underlying molecular processes of duplication and deletion operate.

The potential significance of changes in genome size at the organismal level has been investigated in *Aedes*. As might be expected, there is a strong correlation between genome size and total length of metaphase chromosomes (Rao and Rai, 1987b). However, no apparent association exists between systematic relationships and genome size. Within *Ae. albopictus,* differences in genome content do not cluster geographically, are not associated with island-dwelling versus continent-dwelling populations, nor are they associated with recent colonization (Kumar and Rai, 1990b). Thus it is unlikely that the DNA sequences involved in rapid changes in genome size are directly responsible for speciation events. Ferrari and Rai (1989) studied the association between DNA content and two phenotypic traits, wing length and development time, related to fitness. In 9 of 10 *Ae. albopictus* laboratory strains compared, they found a significant relationship between DNA content and development time. This implies that differences in DNA content may indirectly affect phenotypic traits subject to selection, at least in the laboratory, but its effect on fitness of natural populations of mosquitoes is still unclear.

2. *Repetitive DNA*

DNA reassociation studies from the late 1960s led to the discovery of repeated sequences in eukaryotic genomes (reviewed in Britten and Kohne, 1968) and have been used since to determine the amount and organization of highly repeated, moderately repeated, and unique genomic sequences. Reassociation kinetics are estimated by monitoring the rate at which denatured DNA from a given species reassociates in solution. The rate of reassociation depends on the initial concentration of genomic sequences and the time of incubation under defined conditions. Repeated sequences reassociate much faster than single-copy sequences. The data can be expressed as a C_0t curve, the log of the product of initial DNA concentration (C_0) and incubation time (t) plotted against the proportion of DNA remaining single stranded. Nonlinear regression analysis of the C_0t curve allows the relative amounts of highly repeated, moderately repeated, and unique components of the genome to be determined. Comparison of the reassociation rates of long and short DNA fragments reveals the overall pattern of interspersion of repeated and unique sequences—long-period interspersion, the alternation of repeats about 5 kb long with the unique sequence at least 13 kb long; or short-period interspersion, the alternation of repeats

vidual highly repeated sequence families among and within mosquito species. McLain *et al.* (1986) prepared a plasmid library with *Eco*RI-digested genomic DNA from *Aedes malayensis,* from which they selected nine highly repeated sequences for further analysis. Copy number variation for each repeat family was examined by densitometry following hybridization of cloned repeats to DNA dot blots. Each DNA dot contained known amounts of genomic DNA from *Ae. malayensis* and five other sibling species in the *Ae. scutellaris* subgroup. Comparison of signal intensity following low- versus high-stringency hybridization provided additional information on repeat sequence divergence between species. Copy number varied extensively, but six of the nine clones hybridized at high stringency to genomic DNA of all species and the remaining three hybridized strongly at reduced stringency.

Using the same approach, McLain *et al.* (1987) confirmed and extended these results. They isolated eight repeat-containing clones from the Oahu strain of *Ae. albopictus* and compared their sequence and abundance by dot–blot hybridization to species of the *Ae. scutellaris* subgroup, three species of the *Ae. albopictus* subgroup, and 15 strains of *Ae. albopictus* from widely separated geographic areas. Care was taken to ensure that the different clones did not cross-hybridize and that, at least on the basis of restriction enzyme analysis, the clones did not contain tandem repeats. They found that differences in copy number were as large within *Ae. albopictus* as between species. Again, three clones that did not hybridize to some *Ae. albopictus* strains and other species at high stringency hybridized at lower stringency. Their observation of significant intraspecies variation in copy number of some highly repeated DNA sequences clearly suggests caution in the use of highly repeated DNA for species-diagnostic purposes, at least in *Aedes* species.

Kumar and Rai (1991a; 1991b) have expanded on these studies by examining the *in situ* and Southern hybridization patterns of a 5.2-kb cloned repetitive fragment from *Ae. albopictus. In situ* hybridization to mitotic and meiotic chromosomes of several *Aedes* species produced dispersed patterns on all chromosomes of each species, though hybridization intensity was less in *Aedes aegypti.* Southern analysis revealed the presence of homologous sequences in the DNA from representatives of diverse mosquito species as well as a Chaoborid. Nevertheless, the hybridization patterns were unique to each species, although closely related species shared some bands. In contrast, no intraspecific variation was detected. The conservation of this repetitive DNA throughout the evolutionary radiation of mosquitoes in spite of structural reorganizations suggests a function based on nucleotide sequence.

The hybridization pattern of cloned repeats has also been looked at between sibling species in the *An. gambiae* complex (Besansky, 1990b; N. Besansky, unpublished). An *An. gambiae* genomic library was prepared in EMBL3 phage by *Sau*3A partial digestion. Repeat-containing clones were picked at random following hybridization of the library with radiolabeled genomic DNA. Each of 19 repeats was hybridized to Southern blots of genomic DNA from several East and West African laboratory strains of *An. gambiae* and *An. arabiensis* and in some cases, also, one strain of *Anopheles melas* and a congener outside of the complex, *Anopheles stephensi*. All repeats, regardless of copy number, hybridized at high stringency to all members of the complex, whereas none that was tested hybridized to *An. stephensi* (low-stringency hybridization was not attempted). Four clones appeared to contain satellite-type, simple sequence tandem repeats on the basis of (1) regular, additive ladder patterns generated on Southern blots and (2) the insensitivity of the cloned sequence to digestion with any of a panel of seven restriction enzymes recognizing 6-bp targets. The ladder patterns for each clone were identical for *An. gambiae* and *An. arabiensis*. Another 13 clones probably represented dispersed, highly repetitive sequences because they generated smears of varying intensities on Southern blots from members of the complex. By contrast, four unrelated middle repetitive sequences, three of which were isolated from the same EMBL3 insert, produced discrete patterns of bands that differed among members of the complex.

Employing a different strategy, Cockburn and Mitchell (1989) probed phage libraries (described above) from different mosquito species and genera with individual repeat-containing phage clones to determine the proportion and intensity of hybridizing plaques. In general, most anophelines shared repeated DNA families with the exception of two species-specific families identified in the *An. quadrimaculatus* complex. Although cross-generic hybridizations were generally unsuccessful, one *Aedes* clone hybridized to *Culex* and not *Anopheles* and one *Anopheles* clone hybridized to *Aedes* (hybridization to *Culex* was not determined). Apart from the *An. quadrimaculatus* clones, hybridization at reduced stringency was not attempted.

Several cautionary notes apply to all experiments involving the probing of genomic DNA with clones containing uncharacterized repetitive DNA. It is quite possible that any given repetitive clone may contain multiple related or unrelated repeated sequences within it. For example, one clone from an *An. gambiae* EMBL3 phage library proved to contain three unrelated non-LTR retrotransposons (Besansky, 1990a; J. Bedell and N. Besansky, unpublished). This possibility depends

upon the interspersion pattern of the genome and insert size of the clone, among other things, but should be tested before firm conclusions are drawn. Additionally, the clone of interest could contain a sequence from a member of one of the many conserved multigene families, such as rDNA, 5S RNA, histones, actins, tubulins, or mtDNA. Finally, hybridization stringency should be very carefully controlled. At moderate stringency, high levels of apparently random hybridization can be observed between sequences with skewed G + C contents or long stretches of simple sequence repeats. Such simple or biased sequence composition may be common not only to many highly repeated sequences that are not homologous, but may also be found in regulatory domains.

In summary, the data suggest that individual families of highly repeated sequences, at least in *Aedes,* may evolve particularly rapidly with respect to copy number, without regard to genome size or phylogenetic relationships. This is underscored by the fact that copy number variation is as extensive within *Ae. albopictus* as between different aedine species. On the other hand, many of these same *Aedes* sequence families apparently evolve quite slowly at the nucleotide sequence level, as suggested by their ability to hybridize at high stringency even to *Anopheles* sequences. This might suggest a sequence-dependent function for such families. Yet, the reciprocal relationship does not seem to hold. That is, most anopheline highly repeated sequence families that have been studied have no homology to sequences outside the genus and limited homology between *Anopheles* species from different series within the same subgenus. With the current limited data, it is difficult to generalize from the above observations. More must be learned about the roles of different types of repeated sequences in the genome, the different factors driving and constraining their evolution, and the phylogenetic relationships among the species examined.

B. Genome Mapping

In some mosquito species, invariably those of medical importance, considerable effort has been directed toward assigning linkage relationships among morphological and isozyme markers and associating these with chromosomes by crossing experiments. In *An. quadrimaculatus,* at least 26 markers have been mapped to specific chromosomes or chromosome arms and another 12 have been assigned only as autosomal (Narang *et al.,* 1990). In *An. albimanus,* at least 51 markers have been assigned to chromosomes and another 12 are only known to be autosomal (Narang and Seawright, 1990). Very extensive linkage

maps have also been prepared from *Culex* and *Aedes* mosquitoes, particularly *Ae. aegypti* (e.g., Steiner *et al.*, 1982; Munsterman 1990a,b). Genetic maps can guide the understanding of genome organization and evolutionary relationships among mosquitoes. To the extent that they can be refined, they will also save much time and effort on the part of researchers interested in cloning the genes controlling a variety of phenotypic traits. Molecular cloning techniques have had an important impact on genetic mapping, and have brought the goal of detailed linkage maps of mosquito chromosomes within reach.

All mosquitoes possess polytene chromosomes in certain tissues and developmental stages, including the salivary glands of larvae and the ovarian nurse cells of adult females. Polytene chromosomes represent repeated chromosomal replication in the absence of nuclear division, leading to an increase in DNA content as high as 16,000-fold (Ashburner, 1980). Homologous chromosomes are paired and each chromatid remains aligned with its sisters, causing a thickening of the chromosome and revealing an intricate band/interband pattern that can be mapped and correlated with the genetic map. Unfortunately, the polytene chromosomes of *Aedes* mosquitoes are of very poor quality cytogenetically because they are so difficult to spread, probably due to the fact that their highly repeated dispersed DNA sequences promote extensive ectopic pairing. The chromosomes of *Culex* mosquitoes, whose genomes also contain high levels of repeated DNA, are only slightly better (White, 1980). On the other hand, excellent polytene chromosome preparations can be made from most anophelines, a considerable advantage in linking physical and genetic maps.

With the recent availability of cloned probes and technical improvements of *in situ* hybridization protocols (Reiss, 1990; Graziosi *et al.*, 1990; V. Kumar, V. Petrarca and F. Collins, unpublished), both known and anonymous sequences can be assigned to particular polytene chromosome regions in anophelines. The polytene chromosomes of *An. gambiae* are central to an elegant effort to map the genome of this mosquito (Zheng *et al.*, 1991). This effort relies upon a technique developed for *Drosophila* (Saunders *et al.*, 1989) that involves amplification by PCR of DNA obtained by microdissection of each of the numbered divisions of the *An. gambiae* ovarian nurse cell polytene chromosome complement, thereby creating individual division-specific "libraries." One application of division-specific libraries is the physical mapping of cloned segments of DNA without having to prepare polytene chromosomes, perform *in situ* hybridization, or interpret the banding pattern. Each division-specific library is spotted onto a nylon membrane in order of its chromosomal location. Any cloned sequence,

random or known, can then be labeled and used to probe the chromosomal dot–blot. In addition, each of these libraries can be used as probes to produce division-specific subsets of phage or cosmid genomic libraries as a preliminary step in chromosomal walks across defined genomic divisions (e.g., Sidén-Kiamos *et al.*, 1990).

RFLPs can also be used to map the approximate genetic location of genes. Mapping entails following the segregation of RFLPs in relation to other genetic markers in crossing experiments with inbred strains, or by pedigree analysis as is done in mapping human genes. The RFLPs detected usually result from the gain or loss of a restriction site through base substitution or small insertion/deletions. Variability in the DNA of certain strains of mosquitoes appears to be high enough to support this approach. In ongoing surveys of the G3 colony of *An. gambiae,* at least 63 *Eco*RI RFLPs have been identified at anonymous loci (P. Romans, unpublished), and RFLPs have also been found for the vitellogenin, dopa decarboxylase, diphenol oxidase, α-amylase, and several other loci (P. Romans, unpublished; Romans, 1991). The cytogenetic distribution of these RFLPs has been shown to cover all five chromosome arms by *in situ* hybridization.

An advantage of RFLP analysis is that a few crossing experiments can provide an enormous amount of information because (1) the genomic DNA extracted from the progeny can be apportioned and digested with a variety of enzymes to maximize the chance of detecting polymorphisms and (2) a given blot can be stripped and reprobed repeatedly with different clones. In fact, the only limitations are the amount of interstrain variability and the availability of probes to specific genomic regions. No prior information about the approximate map location of the gene of interest is required. Both *Ae. aegypti* and *An. gambiae* have shown considerable variability between strains; 26 of 33 clones examined in the former species and 64 of 72 unique sequence phage clones examined in the latter generated strain-specific RFLPs (D. W. Severson, A. Mori, D. M. Helke, and B. M. Christensen, unpublished; P. Romans, unpublished). One serious drawback of an RFLP map of *Ae. aegypti* is the difficulty of obtaining a high-density map. Given a genome size of 780 mb (Rao and Rai, 1987b) and a genetic map length of 220 cM (Munstermann, 1990a), each centimorgan corresponds to about 3 mb, but an average resolution of much less than 5 cM may not be very feasible (M. Ashburner, personal communication). Given a map of sufficient density surrounding the gene of interest, the nearest cloned probes can be used in chromosome walks or jumps through libraries to isolate genes defined by features such as chromosomal deletions, inversions, or translocations. The polytene chromo-

some division-specific "libraries" for *An. gambiae* will be particularly important in such efforts because they can be used to partition genomic libraries prior to initiating chromosome walks.

Another source of highly polymorphic genetic markers is provided by microsatellite sequences, composed of variable numbers of simple sequence repeats. These are highly abundant and dispersed throughout the genome; $(GT)_n$, the most frequent repeat unit, occurs about once every 30 kb in mice, humans, and rats (see Jacob *et al.*, 1991). Length variation at individual loci can be detected by PCR amplification with primers that anneal to flanking unique sequences. This powerful approach has been successfully used in the mapping of a complex, polygenic trait in the rat (Jacob *et al.*, 1991), with a substantially larger genome size and longer generation time than any mosquito species. If microsatellite sequences behave similarly in mosquitoes, it should be quite feasible to use them to construct a relatively dense linkage map for mosquito genomes. Several clones containing $(GT)_n$ microsatellite sequences have already been isolated from *An. gambiae* (L. Zheng, personal communication), and high priority is being placed on generating high-density (at least 1 cM) microsatellite linkage maps for both *Ae. aegypti* and *An. gambiae*.

IV. Specific Physiological Systems

A. Resistance to Insecticides and Other Toxic Substances

Resistance to various insecticides by *Anopheles, Aedes,* and *Culex* has long been known (Brown, 1967). These traits have served as important functional markers used to assign linkage groups to particular chromosomes or chromosome arms. Genetic and biochemical experiments revealed their mode and pattern of inheritance and suggested a general biochemical or physiological mechanism of resistance without defining its molecular basis. Insects employ several general mechanisms of resistance, including behavioral avoidance, decreased cuticular penetration, decreased sensitivity of the insecticide target site, and increased detoxification (Georghiou, 1986). Recent molecular genetic experiments on resistance mechanisms have documented three types of changes at the DNA level: point mutations in structural genes, changes in regulatory genes, and DNA amplification (Feyereisen *et al.*, 1990).

Among mosquitoes, *Culex* species have been the principal subjects of published molecular studies of insecticide resistance. In *Culex,* a common mechanism of resistance to organophosphate (OP) insecticides is

increased detoxification, due to overproduction of one or both of the highly active, nonspecific esterases A and B (Mouches *et al.*, 1987). One esterase B allozyme, B1, was purified from an OP-resistant strain (Tem-R) of *Culex quinquefasciatus* (Fournier *et al.*, 1987). In this strain, esterase B1 was 500 times more abundant than in a susceptible strain (Lab-S) of the same species (Mouches *et al.*, 1987). Antiserum raised to esterases B1 was used to screen a cDNA expression library prepared from Tem-R mRNA, and a clone was isolated and used to show a 250-fold difference in hybridization intensity between the Tem-R and Lab-S DNA, thus demonstrating that the overproduction of esterase B1 was due to gene amplification (Mouches *et al.*, 1986). Since then, independent amplifications of esterase B alleles *B1, B2, B3,* and *B4* in both field and laboratory strains of OP-resistant *Culex* mosquitoes have been shown to be responsible for increased esterase production (Raymond *et al.*, 1989; Pasteur *et al.*, 1990).

The nature of the amplification event has been studied for esterase B1 from the Tem-R strain (Mouches *et al.*, 1990). Overlapping clones, including the esterase B1 gene and its flanking regions, were isolated from a recombinant phage library and were used to construct a restriction map of the amplification unit from Tem-R genomic sequences. The conserved part of the genomic amplification unit, "the core," was identified by the pattern of hybridization; intensely hybridizing single bands were obtained from sequences inside the core, versus multiple bands from sequences outside the 25-kb core. Thus a large segment of flanking DNA had been coamplified along with a single 2.8-kb esterase gene. Restriction mapping of esterase B2 sequences in genomic DNA from six different resistant strains of *C. pipiens* showed that, like the esterase B1 amplification unit, the esterase B2 amplification unit consists of about 30 kb of DNA containing a single esterase gene (Raymond *et al.*, 1991).

Interestingly, the esterase B1 gene as well as the amplification core are bordered by repetitive elements. The 5′-end regions of both core and esterase genes contain a 2- to 3-kb sequence, *CE1,* which is moderately repeated in the genomes of resistant and susceptible strains of *C. pipiens* as well as its sibling species *C. quinquefasciatus,* but is absent in *Culex tarsalis.* Extending downstream of the esterase gene to the 3′ end region of the amplification core is another repeated element, *CE2.* It is a composite of a 2.2-kb highly repetitive DNA sequence termed Juan, and a 4.4-kb sequence interrupting Juan. Whereas Juan is found highly repeated in the genomes of resistant and susceptible strains of *C. pipiens, C. quinquefasciatus,* and *C. tarsalis,* the interrupting sequence does not appear to be repeated in susceptible strains.

Whether these elements or other repeated elements play a role in the amplification process awaits the analysis of other amplification units for association with repeated DNA.

The amplification process involving the esterase B alleles is thought to have involved stepwise increases in copy number over time. This is supported by the observation that the level of OP resistance due to esterase B1 in Tem-R mosquitoes has increased significantly over time (cited in Pasteur *et al.*, 1990). Further support is lent by the genomic restriction analysis of the esterase B1 amplification unit. Using cloned fragments from just outside the 5′- and 3′-end boundaries of the conserved amplification core as hybridization probes, Mouches *et al.* (1990) found corresponding genomic sequences amplified 60 and 80 times, rather than the 250-fold amplification characteristic of sequences from within the core. Evidence from *in situ* hybridization to Tem-R meiotic metaphase chromosomes indicates that the esterase B1 amplification units are arrayed tandemly on a single chromosome, and extrachromosomal elements such as double minutes were not detected (Nance *et al.*, 1990). Taken together, the data fit aspects of an "onion skin" model of sequence amplification in which multiple rounds of replication initiate from the same origin within a single replication bubble in a given replication cycle (Stark and Wahl, 1984). This structure can be resolved following recombination between different duplexes into a tandem array. The amount of sequence amplification varies along a bidirectional gradient, with sequences in the middle of the array amplified more than those at the ends. Although a passive role for repeated elements, in which they serve as sites of homologous recombination, can be envisioned in the onion skin model, active transposition plays no part. Until the nature of the amplification units is understood more completely, it is difficult to judge the adequacy of this model, if, in fact, any one model will be adequate.

Although esterase B is not expressed in all tissues or stages of development, every cell type examined, including gametes, contains the amplified gene (Pasteur *et al.*, 1990; Raymond and Pasteur, 1989). Once amplified, the insecticide resistance gene can spread rapidly through populations, even on a global scale (Raymond *et al.*, 1991).

Molecular analysis of other insecticide resistance genes has begun in several different mosquito species. The acetylcholinesterase (AChE) gene is the target site for carbamate and OP insecticides, and insensitive forms of the AChE gene have been found in insecticide-resistant mosquitoes. The AChE gene has been cloned from a phage library of *An. stephensi* DNA, using an AChE cDNA clone from *D. melanogaster* (Hall and Malcolm, 1991). Sequence analysis revealed 69% homol-

ogy at the amino acid level to *D. melanogaster* AChE. *In situ* hybridization to *An. stephensi* polytene chromosomes indicated two locations on different chromosomes, one near the telomere of chromosome 2R and the other close to the centromere of chromosome 3L (Malcolm and Hall, 1990). However, the location is still uncertain because genomic Southern analysis indicates the presence of only one locus. In *An. albimanus*, the AChE gene maps exclusively to the telomeric end of chromosome 2R (Kaiser *et al.*, 1979), which may be the true location of the AChE gene in *An. stephensi* as well. Cloning and characterization of insensitive and sensitive forms of the AChE gene from this mosquito are in progress.

Cytochrome *P*-450s are a multigene family, some of which are involved in insecticide metabolism. A cytochrome *P*-450-containing clone was isolated from an *Ae. aegypti* cosmid library using a degenerate oligonucleotide based on the conserved binding site of rabbit and rat cytochrome *P*-450s (Bonet *et al.*, 1990). Sequencing confirmed the *P*-450 identity of a subclone, which was used to probe Northern blots from pyrethroid-sensitive and -resistant strains of *An. stephensi*. Elevated levels of transcript in the resistant strain led the investigators to propose that pyrethroid resistance was due to enhanced expression rather than a change in substrate specificity of *P*-450. The molecular basis for enhanced expression is under investigation.

Malathion carboxylesterase (MCE), distinct from the nonspecific esterases B discussed above, hydrolyzes the OP insecticide malathion. MCE activity was found to be increased 55-fold in a malathion-resistant strain of *C. tarsalis* compared to a susceptible strain (Whyard *et al.*, 1990). A genomic library from the resistant strain has been prepared and the molecular basis for malathion resistance is currently under study.

Several dot–blot or microtiter plate immunoassays that have recently been developed or improved can rapidly determine the biochemical insecticide resistance mechanisms in individual mosquito specimens (Beyssat-Arnaouty *et al.*, 1989; Dary *et al.*, 1990, 1991). Although useful in their own right, they yield no information about the molecular basis of resistance. There is, however, an assay based on DNA hybridization, developed for the detection of esterase B sequences, which is capable of specifically detecting the amplification of specific insecticide-resistance genes (Agarwal *et al.*, 1986). It is based on the use of a DNA probe derived from the resistance gene being tested; the probe hybridizes strongly to homologous sequences only in genomes where amplification has occurred.

Finally, it seems appropriate to include in this section mention of

other cases of gene amplification in mosquito cell lines in response to exposure to toxic substances, such as the folate analog methotrexate (mtx). The target protein of this drug is the enzyme dihydrofolate reductase (DHFR), which is involved in purine and pyrimidine synthesis. To study the effect of mtx on the DHFR gene and gene product, *Ae. albopictus* cells were selected for mtx resistance by maintenance in successively increasing mtx concentrations (Shotkoski and Fallon, 1990). Cells resistant to the highest level of mtx (Mtx-5011-256 cells) were 3000 times more resistant and contained about 130 times more DHFR protein than the parental, wild-type cells. The resistant cells also had elevated levels of DHFR mRNA. Southern blots of *Eco*RI-digested DNA from wild-type and resistant cells were probed with cDNAs synthesized from a mRNA fraction enriched for DHFR mRNA. The probe detected an 8.5-kb band in resistant but not wild-type cell DNA, strongly implicating DHFR gene amplification. Cloning of this fragment and subsequent sequencing of the DHFR-related sequences revealed a 614-bp DHFR gene with a 56-bp intron conserved in position between mammalian and mosquito genes (Shotkoski and Fallon, 1991). The copy number of DHFR genes per haploid genome in Mtx-5011-256 cells was estimated at 300 by densitometry of dot–blots probed with the DHFR coding sequence. Because the increase in resistance was accompanied by a conversion from diploid to tetraploid and the additional duplication of a single chromosome (Shotkoski and Fallon, 1990), the number of gene copies per cell is at least 1200. The size and organization of the amplification units are being investigated by pulsed-field gel electrophoresis, *in situ* hybridization, and Southern blotting. Preliminary data suggest that the amplification unit comprises at least 110 kb of sequence, and that it is tandemly arranged on 5 of 13 chromosomes (F. A. Shotkoski and A. M. Fallon, unpublished).

Transformation of the *Ae. albopictus* cell line C6/36 resulted in amplified copies of the exogenous sequences (T. J. Monroe, J. O. Carlson, and B. J. Beaty, unpublished), reminiscent of the amplification of endogenous genes. These cells were transformed with a plasmid containing the *Drosophila* heat-shock promoter driving a bacterial hygromycin-resistance gene and selected with hygromycin B. The cellular DNA of some stable transformants, following restriction endonuclease digestion and electrophoresis through agarose gels, contained discrete bands corresponding to plasmid fragments. Densitometry of these bands led to an estimate of 10,000 plasmid copies per haploid genome. *In situ* hybridization of plasmid DNA to metaphase chromosomes revealed large arrays integrated into different sites on the chromosome arms, centromeres, and telomeres in some cells. In addition, plasmid-

carrying minutes and double minutes, some circular, were noted in varying sizes and numbers. Occasionally, whole chromosomes hybridized with plasmid sequences. Furthermore, the rescue of plasmid DNA by transformation of *E. coli* implied that unintegrated plasmids can be maintained. Some of these contained undefined mosquito genomic sequences, resulting from recombination between plasmid and chromosomal DNA. It is not clear if the high copy number of plasmid sequences was selected for by exposure to hygromycin. Monroe *et al.* found a number of stable transformants with only one or a few copies of plasmid per cell, showing that amplification of the plasmid is not always required in the face of the hygromycin selection regimen. The conditions favoring amplification may depend on where in the chromosome the plasmid integrated. An alternative explanation to amplification is suggested by studies of microinjection of mouse embryos with recombinant plasmids. Integration in mammalian cells commonly occurs as a head-to-tail concatemer of multiple copies, although these usually number below 100 as opposed to 10,000 (Gordon and Ruddle, 1985). This process is not well understood. A detailed analysis of more transformants will be helpful in understanding the events resulting in the presence of multiple plasmid sequences in C6/36 cells and how they bear on current molecular models of gene amplification.

B. Oogenesis and Endocrine Regulation

Because of sex, tissue, and stage specificity of oogenesis, its different aspects are being researched as models for regulation of gene expression and as targets for vector control.

The blood meal taken by a female mosquito initiates a complex series of events that culminate in egg production. There is evidence to suggest that distension of the midgut following a blood meal may trigger the activation of the "early" form of the digestive protease trypsin produced by the midgut (Graf and Briegel, 1989). The hydrolysis products from this early trypsin may in turn induce production of the "late" trypsin (C. Barillas-Mury, unpublished). Because late trypsin mRNA is not present prior to the blood meal, and because the increase in message precedes an increase in protein levels, it appears that late trypsin is primarily regulated at the transcriptional level (Barillas-Mury *et al.*, 1991). However, translational regulation may play a role, because the amount of trypsin produced depends on the amount of protein in the blood meal (Briegel and Lea, 1979). Cloning and sequencing of a

cDNA and a genomic clone of late trypsin from *Ae. aegypti* revealed that the gene lacks introns, and the results of Southern blot analysis suggest that there is polymorphism in the regions flanking the coding sequence (Barillas-Mury *et al.,* 1991; C. Barillas-Mury, unpublished). Analysis of the regulatory elements, in progress, has uncovered a region similar to the amino acid regulatory element of yeast, which may respond to the availability of amino acids (C. Barillas-Mury, unpublished).

One of the first events following the blood meal is the increased synthesis and accumulation of ribosomes in the fat body in response to an unknown hormonal signal. In *Ae. aegypti,* within 18 hours after the blood meal, the ribosomes are four times more abundant than before the blood meal (Hotchkin and Fallon, 1987).

Coinciding with the increase in ribosome production is the synthesis of large amounts of the major yolk protein, vitellogenin (reviewed in Raikhel *et al.,* 1990), reaching a peak 24–36 hours after a blood meal in *Ae. aegypti* (Gemmill *et al.,* 1986).

To study the regulation of vitellogenesis at the molecular level, the sequences encoding vitellogenin have been cloned from *Ae. aegypti* (Gemmill *et al.,* 1986) and *An. gambiae* mosquitoes (Romans, 1990). Similar strategies were used in both cases, namely, differential screening of a phage genomic library with first-strand cDNA probes prepared from females 24 hours after a blood meal versus males (*Ae. aegypti*) or non-blood-fed females (*An. gambiae*). From *Ae. aegypti,* two clones specific to vitellogenic females were mapped and cross-hybridized (Gemmill *et al.,* 1986). Although homologous throughout the putative 5.5-kbcoding region, the restriction maps were conserved only in a central 2-kb region. Restriction mapping also indicated that these two genes are located in different regions of the genome. Genomic Southern blots suggest the presence of five different vitellogenin genes in *Ae. aegypti,* though it is not clear if all are expressed. In *An. gambiae,* a given individual contains either four or five vitellogenin genes, all of which map to the same site on chromosome 2R by *in situ* hybridization (Romans, 1990; P. Romans, unpublished). At least four of the genes are in a tandem array with the 6.3-kb coding regions separated by about 3-kb spacers. In contrast to the *Aedes* genes and probably as a consequence of their tandem arrangement, the *An. gambiae* genes are virtually identical at the sequence level throughout the entire coding region. At the amino terminus of genes from both *Aedes* (vgA1) and *Anopheles* is a 16-amino acid signal peptide interrupted by a short intron in the eleventh codon (P. Romans and H. Hagedorn, unpub-

lished; P. Romans, unpublished). The promoter sequences from both genera contain a consensus TATA box, duplicate mRNA cap sites, and several sequence motifs similar to those found for *Drosophila* yolk protein genes, including hormone response elements and one (*Aedes*) or two (*Anopheles*) fat body enhancers (P. Romans, unpublished). In addition, *An. gambiae* contains an ovarian enhancer-like sequence. Transient expression from promoter/*LacZ* fusions will be used to study stage- and tissue-specific transcription in transgenic *Drosophila* and *An. gambiae*.

In addition to vitellogenin, the fat body of *Ae. aegypti* synthesizes another protein of 50 kDa that is secreted into the hemolymph and is internalized by the oocytes (Hays and Raikel, 1990). Like vitellogenin, its synthesis peaks about 24 hours after a blood meal and is undetectable by 48 hours. Both synthesis and secretion of the protein are stimulated by 20-hydroxyecdysone. The entire cDNA was cloned using a combination of immunoscreening of a cDNA expression library in phage, and PCR (W.-L. Cho, K. W. Deitsch, and A. S. Raikhel, unpublished). The cDNA clones hybridize to a 1.5-kb mRNA present only in vitellogenic fat bodies, showing the tissue and stage specificity of the message. Sequence analysis revealed an open reading frame with homology to carboxypeptidases. On this basis the protein, named vitellogenic carboxypeptidase (VCP), is thought to play a role in protein hydrolysis associated with embryogenesis.

As vitellogenin expression terminates, the secretory granules and ribosomes of the fat body are degraded in lysosomes (see Raikhel *et al.*, 1990). A lysosomal enzyme has been purified and characterized from *Ae. aegypti* (Cho and Raikhel, 1990). This enzyme exists as a dimer of two 40-kDa subunits. Based on its substrate utilization and N-terminal sequencing, it has been identified as a mosquito cathepsin D, and is being used to study lysosomal regulation.

C. Immune Mechanisms

Like other insects, mosquitoes possess various defenses against "foreign" invaders; these defenses, by analogy to vertebrate immune systems, have been broadly classified as humoral and cellular immunity, even though the distinction is not always clear (Christensen and Nappi, 1988). Humoral immunity involves the induction of antimicrobial proteins or the melanotic encapsulation of parasites without direct participation by hemocytes. Cellular immunity involves phagocytosis or encapsulation directly mediated by hemocytes. (For recent reviews

of insect immunity, see Christensen and Nappi, 1988; Lackie, 1988; Christensen and Tracy, 1989.)

Variation in the ability of different mosquito species or geographic strains to support parasite infections has been recognized for years, and in many cases the mosquito's system for defense against foreign invaders appears to be responsible for this variation. However, in spite of genetic selection studies suggesting a single major locus controlling susceptibility to infection in several parasite/vector associations, most recently with arboviruses in mosquitoes (Miller and Mitchell, 1991), the molecular bases of these differences in immune response have not been elucidated. The possibility of interrupting parasite transmission by genetic engineering is fuelling molecular studies of mosquito immunity.

1. Humoral Responses

One major component to humoral immunity in insects is the arsenal of hemolymph proteins induced by infecting microbes, wounding, or injection of bacterial cell wall components or peptidoglycan degradation products (Boman and Hultmark, 1987). These proteins include the antibacterial diptericin, attacins, and cecropins, the digestive enzyme lysozyme, and hemolin, thought to initiate an immune response by binding to the bacterial cell wall (Sun *et al.*, 1990). Such antibacterial proteins were deemed ineffective against eukaryotic cells (Boman and Hultmark, 1987). However, recent findings may be challenging this view. For example, *Ae. aegypti* responds not only to *E. coli* and laminarin, a bacterial lipopolysaccharide, but also to the filarial nematode *Brugia pahangi* by producing proteins whose molecular weights in SDS–PAGE gels are close to lysozyme, diptericin, attacins, or cecropins (Chaithong and Townson, 1990). Moreover, hemolymph transferred *in vivo* following these immunizations partially protects against subsequent infection with *B. pahangi*, although it is not clear if any of the induced proteins are directly responsible (Chaithong and Townson, 1990) nor if any are simply products of a wounding response. The genes or portions of genes encoding the *Ae. aegypti* counterparts of attacin, cecropin, and diptericin have been cloned from a genomic library using oligonucleotide probes synthesized on the basis of sequence data from other insects (Knapp and Crampton, 1990). A preliminary sequence has been obtained and subclones have been used for Northern and Southern analyses to determine gene number, organization, and pattern of expression in *Ae. aegypti* strains refractory and susceptible to infection by filarial parasites.

strain of *An. stephensi* could be due to higher levels of the proteolytic enzyme, aminopeptidase, in the midgut lumen or wall (Feldmann *et al.*, 1990).

2. *Cellular Responses*

When invading foreign particles such as bacteria or protozoa are sufficiently small, they may be phagocytosed or trapped in nodules of aggregated hemocytes (Lackie, 1988). If the foreign body is too big to be phagocytosed, hemocytes may play other roles in the immune response. For example, melanotic encapsulation of innoculated *Dirofilaria immitis* microfilariae (mff) is a hemocyte-mediated event in *Ae. aegypti* (Christensen and Tracy, 1989). Ultrastructural studies have shown the lysis of hemocytes in the vicinity of these parasites prior to melanin formation, which occurs near the lysed cells and appears to settle onto the parasite surface (Christensen and Forton, 1986). Phenol oxidase activity is increased in hemocytes during the encapsulation response (Li *et al.*, 1989, 1992). In addition, an 84-kDa polypeptide preferentially expressed during melanotic encapsulation and wound healing is produced by the hemocytes and secreted into the plasma (Beerntsen and Christensen, 1990). In fact, SDS–PAGE, ^{125}I labeling, and lectin binding techniques have shown a significant overall increase in protein concentration in immune-activated hemocytes, some of which are exported to the cell surface (Spray and Christensen, 1991). One 200-kDa surface polypeptide was uniquely expressed in hemocytes from saline- or mff-inoculated mosquitoes. These events are associated with a twofold to threefold increase in the total hemocyte population (Christensen *et al.*, 1989) and a fivefold increase in the proportion of hemocytes whose surface properties allow them to bind lectin, properties that presumably facilitate their recognition and adhesion responses (Nappi and Christensen, 1986). The experiments so far have yielded important clues about the biochemical mechanisms of some aspects of the encapsulation response. Yet, the identity and role of most of the substrates and enzymes involved in the complex sequence of events leading from recognition of the parasite through melanotic encapsulation remain undefined. Work for the future includes the isolation and characterization of the genes encoding and regulating the expression of these proteins.

In *Ae. aegypti,* the genetic basis of another system of defense against the filarial parasites *Brugia malayi* and *Wuchereria bancrofti* was discovered by crossing experiments performed 30 years ago (Macdonald, 1962; Macdonald and Ramachandran, 1965). A single sex-linked reces-

sive locus, f^m, appeared to control susceptibility. Distinct from the melanotic encapsulation response, development of the mff is somehow aborted in the thoracic tissue of resistant strains after penetration of the midgut. Polypeptides synthesized in the thoracic tissue were compared between strains of *Ae. aegypti* refractory and susceptible to infection by *B. malayi* (Wattam and Christensen, 1992). SDS–PAGE gels of radiolabeled polypeptides synthesized *in vivo* showed seven polypeptides unique to blood-fed refractory mosquitoes. The presence of parasites in the blood meal was not necessary to induce their synthesis. All were present 3–24 hours after blood feeding but were undetectable after 48 hours. Wattam and Christensen suggest that these refractory strain-associated proteins may mediate the genetic variation in susceptibility, although there is no evidence as yet that any of them are encoded by the f^m gene. To confirm this suggestion, the f^m gene will need to be isolated and characterized. Work toward this goal is in progress, using RFLP mapping (D. W. Severson, A. Mori, D. M. Helke, and B. M. Christensen, unpublished).

D. Salivation

Another aspect of mosquito physiology that is relevant to the host/parasite relationship is salivation, because most parasites must pass through the salivary glands prior to transmission (James *et al.*, 1991). However, saliva plays many other roles in both sugar and blood feeding (reviewed by James and Rossignol, 1991). Inside the mosquito, enzymes in the saliva aid digestion; externally, the platelet antiaggregating and vasodilatory activity of the saliva facilitate the acquisition of blood (Ribeiro, 1987). The elucidation of these basic processes at the molecular level will enhance our understanding of the parasite's relationship with the salivary gland and may provide salivary gland-specific regulatory DNA sequences that will be useful in engineering pathogen-refractory mosquito strains (Marinotti and James, 1990a).

Several different genes that encode secreted salivary proteins and that are expressed specifically in the salivary glands have been isolated and characterized from *Ae. aegypti*. The first was given the name *Maltase-like I* (*Mal I*) for the similarity of its putative product to a yeast maltase (James *et al.*, 1989). It was isolated from a cDNA library from non-blood-fed females by differentially screening with total abdominal RNA and total salivary gland RNA. A fragment from one of the isolated clones was used to probe developmental Northern blots to

determine stage specificity of expression. A 2.1-kb poly(A)$^+$ RNA was detected only from salivary gland RNA of adult males and females. Sequence analysis of the cDNA clone revealed a 1.8-kb open reading frame followed by a consensus polyadenylation signal. Analysis of the 5′-noncoding sequence from a corresponding genomic clone identified a TATA box as well as two regions of dyad symmetry with the potential for hairpin loop formation. These putative regulatory structures are of particular interest for their potential roles in directing strong stage- and tissue-specific expression in the mosquito. The 67-kDa deduced protein has a possible secretion signal at its N terminus and six possible sites for asparagine-linked glycosylation. Marinotti and James (1990b) have found a 68-kDa soluble glycoprotein, secreted when mosquitoes take a sugar meal, which is an α-glucosidase probably involved in sugar digestion. The correspondence between this enzyme and the properties predicted for the *Mal I* gene product suggest that they are identical.

A salivary gland-specific α-amylase has also been isolated and sequenced (G. L. Grossmann and A. A. James, unpublished). Its expression pattern analyzed by Northern analysis and *in situ* hybridization overlap that seen with the α-glucosidase *Mal I* (James *et al.*, 1989). Interestingly, the 18-bp repeats found at the 5′ end of the *Mal I* gene are also adjacent to the α-amylase coding region, suggesting that they may be involved in regulation of genes encoding carbohydrate-metabolizing enzymes.

Another gene, *D7*, expressed specifically in *Ae. aegypti* salivary glands, has been isolated from a cDNA library using total salivary gland RNA as a probe (James *et al.*, 1991). Oligonucleotides synthesized based on the sequence from *D7* cDNAs were used to isolate homologous clones from salivary gland cDNA and genomic libraries. A Northern blot prepared from RNA from different stages, tissues, and sexes showed that the *D7* cDNA hybridized strongly to a 1.2-kb poly(A)$^+$ RNA from adult female salivary glands and faintly to a message of the same size from adult males. Message was detected up to 48 hours after blood feeding. Sequence comparison of cDNA and genomic clones revealed a TATA box, an open reading frame interrupted by four introns with consensus splice junctions, and a polyadenylation signal. Sequence variation was detected among different cDNAs and between cDNA and genomic clones. It is not yet clear if these differences stem from alternate alleles or multiple gene copies. Polyclonal antibody produced from a recombinant *D7* gene product recognized a 37-kDa protein in salivary glands and saliva from adult females but

not from males, indicating female-specific production of a secreted protein and suggesting its involvement in blood feeding.

The salivary glands are sexually dimorphic in the mosquito, being both larger and more differentiated morphologically in the female. Although the salivary glands are three-lobed in both sexes, the distal lateral and medial lobes of the female appear to be sex specific and composed of much larger secretory cells than those found in the female proximal lateral lobes or in any of the male lobes (James *et al.*, 1989). The structural dimorphism coincides both with dietary differences between males and females, as adults of both sexes feed on nectar whereas only the female takes a blood meal, as well as with spatially segregated gene expression of salivary enzymes (Marinotti *et al.*, 1990; Grossman and James, 1990). To explore salivary gland expression more closely, antisense RNAs have been used to probe tissue sections from male and female salivary glands and identify those cells expressing individual genes. *Mal I* is one gene whose product is thought to be involved in sugar digestion. Northern analysis detected abundant *Mal I* gene expression in adult salivary glands of both sexes of *Ae. aegypti*. By *in situ* hybridization with antisense RNA probes, expression occurs throughout the male salivary gland, but is restricted to the proximal lateral lobes in the female (James *et al.*, 1989). Similar *in situ* hybridization patterns were seen in female salivary glands using probes for the α-amylase, another gene presumably involved in sugar feeding (G. L. Grossman and A. A. James, unpublished). On the other hand, the *D7* gene product is not even detected in males and probably functions in blood-feeding females. By Northern analysis, *D7* is preferentially expressed in adult female salivary glands and is barely detected in the male. By *in situ* hybridization, *D7* expression is barely detected in the three lobes of the male salivary gland and is detected only in the distal lateral and medial lobes of the female salivary glands (James *et al.*, 1991). Furthermore, an apyrase secreted into the saliva from salivary glands of female *Ae. aegypti* and diverse anophelines prevents platelet aggregation and thus aids in blood location and ingestion (Ribeiro, 1987). This enzyme is restricted to the distal lateral and medial lobes of the female salivary glands (Ribeiro *et al.*, 1984); another bacteriolytic enzyme from both male and female salivary glands is restricted in females to the proximal lateral lobes (Rossignol and Lueders, 1986). These observations suggest that the proximal lateral lobes serve functions common to both female and male sugar feeding whereas the distal lateral and medial lobes of the female salivary glands provide factors unique to the blood-

feeding habit of the female (James *et al.*, 1991) and may have evolved together with this capability.

V. Genetic Transformation

Genetic transformation is broadly defined as the uptake and expression of exogenous DNA by cells in culture or in whole organisms. Gene transfer may be transient, reflecting the episomal state of the introduced DNA, or stable following the integration of DNA into the chromosome. Stable integration into embryonic germ line cells can result in a transformed lineage. It is a powerful tool that has allowed the identification and isolation of genes and regulatory sequences as well as a detailed analysis of gene expression, development, and behavior that would be difficult if not impossible to study with classical genetic techniques. Applied to insect vectors of disease by targeting key stages of parasite development in the host (Warburg and Miller, 1991), it could represent the first step in the engineering and ultimate population replacement of a vector by a nonvector strain. However, among insects, *D. melanogaster* is the only species for which efficient methods of stable germ line gene transfer are currently available (reviewed in Ashburner, 1989). This is due to the availability of efficient *Drosophila*-specific transformation vectors, such as those based on the P and hobo transposable elements, as well as a variety of cloned selectable markers and promoter sequences. Initial optimism that P element vectors would function without modification in mosquitoes has faded, and new efforts are focused on a number of alternative approaches. These include efforts to identify and isolate endogenous mobile elements from mosquitoes as well as detailed research on the basis for P and hobo element mobility, with the expectation that by supplying required *Drosophila*-specific cis- or trans-acting factors, these elements could be mobilized in organisms other than *Drosophila*. Another important approach being investigated in mosquitoes is the potential for using the FRT–FLP site-specific recombination system from the 2μm plasmid of the yeast *Saccharomyces cerevisiae* (Meyer-Leon *et al.*, 1984) as a directed recombination system. The system is particularly attractive because this site-specific recombination, which requires only the appropriate FRT recombination site and a source of the FLP recombinase, has been shown to work efficiently in a number of different organisms, including *D. melanogaster* (Golic and Lindquist, 1989) and mammalian cells (O'Gorman *et al.*, 1991). In addition to the basic transformation vectors and recombination tools, a great deal of effort is also being

directed toward the isolation of mosquito-specific promoter and enhancer sequences, selectable marker genes, and mutant mosquito strains that will be required to develop efficient genetic transformation systems for mosquitoes. This process builds on previous genetic research with both mosquitoes and *D. melanogaster* and broadens the understanding of not just mosquito molecular biology but of insect molecular genetics.

A. DNA Delivery

Genetic transformation depends on the successful solution to four separate but interdependent problems: (1) the delivery of DNA into the cell or embryo, (2) the efficient and stable integration of DNA into the chromosome, (3) a suitable marker gene that encodes a dominant phenotypic trait, and (4) a promoter to control expression of the gene. The first problem, how to deliver the exogenous DNA, has been solved for both cultured mosquito cells and embryos. In fact, many methods exist for transfecting mosquito cells, including the use of calcium phosphate, polybrene, lipofection, and electroporation (for review, see Lycett, 1990). With any given method, transfection efficiencies vary from one cell line to another and need to be optimized empirically. Optimal conditions for transfecting the C7-10 clone of *Ae. albopictus* cells using polybrene (Fallon, 1989; Gerenday *et al.*, 1989) and calcium phosphate (Kjer and Fallon, 1991) have been defined. Efficiencies range from 1 in 10,000 cells for polybrene-mediated uptake to as high as 1 in 1000 for the calcium phosphate procedure (Kjer and Fallon, 1991). In both cases only transient expression was tested. Stable transformants of the C6/36 clone of *Ae. albopictus* cells were obtained following the lipofection procedure (Monroe *et al.*, 1990; T. J. Monroe, J. O. Carlson, K. E. Olson, D. L. Clements, and B. J. Beaty, unpublished), and of *Ae. aegypti* MOS20A cells using polybrene with glycerol shock, electroporation, and lipofection (Lycett, 1990; G. J. Lycett, P. Eggleston, and J. M. Crampton, unpublished). Studies in MOS20A cells demonstrated stable transformation at efficies as high as 1 per 500 cells following electroporation (Lycett, 1990).

Three independent laboratories have similarly overcome the more difficult problem of introducing DNA into embryos of *An. gambiae* (Miller *et al.*, 1987), *Ae. triseriatus* (McGrane *et al.*, 1988), and *Ae. aegypti* (Morris *et al.*, 1989) using microinjection. Microinjection of *D. melanogaster* embryos is facilitated by the ability to remove the rigid chorion. Because removal of the mosquito chorion results in the death of the embryo, embryos were injected shortly after oviposition, before

the chorion fully hardened, with glass needles whose tips were 3–5 μm in diameter. The embryos had to be slightly desiccated to prevent leakage of the innoculum, but because excess dehydration was lethal, they were injected under water-saturated halocarbon or paraffin oil. Even with these precautions, the survival of injected embryos through adulthood averaged only 14% in *An. gambiae,* 15% in *Ae. aegypti,* and 6% in *Ae. triseriatus*.

Alternative methods for mass injection of insect embryos are being tested. One such method, initially developed for use in transforming plant cells, involves the use of a particle gun to shoot DNA-coated microparticles into the egg. This technique proved fatal to embryos of *An. albimanus* and does not appear promising, due to the small size yet thick eggshell of the mosquito (Carlson and Cockburn, 1989). However, the obvious labor intensity and poor efficiency of individual microinjections indicate the value of continued effort to develop more efficient methods for introducing DNA into mosquito eggs.

B. DNA Integration

Although random integration of circular or linearized recombinant plasmids into chromosomes is successfully used to transform many diverse cell lines, it is a process that is poorly understood and impossible to control (Handler and O'Brochta, 1991). For example, one common occurrence is the integration of head-to-tail concatemers of multiple copies (Gordon and Ruddle, 1985). Mobile element vectors or site-specific recombination systems improve not only the efficiency and consistency of integration, but also allow some control over the manner in which, or the sites at which, DNA integrates.

For germ line transformation of *D. melanogaster,* vectors based on the P element transposon (reviewed in Engels, 1989) are routinely used. P elements encode a transposase that acts on 31-bp inverted repeats at their termini to effect excision and transposition. Correct processing of transposase message, hence transposition, is limited to the germ line in wild-type P elements. P elements have been engineered such that the transposase coding sequence has been replaced by a multiple cloning site and a promoter controlling a selectable marker gene. If coinjected with an immobilized "helper" plasmid providing preprocessed transposase in trans, the transformation vector can transpose and integrate in somatic and germ cells. In *D. melanogaster,* at least one chromosomal integration event is recovered from 100–200 injected eggs (Handler and O'Brochta, 1991).

The fact that the P element vector has been mobilized in all droso-

philids tested (O'Brochta and Handler, 1988) has encouraged efforts to transform mosquitoes through P-mediated integration. In each case, foreign DNA was successfully introduced into the germ line and some integration events were stable over multiple generations (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). In all cases, however, the efficiency of integration was much lower than expected for a highly efficient vector such as P. For example, of almost 2300 injections, one integration event was recovered from *An. gambiae* (Miller *et al.*, 1987), a rate at least 10-fold lower than expected from *Drosophila* even though survival rates following injection were comparable. Similarly low rates of integration were obtained from *Ae. triseriatus* (McGrane *et al.*, 1988) and *Ae. aegypti* (Morris *et al.*, 1989). Southern analysis indicated that these integration events were not P element mediated. Other transformation experiments using P element constructs and *Ae. albopictus* C6/36 cells or *Ae. aegypti* MOS20A cells also resulted in plasmid integrations independent of P mechanisms, some of which involved large tandem arrays (T. J. Monroe, J. O. Carlson, and B. J. Beaty, unpublished; Lycett, 1990). It is unclear at present what factor(s) block P element mobility in nondrosophilids. As more is learned about P transposase function in heterologous systems, it may be possible to overcome these obstacles. However, a more profitable approach might be to exploit a transposable element endogenous to the mosquito genome rather than to manipulate one adapted to a different organism. Moreover, other classes of transposable elements might be worth exploring. For example, the retrotransposon *copia* from *Drosophila* was deemed unsuitable for use as a transformation vector because of low rates of mobility, but there is precedent for the use of this kind of element for genome manipulation in yeast (Jacobs *et al.*, 1988). A nonviral retrotransposable element from *Drosophila*, the I element, causes hybrid dysgenesis reminiscent of the P–M system, and this type of element has been found in a broad spectrum of eukaryotic organisms.

Any type of transposable element that integrates at high efficiency might make a good candidate for a transformation vector. In *Drosophila*, these elements reside in the middle repetitive component of the genome. Thus, in seeking out endogenous elements from the mosquito, one approach has been the screening of genomic libraries with radiolabeled total genomic DNA to identify repetitive sequences. This approach was used to isolate T1, a non-LTR retrotransposon from *An. gambiae* (Besansky, 1990a). T1 belongs to the LINE-like class of mobile elements that are ubiquitous in eukaryotic genomes, as examples have been found in insects, plants, mammals, frogs, and protozoans

(see Xiong and Eickbush, 1990). They transpose through an RNA intermediate based on their putative reverse transcriptase-encoding sequences, frequent 5′-end truncation, and poly(A)-like tails. However, they are distinguished from retroviral-like elements by the absence of long terminal repeats. T1 is dispersed in about 100 copies in the *An. gambiae* genome and is also found in the genomes of other members of the *An. gambiae* complex, yet is absent from very close relatives belonging to the same series (*Pyretophorus*)—*Anopheles subpictus* and *Anopheles vagus* (N. Besansky, unpublished). T1 is dispersed on all chromosome arms with no apparent target site preference. A high rate of mobility has been inferred both from Southern blot analyses of wild populations of *An. gambiae* from Kenya and from *in situ* hybridizations to polytene chromosomes of *An. gambiae* laboratory strains (Besansky, 1990a; V. Kumar and F. Collins, unpublished).

In the course of characterizing rDNA repeat units from members of the *An. gambiae* complex, two other retrotransposons from *An. gambiae* and *An. arabiensis*, called RT1 and RT2, respectively, were discovered (Paskewitz and Collins, 1989; Besansky *et al.*, 1992). The genomic distribution of these two elements, which do not cross-hybridize on genomic blots, is restricted to the same 17-bp target sequence in a fraction of 28S rDNA units. This is a feature that could be exploited, given an understanding about how the target site is recognized by the integrase. Unfortunately, this information is currently lacking, even in the better known R1 and R2 elements of *Bombyx mori*. The mobility properties of T1, RT1, and RT2 are currently being analyzed to determine the feasibility of using these elements as transformation vectors.

Two different approaches for isolating mobile elements have been taken with *Ae. aegypti* (Crampton *et al.*, 1990a). In the first approach, which was based on the assumption that retrovirus-like transposable elements would be represented as circular DNA molecules in the extrachromosomal fraction of the genome, a plasmid library was generated from extrachromosomal DNA isolated from the MOS20 cell line. When used to probe total genomic Southern blots, one clone, pX16, displayed an intraspecific polymorphism in hybridization pattern, suggesting variation in both chromosomal location and copy number typical of mobile elements. The second approach used oligonucleotide primers corresponding to regions conserved among reverse transcriptases, YXDDML and TAFLHG, in PCR amplifications of *Ae. aegypti* larval and cell line DNA as well as *An. gambiae* DNA (A. Warren and J. M. Crampton, unpublished). Partial sequence data from pX16 and from three cloned PCR products revealed the presence of YXDD boxes

in each case, although the tyrosine (Y) was replaced by a valine (V) in pX16 and the second aspartic acid (D) with an isoleucine (I) in one PCR product. Sequence data from the termini are needed to determine whether these elements fall within the retrovirus-like classes of retrotransposons. However, the presence of a methionine (M) rather than an alanine (A) at the X position suggests that they are all retroviral-like (Xiong and Eickbush, 1988).

Though strategies for directly isolating and analyzing mosquito elements have clearly been fruitful, it is uncertain whether an element identified by such approaches will prove useful as a transformation vector. Alternative approaches, such as the search for and investigation of hybrid dysgenesis-like phenomena or unstable genetic mutations in mosquitoes, should also be undertaken. Preliminary searches for hybrid dysgenesis-like phenomena have been carried out with *Anopheles* species of two different species complexes, the *An. quadrimaculatus* complex (J. A. Seawright, personal communication) and the *An. gambiae* complex (F. H. Collins and V. Finnerty, personal communication). These efforts have not yet identified any biological phenomena that appear to indicate actively transposing mobile elements.

Preliminary experiments with the yeast FLP–FRT site-specific recombination system in the mosquito *Ae. aegypti* have been very promising (Morris *et al.*, 1991). In a series of elegant experiments involving the coinjection of mosquito embryos with target plasmids containing FRT recombination sites and plasmids containing the FLP recombinase gene under the control of the *Drosophila hsp70* promoter, efficient FLP-mediated recombination was observed between tandem FRT sites within a plasmid and between FRT sites on separate plasmids. Synthetic FRT sites tested were functionally equivalent to target sites isolated from the yeast 2μm plasmid. These experiments encourage continued investigation of this and perhaps other site-specific recombinase-based systems as methods for genetically transforming mosquitoes.

C. Promoters

The detection of transformed cells or embryos is critically dependent on a promoter capable of expressing the introduced gene in cooperation with the transcriptional apparatus of the host. Because of the evolutionary conservation of the heat-shock response and heat-shock regulatory sequences, the promoter (*hsp70*) of the *D. melanogaster* 70-kDa heat-shock protein has been used successfully to control expression of genes in cell types as diverse as yeast and mouse (Gerenday *et al.*,

1989, and references therein). The *hsp70* promoter functions effectively in transiently transfected *Ae. albopictus* C7-10 cells using conditions defined for *Drosophila* (Durbin and Fallon, 1985), in contrast to the promoter from the *Drosophila* transposon *copia,* which does not (Swerdel and Fallon, 1987). By characterizing the endogenous heat-shock response based on maximal expression of the *Aedes* homologue *hsp66,* Gerenday *et al.* (1989) discovered that an incubation at 41°C rather than 37°C improved transient expression from the *hsp70* promoter by 10-fold in C7-10 cells. The heat-shock response was also maximized in *Ae. aegypti* Aag-2 cells (Lan and Fallon, 1990) and MOS20 cells (G. J. Lycett, P. Eggleston, and J. M. Crampton, unpublished) at 41°C. This improvement translates to anophelines, because it has recently been shown that almost no mortality was observed in *An. albimanus* larvae at 40°C (Benedict *et al.,* 1991) and that transcript accumulation from the mosquito *hsp70* promoter is undetectable at 37°C, but is high at 40°C (M. Q. Benedict, A. F. Cockburn, and J. A. Seawright, unpublished). Indeed, survival following G418 selection of transgenic *An. gambiae* containing a neomycin-resistance gene under *Drosophila hsp70* control was increased by heat shock at 41°C compared with 37°C (Sakai and Miller, 1992).

During transient transformation, non-heat-shocked cells demonstrated a relatively low background level of gene expression from the *Drosophila hsp70* promoter when compared to that of heat-shocked cells (e.g., Durbin and Fallon, 1985). However, in stably transformed C6/36 cells of *Ae. albopictus,* there was no significant difference in transcription from this promoter following heat shock at 37°C, regardless of whether it was driving selected or unselected genes (Monroe *et al.,* 1990; T. J. Monroe, J. O. Carlson, K. E. Olson, D. L. Clemens, and B. J. Beaty, unpublished). Although heat shock of mammalian (BHK-21) cells at 42°C failed to induce the *Drosophila hsp70* promoter, the inducibility of this promoter in mosquito cells was not tested at this temperature. It is unclear whether constitutive expression resulted from the nature of the integration event or whether stress incurred by handling of the cells inadvertently induced a heat-shock response. The level of expression of antibiotic-resistance genes from the *Drosophila hsp70* promoter was sufficient to allow selection of stable transformants of C6/36 cells, in spite of the lack of inducibility (T. J. Monroe, J. O. Carlson, K. E. Olson, D. L. Clemens, and B. J. Beaty, unpublished). However, following heat shock at 37°C, the level of expression of the *Drosophila hsp70*-regulated genes in both cells and transformed embryos has been insufficient to prevent the production of false positives (T. J. Monroe, J. O. Carlson, K. E. Olson, D. L. Clemens, and B. J.

Beaty, unpublished; Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989).

The application of species-specific promoters could provide the benefits of increased levels and control of gene expression. These will soon become available, because numerous groups have cloned a variety of both constitutively expressed and tissue, sex, and stage-specific genes from the mosquitoes *An. gambiae* and *Ae. aegypti,* including those for actin (C. Salazar, D. Mills Hamm, C. B. Beard, and F. Collins, unpublished), vitellogenin (Gemmill *et al.,* 1986; Romans, 1990), a glucosidase, α-amylase, and the female-enriched protein D7 from salivary glands (James *et al.,* 1989, 1991, G. L. Grossman and A. A. James, unpublished), and the *hsp70* and *hsp82* genes of *An. albimanus* (M. Q. Benedict, A. F. Cockburn, and J. A. Seawright, unpublished). These *An. albimanus* heat-shock genes were cloned from a genomic DNA library using a probe containing the N-terminal coding region of the *Drosophila* homologues. Benedict *et al.* found two contiguous *hsp70* genes at two loci on chromosome 2R, and two *hsp82* genes at one locus on chromosome 3L. Each locus contains a pair of genes whose coding regions are divergently oriented. The heat-shock regulatory elements in the promoter regions were identified by sequence comparisons to previously defined elements from other organisms. These putative heat-shock elements from the *An. albimanus* promoters are more numerous and more similar to the consensus sequence than are those from *Drosophila.*

D. Reporter Genes and Selectable Markers

Transformants may be screened individually, by the expression of an unselected reporter gene that confers a visible phenotype. Such genes are useful for estimating transformation efficiency and also allow the characterization of promoter sequences. Examples of reporter genes include eye color genes such as *rosy* from *Drosophila* or genes from bacteria that encode detectable enzymes such as β-galactosidase (β-gal) or chloramphenicol acetyltransferase (CAT). The genes for both β-gal and CAT have been correctly expressed in cultured mosquito cells (Durbin and Fallon, 1985; Lycett, 1990). CAT activity has been detected in all stages of the mosquito *Ae. aegypti* (embryos, larvae, pupae, and adults) following injection of *hsp70* promoter–CAT gene constructs into embryos, thus CAT is an excellent reporter for hybrid genes in this mosquito (A. Morris and A. James, unpublished). Although β-gal activity has been detected throughout adult *Ae. aegypti* following microinjection of embryos with a *hsp70* promoter–β-gal

gene construct, relatively high background activity might obscure analysis of some tissue-specific promoters with this reporter gene (O. Marrinotti and A. James, unpublished).

Transformants may also be mass selected, by the use of a selectable marker gene that confers resistance to various chemical treatments, such as antibiotics. Two widely used selectable markers derived from bacteria, the neomycin phosphotransferase (*neo*) and hygromycin phosphotransferase (*hyg*) genes, confer resistance to the antibiotics neomycin (or its analog G418) and hygromycin, respectively. Like the genes for CAT and β-gal, the *neo* and *hyg* genes have also been expressed in cultured mosquito cells (Lycett, 1990; T. J. Monroe, J. O. Carlson, K. E. Olson, D. L. Clemens, and B. J. Beaty, unpublished). The usefulness of the *hyg* gene in whole animals is unknown (Handler and O'Brochta, 1991), but the *neo* gene has been expressed in the larval stages of *Aedes* and *Anopheles* mosquitoes (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). Unfortunately, these mosquito species differed dramatically in their sensitivity to the antibiotic, so that effective concentrations had to be carefully established in each case. Moreover, every transformation experiment with the *neo* gene reported to date has been plagued with leaky selection in which cells or mosquitoes apparently resistant to the antibiotic were not actually transformed. (In these experiments, heat shock of the *Drosophila hsp70* promoter driving *neo* was at 37°C.) Although problems associated with the low activity of the *neo* gene product might be partially overcome with the use of a higher temperature to heat shock, a stronger promoter, or a different *neo* gene, the need for alternate selection procedures based on cloned mosquito genes is evident.

A variety of morphological and biochemical mutant strains of mosquitoes are available for transformation, but, with the exception of a few insecticide-resistance genes, the corresponding genes have yet to be cloned or even mapped in many instances. Clearly much more effort needs to be aimed in the direction of cloning dominant gain-of-function genes.

E. Target Genes and Field Application

Overcoming the obstacles to genetic transformation of the mosquito would provide a powerful tool to study of many aspects of basic mosquito biology. However, as part of a program to control mosquito-borne diseases, genetic transformation is only one of a series of problems requiring solutions, including the identification of relevant target genes (not necessarily from the mosquito genome) and the develop-

ment of methods to drive the genetic constructs into natural mosquito populations.

Putative mosquito counterparts of genes involved in developmental control and circadian rhythms have been isolated from *Ae. aegypti* (Crampton *et al.*, 1990c; Eggleston, 1990a). These clones were identified by hybridization to synthetic oligonucleotide probes based on conserved sequences of other organisms. The nine different developmental control genes being analyzed hybridized to the *Antennapedia*-like homeobox sequence from *D. melanogaster* (Crampton *et al.*, 1990c). The characterization of these genes and of their expression is valuable, but their fundamental importance to the fitness of the organism implies that any attempts to introduce defective copies into natural populations call for a strongly counterbalancing driving force. A prevalent opinion, shared by the authors of this review, is that new genetic control strategies based on the premise of population reduction or elimination are as unlikely to succeed in the long run as earlier control attempts involving the release of sterilized male mosquitoes or mosquitoes genetically altered to be relatively infertile when mated with the wild population.

More promising in theory is the strategy of replacing vector populations with those of lesser or no capability of transmitting pathogens. Although this could in principle involve factors that modify the overall dynamic interaction between the mosquito and the human population (such as host-seeking behavior), genetic factors that cause minimal changes in the overall ecology of the vector species are likely to be the least problematic. The most promising genetic modification would appear to be one that affects only the ability of the mosquito population to support development of the pathogen it transmits. In some epidemiological settings, even a very small modification in the vectorial capacity of the natural population may be sufficient to achieve complete elimination of the pathogen (Coluzzi, 1992).

As was mentioned in Section IV,C, various groups are involved in trying to understand the basis for vectorial competence with respect to malaria parasites in anophelines and to filarial worms in aedine mosquitoes, and to map and clone the genes involved. Efforts have also been initiated to characterize *Aedes* clones homologous to attacins, cecropins, and diptericins of other insects. Much of the work on genes with tissue-specific expression, particularly tissues such as salivary glands, where arboviruses undergo cycles of intracellular replication, is motivated by the possibility of a genetic control strategy based on an arbovirus-specific antisense RNA produced in the appropriate tissue under the control of a tissue-specific mosquito promoter. Unfortu-

nately, not much is known, especially at the molecular level, in any of these areas and much more basic research is necessary before any of these approaches can be implemented.

The success of any genetic control strategy will ultimately depend on the availability of a mechanism that favors the spread of the introduced genes through natural populations, such as meiotic drive, reduced heterozygote fitness, parasite-mediated cytoplasmic incompatibility, or replicative transposition (see Curtis and Graves, 1988; Crampton *et al.*, 1990b; Eggleston, 1990b). The spread of an intracellular, *Wolbachia*-like parasite causing a unidirectional mating incompatibility has been documented in *D. simulans* (Turelli and Hoffmann, 1991). Because the incompatibility favors infected females, the infection spread rapidly through populations. Desirable traits, such as those controlling vector competence, could potentially be introduced into infected females, who would then drive the trait to fixation along with the infection. It is worth noting that *Wolbachia*-like parasites have been found in many different insects, including various *Culex* species (see Kitzmiller, 1976). Transposable elements, another potential driving mechanism, also behave like intracellular parasites and, given a high enough rate of transposition, can theoretically spread even an unfavorable trait through a population before natural selection has a chance to act on it, in spite of the sometimes deleterious consequences of transposition (J. Ribiero and M. Kidwell, personal communication). Furthermore, transposable elements have shown themselves capable of transfer across species boundaries (Houck *et al.*, 1991; Mizrokhi and Mazo, 1990), a feature that might be exploited in species complexes with multiple, sympatric vector species, such as the *An. gambiae* complex. That transposable elements are capable of rapidly spreading through natural populations has already been demonstrated by the P element, which invaded populations of *D. melanogaster* worldwide within the past 50 years, probably from the distant relative *Drosophila willistoni* (Anxolabhere *et al.*, 1988). These features have fueled the search for mosquito transposable elements that function like the P element, and they also point out the potential importance of identifying elements not only from the vector species but also from related mosquito species that may be uninvolved in pathogen transmission. Though the scenario for using an engineered element introduced across a species boundary to transform effectively an entire wild population, or even species, clearly falls in the realm of scientific speculation, the potential usefulness of transposable element vectors to the study of basic mosquito biology cannot be overstated.

VI. Concluding Remarks

Most molecular research on mosquitoes to date has been undertaken because of the importance of mosquitoes as vectors of human disease. Indeed, the basic vision that underlies much of the molecular work with members of this insect family is the prospect for genetic control. Nevertheless, the long-range significance of molecular research on mosquitoes will clearly extend far beyond this more narrowly defined objective. Several obvious areas of potential significance to basic research questions are already apparent.

The most important immediate contribution will probably be in the areas of molecular systematics and evolutionary biology. In part, this will be due to the obvious applied importance of this knowledge as a prelude to any type of mosquito control effort, including genetic control strategies. Clearly, a great deal of financial and intellectual effort will be directed toward understanding important mosquito vectors such as *An. gambiae* and *Ae. aegypti,* and much of this work will focus on population genetic and evolutionary processes. Such evolutionary work with mosquitoes, particularly studies involving differentiation of populations and the analysis of closely related cryptic species complexes, should be of importance to evolutionary biologists as well as vector biologists.

It is likely that significant basic findings will also emerge from the present concerted effort to develop the tools for transgenic technology, such as transformation vectors, genome maps, and detailed molecular knowledge of specific physiological systems. The mosquitoes *An. gambiae* and *Ae. aegypti,* and to a lesser extent *C. pipiens,* currently stand out as important model organisms for the group as a whole. Though other species will no doubt also be important, either because of their involvement with specific disease systems or their possession of unique biological characteristics, the community of scientists involved with mosquito research will need to develop a consensus on the importance of a small number of model species such as the above three. The physical resources and effort required to maintain mosquito colonies are considerable, especially for *Anopheles* and *Culex* species, and these requirements in combination with possible institutional restrictions on the importation or maintainance of tropical mosquitoes may require an unusual degree of collaboration among research laboratories engaged in mosquito research.

A final point we wish to emphasize is the extraordinarily broad scope of past and present work with mosquitoes, work that has ranged from

field ecology through physiology and now to molecular biology. Clearly, molecular genetic analysis of mosquitoes is in its infancy relative to many other eukaryotic organisms, but members of the family Culicidae have long been the subject of detailed field and laboratory study. This rich biological background knowledge will not only prove valuable in current genetic control efforts but may also serve to strengthen the potential importance of mosquitoes as basic biological models.

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