DISCUSSION
Species complexes are common in anopheline vectors of malaria (Kitzmiller, 1977). An. subpictus, a minor vector for malaria in Indonesia (Reid, 1968), a suspected vector in Lakshadweep islands (Roy et al., 1978) and incriminated as a vector in coastal villages of Pondicherry in India (Panicker et al., 1981) has been reported as a complex consisting of two sibling species based on chromosomal and morphological evidences (Suquna, 1982).

The existence of more than three types of eggs in An. subpictus (Urbino, 1936) as in An. gambiae, which consists of six sibling species belongs to the same series of An. subpictus and natural occurrence of sporozoite positive of An. subpictus both in coastal (Panicker et al., 1981) and in inland (Kulkarni, 1983) of India, necessitated the search for the existence of more sibling species, if any, in An. subpictus.

In addition to the paracentric inversion reported on the polytene X chromosome bySuquna (1982), a second fixed inversion has been identified on the X chromosome. Based on these two inversions An. subpictus has been classified into four sibling species, species A having the standard arrangement, species B with the inverted arrangement and species C and D having one inversion each.
Sibling species can be identified by the presence of fixed paracentric inversions on the X chromosome only or both X chromosome and autosomes or only in autosomes (Kitzmiller, 1977). In An. gambiae there are three types of X chromosomes, species C, D and melas have the standard arrangement, species A and merus have the Xbcd arrangement and species B has Xbcd arrangement (White, 1974). Therefore, the autosomes have to be screened for inversions to distinguish species A, merus, C, D and melas (White, 1974). In An. culicifacies there are two types of X chromosomes (Green and Miles, 1980), species A and D have the standard arrangement ab (X++) and species B and C have the inverted arrangement (X+) and all the four sibling species could be identified by the presence of inversions on the autosomes (Subbarao et al., 1983; Suguna et al., 1989). But in An. subpictus, the four sibling species have specific X chromosome pattern and therefore it is not necessary to screen fixed inversions on the autosomes.

Attempts to colonise four sibling species of An. subpictus have not been successful so far and in the absence of crossing experiments, hybrid chromosomes were screened for. The absence of hybrid chromosomes in the field population suggests that An. subpictus is a complex consisting of four sibling species.

The male mitotic Y chromosome has also become a diagnostic tool to distinguish the sibling species of a complex. The four sibling
species of \textit{An. culicifacies} could be separated on the Y chromosome morphology, either the short arm or the long arm show reduction in the length (Vasantha \textit{et al.}, 1983; Suguna \textit{et al.}, 1989). In \textit{An. subpictus} sex chromosomes are acrocentric (Avirachan \textit{et al.}, 1969), therefore, the reduction in length is observed on the longer arm of the Y chromosome. Thus, \textit{An. subpictus} could be identified by the polytene X chromosome and the male mitotic Y chromosome.

According to the definition of sibling species, a complex differ much more genetically and biologically and the minute morphological variation, if any, has to be supported by chromosomal evidence (Reid, 1968). Morphological characteristics have always been and still continue to be the most extensively used in classical mosquito taxonomy, because this method is the easiest for quicker observation of the external morphological characters that permits the rapid and direct determination of the species (Chauvet and DeJardin, 1968).

In \textit{An. culicifacies} complex, which consists of four sibling species, no morphological variations have been observed so far. Similarly, in \textit{An. farauti} complex no known reliable morphological characters are available (Sweeney, 1987). In other species complexes, morphological variations were based on egg morphology, immature chaetotaxy and adult female palp. But some of the morphological characters are not consistent, for example, the branches of the mesothoracic hair. The number of branches vary on either side or they overlap and can never be used as a diagnostic morphological characters (White, 1977).
In *An. subpictus* complex, three morphological characters are diagnostic namely, egg morphology, 7th hair of the first abdominal segment of the pupae and the length of apical pale band, subapical dark band and the relative length of palp to proboscis.

In many of the anopheline complexes the number of egg types are not equal to the number of the sibling species. In *An. gambiae*, there are three types of eggs for the six members of the complex (Coluzzi, 1964; White, 1973) and two types of eggs in four members of *An. punctulatus* complex (Bryan, 1974). But, like *An. claviger*, where the two members could be differentiated by two types of eggs (Coluzzi, 1962), the four members of *An. subpictus* complex could be identified by four distinct types of eggs. The differentiation is based on the number of egg ridges on the egg float. Earlier, Christophers (1933) has reported 18-40 egg ridges followed by Suguna (1982) 15-43 and Subbarao et al. (1988a) 21-30. In the present study, it ranged from 16-36. The lowest average number was found in species B (18) followed by D (22), C (26) and A (33). The number of females having the average number of egg ridges were highest for each species and unlike *An. stephensi* the intermediate forms were almost absent (Sweet and Rao, 1937; Subbarao et al., 1987).

In pupal chaetotaxy, the 7th hair of the first abdominal segment was found to be diagnostic and consistent character to identify the four sibling species of *An. subpictus* complex. This is the first time
the 7th hair has been used for this purpose. Identification of the sibling species is more important at the pupal stage. Newly emerged males are used for the preparation of male mitotic chromosomes. But the adult males of the four sibling species of *An. subpictus* are difficult to distinguish. Therefore, the 7th hair of the first abdominal segment becomes more useful and diagnostic. It also helps to distinguish *An. vagus* and *An. culicifacies* which are found to breed along with *An. subpictus*.

Instead of using palpal ratio, the palpal length in relation to proboscis length was found to be much more useful. In *An. subpictus*, the apical pale band is longer than the subapical dark band in species A whereas it is short in species B. In species C and D, both bands are almost equal but the palps were shorter than the proboscis in species C, whereas they are equal in species D.

A comparison of these three characters with the three closely related species, *An. vagus*, *An. indefinitus* and *An. sundaicus* has shown that none of the four sibling species of *An. subpictus* resemble the above species, but like *sundaicus*, the palp may be identical, but could be differentiated by the presence or absence of spots on the legs.

*An. subpictus* breeds both in fresh water and salt water, (Christophers, 1933; Reid, 1968; Rao, 1981). After the
identification of two sibling species in *An. subpictus* (Suguna, 1982), it was observed that species A breeds in fresh water and species B in salt water. Reuben et al. (1984) reported that when the larvae of species A and species B of *An. subpictus* were exposed to 75% sea water, all the fresh water larvae (species A) died whereas, species B survived. But this physiological method of identification is not possible because two more fresh water form species, C and D have been identified in the present study.

Fresh water and salt water species have been identified in *An. gambiae* complex (Coluzzi and Sabatini, 1968, 1969; Davidson and Hunt, 1973). It consists of two salt water and four fresh water forms. Similarly in *An. farauti*, there are two fresh water forms and one salt water form (Bryan, 1973; Mahon and Miethke, 1982). In both these species salt water forms can be distinguished from fresh water form when they are exposed to a dilution of sea water.

*An. subpictus* breeds in all kinds of habitats (Christothers, 1933; Rao, 1981) and even in tree holes (Srivastava, 1989). But in Pondicherry, the study area, it mainly breeds in paddy fields, riverine pools and in back waters. Salinity estimation of the breeding waters showed less salinity in paddy fields. Riverine pools showed a maximum of 0.78% salinity which is almost equalent to 25% sea water. Eventhough it is considered as a fresh water habitats as observed in this study the high salinity in riverine pools is due to
their location near the coast. In brackish water, salinity estimation in the present study showed the highest salinity of 5.3% which is above the salt content of sea water (3.2%) as reported by Reuben et al. (1984).

It was also observed that the four sibling species of An. subpictus showed variations in salinity tolerance in the natural habitats. Species C could not survive beyond 0.6% and species A 0.8%, species D 2.3% and species B 5.3%. This confirms that species A and C are fresh water forms, species B only salt water form and species D can survive both in fresh water and salt water.

It was observed that the two fresh water forms species A and D had their peak in the rainy season (July-October), species C in the cool season (November-December) and species B in the hot dry season (May-June). Whether the same seasonal pattern continues every year has to be studied so that a possible correlation could be suggested to the season, vector prevalence and transmission of malaria.

Biochemical approaches in taxonomy is very recent. Various biochemical keys are given for the identification of species (Cianchi et al., 1985) and the number of enzymes used for these identification is also quite high (Steiner and Joslyn, 1979). In An. gambiae complex each sibling species show a specific pattern to particular enzyme (Miles, 1978). But in some complexes like An. farauti, all the three species could be identified by biochemical means (Sweeney, 1987).
The present study suggested that in An. subpictus complex, the fresh water forms (A, C and D) could be separated from salt water form (B) but an enzyme which could distinguish all the four sibling species is yet to be found.

It was the difference in response to insecticide treatment which suggested that An. gambiae is not a single species but a complex (Davidson, 1964). Much work has not been done with Fenthion, an organophosphate larvicide in An. subpictus. An earlier study has shown that An. subpictus has developed resistance to Fenthion in Pakistan (Rathor et al., 1980). This is the first time that Fenthion susceptibility tests have been carried out with the four sibling species of An. subpictus. All the four species have developed resistance to Fenthion, lowest in species C and highest in species A and the overall resistance level has shown high resistance (71%) in the study area.

During the course of this study it was also found that two doses can be used for each species which can discriminate the heterozygotes from the homozygotes. In the low dose, all susceptible will die but the heterozygotes will survive and in the next dose all the heterozygotes will die and the resistant homozygotes will survive. These two doses also showed variation among the species.
Not only with insecticides, variation in the infection rate to a nematode pathogen, *R. iyengari* was also observed with the four species of *An. subpictus*. It has been reported that the parasitic nematode breeds in paddy fields and *An. subpictus* is one among the mosquitoes that become infected with this parasite. The present study supports that only the fresh water species (A, C and D) of *An. subpictus* complex get the infection and species A having a higher percentage than species C and D. Species B was completely free from this infection, which suggests that the nematode does not survive in salt water.

Eventhough *An. subpictus* has not been considered as an important vector in India, the presence of naturally occurring sporozoite positive in coastal villages of Pondicherry (Panicker et al., 1981) and inland areas of central India (Kulkarni, 1983) poses a problem. Now with the present cytological and morphological characters proposed in this study, the accurate identification of the vector species is possible which requires a further detailed study of the role of the vector sibling species of *An. subpictus* in disease transmission.