Species Complexes in Malaria Vectors in India

MRC is actively engaged in the recognition of new species complexes in malaria vectors and in studying biology, distribution and transmission potential of members of each species complex. Indian anopheline fauna comprises of 58 species, of which 9 act as vectors of malaria. *An. culicifacies, An. fluviatilis, An. stephensi, An. minimus, An. dirus* and *An. sundaicus* are the important 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* are also complexes. *An. subpictus* is not considered a vector but a few sporozite positives have been found in this species in coastal areas of Pondicherry where species B is found. The number of sibling species so far identified among the Indian anophelines is given in Table 1. *An. stephensi* and *An. varuna,* the two malaria vectors in India, have not yet been found as species complexes.

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	3	S, T, U
An. minimus	3	A
An. sundaicus	3	A fourth cytotype, D
An. philippinensis-nivipes	3	*nivipes* A
*An. subpictus**	4	A, B, C, D

*These two complexes have not been studied by MRC.

Recognition of Species Complexes

Pre-mating barriers

Cytotaxonomic studies of natural populations of malaria vectors carried out at MRC led to the recognition of *An. culicifacies, An. fluviatilis* and *An. annularis* as species complexes. Fixed paracentric inversions in ovarian polytene chromosomes were found in the 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

Malaria Research Centre is a WHO Regional Reference Centre for the identification of *An. culicifacies* sibling species and variations

evidence for designating the populations as distinct species (Subbarao *et al.*, 1983, 1994 & Atrie *et al.*, 1999) 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

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 absence of post-mating barriers between species B and C (Subbarao *et al.*, 1988).

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. 29) (Subbarao *et al.*, 1983, 1988 & Vasantha *et al.*, 1991). Structural variations in metaphase chromosomes of

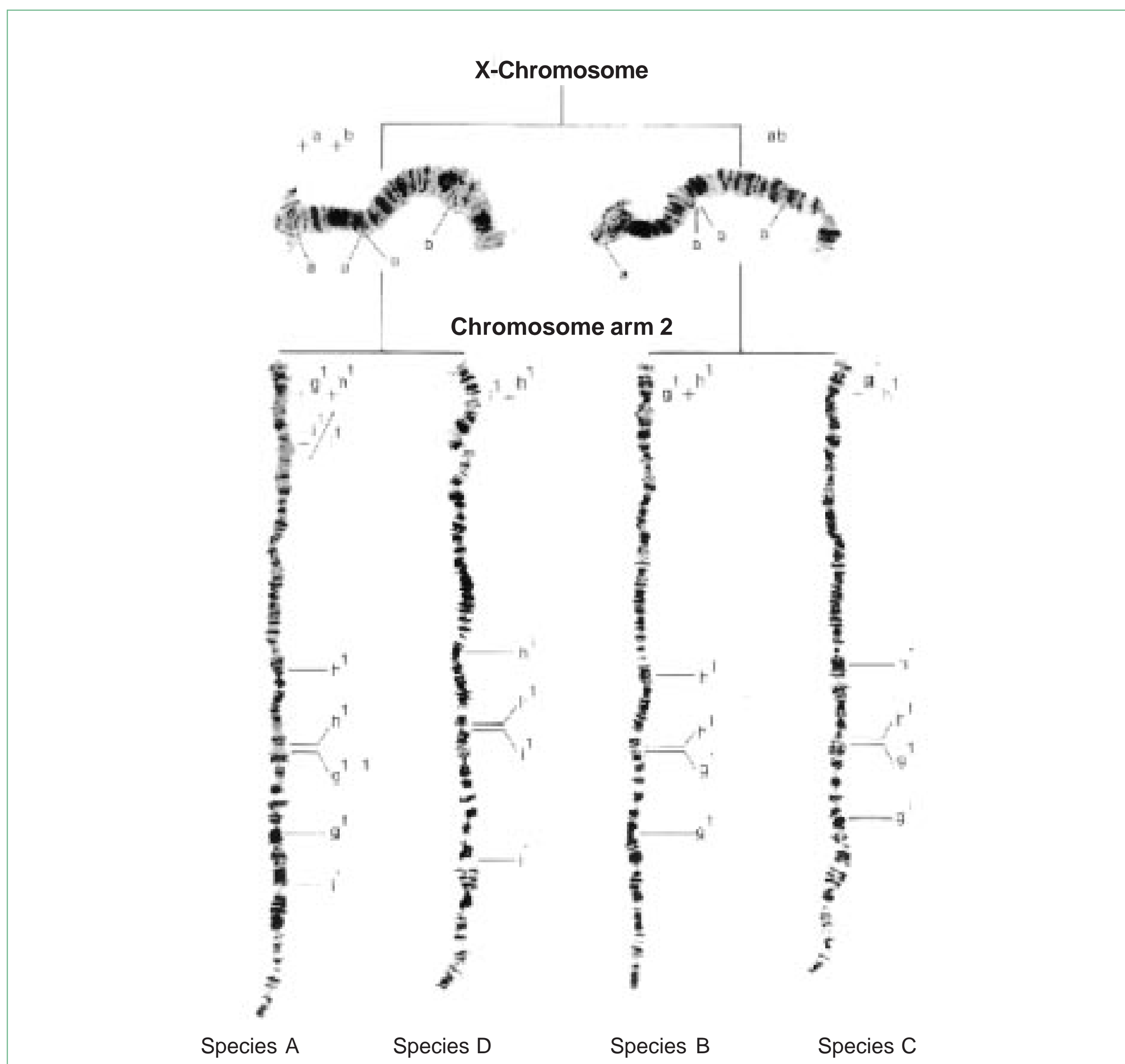

Fig. 29: **Schematic representation of polytene chromosomes of *An. culicifacies* sibling species**

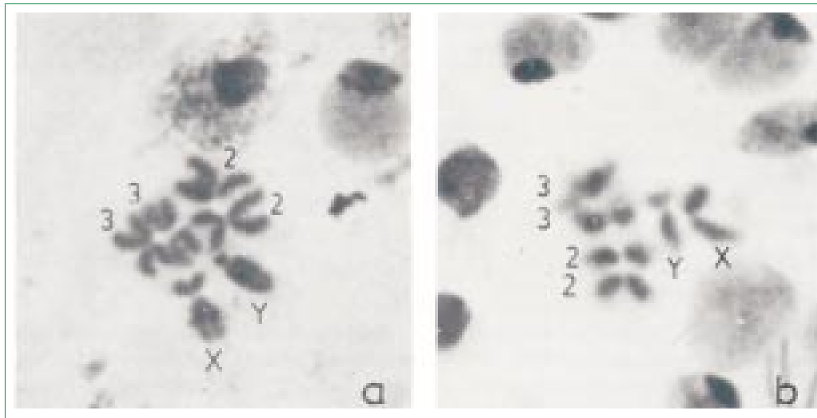

Fig. 30: **Male mitotic karyotypes of *An. culicifacies* from Rameswaram Island: (a) Karyotype with acrocentric Y-chromosome (species B); (b) Karyotype with sub-metacentric Y-chromosome (species E)**

mitotic and meiotic karyotypes (Fig. 30) along with biological variations have been used to differentiate species B and E (Kar *et al*., 1999).

Electrophoretic variations found at lactate dehydrogenase (LDH) locus (Fig. 31) could differentiate species A and D from species B and C (Adak *et al*., 1994). Recently a PCR-based diagnostic assay using primers selected from D2 variable region of the 28S RNA gene, has been developed that distinguishes species A and D from species B, C and E (Fig. 32). Similarly a multiplex PCR has been standardized using two universal primers for D3 region and two allele specific primers, which can differentiate species A/D from species B/C/E (Fig. 33). Another multiplex PCR assay developed using primers from cytochrome oxidase II region distinguishes all 5 species. This assay is being validated with field samples.

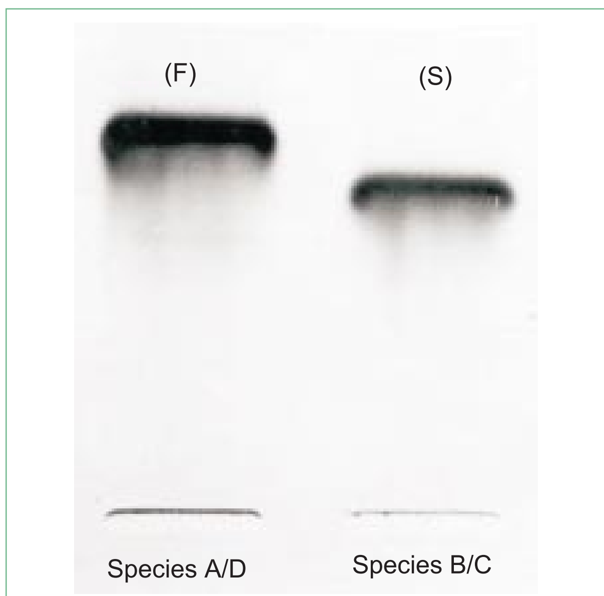

Fig. 31: **Differentiation of members of *An. culicifacies* complex by lactate dehydrogenase enzyme: the two forms of allozyme, i.e. Fast (F) and Slow (S) differentiate species A/D from species B/C of *An. culicifacies* respectively**

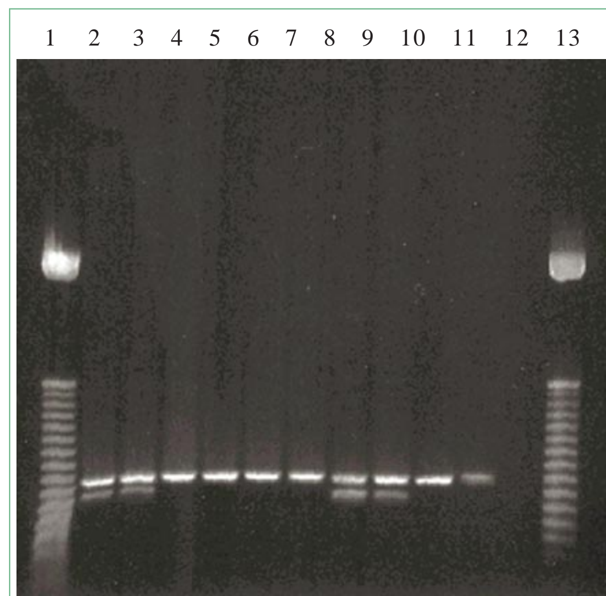

Fig. 32: **PCR assay using primers designed from D2 region of 28S rDNA which differentiates species A/D from species B/C/E of *An. culicifacies*. Lanes 1 &13: DNA ladder; Lanes 2 & 3: species A; Lanes 4 & 5: species B; Lanes 6 & 7: species C; Lanes 8 & 9: species D; Lanes 10 & 11: species E, Lane 12: (–)ve control**

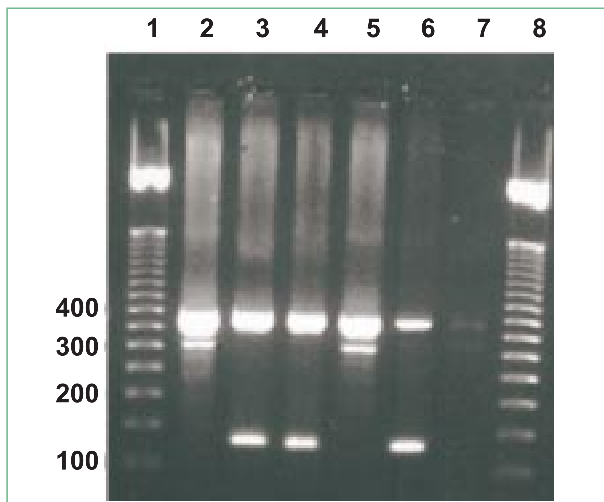

Fig. 33: **PCR products obtained by the primers designed from D3 region of 28S rRNA electrophoresed on 2% agarose gel. Lanes 1 & 8 : 50 bp marker; Lane 2: *An. culicifacies* sp. A; Lane 3 : *An. culicifacies* sp. B; Lane 4 : *An. culicifacies* sp. C; Lane 5 : *An. culicifacies* sp. D; Lane 6 : *An. culicifacies* sp. E from Rameswaram; Lane 7: (–) control**

Fig. 34: **Differentiation of members of *An. fluviatilis* complex: PCR product as seen on 2% agarose gel containing ethidium bromide under UV illumination (Lanes 1-3, species S; Lanes 4-6, species T; Lanes 7-10, species U; Lane 11, negative control, without DNA; Lane 12, 100 bp DNA ladder)**

In the *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). Recently an allele-specific PCR-based diagnostic assay has been developed which can differentiate all the three members of the complex. The assay is based on the differences in nucleotide sequences of D3-domain of 28S ribosomal RNA in species S, T and U (Fig. 34).

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 a new dimension to vector control. Members of the complexes are generally isolated by premating 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.

An. 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. 35. 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 (Subbarao, 1988).

Field studies also demonstrated that the seasonal changes in the prevalence of different sibling species in areas where more than one occurred (Subbarao, *et al* ., 1987). 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 of species C was very low.

Biological variations among species A, B, C, D and E are summarized 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 (Joshi *et al*., 1988) 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 (Subbarao *et al*., 1988, 1992). Species E was found with sporozoites (Kar *et al*., 1999). These species also vary in the rate of development of resistance to different insecticides (Raghavendra *et al*., 1992, 1998).

An. culicifacies s.l. was colonized 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

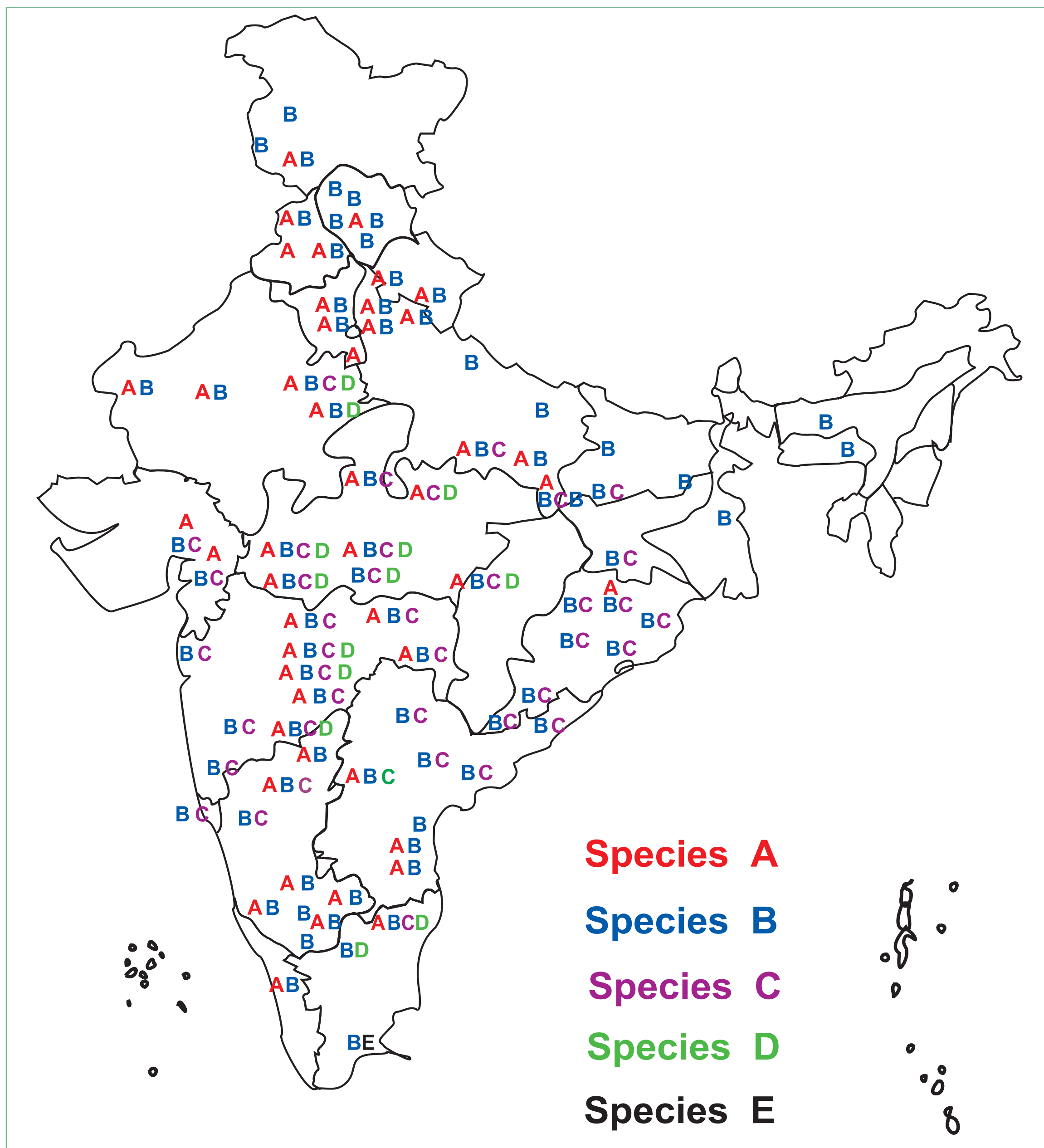

Fig. 35: **Map showing the distribution of members of the *An. culicifacies* complex in India**

Table 2. Biological variations among *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	Upto midnight	–
Peak biting time	2200–2300h	2200–2300h	1800–2100h	1800–2100h	–
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 yrs)	Medium (6–7 yrs)	Fast (4–5 yrs)	–	–
Synthetic pyrethroids	–	Fast (4–5 yrs)	Fast (4–5 yrs)	–	–

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. The effects of crowding which differed significantly among the three sibling species were reflected in higher larval mortalities, longer pupation and emergence times. Species B was the least adversely affected. In 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, variation under laboratory conditions were also studied.

In laboratory studies susceptibility of three members of *An. culicifacies*, species A, B and C were

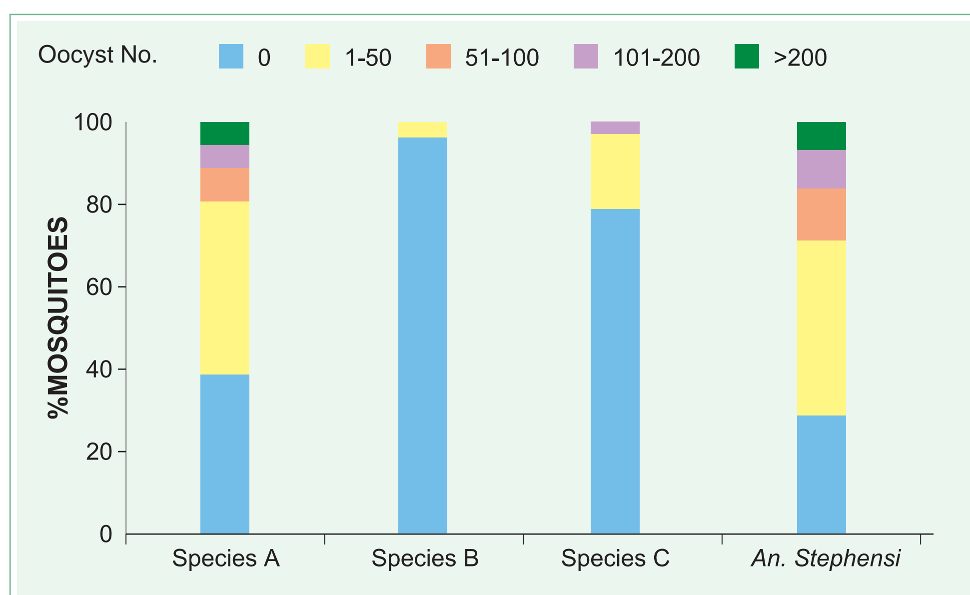

Fig. 36: **Frequency distribution of *P. vivax* oocysts in different members of *An. culicifacies* complex and *An. stephensi***

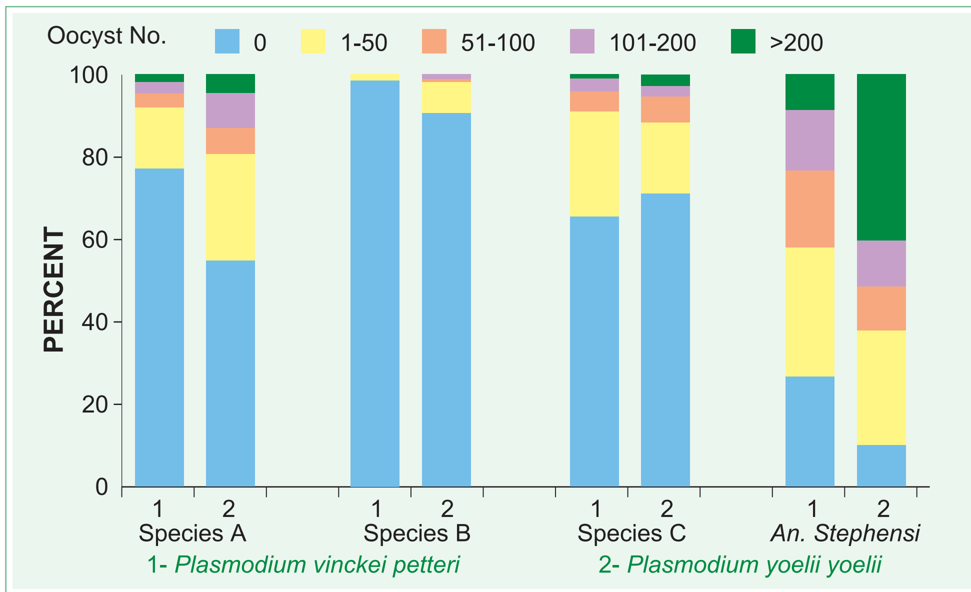

Fig. 37: **Frequency distribution of rodent malaria oocysts (*P. v. petteri* and *P. y. yoelii*) in different members of *An. culicifacies* complex and *An. stephensi***

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 (Figs. 36 & 37) (Adak *et al*., 1999 & Kaur *et al.*, 2000).

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. 38). The strain is partially refractory to *P. falciparum* and rodent malaria parasite *P. vinckei petteri*. 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 are of great interest and research groups involved in the development of transgenic mosquitoes for malarial control are looking for such genes.

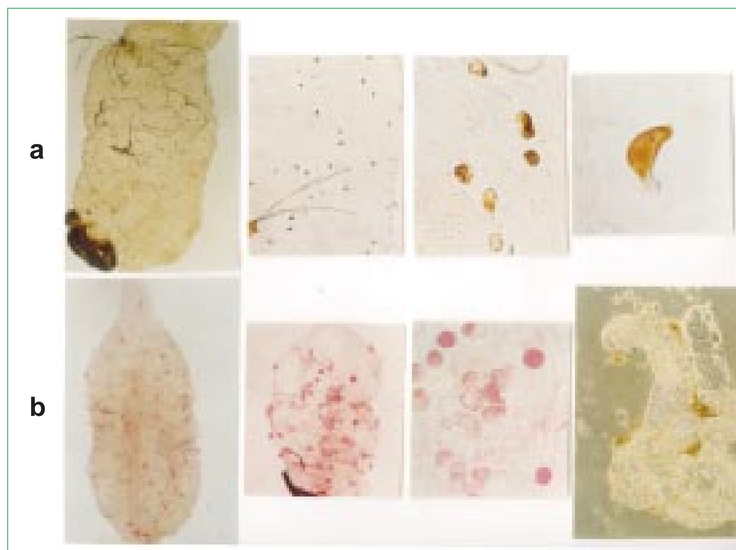

Fig. 38: **(a) Midguts of *An. culicifacies* species B refractory strain showing encapsulated *P. vivax* parasite and (b) Midguts and salivary glands of *An. stephensi* showing normal oocysts and sporozoites**

The laboratory feeding experiments suggest that in addition to encapsulation and melanization 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 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 (Subbarao *et al*., 1988 & Tiwari *et al*., 1994). Broadly it can be stated that as species B is a nonvector, 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 (see malariogenic stratification section for details).

An. 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. 39) and exhibit distinct differences in their biological

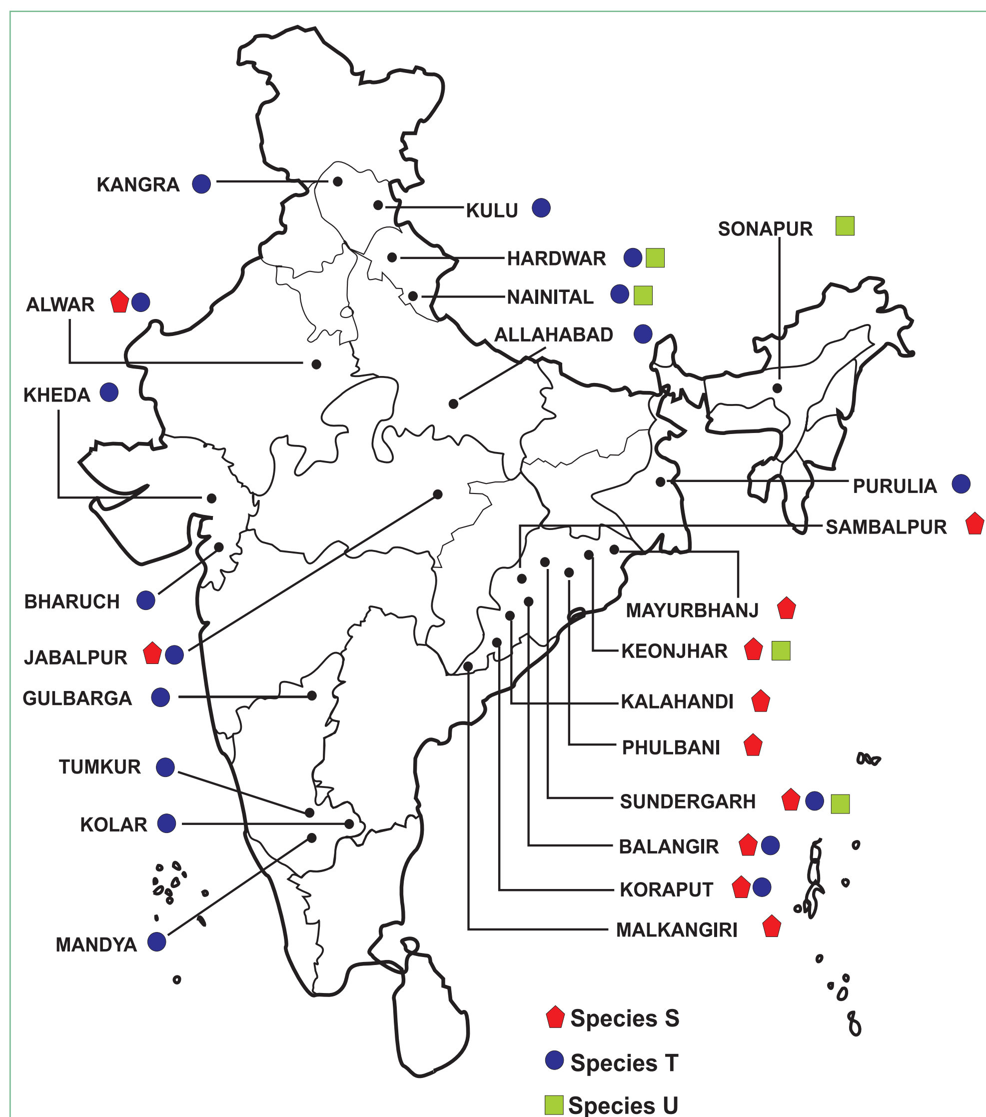

Fig. 39: **Map showing the distribution of members of the *An. fluviatilis* complex in India**

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 habitat	Observed in	
						Ecotypes	Epidemio-logical areas
S	$+q^1+r^1$	Low moderate (1-40)	Anthropo-phagic	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-

characteristics (Table 3). *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 nonvector, 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 gland 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. 40). These studies suggested that the *An. fluviatilis* species T which is not a vector in field, has inherent ability to support normal sporogony. This species is almost zoophagic in field and probably its preference to feed on cattle makes it a nonvector and may act as a vector in the absence of cattle. n

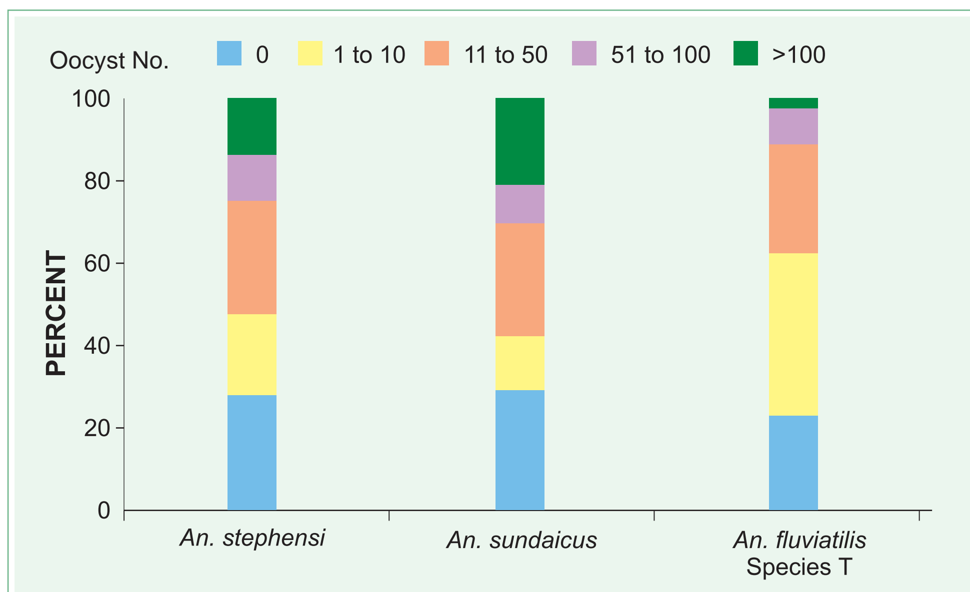

Fig. 40: **Frequency distribution of oocysts in *An. fluviatilis* species T and other two vectors *An. stephensi* and *An. sundaicus***