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REVIEW PAPER

Anopheles culicifacies complex: Geographical distribution of sibling species and existing methods for their identification

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Abstract

Meaningful epidemiological studies and effective vector control programmes depend on efficient methods for differentiating among major vector, poor vector and non-vector of anopheline species complexes. Anopheles culicifacies, has a wide distribution in India, extending to Ethiopia, Yemen, Iran, Afghanistan and Pakistan in the West and Bangladesh, Myanmar, Thailand, Laos and Vietnam in the East. It is also found in Nepal and Southern China to the North and extends to Sri Lanka in the South. This species exist as a complex of five sibling species provisionally designated as A, B, C, D and E. Species A and B are predominant in Northern and Southern part of India whereas species B is recorded all over the Indian subcontinent. Species B and E are found in Sri Lanka to date. Various methods and techniques have been used for identifying sibling species ranging from crossing experiments cytogenetics, isoenzymes, hydrocarbon profile, DNA probes, rDNA-PCR, mt DNA–PCR and RAPD-PCR. Studies of the suitability among different methods delineating the complex of An. culicifacies, the major malaria vector, mainly in the Indian subcontinent and in Sri Lanka have been discussed here.

Key Words : *Anopheles culicifacies, mosquito control*

Introduction

Species complexes are common in Class Insecta (Subbarao and Sharma, 1997, 1988a). Morphologically similar and reproductively isolated populations are known as sibling, cryptic or isomorphic species. About 23 *Anopheles* taxa have been identified so far as species complexes, most notably the *An. gambiae*, *An. funastus* in Africa, *An. quadrimaculatus* in North America, *An. maculipennis* in Europe, *An. culicifacies* in the Indian Subcontinent, *An. dirus, An. annularis, An. leucosphyrus, An. fluviatilis, An. maculates, An. minimus, An. subpictus, An. philippinensis-nivipes* in South – East Asia and they are important vectors of malaria in different parts of the world. Beginning with the earliest studies involving mating incompatibility studies on *An. maculipennis* in the laboratory, investigators discovered that many of the anophelines that vector malaria exists as members of these species complexes that often contain both vector and non-vector species (Besansky, 1999). Unfortunately, members of these species complexes are often morphologically similar creating difficulties in identifying mosquitoes responsible for disease transmission and potentially misleading control efforts. This paper is focused on the geographical distribution of *An. culicifacies* Giles *sensu lato* (Diptera : Culicidae) and the techniques available for the identification of its sibling species.

An. culicifacies is the major vector of malaria in India and contributes for transmission of 60-70% of the 2-3 million malaria cases reported every year. (Manonmani et. al., 2007). It is also the best known and most important vector in Sri Lanka. *An. culicifacies s.l.*has a wide distribution in India, extending to Ethiopia, Yemen, Iran, Afghanistan and Pakistan in the West and Bangladesh, Myanmar, Thailand, Laos and Vietnam in the East. It is also found in Nepal and Southern China to the North and extends to Sri Lanka in the South (Rao, 1984). However, this species exists as an isomorphic or sibling species complex consisting of five reproductively isolated populations and provisionally identified and designated as A, B, C, D and E.

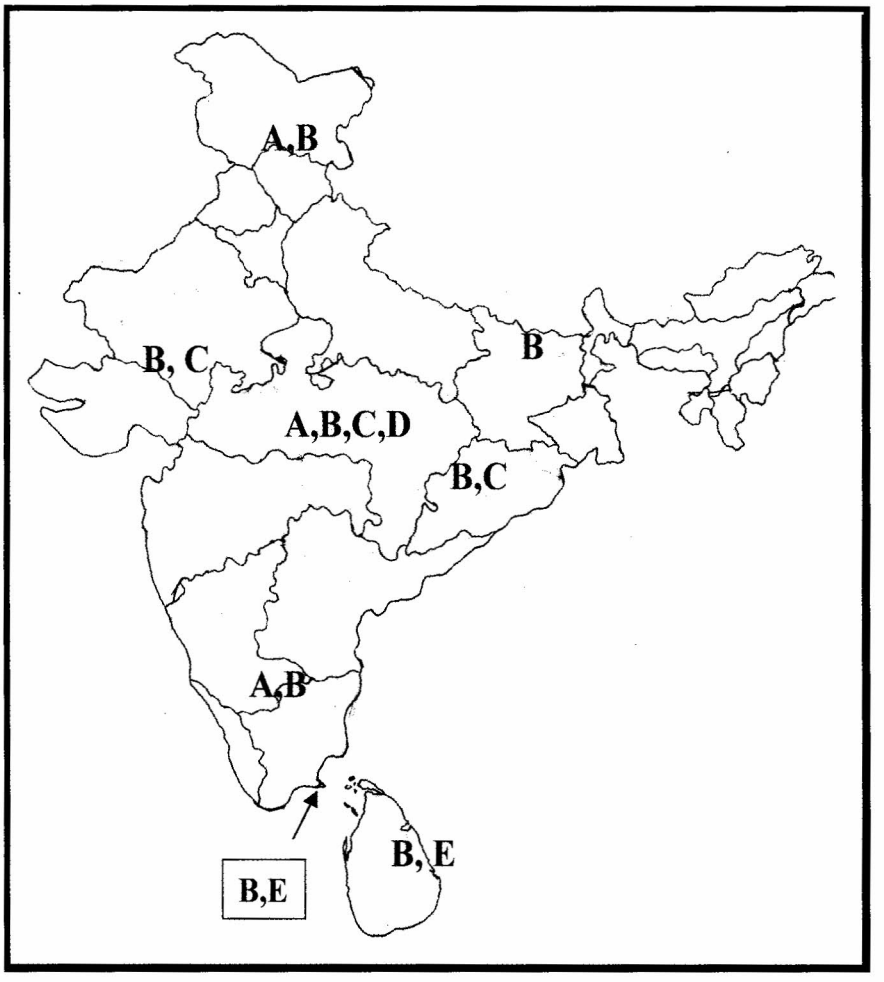

Figure 1. Map showing the distribution of members of the *An. culicifacies* complex the Indian subcontinent and Sri Lanka.

Species A was identified in Yemen Akoh, Beidas and White, 1984) and Iran (Zaim et. al., 1993) and has been found sympatric with species B in Pakistan (Mahmood, Sakai and Akthar, 1984). In India, where all five sibling species are prevalent, species B was found almost throughout the country. Species B was found exclusively in some areas whereas in other areas it was found sympatric with A or

C or D. (Subbarao, et. al., 1988, Suguna et. al., 1989, Subbarao, 1991, Vasantha, Subbarao and Sharma, 1991). Species A and B are sympatric in Northern and Southern India, with the predominance of species A in the North and species B in the South. However, in the Eastern states of North India, species B predominates or is the only species present, species B and C were predominant in the Western and Eastern regions, while species D was found sympatrically with A and B in the North-Western region and with A, B and C in Central India and in a few places in Tamil Nadu state (Figure 1). Baimai, Kijchalao and Rattanarithikul (1996) reported species A and B from Chingmai Province of Thailand.

Diagnostic Methods for Identification of Sibling Species

1. Crossing experiments
2. Cytogenetics
    (i) Polytene chromosomes
    (ii) Mitotic Karyotyping
3. Isoenzymes
4. Hydrocarbon profiles
5. DNA based methods
    (i) DNA probes
    (ii) rDNA cistron
    (iii) mt. DNA
    (iv) RAPD

1. Crossing experiments

Crossing experiments have been carried out in order to ascertain the mating between sibling species in nature and to identify the sibling species of the *An. culicifacies* complex (Miles, 1981, Mahmood et. al., 1984, Subbarao et al., 1988 and 1993). The sterile males from those crosses are reported to have either partially developed reproductive organs or fully developed reproductive organs without the presence of spermatids and spermatozoa. In all of the anopheline species complexes studied so far hybrid male sterility in either one or both of the reciprocal crosses has been

recorded. (Kitzmiller, 1967). Hence, hybrid male sterility is used as the criterion in designing the populations as a separate group.

Table 1. Intraspecies crosses in the *An. culicifacies* complex

Cross ♀	♂	% hatchability	Reproductive organs in hybrid males
A	B	90.0	S
B	A	3.0	S
A	C	85.3	S
C	A	0.0	-
A	D	81.4	N
D	B	76.3	N
A	C	77.2	N
C	B	67.2	N
B	D	0.1	-
D	C	93.2	S
B	D	0.0	-
D	A	95.3	S
Cross ♀	♂	% hatchability	Reproductive organs in hybrid males
R	A	0.7	-
A	RAC	94.3	S
RAC	A	0.0	-
A	RSM	0.0	-
RSM	A	0.7	-
A	R	94.7	N
R	B	98.2	N
C	R	95.1	N
R	C	92.5	N

A,B,C and D = *An.culicifacies* sibling species
R = *An.culicifacies* from Rameshwaram island
RAC = *An.culicifacies* from Rameshwaram island with acrocentric Y - Chromosome.
RAC = *An.culicifacies* from Rameshwaram island with sub-metacentric Y- chromosome .

N = Fully developed reproductive organs with sperm
S = Partially developed or atrophied reproductive organs and no sperm

References:
(1) Subbarao et al.,(1988b)
(2) Subbarao et al.,(1993)

The crosses between species A and species B, species A and species C, species B and species D, species C and species D produced fertile hybrid females and sterile hybrid males. Bidirectional hybrid male sterility between species A and species B and species B and species C was also found. (Subbarao et.al., 1988). Crosses have also been carried out between three strains of *An. culicifacies* isolated from Rameshwaram island (R, RAC, RSM) and sibling species A, B, C. (Subbarao et. al., 1993). Results from reciprocal crosses between species A and *An. culicifacies* s.l. from Rameshwaram island and between species A and R line with acrocentric Y chromosome (RAC) were similar to those from A and B. In the crossing experiments between female species A and the male sub-metacentric line (RSM), egg laying was absent and in the reciprocal cross egg laying was present but the hatching rate was only 0.7%. The crosses between species C and R strain produced fertile hybrid progeny (Table 1). In the *An. culicifacies* species complex both pre-mating and post mating barriers have been found between species A species B and species A and species C whereas only a pre-mating barrier has been observed between species B and species C (Subbarao et. al., 1988, Miles, 1981). It had been reported that the results of crossing experiments between sibling species of *An. culicifacies* under laboratory conditions do not reflect the phenomenon occurring in nature.

2. Cytogenetics

(i) Polytene Chromosomes

Cytogenetics involving the karyotyping of polytene chromosome was one of the earliest tools for the study of anopheline genetics.

Among the disadvantages of this technique is that the polytene chromosome preparations must be made from ovarian tissues or brain cells of fourth instar larvae. This limits the samples to either adult blood fed female mosquitoes or late 4th instar larvae. In addition to the paucity of experienced personnel trained to read polytene chromosome preparations paracentric inversions are not abundant and the number of specimens that can be processed in a given time is also limited. Despite these limitations, this method remains integral for much of the contemporary work.

Table 2. Cytogenetic analysis of *An. culicifacies* species complex

Sibling species	Inversion genotype observable in polytene chromosomes	Metaphase Y chromosome	Reference code
A	$X+^{a}+^{b}$; $2+^{g1}+^{h1};i^{1}/i^{1}$	Sub-metacentric	1,2,3,5,8,9
B	Xab ; $2g^{1}+^{h1}$	Acrocentric/Submetacentric	1,2,3,5,8,9,10
C	Xab ; $2+^{g1}h^{1}$	Acrocentric/Sub-metacentric	3,4,5,6,9
D	$X+^{a}+^{b}$; $2i^{1}+^{h1}$	Sub-metacentric	5,6,7,9
E	Xab;$2g^{1}+h^{1}$	Sub-metacentric	9,10

A, B, C, D and E = *An. culicifacies* sibling species

References:

1. Greens and Miles (1980)
2. Vasantha et. al., (1982)
3. Vasantha et. Al., (1983)
4. Subbarao et. al., (1983)
5. Subbarao et. al.,(1988)
6. Suguna et. al., (1989)
7. Vasantha et. Al., (1991)
8. Subbarao et. al., (1993)
9. Subbbarao et. al., (1998)
10. Surendran et. al., (2000)

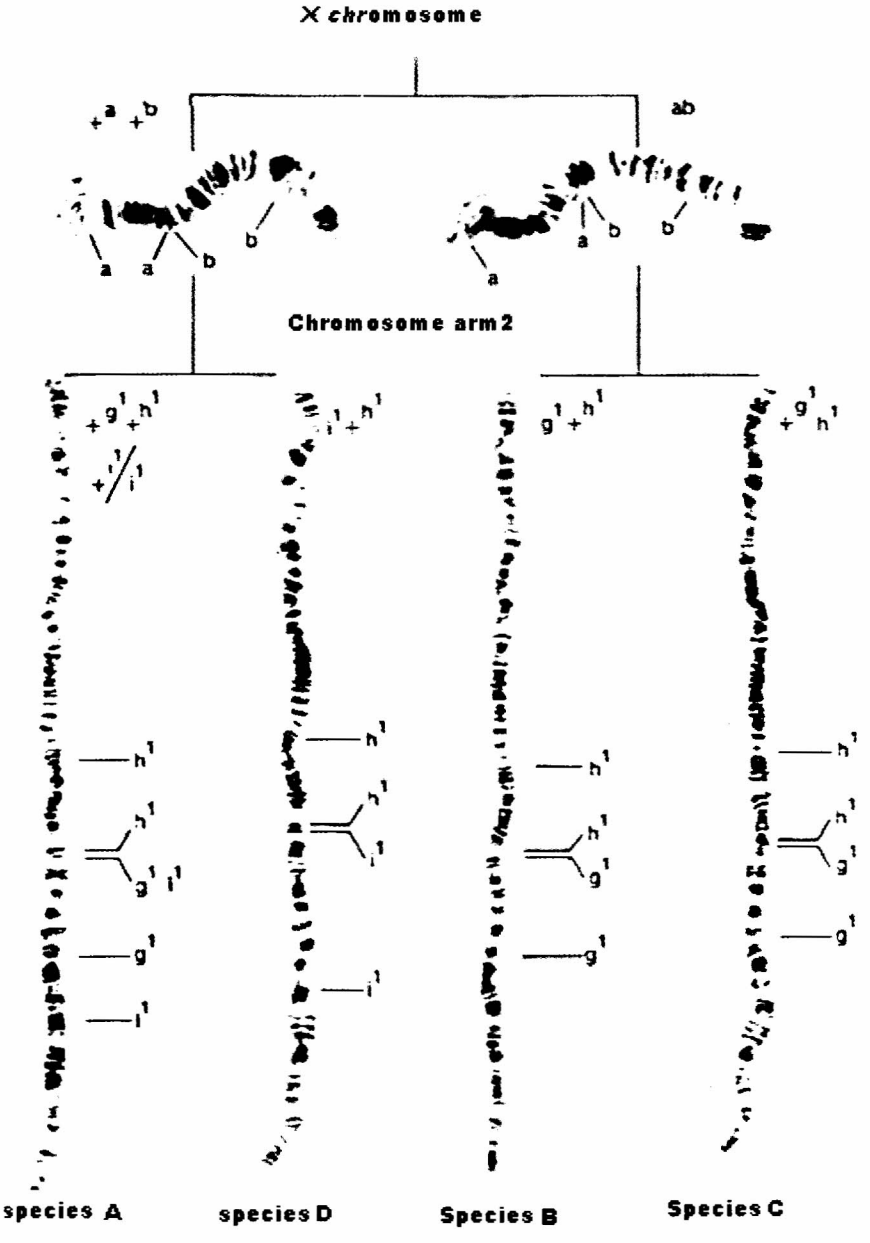

Figure 2 . Schematic representation of polytene chromosomes of *An. culicifacies* sibling species

Source : Subbarao et. al.,1998

Green and Miles (1980) first discovered two distinct polytene X chromosomes in semi – gravid females within *An. culicifacies* population in the village Okhala near India and designated as A and B. The population with standered arrangement, $X+^{a}+^{b}$ was designated as species A and that with Xab arrangement as species B. Subsequently, two species of *An.culicifacies* were described and designated as species C (Subbarao, et. al. 1983) with the arrangement of Xab ; $2+g^{l}h^{l}$ and D with the arrangement of $X+^{a}+^{b};2i^{l}+h^{l}$ (Subbarao et al.,1988, Suguna et. al., 1989; Vasantha Subbarao and Sharma, 1991). Kar et. al., (1999) reported another sibling species in the complex designated as species E. Paracentric inversion sites of the X and 2nd chromosomes of these sibling species are given in Table 2 and Figure 2.

In Sri Lanka, Green and Miles (1980) first reported the presence of sibling species B. Abhayawardene et. al., (1996) carried out a cytogenetic study based on X chromosome and 2nd chromosome paracentric inversions and found that only species B present in Sri Lanka which is a poor vector in India.

There are also technical difficulties in identifying all five species by polytene chromosome examination (Goswami et. al., 2006). Species D can be differentiated from species A only at the population level in areas where the $2i^{l}$ inversions which is diagnostic for species D is polymorphic in species A. In these areas, a deficiency of i^{l} heterozygotes indicates the presence of species D, but individual specimens cannot be identified as species D (Vasantha,Subbarao and Sharma., 1991). Furthermore, species E cannot be differentiated from species B because they have homosequentional polytene chromosome arrangements in both chromosomes. (Table 2).

(ii) Mitotic Karyotyping

This approach involves the use of metaphase chromosomes of brain cells in 3rd or 4th instar larvae therefore semi gravid females are not required. However, specimens have to be taken from indoor resting sites because they have to be reared for F_1 larvae

examination, which limits at least the processing of a large number of field samples.

Differences in the structure of male mitotic Y – chromosome were first reported by Vasantha et al., (1982 and 1983). A and C appeared to have submetacentric Y chromosome whereas B was acrocentric enabling the identification.

Adak et. al., (1997) have demonstrated that the metacentric polymorphism between the three species is not as rigid as first thought as species B and C do exist in acrocentric and submetacentric forms and therefore the differentiation of the members of the complex based on male karyotypes may not be accurate.

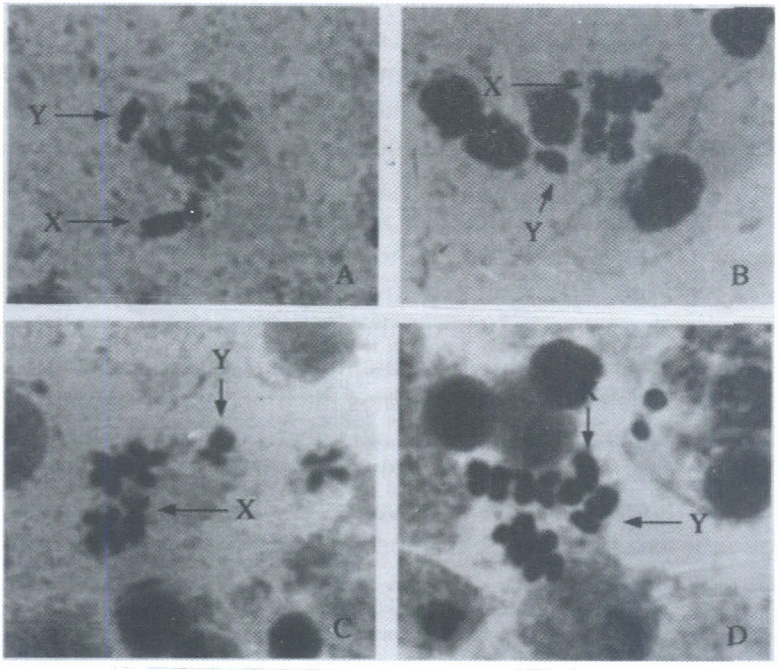

Figure 3. Male mitotic chromosomes of the brain cells of *An. culicifacies* (A) and (B)- acrocentric Y chromosome (X1000). (C) and (D) – Submetacentric Ychromosomes (X1000)

Source : Surendran et. al.,2006

However, studies of *An. culicifacies* from Rameshwaram Island in India, which is in close proximity to Sri Lanka, found evidence for assortive mating correlated with cytogenetic variations. *Plasmodium vivax* sporozoite – positive females had mated only with males of submetacentic Y chromosomes. No malaria infections were found in mothers of acrocentric Y males. These were designated species as B and the submetacentric Y chromosome vectors were designated species E (Kar et al., 1999) Subsequently, Surendran et al., (2000) found two forms of *An. culicifacies* in Sri Lanka and these were designated as species B (with an acrocentric Y chromosome) and species E (with a submetacentric Y chromosome). Centromeric polymorphisms of these sibling species are given in Table 2. Species E is considered as the major vector of malaria in Sri Lanka and species B as the poor vector of malaria. (Surendran et. al., 2006) (Figure 3).

3. Isoenzymes

Isoenzymes or allozymes are different molecular forms of an enzyme coded by different allelic forms of a gene. Therefore, after electrophoresis the enzyme activity can be visualized on a gel and the species can be identified.

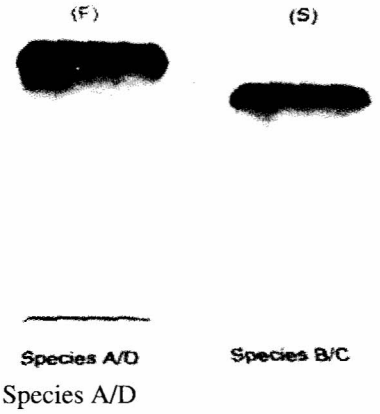

Figure 4. Lactate dehydrogenase allozyme differentiation of species in the *An. culicifacies* complex

Source: Adak et. al .,1994

Adak et. al., (1994) isolated an isoenzyme Lactate Dehydrogenase, which could be used to distinguish species A and D from species B and C in the complex (Figure 4).

4. Hydrocarbon profiles

This approach involves the analysis of cuticular component through Gas Liquid Chromatography (GLC). The profiles of each isomorphic species can be identified from varying retention times of the cuticular hydrocarbon. This technique has many advantages over that of established polytene chromosome and mitotic karyotypic identification. The material could be stored and analysed in any condition and both sexes and all life stages can be identified. However, this approach does involve expertise, sophisticated equipment and more importantly is very time consuming.

Milligan (1986) suggested that the three sibling species A, B and C were found to be significantly different in their cuticular hydrocarbon composition.

5. DNA based techniques

(i) DNA probes

Probes are short and specific stretches of DNA or RNA strands that are radiolabelled or non-radiolabelled and that can hybridize with complementary single strand nucleic acid stretches.

The advantage of this method is that any mosquito stage or part can be used. Therefore specimens can be stored and identified at a later date with other probes or future techniques.

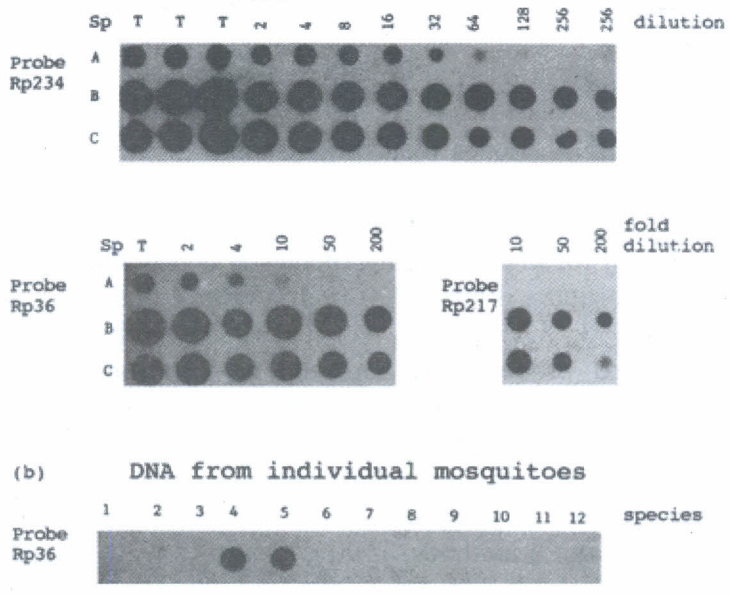

Figure 5. Dot –blot hybridization of DNA extracted from individual mosquitoes to show specificity of DNA probes ; Total DNA (T), approximately 600ng and diluted DNA samples of *Anopheles culicifacies* A, B and C hybridized with probes Rp234, Rp36 and Rp 217. The filters were washed in 0.1 X SSC at 30^0C.

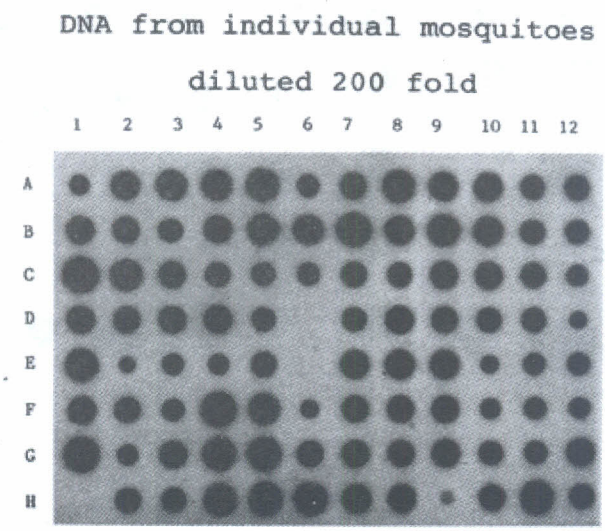

Figure 6. Field evaluation of DNA probes: dot- blot hybridization of DNA extracted from wild –caught individual *Anopheles culicifacies* mosquitoes and control mosquito samples diluted by 200-fold (approximately 3ng) and probed with Rp 36.an identical pattern of hybridization is also given with probe Rp234. controls :*An. culicifacies* sp .A from India (D6,H1), *An. culicifacies* B from India (D7,A12) and *An. tessellatus* (E6). The filter was washed in 0.1xSSC at 30^0C

Development of DNA probes to distinguish the members of *An. culicifacies* was first carried out by our research group in Sri Lanka. Three DNA probes were developed namely Rp36, Rp217, Rp234 which can be used for distinguishing species B and C when DNA from a single mosquito diluted 200-fold (Figures 5 and 6) (De Silva et. al., 1993, Gunasekera et al., 1995). Subsequently, radioactive labeled probes were replaced to a non-radioactive oligo-labelled probe and the DNA extraction procedure was also replaced to a squashed-blot method with only the mosquito head. This gave a safe, quick, easy and reliable identification method for distinguishing species A from species B and C (Figure 7) (De Silva,1996). Using these methods, 1500 mosquitoes were screened for the *An. culicifacies* s.s in Sri Lanka and the results clearly indicated the non existence of sibling species A in Sri Lanka. (Figure 8) (De Silva et al, 1998).

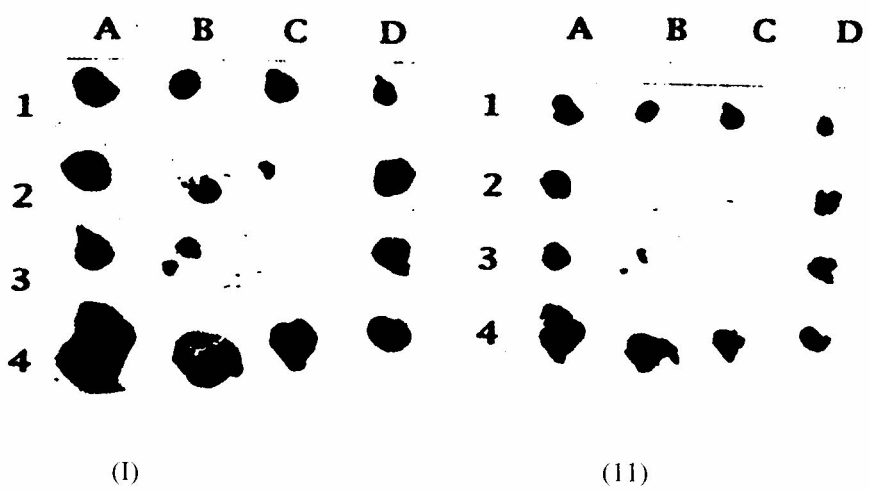

Figure 7. Squash blots of mosquito heads hybridized with ^{32}P-labled Rp217 probe at 37^{0}C.This shows a simple and quick preparation of mosquito DNA for hybridization assays.

(1) Filter washings carried out under low stringency conditions (0.1 x SSC, 0.5% SDS at 30^0C).
(2) Filter washings carried out under low stringency conditions (0.1 x SSC, 0.5% SDS at 60^0C).

Specimen 2B= *An.tessellatus*
2C=*An.subpictus*
3B=*Cx.quinquefasciatus*
3C=*An.culicifacies* species

All others ,*An.culicifacies* species B, Sri Lanka (laboratory stock).

DNA probe techniques have a high throughput potential (Krzynwinski and Besensky, 2003). However, this assay is limited by the fact that it may be unreliable because of its sensitivity both to unequal amounts of target DNA located on a membrane and to variation in copy number across the different geographical regions

(ii) Ribosomal DNA

The intergenic spacer (IGS) and Internal Transcribed Spacers (ITS1 and ITS2) within the nuclear ribosomal genome have become very popular targets for addressing taxonomic issues among anophelines. It has been noted that the nucleotide sequence of these spacer regions are often much more polymorphic between species than within species. This makes this region of the genome useful for delineating molecular differences between criptic species by length or sequence polymorphism.

Table 3. The GenBank accession numbers of different *An. culicifacies* sibling species and their country of isolation.

An.culicifacies siblings	GenBank accession number	Country
B	AY167747	Sri Lanka
E	AY168883	Sri Lanka
E	AJ534645	India
D	AJ534644	India
C	AJ534643	India
B	AJ534247	India
A	AJ534246	India
A	AF479315	Cambodia
B1	AF479314	Cambodia
B2	AF479313	Cambodia
B3	AF479312	Cambodia
B4	AF479311	Cambodia
B5	AF440396	Cambodia
'Bluchistan'	AF402296	Iran
A	AF402297	Iran
Unknown	AY007172	China1
Unknown	AY007168	China2

Polymerase Chain Reaction (PCR) assay developed from ITS2 region which differentiated species A from B has been first reported by Curtis and Townson (2003). The assay has not been evaluated with other species and even for species A and B. It has also not been evaluated on field specimens.

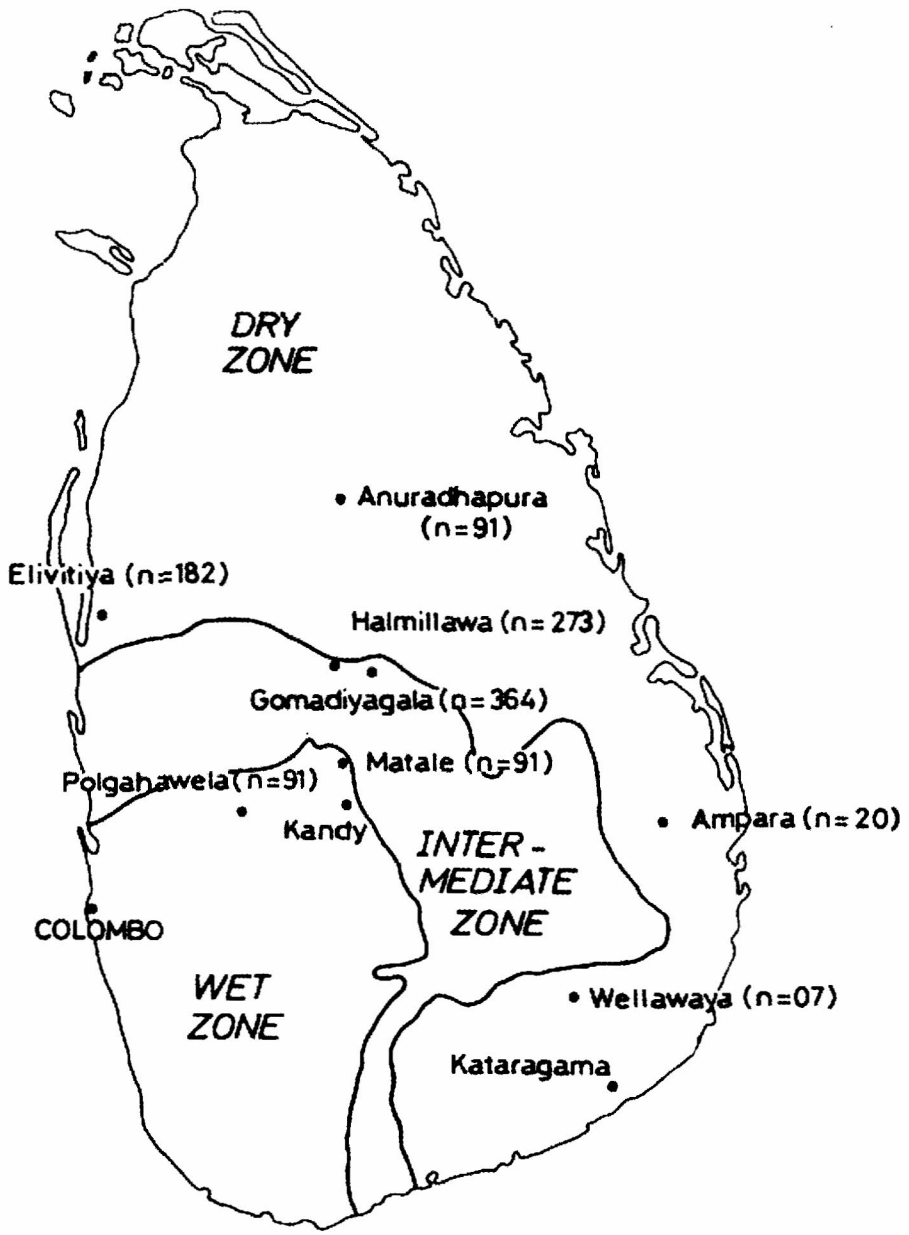

Figure 8. A map of Sri Lanka, showing three climatic zones, study villages and the numbers (n) of mosquitoes analyzed to date for sibling species status A.

ITS2 sequence of species B and E were first deposited in the GenBank by our group and then followed by other workers. Accession numbers and country of origin with respect to different species of the *An. culicifacies* complex are shown in Table 3.

Singh et. al., (2004) developed a PCR assay from D3 Domain of the 28S rDNA cistron to differentiate species A and D from species B, C and E. Recently, Ragavendra, et. al., (2009) developed a multiplex PCR assay from D2 Domain of 28S rDNA cistron to distinguish members of the *Anopheles culicifacies* complex into groups A and D from species B, C and E. In addition, Goswami et. al., (2006) developed ITS2 PCR-RFLP assay again grouped five sibling species into two categories as the D2 and D3 assay. Thus, various attempts to find variation in rDNA among all the five species have not been successful. However, ITS2 PCR-RFLP assay was further improved to a rDNA-PCR assay without restriction digestion by Rsa I, which group the five members to the same categories previously mentioned by Manonmani et. al., (2007).

(iii) mtDNA

Mitochondrial DNA has been used in sibling species identification in various insect taxa as well as in some anophelines (Mitchel et. al., 1992, Narang, et. al., 1993). However, the mitochondrial genome is frequently utilized in phylogenetic and population genetic studies.

A Polymerase Chain Reaction Restriction Fragments Length Polymorphism (PCR-RFLP) technique using variations in the mitochondrial cytochrome II (COII) region developed by Goswami et. al., (2005), distinguishes *An. culicifacies* species E from B and C.

Based on the information gathered from previous studies Goswami et. al., (2006) developed two allele specific PCR assays (AD-PCR and BCE-PCR) using sequence differences in the mitochondrial

CO II subunit. The AD-PCR assay distinguishes species A and D whereas the BCE-PCR assay distinguishes species B, C and E. This assay system is the first and the best available at present to distinguish all sibling species in the *An. culicifacies* species complex (Goswami, et. al., 2006).

However, Surendran et. al., (2006) tested the ability of the CO II PCR-RFLP developed by Goswami et. al., (2006) to differentiate species B and E collected from Sri Lanka. It has been suggested that there were sequence variations within the COII fragment equivalent to those seen in the Indian species B and E specimens and the variation did not correlate with the karyotype. Therefore, the CO II polymerization detected in *An. culicifacies* from Sri Lanka cannot be used as a simple molecular tool to distinguish the sibling species, unlike the situation in India (Surendran, et. al., 2006).

(iv) RAPD

Random Amplified Polymorphic DNA (RAPD) analysis makes use of a set of primers of 8 – 10 nucleotides whose sequence is essentially random. The random primers are fixed individually or in pairs in PCR reaction to amplify fragments of genomic DNA from the organism of interest.

RAPD has been utilized to distinguish between *An. gambiae* and *An. arabiensis* (Wilkerson et. al., 1993). Similarly Favia et. al., (1994) utilized RAPD to differentiate chromosomal forms of *An. gambiae* s.s. This research led first to an RFLP based diagnostic for the M (Mopti) and S (Savana and Bamako) forms which has since been refined into PCR diagnostic. Mukabayire et.al., (1999) have used sequenced–taged amplified RAPD's and single copy markers for differentiating taxa within *An. gambiae*.

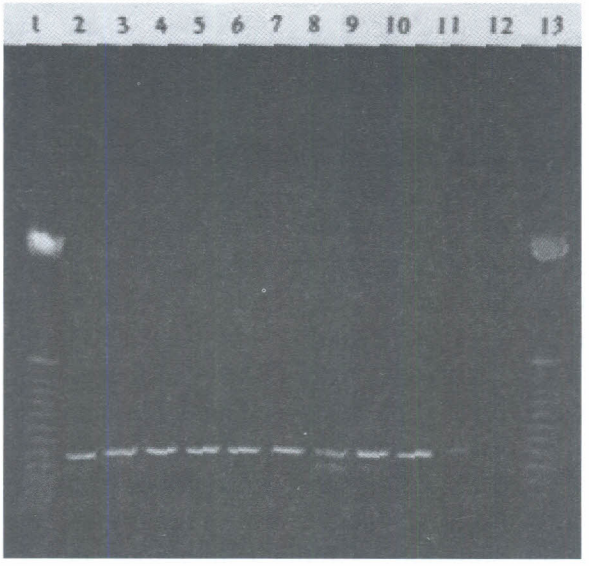

Figure 9. Ethidium bromide stained 1.55 agarose gel, showing fragments amplified by PCR that differentiates species A/D from species B/C/E of *An.culicifacies*.Lanes 1 & 13: 50bp DNA ladders;Lanes 2&3:species A; Lanes 4&5:species B; Lanes 6&7 :species C; Lanes 8&9: species D; Lanes 10&11: species E, Lane 12 (-) ve Control (no DNA)

Source: Ragavendra et al., 2009.

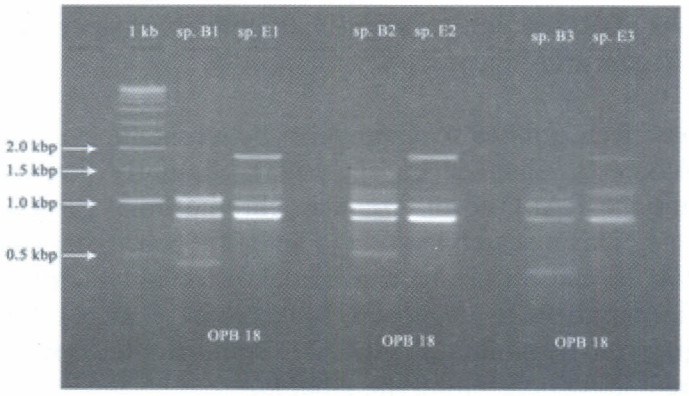

Figure 10 . Mosquito DNA was screened using thirty RAPD primers. Of the thirty primers, sixteen RAPD markers showed clear banding pattern with good resolution. Among these sixteen primers only one primer (OPB 18) was able to discriminate species B and E.

RAPD assay carried out by our group was able to differentiate sibling species B and E of *An.culicifacies* as shown in Figure 9 (Munasinghe et. al., 2004). However the diagnostic band has been further characterized to develop sequence tagged or SCAR assay to improve the reproducibility as a diagnostic tool for these species. The work has been in the experimental stage still. Because of the poor reproducibility and reliability, this method is rarely used for identification of sibling species.

Conclusions

Anopheles culicifacies, a complex of five sibling species is a major vector of malaria in Indian subcontinent and neighboring counties including Sri Lanka. The five species are provisionally designated as A, B, C D and E. Polytene chromosome was initially the only reliable and available method that differentiate A, B, C and D. However polytene chromosome analysis could not be used to differentiate species B and E thus mitotic karyotyping has been applied to differentiate them. Cytogenetic analysis is time consuming and labour intensive. DNA based techniques such as rDNA, COII of mt-DNA, RAPD seem to be good alternatives to the conventional cytogenetic techniques. These techniques too have limitations. Goswami et. al., (2005) developed a two-step multiplex PCR assay based on sequence differences within the COII region that distinguishes all five sibling species and have claimed this as the only method that can be used in all epidemiologic studies until another DNA-based method involving fewer steps is developed. However, Surendran et. al., in 2006 reported non-usability of COII PCR assay to distinguish the species B from species E in Sri Lanka. Random Amplified Polymorphic (RAPD) marker developed by us distinguishes species B from E in Sri Lanka ; this remains to be further characterized and validated.

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