# Malaria: existing methods of vector control and molecular entomology

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In general, the most effective means of malaria vector control is the killing of adult mosquitoes with a residual insecticide applied to bednets or sprayed on house walls and ceilings. Major reductions in all-cause child mortality have been achieved in Africa by these means. In some circumstances, personal protection and larval control may also make a contribution. We discuss the prospects of genetic control by release of sterile male mosquitoes or driving genes for refractoriness to malaria into wild populations. Many major malaria vectors belong to complexes of sibling species which differ in vectorial and biological characteristics. Distinguishing the species by cytogenetic or molecular methods is important for epidemiological studies and could improve the targeting of control.

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Most of the successful attempts at malaria eradication or control have exploited the 'weak link' in the life cycle of *Plasmodium*, represented by the fact that most *Anopheles*, which have picked up an infecting dose of gametocytes, die of natural causes before the process of sporozoite production has been completed. Increasing this mosquito mortality rate, for example with residual insecticides, reduces the number of sporozoite infective mosquitoes almost to zero. Through such means, malaria has been successfully controlled or eradicated from many regions of the world but, in others, it has proved refractory to such efforts. However, improvements in methods for reducing these relatively small numbers of dangerous mosquitoes have recently been made or are now in prospect. Furthermore, molecular and recombinant DNA technology offers new possibilities for malaria vector control and for evaluating the role of mosquitoes in malaria epidemiology. In this article, we review the successes and difficulties with currently available control methods and examine ways in which molecular entomology may contribute to more effective control.

# Insecticides and repellents

## House spraying with DDT or modern insecticides

Most of the dangerous malaria vector species come indoors to bite at night and some rest indoors for the subsequent hours or days. This is the basis for the successful use of residual insecticide, sprayed indoors on walls and ceilings.

DDT is cheap and durable and, long after it was banned for agricultural use because of its tendency to accumulate in outdoor food chains, it continued to be WHO's insecticide of choice for malaria vector control by house spraying, except where there is DDT resistance. As discussed by Curtis[1], there are now reasons to prefer pyrethroids (synthetic and more durable analogues of natural pyrethrum) for this purpose because: (i) they kill mosquitoes more quickly so fewer exit from a sprayed room than is the case with DDT[2]; (ii) they do not accumulate in mammalian tissues, whereas the accumulation of DDT in human breast milk follows from its anti-malarial, as well as its agricultural usage[3]; and (iii) the illicit diversion to agriculture of DDT intended for anti-malarial use may lead to crop-residues above the extremely stringent limits set for agricultural exports.

A link between residues of a DDT derivative and subsequent development of breast cancer was reported by Wolff et al[4], but a meta-analysis of several such studies appears not to substantiate this[5].

## Pyrethroid treated bednets

A more precisely targeted way of using residual pyrethroids is to apply them to bednets or curtains to which mosquitoes are attracted in the course of seeking bed or house occupants. The safety record of pyrethroids when used in such close proximity to humans is re-assuring[6]. The treated net is a form of baited trap, with the human occupants forming the bait. When mosquitoes contact pyrethroid residues, they are either irritated so that they cease trying to penetrate the net to bite or they are killed.

Millions of treated nets are in use in China[7] and Vietnam (Tran Duc Hinh and J.D. Lines, personal communications) with a highly significant impact on malaria transmission and malaria morbidity. In several African countries, significant reductions in all-cause child mortality have recently been reported in large scale trials of treated nets (Table 1) although problems remain in extending the use of treated nets in that continent.

The insecticidal effectiveness of a residue of one of the more modern alpha-cyano pyrethroids can persist on a net in domestic use for a year

**Table 1** Results of studies of impact of insecticide treated bednets on all-cause child mortality in areas of four African countries arranged in order of intensities of malaria transmission.

| Country | Infective bites/person/year | % reduction in child mortality (95% CI) | Reference |
|---------|------------------------------|------------------------------------------|-----------|
| Gambia | 1–10 | 63% (32 to 88%) | 8 |
| Gambia | 1–10 | 25% (2 to 43%) | 9 |
| Kenya | 10–30 | 33% (7 to 51%) | 10 |
| Ghana | 100–300 | 17% (0 to 31%) | 11 |
| Burkina Faso | 300–500 | 15% (–4 to 30%) | 12 |

or more[13]. The residue is somewhat reduced by repeated vigorous washing of nets. In urban areas, where frequent net washing is generally felt to be important, the most practicable solution may be to sell 'dip-it-yourself' kits with sachets or tablets which provide a sufficient dose to bring back the residue or a washed net to the required level after each wash[6,13].

'Olyset' nets, in which a pyrethroid has been incorporated into polyethylene before forming it into fibres from which netting is made, continue to be insecticidally effective for at least 2 years of domestic use. If they prove effective for 3 or 4 years and can be made cheap enough, they could be a practicable alternative to the use of conventional netting for which a system of regular re-treatment has to be put in place.

Despite the proven success of treated nets in malaria control, there remain concerns about their long term impact, particularly if their use should lead to a gradual reduction in naturally acquired immunity as argued by Snow *et al*[14] and elsewhere in this issue. Such a reduction might explain the apparently lesser percentage impact on child mortality where intensity of transmission (and hence initial levels of immunity) are higher (Table 1). The additional use of a vaccine to replace lost immunity may eventually prove possible and necessary.

In earlier decades, reductions in child mortality comparable to those shown in Table 1 were achieved by house spraying in the Pare-Taveta and Kisumu projects in East Africa[15,16]. No direct comparison on a scale sufficient to detect mortality effects has yet been made between house spraying and treated bednets. However, in a recent trial in Tanzania their effects on entomological parameters, incidence of re-infection with malaria and haemoglobin levels in children were found to be similar[17]. However, costings of nets, insecticide and the labour involved in application favoured the bednet method, as also reported by Kere and Kere[18] from a comparison of treated nets with DDT spraying against *Anopheles farauti* in the Solomon Islands. Against that vector, the nets were also decisively more effective than DDT spraying[19].

## Pyrethroid resistance

In view of the present emphasis on pyrethroids for both house spraying and treated bednets, resistance to these compounds is of particular concern. Cross-resistance to pyrethroids and DDT has been reported under the name *kdr* (knock-down resistance) in several insect species[20,21], but other mechanisms of DDT resistance which do not give cross resistance to pyrethroids are more common in anophelines[22]. However, pyrethroid resistance in adult *Anopheles stephensi* is multifactorial and includes a *kdr*-type mechanism[23]. Furthermore, there is a recent report of *kdr*-type resistance in *Anopheles gambiae* in West Africa[24], thought to have been selected by earlier use of DDT and/or agricultural use of pyrethroids, but not by the use of treated nets.

There was no detectable build up of resistance in malaria vectors following the use of pyrethroid-treated nets over 7 years on a large scale in China[25] and in a single village in Tanzania[26]. However, after treated nets or curtains were used for 1 year in four Kenyan villages, there was an increase in the median time of exposure required to kill *An. gambiae*. No further increase was found after 2 more years use of the nets or curtains, but resistance could be enhanced by laboratory selection[27]. In this case, resistance to permethrin did not confer cross resistance to deltamethrin, but in both larval and adult *An. stephensi*, resistance to permethrin extends to cyano-substituted pyrethroids[28,29] and the related compound etofenprox[30].

The above information was mostly obtained by direct bioassays of the killing power of a given pyrethroid exposure to different mosquito strains. Such simple tests have the virtue of picking up cases of resistance due to any of the various biochemical or neurophysiological mechanisms which are known to arise. However, the detection of genes for resistance when they are at low frequency is difficult or impossible by bioassays, but feasible by biochemical and molecular tests for the mechanisms of resistance. Detection of resistance at such a low level could be valuable by allowing an early switch to an alternative, unrelated insecticide to which the population remains susceptible. Changes in a voltage-sensitive sodium ion channel genes are thought to be important in pyrethroid resistance of the *kdr* type[23,24,31] and current research is concentrated on developing PCR-based tests for such forms of resistance. Combined with other biochemical tests for detecting resistance based on abnormal levels of esterase or cytochrome P-450, such methods could be very valuable in detecting the early appearance of resistance.

## The role of synthetic and biological larvicides

Where anopheline breeding sites are limited and readily found, well organized and sustained use of larvicides can suppress the density of

adult mosquito populations sufficiently to control malaria transmission. However, this approach is relatively costly, non-persistent compounds of low mammalian toxicity must be used and resistance can develop fairly rapidly. The organophosphate temephos (Abate) is the standard insecticide for this purpose, and is safe in drinking water. However, there are recorded cases of resistance in seven species of *Anopheles*, including five in the Middle East where larviciding is commonly employed.

Pyriproxyfen, which interferes with larval/pupal/adult metamorphosis, is more durable and cost effective than temephos and other larvicidal agents[32]. A recent trial in Sri Lanka showed a highly significant impact on malaria incidence.

The toxins produced by the bacterium *Bacillus thuringiensis israelensis* (Bti) and *B. sphaericus* are very specifically toxic after ingestion by the larvae of mosquitoes and *Simulium*. Commercially produced Bti is extensively used in nuisance mosquito control in environmentally sensitive areas in developed countries. Although bacterial toxins require frequent re-application, they are now favoured in India[33], one advantage being that their specificity removes the temptation to divert them illicitly from anti-malarial to agricultural use.

Larvivorous fish continue to have some role in countries, such as India, but attempts to find other self-replicating biological control agents for *Anopheles* larvae have not met with much success.

### Domestic protection and repellents

Urban populations in the tropics spend appreciable proportions of their income on protection against mosquitoes[34]. Some of the methods used are effective, *e.g.* house screening or electrically heated vaporising mats containing volatile pyrethroids. A factory in Mumbai (Bombay), India, produces 6 billion of these mats per year. On the other hand, astute advertising finds a market for totally ineffective products such as buzzers or so-called ultrasonic repellers[35]. In between lie products which have some effect but which are not a wise investment, especially for those with low incomes. Thus there is an important role for objective testing and reporting on the effectiveness of anti-mosquito products.

Among repellents for use on skin and/or clothing, deet (dimethyl benzamide = diethyl toluamide) is the most widely available active ingredient. Occasional poisonings have been recorded due to deet, but at no higher rate per package sold than with other common household chemicals[36]. Deet also attacks certain plastics (for example, in spectacle frames) and paint work. No such effects, nor any poisoning, have been reported for a repellent derived from lemon eucalyptus, despite a considerable volume of sales. This repellent was reported in China (data

of Li Zuzi in Curtis *et al*[37]) and Tanzania[38] to be nearly as effective as deet, in contrast with citronella, another plant product with a lemon fragrance, which is markedly less effective and durable[39,40], although it is extensively sold in the USA mixed with lamp oil. Although repellents applied to skin and clothing, or volatile pyrethroids in vaporising mats, *etc.* are of proven efficacy in reducing the numbers of mosquito bites, their impact on malaria has not been quantified.

# Genetic control

## Sterile insect technique

A quarter of a century ago there was much enthusiasm for testing the sterile insect technique (SIT) against mosquitoes[41], including anophelines[42]. However, due to ill-informed press and political campaigns[43] the idea went into eclipse. Since then, screw worm flies and Mediterranean fruit flies have been eradicated from whole countries in Latin America by SIT and, as a result, enthusiasm for this method of eradication for some *Anopheles* populations seems to be undergoing a modest revival[44].

The earlier work had underlined the problem of immigrant, already inseminated females which would not re-mate with sterile males on arrival in the release area. It also emphasised the need for a reliable method for eliminating biting females from batches of males being prepared for release. In successful SIT programmes the problem of immigration has been overcome by massive rolling programmes of release from factories, some with capacities in excess of 100 million insects per week. The same scale of investment is unlikely in a public health programme where the economic benefits are less tangible. However, more modest rearing facilities could well be enough to eradicate residual urban populations left after intensive application of conventional larvicidal control, where the surrounding countryside is occupied by a different anopheline species (*e.g.* south Indian urban *An. stephensi* surrounded by rural *An. culicifacies*).

Sex separation can be conveniently based on a dominant insecticide resistance gene translocated on to the Y chromosome, with the untranslocated allele being for insecticide susceptibility. Thus, the homozygous susceptible females can be killed in the aquatic stages leaving the heterozygous males. Such systems have been produced for several anopheline species and for Mediterranean fruit flies. In one case, an inversion was introduced to inhibit crossing over between the resistance gene and the translocation breakpoint[45]. Such a stabilising system would

seem to be essential for any large scale programme in which rare recombinants could cumulatively blunt the effectiveness of the system[46]. It is hoped to select an appropriate inversion by *in situ* hybridisation to an existing clone for a dieldrin resistance gene[47].

### Genetic transformation to render mosquitoes unable to transmit malaria

The SIT depends on dominant lethal mutations transmitted by the released males into the eggs laid by their wild mates. An alternative would be to use genes which are not lethal to the eggs, but which result in adults which are harmless to humans either because they cannot be infected by *Plasmodium* or because they prefer animals to man for their blood meals.

*Anopheles* strains which are non-susceptible (refractory) to some species or strains of *Plasmodium* have been found in nature or have been selected by conventional animal breeding techniques. For example, it has been possible to select a strain of the major African malaria vector, *An. gambiae*, that encapsulates and kills the malaria parasites within a melanin-rich capsule in the mosquito midgut. Genetic mapping has revealed one major and two minor loci controlling this encapsulation reaction[48]. In several other cases, refractoriness appears to determined by more than one gene locus. Where a reliably refractory phenotype requires the concerted action of alleles at several loci, this would make the driving of the corresponding genotypes into wild populations more difficult, if not impossible.

An alternative approach has been to attempt to identify the specific mosquito cell surface receptors that are essential to the malaria parasite's development and then develop constructs that block the receptor-mediated interaction[49]. Others have focused on detailed studies of the innate immunity mechanisms of mosquitoes with a view to developing parasite-killing constructs but, while these approaches have yielded interesting scientific data, as yet no genetic constructs of potential value in control have emerged. At one stage, it was suggested that genetic constructs could be made containing genes or parts of genes for mammalian antibodies to surface proteins of the ookinete stage of the malaria parasite. These constructs would then express the ookinete blocking antibody in the mosquito midgut. Although experiments have demonstrated that mammalian antibodies can indeed block parasite development in the midgut, there are practical problems in handling genetic constructs for such large proteins.

An alternative idea from Bob Sinden and Julian Crampton has been to develop constructs that contain antigen genes that can be expressed in the salivary glands of mosquitoes. The mosquito, thereby, would serve

as a vaccine delivery system. This concept has been tested using *Aedes aegypti* and a rodent malaria antigen, Pbs21, that is specific to the sexual stages of the parasite. Antibodies to this effectively block transmission. An expression vector construct has been made in which the Pbs21 gene is under the control of a baculovirus promoter. The gene has been transiently expressed in mosquito cells in culture and the recombinant Pbs21 protein shown to induce a transmission-blocking immunity in mice. It has also been possible to transfect cultured salivary glands using a liposome-based transfection agent and demonstrate gene expression in this *in vitro* system. The next step is to produce a transgenic mosquito capable of delivering a small amount of this antigen at the time it takes its blood meal (Stowell, Crampton and Sinden, personal communication). Two problems that would have to be faced if this method is ever going to be used are: (i) the acceptability to human populations of involuntary vaccination; and (ii) the wide variation in dose of vaccine that would be received by different individuals.

## Means of genetically transforming insects in the laboratory and spreading genes in wild populations

A variety of methods have been suggested for introducing desirable genes into mosquitoes in the laboratory and spreading these genes in natural populations. The working of some of the latter proposed methods is illustrated in simplified form in Table 2 and described more fully below. Mass release of males carrying refractoriness genes, but no specific means of giving these genes a selective advantage in the field (Table 2A), would require even more massive rearing facilities than the SIT, because the latter method has the advantage that the population of competing wild males would steadily decrease. Therefore, the practical application of such techniques would seem to demand a means whereby a relatively small seeding release would lead eventually to increasing frequencies of the desired genes and the spreading of them into new areas and into wild type immigrants entering the release area.

Various genetic systems for this have been proposed; of these the use of transposable genetic elements[50] or *Wolbachia* bacteria[51] seem the most hopeful.

In *Drosophila*, one transposable genetic element, the P-element, has apparently become fixed in virtually every wild population of this fly within a 50 year period, despite reduced fitness in its carriers and a decline in transposition frequency during its spread in populations. Hence, the idea of linking some desirable trait, such as refractoriness to the malaria parasite, to a transposable genetic element and introducing it into mosquito populations has attracted much research interest. There

**Table 2** Simple model to compare possible methods of spreading a refractoriness gene in a wild population. It is assumed that releases establish a frequency p of the refractoriness gene (R), leaving q of the susceptibility allele (S), (p + q = 1)

**(A) No driving system**

| | | Male gametes | | $q_1$, susceptibility frequency at next generation |
|---|---|---|---|---|
| | | S (q) | R (p) | |
| Female | S (q) | SS (q²) | RS (pq) | $q^2 + pq = q$ |
| gametes | R (p) | RS (pq) | RR (p²) | (Hardy Weinberg) |

**(B) Transposon (T) linked to R**

| | | Male parents | | |
|---|---|---|---|---|
| | | SS (q) | TT (p) | |
| Female | SS (q) | SS (q²) | TS → *TT (pq) | $q^2$ |
| parents | TT (p) | TS → *TT(pq) | TT (p²) | |

*Transposition (assumed to occur in all heterozygotes for T)

**(C) Wolbachia infected (I) mosquitoes with the R gene and uninfected (U) ones with S**

| | | Male parents | | |
|---|---|---|---|---|
| | | U (q) | I (p) | |
| Female | U (q) | U (q²) | sterile (pq) | $q^2/(1 - pq)$ |
| parents | I (p) | I (pq) | I (p²) | |

have been several studies of the population genetics of these elements and their application to driving desired genes into vector populations has been modelled (*see* Table 2B and Kidwell & Ribeiro[50] for a more comprehensive treatment).

Several of the transposable elements are currently the subject of attempts to transform insects genetically in the laboratory, for example members of the *hAT* group, particularly constructs based on *Hermes*, as well as various *mariner/Tc1*-like elements. One of the latter, *Minos*, a transposable element from *Drosophila hydei*, has been successfully used as a gene vector in the Medfly, the first example of a non-drosophilid insect being genetically transformed through transpositional recombination involving a gene vector[52]. Recently, Jasinskiene et al[53] has reported that a gene vector based on *Hermes* has been used to transfect mosquitoes and to produce a number of apparently stable transformed lines expressing the introduced gene. This is the first example of a transgenic mosquito in which the integrated foreign gene is transmitted in a stable manner through the germ line, an essential prerequisite for construction of genetically manipulated control agents.

Instability of gene vectors based on transposable elements may occur where there are related endogenous elements. This could be countered

by constructing suicide vectors that partially self-destruct following integration, thereby preventing their further transposition; this property would be useful for an experimental laboratory transfection system, but it would deprive the element of its capacity to spread in natural populations. It remains uncertain whether any of the above described transposable elements could be used to drive genes into natural populations.

*Wolbachia* are maternally inherited and cause sterility in matings of male insects infected with these bacteria to uninfected females. The reciprocal cross is fertile. Thus, uninfected females are at risk of failing to pass on their uninfected cytoplasm, whereas infected females are at no such risk (Table 2C). Again, in *Drosophila*, there is evidence for the spontaneous spreading, over very large areas, of *Wolbachia*[54]. It may be possible to introduce functional genes into *Wolbachia* or other maternally inherited entities so that these would 'hitch hike' with the spreading *Wolbachia*; a mitochondrial variant was observed to do this in the above mentioned spontaneous spreading process. The feasibility of using transgenic endosymbionts to alter the disease carrying capacity of a disease vector (*Rhodnius prolixus*) has been demonstrated[55].

In addition to transposable elements and *Wolbachia*, candidates for the introduction and spread of desirable genes may be found among the RNA and DNA virus gene-delivery vehicles currently being studied[56,57]. One transfection system, based on a mosquito parvovirus, AeDNV, has already been successfully tested in mosquito cell cultures and it has been suggested that a similar construct could carry genes expressing refractoriness and be used to infect wild mosquito populations, although it would not have the same driving potential as a transposable genetic element or *Wolbachia*.

In a programme using one of these driving factors, even rare events which dissociate the driving factor from the refractoriness gene could lead to failure to achieve anything useful if (as seems probable) the driver without the gene would have a selective advantage over the driver with the gene.

Finally, it should be noted that transgenesis has become the focus of much public concern about attendant ecological and health risks. These concerns, currently directed at transgenic soya beans and tomatoes, may be far more strident and influential where transgenic mosquitoes are involved.

## Molecular taxonomy and mosquito control

Sir Ronald Ross first introduced the concept of controlling malaria by controlling the larvae of the vector mosquitoes. It was soon realised that, in a given geographical area, only certain species of *Anopheles* were

**Table 3** *Anopheles* sibling species complexes: why identification is important and how it is done

| Complex | Geographic region | No. of species | Notes | Identification |
|---------|-------------------|----------------|-------|----------------|
| *An. gambiae* | Afrotropical | 6 + 3 'forms' | Two major vectors and one non-vector | A,B,C,D |
| *An. culicifacies* | Predominantly oriental but into Afrotropical + Palaearctic | 4 | One major vector and one non-vector in India | A,B,C,D |
| *An. dirus* | Oriental | 7 | Major differences in biology and vectorial importance | A,B,C,D |
| *An. punctulatus* | Australasian | 10 | (As for *An. dirus*) | A,B,C |
| *An. maculipennis* | Palaearctic and Nearctic | 16 | Some important, others poor vectors | A,B,D |
| *An. nuneztovari* | Neotropical | 3 | ? | A, ? |

*Identification method*: A, polytene chromosomes; B, isoenzymes; C, DNA probes; and D, PCR or other sequence differences.

important in malaria transmission, hence larval control could be focused on these species and there followed a great flowering of taxonomic research into mosquitoes. This straightforward picture became confused during the early 1920s with the discovery in southern Europe of 'anophelism without malaria', areas where, though conditions seemed suitable and the vector species *An. maculipennis* was present, yet malaria did not occur. On closer study, it emerged that *An. maculipennis* was not a single species but a group of species, which were morphologically distinct only in their eggs, but which showed important differences in their tendency to bite humans or animals[58].

Many other examples have subsequently come to light among malaria vectors through laboratory crossing experiments, studies of the giant chromosomes (cytotaxonomy) and, more recently, the use of molecular techniques. Each of the major malaria vectors in Europe, Africa, the Middle East, Indian sub-continent, South-East Asia, China, Australasia and the Americas has subsequently been shown to constitute, a sibling species complex (Table 3). Such discoveries are not only of interest to evolutionary biologists, but also of considerable importance to the understanding of malaria epidemiology and, potentially, better targeting of control, since in all the major sibling species complexes of malaria vectors there are differences in vectorial importance, biting behaviour and larval ecology between the constituent species.

For these reasons considerable effort has been devoted to the development of techniques for identification of individual species within species complexes. The morphology of the giant (polytene) chromosomes has been a gold-standard in such work but the method is somewhat cumbersome for detailed field research and applicable only to
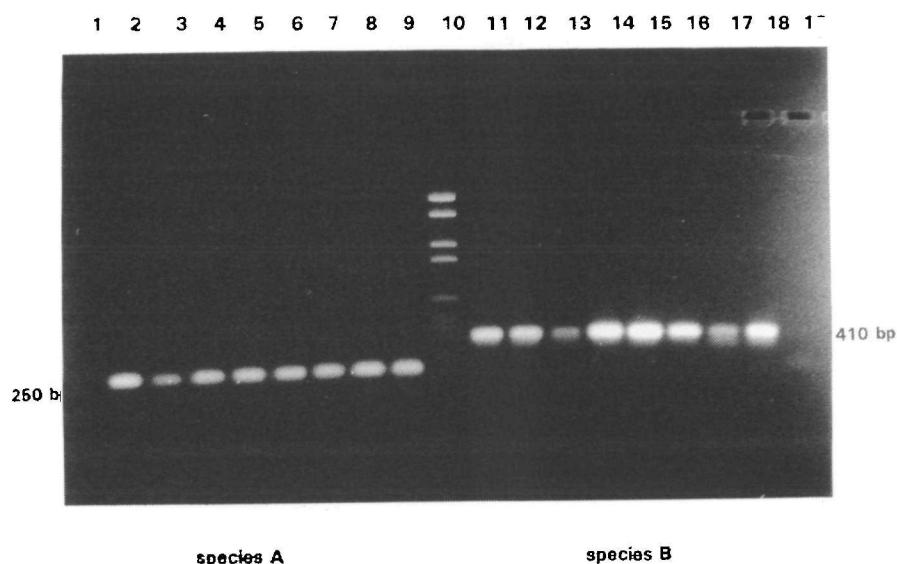
Fig. 1 PCR-based assays are increasingly used to distinguish malaria vector species which cannot be differentiated on morphological grounds. Here, a PCR assay based on DNA sequence differences in the ITS-2 spacer region of the nuclear ribosomal genes distinguishes two species of the *An. culicifacies* complex, which includes the major vectors of malaria in rural areas of the Middle East and Indian sub-continent. Species A, the most important malaria vector in India, is readily distinguished from species B which is not a vector.

fourth instar larvae, or more commonly, semi-gravid females. The most important advances have come from the application of recombinant DNA technology providing, first DNA probes based on high copy number repeat sequences, then, more recently, PCR-based techniques that rely on sequence differences, particularly in the nuclear ribosomal genes[59]. These are available for all members of the *An. gambiae* complex, the major malaria vectors in Africa, and are currently being developed for many other groups including *An. culicifacies*, the complex that includes the major malaria vectors of rural India (Fig. 1). PCR-based techniques are also being employed to study allelic variation at microsatellite loci and in mitochondrial genes in malaria vectors. These studies yield information on population structure and gene flow within individual species that should contribute to an understanding of the potential rate of spread of insecticide resistance and would be essential for those contemplating field releases of sterile or genetically transformed mosquitoes. One of the conclusions from this recent work is that, within tropical Africa, *An. gambiae* behaves largely as a single population over a range extending some 6000 km, with gene flow only weakly restricted[60].

A global *Anopheles* genome initiative is focusing on *An. gambiae* and rapidly expanding our knowledge of the genetics[61] of this, the most important malaria vector. It remains to be seen whether these advances in fundamental knowledge lead to more effective methods for malaria control.

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