

Phylogenetic study based on 28S rRNA gene sequencing of *Wuchereria bancrofti* isolated from the filaria endemic areas of Bankura district, West Bengal, India

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Abstract Lymphatic filariasis is one of the major public health concern in India, and Bankura district of West Bengal is one of the main filaria prone area of the country. *Wuchereria bancrofti* is the causative organism and *Culex quinquefasciatus* is the main vector of lymphatic filariasis in India. In the present study, infection and infectivity rate of filarial vector *C. quinquefasciatus* were determined. The molecular characterization, DNA fingerprinting and phylogenetic analysis of *W. bancrofti* was done. In the study area, overall vector infection and infectivity rates were 4.83 and 0.97%, respectively. The infection and infectivity rate were found to be higher in rainy season and lower in summer season. The AT and GC content of *W. bancrofti* SNC Bankura (Accession No. JF930705) were 55.59 and 44.41%, respectively. The phylogenetic tree was prepared following neighbour joining, maximum parsimony, minimum evolutionary and UPGMA methods. The study revealed that *W. bancrofti* SNC Bankura (JF930705) showed 100% similarity with *W. bancrofti* (Accession No. EU370161). The cluster containing, *W. bancrofti* SNC Bankura (JF930705) and *W. bancrofti* (EU370161) branched with *Brugia pahangi* (M15309) with 62% bootstrap value. *W. bancrofti* SNC Bankura (JF930705), *W. bancrofti* (EU370161) and *B. pahangi* (M15309) branched

with *Dirofilaria immitis* (AF188120) with 78% bootstrap value.

Keywords *Wuchereria bancrofti* · Phylogenetic tree · DNA fingerprinting · Infection and infectivity rate

Introduction

Lymphatic filariasis seldom captures any newspaper as it produces neither mortality nor epidemics or epizootics drawing alarming public attention, still this disease, in its various forms remains a public health problem of considerable magnitude in almost all the tropical and subtropical countries (WHO 1992, 1999, 2016). Worldwide 947 million people are at risk of lymphatic filariasis in 54 countries (WHO 2016). Lymphatic filariasis is caused by three types of nematodes belonging two different genera, i.e.; *W. bancrofti* producing bancroftian filariasis, *Brugia malayi* causing of Malayan filariasis and *B. timori* causing timor filariasis. Both the genera are included under the superfamily Filarioidea (Baylis 1939). Zoo geographical distribution indicates that *W. bancrofti* is present throughout the tropical regions of Asia, Africa, the Pacific and the Americas (Hawking 1976a, b). The nocturnally periodic form of this filarial parasite transmitted by *C. quinquefasciatus*, occurs in almost all the tropical and subtropical countries in a considerably widespread but focal distribution pattern. It disappeared from the North America and Australia as well as from some islands in the Caribbean and has greatly been reduced in the last 25 years in Americans, especially in Brazil. But its prevalence is on the gradual increase in the growing towns of Asia except Republic of China and Japan. The rural forms, adapted to anophelines are common in East and West Africa. The day biting

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mosquitoes transmit the diurnally sub-periodic form in the rural areas of South Pacific, the diurnally sub-periodic *W. bancrofti* is found in two small communities of Nicobar Island. A nocturnally sub-periodic form is found in the jungle areas of Thailand (Manson-Bahr and Bell 1995).

Lymphatic filariasis is a serious socio-economic and public health problem in India where 45% population live in areas known to be endemic for this disease. In this country 40 million people are infected taking nearly one-third of global burden of lymphatic filariasis (Michael et al. 1996; UNEWS 2014). *Wuchereria bancrofti* is the main causative agent of filariasis in India. Major parts of Bankura district have been affected by filariasis till date (Rudra and Chandra 2000; Mandal et al. 2016). The present study was conducted in Bankura, West Bengal, India to know the infection and infectivity rates of the filarial vector and to characterize the parasite *W. bancrofti* by phylogenetic analysis.

Materials and methods

Collection of mosquitoes

Adult *Culex* mosquitoes were collected from human habitations and cattlesheds of different villages [Dhulai, Sahapur, Dhansimla, Masterdanga, Kochdih, Kesosole, Sukhosole, Dakhinsole, Manikbazar] of Bankura from 6.00 a.m. to 8.00 a.m. following standard methodology (Service 1963) during October 2013–November 2014. The test tube was brought slowly and perpendicularly onto the mosquito and then it was imprisoned in the opening of the test tube. After the mosquito having taken, the tube was closed with a piece of cotton wool and was pushed with the mosquito towards the bottom of tube. A space was given between bottom of the test tube and the wool plug so that the mosquito could move freely. The operation was repeated until there were five or six mosquitoes in the long test tubes, each isolated from others by some layer of cotton wool. The tubes were taken to the laboratory where mosquitoes were transferred into mosquito cage for further study. Mosquitoes were placed on separate clean glass slides. Legs and wings were removed. The head, thorax and abdomen of each of the mosquitoes were separated by needles and each part was taken in separate drops of distilled water on the slide and teased apart by two fine entomological needles. The slides were then examined very minutely under compound microscope to see whether those mosquitoes were naturally infected by any kind of filarial parasites. Head, thorax and abdominal parts of each dissected mosquito were examined separately. The slides which were positive for filarial parasites were kept separate and allowed to be semidried in air. The slides were then

stained with Leishman's stain and observed under microscope. The infection rate (1st, 2nd stage larvae of *W. bancrofti*) and infectivity rate (3rd stage larvae of *W. bancrofti*) of vectors were noted.

DNA isolation and PCR amplification of 28S rDNA fragment from *W. bancrofti*, and its sequencing

Genomic DNA was extracted from the microfilariae (≈ 2000 in number) following phenol/chloroform method of Datta et al. (2007). The mosquitoes infected by *W. bancrofti* larvae were dried and homogenized thoroughly and then digested in 500 μ l lysis buffer [20 mM Tris–HCl, 50 mM EDTA, 0.5% SDS, 100 mM NaCl, 1% β mercaptoethanol (v/v), pH 8.0] and proteinase-K (0.1 mg/ml) and then incubated at 55 °C for almost 3 h. The DNA pellet was obtained by phenol–chloroform–isoamyl alcohol extraction (25:24:1) and ethanol precipitation and then resuspended in 20 μ l sterile nuclease-free water. The PCR was carried out using a thermal cycler in 50 μ l of reaction mixture [PCR buffer (1 \times) containing (NH₄)₂SO₄, dNTP mix (2 mM), MgCl₂ (2 mM), 1 μ M each of forward and reverse primers, 1.5 units of Taq polymerase and template DNA]. Denaturation was done at 95 °C for 3 min followed by 35 thermal cycles at [94 °C for 45 s, 51 °C for 1 min and 72 °C for 1 min]. Final extension was performed at 72 °C for 7 min. For amplification of the filaria-specific 28S rDNA, the primer pairs BD1A F [5'-atgaaaggcgttgatatag-3'] and BD1A R [5'- gcaagccatgcaagcgttgag-3'] (Smith and Rajan 2000) were taken. The PCR products were loaded on 1.5% agarose gel and sequenced following standard methodology (Lou and Golding 2007). The BLAST analysis of the obtained partial 28S rRNA gene sequence was done using BLAST-x program at NCBI. The sequence data were then aligned using the “ClustalW Submission Form” (<http://www.ebi.ac.uk/clustalw>) and analyzed by ClustalW (Thompson et al. 1994). Evolutionary distances were calculated following Jukes and Cantor (1969) and the phylogenetic tree was prepared following the “neighborjoining” method, minimum evolution (ME), and maximum parsimony (MP) methods described in MEGA version 3.1 (Kumar et al. 2004; Saitou and Nei 1987).

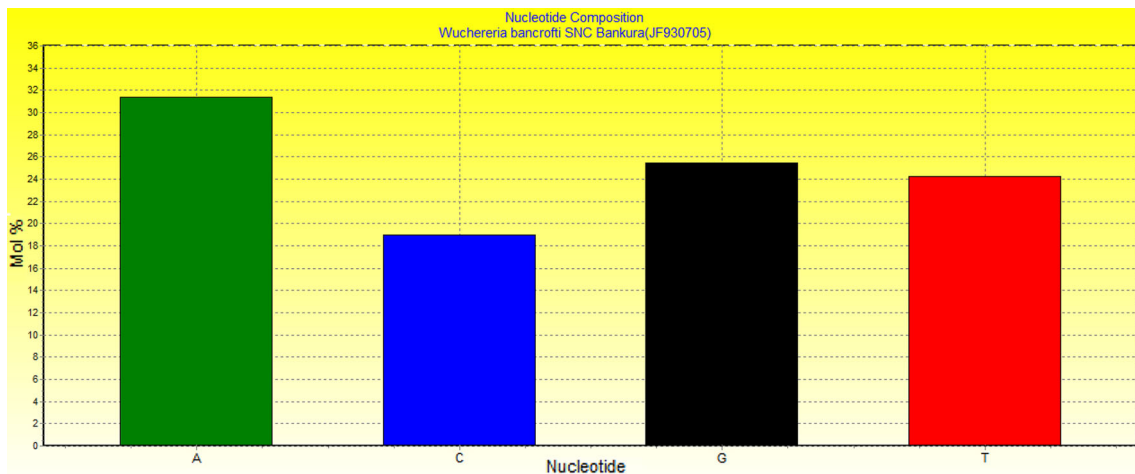
Results

Overall vector infection and infectivity rates among the human-house-invading mosquitoes were 4.83 and 0.97%, respectively (Table 1). A greater number of infected and infective vectors were encountered in the rainy season (6.45 and 1.71%, respectively) and lower infection and infectivity rate were found in the summer season (3.07 and

Table 1 Per man hour density (PMD), infection rate and infectivity rate of *Culex quinquefasciatus* in Bankura district, West Bengal, India

Season	PMD (\pm SE)	Infection rate (\pm SE)	Infectivity rate (\pm SE)
Summer	42.875 \pm 0.23	3.0675 \pm 0.025	0.393 \pm 0.01
Rainy	57.875 \pm 0.33	6.445 \pm 0.03	1.705 \pm 0.02
Winter	39.875 \pm 0.15	4.982 \pm 0.023	0.813 \pm 0.05
Average	46.875 \pm 0.25	4.832 \pm 0.025	0.970 \pm 0.028

SE standard error

**Fig. 1** Bar diagram showing mol% of A, T, G, C in the 28S rRNA gene sequence of *W. bancrofti* SNC Bankura (*JF930705*)

0.39%, respectively). Overall per man hour density (PMD) of *C. quinquefasciatus* in the study area was found to be 46.88. PMD was higher in rainy season and comparatively lower in winter season (Table 1).

The molecular analysis revealed that the mol% of A, T, G and C content of the 28S rDNA sequence of *W. bancrofti* SNC Bankura were shown in Fig. 1. The A+T content and G+C content of the specimen *W. bancrofti* SNC Bankura (GenBank Accession Number *JF930705*) were 55.59 and 44.41%, respectively. The DNA finger printing of *W. bancrofti* SNC Bankura (*JF930705*) was shown in Fig. 2. Very high similarity is shared with *W. bancrofti* (Accession number EU370161), followed by *B. pahangi* (Accession number M15309), *O. gibsoni* (Accession number M15308) and *Dirofilaria immitis* (Accession number AF188120). When compared with non-filarial nematodes, a relatively lower similarity was observed. These findings are reflected in the phylogenetic trees generated using the NJ, ME, MP and UPGMA methods (Fig. 3). In Neighbour joining tree and minimum evolution tree, *W. bancrofti* SNC Bankura (*JF930705*) branched with *W. bancrofti* (EU370161) with 98% bootstrap value. The cluster containing, *W. bancrofti* SNC Bankura (*JF930705*) and *W. bancrofti* (EU370161) branched with *B. pahangi* (M15309) with 62% bootstrap value. *W. bancrofti* SNC Bankura (*JF930705*), *W. bancrofti* (EU370161) and *B. pahangi* (M15309) branched

with *D. immitis* (AF188120) with 78% bootstrap value. In all the phylogenetic trees it was clearly observed that *W. bancrofti* SNC Bankura (*JF930705*) showed closest relationship with *W. bancrofti* (*JF930705*) (Fig. 3).

Discussion

Both vector infection and infectivity rates were quite high in the present study areas which were found to be in concordance with the observation of Kumar and Singh (1999) where the infection rate of *C. quinquefasciatus* mosquito was 27.3% reflecting much higher infection and infectivity rates in respect to filarial transmission. Vector infection and infectivity rate of the present study area were found to be higher than of Katwa, Burdwan (Das et al. 2003) but lower than in Andhra Pradesh (Murty et al. 2002). The overall microfilarial infection rate obtained in the present study was comparable to reports from filariasis endemic countries (WHO 1999; Pedersen and Mukolo 2012) but is much higher than that obtained in Nigeria, an endemic area of filariasis (Awolola et al. 2006). In the present study, the vector infection and infectivity rates were higher in the rainy season indicating rainy season as the most favourable period for disease transmission. (Table 1). Statistical analysis (Z test) revealed significant difference in infection

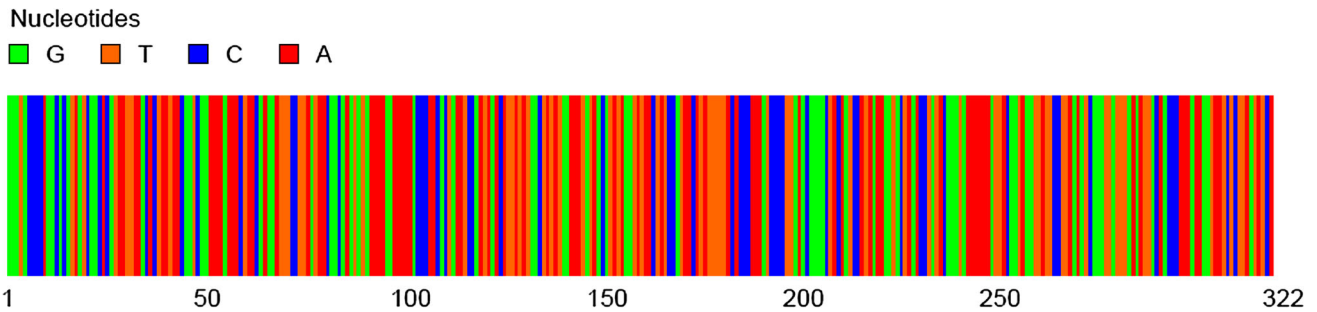


Fig. 2 DNA finger printing of 28S rRNA gene sequence of *W. bancrofti* SNC Bankura (*JF930705*)

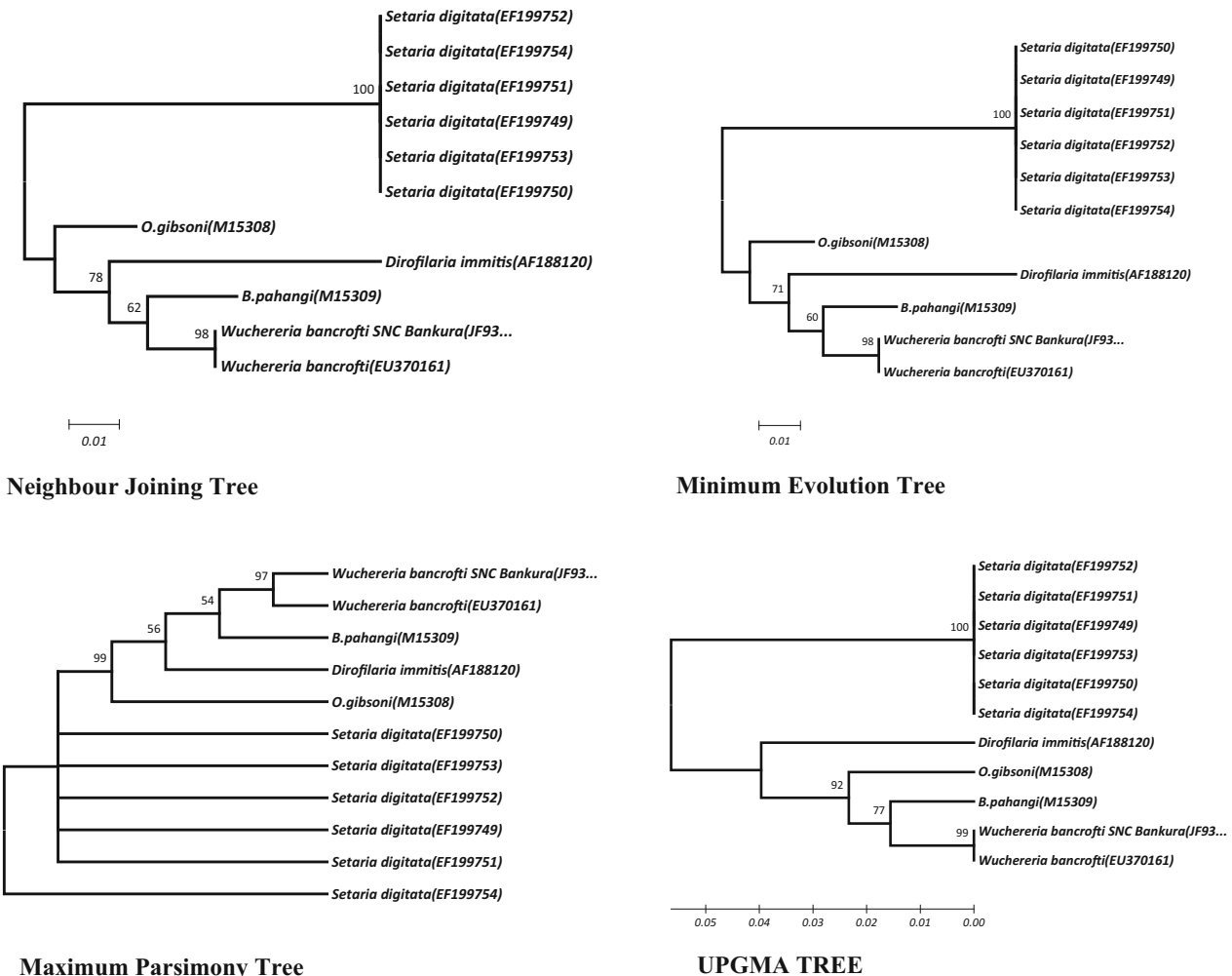


Fig. 3 Phylogenetic trees based on 28S rRNA gene sequences, constructed using neighbour joining, minimum evolution, maximum parsimony and UPGMA methods by Kimura-2-distances in MEGA 4 programme. *Numbers on the nodes* are bootstrap values

and infectivity rate in different seasons ($p < 0.05$) which was found to be similar to the findings of Chandra et al. (2007) and Dixit et al. (2009). Per man hour density of the study area during the study period was quite higher than previous records (Rudra and Chandra 1998). PMD of filarial vector, *C. quinquefasciatus* was higher ($p < 0.05$) in the rainy season. Per man hour density of the study area

of Bankura was quite higher than North 24 Parganas (De and Chandra 1994), Madhya Pradesh (Chand et al. 1996) and Digha (Chandra et al. 2007). The infection and infectivity rates of the vector mosquito greatly varied in the world scenario. Along with the environmental factors and the standard of hygiene including sanitary systems, the density of vector population might be important

determinants of the level of disease in case of filariasis (Azmi et al. 2015). The phylogeny obtained in this study is in concordance with previous findings. Xie et al. (1994) studied the phylogenetic relationship based on the 5S rRNA gene spacer region and observed the presence of *Wuchereria* in the *Brugia-Wuchereria* group. A similar phylogeny was obtained in a different study of Casiraghi et al. (2001), who constructed the trees using the cytochrome oxidase subunit I (COI). Fong et al. (2008) constructed different phylogenetic trees and compared the total nucleotide numbers among the filarial worms to establish the close phylogenetic relationship among the filarial worms. The bootstrap value of the node branching *W. bancrofti* SNC Bankura (JF930705) and *W. bancrofti* (EU370161) was 98%, which suggested that the branching was quite prominent and the node is well supported. The higher bootstrap values represented within the phylogenetic trees of *W. bancrofti* SNC Bankura (JF930705) constructed by different methods also suggested that *W. bancrofti* SNC Bankura (JF930705) was highly similar with other *W. bancrofti* but quite different from other filarial worms such as *B. pahangi* and *D. immitis* while considering the partial 28S rRNA gene sequences.

Conclusion

The result related to the infection and infectivity rate of *W. bancrofti* in filarial vector *C. quinquefasciatus* in Bankura was quite higher which proved the study area to be endemic for filariasis. Peak filarial transmission was found during rainy season in the study area. The molecular-based phylogeny of *W. bancrofti* SNC Bankura (JF930705) is congruous with its traditional classification which is based on morphological characters where it is found to be similar with other microfilariae causing filariasis. In this study the molecular analysis using real-time PCR has great potential as a diagnosis and monitoring tool in filariasis elimination programmes due to its high-throughput capacity and decreased risk of cross-contamination between test samples.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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