Scanning Electron Microscopy and Molecular Characterization of *Aedes albopictus* (Skuse)

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Abstract Scanning electron microscopy and molecular characterization of *Aedes albopictus* larvae were done. Analysis of 28S rRNA gene sequence of *Aedes albopictus* Burdwan-SNC1 (KC208593) revealed comparatively higher GC content. Phylogenetic tree was prepared using the Neighbor-Joining (NJ) method. Phylogenetic tree revealed that *Aedes albopictus* Burdwan-SNC1 (KC208593) branched with the cluster containing of *Aedes palauensis* A26, *Aedes hensilli* A25, *Aedes riversi* A51A, *Aedes dybasi* A28, *Aedes galloisi* A77, *Aedes flavopictus* A47, *Aedes albopictus* A22, *Aedes albopictus* A34, *Aedes albopictus* MQSRNAGN and also branched with the cluster containing *Aedes aegypti* A20 and *Aedes wadai* A85. Scanning electron micrographs of *Aedes albopictus* larvae revealed that the structural complexity and number of both comb scales and pecten spines increased with larval growth and development. Similar results were revealed by anal papillae of abdominal segments.

Keywords *Aedes albopictus* ·

Scanning electron microscopy · 28S rRNA sequence ·
Phylogenetic tree

Introduction

Majority of the mosquitoes are hematophagus in nature, which allows them to spread several pathogenic diseases. Among them one of the major public health concerns over the years is Dengue fever. Along with it, complications like Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) have also posed tremendous threats. Dengue fever is a public health problem of global importance, producing a spectrum of disease spanning febrile arthralgia to hemorrhagic death. The burden of the disease is shocking worldwide. Dengue is an emerging infectious disease that is estimated to affect 50–100 million individuals each year in tropical and subtropical areas [1]. Cases across the Americas, south-east Asia and western Pacific have exceeded 1.2 million cases in 2008 and over 2.3 million in 2010 (based on official data submitted by Member States of WHO). Recently the number of reported cases has continued to increase. In 2010, 1.6 million cases of dengue were reported in the Americas alone, of which 49,000 cases were severe dengue. In 2013, cases have occurred in Florida (United States of America) and Yunnan province of China. In Asia, Singapore has reported an increase in cases after a lapse of several years and outbreaks have also been reported in Laos. An estimated 5,00,000 people with severe dengue require hospitalization each year, a large proportion of whom are children. According to WHO about 2.5 % of the affected people die. Since 1967, many outbreaks of dengue have been investigated from India, with two major outbreaks reported from Delhi in 1996 and 2003 [2]. The 1996 outbreak was one of the largest one in Delhi, indicating a serious resurgence of the disease [3]. Gwalior, Madhya Pradesh, also saw a serious outbreak of febrile illness during October, 2003 to December, 2003 among which the major symptoms of

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patients included severe headache, fever, myalgia and vomiting etc [4]. Reports are available on the prevalence of *Aedes aegypti* throughout the year and *Aedes albopictus* in majority of the surveyed localities of Delhi with dengue virus infection in both *Aedes aegypti* and *Aedes albopictus* tested localities. This is the first report of dengue virus in *Aedes albopictus* from north India [5]. Another comprehensive study conducted in Kolkata suggested that dengue affected cases in 2012 outnumbered those of 2010 and 2011. Moreover it showed incidence within the age group of 11–30 years, with males being the target gender. Monsoon and post monsoon period showed the maximum of the dengue cases [6].

The vector of Dengue belongs to genus “*Aedes*” (Subfamily: Culicinae). Two important species *Aedes aegypti* and *Aedes albopictus*, are involved in the spread of DHF in north and south America, Africa, Europe [7–11] both of them are well adapted to breed in various artificial containers. *Aedes albopictus* is capable of breeding in a wide range of container types and water holding containers. General breeding sites, such as tree holes, coconut shells, fruit peels, water jars, earthen pots, cemented tanks, unused and discarded tyres, and old boats or cars holding water have been found to contain *Aedes albopictus* larvae [12–14]. Certain areas showed the presence of both the species while sampling, which suggests convergent habitat segregation [15]. Present piece of work has been designed to determine the molecular characterization and Scanning Electron Microscopy of *Aedes Albopictus* third instar larva collected from rural areas of Burdwan with the aim to record the specific morphological features of *Aedes albopictus* larvae found in different localities of Burdwan as well as to determine the phylogenetic status of these mosquito species on the basis of 28S rRNA gene sequence alignment and phylogenetic tree construction to understand the bionomics of this vector species for effective control measures.

Material and Methods

Mosquito-Culture and Collection of Larva

Aedes albopictus third instar larvae were collected with the help of plankton net from various containers and tyres, cemented tanks, water logged areas with rain water in and around Burdwan district of West Bengal, India. Larvae were reared up to adult emergence in a mixture of breeding habitat water and tap water (1:1 v/v) in white enamel trays at 27 ± 1 °C and 80 ± 5 % relative humidity (RH) for 2–4 days. The larvae were fed on phyto- and zooplanktons during culture or experiments. The adults were maintained on honey soaked cotton pads at 27 – 28 °C and 80 ± 5 %

RH in cages (60 cm^3) in the Parasitology and Microbiology Research Laboratory, Department of Zoology, Burdwan University.

Preparation of Scanning Electron Micrograph

The third instar larvae were fixed in 2.5 % Glutaraldehyde for 45 min. The specimens were then dehydrated through graded alcohols (50, 70, 90 %) and finally with absolute alcohol for 10 min. Finally they were subjected to critical point drying (C.P.D.). The specimens were gold coated and observed under scanning electron microscope. Measurements of different parts of larva were taken [16, 17].

PCR Amplification and Sequencing of 28S rRNA Gene

After isolation of DNA, PCR amplification of *Ae. albopictus* DNA was done. Using 28S rRNA primers, the ~400 bp fragment was amplified using Taq DNA Polymerase. Universal primers were used. The PCR products were loaded on 1.5 % agarose gel. The PCR conditions are as follows:

- Step 1 At 94 °C for 5 min for one cycle
- Step 2 At 94 °C- 30 s
- Step 3 At 54 °C- 35 s for 35 cycles
- Step 4 At 72 °C for 30 s
- Step 5 At 72 °C for 10 min

The PCR product was purified, bi-directionally sequenced and aligned.

Sequence Analysis and Phylogenetic Tree Preparation

Phylogenetic affiliation of the *Ae. albopictus* under study was done by 28S rRNA gene sequence analysis. 28S rRNA gene sequence was chosen for phylogenetic analysis because it is a evolutionary conserved sequence. Nucleotide BLAST (BLASTN) was done to investigate the most similar sequences from NCBI database. *Aedes riversi*, *Aedes flavopictus* have been described to date in the Ryuku Archipelago, Japan and their geographic distributions have been known to overlap. *Aedes riversi* and *Aedes flavopictus* are adapted to a forest environment whereas *Aedes galliosi* and *Aedes wadai*, which are a very rare group of species are confined more to the northern part of Japan [18]. Though these vectors cohabit domestic environments, still they have different roles in DHF/DF transmission [19]. Similarly *Aedes hensili* is responsible for transmission of Zika virus which was found from the blood of the people of Africa [20]. *Aedes (Stegomyia) dybasi* is an unique species, since it inhabits in pitchers of carnivorous plant, *Nepenthes mirabilis* Druce, as larval habitats. Traits of *Ae. dybasi* were compared with two sympatric, container-breeding

Fig. 1 Neighbor-joining tree constructed based on *Aedes albopictus* Burdwan-SNC1 (KC208593) 28S ribosomal RNA partial gene sequence as reference sequence along with few other 28S rRNA gene sequences retrieved from NCBI

Fig. 2 Restriction map of 28S rRNA gene sequence of *Aedes albopictus* isolate Burdwan-SNC 1 (KC208593)

species, *Aedes palauensis*, a probable ancestor of *Ae. dybasi*, and *Aedes hensilli* [21]. Phylogenetic tree was constructed taking those isolates which had the most similar sequences and which were ecologically relevant. The sequence data was aligned using the 'ClustalW Submission Form' and analyzed by ClustalW [22]. Evolutionary distances were calculated using the method of Jukes and Cantor [23] and the topology was inferred using the 'neighbor-joining' method [24]. 28S rRNA gene sequence of *Armigeres subalbatus* was taken as an out-group. To assign the taxonomical affiliation of *Ae. albopictus*, phylogenetic tree was prepared following Tamura et al. [25].

Results and Discussion

Analysis of 28S rRNA gene sequence of *Ae. albopictus* Burdwan-SNC1 (KC208593) revealed that it has higher GC content (60.56 %) than other isolates of *Aedes flavopictus* A47 (56.08 %), *Aedes albopictus* A34 (56.72 %), *Aedes albopictus* A22 (56.86 %), *Ae. albopictus* MQSRNAGN (L22060) (52.45 %), *Aedes palauensis* A26 (56.54 %), *Aedes hensilli* A25 (56.82 %), *Aedes riversi*

Fig. 3 VIII abdominal segment of 3rd instar larva of *Aedes albopictus* showing comb scales and pecten teeth

A51A (56.665 %), *Aedes dybasi* A28 (56.86 %), *Aedes aegypti* A20 (56.36), *Aedes galloisi* A77 (56.55 %), *Aedes wadai* A85 (56.75 %). AT content of *Aedes albopictus* Burdwan-SNC1 (KC208593) was found to be 39.44 %. Taking *Aedes albopictus* Burdwan-SNC1 (KC208593)

Fig. 4 VIII abdominal segment of 3rd instar larva of *Aedes albopictus* showing anal papillae

Fig. 5 VIII abdominal segment of 3rd instar larva of *Aedes albopictus* showing tips of the abdomen, spiracles, anal papillae

28S rRNA gene sequence as reference sequence, the phylogenetic tree was prepared and displayed in Fig. 1. The phylogenetic tree analysis showed that *Aedes albopictus* Burdwan-SNC1 (KC208593) branched with the cluster containing *Aedes palauensis* A26, *Aedes hensilli* A25, *Aedes riversi* A51A, *Aedes dybasi* A28, *Aedes galloisi* A77, *Aedes flavopictus* A47, *Aedes albopictus* A22, *Aedes albopictus* A34, *Aedes albopictus* MQSRNAGN (Fig. 2) and also branched with the cluster containing *Aedes aegypti* A20 and *Aedes wadai* (A85). The branch lengths have also been shown. The Restriction enzyme map on the 28SrRNA nucleotide sequence of *Aedes albopictus* Burdwan-SNC1 (KC208593) has been shown in Fig. 2.

Scanning electron micrographs of *Ae. albopictus* larva have been shown in Figs. 3, 4 and 5. It has been revealed that the structural complexity and the number of comb scales was positively correlated with the development and

instar of the larva. As also noticed by Stefan and Francisco [26] the number of comb scales varied in different instars as well as in different specimens collected from different areas. Usually the comb scales were four in number in the 1st instar larvae while six to seven were recorded from the 3rd instar larvae of the collected specimens (Fig. 3). They are arranged in a row in first instar, gradually achieving a semilunar pattern while developing from 1st to 4th larval instar. Each of the comb spines has a long median denticle (close to the siphon) and subapical denticles having pointed tips (Fig. 3). The respiratory siphon bears a row of pecten teeth on both sides and basal denticles. They are arranged in a row orienting towards the spiracle. The spines proximal to the spiracle are usually longer and the distal ones are comparatively smaller. The number of pecten teeth showed an increase in successive instars [26]. Their number in the 3rd instar larva varies from 12 to 13. The denticles of the pecten teeth are variable in size in every larval stage. At the terminal portion of the *Ae. albopictus* larva is the anal segment (Figs. 4, 5). Anal papillae are seen on the anal segment. Three anal papillae of more or less equal size were seen in all the larval instars which also gradually increased in length with successive larval duration and development.

Conclusion

Scanning Electron micrograph of the larval stage of *Aedes albopictus* revealed the structural peculiarity of this vector and showed that both comb scales and pecten spines were increased in number with the development and growth of larva. 28S rRNA gene sequence revealed the higher GC content of this species occurring in Burdwan which reflects the regional specificity of these vectors. Phylogenetic tree clearly reveals that this species branched with other *Aedes* spp which have great medical importance in different regions of the world.

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