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Molecular Phylogenetic Studies of the Genus *Brugia*

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MOLECULAR PHYLOGENETIC STUDIES ON *BRUGIA* FILARIAE USING *HHA I* REPEAT SEQUENCES

XIE H.*, BAIN O.** and WILLIAMS S. A.*,***

Summary :

This paper is the first molecular phylogenetic study on *Brugia* parasites (family Onchocercidae) which includes 6 of the 10 species of this genus : *B. beaveri* Ash et Little, 1964; *B. buckleyi* Dissanaïke et Paramanathan, 1961; *B. malayi* (Brug, 1927) Buckley, 1960; *B. pahangi*, (Buckley et Edeson, 1956) Buckley, 1960; *B. patei* (Buckley, Nelson et Heisch, 1958) Buckley, 1960 and *B. timori* Partono et al., 1977. *Hha I* repeat sequences are 322 nucleotides long, highly repeated, tandemly arranged and unique to the nuclear genomes of the genus *Brugia*. *Hha I* repeat sequence data was collected by PCR, cloning and dideoxy sequencing. The *Hha I* repeat sequences were aligned and analyzed by maximum parsimony algorithms, distance methods and maximum likelihood methods to construct phylogenetic trees. Bootstrap analysis was used to test the robustness of the different phylogenetic reconstructions. The data indicated that the *Hha I* repeat sequences are highly conserved within species yet differ significantly between species. The various tree-building methods gave identical results. Bootstrap analyses on the *Hha I* repeat sequence data set identified at least two clades : the *B. pahangi*-*B. beaveri* clade and the *B. malayi*-*B. timori*-*B. buckleyi* clade; the first clade includes parasites of carnivores from Asia and America; the second includes species from primates and lagomorphs from Asiatic region. It was also noted that the *Hha I* repeat sequences obtained from *B. malayi* were identical to those obtained from *B. timori*, indicating very recent speciation.

KEY WORDS : evolution, phylogenetics, PCR, molecular cloning, *Hha I* repeats, *Brugia malayi*, *B. pahangi*, *B. beaveri*, *B. timori*, *B. patei*, *B. buckleyi*.

There are ten species in the genus *Brugia*, six of which were available for study in this paper : *B. beaveri* Ash and Little, 1964; *B. buckleyi* Dissanaïke and Paramanathan, 1961; *B. malayi* (Brug, 1927) Buckley, 1960; *B. pahangi* (Buckley and Edeson, 1956) Buckley, 1960; *B. patei*

Résumé : ETUDES PHYLOGÉNÉTIQUES MOLÉCULAIRES DES FILAIRES DU GENRE *BRUGIA* À L'AIDE DE LA SÉQUENCE RÉPÉTÉE *HHA I*

Cet article est la première étude phylogénétique moléculaire sur les filaires du genre *Brugia* (Onchocercidae); elle inclut six des 10 espèces du genre : *B. beaveri* Ash et Little, 1964; *B. buckleyi* Dissanaïke et Paramanathan, 1961; *B. malayi* (Brug, 1927) Buckley, 1960; *B. pahangi* (Buckley et Edeson, 1956) Buckley, 1960; *B. patei* (Buckley, Nelson et Heisch, 1958) Buckley, 1960 et *B. timori* Partono et al., 1977. Les répétitions *HHA I* sont longues de 322 nucléotides, hautement répétées, arrangées en copies directes et propres aux génomes nucléaires des espèces du genre *Brugia*. Les répétitions *HHA I* ont été amplifiées par PCR, clonées et séquencées. Les séquences obtenues ont été alignées puis leur phylogénie reconstruite par les méthodes de parcimonie, de distance et de vraisemblance. Des analyses de bootstrap ont été utilisées pour tester la robustesse des différentes reconstructions phylogénétiques. Les données indiquent que les séquences de la répétition *HHA I* sont fortement conservées dans une même espèce alors qu'elles diffèrent significativement d'une espèce à l'autre. Les différents arbres construits par les trois méthodes ont donné des résultats identiques. Les analyses de bootstrap de l'ensemble des séquences de la répétition *HHA I* ont révélé au moins deux clades : le clade *B. pahangi*-*B. beaveri*, et le clade *B. malayi*-*B. timori*-*B. buckleyi*; le premier clade groupe deux espèces parasites de carnivores, d'Asie et d'Amérique; le deuxième des espèces de primates et de lagomorphes de région asiatique. Il est à noter que la séquence de la répétition *HHA I* de *B. malayi* est identique à celle obtenue pour *B. timori*, ce qui suggère une spéciation très récente.

MOTS CLES : évolution, phylogénèse, PCR, clonage moléculaire, répétitions *HHA I*, *Brugia malayi*, *B. pahangi*, *B. beaveri*, *B. timori*, *B. patei*, *B. buckleyi*.

(Buckley, Nelson and Heisch, 1958) Buckley, 1960 and *B. timori* Partono et al., 1977. The morphology and biology of the *Brugia* species are relatively well known, but the phylogenetic relationships among them are yet to be understood. It is important to understand the evolutionary relationships of the various filarial parasites, since this knowledge can help parasitologists in the design of model systems for species that are difficult to maintain in the laboratory. Furthermore, this study may provide data for morphologists (who base their classification systems entirely on parasite morphology) with a new means to reference their own conclusions. It is very difficult even for morphologists to identify the different species of *Brugia* at the infective stage (L3 larvae). To resolve fine taxonomical distinctions at the DNA

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level, highly repetitive DNA elements and other less conserved sequences can be very useful (Williams *et al.*, 1988). Because most highly repeated DNA sequences do not appear to code for any protein or RNA product, these sequences are able to evolve at a much faster rate than the coding region sequences of genes. McReynolds *et al.* (1986) did digests of purified *B. malayi* genomic DNA with a variety of restriction endonucleases. Repeated DNA sequences of 322 bp were observed when the DNA was digested with either *Hba* I, *Alu* I or *Rsa* I. The 322 bp repeat sequence was designated the "*Hba* I repeat". By subsequent dot hybridization and DNA renaturation kinetics experiments, it was determined that the *Hba* I repeats comprise about 12% of the DNA of *B. malayi* with a copy number of about 30,000 per haploid genome. This repeat is absent in other genera, including the very closely related genus *Wuchereria* (Williams *et al.*, 1988). Since the ribosomal DNA spacer region sequences were unable to resolve the phylogenetic relationships among the *Brugia* species in a previous study (Xie *et al.*, 1994), *Hba* I repeat sequences were used to investigate the phylogenetic relationships among the six species in the genus *Brugia*.

MATERIALS AND METHODS

PARASITE MATERIALS

Adult *B. pahangi* and *B. malayi* were obtained from Dr. J. McCall (TRS Laboratory, Athens, GA, U.S.A.). Adult *B. malayi* worms preserved in 70% ethanol were provided by Dr. O. Bain (Paris, France). *B. patei* adults were kindly provided by Drs. U.R. Rao and A.C. Vickery (University of Southern Florida, Gainesville, U.S.A.). *B. beaveri* adults preserved in 100mM EDTA were kindly provided by Dr. T.C. Orihel (Tulane University Medical Center, New Orleans, U.S.A.). *B. buckleyi* adults preserved in 70% ethanol were kindly provided by Dr. A.S. Dissanaike (Colombo, Sri Lanka). *B. timori* microfilariae preserved in 100mM EDTA were kindly provided by Dr. F. Partono (University of Indonesia, Jakarta, Indonesia). Morphological analyses were conducted to verify the identity of some of the specimens.

EXPERIMENTAL PROCEDURES

Extraction of filarial parasite DNA

Individual adult filarial parasites were picked from 70% ethanol solution and evaporated to dryness. The worms were resuspended in 50 µl of H₂O and boiled

for 10 minutes to release DNA. These preparations were used as the PCR templates. The same method was used to prepare PCR template from *B. timori* microfilariae.

Amplification of Hba I repeat DNA sequences from filarial parasite DNA

Two PCR primers matching the consensus sequences of the conserved regions of the *Hba* I repeat were synthesized by the phosphoramidite method using the Applied Biosystem 391A DNA synthesizer (Foster City, CA, U.S.A.). The sequences of the two primers were :

Primer 1: 5'-GCGCATAAATTCATCAGC-3';

Primer 2: 5'-GCAAACTTAATTACAAAAGC-3'.

All PCR reagents except primers, templates and double distilled water (ddH₂O) were from the GeneAmp PCR kit purchased from Perkin-Elmer Cetus (Norwalk, CT, U.S.A.). The PCR reaction contents included : 5 µl of 10x reaction buffer, 8 µl of 1.25mM dNTP mixture, 20 pmoles of each primer, 1-2 µl template DNA solution, 1 unit of *Taq* polymerase and ddH₂O added to a total volume of 50 µl. The PCR cycling programs used were: 93°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute for a total of 30 cycles (Model 480 thermal cycler, Perkin-Elmer Cetus, Norwalk, CT, U.S.A.); and 93°C, 0.5 minute; 55°C, 0.5 minute; 72°C, 0.5 minute for a total of 30 cycles (System 9600 thermal cycler, Perkin-Elmer Cetus, Norwalk, CT, U.S.A.).

Cloning and sequencing of the PCR amplified Hba I repeat sequences and sequence data analysis

The details of the procedures were described in our previous paper (Xie *et al.*, 1994). Please refer to it for more information.

RESULTS

DNA SEQUENCE COLLECTION AND ALIGNMENT

The head-to-tail tandem organization of *Hba* I repeats is shown in Fig. 1. At least three sequences were obtained for each of the six species. These sequences plus previously unpublished sequence data from our database were used for deriving consensus sequences. *Hba* I repeat sequences within each species share 99% similarity (data not shown). Consensus sequences for each of the six species were derived and aligned to one another. The portion of the aligned sequence data matrix which is the input for the tree reconstruction algorithms is shown in Table I.

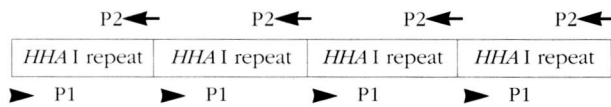


Fig. 1 – *Hba* I repeat structure. PCR primer positions are illustrated by arrows. P1 is *Hba* I primer 1; P2 is *Hba* I primer 2.

PHYLOGENETIC TREE RECONSTRUCTION

Analysis by maximum parsimony method (Swofford, 1993)

Search settings :

Exhaustive search option was used. Gapmode was set to newstate. Branches having maximum length

zero was collapsed to yield polytomies; topological constraints were not enforced; trees were unrooted; multi-state taxa was interpreted as polymorphism; character-state optimization was by accelerated transformation.

Search results :

One shortest tree of length 87 was found in this search (Fig. 2), which is one step shorter than the next two trees. The next two trees are both 88 steps long. They differ from the shortest tree in their relative positions of *B. buckleyi* and *B. timori*. The statistics of the shortest tree are the following (Kluge and Farris, 1969; Farris, 1989): Consistency index (CI) =

```
Constant : * * * * *
Uninf. : * * * * *
B.beaveri A A T T A A T G A T G G - T T T A A T T C A - A T T T A A G A A T T A A A A T T A A A T T T T A T T T C A A A T T
B.pahangi A A T T A A T G A T G G T T T T A A T T C A - A T T T A A G A A T T A A A A T T A A A T T T A A A T T C A A A T T
B.malayi A A T T A A T C A T G A T T T T A A T T C A - A T T T A A G A A T T T A A A A T T A A A T T T A A A T T C A A A T T
B.timori A A T T A A T C A T G A T T T T A A T T C A - A T T T A A G A A T T T A A A A T T A A A T T T A A A T T C A A A T T
B.patei A A T T A A T C A T G G T T T T A A T T C A - A T T T A T G A A T T A A A T T T A A A T T T A A A T T C A A A T T
B.buckleyi A A T T A A T C A T G A T T T T A A T T C A T G T T T A A G A A T T A A A A T T A A A T T T A A A T T C A A A T T
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```
Constant : * * * * *
Uninf. : * * * * *
B.beaveri T A A A T T T T G A A - T T T T T T A A A A A T T T T A A A A T T T A T G A T A G T T T T C R T A C A T T T T A A C
B.pahangi T A A A T T T T G A A - T T T T T T A A A A A T C T T A A A A T T T G T G A T A G T T T T C T T A C A T T T T A A C
B.malayi T A A A T T T T G A A - T T T T T T A A A A A T T T T A A A A T T T G T T A T A G T T T T C T T A C A T T A G A C
B.timori T A A A T T T T G A A - T T T T T T A A A A A T T T T A A A A T T T G T T R T A G T T T T C T T A C A T T A G A C
B.patei T A A T T T T T G A A - T T T T T T G A A A A T T T T - A A A T T T G T T A T A G T T T T C T T A C A T T A A A C
B.buckleyi T A A A T T T T G A A A T T T T T T A A A A A T T T T A A A A T T T T T A T A A T T T T C C T A C A C T A G A C
```

```
Constant : * * * * *
Uninf. : * * * * *
B.beaveri A A G A A A A T T G G T T C T A A A T T A T C A A W T T T A W T T T T A A T T A W G T G C S A A A A C C A T T A A
B.pahangi A T G A A A A T T G G T T C T A A A T T A T C A A T T T T A A T T T T W A T T A A G T S T C A A A A G T G C T A A
B.malayi A A G G A A A T T G G T T C T A A T T T A T C A A T T T T A A T T T T A A T T A A G T G C C A A A A C T R C T - A
B.timori A A G G A A A T T G G T T C T A A T T T A T C A A T T T A A T T T A A T T A A G T G C C A A A A C T A C T - A
B.patei A G G A A A A T T G G T T T T A A T T T A T C A A T T T T A A T T T T A A T T A A G T G C C A A A A C T A C T - A
B.buckleyi A A G G A A A T T G G T T C T A A T T T A T C A A T T T T A A T T T T A A T T A A G T G C C A A A A C T G C T A A
```

```
Constant : * * * * *
Uninf. : * * * * *
B.beaveri A A A T A G C A A T A T T T T G A A C T T A A C T G A T T A T G T T G T A T G C A T T G C A C Y A G G G C G G G T
B.pahangi A A A T A G C A A T A T T T T G A A G T T A A T T C A T T A T G T T T T A T G C A T T G C A C T G G G G C R K G T
B.malayi A A A A A A G C T T A T T T T G A A M T T A A T T G A C T A T G T T A C G T G M A T T G T A C C A G T G C T G G T
B.timori A A A A A A G C T T A T T T T G A A A T T A A T T G A C T A T G T T A C G T G A A T T G T A C C A G T G C T G G T
B.patei A A A A A A G C T T A T T T T G A A C T T A A T T G A C T A T G T T A C G T G C A T T G T A C C A G T G C T G G T
B.buckleyi A A A A A A G C T T A T T T T G A A C T T A A T T G A C T A C G T T A C G T G C A T T G T A C C A G T G C T G G C
```

```
Constant : * * * * *
Uninf. : * * * * *
B.beaveri C A T A Y A T G G T G T G G T C A T T T T A T A G T T T A
B.pahangi C A T A T A T G G T - T G G T C A T T T T A T A G T T T A
B.malayi C G T A T A T T G C G T T G T C A T T T T A T A G T T T A
B.timori C G T A T A T T G C G T C G T C A T T T T A T A G T T T A
B.patei C A T A T A T T G T A T T T T C A T T T T A G A G T T A A
B.buckleyi C G T A T A T T G C G T T G T C A A T T T A T A G T T T A
```

Table I. – *Hba* I repeat DNA sequence data matrix used for phylogenetic tree reconstruction. There are 257 nucleotide positions in the data matrix. Twenty seven of them (about 10%) are informative. (“-” : gap; Constant: invariable site; Uninf.: uninformative site, as calculated by PAUP 3.1)

0.977; CI excluding uninformative characters = 0.708; Retention index (RI) = 0.931; Rescaled consistency index (RC) = 0.910.

Analysis by Neighbor-joining method (Saitou and Nei, 1987) and by Maximum likelihood method (Felsenstein, 1981 and 1993)

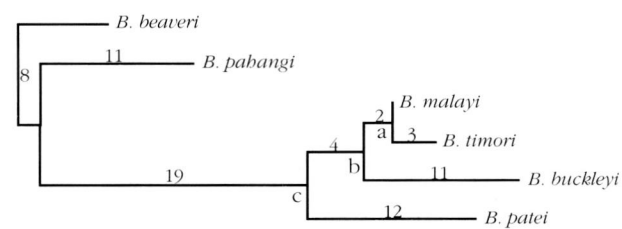


Fig. 2. – Exhaustive search tree found using PAUP 3.1. The numbers above the branches indicate the branch lengths calculated by PAUP 3.1. Small case letter 'a', 'b' and 'c' indicate the clades identified by the bootstrapping analysis.

Both neighbor-joining and maximum likelihood methods yield trees identical to the tree generated by the parsimony analysis (Fig. 2). They are not shown here.

Analysis by bootstrap methods (Felsenstein, 1985)

Bootstrap analyses were done with parsimony algorithm, neighbor-joining algorithm and with maximum likelihood algorithm (Table II and Fig. 2). All the three methods give very similar results. It is clear from these analyses that the *B. malayi*-*B. timori*-*B. buckleyi* clade, *B. malayi*-*B. timori*-*B. buckleyi*-*B. patei* clade and the *B. pahangi*-*B. beaveri* clade are strongly supported (Fig. 2 and Table II).

Clade	a	b	c
Parsimony	78%	98%	100%
Neighbor-joining	83%	94%	100%
Maximum likelihood	90%	95%	100%

Table II. – Summary of the bootstrap values. a, b and c are the clades noted in Figure 2.

DISCUSSION

DNA SEQUENCE DATA COLLECTION

The *Hba* I repeat sequences were chosen to examine the phylogenetic relationships of the *Brugia* species because it was shown that *Hba* I repeats possess species-specific regions within the repeat sequences. Most of the parasite materials

were available only in very limited quantities for this study, so it was not practical to use traditional methods to extract genomic DNA and to perform endonuclease digestion and cloning. A very effective method was developed using PCR technology. This method proved very efficient and reliable. For *Hba* I repeat sequences, no difference was seen in sequence data collected by the PCR cloning method and the traditional genomic DNA cloning method (data not shown). To further test the error rate of *Taq* polymerase used in PCR reactions, one already sequenced *Hba* I repeat clone was subjected to PCR and direct cloning procedures. Four clones were randomly selected and sequenced using the CircumVent Thermal Cycle Sequencing Kit. No nucleotide misincorporation was detected from the 1200 bases sequenced.

TREE RECONSTRUCTION ALGORITHMS

In this study, three main categories of tree reconstruction methods were used: parsimony, distance and maximum likelihood analyses. The *Hba* I repeat sequence data set gave very similar tree topologies no matter which phylogenetic reconstruction algorithms were used.

Parsimony analysis : Because the sequence region studied was non-coding, all of the characters were equally weighted. The same tree was obtained even when transversions were weighted twice as heavy as transitions (data not shown). Different sequence addition methods were tried and all gave the same topology. Multi-state taxa were generally treated as polymorphism according to the sequence data. However, the same topologies were still obtained even if multi-state taxa were treated as uncertainty, in which case the consistency indices were improved dramatically (data not shown). This observation is due to the presence of a large amount of nucleotide polymorphism in the data set. The tree length distribution is heavily skewed (data not shown) indicating that a significant degree of phylogenetic information is contained in the data set (Hillis, 1991). The exhaustive search yielded one shortest tree (Fig. 2) which unambiguously resolved the branching order of the six species of *Brugia*. However, this tree was not rooted because there is no outgroup available (*Hba* I repeats were only found in *Brugia* species).

Distance analysis and maximum likelihood analysis : Distance methods including UPGMA (Sneath and Sokal, 1973), neighbor-joining, Fitch-Margoliash (Fitch-Margoliash, 1967) methods and the maximum likelihood analysis were used in this analysis. Distance matrices were calculated with the maximum

likelihood method to transform the observed pairwise distances. All of the methods gave identical tree topologies (data not shown). These results are in good agreement with the exhaustive search tree obtained by the parsimony method (Fig. 2).

CONFIDENCE LIMITS ON PHYLOGENIES AND THE HYPOTHESIS

In this study, algorithms from all three major categories were applied to the bootstrapped replicates. All three bootstrap analyses (Table II) support the grouping of the *B. malayi*-*B. timori*-*B. buckleyi* clade, *B. malayi*-*B. timori*-*B. buckleyi*-*B. patei* and the *B. beaveri*-*B. pabangi* clade which clearly identified the branching pattern of the six *Brugia* species (Fig. 2).

The *Hba* I repeat sequence data in this study showed distinctive patterns with respect to individual species (Except *B. malayi* and *B. timori*, Table I). This is a good example of the congruence among morphological data. *B. timori* and *B. malayi* were shown to be very closely related from both the *Hba* I repeat data and the 5S rDNA spacer region sequence data (Xie *et al.*, 1994). Both the 5S spacer region sequences and the *Hba* I repeat sequences from the two species are virtually identical. Morphologically (Partono *et al.*, 1977), these two species distinguish themselves from the other *Brugia* species by having a left to right spicular ratio of 3.0 (in the males) which is the biggest in the genus. The microfilariae of *B. timori* differ from the microfilariae of *B. malayi* in having a greater total body length and a long cephalic space. Overall, these two species are the most closely related among the *Brugia* species morphologically and molecularly. These two species may have been separated too recently to show any DNA sequence variation between them.

It should be noted that *B. malayi* has a relatively wide distribution in Southeast Asia and has marked intraspecific variations both morphologically (Bain *et al.*, 1988) and biologically (e.g. periodicity). *Hba* I repeat sequences from *B. malayi* were also collected with regard to different geographical origin with different morphology (strains from China and India), to their periodicity and to their host specificity by Freedman (1991) and Xie (1993). The molecular data failed to reveal any variations among the different strains of *B. malayi*.

It should also be noted that of the three species from carnivores (*B. pabangi*, *B. patei* and *B. beaveri*), only *B. pabangi* (localized in the oriental region) and *B. beaveri* (localized in the nearctic region) are closely connected; Oriental species from humans and other mammals (*B. malayi* and *B. timori*) are closely

connected to the species from Lagomorphs (*B. buckleyi*). More studies are necessary if one is to elucidate the more detailed evolution pathways of the *Brugia* species.

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