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SHORT COMMUNICATION

Molecular differentiation of nocturnally periodic and diurnally sub-periodic *Wuchereria bancrofti* by Randomly Amplified Polymorphic DNA (RAPD)

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Abstract *Wuchereria bancrofti*, a nematode parasite, is responsible for causing 90% of lymphatic filariasis infection in the world. In India, *W. bancrofti* exists in two physiological forms, nocturnally periodic in the main land and diurnally sub-periodic in Car Nicobar group of islands. Differentiation of these two parasitic forms by conventional microscopic methods difficult requiring good skill and hence tedious. Therefore, we developed a simple and rapid Random Amplified Polymorphic DNA (RAPD) assay to differentiate these parasitic forms. Also, the phylogenetic relationship between periodic and sub-periodic *W. bancrofti* and also *Brugia malayi* populations was analyzed using RAPD profile generated. Distinct RAPD profiles were observed among the three parasites with the formation of three distinct clusters in the phylogenetic tree. Substantial genetic diversity (Nei's genetic diversity H) was observed among periodic (H = 0.0577) and sub-periodic (H = 0.1415) *W. bancrofti* populations.

Keywords Filariasis · Sub-periodic · *Wuchereria bancrofti* · RAPD · Genetic diversity

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Introduction

Lymphatic filariasis has been the major public health problem in tropical and sub-tropical countries. Globally, 1,307 million people in 83 countries are exposed to the risk of infection with LF (WHO 2006). More than 90% of the LF infections are caused by *Wuchereria bancrofti*, and the remaining 10% are caused by *Brugia malayi* and *B. timori* (Ottesen et al. 1999). In India, an estimated 554.2 million people are at risk of this infection (WHO 2007) and about 40 million people are already affected by this disease. *W. bancrofti* exists as three physiological forms, that differ from each other in the periodicity of appearance of microfilaria (Mf) in the peripheral blood of the human host, and are (a) nocturnally periodic, (b) nocturnally sub-periodic, and (c) diurnally sub-periodic (Sasa 1976).

The nocturnally periodic form is barely present in the blood at non-peak times; while sub-periodic form appears at all times but occur at higher densities during peak times (10.00 pm and 02.00 am). Nocturnal periodicity is a feature of LF infection essentially everywhere in the world (Sasa 1976). The sub-periodic form, mainly seen in eastern pacific islands is transmitted by *Aedes* species (Chow 1973; Hawking and Denham 1976). Sub-periodic strains exist as two forms viz., (a) nocturnally sub-periodic form with circulating levels of Mf greatest at night (Grove 1983), and (b) diurnally sub-periodic strains with circulating levels of Mf greatest during the day and are reported from south pacific and Andaman and Nicobar islands (Dondero and Sivanandam 1971; Tewari et al. 1995; Shriram et al. 1996). These observations are consistent with the hypothesis that the Mf abundance cycle in the bloodstream has evolved to maximize transmission to mosquitoes.

In India, two physiological forms of *W. bancrofti* viz., nocturnally periodic and diurnally sub-periodic forms

occur. Nocturnally periodic form is prevalent in the Indian main land and transmitted by *Culex quinquefasciatus*, while diurnally sub-periodic form occurs in the islands of Car Nicobar archipelago, Indian Ocean (Basu 1958; Kalra 1974), where it is transmitted by *Ochlerotatus nievies* (Kalra 1974). However, there are no studies to show whether the diurnally sub-periodic form is indeed entirely absent from the mainland India. Given the recent increase in the movement of human population between mainland India and Andaman Nicobar islands during the last few decades, it is possible that the sub-periodic form might have spread to the mainland India. Also, probably it is not reported in mainland merely because there were no serious efforts to look for this form in the main land. As stated above the two physiological forms are morphologically very similar and can be differentiated through microscopic examination of blood smear only by a highly skilled person, which is generally lacking in the routine surveys. Also, morphological and morphometric studies are time-consuming, laborious, and consequently not suitable for large-scale application. Hence, there is a need to identify/develop an alternate method for their differentiation. Molecular biological methods have been helpful in diagnosis and population studies (Gasser et al. 2004; Hoti et al. 2008). However, there are no reports of genetic markers to differentiate between the two forms of *W. bancrofti* so far. Random Amplified Polymorphic DNA (RAPD) was found to be useful for studying genetic diversity of filarial parasites (Pradeep Kumar et al. 2002; Thangadurai et al. 2006, Nuchprayoon et al. 2007, Dhamodharan et al. 2008). These markers were found to be useful for analyzing the inter- and intra-specific genetic variations and for finding phylogenetic relationships among *W. bancrofti* populations collected from different geographical locations in India (Thangadurai et al. 2006). More recently, Nuchprayoon et al. (2007) employed this method for differentiating nocturnally sub-periodic form of *W. bancrofti* occurring in Thailand and nocturnally periodic form existing in Myanmar. We present here a method to differentiate nocturnally periodic and diurnally sub-periodic forms of *W. bancrofti* occurring in the main land and Car Nicobar islands of India, respectively. We also report the genetic variation within the populations of the two forms of *W. bancrofti* and *B. malayi* by RAPD analysis.

Materials and methods

Study area and sample collection

Blood samples (smear) were collected from microfilaraemic individuals residing in three regions of India (Fig. 1) viz., Pondicherry located at 11° 56′ N, Longitude—79° 50′ E on the south east peninsula of India and endemic for nocturnally periodic *W. bancrofti*, Orissa state—located at 21° 56′′6′N, 86° 43′′ 17′E, in the middle east of the country and is endemic for both nocturnally periodic *W. bancrofti* and *B. malayi*, and Car Nicobar islands—located at 6–10°N 92–94°E in the Bay of Bengal and endemic for sub-periodic *W. bancrofti*.

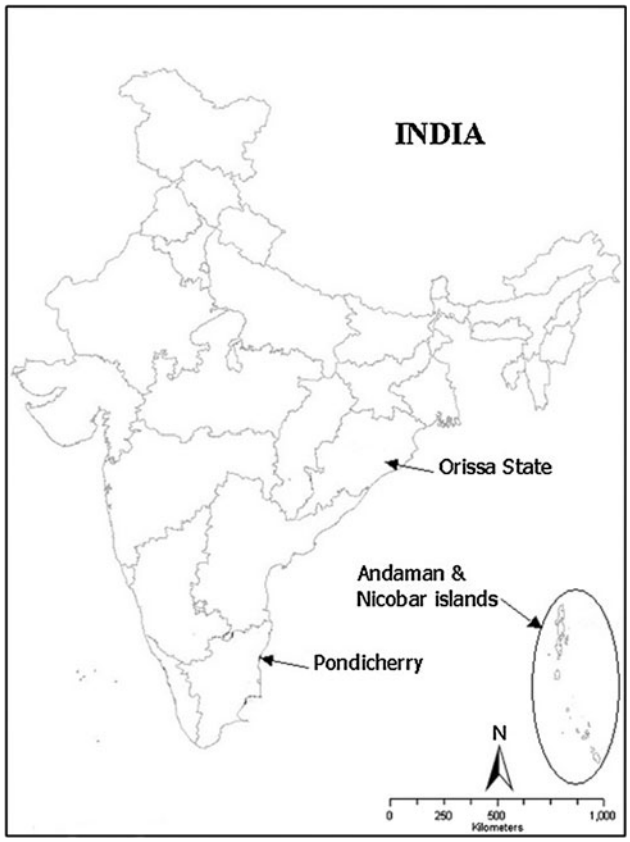

Fig. 1 Map of India showing the study areas from where the blood samples from microfilariae carriers were collected

Thick blood smears were collected during the year 2003 from microfilaria (mf) carriers. The slides were stained by Jaswant Singh Bhattacharji (JSB) stain (Singh and Bhattacharji 1944), and those found to have mf were stored in a wooden slide box at room temperature until further use. Ten populations (all mf in the blood smear on a slide collected from an individual) of nocturnally periodic *W. bancrofti* from Pondicherry, 10 sub-periodic *W. bancrofti* populations from Car Nicobar, and 10 *B. malayi* populations from Orissa were used in this study. The nocturnally periodic and diurnally sub-periodic *W. bancrofti* populations were differentiated by their geographic origin and periodicity in documented clinical histories. Nocturnally periodic *Brugia malayi* was morphologically differentiated by microscopic examination of tail nuclei (Sasa 1976). The study was approved by the ethical committee of the institute

and written informed consent was obtained from Mf carrier before collection of blood smear.

Isolation of microfilaria and DNA extraction

Mf from the positive slides were purified and isolated by using the method reported earlier (Bisht et al. 2006). The genomic DNA was extracted from 10 numbers of mf. Briefly, the mf were treated with digestion buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 20 mM EDTA, Proteinase K (1 mg/ml), and SDS (1%) at 56°C over night, and the DNA was extracted by DNA standard Phenol–Chloroform procedure (Hoti et al. 2003). Concentration of the extracted DNA was estimated spectrophotometrically (Genequant, Amersahm, USA) and used for RAPD analysis.

RAPD-DNA fingerprinting

The RAPD-PCR was performed in a thermocycler (BioRad, USA) using RAPD primer-1 (Amersham-Pharmacia, USA), which was found to be useful for differentiating *W. bancrofti* populations in an earlier study (Patra et al. 2007). The PCR mixture (30 µl final volume) contained ~25 ng of genomic DNA as template, 100 pmol of primer, 1 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 1 U of *Taq* DNA polymerase (Finzyme, Finland) in 1× PCR buffer. The thermal conditions used for the PCR were: initial denaturation for 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 40°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The amplicons were electrophoresed on a 1% agarose gel, stained with ethidium bromide and documented using Geldoc Mega (BioSystematica, UK). Molecular weight of each band was determined by using Alphatech gel documentation system (USA).

Genetic analysis

All bands obtained on RAPD fingerprints were scored based on their presence or absence, and the data obtained was transferred into a binary code with 1 or 0, respectively. This data was used to calculate the percentage of polymorphic loci, genetic distance, and genetic identity (Nei's unbiased distance estimate; Nei 1978). Cluster analysis was performed with the Unweighted Pair Group Method with Arithmetic average (UPGMA) using 'PopGene 32' software (Yeh and Yang 1999). The Nei's gene diversity (H) (Nei 1978) between groups of parasite populations was estimated to understand the genetic variability. Phylogenetic tree was constructed using Mega.4 (Timura et al. 2007) by using Nei's unbiased genetic distance matrix.

Results and discussion

RAPD profiles were generated for 10 populations each of nocturnally periodic and diurnally sub-periodic *W. bancrofti* and nocturnally periodic *B. malayi* (Fig. 2). The profiles of the two forms of *W. bancrofti* varied significantly from each other, both in number, as well as in size of bands amplified. A total of 26 bands ranging in size from 100–3,200 bp were generated for different populations of which 6 were common to all populations. Polymorphic fragments were observed among the three groups of parasite populations. Percentage of polymorphic loci was estimated to be highest (38.46%) in diurnally sub-periodic *W. bancrofti* populations from Nicobar islands and lowest (3.85%) in *B. malayi* from Orissa State. Genetic diversity was found to be highest (0.1415) in diurnally sub-periodic *W. bancrofti* populations.

Phylogenetic tree was constructed (Fig. 3) using the UPGMA and NJ methods based on Nei's genetic distance for all populations. The phylogenetic tree obtained by using UPGMA was in concordance with that obtained by using the NJ method in describing relationships between species. The parasite populations branched into three distinct clusters in the tree corresponding to nocturnally periodic, diurnally sub-periodic forms of *W. bancrofti* and nocturnally periodic *B. malayi*. Thus, genetically there was a clear separation among the three groups of parasite populations. Further, the populations of the two forms of *W. bancrofti* branched together as two sub-branches, whereas *B. malayi* populations branched separately as an ancient group.

RAPD profiles generated were also used to analyze the intra-population genetic diversity among 10 populations each of nocturnally periodic and diurnally sub-periodic

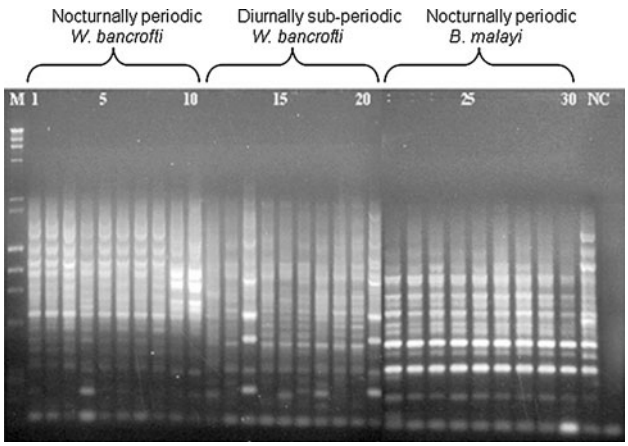

Fig. 2 RAPD profile of human lymphatic filarial parasites populations from India. *Lanes 1–10* nocturnally periodic *W. bancrofti*, *Lanes 11–20* Diurnally sub-periodic *W. bancrofti*, *Lanes 21–30* nocturnally periodic *B. malayi*, *Lane M* Marker (Lambda/Hind III-Phi X 174/Hae III fragments) and *C* Negative Control (without Template DNA)

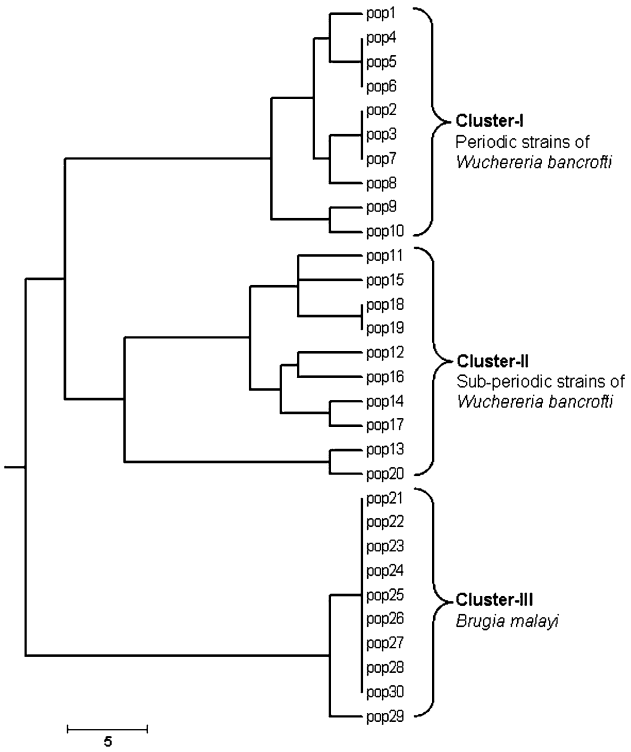

Fig. 3 Phylogenetic tree (UPGMA) of individual populations of nocturnally periodic and diurnally sub-periodic *W. bancrofti* and nocturnally periodic *B. malayi* (*WB W. bancrofti*, *SWB* sub-periodic *W. bancrofti*, *BM B. malayi*). Clusters I, II and III indicate the parasite populations grouped based on species/physiological forms

W. bancrofti and also nocturnally periodic *B. malayi*. The results of the analysis are presented in Table. 1. The phylogenetic tree (Fig. 3) also clearly revealed the genetic variation within populations of different parasite forms. The two forms of *W. bancrofti* further branched into three minor branches each, while *B. malayi* populations remained as a single major cluster without branching further. This indicates that *W. bancrofti* populations of the two forms are genetically heterogeneous while *B. malayi* populations are homogeneous. This is also reflected in the values of genetic distance and genetic diversity. The genetic distance within the parasite populations was highest for nocturnally periodic *W. bancrofti* (0.000–0.4249) than diurnally sub-periodic *W. bancrofti* (0.000–0.1671, respectively). The genetic distance between nocturnally periodic *B. malayi* populations was lowest (0.000–0.0392) when compared to that within *W. bancrofti* populations. The gene diversity within the groups also showed similar pattern (Tables 1 and 2).

It is well known that the sub-periodic form of *W. bancrofti* is present only in the remotely located Andaman and Nicobar islands, while the periodic form is present in the main land India. However, there are no studies to show whether the sub-periodic form is indeed entirely absent from the mainland India. Given the recent increase in the movement of human population between mainland and Andaman Nicobar islands due to improved Sea and Air

Table 1 Polymorphic loci, genetic distance and gene diversity among 3 groups of human lymphatic filarial parasites

Sample ID	Polymorphic loci		Gene diversity (H)	*Genetic distance
	Number	%		
W. bancrofti—nocturnally periodic	5	19.23	0.0577 ± 0.1316	0.000–0.1671
W. bancrofti—diurnally sub-periodic	10	38.46	0.1415 ± 0.1929	0.000–0.4249
B. malayi—nocturnally periodic	1	3.85	0.0069 ± 0.0353	0.000–0.0392
Over all	19	73.08	0.2424 ± 0.2024	0.000–0.6931

H = Nei's genetic diversity

Genetic distance: A measure of the allelic substitutions per locus that have occurred during the separate evolution of two populations or species

This measure assumes that genetic differences arise due to mutations and genetic drift

Genetic diversity: Proportion of polymorphic loci across the genome

* Range of genetic distance within groups of filarial parasites

Table 2 Genetic distance (below diagonal) and genetic identity (Above diagonal) among 3 groups of human lymphatic filarial parasites

Parasite populations	*W. bancrofti* nocturnally periodic	*W. bancrofti* diurnally sub-periodic	*B. malayi* nocturnally periodic
W. bancrofti nocturnally periodic	–	0.7697	0.7085
W. bancrofti diurnally sub-periodic	0.2617	–	0.6873
B. malayi nocturnally periodic	0.3446	0.3750	–

Genetic identity: The relatedness of two populations as represented by the percentage of allele they share

transport facilities, it is possible that the sub-periodic form might have spread to some of the areas of the mainland India. It is also possible that it is not reported in the mainland merely because there were no serious efforts to look for this form in the main land. The differentiation of the two forms requires skill, which is lacking in the routine examination of blood smears. Hence, there is a need to look into this issue for which better tools, such as molecular tools, are required. The present study revealed that the RAPD-PCR is able to differentiate between the two forms of *W. bancrofti*, which can further be used for designing PCR assays for their differentiation. Employing such assays in the surveillance may help in detecting the prevalence of sup-periodic form in the mainland India. Nuchprayoon et al. (2007) were able to differentiate between the Thai and Myanmar strains of *W. bancrofti* based on RAPD profiles, as it was not possible to rely on morphological criteria alone. They also observed that RAPD profiles revealed a significant diversity between these two strains.

RAPD is a simple molecular tool for the analysis of genetic variation of filarial parasites (Hoti et al. 2008) and other organisms (Wilkerson et al. 1993; Lescuyer et al. 1997; Steindel et al. 1993). The potential of RAPD has also been exploited in taxonomy and population genetics of several organisms by other workers (Bandi et al. 1993; Shiff et al. 2000). This method also reveals the inter-specific phylogenetic relationships at the molecular level. The RAPD- profile generated in this study was also able to show genetic diversity between the two physiological forms of *W. bancrofti* as also *B. malayi*. It can be concluded that RAPD-PCR may be useful for differentiating the nocturnally periodic and diurnally sub-periodic forms of *W. bancrofti* in India.

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