1

Vector Biology and Control

1.1 Studies on Anopheline Species Complexes

1.1.1 The Culicifacies Complex

Bionomics and Distribution Pattern

Anopheles culicifacies populations from malaria endemic districts, namely Ranchi and Gumla (Jharkhand state) were examined for sibling species composition. In District Ranchi, cytological examination of the samples collected from villages under Primary Health Centres, Angada and Bharno revealed the prevalence of species B and C with predominance of the former, whereas in District Gumla, *An. culicifacies* species B and C were found in almost equal proportion in the villages surveyed. Similarly, in District Bastar (Chhattisgarh state) species B and C were sympatric with predominance of the latter (87.5%). In a longitudinal study in District Jabalpur (Madhya Pradesh), examination of *An. culicifacies* populations for the second successive year revealed the prevalence of species B, C and D in the study villages with predominance of species C (>70%). The established vector species (C & D) together constituted >90% of the total *An. culicifacies* population, whereas proportion of non-vector species B was low suggesting high malariogenic potential of the study area. In all above mentioned districts, *An. culicifacies* sibling species were found to be predominantly zoophagic as revealed by blood meal source analysis.

1.1.2 Fluviatilis and Minimus Complexes (Minimus Group)

Bionomics and Distribution Pattern

An. fluviatilis populations from malaria endemic districts, namely Damtari, Surguja and Korba (Chhattisgarh state) were analyzed for sibling species composition and host preference. In all these districts, only species T was prevalent and was found to be polymorphic for q^1 inversion (Table 1.1.1). In the study areas, this species was found resting predominantly in cattlesheds or mixed dwellings and was totally zoophagic, which indicates its limited role in malaria transmission in the above mentioned districts.

Table 1.1.1. *An. fluviatilis* sibling species composition in the study districts of Chhattisgarh state

Districts	Total identified	Sibling species						HBI
		S	T			U	V	
			$+q^1$	$+q^1/q^1$	q^1			
Dhamtari	34	0	1	7	26	0	0	0
Surguja	33	0	0	4	29	0	0	0
Korba	23	0	0	2	21	0	0	0

$+q^1$, $+q^1/q^1$ and q^1 — Chromosomal inversion genotypes; HBI— Human blood index.

Similarly, in Ranchi and Gumla districts, only *An. fluviatilis* species T was found prevalent in the study areas and was totally zoophagic. Species T in these districts was sympatric with *An. culicifacies* sibling species B and C.

Identification of *An. fluviatilis* Species 'T' as Malaria Vector

An. fluviatilis species 'T' is regarded as a non-vector due to its zoophagic nature. However, for the first time, it was found that *An. fluviatilis* which were malaria sporozite positive as determined by sporozoite enzyme linked immunosorbent assay (ELISA) are *An. fluviatilis* species T. We characterized four specimens of sporozoite positive *An. fluviatilis* from Jabalpur and two specimens from Ranchi by sequencing ITS2. It was found that their ITS2 sequences are homologous to *An. fluviatilis* T. This is the first record of *An. fluviatilis* T incriminated as a malaria vector.

Molecular Characterization of *An. fluviatilis* and *An. minimus* from Jalpaiguri

A total of 43 specimens of morphologically identified *An. minimus* and 125 specimens of *An. fluviatilis* were characterized for sibling species through polymerase chain reaction (PCR) assay and PCR-RFLP. All *An. minimus* samples were identified as *An. minimus* A (sensu stricto). Out of 125 *An. fluviatilis* examined, 118 were identified as species T, and rest seven were identified as *An. minimus* A. Later these were confirmed to be *An. minimus* A based on their ITS2 sequences. Some of the *An. fluviatilis* samples were also sequenced for ITS2 rDNA for confirmation of PCR results.

"Anopheles fluviatilis species T was incriminated as malaria vector for the first time which was earlier thought to be a non-vector"

1.1.3 The Annularis Complex

Identification of *An. annularis* Species 'A' as Malaria Vector

Five specimens of *An. annularis* from District Ranchi, Jharkhand, which were found to harbour *Plasmodium falciparum* sporozoites were identified as *An. annularis* species A based on ITS2 sequence analysis.

1.2 Vector-Parasite Interactions

1.2.1 Susceptibility of *Anopheles fluviatilis* Species 'T' and 'U' to *Plasmodium vivax*

Susceptibility of *An. fluviatilis* species T and U to *P. vivax* was ascertained by feeding the mosquitoes on *P. vivax*-infected blood samples through artificial membrane. The *An. stephensi* was used as positive control. The oocyst rates in *Anopheles fluviatilis* species T, U and *An. stephensi* were 42.37, 45.63 and 58.78%, respectively. The geometric mean number of oocysts were 17.70, 17.44 and 17.70, respectively. The oocyst rates and geometric mean number of oocysts in these species didn't differ significantly. The sporozoite rate in *An. fluviatilis* species T and U was 39.5 and 32.03%, respectively as against 54.95% in *An. stephensi*. Statistically there was no significant difference in sporozoite rates between species T and *An. stephensi* but significantly lower sporozoite rate was recorded in species U as compared to *An. stephensi* ($\chi^2 = 0.0340$, df = 1, $p < 0.01$).

The overall proportions of mosquitoes with oocyst count ranges of 0, 1–50, 51–100 and 101–200, for each species infected with *P. vivax* are shown in Fig. 1.2.1. On the basis of above finding, it may be concluded that *An. fluviatilis* species T and U are susceptible to *P. vivax*. Thus, it may be extrapolated that *An. fluviatilis* species T and U may act as vectors when they are in high density in an endemic area and/or in the absence of other vector species.

1.2.2 Phenoloxidase Activity in Different Members of the Culicifacies Complex

The members of Culicifacies Complex differ in their susceptibility to plasmodial sporogonic

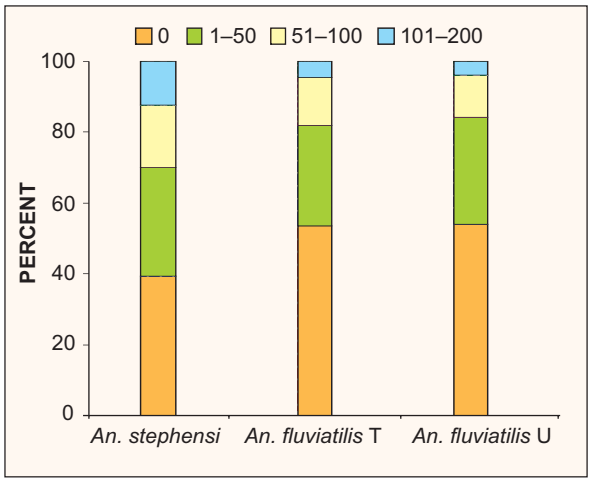

Fig. 1.2.1: Proportion of oocyst densities in species T and U of Fluviatilis Complex against *P. vivax*

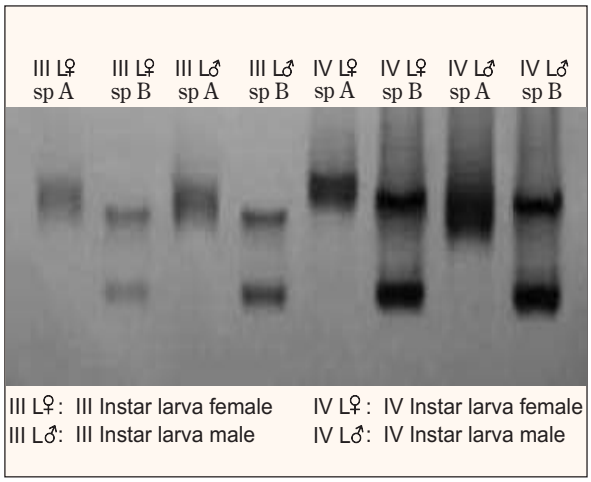

Fig. 1.2.2: Comparison of PO activity in III & IV instar male and female larvae of *An. culicifacies* species A and B

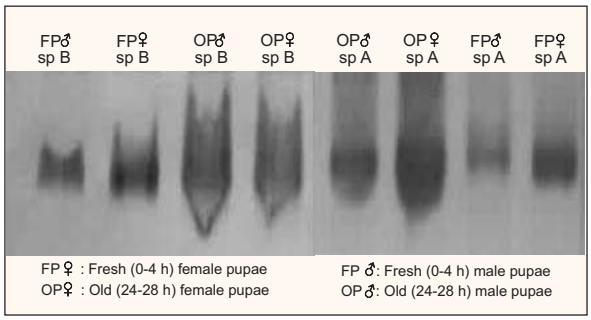

Fig. 1.2.3: Comparison of PO activity in fresh and old male and female pupae of *An. culicifacies* A and B

success. Encapsulation of invading parasite is one of the mechanisms by which sporogony is aborted in some strains of *An. culicifaces* B. The phenoloxidase (PO) is the prime enzyme (14.18.1.1) responsible for melanisation, which is secreted by fat bodies and/or hemocytes in its inactive form, the prophenoloxidase (PPO), and further activated by serine proteases. We studied the differential activity and isozymes of PO in the members of *An. culicifacies.* For the purpose, cyclic colonies of *An. culicifacies* sibling species A (Dehra strain) and species B (Haldwani strain) were used.

Qualitative Assay

Qualitative assay of enzyme phenoloxidase was done using PAGE to study the isozyme profile of both the sibling species. Tyrosine (mono-phenol compound) and DOPA (a di-phenol compound) were used as substrate to visualize the enzyme on the polyacrylamide gel for mono and di-phenoloxidase activity respectively. Third and fourth instar larvae of *An. culicifacies* B showed two bands of enzyme on substrate staining with more or less identical enzyme activity. Both the bands were apart by very little gap when resolved on 5% SDS PAGE, which inferred slight difference in their molecular weight. These bands may be marked as slow and fast isozymes of phenoloxidase enzyme (Fig. 1.2.2). Same isozyme pattern was observed in both the sexes of III and IV instar larvae. One broad and light band of PO was observed in fresh (0–4 h old) and old pupae (24–28 h old) of *An. culicifacies* B. This may be due to high amount of protein loaded per well because of less activity of PO in pupae as shown in Fig. 1.2.3.

On the other hand, *An. culicifacies* A showed only one darker and broad band of PO in III and IV instar male and female larvae (Fig. 1.2.2). The Rf (Resolution factor) value of isozyme present in *An. culicifacies* A is nearly equal to that of slow isozyme present in species B. So, it may be concluded that species B has two isozymes of PO— slow and fast, while species A has only one, i.e. slow. Similarly, slow isozymes of PO were also found in fresh (0-4 h) as well as old (24–28 h) male and female pupae of *An. culicifacies* A (Fig. 1.2.3). The isozymes of PO found in different develop-

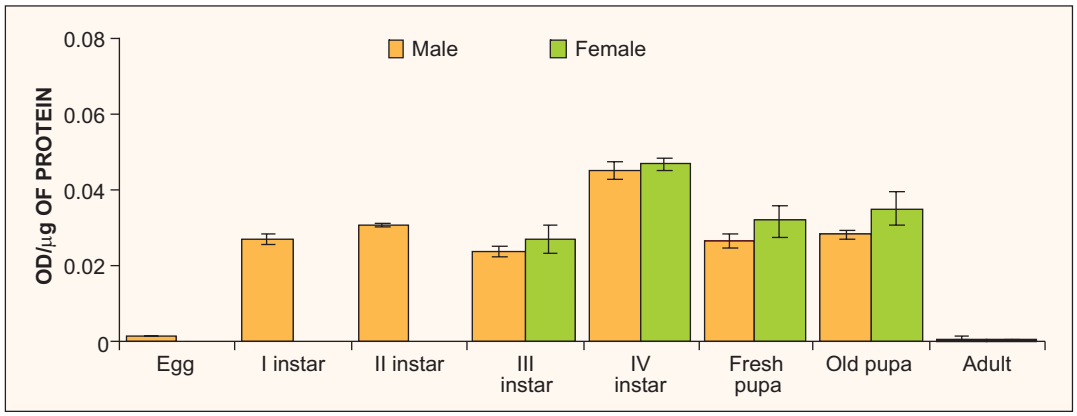

Fig 1.2.4: Quantitative assay of phenoloxidase activity in different developmental stages of male and female *An. culicifacies* A

mental stages of *An. culicifacies* species A and B showed activity for mono- as well as diphenoloxidase.

Quantitative Assay

Enzyme activity was measured in optical density (OD) per microgram of protein. Activity of PO was analyzed among different developmental stages of *An. culicifacies* species A. Increase in enzyme activity was observed with the development. First, second and third instar larvae showed more or less equal activity. On the other hand, IV instar larvae showed significantly higher enzyme activity when compared to III instar larvae ($p < 0.0001$, both for male and female). While, in the later stages of development a steep decrease in enzyme activity was observed in pupae (~1.5 fold) and 4–6 days old adult (~60 fold) (Fig. 1.2.4).

Activity of PO was also analyzed among different developmental stages of *An. culicifacies* B where a significant increase in PO activity was noticed among III and IV instar larvae ($p > 0.001$, both for male and female). A sudden decrease (~3 fold) was observed in pupal stage (Fig. 1.2.5). Rest of the developmental stages could not be analyzed because of non-availability of biological material due to contamination in the colony.

On the basis of above finding one may infer that the IV instar larvae showed highest enzyme activity as compared to other developmental stages in both the species and sexes. In comparison to males, females showed slightly high enzyme activity in all developmental stages. *An. culicifacies* species B showed high PO activity as compared to species A.

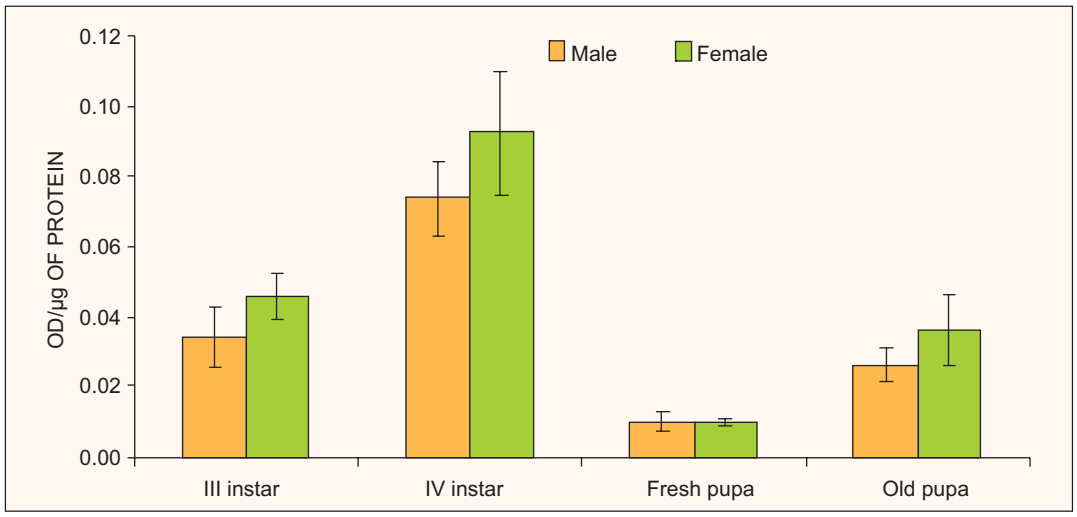

Fig. 1.2.5: Quantitative assay of phenoloxidase activity in different developmental stages of male and female *An. culicifacies* B

1.2.3 Nitric Oxide Synthase (NOS) in Malaria Vectors

Molecular Characterization of NOS in *An. stephensi*

Nitric oxide (NO) is a ubiquitous free radical produced by Nitric oxide synthase (NOS), that plays a protective role against the invading pathogen as well as a role of a biological messenger. In *An. stephensi*, one of the major malaria vectors in India, the NOS gene is encoded by 19 exons, spanning 33 kilobases with an open reading frame of 1247 amino acids. The induction of NO during *P. falciparum* and *P. berghei* infections in *An. stephensi* and the importance of promoter polymorphisms in altering gene expression levels prompted us to study the genetic diversity in NOS promoter by employing sequencing methods, as well as to measure the induction of NO synthesis on *P. vivax* infection in *An. stephensi* using Griess method. No variation was observed in the ~300 bp NOS promoter region of *An. stephensi*, analyzed from different regions of the Indian subcontinent, but a difference at four nucleotide positions was observed when compared to previously reported sequence by Luckhart *et al.* 1999.

A highly significant elevation in NO concentration was observed in *P. vivax* infected mosquitoes when compared to unfed ones (p = 0.0001) (Fig. 1.2.6). In addition, variation in NO levels was observed among unfed mosquitoes collected from different regions of India

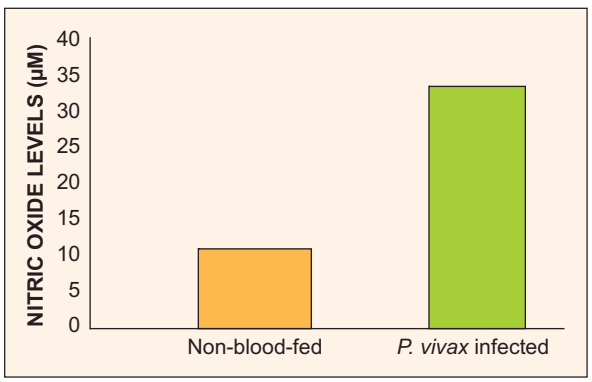

Fig. 1.2.6: A graphical representation of nitric oxide levels, measured using Griess method in *P. vivax* infected and non-blood-fed *An. stephensi* samples

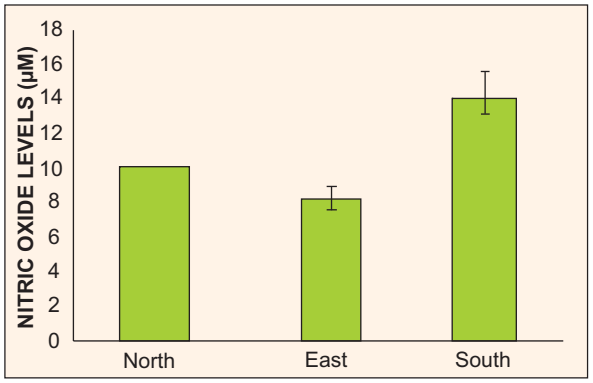

Fig. 1.2.7: A graphical representation of nitric oxide levels measured using Griess method in *An. stephensi* samples collected from North, East and South Zones of the Indian subcontinent

(Delhi, Punjab, Hardwar, Chennai and Kolkata). Moreover, NO concentration was found to be significantly different amongst unfed *An. stephensi* populations of North, East and South zones of India (North *vs* South, p = 0.0005; North *vs* East, p = 0.015; South *vs* East, p = 0.0006) (Fig. 1.2.7). A substantial increase in the NO levels was observed in *An. stephensi* as early as 2 h post-vivax infection up to two days and decline thereafter (Fig. 1.2.8). Taken together, from the above results, it can be concluded that the elevated levels of NO as early as 2 h post-blood meal could be a consequence of NOS induction in response to the parasite invasion of the midgut, suggesting that the parasite (asexual blood stages and gametocytes) itself is the prime target of NO mediated destruction. Moreover, the apt explanation for increased NO levels up to two days post-blood meal could be that NO may be responsible for maximum interference during this sexual phase of parasite development. The intrapopulation variation in NO concentration could be attributed to the variation at the gene level. Therefore, NO levels could be of biological importance, adversely affecting *P. vivax* development.

1.2.4 Biochemical Determination of Nitric Oxide Metabolites, Nitrate and Nitrite in *An. culicifacies* by HPLC

The diverse physiological and pathological role of nitric oxide in innate immune defenses against many intra and extracellular patho-

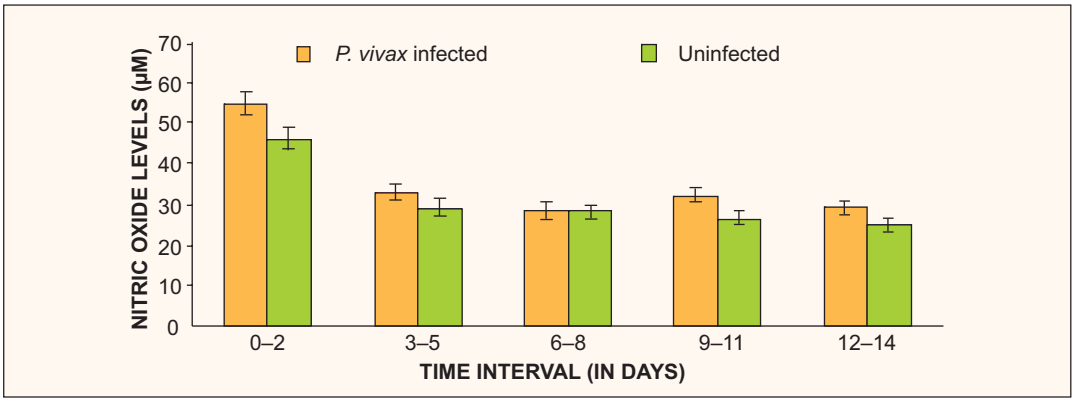

Fig. 1.2.8: A graphical representation of NO levels in *P. vivax* infected and uninfected blood-fed *An. stephensi* samples collected at regular intervals post-blood meal. NO levels were measured using Griess method

gens, have led to the development of various methods for determining nitric oxide (NO) synthesis. NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-) are produced by the action of an inducible *An. culicifacies* NO synthase (AcNOS) in mosquito midguts and may be central to antiparasitic arsenal of these mosquitoes.

While exploring a plausible mechanism of refractoriness based on nitric oxide synthase physiology among the sibling species of *An. culicifacies*, a sensitive, specific and cost-effective high performance liquid chromatography (HPLC) method was developed, which is not influenced by the presence of biogenic amines, for the determination of NO_2^- and NO_3^- from mosquito midguts and haemolymph. This method is based on extraction, efficiency, assay reproducibility and contaminant minimization. It entails de-proteinization by centrifugal ultra filtration through ultracel 3K filter and analysis by high performance anion exchange liquid chromatography (Sphereclone, 5µ SAX column) with UV detection at 214 nm. The lower detection limit of the assay procedure is 50 pmoles in all midgut and haemolymph samples. Retention times for NO_2^- and NO_3^- in standards and in midgut samples were 3.42 and 4.53 min respectively (Fig. 1.2.9). Assay linearity for standards ranged between 50 nM and 1 mM. Recoveries of NO_2^- and NO_3^- from spiked samples (1–100 µM) and from the extracted standards (1–100 µM) were calculated to be 100%. Intraassay and interassay variations and relative standard deviations (RSDs) for NO_2^- and NO_3^- in spiked and unspiked midgut samples were 5.7% or less (Tables 1.2.1 and 1.2.2). Increased levels NO_2^- and NO_3^- in haemolymph and midguts (Figs. 1.2.10 and 1.2.11) of *An. culicifacies* sibling species B in comparison to species A reflect towards a mechanism of refractoriness based on AcNOS physiology.

Table 1.2.1. Recoveries of NO_2^- and NO_3^+ in extracted KNO_2/KNO_3 standards (Mean ± SD, n = 8)

Concentration	Recovery	
	NO_2^-	NO_3^-
48 nM	90.7 ± 1.9	28.3 ± 3.4
97 nM	94.5 ± 6.2	54.8 ± 4.3
195 nM	94.7 ± 5.9	87.8 ± 5.1
390 nM	93.4 ± 4.0	80.2 ± 1.5
780 nM	93.6 ± 1.9	93.7 ± 4.5
1.56 µM	93.7 ± 2.9	87.9 ± 3.9
3.12 µM	91.8 ± 1.6	95.2 ± 2.9
6.25 µM	98.5 ± 2.4	98.5 ± 3.1
12.5 µM	97.1 ± 1.8	94.8 ± 1.9
25 µM	99.0 ± 5.4	100.5 ± 3.2
50 µM	100 ± 5.8	97.3 ± 2.9
100 µM	97.1 ± 2.4	99.2 ± 4.9

"HPLC was performed for the determination of NO_2 & NO_3 from mosquito mid guts and haemolymph"

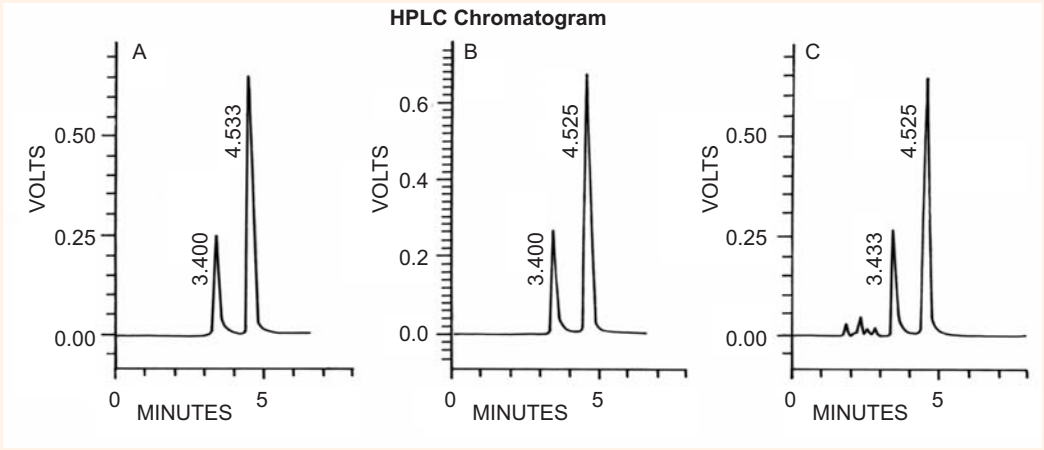

Fig.1.2.9: HPLC analysis of nitrite and nitrate. Chromatograms of an aqueous standard containing 25 µM nitrite and nitrate (A); washed and spiked *An. culicifacies* midguts obtained under control (B); and *An. culicifacies* haemolymph (C)

Table 1.2.2. Relative standard deviations (RSDs) and recoveries for NO_2^- and NO_3^- from mosquito mid-gut spiked* with 1–100 µM KNO_2/KNO_3 (mean ± SD, n=6)

Concentration (µm)	NO_2^-			NO_3^-		
	Intra assay RSD (%)	Inter assay RSD (%)	Recovery (%)	Intra assay RSD (%)	Inter assay RSD (%)	Recovery (%)
0	8.3	8.9	–	4.2	5.6	–
1	8.8	9.3	94.4 ± 4.4	8.7	9.9	98.9 ± 2.2
2.5	7.1	9.8	98.2 ± 6.6	7.2	8.5	98.5 ± 2.4
5	6.7	9.9	95.2 ± 2.9	3.9	5.9	97.1 ± 1.8
10	5.1	9.2	98.5 ± 3.1	3.1	2.9	99 ± 5.4
25	4.5	5.3	99.8 ± 1.9	2.2	3.0	100 ± 5.8
50	5.5	4.9	100.5 ± 3.2	2.9	2.6	94.5 ± 6.2
100	3.9	4.7	97.3 ± 2.9	1.2	4.1	94.7 ± 5.9

*Unspiked midgut samples, i.e. midgut samples to which exogenous NO_2^- and NO_3^- has not been added

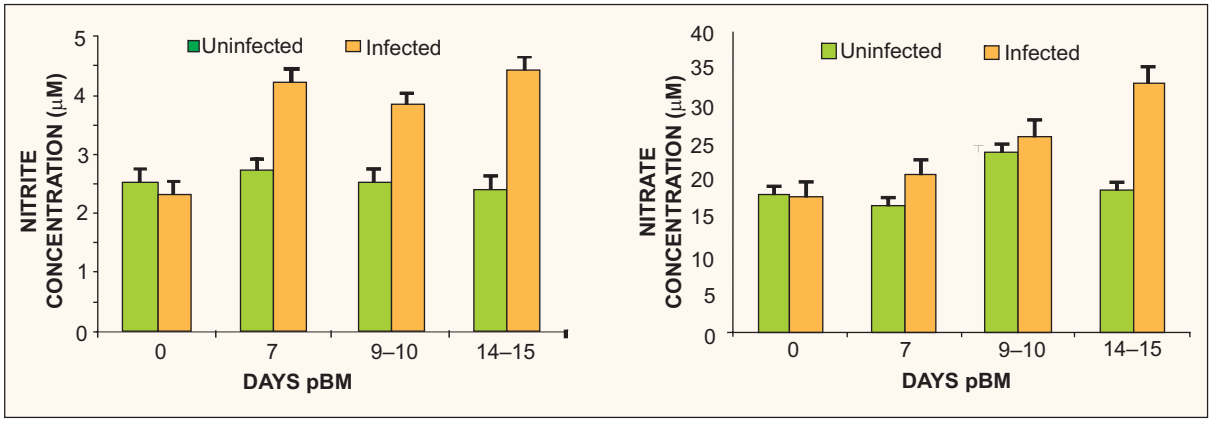

Fig. 1.2.10: Haemolymph nitrite/nitrate of blood-fed uninfected and blood-fed *P. vivax* infected *An. culicifaces* species B was determined at 7, 9–10 and 14–15 days pBM using a high performance anion liquid chromatography method. Means were analysed by using a paired *t*-test (α = 0.075); p-values are represented above the bars

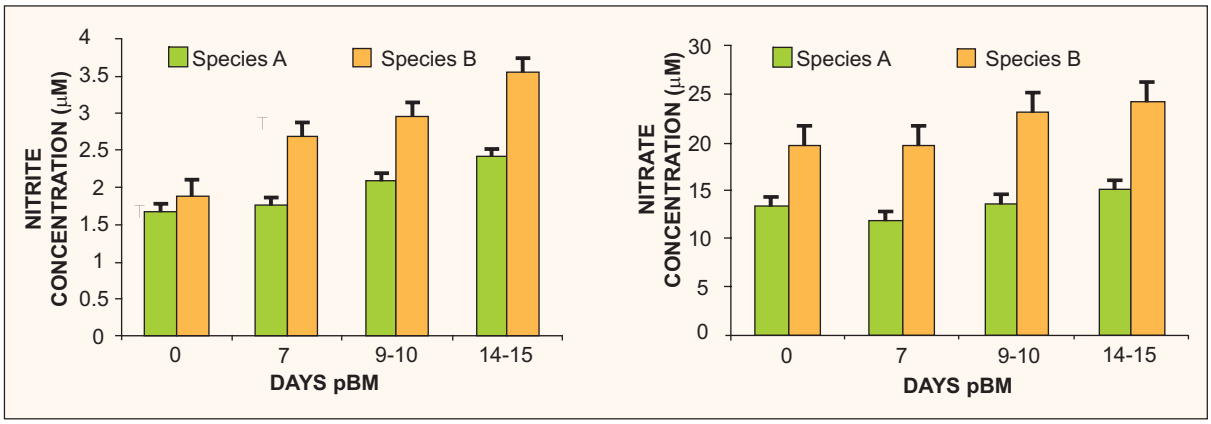

Fig. 1.2.11: Midgut nitrite/nitrate of blood fed *An. culicifaces* species A and species B was determined at 7, 9–10 and 14–15 days pBM using a high performance anion liquid chromatography method. Means were analysed by using a paired *t*-test (α= 0.075); p-values are represented above the bars

The procedure is suitable for the routine determination of nitrite and nitrate. It has proved a sensitive, accurate and reproducible method. The principal strength of this procedure is its simplicity. This anion HPLC method coupled with ultrafiltration to reduce protein and salt contaminants has not been used earlier to measure midgut and haemolymph nitrite and nitrate concentrations in mosquitoes. This method can be used for the detection, identification and quantitative measurement of all nitric oxide metabolites, namely nitrite and nitrate, thus, making it an effective tool for diagnostic purposes and useful for identifying the AcNOS gene products that may impart refractory phenotype that is associated with the immune response to malaria parasites. Such responses may be important for the vectorial capacity of the mosquito and understanding of parasite-vector interactions and mechanism of refractoriness. This procedure may also be suitable for routine determination of NO_2^- and NO_3^- in various other biological fluids/samples.

Different types of surfaces used for evaluation of chlorfenapyr indoor residual spraying

1.3 Vector Control

1.3.1 External Audit of DDT Use in Different States of India

The project was undertaken with a view to find out the rationale of DDT use in different states of India. Field visits were undertaken in 10 districts of five states (Assam, Uttar Pradesh, Rajasthan, Andhra Pradesh and Orissa) for evaluation of proper use of DDT as per norms and deficiencies, if any, for remedial measures. The advance plan, dosage, requirement of insecticide, receipt of insecticide, spray quality, coverage and acceptability of DDT were assessed through questionnaires, cone bioassays and insecticide susceptibility tests.

The vector species was found susceptible to DDT in Assam and Uttar Pradesh and to some extent in Rajasthan, while it was resistant to DDT in Andhra Pradesh and Orissa. The coverage of houses and rooms ranged from 12.5 to 80%. But the results of cone bioassay (to assess the quality and impact of spray on walls) were unsatisfactory as the mortality of vector species ranged from 5 to 50% only. However, in

Uttar Pradesh the results were found satisfactory (74–90% mortality after 27 days). It shows that DDT spray did not provide required residual impact except in two districts of Uttar Pradesh. It was found that strict supervision, data management and training of spray workers is still required for effective IRS. Pilferage of DDT was negligible. Suggestions for remedial measures have been given to the National Vector Borne Disease Control Programme.

1.3.2 Evaluation (Phase-I) of Chlorfenapyr (Pyrrole Insecticide) against Susceptible and Resistant Strains of Mosquito Species

Chlorfenapyr, a pyrrole group insecticide, is a pro-insecticide which acts by inhibiting the reaction of conversion of mitochondrial ADP to ATP. Chlorfenapyr 10% SC was sprayed on different substrates, namely mud, mud-coated with lime, wood, cement, cement-coated with distemper in doses ranging from 0.125 to 4%. *An. culicifacies*, *An. stephensi* and *Cx. quinquefasciatus* were exposed to the surfaces in the laboratory. Doses up to 1% on all the surfaces have resulted in decrease in efficacy within 2 to 3 weeks and bioassays with these doses were discontinued. Further, studies were carried out with 2, 3 and 4% corresponding to 400, 600, 800 mg/m^2 respectively impregnated surfaces. Cone bioassays were performed on the surfaces by exposing 3-day-old sugar-fed mosquitoes on different species, namely *An. culicifacies, An. stephensi, Cx. quinquefasciatus* for 30 min and percent mortality was recorded after 24 h holding period. Chlorfenapyr was found effective in causing mortality of mosquitoes. However, there was no mortality during the exposure period, but mortalities could be observed within 48 h of holding period. The determined diagnostic concentration of chlorfenapyr for assessing susceptibility in field mosquitoes was 5% with 2 h exposure and 48 h holding. Chlorfenapyr @ 400 mg/m^2 was found to be effective up to 28 weeks against *An. culicifacies*; up to 34 weeks against *An. stephensi* while against *Cx. quinquefasciatus* two surfaces, namely mud+ lime and wood have shown consistent results and with other surfaces variable persistence of effectiveness was observed. No cross-resistance with laboratory selected insecticide-resistant lines was noticed with chlorfenapyr. The result indicated that chlorfenapyr has a residual efficacy of six months and above. Hence, this insecticide can be used as an effective insecticide for residual spraying for vector control and insecticide resistance management which has a novel mode of action.

"Chlorfenapyr can be used as an effective insecticide for indoor residual spray against malaria vectors"

1.3.3 Bioefficacy Studies on Interceptor® Long-lasting Insecticidal Nets Impregnated with Alpha-cypermethrin

A one year laboratory study on the efficacy of Alpha-cypermethrin-treated LNs was undertaken. The study was carried out on cold wash-shade dried and cold wash-sunlight dried nets. The bioavailability was assesed by conducting WHO cone bioassays and ring net bioassays. In bioassays, 93% mortality on unwashed and 100% on washed nets up to 30 washes was registered. Slight variations observed in the mortalities were not significant for the over all bioefficacy of the net. However, with the sunlight dried nets, the effect on *Cx. quinquefasciatus* was relatively less as compared to *An. stephensi*. Tunnel tests for bioefficacy on washed nets are in progress.

1.3.4 Evaluation of ZeroFly®, an Insecticide Incorporated Plastic Sheeting against Mosquitoes with Particular Reference to Malaria Vectors

This study was initiated in the month of August 2006 in labour camps in Delhi and Noida. In both the localities Zerofly plastic sheets, incorporated with deltamethrin @ 265 mg

Table 1.3.1. Malaria incidence (active case detection) in Zerofly and control plastic sheets used in labour camps in Noida

Period	Labour Camps	BSE	Total (+)ve	*Pf*	SPR	SFR	PI
July 2006	E (Zerofly)	9	3	0	33.3	0	11.1
(Pre-intervention)	C (Untreated)	8	1	0	12.5	0	3.8
August 06 to July 07	E (Zerofly)	86	1	0	1.1	0	3.7
(Post-intervention)	C (Untreated)	112	12	7	16.9	6.2	73.0

a.i./m^2, were fixed at a distance of at least one km from the control localities where plastic sheets without insecticide (untreated) were fixed. In addition to two localities, the study was also carried out in RAC Police Camp in Delhi, where the company provided plastic sheets during the month of December 2006. Bioassay tests on Zerofly sheets with 3-minute exposure period resulted in 100% mortalities in *An. culicifacies* and *An. stephensi.* The effect of Zerofly sheets persisted at 100% mortality level against *An. culicifacies* even after one year of use under field conditions. Fortnightly monitoring of entomological parameters showed almost complete reduction in the indoor resting density of vector and non-vector mosquitoes in the labour camps provided with Zerofly plastic sheetings as compared with the camp provided with untreated plastic sheetings. In labour camp at a construction site in Noida, man hour density (MHD) of malaria vectors *An. culicifacies* and *An. stephensi* during post-intervention period was in the range of 0–5 in the experimental area as compared to 0–12 and 0–15.5 in the control area (Fig. 1.3.1) respectively.

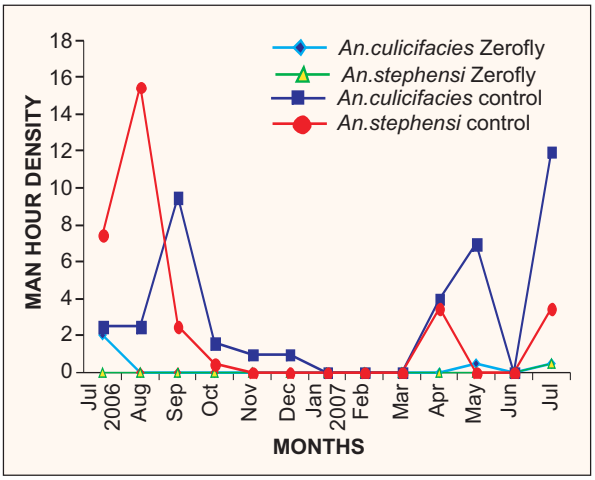

Fig. 1.3.1: Impact of Zerofly sheeting on indoor resting density of malaria vectors in labour camp at Noida

Similarly, in JJ cluster of agricultural labour in the Jamuna belt area in Delhi, MHD of malaria vectors *An. culicifacies* and *An. stephensi* ranged between 0 and 5 in the experimental area as compared to 0 and 13, and 0 and 15 in the control areas respectively. Parasite incidence (PI, cases per thousand population) at the construction site labour camp in Noida was 3.7 in the experimental area as compared to 73 in the control area (Table 1.3.1). Similarly, in the JJ cluster in Delhi the parasite incidence in the experimental and control areas was 42 and 62.7, respectively. In RAC Police Camp, intervention with Zerofly sheeting revealed reduction of culicine density in the experimental tents as compared to the control tents. Survey about the perception of the users about side-effects and benefits revealed a highly positive response in favour of the benefits of Zerofly sheetings and no adverse events were reported by the users.

1.3.5 Follow-up Study on the Long-lasting Efficacy of Olyset® Net against Malaria Vectors and Incidence of Malaria in a Village of District Gautam Budh Nagar, Uttar Pradesh

Follow up study on the long-lasting efficacy of Olyset nets was continued in the three villages, viz. Khandera (Olyset net), Beel (untreated net) and Anandpur (without net) in

Distt. Gautam Budh Nagar, U.P., beyond the initial trial period of one year. The cone bioassays with *An. culicifacies* carried out on Olyset net collected randomly from field after three years of use and with different number of washes revealed no significant difference in the efficacy of nets after different number of washes. However, the median knockdown time on fresh nets was 6.8 min as compared to 12-13.5 min on nets with different number of washes. This indicate that washing of net did not reduce the effect of Olyset net, but the overall knockdown time was increased after three years of use. Fortnightly monitoring of the man hour density (MHD) of mosquitoes and surveillance of malaria incidence was carried out. Data of MHD and malaria incidence of each fortnight in the three villages were pooled and recorded month wise. Results revealed a marked difference in the indoor resting density of mosquitoes, particularly the major malaria vector, *An. culicifacies* in the Olyset net village, when compared with untreated net and without net villages. Average MHD of this species in human dwellings with Olyset nets was 34.3 as compared to 37.8 and 33.7 in untreated net and without net villages during 2003–04 in the pre-intervention year. The average MHD of *An. culicifacies* in the Olyset net village declined from 10.3 to 8.3 during 2005–06 and 2006–07 but during 2007–08 the average MHD was 20 per man hour. The average MHD during 2004–05, 2005–06 and 2006–07 in untreated net village was 22.6, 21.1 and 26.5, respectively, whereas in the without net village it was 39.6, 24.6 and 27.7, respectively. The data clearly indicate the impact of Olyset nets in reducing the density of *An. culicifacies* during the first two years of post-intervention period, while the difference in the MHD during the third year was much less.

Epidemiological results from the three villages revealed considerable difference in the prevalence of malaria. Parasite incidence (cases per thousand population) in village with Olyset nets during 2003–04 (pre intervention year) was 39.5, which declined to 1.5, 0 and 2.5 during 2004–05, 2005–06 and 2006–07 respectively in the post-intervention years. The PI in the untreated net village during 2003–04 (pre-intervention) was 44 as compared to 6.1, 3.8 and 8.8 during 2004–05, 2005–06 and 2006–07 respectively. Whereas in the without net village, the PI during 2003–04 (pre-intervention) was 19 and during intervention years 2004–05, 2005–06, 2006–07 was 19.5, 11.5 and 10 respectively. Similar trend in *Pf* per thousand population in the three villages was also observed. Results indicate long-lasting impact of Olyset nets. Results also revealed in reduction of mosquito nuisance in the Olyset-net village. Follow up observation on the effect of Olyset net are still in progress.

"Usage of Olyset nets reduced the parasite incidence from 39.5 in pre-intervention to <2.5 in post-intervention"

1.3.6 Phase III Evaluation of PermaNet® 2.0 against Malaria Vectors and Disease Transmission

Permanet 2.0, a deltamethrin-treated LN have already undergone Phase II entomological evaluations to demonstrate bio-efficacy and wash-resistance in field against malaria vectors in three different areas in India. The present study (Phase III) field evaluation of Permanet 2.0 against malaria vectors and disease prevalence was initiated in the endemic areas of District Gautam Budh Nagar, in western Uttar Pradesh in April 2007 and in tribal areas of Orissa in August 2007, following uniform protocol of NIMR (ICMR).

Three villages with similar malaria endemicity, topography and mosquito prevalence in District Gautam Budh Nagar in Uttar Pradesh, where malaria is transmitted mainly by *An. culicifacies* and *An. stephensi* and three clusters of small villages in tribal areas in Sundargarh district, Orissa, where *An. fluviatilis* and *An.*

culicifacies, the major vectors of malaria have been selected for the Phase III evaluation of Permanet. Entomological and epidemiological parameters as per uniform protocol were monitored following standard procedures.

Results revealed that the MHD of *An. culicifacies* during pre-intervention period in Permanet, untreated net and without net villages ranged from 10–13, 24–27 and 8–12, respectively. With the commencement of intervention, there was sharp decline in density of *An. culicifacies* in June 2007, whereas the density in the untreated and without net control villages did not decline in June 2007. However, there was an increase in the resting density of *An. culicifacies* in all the villages during the monsoon and post-monsoon period of August to November 2007, but the build-up of *An. culicifacies* density was much higher in the control villages as compared to Permanet village. The parity rate of *An. culicifacies* was low in Permanet village as compared to untreated net and without net villages. The parity rate of *An. culicifacies* in June 2007 in the first month during post-intervention period in Permanet, untreated net and without net villages was 20, 66.6 and 60% respectively.

"Malaria incidence (cases/1000) in Permanet used village was (0.84) much less than the untreated net (5.19) and without net (13.46) villages"

Comparison of malaria incidence data showed that during pre-intervention period of April–May 2007, the PI in the Permanet villages was 2.5 and in the villages with untreated nets and without nets was 1.7 and 2.9 respectively. There was no significant difference ($p > 0.05$) in the malaria endemicity in the Permanet and control villages. During intervention phase, the malaria incidence in the Permanet net, untreated net and without net villages was 0.84, 5.19 and 13.46, respectively.

The compliance rate of the net usage in the Permanet and control villages was ascertained through random checking of houses and recording of people sleeping under mosquito nets. There was 85–99% compliance rate of net usage in the study population during different months. The community perceptions on adverse effects and collateral benefits of Permanet usage was assessed by conducting cross-sectional survey among the users (n = 394, M–220, F–194). Almost every respondent asserted that they are sleeping under the treated nets. There were minimal complaints of skin irritation (0.5%) and eye irritation (0.25%). However, these effects were only temporary, lasting for few hours of the first usage. Majority of the respondents enthusiastically reported that Permanets provided them relief not only from mosquitoes but also from other household pests such as headlice, bed-bugs, cockroaches, ants and houseflies.

1.3.7 Field Evaluation of Bacticide DT (Dispersible Tablets), a Formulation of *Bacillus thuringiensis* var. *israelensis* H-14, Strain 164 against Larvae of Mosquito Vectors

A multicentric trial of Bacticide DT formulation was carried out in urban areas to see persistence in domestic and peridomestic breeding containers, such as desert coolers, containers, water tanks which are potential mosquito breeding habitats of *Ae. aegypti* and *An. stephensi* and also against *Cx. quinquefasciatus*. The study was carried out in urban/periurban

Application of bacticide

areas of Raipur (Chhattisgarh), Hardwar (Uttarakhand) and Sonepat (Haryana) areas. The methodology was based on the common protocol developed by NIMR for evaluation of biolarvicide. The Bacticide DT (400 mg) was evaluated in natural breeding habitats of *Anopheles*, *Culex* and *Aedes* species. Application

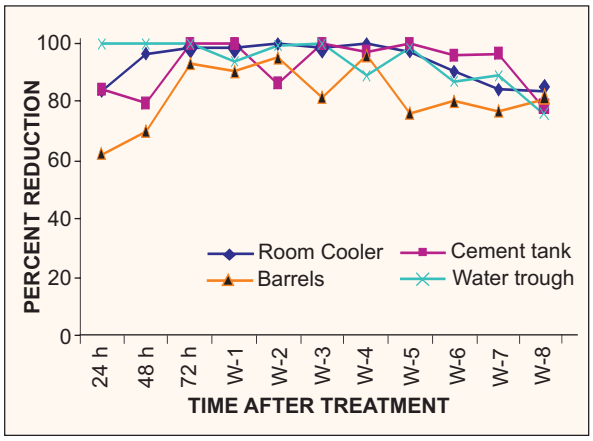

Fig. 1.3.2: Effect of Bacticide DT 400 mg on late instars of *Ae. aegypti* in different breeding habitats in Raipur

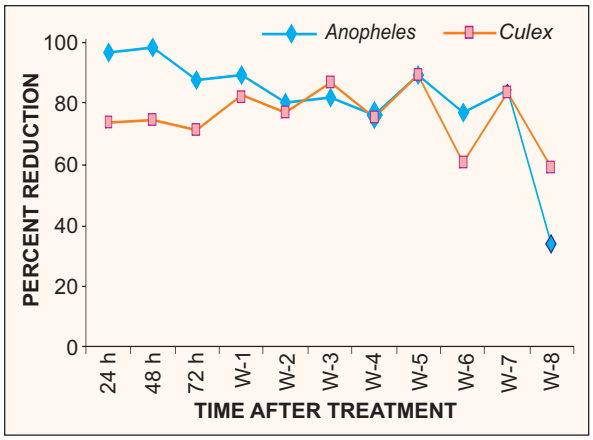

Fig. 1.3.3: Effect of Bacticide DT 400 mg on late instars of *Ae. stephensi* and *Cx. quinquefasciatus* in different breeding habitats in Sonepat

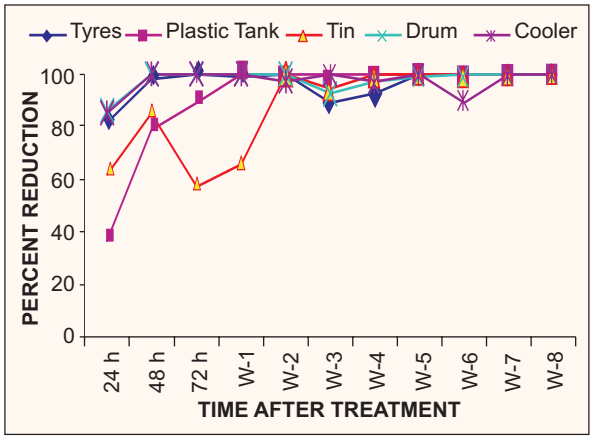

Fig. 1.3.4: Effect of Bacticide DT 400 mg on late instars of *Ae. aegypti* in different breeding habitats in Hardwar

of one dispersible tablet (400 mg)/m^2 of water surface or one tablet per container with <50 litre of water produced >80% reduction of late instars up to two weeks . In container habitats of *Anopheles* and *Aedes*, where water was >20 litre, the effect was up to four weeks (>80% reduction) (Figs. 1.3.2 to 1.3.4). Bacticide DT formulation was more effective against *Aedes* compared to *Anopheles* and *Culex* species.

1.3.8 Field Evaluation (Phase III) of Bacticide WP, a Formulation of *Bacillus thuringiensis* var. *israelensis* H-14, Strain 164 against Larvae of Mosquito Vectors

This study was carried out to evaluate the effectiveness of Bacticide WP formulation for control *of An. culicifacies, An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus* in a locality as a multicentric trial (Table 1.3.2). Bacticide WP was evaluated at the dose of 200 mg/m^2 in

Table 1.3.2. Habitats and target species selected for the trial at various sites

Site	Trial habitats	Target species
Raipur	Cement tanks	*An. stephensi, An. subpictus* and *Cx. quinquefasciatus*
	Coolers	*Ae. aegypti, An. stephensi*
	Drains	*Cx. quinquefasciatus,* Anopheline species
	Pools	*An. subpictus, Cx. quinquefasciatus*
Sonepat	Cement tanks	*An. stephensi*
	Pools	*An. subpictus*
	Tanks and drains	*Cx. quinquefasciatus*
Mathura	Pits, Pools	*An. culicifacies*
	Drains	*Cx. quinquefasciatus,* Anopheline species

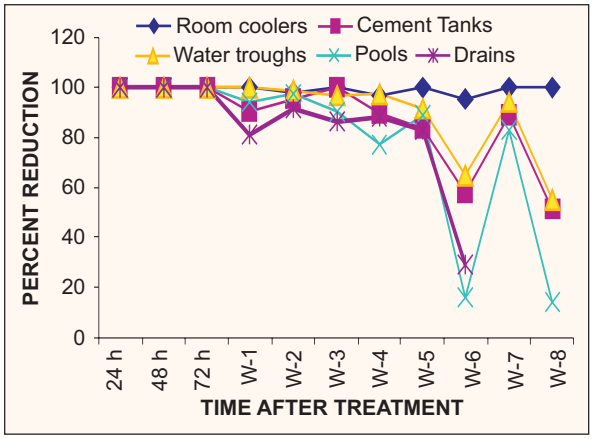

Fig. 1.3.5: Effect of Bacticide WP @ 200 mg/m² on late instars of anophelines in Raipur

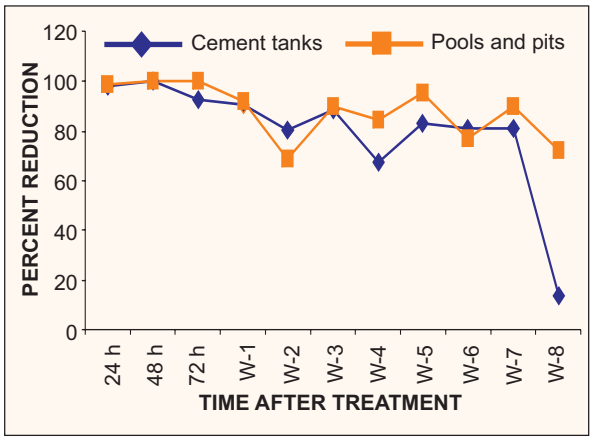

Fig. 1.3.6: Effect of Bacticide WP @ 200 mg/m² on late instars of anophelines in Sonepat

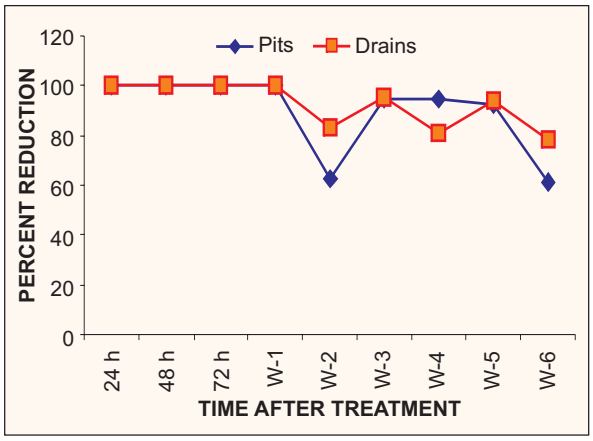

Fig. 1.3.7: Effect of Bacticide WP @ 200 mg/m² on late instars of anophelines in Mathura

natural breeding habitats against *An. stephensi*, *An. subpictus*, *Cx. quinquefasciatus* at Raipur and Sonepat. It was also evaluated against *Ae. aegypti* in Raipur. In Mathura, it was evaluated against *An. culicifacies* and also against *Cx. quinquefasciatus*.

Results showed within a week maximum of 100 percent reduction of late instar larvae of target species *An. stephensi* in coolers, cemented tanks, *An. subpictus* in pits and pools, cemented tanks, clean water drains and *Ae. aegypti* in coolers during post-treatment period. Bacticide WP was effective (>80% reduction) in clean water small habitats for two weeks. (Figs. 1.3.5 to 1.3.7). Against *Cx. quinquefasciatus* in surface drains with organic matter, the reduction was >80% for seven days and in small containers such as coolers, tanks the effect was (>80%) for two weeks. In general, the formulation was more effective against *Aedes* compared to *Anopheles* and *Culex*.

1.3.9 Multicentric Study on Efficacy Trial with Enhanced Dose of Fenthion 82.5% E.C. against Mosquitoes in Polluted Water

At present fenthion is used in polluted water under NVBDCP at a dose of 100 ml/ha. However, a recent study has shown ineffectiveness of fenthion @100 ml/ha against mosquitoes, which breed in polluted water. Since currently used dose of 100 ml/ha of Fenthion (E.C. 82.5%) per week was found to be ineffective to control larval breeding of *Cx. quinquefasciatus* in polluted water habitats, on the recommendation of NVBDCP expert group meeting held on 6 June 2007, a multicentric study was initiated by NIMR during August 2007–March 2008 to evaluate the efficacy of fenthion against *Culex* larvae using enhanced doses of 150 ml and 200 ml/ha in Delhi, Chennai and Raipur.

In Delhi, four different localities representing South, East, North and West Delhi areas having cesspools, which support *Cx. quinquefasciatus* breeding were selected. Fenthion has been in use in Delhi for the last 30 years and an additional area included in the study is from Noida having stagnant polluted drains with prolific breeding of *Culex* mosquitoes. In this area fenthion was never used in control programme.

Weekly application of enhanced dose (150 ml/ha) of fenthion for three weeks in cesspools in different areas of Delhi showed considerable variations in its efficacy. In South Delhi, >80% reduction in the density of III+IV instar larvae was observed on Day 16, i.e. after three applications. In East Delhi, >80% reduction was observed immediately after the spray on three occasions. In North Delhi, the reduction always remained below 71%. In West Delhi, >80% reduction was observed on Day 9 onwards.

Similar weekly application of fenthion (200 ml/ha) for three weeks in cesspools in South Delhi registered >80% reduction immediately after the spray on three occasions, but it declined to 43% on Day 7 and 64% on Day 14, however, after third application >80% reduction was observed up to Day 21. In North Delhi also, >80% reduction was observed immediately after the spray on three occasions, but the percentage reduction declined to 79% on Day 7, 72% on Day 14 and 61.5% on Day 21. In East and West Delhi areas, however, >80% reduction was observed up to three weeks.

In Noida, fenthion treatment at 150 ml/ha caused 73.8 to 90.7% reduction from Day 1 to Day 3, but it declined to 67% on Day 7. Further applications registered >80% reduction throughout next two weeks. Higher dose of 200 ml/ha was effective for the entire period of three weeks as the percentage reduction ranged from 88 to 97.

In Chennai, fenthion has been in use for the last 30 years, the selected areas for the fenthion trial included cesspools, stagnant polluted drains and moderately polluted unused wells from different parts of the city. Single application of 150 ml/ha of fenthion in drains caused <80% reduction up to one week, however, 200 ml/ha caused 71% reduction on Day 1, which increased to 89% on Day 3 but again it declined to 17% on Day 7. In cesspools, 150 ml/ha of fenthion produced 93–95% reduction on Day 2 and 3, but it reduced to 63% on Day 7. Similar treatment of cesspools with 200 ml/ha caused >80% reduction for the first three days then it declined to 73% on Day 7. Both the doses of fenthion, however, registered >80% control of *Culex* breeding for a period of one week in moderately polluted unused wells.

In Raipur, where fenthion was never used in urban malaria control, single spray of 150 ml/ha of fenthion in polluted drains registered >90% reduction up to 14 days. In another area having highly polluted drains, application of 200 ml/ha caused 98–100% reduction for one week. In pools, 150 ml/ha caused >90% reduction up to Day 3 and it declined to 79% on Day 7. However, at 200 ml/ha treatment in pools, >93% reduction was observed for a period of one week and >80% reduction up to three weeks. Application of both the doses of fenthion in moderately polluted cemented tanks, which supported *Anopheles* breeding, produced 100% mortality.

1.3.10 Multicentric Study on Evaluation of Mosquito Larvicide Temephos for Use in Polluted Water

Mosquito larvicides, viz. temephos and fenthion are used in vector control programme against mosquito vectors under urban malaria scheme in India. At present temephos is used in clean and potable water under NVBDCP due to its very low mammalian toxicity. National Programme Technical Advisory Committee of NVBDCP recommended a study on the efficacy of temephos in polluted water. In view of this recommendation, a multicentric study was initiated by NIMR to determine the effective doses of temephos for mosquito larval control in polluted water. The study was carried out at three urban field sites, namely Delhi, Raipur and Ranchi.

In Delhi, a treatment of temephos in highly polluted cesspools in Rohini showed only a dose of 200 g/ha could cause >80% reduction

in *Cx. quinquefasciatus* larval density for a period of one week. In Gautam Budh Nagar, 25 g/ha of temephos was effective in reducing larval density >80% for one week in clean water. With polluted cesspools 100 g/ha was effective for seven days, while 200 g/ha produced >80% reduction up to 14 days. However, treatment of polluted ain with 100 and 200 g/ha caused effective control for seven days.

In Raipur, breeding in choked drains was reduced to >80% with a dose of 50 and 100 g/ha and with 200 g/ha similar effect was for two weeks. In Ranchi, treatment of 100 and 200 g/ha produced >80% reduction for one week. pH of water in habitats of Delhi, Raipur, and Ranchi varied between 7.0 and 8.0. This range of pH did not influence the toxicity of temephos. Overall results from the multicentric trial indicate 200 g/ha of temephos 50% EC is effective for larval control for seven days in highly polluted water, while in moderate polluted water, the effect extended up to 14 days in Delhi and Raipur but not in Ranchi. Thus, 4 to 8 fold increase in temephos dosage in clean water by the national programme is required to effectively control *Cx. quinquefasciatus* breeding in polluted water.

Launching of the experiment on efficacy of attracticide in Delhi

1.4 Vector Surveillance

1.4.1 Application of Attracticide (Oviposition Pheromone in Combination with Insect Growth Regulator) for Surveillance and Control of Dengue and Chikungunya Mosquitoes

The experiment was initiated in Delhi in October 2007 and in Bengaluru in December 2007. In Delhi, about 6500 ovitraps were placed in five localities, viz. Mayur Kunj (Trilok Puri), Valmiki Colony (Panchkuian Road), Netaji Nagar, R.K. Puram and Railway Colony (Tughlakabad). In Bengaluru, about 6000 ovitraps were placed in three localities, viz. Ashok Nagar, Kanteerava Nagar and Sanjay Gandhi Nagar + Narayanpura. The experimental ovitraps contained 395 ml water treated with 5 mg C-21, IGR and solvent. Untreated ovitraps contained 400 ml water with solvent only. The experiments will also to be carried out in Kerala where baseline data hav been collected for selection of study sites.

Before starting the experiment, a meeting with community was organized at Delhi and Bengaluru to make them aware about this experiment taking place. Experiment at Delhi in R.K. Puram location was inaugurated by Mr. Deepak Gupta, IAS, Special Secretary, Ministry of Health & Family Welfare. Training to the newly appointed supervisors and surveillance workers was provided to carry out the experiment and to check the mosquito breeding. A surveillance worker was asked to check ovitraps and record the breeding in about 50

houses in a day. Thus, a surveillance worker covered about 250 houses in a week. The supervisors monitored the work of the surveillance workers, collected data from the field for processing on computers and provided IEC to the community regarding the experiment being carried out. The study is in progress.

1.5 Insecticide Resistance

1.5.1 Molecular Basis of kdr Resistance in *An. culicifacies* and Development of PCR-based Methods for kdr Genotyping

An. culicifacies, the most important malaria vector in India, is resistant to DDT and is developing resistance to pyrethroids—only alternative so far available for the impregnation of bednet. The presence of *kdr*-based resistance in vector is a threat to the success of the pyrethroid-impregnated bednet programme. The NIMR established the presence of *kdr* mutation in the field population of *An. culicifacies*, and developed PCR-based methods for *kdr* genotyping in field populations.

To identify the presence of *kdr*-based mechanism of knockdown resistance in *An. culicifacies*, we sequenced 1.4 kb span encompassing S4–S6 region of domain II of para type voltage gated sodium channel which revealed a single point mutation Leu-Phe at *kdr* locus in few specimens of DDT and pyrethoid-resistant *An. culicifacies*. We developed and tested three molecular methods for *kdr* genotyping, viz. Allele Specific PCR (ASPCR), amplification refractory mutation system (ARMS) and primer introduced restriction assay (PIRA) (Figs. 1.5.1–1.5.3). The results were validated following DNA sequencing of samples.

"Three high throughput molecular assays were developed for kdr genotyping in Anopheles culicifacies"

The presence of Leu-Phe mutation at *kdr* locus was demonstrated in some DDT and pyrethroid resistant *An. culicifacies* from Surat district of Gujarat, India. We didn't find Leu-Ser mutation at *kdr* locus, as reported in *An. gambiae*, and Leu-His mutation at position 29 of exon I of VGSC (upstream to the *kdr* locus) as has been reported in Iranian *An. culicifacies*

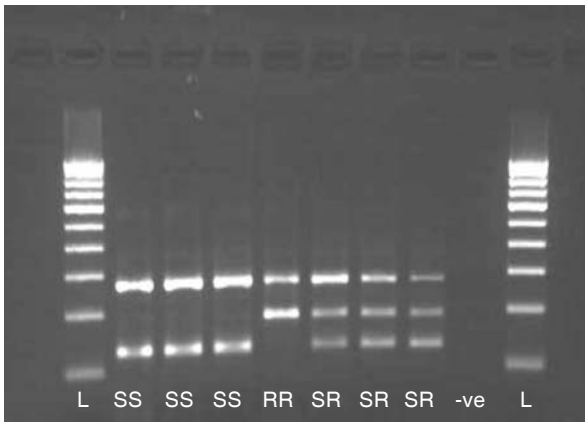

Fig. 1.5.1: Allele-specific PCR (ASPCR) assay for *kdr* genotyping

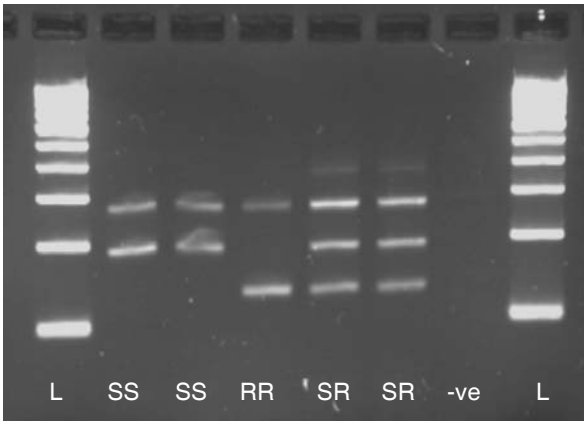

Fig. 1.5.2: Amplification refractory mutation system (ARMS) assay for *kdr* genotyping

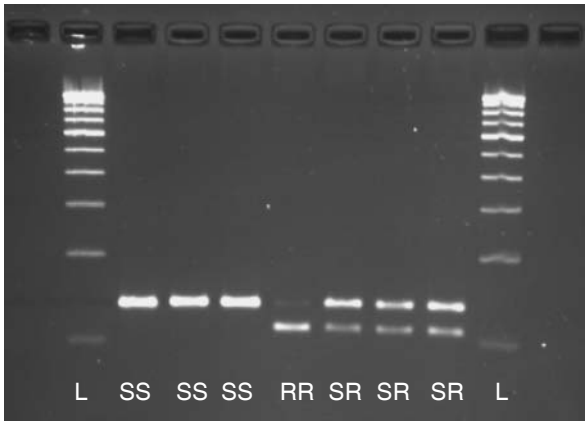

Fig. 1.5.3: Primer induced restriction assay (PIRA) for *kdr* genotyping

s.l. The genotyping of a DDT and pyrethroids-resistant *An. culicifacies* population from Surat, India comprising of species B and C was done using three PCR-based assays which revealed a low frequency of *kdr* allele mostly in heterozygous conditions. The reliability of three PCR-based assays was confirmed following sequencing all the samples genotyped as homozygous and heterozygous resistant, and some randomly selected homozygous susceptible samples. All the three PCR based assays were found to be specific and no descrepancy in result was noticed.

1.6 Evolutionary Genetics

1.6.1 Evolutionary Genetics of *Anopheles gambiae* X-chromosome

Understanding the genetic architecture of individual taxa of medical importance is the first step for designing disease preventive strategies. To understand the genetic details and evolutionary perspective of the model malaria vector, *An. gambiae* and to use the information in other species of local importance, we scanned the published X-chromosome sequence for detailed characterization and obtain evolutionary status of different genes. The telocentric X-chromosome contains 106 genes of known functions and 982 novel genes. We considered the known genes and first classified them based on size (in nucleotide base pairs) (Fig. 1.6.1). Majorities of both the known and novel genes are with introns. The known genes are strictly biased towards less number of introns; about half of the total known genes have only one or two introns (Fig. 1.6.2). The extreme sized (either long or short) genes were found to be most prevalent (58% short and 23% large). Statistically significant positive correlations between gene length and intron length as well as with intron number and intron length were obtained signifying the role of introns in contributing to the overall size of the known genes of X-chromosome in *An. gambiae*. We compared each individual gene of *An. gambiae* with 33 other taxa having whole genome sequence information. In general, the mosquito *Ae. aegypti* was found to be genetically closest and the yeast *Saccharomyces cerevisiae* as most distant taxa to *An. gambiae* (Fig. 1.6.3). Further, only about a quarter of the known genes of X-chromo-

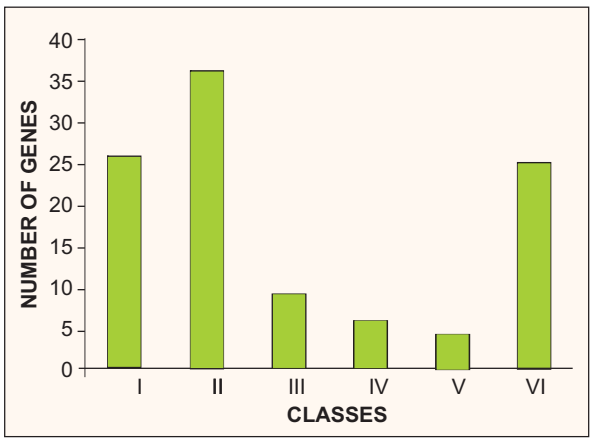

Fig. 1.6.1: Classification of known genes of *An. gambiae* X-chromosome based on size (nucleotide base pair)

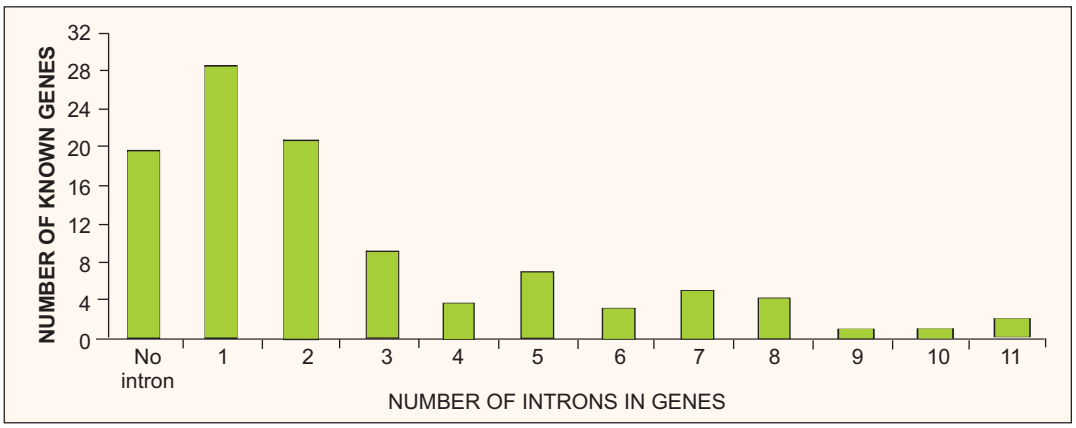

Fig. 1.6.2: Distribution of *An. gambiae* X-chromosome known genes according to the number of introns

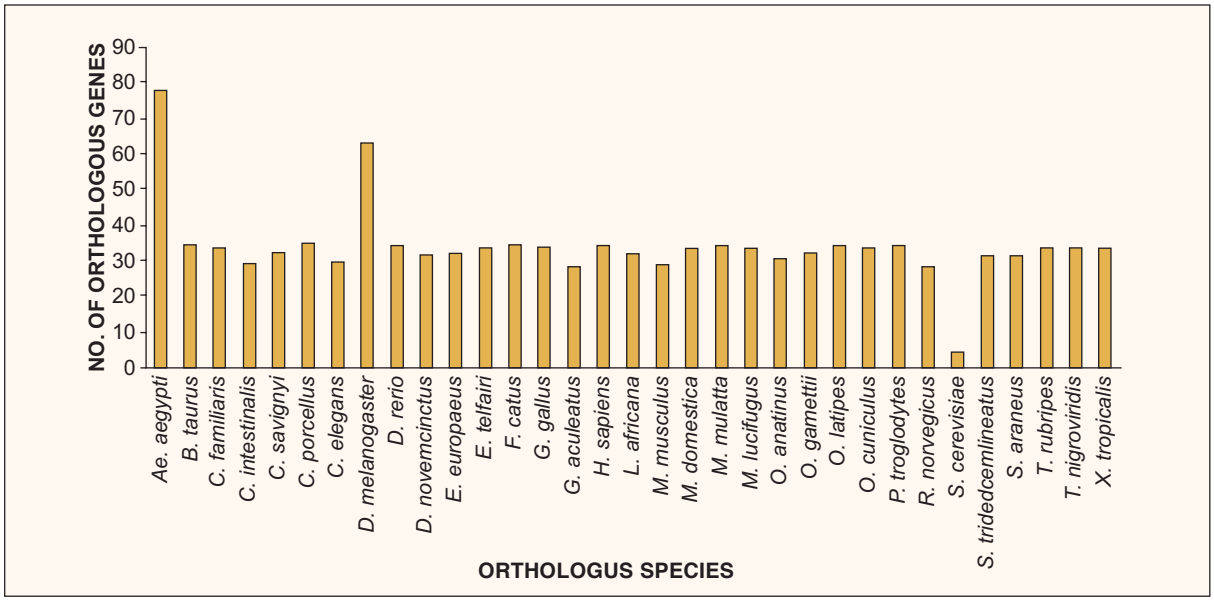

Fig. 1.6.3: Distribution of different taxa showing number of shared genes with *An. gambiae*

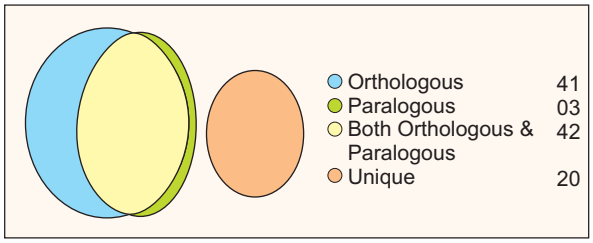

Fig. 1.6.4: Distribution of different gene types (based on homology prediction) in X-chromosome of *An. gambiae*

some were unique to *An. gambiae* and majorities have orthologs in different taxa (Fig. 1.6.4). A phylogenetic tree was constructed based on a single gene found to be highly orthologous across all the 34 taxa. Evolutionary relationships among 13 different taxa were inferred which corroborate the previous and present findings on genetic relation-

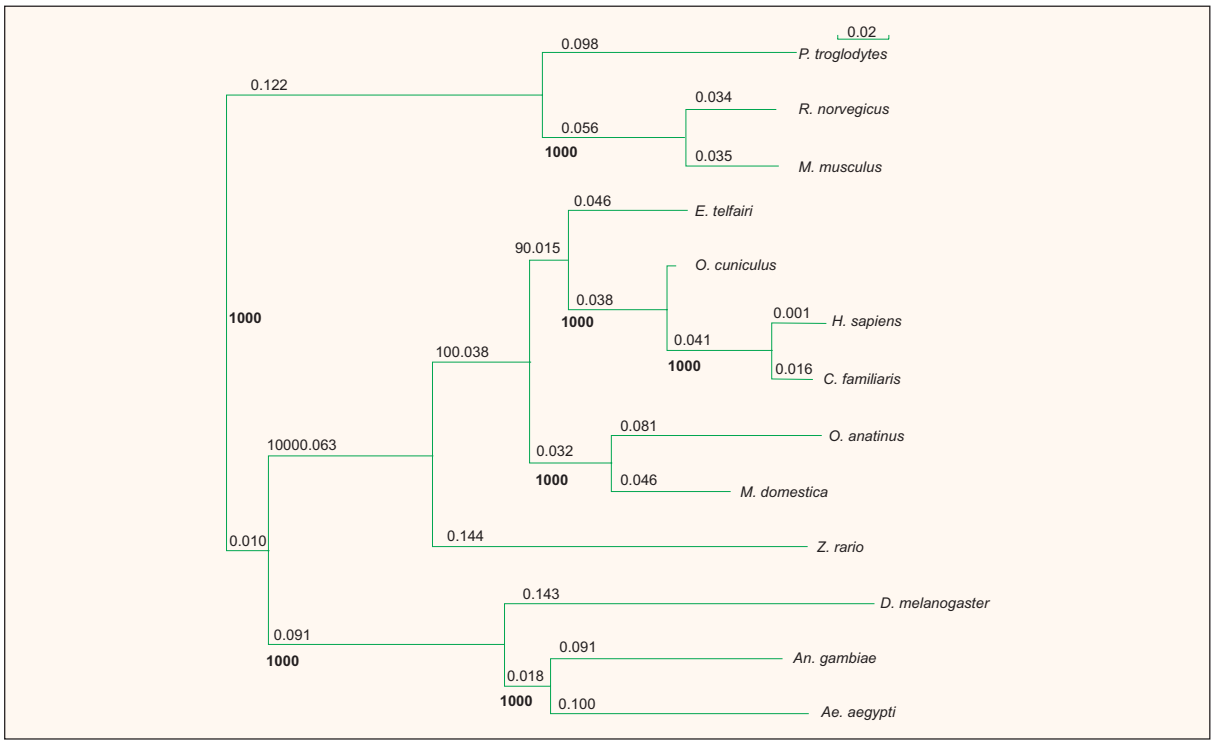

Fig. 1.6.5: Un-rooted neighbour-joining (NJ) phylogenetic tree with bootstrap values (in bold font) in 13 different taxa. The figures (in each horizontal line) indicate the lengths of each branch leading towards taxa

ships across various taxa (Fig. 1.6.5). The study not only provides fine-scale views to the genetic architecture of the X-chromosome of the model malaria vector of African importance, but also reveals several interesting features on evolutionary insights into genes and taxa of different taxonomic status. The information is of great importance, especially to the population geneticists, to understand genetic diversity and infer the respective roles of demography and natural selection in evolution of genes in different *Anopheles* species populations of local importance. ❑

"Comparative genetic studies of An. gambiae X-chromosome genes with other sequenced taxa provide evidence of genetic relatedness"