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Morphological and genetic variation of *Wuchereria bancrofti* microfilariae in carriers in Thailand, Lao PDR and Myanmar: evaluation using Giemsa-stained thick blood films

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Research Paper

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Abstract

There is geographical variation in the morphology and genetics of *Wuchereria bancrofti*, the major cause of human lymphatic filariasis. This study aims to compare morphological and genetic variation of *W. bancrofti* microfilariae recovered from carriers in Lao PDR, Myanmar and Thailand. Six morphological parameters (body length, cephalic space length and width, length of head to nerve ring, body width at nerve ring, Innenkörper length and number of column nuclei between the cephalic space and nerve ring) were evaluated from microfilariae in Giemsa-stained thick blood films. A portion of the cytochrome c oxidase subunit 1 gene of mitochondrial DNA was sequenced and analysed. *Wuchereria bancrofti* microfilariae showed a wide variation in their morphology and morphometry among three countries. Phylogenetic analysis confirmed that all microfilariae belonged to *W. bancrofti*. Higher mutation frequencies were observed in samples from Myanmar, relative to Thailand and Lao PDR. This study highlights the morphological disparities of microfilariae and genetic variability within *W. bancrofti* among three geographical locations. We found that reported morphometric differences between localities were less clear-cut than previously thought. Further studies are needed to determine the microfilarial periodicity in Lao PDR.

Introduction

Lymphatic filariasis is still a major public health problem in tropical and subtropical countries. Approximately 856 million people in 52 countries worldwide remain at risk (WHO, 2016). The disease is caused by mosquito-transmitted filarial worms, especially *Wuchereria bancrofti* (>90% of cases). *Brugia malayi* and *B. timori* cause about 10% of cases (WHO, 2016). Various genera of mosquitoes – that is, *Anopheles*, *Culex*, *Aedes*, *Mansonia* and *Ochlerotatus* act as vectors, depending on the geographical location and filarial species/strain (WHO, 2013). *Wuchereria bancrofti* can be physiologically classified into three strains according to the periodicity of microfilariae in peripheral blood: nocturnally periodic (urban and rural types), nocturnally sub-periodic and diurnal sub-periodic (the Pacific type) (Sucharit & Harinasuta, 1981; Jitpakdi *et al*., 1999). In Thailand and Myanmar, only two types (nocturnally periodic and nocturnally sub-periodic) have been recognized (Jitpakdi *et al*., 1999). Data relating to morphometry, genetic diversity and periodicity of microfilariae are lacking for Lao PDR. Therefore, it is important to study bancroftian filarial epidemiology in this country (Sudomo *et al*., 2010). Apart from the species of mosquito vector used and the periodicity of microfilariae in peripheral blood, nocturnally periodic and nocturnally sub-periodic strains can also be clearly differentiated using morphological parameters (Jitpakdi *et al*., 1999). The number of nuclei between the cephalic space and the nerve ring and most body dimensions, are significantly lower in microfilariae and infective larvae of the nocturnally periodic type relative to the nocturnally sub-periodic type (Jitpakdi *et al*., 1999).

Genetic variation of nematodes is an important factor that may influence the efficacy of available drugs as well as the development and spread of drug resistance among parasite populations (Anderson & Jaenike, 1997). Previous studies based on sequences of the mitochondrial cytochrome c oxidase subunit 1 (*cox*-1) gene in Papua New Guinea (Small *et al*., 2013) and Ghana (de Souza *et al*., 2014) indicated that *W. bancrofti* exhibits high levels of genetic diversity. This was very evident also from a comparison of complete mitochondrial genomes from isolates of *W. bancrofti* from India, West Africa and Papua New Guinea (Ramesh *et al*., 2012). Thus, an understanding of genetic variation may provide useful tools for discerning

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Table 1. Details of filariasis carriers and geographical sources of samples.

Sample no.	Location	Year	Age	Sex	Species (according to morphometry)
1	Attapeu, Lao PDR	2007	60	F	*W. bancrofti*
2	Attapeu, Lao PDR	2007	56	F	*W. bancrofti*
3	Attapeu, Lao PDR	2007	45	F	*W. bancrofti*
4	Mawlamayine, Myanmar (NP)	1999	20	M	*W. bancrofti*
5	Rangoon, Myanmar (NP)	1999	21	M	*W. bancrofti*
6	Mawlamayine, Myanmar (NP)	1999	24	M	*W. bancrofti*
7	Hui La Pring, Pawo, Maesod Tak, Thailand (NSP)	1999	25	M	*W. bancrofti*
8	Hui La Pring, Pawo, Maesod Tak, Thailand (NSP)	1999	30	M	*W. bancrofti*
9	Hui La Pring, Pawo, Maesod Tak, Thailand (NSP)	1999	26	F	*W. bancrofti*

Strain identity, where known from previous work, is indicated. NP, nocturnal periodic strain; NSP, nocturnal sub periodic strain.

epidemiological patterns and for designing strategies for prevention and control. Here, we compared morphometric measurements and genetic variation of *W. bancrofti* microfilariae recovered from three adjacent Asian countries – Lao PDR, Myanmar and Thailand. Morphometric data were obtained from microfilariae in Giemsa-stained thick blood films from *W. bancrofti*-infected carriers. We found that reported morphometric differences between localities were less clear-cut than previously thought. These blood films were also a source of DNA for amplification and sequencing of the mitochondrial *cox*-1 gene.

Materials and methods

Patients and specimens

Giemsa-stained thick blood films from nine human carriers of bancroftian filariasis were obtained in 1999 or 2007 and stored at room temperature until used in 2016 (table 1). Three samples were obtained from patients in Attapeu Province southern Lao PDR; three samples were obtained from infected Myanmar immigrants (nocturnally periodic strain); two from Mawlamyine, south-eastern Myanmar and one from Yangon in lower Myanmar. The remaining three samples (nocturnally subperiodic strain) were obtained from Karen ethnic people, Mae Sot District, Tak Province in western Thailand, near the Myanmar border. Details of carriers and source of samples are summarized in table 1.

Morphometric measurements and statistical analyses

A total of 254 microfilariae were examined at a final magnification of 100× using a compound microscope. One hundred microfilariae obtained from three Lao PDR carriers, 97 microfilariae obtained from three Myanmar and 57 microfilariae obtained from three Thai carriers were measured using camera-lucida drawings by two experienced laboratory personnel. Six morphological parameters were measured for each microfilaria: body length, cephalic space length and width, length of head to nerve ring, body width at nerve ring and Innenkörper length. In addition, the number of column nuclei between the cephalic space and the nerve ring were counted. Statistical analyses were carried out in IBM SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, IL). One-way analysis of variance with Tukey's multiple comparison tests was used to identify significant ($P < 0.05$) differences between morphometric parameters among the three countries.

Polymerase chain reaction (PCR) amplification, DNA sequencing and sequence analysis

DNA was extracted directly from individual slides positive for *W. bancrofti* using a commercial DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) according to the manufacturer's instructions. Initial attempts to amplify relatively long portions of the *cox*-1 gene were unsuccessful. Assuming that the DNA had been degraded by Giemsa staining or long storage, we then amplified short DNA fragments using two primer pairs (approximate product sizes 151 and 139 bp), which were designed using the Primer Express Software Version 3.0 (Life Technologies, CA, USA) based on the *cox*-1 sequence of *W. bancrofti* (GenBank accession number HQ184469). The two regions that were amplified overlapped to give a contig of 244 bp (208 bp after trimming the primer sequences). Details of primers used are given in table 2. Each PCR was performed according to the previously published method (Jongthawin *et al.*, 2015). The thermocycling conditions for both sets of primers were as follows: 94°C for 5 min; ten cycles of 95°C for 30 s, 45°C for 30 s and 72°C for 30 s; 25 cycles of 95°C for 30 s, 50° C for 30 s and 72°C for 30 s; with a final step at 72°C for 10 min. Amplicons were separated by 1.5% agarose gel-electrophoresis and 151 and 139 bp fragments were excised and sequenced using the Applied Biosystems 3730 × I DNA Analyzer and ABI Big Dye sequencing kit, version 3.1 (Foster City, CA, USA). DNA sequencing was conducted at First BASE Laboratories Sdn Bhd (Selangor, Malaysia) in both directions, using the PCR primers as sequencing primers.

The identity of the partial *cox*-1 gene sequence from each sample was checked using the BLAST-N search tool (National Center for Biotechnology Information, Bethesda, MD, USA). The new *cox*-1 sequences from the three countries were aligned with sequences of filarial nematodes from the GenBank database (alignment length was 208 bp) using the BioEdit sequence alignment editor (Hall, 1999). Phylogenetic relationships were analysed using the maximum likelihood (ML) method implemented in MEGA v6 (Tamura *et al.*, 2013). The best substitution model for *cox*-1 was Hasegawa–Kishino–Yano (TN93) + G. Support for clusters in each tree was calculated using 1000 bootstrap replications. Pairwise genetic distance values, which describe

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Table 2. Details of primers used in this study.

PCR assay	Sequences	Amplicon sizes
W. bancrofti cox-1	Wb *cox*1_F3 = 5′ CTTTGAGGGTAGAAGGT ′3 Wb *cox*1_R4 = 5′ CTAGCCTGATCCAAAG ′3	151 bp
	Wb *cox*1_F2 = 5′ CTACTCAGAATATGCGTTC ′3 Wb *cox*1_R2 = 5′ CGGTCAAACAACAAAAACA ′3	139 bp

the number of differences (corrected according to the model used) between two DNA sequences, were calculated using the Tamura–Nei model (Tamura & Nei, 1993).

Results

Comparison of morphometric data of W. bancrofti *microfilariae from Lao PDR, Myanmar and Thailand*

Representative images of *W. bancrofti* microfilariae from Thailand, Myanmar and Lao PDR are shown in fig. 1. Microfilariae from the three countries were sheathed, lying in graceful coils without secondary kinking. Somatic nuclei were discrete, overlapping where crowded but with distinct borders, countable and no nuclei were present at the tip of the tail. Comparisons of measurement and count parameters of microfilariae from the three countries are shown in table 3. The body length differed significantly by country among specimens from Lao PDR, Myanmar and Thailand, with those of the Thailand type being the longest. Similarly, Innenkörper length and the number of nuclei between the cephalic space and nerve ring also differed significantly among countries (table 3). Microfilariae from Lao PDR had a significantly longer and wider cephalic space and a greater body width at the nerve ring than the two other types. Microfilariae from Myanmar had significantly shorter head to nerve ring lengths than did those from the other countries (table 3).

Molecular phylogenetic analysis of W. bancrofti *microfilariae*

A contig, 244 bp in length, of *cox*-1 sequence was obtained from nine samples of microfilariae. After trimming the primer sequences, the final length was 208 bp. Eight variable sites were found among these sequences from three countries, and genetic distances ranged from 0.00 to 0.027 (see supplementary fig. S1). To verify the species, phylogenetic relationships of roundworms in the family Onchocercidae were reconstructed using the ML method (fig. 2). All partial *cox*-1 sequences of microfilariae obtained in this study were located in a clade containing *W. bancrofti* sequences from various geographical localities including Mali (GenBank accession number JN367461), Papua New Guinea (JF775522), India (JQ316200) and Italy (AM749235), with 98–100% similarity (BLAST-N search), confirming that the microfilariae specimens obtained from Lao PDR, Myanmar and Thailand were *W. bancrofti*. These new partial *cox*-1 sequences have been deposited in the GenBank database under the accession numbers MK138550–MK138558.

Discussion

Morphology of our samples from Lao PDR, Thailand and Myanmar indicate that the microfilariae belong to *W. bancrofti*.

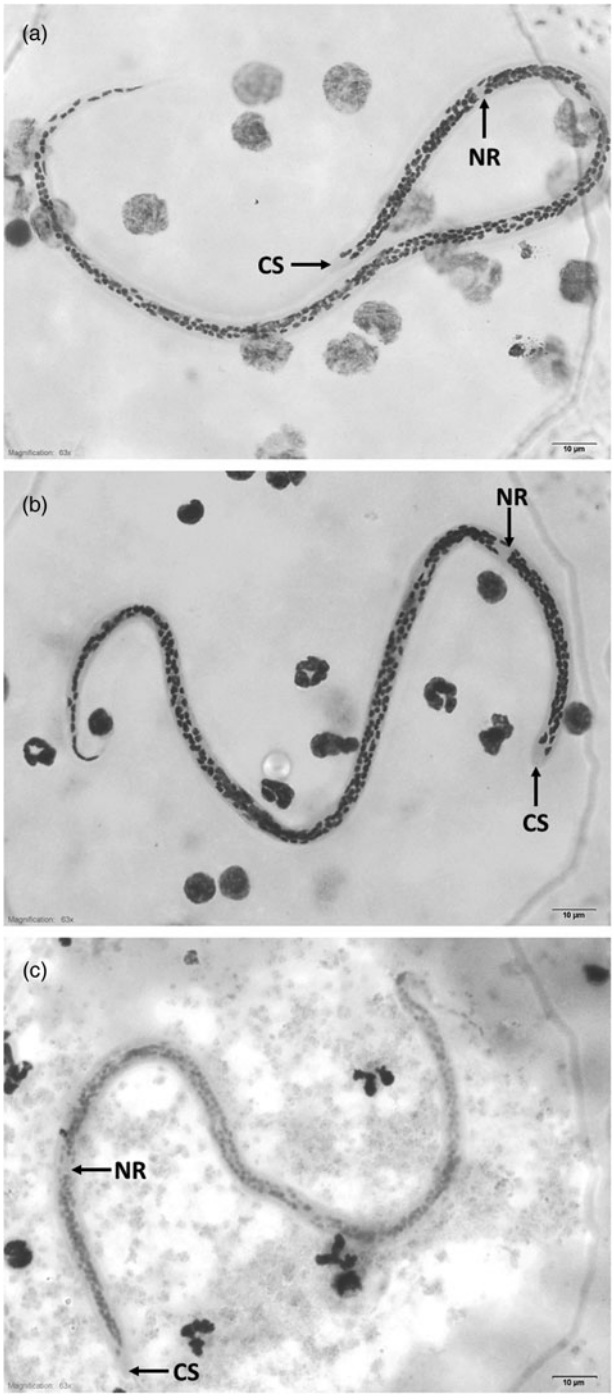

Fig. 1. A typical microfilaria from Thailand (a), Myanmar (b) and Lao PDR (c), observed at a final magnification of 100×. Scale bars: 10 µm. Abbreviations: CS, cephalic space; NR, nerve ring.

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Table 3. Morphometric measurements and counts of column nuclei between the cephalic space and nerve ring of microfilariae of *W. bancrofti* from Lao PDR, Myanmar and Thailand.

Measurements	Lao PDR (*n* = 100)	Myanmar (nocturnal periodic strain) (*n* = 97)	Thailand (nocturnal sub-periodic strain) (*n* = 50)
Body length	275.96 ± 24.77[a]	265.15 ± 21.68[a]	292.68 ± 12.36[a]
	(200–340)	(200–304.39)	(270–322.22)
Cephalic space length	5.42 ± 0.9[b]	4.38 ± 0.72	4.52 ± 0.94
	(3.57–8)	(3.33–6)	(3.33–8.33)
Cephalic space width	4.63 ± 0.94[b]	3.94 ± 0.65	3.9 ± 0.71
	(2.86–6.67)	(2.50–5)	(2.50–5.56)
Body width at nerve ring	5.83 ± 1.41[b]	4.30 ± 0.85	4.48 ± 0.75
	(3.33–8.33)	(2.50–6)	(3.33–5.83)
Innenkörper length	32.19 ± 11.12[a]	37.59 ± 5.53[a]	44.58 ± 7.58[a]
	(10–64.29)	(20.00–56.67)	(25–74.44)
Head to nerve ring	54.26 ± 5.77	50.64 ± 5.51[c]	53.74 ± 6.28
	(40–70)	(33.33–76.92)	(33.33–60.83)
Number of nuclei between cephalic space and nerve ring	54.74 ± 7.37[a]	52.20 ± 5.66[a]	72.06 ± 6.91[a]
	(40–76)	(40–72)	(62–95)

Measurements in μm ± SD. Range in parentheses.
[a]Significant difference among Lao PDR, Myanmar and Thailand.
[b]Significant difference between Lao PDR and each of the other two countries.
[c]Significant difference between Myanmar and each of the other two countries.

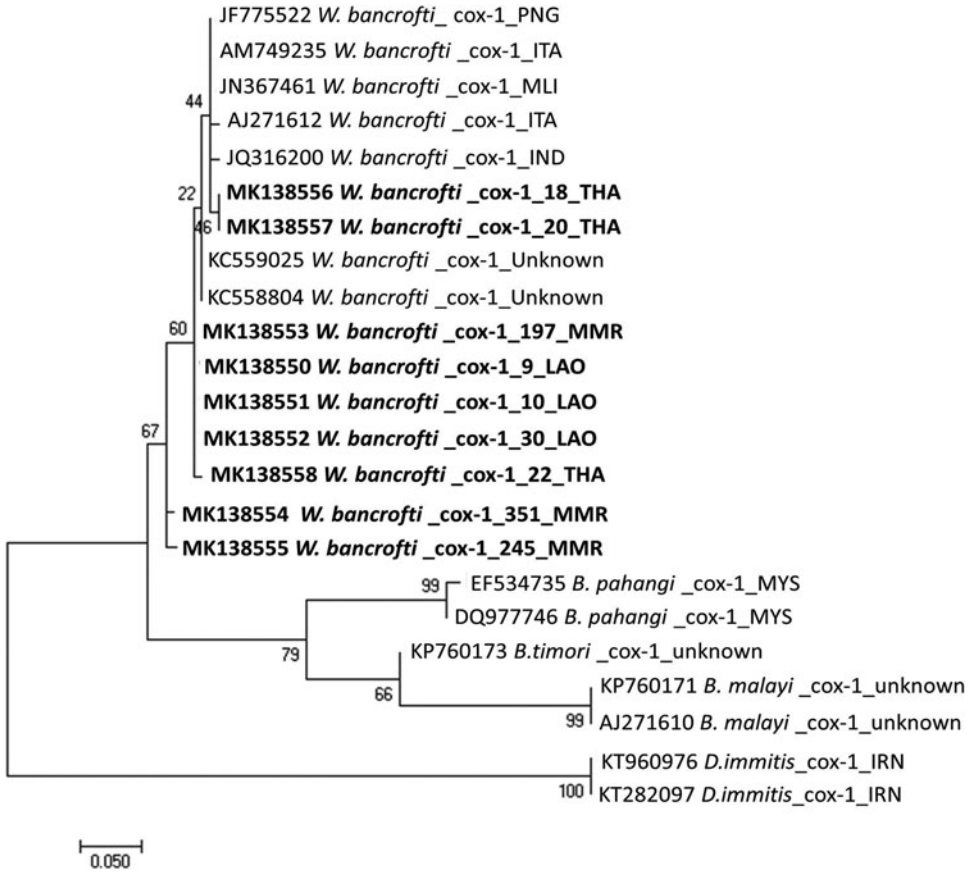

Fig. 2. The ML tree reconstructed from *cox*-1 gene sequences of *W. bancrofti* and other related species. Bootstrap scores (percentages of 1000 replications) are presented for each node. Sequences obtained in this study are indicated in bold font with accession numbers (MK138550–MK138558), species name, sample codes and country codes (ISO 3166-1 alpha-3 code). Other sequences from the DNA database are shown with accession numbers, species names and country codes. Scale bar indicated nucleotide substiutions per site.

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Microfilariae of this species are generally 244–296 μm in length and 7.5–10 μm in width. Other conspicuous features distinguish them from microfilariae of *B. malayi*: nuclei do not reach the tail end, there are no terminal nuclei and nuclei are regularly spaced and well dispersed. In addition, the body is bigger and wider than in *B. malayi*, exhibits graceful sweeping curves, a short head space and is bluntly rounded anteriorly and pointed caudally (Beaver *et al*., 1984).

Wuchereria bancrofti in the Karen ethnic group living near the Thai–Myanmar border is generally regarded as the nocturnally sub-periodic type (Thailand type), whereas the Myanmar strain is the nocturnally periodic type (Jitpakdi *et al*., 1999). The types are said to differ in morphology and use different mosquito vectors (Jitpakdi *et al*., 1999). The body length and cephalic space (length and width) of the Thai nocturnally sub-periodic strain of *W. bancrofti* were significantly larger than those of the Myanmar nocturnally periodic strain (Nuchprayoon *et al*., 2007).

Morphometric findings in the present study indicate that there is a wide geographical variation in length and width of microfilariae and in the number of nuclei between the cephalic space and nerve ring. This might result from either natural variability or reflect subspecies variations, which are currently undescribed in these countries. Analysis of many *W. bancrofti* populations from various geographical localities will be needed to clarify the situation. According to data in the previous study (Jitpakdi *et al*., 1999), the microfilariae we obtained from Thailand and Myanmar morphologically conform to the nocturnally sub-periodic and nocturnally periodic types, respectively. However, there are some discrepancies between our observations and those of Jitpakdi *et al*. (1999) and Nuchprayoon *et al*. (2007). We found the body dimensions and the numbers of nuclei between the cephalic space and nerve ring of both types to be smaller than previously observed (Jitpakdi *et al*., 1999). In contrast, the Innenkörper length of microfilariae from Thailand was larger than reported by Nuchprayoon *et al*. (2007).

Little is known about the periodicity of *W. bancrofti* microfilariae in Lao PDR. We observed that *W. bancrofti* microfilariae from Lao PDR have morphometric parameters overlapping those of Thailand and Myanmar strains. Several parameters relating to body dimension were very similar to those of the nocturnally sub-periodic type (Thailand strain), but the number of nuclei between cephalic space and nerve ring was closest to that in the nocturnally periodic type (Myanmar strain). In the absence of parasitological knowledge about microfilarial periodicity of *W. bancrofti* from Lao PDR, the differences in morphometric data need further investigation.

Random amplified polymorphic DNA (RAPD) markers can be used for differentiating Thailand and Myanmar strains of *W. bancrofti* (Nuchprayoon *et al*., 2007). Here, we successfully PCR-amplified DNA extracted from Giemsa-stained blood films using two primer pairs targeting short, overlapping fragments of the mitochondrial *cox*-1 gene. The samples had been stored at room temperature for nine or 17 years. Phylogenetic relationships among *W. bancrofti* and other related species based on their partial *cox*-1 sequences were constructed. All new *cox*-1 sequences (MK138550–MK138558) were located among known sequences of *W. bancrofti cox*-1 from the GenBank database, confirming them as belonging to *W. bancrofti*. However, the phylogeny we reconstructed differed from that based on RAPD markers by Nuchprayoon *et al*. (2007). Our *W. bancrofti cox*-1 sequences from Lao PDR, Myanmar and Thailand were located in the same clade, while the relationship inferred using RAPD markers identified two very distinct clusters (Nuchprayoon *et al*., 2007). This could be due to the use of different molecular methods in the two studies or possibly the *cox*-1 sequences do not allow easy discrimination between nocturnally periodic and nocturnally sub-periodic strains.

In conclusion, this study highlights the morphological differences among microfilariae and also the genetic variability within *W. bancrofti* from Lao PDR, Myanmar and Thailand. The obtained data are a contribution towards the understanding of bancroftian filariasis epidemiology in these countries. Further studies are needed to validate the biological data and assess the microfilarial periodicity in Lao PDR.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19000865.

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Conflicts of interest. None.

Ethical standards. The protocol was approved and registered by the Khon Kaen University Ethics Committee for Human Research, Khon Kaen, Thailand (HE601114).

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