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DNA PROBES FOR THE IDENTIFICATION OF MOSQUITO SPECIES

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Introduction

Mosquitoes are known to transmit a number of diseases to humans. The disease malaria for example, is transmitted to man through the bite of a mosquito infected with a protozoan parasite known as *Plasmodium.* The mosquito vector known as *Anopheles culicifacies* is mainly responsible for malaria in Sri Lanka. *An.culicifacies* is also a major vector of malaria in India and Pakistan.

Most mosquito species exist as a species complex comprising several sibling species within the same taxon. Sibling species arise due to reproductive isolation; they either do not cross-mate with other siblings or even if they cross-mate they do not produce viable offspring. However, their external features do not help to distinguish sibling species because they all look alike (isomorphic) and therefore the conventional taxonomic keys based on morphological features cannot be used to identify these siblings. In *An. culicifacies,* five sibling species have been identified up to now in India by the analysis of polytene chromosomes found in mosquito cells. Despite the similarity of these sibling species in their morphological features, differences between species have been found with respect to their seasonal prevalence, vectorial capacity and their response to insecticides. For example some sibling species are found to be more resistant to DDT insecticide, than the others. Each sibling species may therefore play a different role in transmission of malaria, due to Such behavioural differences amongst them. Hence it is very important to identify the sibling species of *An.culicifacies* present in areas where malaria is prevalent in Sri Lanka, in order to implement a successful vector control programme. For instance, if malaria vector species present in an area is known, the most suitable insecticide for that species could be sprayed. Proper use of insecticides will not only help to control the vector of malaria but also to reduce the wastage of resources.

Current Cyto-taxonomic Method of sibling species identification

The sibling species of the *An. culicifacies* are currently identified by staining and examination of the banding patterns of the polytene chromosomes present in the salivary glands or the ovaries of the mosquito. This is a technically difficult, time consuming method and certain stages of the life cycle can only be identified. A mosquito has several life cycle stages such as larvae, pupae and adult. Only fresh semi-gravid adult or mature larvae are identified by this procedure. Hence, many samples collected from the field cannot be identified due to this limitation.

DNA probes for mosquito sibling species identification

Recombinant DNA technology has contributed to the development of novel strategies

including DNA probes for the identification of mosquito vector species.

The use of DNA probes is an attractive alternative to the conventional techniques of vector identification due to the convenience, accuracy, sensitivity and reliability. In general, these techniques are not sex specific or life cycle stage specific and a large number of samples can be analysed at the same time. DNA probes are developed based on the differences found in the DNA of the members of a species complex.

What is DNA ?

DNA (deoxyribonucleic acid) is the genetic material found in an organism and contains the blueprint of life. It is found in the chromosomes present in the nucleus of cells. DNA is a giant molecule or a polymer made up of units called nucleotides (Fig.1). Each nucleotide is made up of three components; a phosphate group, a sugar and a nitrogenous base. There are four bases in the DNA; adenine (A), guanine (G), cytosine (C) and thymine (T) and hence a DNA molecule contains only four different nucleotides. One chromosome may contain as much as a billion nucleotides and the order of these nucleotide bases vary between regions giving rise to many different base sequences within the molecule. The genetic information in the DNA is encoded by this nucleotide base sequence of a DNA molecule. In other words, `the language of DNA' has four different letters and by changing the order or the sequence of the four bases (letters) the information in the DNA can be varied giving rise to a large number of different DNA molecules carrying various messages.

DNA exists in the cell as double stranded molecules in helical form. The two strands are held together by specific H-bonding between adenine and thymine and cytosine and guanine base pairs (Fig.2). Hence, the two strands of the DNA are said to be 'complementary' to each other and if the nucleotide base sequence of

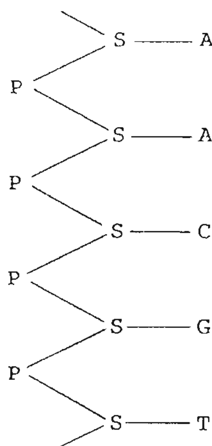

Fig. 1 : The DNA molecule is a polymer of nucleotides.
The nucleotide molecules are linked together producing an alternating sequence of phosphate (P) and sugar (S) residues, which is the backbone of the DNA molecule. Four types of bases (A,G,T and C) bind to the backbone via sugar residues.

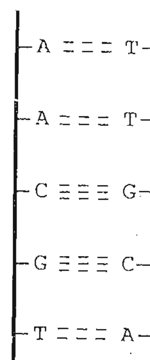

Fig: 2: Hydrogen bonding between the complementary strands of DNA.

Bases A and T bind to each other using 2 bonds, whereas G to C binding uses 3 bonds (Broken lines show hydrogen bonds).

one strand of the DNA is known, one can predict the sequence of the other strand. Since DNA is the primary source of variation among animal species, DNA sequences unique to an animal species, are found within the genome of the species. Such sequences can be isolated or cloned and developed as DNA probes for species identification.

What is a DNA probe?

A DNA probe is a short DNA fragment or a short sequence of nucleotides that is labelled with either a radioisotope or a non-radioactive chemical that can be detected by its signal and is used to detect a DNA sequence which is complementary to that of the probe. The probe binds to the target sequence only by complementary base pairing and this process of binding is referred to as 'nucleic acid hybridization' (Fig.3). During this process, the double stranded DNA molecules are denatured to separate the two strands and allowed to hybridize with the labelled single stranded DNA probe. Short DNA fragments, isolated or cloned from an animal genome, or synthesized in a laboratory, are usually developed as DNA probes.

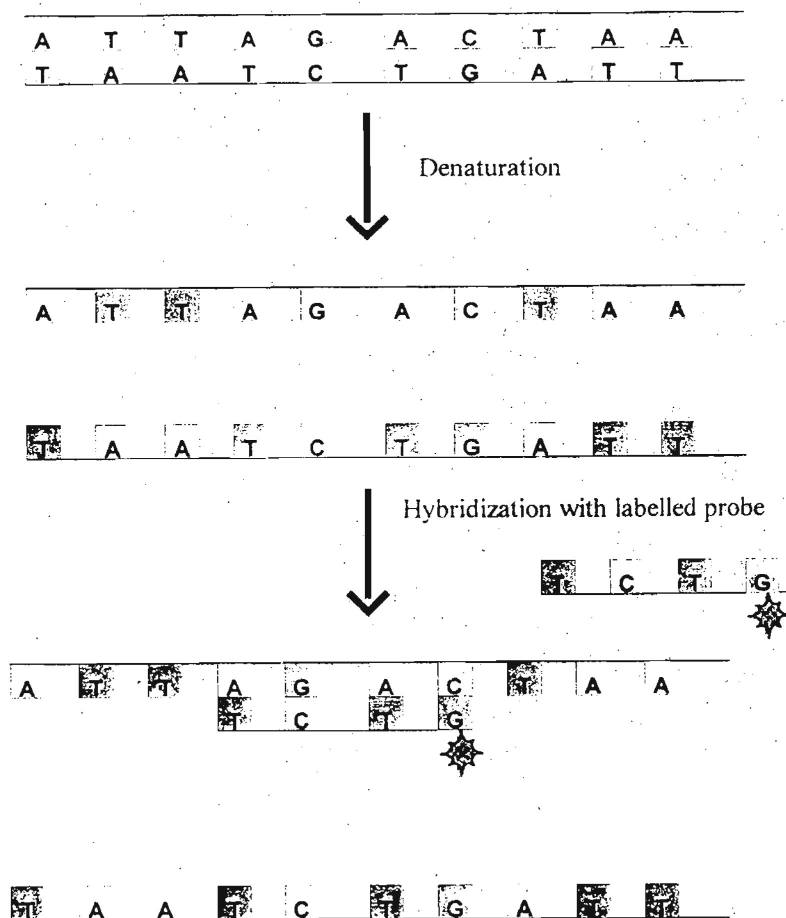

Fig. 3: Nucleic acid hybridization.
A single stranded labelled DNA base sequence containing usually 15 or more bases is used as the probe.

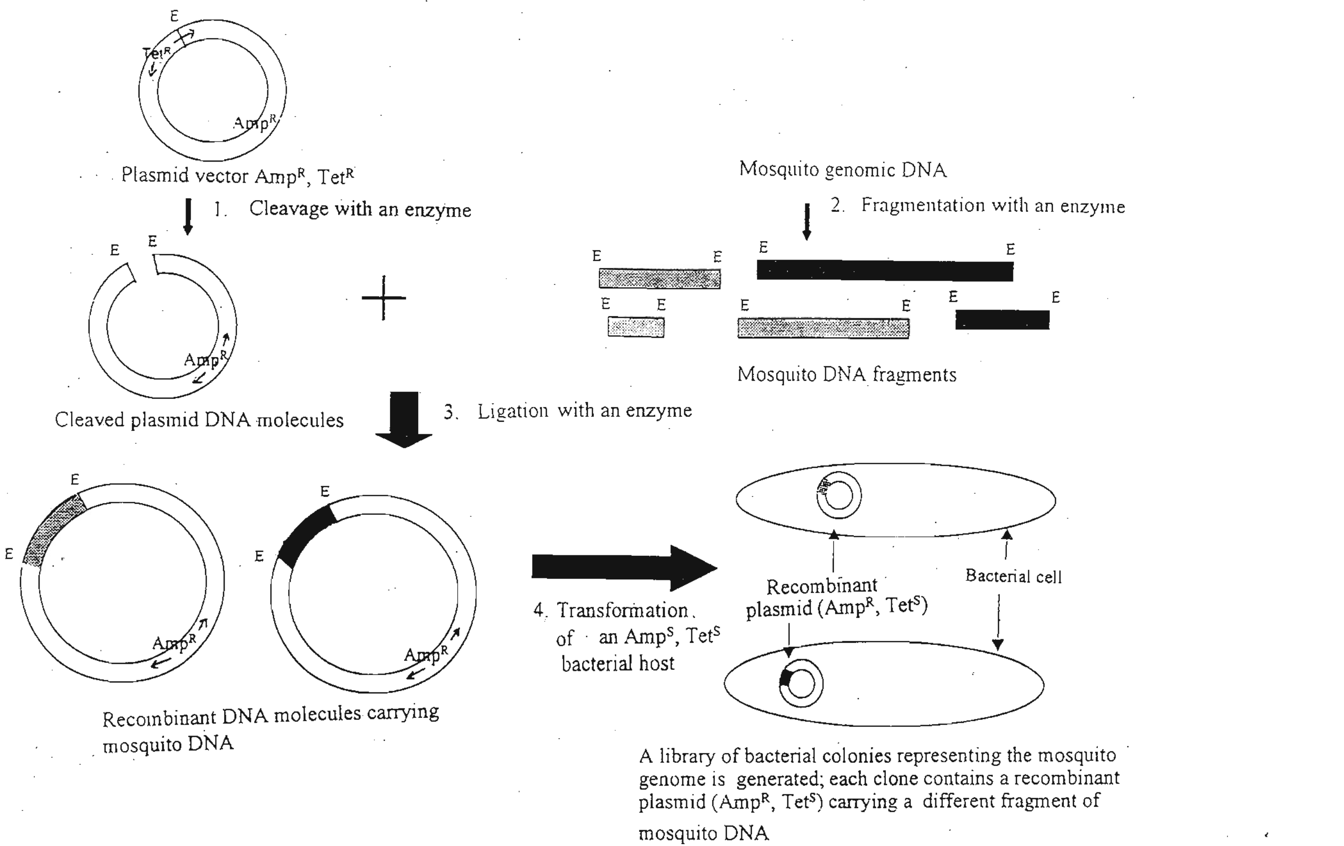

Fig. 4 - The steps in the construction of a mosquito genomic library

i. A plasmid vector containing genes for ampicillin and tetracycline resistance (Amp^R, Tet^R, respectively) is cleaved at a site (E) within the tetracycline resistance gene using an enzyme.

ii. The genomic DNA extracted from the mosquito species is also fragmented using an enzyme.

iii. The collection of cleaved plasmid DNA molecules generated and ligated to mosquito DNA fragments. A large number of recombinant DNA molecules containing mosquito DNA inserts are generated and each molecule contains a different DNA fragment of the mosquito genome. The tetracycline resistance gene is destroyed in each recombinant plasmid DNA molecule due to the insertion of a mosquito DNA fragment into this gene.

iv. This collection of recombinant plasmid DNA molecules is introduced into ampicillin and teracycline sensitive (Amp^S, Tet^S, respectively) bacterial host cells and grown in ampicillin medium. The bacterial clones containing recombinant plasmids are able to grow in ampicillin, however, they are unable to grow in tetracycline medium, and therefore can be identified.

Cloning of DNA fragments

Due to advances made in recombinant DNA technology it is now possible to separate a particular DNA fragment from all the other DNA extracted from the organism and make a large number of copies of that isolated DNA fragment. This process is known as 'DNA cloning'. Isolation of DNA fragments from the *An. culicifacies* genome is carried out by constructing a DNA library of the *An. culicifacies* mosquito genome. As the starting material for library construction, total genomic DNA is isolated from this mosquito species. Large chromosomal DNA molecules are then fragmented and ligated (using enzymes) into a cloning vector known as a 'plasmid'. A plasmid is a small, circular, double stranded DNA molecule, which is naturally present in many bacterial cells in addition to their chromosomal DNA. A plasmid is able to replicate independently inside a bacterial cell and in doing so, the genetic information present in the plasmid are expressed in the bacterial cell. For example, many plasmids carry genes for resistance to certain antibiotics, such as, ampicillin and tetracycline. The entry of such a plasmid into a bacterial cell would allow the bacterium to grow in a medium that contains ampicillin and tetracycline, as soon as the antibiotic resistant genes are expressed in the cell.

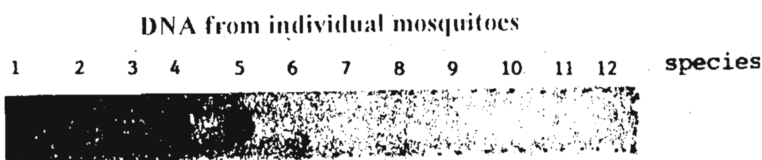

Fig. 5 : The DNA probe Rp 36 specifically identifies the *An. culicifacies* mosquito species.

The nucleic acid hybridization of genomic DNA extracts of various mosquito species (1-12) with radioisotope labelled DNA probe revealed hybridization signals only from *An. culificacies*, female (4) and *An. culicifacies*, male (5). (Experiments also revealed that the probe hybridizes with all three tested *An. culicifacies* sibling species; A, B and C).

1. *Aedes aegypti*
2. *Aedes togoi*
3. *Anopheles aconitus*
4. *Anopheles culicifacies (female)*
5. *Anopheles culicifacies (male)*
6. *Anopheles jamesi*
7. *Anopheles kaweri*
8. *Anopheles nigerrimus*
9. *Anopheles subpictus*
10. *Anopheles vagus*
11 *Culex quinquefaciatus*

The plasmid DNA molecules can easily be separated from the cell and taken into a test tube and purified from the bacterial chromosomal DNA. A Genetic Engineer can then make various changes in these plasmid DNA molecules. For example a plasmid can be cut and pasted to a piece of mosquito DNA using enzymes, and the recombinant DNA molecule so generated can be re-introduced to a bacterial cell. The characteristics of the bacterial cell will now be changed according to the genetic information present in the new DNA molecule. This process is referred to as 'transformation of bacteria'. Since a plasmid can be used to transfer one animal's DNA to another animal, it is known as a DNA 'vector'.

If we introduce the collection of recombinant DNA molecules generated by the ligation of mosquito genomic DNA fragments with cleaved plasmid DNA molecules into bacterial cells, millions of bacterial colonies harbouring recombinant plasmids are observed. Each such colony is referred to as a 'clone'. All the cells in a single clone contain copies of only one mosquito DNA fragment, since a colony starts from a single bacterial cell containing usually only one recombinant plasmid DNA molecule. Here, the plasmid replicates independently inside this bacterial cell and during the division of the bacterium, the daughter cells receive copies of the mosquito DNA fragment present in the plasmid. During bacterial transformation it is not only one recombinant DNA molecule that is introduced into bacteria but a whole collection of recombinants, each containing different regions of the mosquito genome. Each of these would enter a bacterial cell and give rise to a colony. Hence, this collection or the total pool of DNA clones is referred to as a 'genomic library' as it is assumed to be representing the complete genome of the mosquito. The main steps in the construction of such a library is illustrated in Fig.4. Next, we have to screen this genomic library to identify and isolate a DNA fragment that can be used as a DNA probe for the identification of the mosquito species.

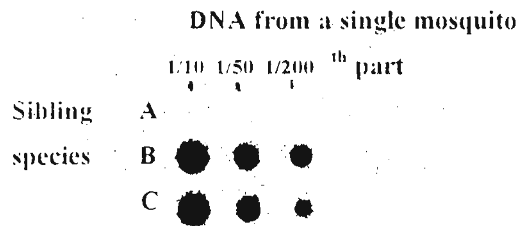

Fig. 6: The DNA probe Rp 217 distinguishes *An.culicifacies* sibling species A from species B and C.

The nucleic acid hybridization of DNA extracts of *An.culicifacies* sibling species A, B and C with the radioisotope labelled DNA probe did not reveal a hybridization signal with sibling species A. However, the sibling species B and C gave a hybridization signal with a minute quantity of DNA (1/200th part).

Screening of DNA libraries

Two important features that should be present in any species identification DNA probe are high sensitivity and specificity. In an animal genome there are sequences, which are present only once in the genome (single copy), as well as sequences that are repeated several times (multicopy; repetitive) in the genome. In order to develop a highly sensitive DNA probe for species identification it is very important to isolate a DNA fragment which is repeated in the genome, so that a minute amount of

mosquito sample (eg: a part of the head, a leg) is sufficient for its identification. Repetitive DNA sequences can be isolated from a DNA library by screening the library using a DNA probe made from a total mixture of genomic DNA fragments of the mosquito species. If a library DNA clone contains a mosquito DNA insert that is repeated in the genome, that clone would give a highly intense hybridization signal very quickly during the screening procedure. This is because a large number of copies of a repeat sequence is present in the total mixture of genomic DNA fragments labelled, and all these copies would quickly go and bind with the clone containing a complementary sequence as soon as it is met. Such positive clones are then isolated and the DNA inserts are then examined for their specificity i.e. a characteristic specific to a particular species so that one species can be distinguished from the other species. Here, we check to see whether the cloned DNA fragment hybridizes or not with the other mosquito species.

Using the procedures outlined above, our research group (B.G.N.K. de Silva, W. Abeywickreme and E.H. Karunanyake) was successful in developing the first DNA probe in the world that can distinguish all life cycle stages of *An. culicifacies* from other mosquito species (Fig.5). We also have developed a DNA probe technique that can distinguish *An. culicifacies* sibling species A from B and C and only a minute quantity of DNA is needed for this assay (Fig.6). The DNA probes developed in this study have also been tested and successfully applied in the field. The field investigations using these DNA probes revealed the absence of *An.culicifacies* sibling species A in Sri Lanka.

Next Vidurava issue, Vol. 21, Number 1

This issue will be published on "Science Communication". Contents will cover,

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