Species Complexes in Malaria Vectors in India

Species complexes are of common occurrence among *Anopheles* taxa. Since the discovery of *Anopheles maculipennis* as species complex in Europe, about 30 *Anopheles* taxa have been identified so far as species complexes and they are important vectors of malaria in different parts of the world. Members of a species complex, commonly known as sibling species/isomorphic or cryptic species, are reproductively isolated evolutionary units with distinct gene pools, and hence, differ in biological characteristics which determine their potential in the transmission of disease. Failure to recognize sibling species of anopheline taxa may result in failure to distinguish between a vector and a non-vector; hence the assessment of the impact of control measures may be seriously misleading if they are carried out on a morphologically defined taxon which could be a mixture of two or more sibling species. In addition, differences in the biological characteristics of the members of complexes have an important bearing on the malaria transmission dynamics.

NIMR is actively engaged in recognition of new species complexes in malaria vectors and in studying biology, distribution and transmission potential of the members of each species complex. Indian anopheline fauna comprises of 58 species, of which nine are vectors of malaria. *An. culicifacies, An. fluviatilis, An. stephensi, An. minimus, An. dirus* and *An. sundaicus* are the major malaria vectors. All these vectors except *An. stephensi*, are species complexes—each of these morphological species comprises a number of morphologically indistinguishable biological species commonly known as sibling species or cryptic species or isomorphic species. Among the vectors of secondary importance *An. annularis* and *An. (philippinensis) nivipes* specimens are species complexes. *An. subpictus* is not considered a vector but a few sporozite positive specimens have been found in this species in coastal areas of Puducherry where species B is found. The number of sibling species so far identified among the Indian anophelines is given in Table 1.

Recognition of Species Complexes

Pre-mating Barriers

The assortative mating observed between sibling species in nature is mainly due to pre-mating barriers which involve seasonal/ethological/mechanical isolating mechanisms. Cytotaxonomic studies of natural populations of malaria vectors carried out at NIMR led to the recognition of *An. culicifacies, An. fluviatilis* and *An. annularis* as species complexes. Fixed paracentric inversions in the ovarian polytene

Table 1. Species complexes among Indian anophelines

Species	No. of sibling species identified	Sibling species found in India
An. annularis	2	A, B
An. culicifacies	5	A, B, C, D, E
*An. dirus**	7	D, E (D in northeastern states and E in Karnataka)
An. fluviatilis	4	S, T, U, V
An. minimus	3	A
An. sundaicus	4	A new cytotype D
An. philippinensis-nivipes	3	*nivipes* A
*An. subpictus**	4	A, B, C, D

*These two complexes have not been studied by NIMR.

National Institute of Malaria Research is a WHO Regional Reference Centre for the identification of *Anopheles culicifacies* sibling species and variations

chromosomes were found in natural populations. Presence of alternate arrangements of an inversion—homozygous, standard and inverted, in a population with a total absence of the inversion heterozygotes indicated assortative mating (reproductive isolation). This was taken as evidence for designating the populations as distinct species (Atrie *et al* 1999; Subbarao *et al* 1983, 1994) and the fixed paracentric inversions are used to identify the species. This technique was used extensively to study the biological characteristics of sibling species of these complexes.

Post-mating Barriers

The pre-mating barriers generally break down in the laboratory and sibling species mate at random and produce hybrid progeny. Genetic differences between species are expressed in the form of non-viability of hybrid progeny or hybrid sterility which represent post-mating barriers.

In addition to pre-mating isolating mechanism observed in natural population, post-mating barriers were found to exist between the sibling species of *An. culicifacies* complex. Genetic crosses revealed bi-directional hybrid male sterility between species A and B demonstrating post-mating barriers between these species. In contrast, F_1 hybrid males of reciprocal crosses between species B and C were found fully fertile indicating the absence of post-mating barriers between species B and C. Similarly, fully fertile hybrid males were observed in reciprocal crosses between species T and U of *An. fluviatilis* complex.

Diagnostic Methods for the Identification of Sibling Species

Different methods for the identification of sibling species have been developed which are being used in various studies depending on the feasibility. In case of *An. culicifacies* complex, paracentric inversions readable on the polytene chromosomes have been identified which differentiate most of the members—species A, B, C and D at the population level (Fig. 30) (Subbarao *et al* 1983, 1988 & Vasantha *et al* 1991). Structural variations in metaphase chromosomes of mitotic and meiotic karyotypes (Fig. 31) along with biological variations have been used to differentiate species B and E.

Electrophoretic variations found at lactate dehydrogenase (LDH) locus (Fig. 32) could differentiate species A and D from species B and C. An allele-specific polymerase chain reaction (ASPCR) assay targeted to the D3 domain of 28S ribosomal DNA was developed which discriminates *An. culicifacies* species A and D from species B, C and E (Singh *et al* 2004). Similarly, a PCR-RFLP method targeting mitochondrial cytochromes oxidase subunit II and ITS2 of ribosomal DNA was developed which could differentiate species A and D from species B, C and E (Goswami *et al* 2005) (Fig. 33). Recently, two allele-

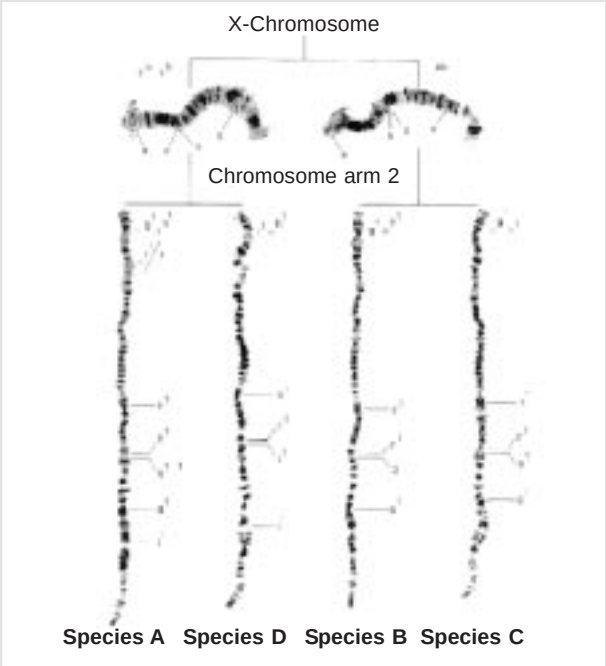

Fig. 30: Schematic representation of polytene chromosomes of *An. culicifacies* sibling species

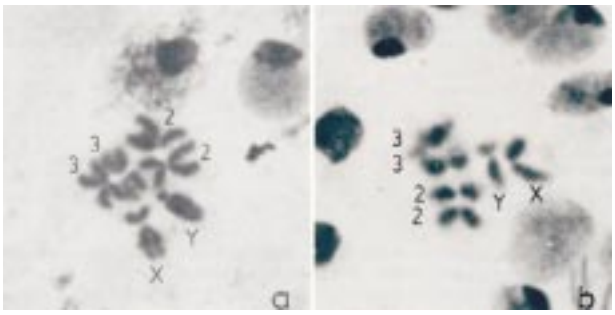

Fig. 31: Male mitotic karyotypes of *An. culicifacies* from Rameswaram Island: (a) Karyotype with acrocentric Y-chromosome (species B); (b) Karyotype with sub-metacentric Y-chromosome (species E)

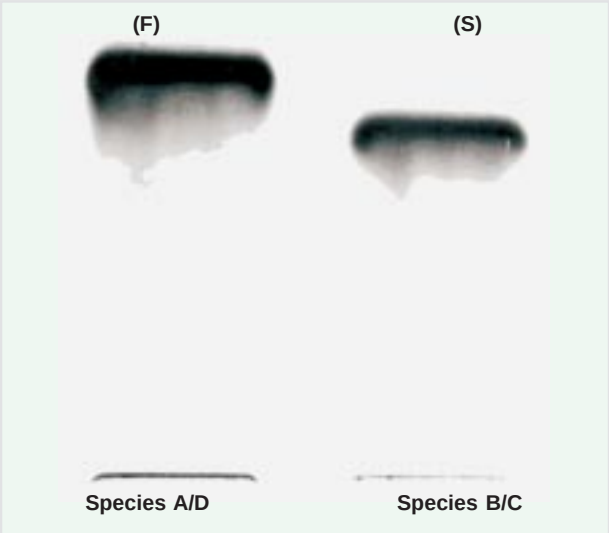

Fig. 32: Differentiation of the members of *An. culicifacies* complex by lactate dehydrogenase enzyme: the two forms of allozyme, i.e. Fast (F) and Slow (S) differentiate species A/D from species B/C of *An. culicifacies* respectively

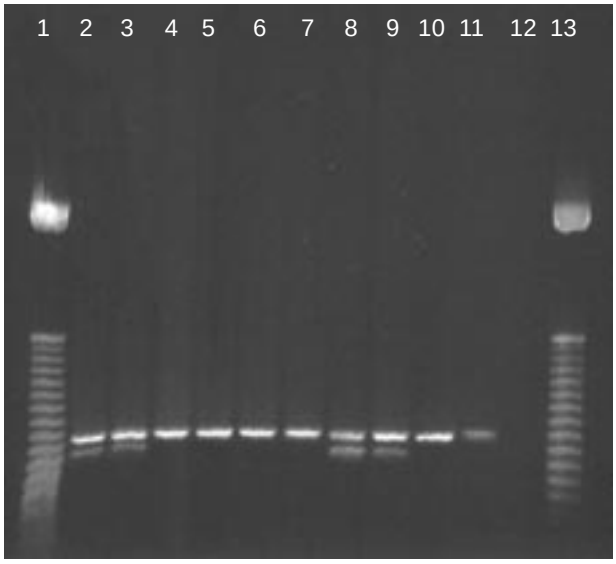

Fig. 33: PCR assay using primers designed from D2 region of 28S rDNA which differentiates species A and D from species B, C and E of *An. culicifacies*. Lanes 1 &13: DNA ladder; Lanes 2 & 3: species A; Lanes 4 & 5: species B; Lanes 6 & 7: species C; Lanes 8 & 9: species D; Lanes 10 & 11: species E, Lane 12: negative control

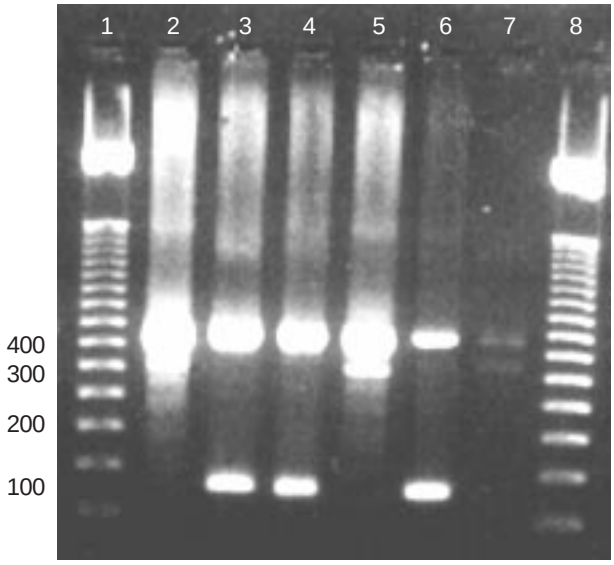

Fig. 34: PCR products obtained by the primers designed from D3 region of 28S rRNA electrophoresed on 2% agarose gel. Lanes 1 & 8: 50 bp marker; Lane 2: *An. culicifacies* sp. A; Lane 3: *An. culicifacies* sp. B; Lane 4: *An. culicifacies* sp. C; Lane 5: *An. culicifacies* sp. D; Lane 6: *An. culicifacies* sp. E from Rameswaram; Lane 7: Negative control

specific PCR assays (AD-PCR and BCE-PCR) using sequence differences in the mitochondrial cytochrome oxidase II (CO II) subunit have been developed. With a combination of two PCR assays, namely the D3-PCR/ITS2-RsaI assay, followed by either the AD-PCR or the BCE-PCR assay, it is possible to identify individual specimens of any of the species of *An. culicifacies* complex (Goswami *et al* 2006) (Fig. 34).

In *An. fluviatilis* complex, species S, T and U were identified by fixed inversions on polytene chromosome arm 2 which are species-specific (Subbarao *et al* 1994). The newly discovered species V in this complex can be identified by two fixed paracentric inversions on polytene chromosomes 2 and 3. Recently, an allele-specific PCR-based diagnostic assay has been developed which can differentiate all the three members of the complex (Fig. 35). The assay is based on the differences in nucleotide sequences of D3-domain of 28S ribosomal RNA in species S, T and U (Singh *et al* 2004).

In case of *An. annularis* complex, for species A and B the only method available is polytene chromosome examination for fixed paracentric inversions (Atrie *et al* 1999). *An. minimus* populations from northeastern states of India were identified as species A by using diagnostic Octanol dehydrogenase electromorphs. Polytene chromosome examination also identifies *An. sundaicus* sibling species. A new cytotype found in Andaman & Nicobar Islands can easily be distinguished from species A, B and C found in other southeast Asian countries by this method.

The discovery of species complexes adds new dimensions to vector control. Members of the complexes are generally isolated by pre-mating

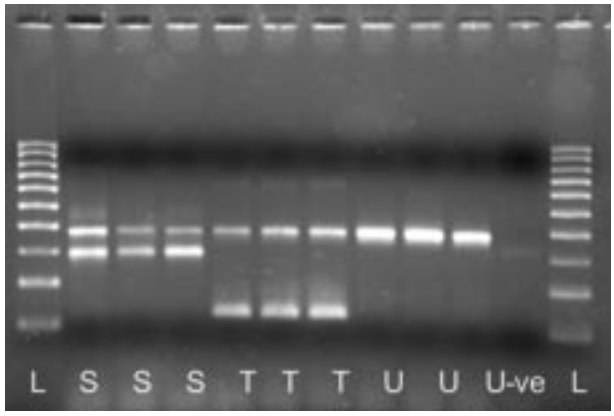

Fig. 35: Differentiation of the members of *An. fluviatilis* complex: PCR product as seen on 2% agarose gel containing ethidium bromide under UV illumination (L= 100 bp DNA ladder, S= Species S, T= Species T, U= Species U, –ve= Negative control without DNA)

barriers. Hence, the genetic structure of each species differs from the other and thus have to be taken into account for all types of control strategies.

Anopheles culicifacies Complex

In India, all five species of *An. culicifacies* complex have been found. Sites surveyed and the distribution of the species is given in Fig. 36. Species B was found almost throughout the country wherever *An. culicifacies* was encountered. In some areas, species B was found exclusively, whereas in other areas it was found sympatric with other species.

Field studies also demonstrated that the seasonal changes in the prevalence of different

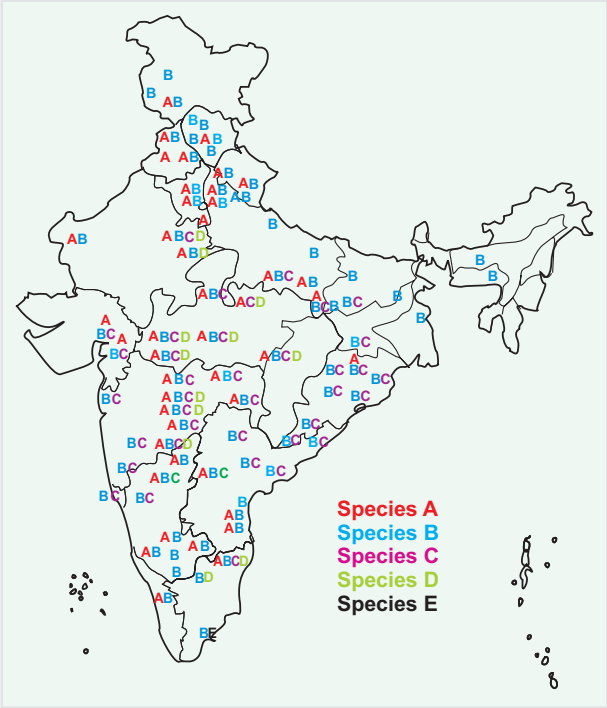

Fig. 36: Map showing the distribution of members of the *An. culicifacies* complex in India

sibling species in areas where more than one species occurred. In Alwar, Rajasthan, where four sibling species (A, B, C and D) were prevalent (Species E was not discovered at that time), all four species were found throughout the year with varying proportion. Species B increased in post-monsoon months while the proportion of species D remained the same throughout the year and densities of species C was very low.

Biological variations among species A, B, C, D and E are summarised in Table 2. Feeding preference, which is an important character that influences the vectorial potential is for cattle for species A, B, C and D while species E is highly anthropophagic. Incrimination studies using immunoradiometric analysis revealed species A, C and D to be vectors of *P. vivax* and *P. falciparum* malaria and species B to be a poor vector, if at all. Species E was found with sporozoites. These species also vary in the rate of development of resistance to different insecticides.

An. culicifacies s.l. was colonised in the laboratory for the first time in India in 1977 (Ansari *et al* 1977). After the discovery of sibling species in this taxon, laboratory colonies of species A, B and C were established from the cytologically identified field-collected isofemale progeny. Distinct differences were observed in laboratory studies with reference to insemination rates, fecundity, longevity, *etc.* among species A, B and C. Under laboratory conditions, the insemination rates were relatively low (< 60%) with species C showing the highest rates. Oviposition in all the three species was confined to the period of 2000 to 0800 hrs. The frequency of egg deposition during the seven-gonotrophic cycles showed normal distribution pattern in all the three sibling species. The egg hatching rate was >70% being maximum in species C. Species A had higher larval mortality rates, longer pupation time and longer emergence time than the other two species (Fig. 37). The effects of crowding which differed significantly among the three sibling species were reflected in higher larval mortalities, longer pupation and emergence time. Species B was the least adversely affected. In

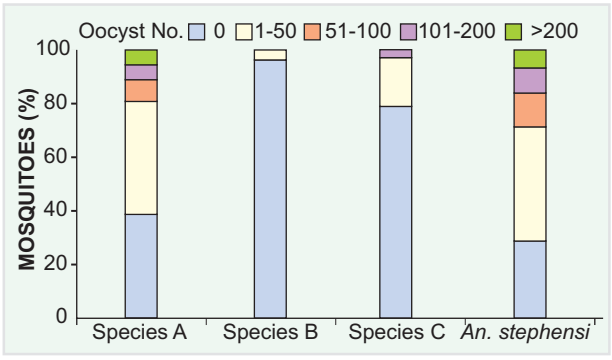

Fig. 37: Frequency distribution of *P. vivax* oocysts in different members of *An. culicifacies* complex and *An. stephensi*

Table 2. Biological variations among the *An. culicifacies* sibling species

	Sibling species				
	A	B	C	D	E
Anthropophilic Index (%)	0–4	0–1	0–3	0–1	High
Biting activity	All night	All night	All night	Up to midnight	–
Peak biting time	2200–2300 hrs	2200–2300 hrs	1800–2100 hrs	1800–2100 hrs	–
Vector potential	Vector	Non/poor vector	Vector	Vector	Vector
Sporozoite rate (%)	0.51	0.04	0.3	0.4	20
Resistance					
DDT	Slow	Fast	Fast	–	–
Malathion	Slow (9–10 yr)	Medium (6–7 yr)	Fast (4–5 yr)	–	–
Synthetic pyrethroids	–	Fast (4–5 yr)	Fast (4–5 yr)	–	–

general, food availability had a greater impact than larval density per se. Horizontal life-table experiments showed: (i) the adult survivorship patterns were similar among the three sibling species, but B had higher longevity than others; (ii) the longevity of males was shortest in species B; and (iii) the gross and the net reproduction rates as well as intrinsic rates of increase were highest in species B and lowest in species A. Species C had a significantly longer generation time than A and B. In addition to the biological variations examined in field population, variations under laboratory conditions were also studied.

In laboratory studies, susceptibility of three members of *An. culicifacies*, species A, B and C were determined against malaria parasites, *P. vivax, P. vinckei petteri* and *P. yoelii yoelii*, where it was found that species A had significantly higher oocyst load, oocyst rate and sporozoite rate as compared to species B and C. Species B was found least susceptible (Fig. 38).

A strain of *An. culicifacies* species B exhibiting complete refractoriness to *P. vivax* sporogony was isolated. In this line, late ookinetes are encapsulated with melanin like pigment within the midgut epithelia and further sporogony is completely aborted (Fig. 39). The strain is partially refractory to *P. falciparum* and rodent malaria parasite, *P. vinckei petteri* (Adak *et al* 2006). Genetic analysis revealed that the gene for refractoriness is dominant and autosomal. This strain is now being used to study the host parasite interaction at genetic, biochemical and molecular level. The gene(s) of this kind is(are) of great interest and research groups involved in the development of transgenic mosquitoes for malaria control are looking for such genes.

Molecular characterisation of a serine protease (*acsp*30) – encoding gene from *An. culicifacies* revealed that it was expressed in high abundance in the refractory (R) strain compared to the susceptible (S) strain. Gene organisation and primary sequence of *acsp*30 were found identical in R and S strains suggesting a divergent regulatory sequences of *acsp*30 in these strains. To examine this further, the

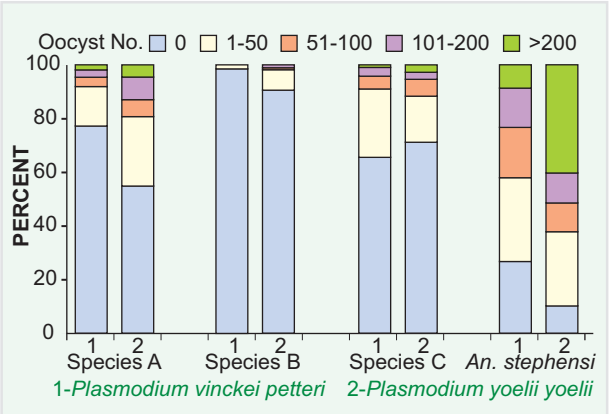

Fig. 38: Frequency distribution of rodent malaria oocysts (*P. v. petteri* and *P. y. yoelii*) in different members of *An. culicifacies* complex and *An. stephensi*

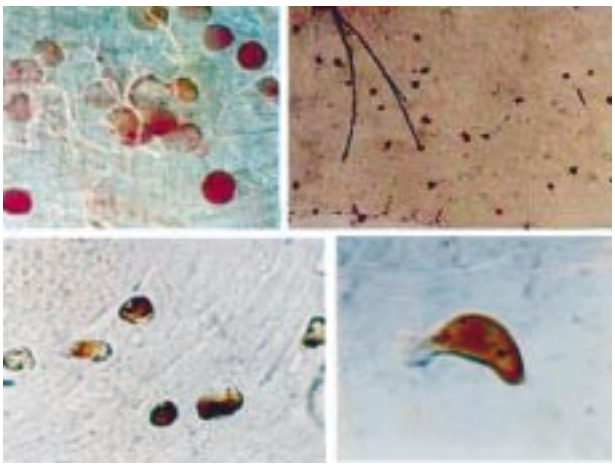

Fig. 39: Midguts of *An. culicifacies* species B refractory strain showing encapsulated *P. vivax* parasite

upstream regulatory sequences of *acsp*30 were isolated, cloned and evaluated for the presence of promoter activity. The 702 bp upstream region of *acsp*30 from the strains revealed sequences divergence. The promoter activity was found to be 1.5 fold higher in the R strain than in the S strain (Rodrigues *et al* 2007). Therefore, the specified upregulation of *acsp*30 in the R strain only in response to *Plasmodium* infection suggests its role in contributing the refractory phenotype to the *An. culicifacies* mosquito population.

Further, the role of prophenoloxidases, which are the key components in recognition and melanisation of invading organisms was investigated. The transcript levels of prophenoloxidase–encoding AcPPO6A gene were found to be higher in naive adult refractory female mosquitoes as compared to female susceptible mosquitoes. Upregulation of AcPPO6A transcription in response to parasite challenge exclusively in refractory strains suggests its role in melanotic encapsulation of the parasite (Rodrigues *et al* 2008).

The laboratory feeding experiments suggest that in addition to encapsulation and melanisation of oocysts in species B, there appears to be another more common genetic/physiological mechanism in species B, *i.e.* parasites in the ingested blood in the gut are destroyed before they enter into the midgut epithelium. Studies on life-table parameters such as longevity did not indicate that species B falls short of requirements to be an effective vector. Further, species A, B, C and D were all predominantly zoophilic. Thus, the genetic and physiological background is the main factor for species B to be a non-vector.

In epidemiological studies, a good correlation was observed between sibling species prevalence and malaria incidence. Broadly, it can be stated that as species B is a non-vector, there would be no malaria in areas where only species B is found. Also in areas where other sibling species are found in low proportions along with species B the incidence of malaria would be low. These findings coupled with geographical distribution of sibling species were used

to stratify the country and to recommend control strategies.

Anopheles fluviatilis Complex

Mapping geographic distribution of *An. fluviatilis* sibling species and studies on their bionomics and role in malaria transmission have been carried out by conducting spot surveys and longitudinal studies in different parts of India. Results revealed that species S, T and U have definite distribution pattern (Fig. 40) and exhibit distinct differences in their biological characteristics (Table 3). Species T is most widely distributed in India whereas species S is predominantly found in Orissa state. *An. fluviatilis* species T and U prefer to rest in cattlesheds and are primarily zoophagic (Nanda *et al* 1996). These species appear to be playing very minor role in malaria transmission (Sharma *et al* 1995; Shukla *et al* 1998). In contrast, species S prefers to rest in human dwellings and is highly anthropophagic. Vector incrimination studies have shown that species S is a very efficient vector of malaria in areas of its distribution (Subbarao 1998; Nanda *et al* 2000).

Susceptibility of *An. fluviatilis* species T, a non-vector, was compared with two established malaria vectors, *An. stephensi* and *An. sundaicus* in laboratory feeding experiments by feeding them artificially (through 'Parafilm' membrane) on the *P. vivax*-infected blood having mature gametocytes. Examination of gut of mosquitoes on Day 6 and salivary glands after 9th day of infective feeding (incubation temperature 27°C) revealed that all the three species had high oocyst and sporozoite rates and there were no significant differences in these rates among all the three species (Fig. 41) (Adak *et al* 2005). Similar results were obtained with *An. fluviatilis* species U in laboratory feeding experiments. These studies suggested that the *An. fluviatilis* species T and U which are not vectors in field, have inherent ability to support normal sporogony. These species are almost zoophagic in field and probably their preference to feed on cattle makes them poor vectors and may act as vector in the absence of cattle.

In recent years, cytogenetic studies carried out on *An. fluviatilis* population from villages under Laksar

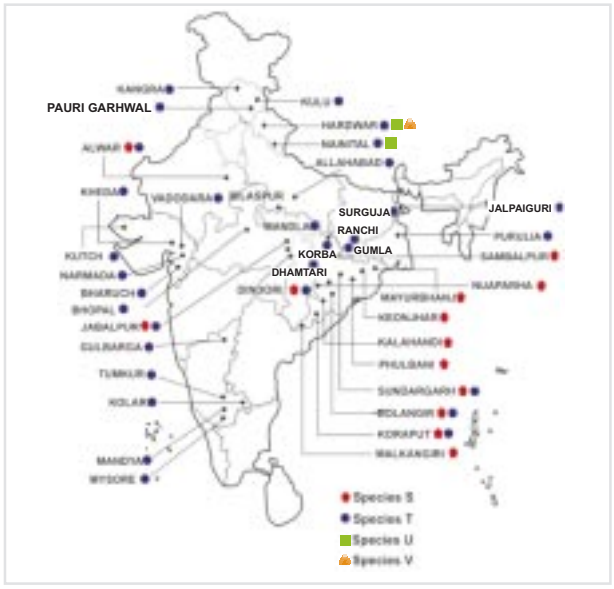

Fig. 40: Map showing the distribution of the members of the *An. fluviatilis* complex in India

PHC of District Hardwar (Uttarakhand) revealed existence of a new species in *An. fluviatilis* complex. The new cytotype observed differs from the reported species of the Fluviatilis Complex by two fixed paracentric inversions S^1 and S in polytene chromosomes 2 and 3, respectively. Longitudinal study carried out in study villages showed that the new

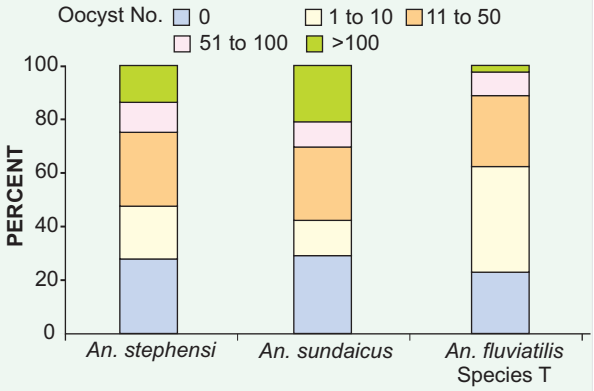

Fig. 41: Frequency distribution of oocysts in *An. fluviatilis* species T and other two vectors *An. stephensi* and *An. sundaicus*

Table 3. Biological differences and diagnostic characters of *An. fluviatilis* sibling species

Species	Inversion genotypes on Chromosome arm 2	Densities (MHD)	Feeding preference	Sporozoite positives	Preferred adult habitats	Observed in	
						Ecotypes	Epidemiological areas
S	$+q^1+r^1$	Low moderate (1–40)	Anthropophagic	Found	Human dwelling	Hilly forest & foothills	Hyper-endemic
T	q^1+r^1	High (up to 200)	Almost totally zoophagic	Not found	Cattlesheds	Foothills & plains	Hypo-meso-endemic
U	$+q^1r^1$	-do-	-do-	-do-	-do-	-do-	-do-

cytotype was sympatric with species T and U in all the seasons. The presense of two fixed paracentric inversions in polytene chromosomes with total absence of inversion heterozygotes unequivocally establishes this cytological variant as a new species (species V) in the *An. fluviatilis* complex. Analysing the DNA sequences of D3 domain of 28S rDNA has also shown that species V is distinctly different from species S, T and U. Preliminary observations made on the biological characteristics of species V revealed that it rests predominantly in human and mixed dwellings and has an anthropophagic index of about 4%. Studies are in progress to ascertain the role of species V in malaria transmission.

Anopheles minimus Complex

Out of the three recognized species of *An. minimus*—species A, C and E, only species A has been recorded from India based on isozyme analysis (Adak *et al* unpublished report). Mapping the distribution of *An. minimus* sibling species using molecular tools also revealed prevalence of species A in India. Recently, *An. fluviatilis* S was made synonym of *An. minimus* C by certain investigators, as a result the distribution of *An. minimus* C was shown in India. However, further molecular studies by NIMR revoked the synonymy of *An. fluviatilis* S with *An. minimus* C. Pair-wise distance and phylogenetic analysis using ITS2 sequences of the members of the Minimus and Fluviatilis Complexes revealed that *An. fluviatilis* S and *An. minimus* C are genetically distant and independent species (Singh *et al* 2006) (Fig. 42).

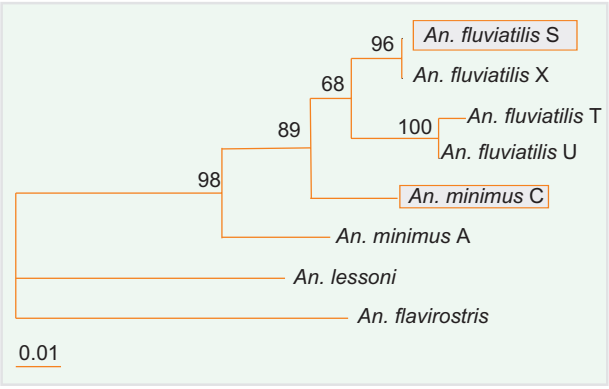

Fig. 42: Maximum Likelihood (ML) tree inferred from ITS2 sequences from the members of Fluviatilis and Minimus Complexes. Numbers above the branches are bootstrap values. Scale bar represents 0.01 nucleotide substitutions per site. *Anopheles lessoni* and *An. flavirostris* are taken as outgroup

Anopheles sundaicus Complex

In India, *An. sundaicus* is abundantly found only in Andaman and Nicobar Islands, where this species is the sole malaria vector. Studies carried out in Thailand and Indonesia have established *An. sundaicus* as a complex of three isomorphic species (species A, B and C) identifiable on the basis of

Table 4. Comparison of polytene and mitotic chromosomes of cytotype D with other forms in *An. sundaicus* complex

Forms	Polytene banding patterns				Mitotic chromosomes	
	Xa	Xb	2a	2b	Y	Ch
A	+	–	+	–	Y_1	Normal
B	–	+	+	–	Y_2	Normal
C	–	+	–	+	Y_1	Large
D	+	–	–	+	Y_1	

Note : +presence; – absence; Y_1—telocentric with 2 heterochromatin blocks; Y_2— telocentric but longer than Y_1 and with three heterochromatin blocks; Ch—pericentromeric heterochromatic blocks in autosome 2.

cytological variations together with enzyme polymorphism analysis. Another species in *An. sundaicus* complex has been identified from Malaysia on the basis of sequence variations in the regions of cytochrome b and cytochrome oxidase 1 of the mitochondrial DNA and this has been designated as *An. sundaicus sensu stricto*.

Cytogenetic characterization of *An. sundaicus* population from Car Nicobar Island, India was carried out. All the samples screened for ovarian polytene chromosomes had X-chromosome of Xa type as reported in case of species A and chromosome 2 (2b type) similar to that in species C. This combination revealed the existence of a new cytogenetic variant, *i.e.* cytotype D, reported for the first time in the Indian subcontinent. Examination of male and female mitotic karyotypes further substantiated these results (Table 4).

The above observations were strongly supported by molecular studies carried out on *An. sundaicus* samples from four geographically isolated areas — Teressa, Nancowry, Car Nicobar and Katchal islands. PCR-amplification and nucleotide sequence analyses were performed for ITS 2 and domain-3 (D3) of 28S rRNA. The ITS2 region of *An. sundaicus* from all four islands was identical but different from *An. sundaicus* A of Vietnam and *An. sundaicus sensu stricto* of Malaysia. Similarly, the D3 sequences, reported for the first time for a species of the Sundaicus Complex, were identical among all samples analysed from the four islands. These observations suggested the existence of *An. sundaicus* D in Andaman and Nicobar Islands.

Examination of *An. sundaicus* populations from other Andaman and Nicobar Islands revealed the prevalence of only cytotype D both in fresh water and brackish water areas, indicating wide adaptability of this form to different habitats.

Anopheles annularis Complex

An. annularis has wide distribution in India and is considered an important vector in certain parts of Orissa state. It is a secondary vector in certain localities and is sometimes found abundantly.

Cytogenetic studies carried out in different parts of India established *An. annularis* as a species complex comprising species A and B. These species were identified on the basis of differences in banding pattern on arm 2 of the polytene chromosomes complement. Species A is characterized by $+j^1$ arrangement and species B by j^1 arrangement on chromosome arm 2. The X-chromosome and the autosomal arms 3, 4 and 5 are homosequential in both the species. The male and female mitotic karyotypes have also been found to be the same in species A and B. Recently, ribosomal DNA PCR-RFLP methods have been developed, based on sequence analysis of ITS2 and domain 3 (D3) of *An. annularis* that can differentiate species A from B (Alam *et al* 2007).

Mapping the distribution of *An. annularis* sibling species in different geographical areas of India revealed the prevalence of species A in Districts Alwar (Rajasthan); Ghaziabad & Shahjahanpur (Uttar Pradesh); Sonepat (Haryana); Sundargarh and Koraput (Orissa); and Kamrup (Assam). Whereas, species B has been reported only from Districts Shahjahanpur and Ghaziabad in Uttar Pradesh state where it was found sympatric with species A. Blood samples from the gut of mosquitoes that were identified to sibling species in the above mentioned districts were analysed for determining the blood meal source. Almost all the samples belonging to species A and B had bovine blood strongly indicating that these species are primarily zoophagic.

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