

Cloning and comparison of repeated DNA sequences from the human filarial parasite *Brugia malayi* and the animal parasite *Brugia pahangi*

(DNA probe/diagnosis/hybridization/nematodes/speciation)

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ABSTRACT A 320-base-pair repeated sequence was observed when DNA samples from the filarial parasites *Brugia malayi* and *Brugia pahangi* were digested with the restriction endonuclease *Hha* I. A 640-base-pair dimer of the repeated sequence from *B. malayi* was inserted into the plasmid pBR322. When dot hybridization was used, the copy number of the repeat in *B. malayi* was found to be about 30,000. The 320-base-pair *Hha* I repeated sequences are arranged in direct tandem arrays and comprise about 12% of the genome. *B. pahangi* has a related repeated sequence that cross-hybridizes with the cloned *B. malayi* *Hha* I repeat. Dot hybridization with the cloned repeat shows that the sequence is present in *B. malayi* and in *B. pahangi* but not in four other species of filarial parasites. The cloned repeated DNA sequence is an extremely sensitive probe for detection of *Brugia* in blood samples. Hybridization with the cloned repeat permits the detection of DNA isolated from a single parasite in an aliquot of blood from animals infected with *B. malayi*. There are differences in the restriction sites present in the repeated sequences that can be used to differentiate between the two *Brugia* species. The *B. malayi* repeated DNA sequence is cleaved by *Alu* I and *Rsa* I but the *B. pahangi* sequence is not. A comparison of repeated sequences between the two species by DNA sequence analysis indicates that some regions of individual repeats are over 95% homologous, while other short regions are only 60-65% homologous. These differences in DNA sequence will allow the construction of species-specific hybridization probes.

Filarial nematodes cause chronic infections in about 400-million people living in tropical regions of the world (1). The parasites are transmitted to the human host by blood-sucking arthropod vectors such as mosquitoes or black flies. There are at least seven species of filarial parasites that infect humans. These have different insect vectors, variable host ranges, and different degrees of pathogenicity (2). *Onchocerca volvulus*, for example, is transmitted by the black fly and can cause a pathological eye condition that leads to blindness. *Brugia malayi* and *Wuchereria bancrofti* are transmitted by a mosquito vector and can cause lymphatic blockage that leads to elephantiasis.

To control a parasitic disease effectively, the nature and scope of the parasite problem in an endemic region must be assessed. Collection of detailed and accurate epidemiological data is hampered, however, by difficulties encountered in detecting and identifying filarial parasites in human, animal, and insect populations (3). There is currently no fast, reliable, and sensitive biochemical or immunological method for distinguishing closely related species or subspecies of filarial parasites. The difficulty in distinguishing the human filarial parasite *B. malayi* from the animal parasite *Brugia pahangi*

in regions where both species are endemic is an example of this problem.

This paper describes the cloning and characterization of members of a DNA repeated-sequence family from both *B. malayi* and *B. pahangi*. These repeats can be used in a very sensitive DNA hybridization assay to detect *Brugia* parasites. Differences in the restriction-enzyme cleavage sites in the repeated DNA allow *B. malayi* to be distinguished from *B. pahangi*. Additionally, the DNA repeated-sequence data demonstrates the presence of nucleotide differences between the two species that should prove useful in the construction of species-specific DNA hybridization probes.

MATERIALS AND METHODS

Isolation of DNA from Parasites. Frozen *B. malayi* adults were provided by Eric Otteson (National Institutes of Health). Adult *Onchocerca volvulus* parasites were obtained from Jeffrey Williams (Michigan State University). Infected blood samples and all other parasites were supplied by John McCall (TRS Laboratory, Athens, GA) (4, 5). DNA was isolated from frozen adult female worms or from microfilariae in whole blood by the proteinase K-digestion method of Emmons *et al.* (6) followed by phenol extraction. For the Southern blots and cloning experiments, the DNA was further purified by ethidium bromide/CsCl centrifugation (7), which removed a band of white material, possibly from the cuticle, that was more dense than the DNA.

Cloning of Repeated DNA from *B. malayi*. All restriction endonucleases, methylases, ligases, linkers, and ³⁵S-labeled dideoxy sequencing reagents used in these experiments were prepared at New England Biolabs and used as described by the supplier. *B. malayi* DNA was cleaved with *Alu* I, protected with *Eco*RI methylase, and then ligated to 5'-phosphorylated *Eco*RI linkers with T4 DNA ligase. Cohesive *Eco*RI ends were generated by digestion with *Eco*RI. The linkers were separated from the *Brugia* DNA by electrophoresis on a 1.5% low melt agarose gel stained with ethidium bromide. The 320-base-pair (bp) and 640-bp restriction fragments were cut out and the DNA isolated (8). In two separate reactions, either the 640-bp or the 320-bp fragments were ligated to *Eco*RI cleaved pBR322 that had been dephosphorylated by treatment with bacterial alkaline phosphatase (9). *Escherichia coli* RRI cells were transformed with the recombinant plasmids, selected for growth on ampicillin, and screened for repeated sequences by hybridization with radioactively labeled *B. malayi* DNA (10). Plasmid DNA (pBma68), which was isolated (7) from the clone that hybridized to the genomic probe, was characterized by restriction enzyme digestion.

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Abbreviation: bp, base pair(s).

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Southern and Dot Hybridizations. Southern hybridizations were performed as described (11) except that the DNA was partially depurinated (12) prior to transfer. The baked nitrocellulose filters were incubated in $10\times$ Denhardt's solution ($1\times$ Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinyl pyrrolidone) (13) and then hybridized to pBma68 nick-translated (14) to a specific activity of 10^7 cpm/ μ g with [α - 32 P]dATP. The filter was washed with $2\times$ NaCl/Cit twice at room temperature and once for 30 min at 60°C ($1\times$ NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). For dot hybridizations, DNA was denatured in 0.3 M NaOH for 10 min at room temperature. An equal volume of 2 M ammonium acetate was added, and the samples were incubated on ice and then were spotted onto nitrocellulose filters (15). The filters were baked, hybridized, and washed as described for Southern hybridizations.

Detection of Microfilariae in Blood by DNA Hybridization. DNA was extracted from 1.0-ml samples of whole blood by using an abbreviated protocol that did not include a CsCl centrifugation step (6). Blood samples were obtained from uninfected cats, cats infected with *B. malayi* (14,500 microfilariae/ml), and dogs infected with *Dirofilaria immitis* (53,900 microfilariae/ml). The samples were extracted with phenol and chloroform until all of the heme color was removed. Aliquots of the sample (including 200 ng of calf thymus DNA added as carrier) were spotted onto nitrocellulose filters and hybridized with the pBma68 probe as described above for dot hybridizations.

Subcloning *B. malayi* Repeated DNA in M13mp8. Purified, 640-bp repeated DNA isolated from the pBma68 clone was cleaved in a partial *Dra* I digest, ligated to *Sma* I cut M13mp8, and transformed into *E. coli* strain JM101. Plaque filter hybridization was performed as described by Messing (16) by using nick-translated pBma68 as the hybridization probe. Clones that hybridized to the probe were plaque purified and single-stranded virion DNA was prepared for sequencing.

Cloning of Repeated DNA from *B. pahangi*. *B. pahangi* genomic DNA was cleaved with *Hin*PI (an isoschizomer of *Hha* I), ligated to *Acc* I-cleaved M13mp8, and transformed into *E. coli* strain JM101. Filter hybridization of the plaques to duplicate filters was performed as described (16). One set of filters was hybridized to nick-translated (14) *B. malayi* repeat (pBma68) and the duplicate set to nick-translated *B. pahangi* genomic DNA. Clones that hybridized to both probes were selected as putative *B. pahangi* repeats related to the cloned *B. malayi* sequence. Single-stranded virion DNA was isolated from the positive clones as template for sequence analysis.

DNA Sequence Analysis. DNA sequence analysis was performed using the Sanger dideoxy method as described by Messing (16) except that deoxyadenosine 5'-[α - 35 S]thio]triphosphate was used instead of [α - 32 P]dATP (17).

RESULTS

A search was made for repeated DNA sequences in *B. malayi* by digestion of the purified DNA with a variety of restriction endonucleases. Repeated sequences of 320 bp and 640 bp were observed when genomic DNA was digested with either *Hha* I, *Alu* I, or *Rsa* I (Fig. 1). Simultaneous digestion of *B. malayi* DNA with two restriction endonucleases allowed construction of a restriction map of the repeated sequence (Fig. 1). The results of the double digestions suggest that all three enzymes define the same sequence, which is arranged as a direct tandem repeat. There is, however, heterogeneity in the restriction sites in the repeated sequences. When an excess of restriction endonuclease is used in the single-enzyme digests, dimers of the 320-bp band are observed. Uncut monomer is also seen in the double enzyme digests (Fig. 1). This suggests that *Hha* I, *Alu* I, and *Rsa* I sites are

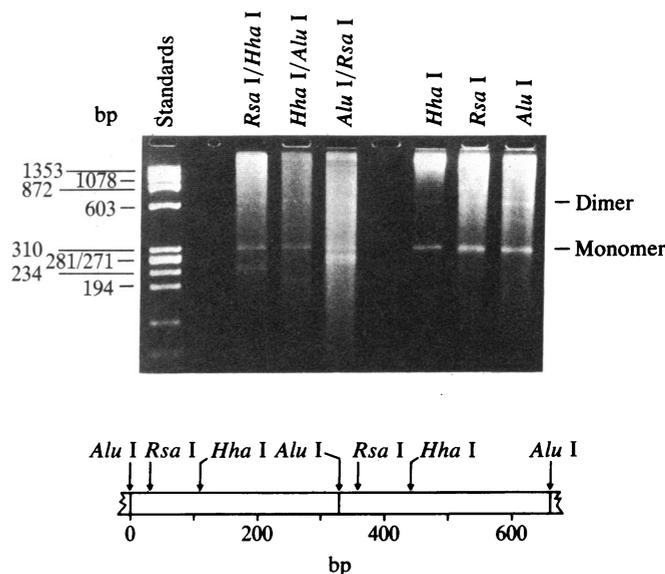


FIG. 1. Restriction map of the *B. malayi* *Hha* I repeated sequence. The genomic DNA was digested with one enzyme (*Hha* I, *Rsa* I, or *Alu* I) or with a combination of two of these enzymes. The digests were analyzed by electrophoresis on a 3% (wt/vol) agarose gel and visualized with 0.5 μ g of ethidium bromide/ml. The standard is ϕ X174 DNA digested with *Hae* III. The bottom of the figure shows the genomic restriction map of the *Hha* I 320-bp repeat.

present in most, but not all, members of the repeat family in *B. malayi*.

A dimer of the *B. malayi* repeat was cloned in pBR322. The plasmid pBma68 contains two inserts, 560 bp and 640 bp, which are excised when digested with *Eco*RI (Fig. 2). Hybridization of radioactively labeled genomic *B. malayi* DNA to the two excised fragments showed that only the 640-bp insert was a repeated sequence. A comparison of the restriction maps of the genomic and cloned repeats (Figs. 1 and 2) shows a similar arrangement of restriction sites. The genomic sequence has restriction endonuclease sites that are

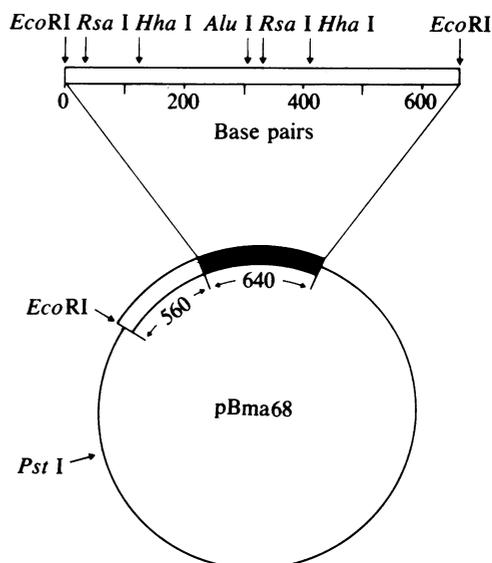


FIG. 2. Restriction map of the *Hha* I repeated sequence dimer cloned from *B. malayi*. Of the two inserts, only the 640-bp insert, shown in black, contains repeated sequence DNA. The *Pst* I site of pBR322 is included to show the orientation of the inserted DNA fragments. The restriction map of the repeated insert is shown at the top of the figure.

multiples of 320 bp apart. The cloned sequence is a dimer of the 320-bp repeat.

To determine if the plasmid with the cloned repeated sequence (pBma68) contained a species-specific repeat, it was hybridized to DNA from other filarial parasites. Only *B. malayi* DNA and *B. pahangi* DNA hybridized to the cloned repeat (Fig. 3). Species representing four other genera of parasites: *D. immitis*, *Dipetalonema viteae*, *Litomosoides carinii*, and *O. volvulus* showed no detectable cross hybridization at a level of sensitivity of 0.1%.

An analysis of the restriction sites in the *Hha* I repeated sequence revealed differences between the two *Brugia* species. Genomic DNA isolated from *B. malayi* and *B. pahangi* was digested with *Alu* I, *Rsa* I, *Hha* I, and *Msp* I and separated by gel electrophoresis. The repeats were visualized by hybridization to pBma68 (Fig. 4). *Alu* I and *Rsa* I cleave *B. malayi* DNA to give multiples of the 320-bp repeated sequence. However, the repeated sequence in *B. pahangi* is not cleaved by these two enzymes. On the other hand, *Msp* I cleaves 10 times as many *B. pahangi* repeats as *B. malayi* repeats. Since *Hha* I cleaves most members of the repeated sequence family in both species, the family is designated the *Hha* I repeat family. Many of the digestions of both *B. malayi* and *B. pahangi* DNA (Fig. 4) reveal a ladder of 320-bp repeats. Such a pattern suggests that most members of the *Hha* I repeat family are organized in direct tandem arrays.

The copy number of the *Hha* I repeated sequence and the genome size of *B. malayi* were estimated by comparing the intensity of hybridization of different cloned inserts to genomic DNA (Table 1). The genomic hybridization was compared to standards that contained serial dilutions of the unlabeled insert in the presence of carrier DNA (15). The copy number of the 320-bp *Hha* I repeat in *B. malayi* was estimated to be 30,000. The size of the *B. malayi* genome calculated from this experiment was 80 million bp, the same as the free-living nematode *Caenorhabditis elegans* (18). The *Hha* I repeat family comprises about 12% of the *B. malayi* genome or about 10-million bp.

Dot hybridization using the cloned *Hha* I repeated sequence as a probe was shown to be a very sensitive method for detecting microfilariae in the blood of infected animals. DNA was isolated from 1.0 ml of blood from cats infected with *B. malayi*, from dogs infected with *D. immitis*, and from uninfected cats. The cloned 640-bp repeated DNA served as the positive control. DNA isolated from the equivalent of a few microliters of blood was spotted onto nitrocellulose filters. The most concentrated samples (see 10⁰ dilutions, Fig. 5) from *B. malayi* contained the equivalent of 42 microfilariae and from *D. immitis* 72 microfilariae. Hybridization was detectable in a 1:100 dilution of the *B. malayi* sample that contained DNA from less than one microfilaria. The intensity of this hybridization dot was equivalent to 10 pg of the 640-bp repeated DNA. The hybridization was specific for *B. malayi* DNA and the control containing the 640-bp repeated DNA. There was no detectable hybridization to the uninfected

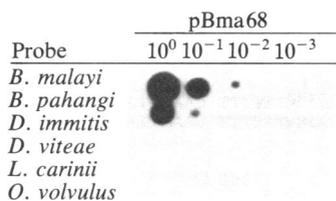


FIG. 3. Autoradiograph of cloned *Brugia* repeated DNA hybridized to DNA isolated from different species of filarial parasites. The radioactive pBma68 probe was hybridized to various concentrations of DNA isolated from six different species. The 10⁰ dilution contained 225 ng of DNA from each species.

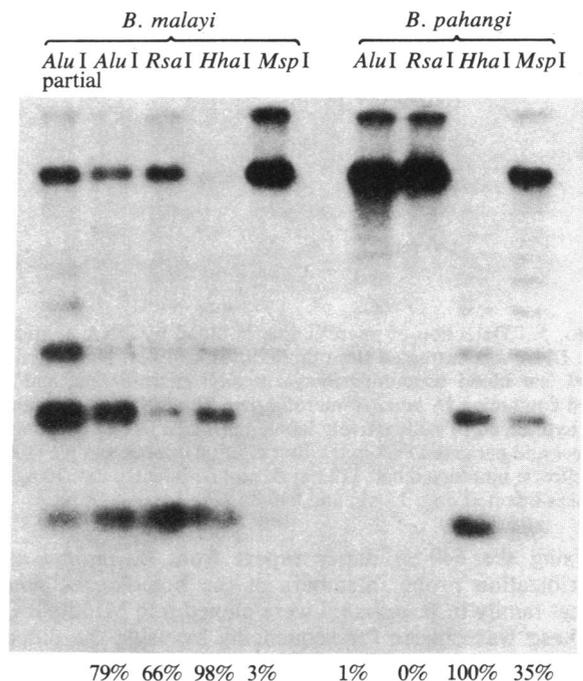


FIG. 4. Restriction analysis of repeated DNA from *B. malayi* and *B. pahangi*. Genomic DNA was digested with *Alu* I, *Rsa* I, *Hha* I, or *Msp* I, separated on a 1.2% agarose gel, denatured, transferred to nitrocellulose, and then hybridized with nick-translated cloned repeated DNA (pBma68). The figure is an autoradiograph of the hybridization. Standards, not shown, indicate that the lowest band is the 320-bp monomer. The number at the bottom of each lane reflects the degree of cleavage of the *Hha* I repeat family by the various restriction endonucleases. The absorbance of the monomer, dimer, and trimer bands in each lane is summed and then divided by the total absorbance in that lane to give a percentage value.

blood or to blood containing *D. immitis* (Fig. 5). The absence of hybridization to the control samples demonstrates that cat DNA, dog DNA, and calf thymus DNA (which was used as carrier) do not hybridize to the cloned repeat.

The DNA sequence of the 640-bp repeat from pBma68 was determined by subcloning into M13mp8. Regions of overlap from nine subclones allowed the construction of the entire 640-bp sequence. The two copies of the *Hha* I repeat from *B. malayi* are virtually identical, except that one of the two copies has an 11-bp deletion. The full length copy is 322 bp long and is 79% A-T (Fig. 6). The DNA sequence confirms the location of the *Alu* I, *Rsa* I, and *Hha* I sites shown in the restriction map (Figs. 1 and 2).

Table 1. Genomic frequency of DNA segments cloned from *B. malayi*

Plasmid	Insert size, bp	Frequency per genome	Copy number per genome
pBma68	640	1.2 × 10 ⁻¹	15,000*
pBma31	320	1.5 × 10 ⁻⁵	4
pBma33	290	4.0 × 10 ⁻⁶	1
pBma61	640	8.0 × 10 ⁻⁶	1

Four different clones containing *B. malayi* DNA inserted into pBR322 were cleaved with *Eco*RI and the inserts isolated. Serial dilutions of the insert DNA were hybridized with the homologous plasmid as probe. A scan of the autoradiograph was used to calculate the frequency of each insert fragment in the *B. malayi* genome. The copy number calculations are based on the assumption that the inserts in pBma33 and pBma61 are single copy (15).

*15,000 copies of the 640-bp dimer equal 30,000 copies of the 320-bp monomer.

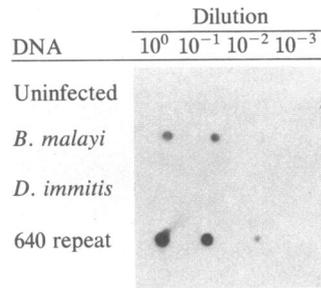


FIG. 5. Detection of microfilariae in blood by DNA hybridization. DNA was extracted from three blood samples: uninfected cat blood, cat blood containing *Brugia malayi* microfilariae, and dog blood containing *D. immitis* microfilariae. The DNA was denatured and hybridized to radioactively labeled pBma68 DNA. The amount of host and parasite DNA in the first dilution of each sample (10^0) is as follows: uninfected cat, 112 ng; *B. malayi*-infected cat, 70 ng; *D. immitis*-infected dog, 15 ng; and 640-bp *Hha* I repeat, 1 ng.

Using the 640-bp dimer repeat from *B. malayi* as a hybridization probe, members of the homologous *Hha* I repeat family in *B. pahangi* were cloned into M13mp8. One of these was chosen for sequencing by using the dideoxy chain termination method. The *B. pahangi Hha* I repeat is also 322 bp long and is 80% A·T. The DNA sequence confirms the absence of the *Alu* I and *Rsa* I sites in the *B. pahangi* repeat. A comparison of the *Hha* I repeated sequences from the two *Brugia* species shows that they are highly homologous (Fig. 6). There are two large regions of homology; region 155–230 has only 8 differences (89% homology), and region 276–139 has 11 differences (94% homology). There are 2 smaller regions of the repeat that have diverged significantly; region 140–154 has 6 differences (60% homology), and region 231–275 has 16 differences (64% homology). These two regions of divergence can be seen graphically in Fig. 7. It is interesting to note that the second region of nucleotide difference (231–275) has a much higher G·C content (43% for *B. pahangi* and 47% for *B. malayi*) than the total repeat (20% G·C).

DISCUSSION

The *Hha* I repeated DNA family found in *B. malayi* and *B. pahangi* is arranged as a direct tandem repeat of 320-bp. The repeated sequence can be observed by gel electrophoresis when *B. malayi* genomic DNA is cleaved with *Alu* I, *Rsa* I, or *Hha* I or when *B. pahangi* genomic DNA is cleaved with *Hha* I or *Msp* I (Figs. 1 and 3). A 640-bp dimer of the repeat

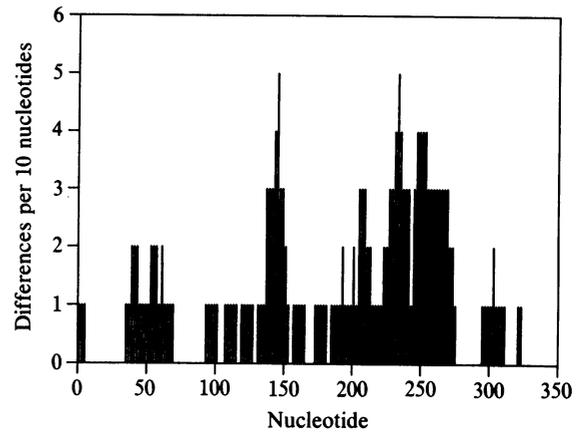


FIG. 7. Bar graph plot of nucleotide sequence differences between the *B. malayi* and *B. pahangi* repeats shown in Fig. 6. The number of differences between successive blocks of 10 nucleotides is plotted on the y axis and the nucleotide number is plotted on the x axis. The nucleotide number is the first nucleotide in each block of 10. The blocks start with alternating nucleotides beginning with nucleotide 1. For example, the blocks beginning with nucleotides 1, 3, and 5 have one difference each, while the block beginning with nucleotide 7 has no differences.

from *B. malayi* was inserted into the plasmid pBR322. This clone, pBma68, was used as a hybridization probe to determine copy number of the repeated sequence in *B. malayi*. There are approximately 30,000 copies of the 320-bp repeat in *B. malayi* which comprise about 12% of the genome (Table 1).

A survey was made of other filarial parasites to see if they also contained the cloned *B. malayi* repeat. One-tenth of the level of hybridization with pBma68 was observed with DNA from *B. pahangi*, but no hybridization was seen with DNA from four other species: *L. carinii* (parasite of cotton rats), *D. viteae* (parasite of gerbils), *D. immitis* (dog heartworm), and *O. volvulus* (parasite of humans) (Fig. 3). These results suggest that the *Hha* I repeat is *Brugia* specific. This *Hha* I repeat is probably the result of the amplification of an ancestral *Brugia* sequence after the genus diverged from other filarial parasites.

A detailed analysis of the *Brugia Hha* I repeat by using restriction-endonuclease digestion and DNA sequencing was done to find species-specific differences between *B. malayi* and *B. pahangi*. The restriction sites in the repeated sequence of the two species were compared by Southern blot analysis by using the plasmid containing the cloned *B. malayi* repeat

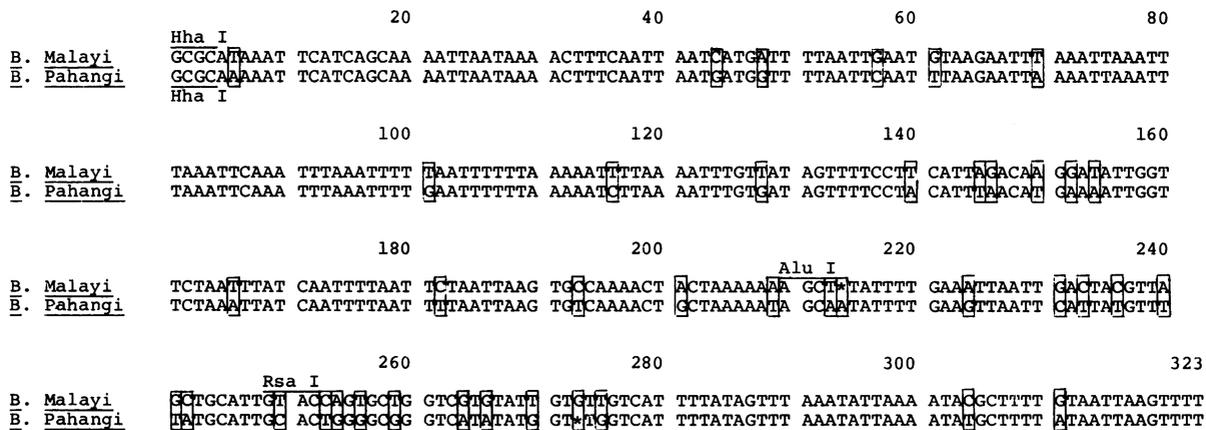


FIG. 6. Nucleotide sequence data from one copy of the *Hha* I-repeat family cloned from *B. malayi* and one from *B. pahangi*. Key restriction endonuclease sites are overlined or underlined. Each nucleotide position that differs between the two species is boxed. The sequences are aligned beginning with the *Hha* I site. The two single-base-pair deletions, inserted to maximize homology, are indicated by asterisks.

(pBma68) as the hybridization probe. The repeated sequences in both species are cleaved by *Hha* I. Most of the *B. malayi* repeated sequences are cleaved by *Alu* I and *Rsa* I, but the repeats in *B. pahangi* are not cut by these two enzymes. When *Brugia* DNA was digested with *Msp* I, however, many of the *B. pahangi* repeated sequences were cleaved while the *B. malayi* repeats were not (Fig. 4).

The DNA sequence of a *Hha* I repeat from each of the two *Brugia* species was determined. The recognition site for *Hha* I is present in both species, however, only the *B. malayi* repeat contains the *Alu* I and *Rsa* I recognition sites (Fig. 6). The differences in the DNA sequences are not random but are clustered in two regions, 140–154 and 231–275. These two regions have only about 60–65% homology, while the remainder of the sequence is 93% homologous. The localized regions of divergence, especially the region around nucleotide 250, offer the possibility of synthesis of DNA hybridization probes that will be species specific.

Repeated DNA sequences have been used by other investigators as hybridization probes for parasite detection and identification. Examples of this approach include the following: restriction endonuclease polymorphisms in the ribosomal genes of *Schistosoma mansoni* (19), strain variation in the kinetoplast DNA of *Leishmania* (20), and a species-specific tandem repeat in *Trypanosoma cruzi* (21, 22). Repeated DNA sequences have been observed in a variety of helminths by restriction enzyme digestion of genomic DNA (23). However, prior to this report, there have been no studies on the use of cloned DNA sequences as hybridization probes in filarial nematodes. The cloned *Hha* I repeated sequence described here possesses several features that make it an excellent candidate for use as a probe in a parasite detection and identification assay. It is specific for the genus *Brugia* and has a high copy number that makes it an extremely sensitive probe for parasite detection. Within this genus, differences in the restriction sites enable *B. malayi* to be distinguished from *B. pahangi*. DNA sequence analysis comparing cloned *Hha* I repeats from each species has identified two short segments of the repeat that have diagnostic potential.

The most common method, currently, for detecting and identifying filarial parasites is microscopic examination of blood from an infected host. However, in the case of *B. malayi* and *B. pahangi*, the circulating microfilariae are difficult to distinguish morphologically (24). Differences have been observed in the electrophoretic mobility of isozymes from *B. malayi* and *B. pahangi* (25, 26). Stage-specific monoclonal antibodies have been prepared against *B. malayi* antigens, but have not been used to distinguish *B. malayi* from *B. pahangi* (27).

B. malayi and *B. pahangi* are present in overlapping geographical areas in Southeast Asia. The similar morphologies of the two species make identification very difficult. Definitive characterization rests on minor morphological differences in adult male worms that must be obtained by biopsy or at autopsy (3). *B. pahangi* is known to infect a wide range of animal species including dogs, cats, monkeys, tigers, and otters. *B. malayi*, which infects primarily humans, has also been found in cats and monkeys (3). It is not known if humans serve as a natural host for *B. pahangi*, although experimental infection of man has been reported (28). DNA hybridization probes specific for *B. pahangi* could aid in determining if it is a natural human pathogen. Hybridization can be a sensitive means of parasite detection as was shown by the dot hybridization of DNA from infected blood samples, where as little as 10 pg of parasite DNA could be detected (Fig. 5). The availability of *B. malayi* and *B. pahangi* species-specific DNA probes for use in a fast, reliable, and

sensitive assay will greatly aid in the collection of accurate epidemiological data regarding these parasites. Such data will be crucial in evaluating strategies for controlling filariasis.

The data presented here show that differences between the *Hha* I repeated sequences of *B. malayi* and *B. pahangi* can be used to distinguish the two species by restriction site polymorphisms and by differences in specific regions of the DNA sequence.

Note Added in Proof. From the DNA sequence information we have constructed two oligonucleotide probes, one specific for *B. pahangi* and the other specific for *B. malayi*.

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