Molecular entomology and prospects for malaria control

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During the past decade, the techniques of molecular and cell biology have been embraced by many scientists doing research on anopheline vectors of malaria parasites. Some of the most important research advances in molecular entomology have concerned the development of sophisticated molecular tools for procedures such as genetic and physical mapping and germ line transformation. Major advances have also been made in the study of specific biological processes such as insect defence against pathogens and the manner in which malaria parasites and their anopheline hosts interact during sporogony. One of the most important highlights of this research trend has been the emergence during the past year of a formal international *Anopheles gambiae* genome project, which at present includes investigators in several laboratories in Europe and the USA. Although much of this molecular research is directed towards the development of malaria control strategies that are probably many years from implementation, there are some important areas of molecular entomology that may have a more near-term impact on malaria control. We highlight developments over the past decade in three such areas that we believe can make important contributions to the development of near-term malaria control strategies. These areas are anopheline species identification, the detection and monitoring of insecticide susceptibility/resistance in wild anopheline populations and the determination of the genetic structure of anopheline populations.

Keywords: *Anopheles;* molecular entomology; drug resistance, genetics; genetic techniques; research trends; review literature.

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Introduction

Plasmodium falciparum malaria remains one of the three most important pathogen-specific causes of human mortality in the world today. The 1998 *World Health Report* stated that there are now more cases of malaria in the world, perhaps 300–500 million per year (a major underestimate in the minds of many) than there were in 1954 (then estimated at 250 million). More importantly, the annual number of deaths caused by malaria, estimated at between 1.5 and 2.7 million in 1997, seems to have remained stable or even risen over this period (*1*). The problem of malaria has been exacerbated in recent years by the development and rapid spread of resistance in *P. falciparum* to the more commonly used and affordable antimalarial drugs. Chloroquine resistance, which first appeared in East Africa in the late 1970s, has now spread throughout most of the continent, and resistance to

pyrimethamine–sulfadoxine (Fansidar) has followed rapidly. The emergence of insecticide resistance in African malaria vectors (see below) threatens to exacerbate further the problem.

In 1990, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), together with the John D. and Catherine T. MacArthur Foundation and the University of Arizona, convened a meeting in Tucson, Arizona. At this meeting, 36 specialists in entomology, genetics and biochemistry were brought together to discuss the prospects for malaria control by genetic modification of the vector competence of natural vector populations (*2*). In the decade since that meeting, an extraordinary amount of molecular research has been done on malaria vectors, particularly *Anopheles gambiae*, the principal vector of malaria in sub-Saharan Africa. A fine-scale *A. gambiae* genetic map based on microsatellite markers and other sequenced tagged sites has been developed (*3, 4*) and used to map both morphological markers (*5*) and genes affecting parasite development (*6*). These markers have also formed the basis of a rapidly increasing number of population genetic and ecological studies of *A. gambiae* and its sibling species. Studies of innate immunity in *Anopheles* have revealed an extraordinarily complex defence system, some of whose elements are responsive to malaria parasites (*7–*

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19). Several groups are also actively examining the complex interactions between the malaria parasite and its mosquito vector during both the midgut and the salivary gland phases of sporogony (*20–31*). Other investigators are characterizing genes expressed in the midgut, fat body and salivary glands, with the long-range goal of developing *Plasmodium*-inhibiting constructs that can be expressed specifically in these tissues (*32, 33*).

Indeed, the past year has resulted in two additional important developments in malaria vector research. An efficient technique for *Anopheles* germ line transformation has been developed (*34, 35*), and an informal genome project for *A. gambiae* has been launched. More than 25 000 *Anopheles* sequences are now in GenBank, more than 6000 of which are cDNAs (*36*). Around 17 000 of these entries are the end-sequences of a fivefold coverage bacterial artificial chromosome genomic library of *A. gambiae* (X. Wang, Z. Ke and F. H. Collins, unpublished data), sequenced by the French national genomics centre Génescope. The combined total BAC end-sequence is more than 14 megabases (http://bioweb.pasteur.fr/BBMI), an amount equivalent to about 5.4% of the 260-megabase *A. gambiae* genome (*37*). End-sequencing has also begun on a second bacterial artificial chromosome library, containing about 30 000 clones with an almost 20-fold genome coverage (F. H. Collins and H. Zhang, unpublished data). The National Institute of Allergy and Infectious Diseases (National Institutes of Health) has recently reviewed proposals to initiate genomics projects for several new organisms, one of which is *A. gambiae* (www.niaid.nih.gov/dmid/genomes/priorities.htm). Thus building on the solid foundation of molecular work already completed, excellent prospects exist that a formal international *A. gambiae* genome project will soon be launched. The results of such a project, combined with the ongoing human and *P. falciparum* genome projects, will set the stage for a much deeper understanding of the interactions among the parasite, vector and human host that will almost certainly lead to new approaches for preventing and curing malaria.

Although most scientists in the vector research community have welcomed these developments, some still remain sceptical that molecular research on vectors will have any major impact on malaria control in the near future, and that such projects draw funds that could be better used implementing currently available methods for malaria control (*38*). There is also a somewhat justifiable concern that much of the current molecular research is too focused on a single molecular-based malaria control strategy, the interruption of malaria parasite transmission by altering the vector competence of natural vector populations (M. Coluzzi, personal communication).

Methods

Rather than attempt to review all recent progress in molecular studies of malaria vectors — some aspects

of which were briefly mentioned above — we focus on research in three areas that we believe can have important near-term impacts on vector-based malaria control programmes. These areas are molecular assays for mosquito species identification, studies of the molecular basis of insecticide resistance, and improved molecular tools for the study of vector population structure and gene flow. Most of the cited literature was identified from searches of databases and from the literature that the authors track for their own research purposes.

Discussion

Identification of vector species

Species identification is clearly critical to any vector control programme that seeks to be efficient as well as effective, yet it has been apparent since as early as the 1920s that some malaria vectors are members of morphologically indistinguishable groups of species (*39*). For example, in a study of benzene hexachloride insecticide resistance among the malaria vectors in Zimbabwe in the late 1970s, bioassays suggested that most if not all wild specimens of the *A. gambiae* species complex were susceptible to benzene hexachloride. However, when the specimens tested in these bioassays were subsequently identified to species by a diagnostic isoenzyme assay, it was found that only *A. quadriannulatus* (constituting most of the test samples) was killed by benzene hexachloride. The few *A. arabiensis* in the samples survived exposure to the insecticide (*40*). This was an important finding, as only *A. arabiensis* is a vector in Zimbabwe; *A. quadriannulatus* is a highly zoophilic species that is not involved in transmission.

Within the past decade or so it has become apparent that most major vectors are members of such cryptic species complexes. Until the late 1980s, methods for identifying individual members of such complexes were too labour intensive to be used routinely in field studies of vector biology or malaria transmission. Thus, research even on vectors known to be a member of such complexes continued to lead to publications in which the species was identified only to the nominal taxon of the complex. The unfortunate consequence of such work was that because the species under study could be any one species or a mixture of species in a cryptic species complex, the resulting literature was at best imprecise and at worst misleading. The number of malaria vectors that are members of such complexes continues to increase as more vector populations are subject to careful population analysis (see below), suggesting that much of the scientific literature describing the ecology of malaria vectors and their involvement in transmission is to some extent compromised.

Hybridization assays. Most of the first DNA-based assays for identifying cryptic species (ignoring for this discussion analysis of chromosome structure) were based on hybridization assays that detected species-specific differences in highly repetitive se-

quences. Although somewhat time consuming and technical, these assays were more efficient than previous methods, such as polytene chromosome analysis and isoenzyme gel electrophoresis. The approach, however, generally had two major limitations. The species composition of the cryptic species complex under study had to be known before the assay could be developed, and each species in the complex had to differ from the others in the abundance of one or more repeat sequences. Despite these limitations, repeat sequence hybridization assays were developed and used for field studies of the *A. gambiae*, *A. dirus* and *A. punctulatus* complexes (*41–47*).

Polymerase chain reaction methods. With the development and refinement of polymerase chain reaction (PCR) technology in the mid and late 1980s, PCR-based assays became more popular. Although randomly amplified polymorphic DNA–PCR has been used in *Anopheles* species identification (*48–50*), species-diagnostic PCR assays are more commonly targeted at specific regions of repeat gene families, such as ribosomal DNA (rDNA) that were found to differ among cryptic species. Initially, assays were based on species-specific differences in the nucleotide sequences of rapidly evolving regions such as rDNA internal transcribed spacers (ITS1 and ITS2) or intergenic spacer regions. Thus prior knowledge of the composition of the species complex was required. For example, a diagnostic assay that distinguishes cryptic species in the *A. gambiae* complex is based on differences in the intergenic spacer (*51–54*). Similar diagnostic assays based on differences in the ITS1 or ITS2 sequences have been developed for the *A. hermsi*/*A. freeborni* cryptic pair of species (*55*), the *A. quadrimaculatus* complex (*56*), the European *A. maculipennis* complex (*57*) and the *A. dirus* complex (*58*). Generally, because such spacer regions evolve and diverge rapidly, it is usually possible to find diagnostic differences between even the most closely related of species.

PCR amplification of regions of the rDNA, followed by restriction enzyme digestion or by single DNA strand conformational polymorphisms have also been used to develop assays for different chromosomal forms of *A. gambiae* (*59*), the *A. punctulatus* complex (*60*), the *A. funestus* group (*61*) and the *A. minimus* group (*62*). Although both these approaches are technically a little more complicated than simple rDNA-PCR (particularly single DNA strand conformational polymorphisms), they do offer several advantages, the most important of which is that sequencing is not a prerequisite to assay development. Because sequencing is not required, the assays can be used as population screening tools to survey for variation that might be indicative of the presence of cryptic species.

Although identification of cryptic species within a complex has traditionally been done by observing mating incompatibilities, it can also be inferred indirectly by showing combinations of molecular and/or biological markers that group nonrandomly in a single population. Drawing inferences about species identity in allopatric populations that exhibit such nonrandomly grouped markers is not a straightforward issue, but it is the failure to identify cryptic species found in the same geographical areas that poses the greatest problem for the study and control of malaria transmission.

There is little doubt that these new species identification tools are having an impact on vector studies. A brief survey of abstracts of field studies of *A. gambiae* complex species revealed that 21 of 30 articles (70%) published in the mid 1980s identified the species only as *A. gambiae s.l.* whereas only 12 of 30 articles (40%) published in the late 1990s failed to identify the mosquito species.

Insecticide resistance

Insecticides play a central role in controlling the mosquito vectors of malaria and will continue to do so in the foreseeable future. However, the ubiquitous use of a limited number of insecticides for both agricultural pests and vectors of human diseases has led to insecticide resistance. By 1992 more than 55 different species of anopheline mosquitoes were found to be resistant to one or more of the commonly used insecticides (*63*). To prolong the effectiveness of the currently available insecticides and thereby prevent control failure, it is vital to detect the emergence of resistance at an early stage so that appropriate action can be taken. Traditionally, detection has been based on insecticide susceptibility tests with WHO test kits, accompanied by biochemical assays to identify the underlying resistance mechanism where available. Recently, several PCR-based detection methods have been developed to detect target site resistance, but further work is urgently needed to identify the primary resistance alleles conferring metabolic resistance to insecticides. In addition, a clear understanding of the molecular basis of resistance to insecticides in mosquitoes will aid the development of new alternatives to the existing control measures.

Chemical control of the mosquito vectors of malaria can target the larval or adult stage of the mosquito's life cycle. In urban areas of India, control of the vector *A. stephensi* is based on larviciding and removal of breeding sites; in areas with seasonal malaria, applying larvicides to the mosquito breeding grounds after the rains can be an effective means of control. However, the larvae of many of the major malaria vectors live in transient aquatic habitats produced during the rainy season or by agricultural irrigation. Most of these sites are too plentiful or too temporary to be efficiently removed or treated. Thus most efforts at vector control target the adult mosquitoes, either by indoor residual spraying with insecticides or by the use of insecticide-impregnated bednets and curtains.

Types of insecticides. The major insecticides used for indoor house spraying are dichlorodiphenyltricholoroethane (DDT), synthetic pyrethroids and, to a lesser extent, malathion. House spraying with DDT achieved spectacular success in reducing the

incidence of malaria in large areas of tropical Asia and Latin America and in some African countries during the 1950s and 1960s (*64*), and successful control is still achieved by this method in many regions today. However, the emergence of DDT resistance and the intense pressure from environmentalists threaten the use of this insecticide in malaria control. Malathion is a poor alternative to DDT. It has an unpleasant odour, which contributes to a high refusal rate, and it also has a shorter residual activity, higher cost and needs to be applied twice as frequently as DDT (*65, 66*). Furthermore, malathion resistance has been detected in *A. stephensi, A. arabiensis* and *A. culicifacies* (*67–69*). Thus the cessation of DDT spraying would increase the use of pyrethroid insecticides and thereby increase the selection for pyrethroid resistance in malaria vectors.

At present, synthetic pyrethroids are the only class of insecticide suitable for impregnation of bednets because of their low toxicity to mammals and rapid action. Other alternatives are being investigated (*70*), but there are fears that the emergence of pyrethroid resistance in the *Anopheles* vectors will threaten the sustainability of this control measure. Two foci of pyrethroid resistance have already been detected in *A. gambiae*: one large focus in Burkina Faso and Côte d'Ivoire (*71*) and a second smaller focus in western Kenya (*72*). In addition, permethrin-resistant populations of *A. funestus* (*73*), *A. albimanus* (*74*), *A. stephensi* (*75*) and *A. subpictus* (*76*) have been reported. Given the predicted increases in use of pyrethroid insecticide-impregnated bednets, the incidence of pyrethroid resistance is likely to increase and compromise this widely advocated control strategy.

The common target site for DDT and pyrethroid insecticides is the voltage-gated sodium channel in the insects' nervous system. Two different mutant alleles of this gene have been detected within the two foci of pyrethroid resistant *A. gambiae* described above (*71, 77*). Both of these mutations have previously been detected in DDT- or pyrethroid-resistant strains of other insect taxa (*78, 79*). PCR-based diagnostic assays have been developed to detect these mutations in field populations of *A. gambiae*, and work is currently in progress to determine whether the frequency of these resistance alleles has increased in response to the widespread use of permethrin for malaria control.

PCR detection of resistance-associated mutations. Diagnostic PCR assays have been developed to detect resistance-associated mutations in the target sites of other insecticide classes. For example, a single amino acid substitution in the insect's γ-aminobutyric acid receptor results in resistance to cyclodiene insecticides in many insect species (*80*). A PCR assay developed to detect this mutation has been successfully adapted to detect mutant *Anopheles* γ-aminobutyric acid alleles (J. Hemingway and J. Morgan, personal communication). Cyclodiene insecticides have been largely withdrawn from use but the resistance alleles are still found at a relatively high frequency in field populations. This is of concern because novel insecticides, such as the fipronils, also target the γ-aminobutyric acid receptor, and there are fears that their efficacy in the field could be reduced by the past use of cyclodiene insecticides (*81*).

Metabolic resistance. Metabolic resistance to DDT and pyrethroids in malaria vectors is widely reported but poorly understood. This is largely because the three major enzyme families, the cytochrome *P*-450s, glutathione *S*-transferases and esterases, responsible for most of the insecticide metabolism in insects are each comprised of many enzymes with overlapping physical and catalytic properties, and pure preparations of individual enzymes can be difficult to obtain. Even in cases where the enzymes responsible for increased metabolism have been isolated, the resistance-associated mutation responsible for the increased metabolism can be difficult to identify. This is especially true for cytochrome *P*-450 and glutathione *S*-transferase–based resistance mechanisms, where resistance is widely reported to be due to a mutation in a regulatory gene, resulting in increased production of one or more enzymes capable of metabolizing the insecticide (*82–84*).

Several glutathione *S*-transferase and cytochrome *P*-450 genes have been cloned from *Anopheles* (*85–87*), but the role of these genes in insecticide metabolism is unclear. Given that the *Drosophila* genome is predicted to contain 94 cytochrome *P*-450 genes and 42 glutathione *S*-transferase genes (*88*), the search for the *Anopheles* genes involved in resistance could be protracted.

An alternative approach, which is currently being employed by the laboratories of F. H. Collins (Notre Dame, USA) and J. Hemingway (Cardiff, Wales) to study the molecular basis of metabolic resistance to permethrin and DDT in *A. gambiae*, is to use genetic techniques to map the major genes involved in metabolic resistance. The microsatellite markers developed by Zheng et al. (*3*) have been used to map two loci linked to inheritance of resistance to pyrethroids (*rtp1* and *rtp2*; resistance to permethrin 1 and 2 respectively) and two separate loci linked to DDT resistance (H. Ranson, J. Hemingway and F. H. Collins, unpublished data). The next stage of the project is to identify additional microsatellite markers to enable fine scale mapping and eventual isolation of the resistance genes by positional cloning.

A cytochrome *P*-450–based cross-resistance to DDT and permethrin, which is expressed only in female mosquitoes, has recently been reported in *A. albimanus* (*89*). Females from both pyrethroid-resistant and susceptible populations of *A. gambiae* from Kenya were also significantly more tolerant of the insecticide than males. In addition, genetic crosses between these two strains suggest that a non-chromosomal, female parent-transmitted effect may be involved in permethrin resistance (H. Ranson, J.M. Vulule and F.H. Collins, unpublished data).

Molecular basis of resistance. Characterization of the molecular basis of resistance to insecticides is of importance to maximize the efficacy of the

currently available insecticides in malaria control programmes. Traditionally, detection of resistance is based on insecticide susceptibility tests in which insects are exposed to papers impregnated with a discriminating concentration of insecticide for a fixed time. This approach is arguably the most reliable and simplest to perform in the field, but it has many limitations (*90*). In particular, each mosquito can be exposed to only one dose of one insecticide, and, under field conditions, it can be difficult to obtain sufficient mosquitoes to accurately assess the resistance status of the population. The latter limitation is particularly important when the goal is to detect resistance when it is first emerging in a population and thus is present at low frequencies.

The development of a series of biochemical assays for the common resistance mechanisms has enabled individual mosquitoes to be tested for a range of resistance mechanisms (*91*). Assays are available to detect insensitive acetylcholinesterase and for all the major metabolic resistance mechanisms. The assays for glutathione *S*-transferase and esterase-based resistance work by detecting a colour change after incubation of mosquito homogenate with a model substrate for the particular enzyme family being assayed. The sensitivity, and hence reliability, of these assays is limited by the choice of model substrate. Without knowing the substrate specificity of the enzymes responsible for insecticide metabolism, the choice of substrate is restricted to those that are recognized by most members of the enzyme family. The only assay currently available to detect cytochrome *P*-450-based resistance, for example, is based on comparison of total haem content between field populations and those susceptible in the laboratory (*92*), and hence upregulation of a single member of this large family may go undetected by this assay. As the individual enzymes responsible for insecticide metabolism in the field are characterized, a series of substrates more diagnostic of resistance can be developed.

Allele-specific PCR assays. Allele-specific PCR assays have been developed to detect several resistance alleles as described above. These assays are not as readily adapted for field application as the bioassays, but they have the advantage of detecting heterozygotes, which may be missed by phenotypic measurements. PCR detection enables the resistance alleles to be traced as they spread through a population. While the genes responsible for metabolic resistance to permethrin and DDT in *Anopheles* still await detection, it may be possible to monitor the spread of these resistance mechanisms by molecular methods, provided that genetic markers sufficiently tightly linked to the resistance loci are identified.

Thus, as our knowledge of the molecular mechanisms of insecticide resistance increases, our means of detecting and monitoring resistance in the field is expected to improve. These methods should not be seen as a replacement for bioassays, as this remains the only reliable way of detecting novel mechanisms of resistance as they emerge in the field.

Once resistance has been detected, appropriate action needs to be taken promptly to avoid the resistance alleles becoming fixed in the population. A large-scale trial of the use of rotations or mosaics of insecticides compared with single use of DDT or pyrethroid is currently underway in Mexico (*93*). Changes in frequencies of resistance genes are being monitored over a four-year period (*94*). Information generated from such large-scale trials, combined with predictions of patterns of cross-resistance to insecticides based on our knowledge of the molecular mechanisms involved, may allow us to establish rational strategies for long-term insecticide use in malaria control.

Finally, knowledge of the molecular basis of insecticide resistance will aid research into the design of new insecticides. First, new insecticide candidates could be tested against resistance mechanisms present in the field. Although this is currently possible by testing novel compounds on strains resistant in the laboratory, a more convenient approach that enabled the effect of existing resistance mechanisms to be assayed *in vitro* would be welcome. Second, in the case of metabolic resistance, it may be possible to develop synergists, to be applied in conjunction with the insecticide, which overcome the resistance mechanism. This would require the determination of the three-dimensional structure of the enzymes responsible for increases in metabolism and the manner in which these enzymes interact with their insecticide targets, so that specific inhibitors could be developed.

Vector population genetics

Many species of malaria vectors have excellent polytene chromosomes either in the salivary glands of fourth instar larvae or in the ovarian nurse cells, and because polymorphic paracentric inversions are relatively common in *Anopheles*, these chromosomes have been much studied at the population level. Several excellent studies have also relied on polymorphic isoenzymes as markers. Some of these studies have revealed the existence of cryptic taxa, which have been confirmed later by other techniques. The technical difficulties associated with the use of chromosomes and isoenzymes as population genetic markers, however, has led to a shift toward the use of molecular markers in population genetic studies, especially those that can be detected by PCR. The most popular markers thus far have been microsatellite loci, although several studies have also incorporated single DNA strand conformational polymorphisms and randomly amplified polymorphic DNA–PCR products and DNA sequences, notably mitochondrial DNA (mtDNA) and rDNA spacers (*95–101*). Because microsatellite loci generally have higher mutation rates than other regions of the genome and thus are highly polymorphic in populations, they are valuable for population studies. However, because alleles of identical size can result from convergence as well as common descent, microsatellite data alone (not supported by data from

other types of genetic loci) should generally be interpreted conservatively.

Clearly, several important applied strategic questions underlie most studies of *Anopheles* population structure today. Genetic strategies are being seriously reconsidered for vector control (or more properly for the control of pathogen transmission), and a good understanding of the extent of population structuring is essential for such strategies. Also, as insecticides remain one of the main tools for mosquito control, the ability to detect the emergence of resistance and to predict its spread throughout mosquito populations is of high priority. And of course, population genetic studies can be one of the first indicators that a presumed monotypic population actually consists of two or more taxa between which gene flow is restricted.

Microsatellite loci for vectors. By far the most widely studied malaria vectors are the members of the *A. gambiae* species complex, in large measure because the tools (e.g. microsatellites) developed for genetic mapping of this mosquito's genome were rapidly applied to questions concerning population genetics. However, microsatellite loci have now been developed for other vector species, including *A. funestus* (*102*), *A. maculatus* (*103, 104*) and *A. dirus* (*105, 106*). Such studies, in association with related studies at the population level by using polymorphic chromosomal inversions and other molecular markers, have revealed an extraordinary level of structure within the *A. gambiae* complex, and in particular within the taxon *A. gambiae* itself, where five so-called "chromosomal forms" (Bamako, Mopti, Savannah, Forest and Bissau) have been identified (*107, 108*).

Population genetic studies. Population genetic studies of mosquitoes give information about the level of gene exchange between populations. Such information is useful in inferring dispersion patterns of the vectors, the knowledge of which is critical in vector control. If levels of gene flow observed between populations in different geographical locales are significantly higher than those that can be attributed to chance alone, the implications are that genes of interest if present in one population will, over time, move to the other populations. If in the mosquito, for example, the genes are those responsible for insecticide resistance or vector competence, it is possible to predict not only how far but also how fast such resistance/competence will spread.

Smallest breeding unit. In terms of rational understanding of gene flow and vector control studies, the population geneticist's concern would be to establish the smallest breeding unit (the deme) beyond which gene exchange would not occur. Studies in Kenya have suggested that the distance associated with a deme for the savanna form of *A. gambiae* is at least 50 km in diameter (*109*). Interestingly, distance may not necessarily be a major barrier to gene flow in this form: *A. gambiae* savanna from western Kenya was only slightly differentiated from that from west Africa, 5000 km away, but showed significant genetic isolation from popula-

tions on the Kenya coast, 700 km away (*110–114*). It has been suggested that the eastern arm of the high elevation rift valley, which separates western and coastal Kenya, may be a barrier to gene flow. It is thought that the temperate plateaux of this rift system, in which human settlement is sparse, causes a discontinuity of this highly anthropophilic species. However, the western arm of the rift system is settled almost uniformly. Results from studies of *A. arabiensis* populations are somewhat different but consistent with the biology of this species. Lack of human settlement would not cause discontinuity of this species because it is partially zoophilic. Kamau et al. (*114*) found that populations from both sides of the eastern arm of the rift valley experience major levels of gene exchange. If there is no barrier to gene flow between certain populations, and the allele for resistance to a particular insecticide becomes present in one area through a mutational process, it is only a matter of time before it spreads to the other populations. The use of the insecticide to which resistance developed in one area would thus be ineffective in time in the other areas. Being aware of this enables workers in vector control to be extra vigilant for the spread of insecticide resistance so that appropriate action can be taken early enough. Knowledge of the genetic structure of the population is therefore important in the effective management of vector populations through rational use of insecticides. Alongside this, vector dispersal patterns give an indication of the ability of the mosquito vector to disperse drug-resistant strains of the malaria parasites. This again enables workers in malaria control to know in which areas they need to be on the lookout for the development of such resistance.

Control of malaria using transgenic technology. Elsewhere it has been suggested that malaria can be controlled by the use of transgenic technology. This would entail the introduction of genes responsible for refractoriness to infection into natural populations of the mosquito vector by the malaria parasite. Considerable progress has been made towards the identification of mosquito refractory mechanisms (*6, 115, 116*), although the study of possible mechanisms for introducing refractory genes into natural populations of vectors has not advanced much beyond the theoretical stage (*117*). However, the success of a programme for malaria control based on genetically modified vector populations also requires a thorough understanding of the genetic population structure of the vector and of barriers to gene flow. This understanding is critical in the determination of the spatial and temporal scale required for such gene introductions. Focal gene introductions would be unsuitable for populations experiencing little or no gene exchange, as the genes of interest would not spread.

Population bottlenecks. One question that a population geneticist seeks to answer is whether or not populations experience bottlenecks. Population bottlenecks occur when the size of the population is reduced dramatically through some events. Subse-

quent population expansion is from the few remaining individuals. Severe bottlenecks would be expected in mosquito populations that survive through adverse seasons (such as the dry savannas of sub-Sahelian Africa) in small persistently breeding pockets. Where populations persist as aestivating adults, such bottlenecks need not be experienced. Lehmann et al. (*118*) found the effective population sizes for *A. gambiae* in western and coastal Kenya to be at least 10^3 individuals, whereas Taylor et al. (*119*) estimated the effective population size of *A. arabiensis* in the even drier northern savannas to be at least 400 individuals. (Such numbers do not represent the absolute number of females surviving the bottlenecks but rather the effective number of females contributing their alleles to the subsequent generation at which allele frequencies are measured.) These findings suggest that a substantial number of mosquitoes survive during the dry season, possibly staying dormant in animal burrows. In situations where severe population bottlenecks occur, however, some allele frequencies will change whereas other alleles may disappear altogether. This means that refractory genes introduced into a population could be lost, creating the need to reintroduce them. This information is important in determining the temporal scale of gene introductions and impacts on strategies for vector control.

Population genetic tools are also being used to answer other ecological questions, such as whether members of species complexes partition different components of their environment, such as larval breeding habitats (*120*), or whether *Anopheles* species that lay eggs in small pools disperse their egg batches over many pools or lay the entire batch in a single pool (J. Meese and D. W. Severson, personal communication).

The use of genetic/molecular markers to estimate levels of genetic variability in a population depends on the assumption that they are selectively neutral. However, by processes such as genetic hitchhiking, some alleles of putatively neutral markers may show close linkage to genes that are actually under selection. Such alleles may then be used as indicators of the selective processes at closely linked loci. This approach (building on the markers for the *rtp1* and *rtp2* genes mentioned previously) is now being used to assess whether permethrin resistance in western Kenya is spreading from the site where it was originally found to a new and larger study area of permethrin-impregnated bednets (J. Vulule, unpublished data). More generally, a genome-wide scan for the association of particular alleles with other traits of potential relevance to transmission (such as activity cycles, resting behaviour, host attraction or susceptibility to infection) could be used to look for markers hitchhiking with a locus under selection. Once a stable linkage is found, such indicator alleles in other areas where mosquitoes are sampled could be used as screening tools for that specific vector activity. Therefore, without resorting to expensive and time-consuming ecological and behaviour studies, simple genetic assays could provide basic information needed for more targeted vector control. For example, the presence of indicator loci with significantly reduced allelic variation in early-evening outdoor biting vectors rather than late-night indoor biters could provide early indication of behavioural selection towards avoidance of a late-night control strategy such as insecticide-impregnated bednets.

Conclusions

Important applied advances in research have been made in the areas of *Anopheles* species identification, the determination of the molecular basis of many types of insecticide resistance and the understanding of such population genetic phenomena as deme size, gene flow, effective population size and within-taxon population structure indicative of the presence of emerging or cryptic species. Tools are now available that will enable both research scientists in the field and professionals concerned with malaria control to understand better the biology of malaria parasite transmission and to use this knowledge in devising more efficient vector-targeted control programmes. The potential value and importance of using tools that permit better identification of the vector species involved in transmission is obvious. Although some vectors, such as *A. albimanus* in central America and the Caribbean, are not members of species complexes (and can thus be identified by simple morphological assays), most major vectors seem to be members of complexes of morphologically identical species. Moreover, many such complexes include members that make significantly different contributions to malaria transmission, usually because of major differences in biological characteristics, such as blood meal preference. When members of such complexes are found in the same geographical area, it is important not only to carry out careful studies of their biologies and involvements in transmission but also to integrate this information into control programmes. Lessons learned from the now well-studied complexes, such as the *A. maculipennis* complex in Europe and the *A. gambiae* complex in Africa, are likely to apply far more widely.

With the rapid worldwide spread of parasite resistance to the more affordable and previously widely used antimalarials such as chloroquine, the malaria control community has been returning to strategies based on insecticides, especially the use of bednets impregnated with pyrethroid insecticides. In fact, programmes for insecticide-impregnated bednets are now being implemented as key components of countrywide control programmes in many malarious countries in the world. Monitoring of insecticide resistance is an important component of most of these control programmes, but sampling of sufficient specimens collected in the field can be difficult in areas where such control programmes are in progress. It is generally recognized that detecting resistance at low frequencies in populations and

shifting to the use of alternative insecticides to which the emerging resistance mechanism does not confer cross-resistance, can be valuable in forestalling the development of high levels of resistance in populations; bioassays are poorly suited to provide such data. Thus the development of molecular and biochemical assays that can detect resistance genotypes in populations at frequencies far below those possible with bioassays should be integrated into all insecticide-based malaria control programmes where such assays could play a role. Moreover, specialists in control programmes who begin to find bioassay evidence for the emergence of resistance should make every effort to recruit molecular biologists to study the mechanisms of resistance and to devise methods for rapid detection in single specimens. Finally, although selection of alternative insecticides in the face of emerging resistance can be made empirically on the basis of bioassays, an understanding of the molecular basis of resistance can often provide a more rational basis for such a choice.

Although the argument in support of many genetic studies in *Anopheles* populations is frequently the potential value of such knowledge in the context of "future genetic control programmes", population genetics is also of more immediate value to the specialist in malaria control. First, and perhaps most important, it can provide a clear picture of the presence or absence of substructure within given vector populations. With vectors that have not been extensively studied by using more conventional markers, such as polytene chromosome inversions or isoenzyme allele frequencies, molecular genetic studies of populations may provide the first clue that a presumed monotypic taxon consists of two or more taxa located in the same geographical area that have little or no between-taxa gene flow. Such findings can then lead to the development of more rapid assays for taxon identification and the subsequent study of such taxa for potential differences in their involvement in transmission. Variables in population structure can also provide information that can be used to predict whether insecticide resistance is likely to spread rapidly to new locales where the insecticide is also being used or whether there are barriers to gene flow that are likely to curtail such spread.

In summary, we believe that recent molecular developments in vector species identification, vector population genetics and the detection and understanding of insecticide resistance are producing assays and information that should enable professionals in malaria control to improve significantly the efficiency of vector-targeted control efforts. Assays currently exist and more are being developed that can help guide decisions related to control efforts, especially those based on insecticide use. We strongly recommend that the malaria control community makes every effort to establish links with the international groups currently developing these new assays. Professionals in malaria control can play an important part in guiding the research direction of such international groups, and these groups can greatly assist control efforts by providing training and emphasizing to their funding agencies the importance of such training. ■

Acknowledgements

Résumé

Entomologie moléculaire et perspectives en matière de lutte antipaludique

Le développement de l'entomologie moléculaire a été fortement stimulé par une réunion scientifique parrainée par l'OMS/Programme spécial de recherche et de formation concernant les maladies tropicales (OMS/TDR), la Fondation John D. and Catherine T. MacArthur et l'Université d'Arizona à Tucson, qui a eu lieu en 1990 à Tucson (Arizona). Les résultats de cette réunion ont été présentés dans le document OMS/TDR *Prospects for Malaria Control by Genetic Manipulation of its Vectors*, qui a servi par la suite à définir les grands axes de recherche pour le Comité d'orientation sur l'entomologie moléculaire nouvellement créé. Grâce en grande partie au financement assuré par ce comité, d'importants progrès ont été réalisés ces dix dernières années dans l'étude moléculaire des vecteurs du paludisme, notamment la mise au point d'outils moléculaires sophistiqués pour des opérations comme l'établissement de la carte génétique et physique et la transformation de la lignée germinale. D'importants progrès ont également été réalisés dans l'étude de processus biologiques spécifiques tels que les mécanismes de défense des insectes contre les agents pathogènes et la façon dont les parasites du paludisme et les anophèles hôtes interagissent au cours de la sporogonie. L'un des événements marquants de cette tendance de la recherche a été l'émergence, l'année dernière, d'un projet international officiel de cartographie du génome d'*Anopheles gambiae*, auquel participent actuellement des chercheurs de plusieurs laboratoires d'Europe et des Etats-Unis d'Amérique. Bien qu'une grande partie de ces travaux soient axés sur la recherche fondamentale ou sur l'élaboration de stratégies de lutte antipaludique qui ne seront probablement pas applicables avant de nombreuses années, il existe certains domaines importants de l'entomologie moléculaire qui peuvent avoir des retombées plus immédiates sur la lutte antipaludique.

Nous décrivons ici les acquisitions majeures de la dernière décennie dans trois domaines que nous estimons susceptibles de contribuer de façon importante à l'élaboration de stratégies de lutte applicables dans un avenir proche. Il s'agit de l'identification des espèces anophéliennes, de la détection et de la surveillance de la sensibilité ou de la résistance aux insecticides chez les populations sauvages d'anophèles, et de la détermination de la structure génétique des populations anophéliennes. Nous voulons non seulement souligner quelles sont les avancées majeures dans ces domaines, mais également indiquer la façon dont ces résultats peuvent intéresser les spécialistes de la lutte antipaludique. Nous pensons que les progrès récemment réalisés au niveau moléculaire dans l'identification des espèces vectrices, la détection et la compréhension de la résistance aux insecticides et la génétique des populations de vecteurs

déboucheront sur des tests et des informations qui devraient permettre aux professionnels spécialisés dans la lutte antivectorielle d'améliorer sensiblement l'efficacité des mesures axées sur les vecteurs. Il existe déjà des tests, et d'autres sont en cours de mise au point, qui pourront aider à orienter les choix en matière d'activités de lutte, en particulier celles qui reposent sur l'emploi d'insecticides. Nous recommandons vivement que les acteurs de la lutte antipaludique s'efforcent d'établir des liens avec les groupes internationaux qui travaillent actuellement sur ces nouveaux tests. Les spécialistes de la lutte antipaludique peuvent prendre une part importante à l'orientation des travaux de recherche de ces groupes, lesquels peuvent à leur tour contribuer aux efforts de lutte en assurant des activités de formation, et en insistant auprès de leurs organismes de financement sur l'importance d'une telle formation.

Resumen

Entomología molecular y perspectivas de la lucha contra el paludismo

El desarrollo de la entomología molecular recibió un fuerte impulso en 1990 en una reunión científica celebrada en Tucson, Arizona (Estados Unidos) y patrocinada por el Programa Especial de Investigaciones y Enseñanzas sobre Enfermedades Tropicales de la Organización Mundial de la Salud (OMS/TDR), la Fundación John D. y Catherine T. MacArthur y la Universidad de Arizona en Tucson. Las conclusiones de esta reunión se resumieron en el documento OMS/TDR *Prospects for Malaria Control by Genetic Manipulation of its Vectors* [Perspectivas de la lucha antipalúdica basada en la manipulación genética de sus vectores], que sería utilizado posteriormente para definir las prioridades básicas de investigación del recién establecido Comité Directivo OMS/TDR de Entomología Molecular. Estimulados en gran parte por fondos proporcionados por este nuevo comité directivo, en el último decenio los estudios moleculares sobre los vectores del paludismo han experimentado grandes progresos. Muchos de esos avances guardan relación con el desarrollo de herramientas moleculares complejas para procedimientos tales como la cartografía genética y física y la transformación de líneas germinales. Se han logrado también importantes avances en el estudio de procesos biológicos específicos como las defensas del insecto contra agentes patógenos y los mecanismos de interacción entre los parásitos del paludismo y sus huéspedes anofelinos durante la esporogonia. Uno de los aspectos más notables de esta línea de investigación ha sido el inicio durante el último año de un proyecto internacional oficial de estudio del genoma de *Anopheles gambiae*, en el que actualmente trabajan investigadores de varios laboratorios de Europa y de los Estados Unidos. Aunque muchas de esas investigaciones moleculares son de carácter teórico o tienen por objeto la formulación de estrategias de lucha contra el paludismo que probablemente tardarán muchos años en aplicarse, la entomología molecular presenta algunas áreas relevan-

tes donde los avances podrían tener repercusión a más corto plazo en la lucha contra la enfermedad. Destacamos aquí los avances registrados durante la pasada década en tres de esas áreas, que consideramos que podrían impulsar considerablemente el desarrollo de estrategias de control del paludismo a corto plazo. Esas áreas son la identificación de especies anofelinas, la detección y vigilancia de la sensibilidad/resistencia a los insecticidas en poblaciones salvajes de anofeles y la determinación de la estructura genética de las poblaciones de anofeles. Procuramos destacar no sólo los principales progresos de las investigaciones en esos ámbitos, sino también el valor que esos avances encierran para los profesionales implicados en la lucha contra el paludismo. Consideramos que los recientes progresos en la identificación molecular de especies de vectores, la detección de la resistencia a los insecticidas y el conocimiento de los mecanismos que la determinan y de la genética de las poblaciones de vectores están generando pruebas y datos que deberían permitir a los profesionales implicados en la lucha antivectorial mejorar sustancialmente la eficiencia de esas actividades de lucha. Se dispone ya de pruebas, a las que se sumarán otras ahora en desarrollo, que pueden servir de orientación para tomar decisiones en materia de lucha antivectorial, especialmente en lo relativo al uso de insecticidas. Recomendamos firmemente que la comunidad activa en el control del paludismo haga todo lo posible para establecer contacto con los grupos internacionales que están desarrollando las nuevas pruebas. Los profesionales que trabajan en ese terreno pueden desempeñar un papel importante orientando los esfuerzos de investigación de tales grupos internacionales, y estos grupos pueden a su vez contribuir enormemente a las actividades de control proporcionando capacitación y resaltando ante los organismos que los financian la importancia de esa capacitación.

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