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Molecular Phylogenetic Studies on Filarial Parasites Based on 5S Ribosomal Spacer Sequences

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MOLECULAR PHYLOGENETIC STUDIES ON FILARIAL PARASITES BASED ON 5S RIBOSOMAL SPACER SEQUENCES

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Summary :
This paper is the first large-scale molecular phylogenetic study on filarial parasites (family Onchocercidae) which includes 16 species of 6 genera : Brugia beaveri Ash et Little, 1964 ; B. buckleyi Dissanaike et Paramananthan, 1961 ; B. malayi (Brug, 1927) Buckley, 1960 ; B. pahangi (Buckley et Edeson, 1956) Buckley, 1960 ; B. patei (Buckley, Nelson et Heisch, 1958) Buckley, 1960 ; B. timori Partono et al, 1977 ; Wuchereria bancrofti (Cobbold, 1877) Seurat, 1921 ; W. kalimantani Palmieri , Purnomo, Dennis and Marwoto, 1980 ; Mansonella perstans (Manson, 1891) Eberhard et Orihel, 1984 ; Loa loa, Stiles, 1905 ; Onchocerca volvulus (Leuckart, 1983) Railliet et Henry, 1910 ; O. ochengi Bwangamoi, 1969 ; O. gutturosa Neumann, 1910 ; Dirofilaria immitis (Leidy, 1856) Railliet et Henry, 1911 ; Acanthocheilonema viteae (Krepkogorskaya, 1933) Bain, Baker et Chabaud, 1982 and Litomosoides sigmodontis Chandler, 1931. 5S rRNA gene spacer region sequence data were collected by PCR, cloning and dideoxy sequencing. The 5S rRNA gene spacer region 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 5S spacer region sequences are highly conserved within species yet differ significantly between species. Spliced leader sequences were observed in all of the 5S rDNA spacers with no sequence variation, although flanking region sequence and length heterogeneity was observed even within species. All of the various tree-building methods gave very similar results. This study identified four clades which are strongly supported by bootstrap analysis: the Brugia clade; the Wuchereria clade; the Brugia-Wuchereria clade and the Onchocerca clade. The analyses indicated that L. sigmodontis and A. viteae may be the most primitive among the 16 species studied. The data did not show any close relationship between Loa loa and D. immitis presently classified in the same subfamily, and the constitution of the Dirofilariinae subfamily is questionable.

Résumé : ÉTUDES PHYLOGÉNÉTIQUES MOLÉCULAIRES DES FILAIRES À PARTIR DE SÉQUENCES DU « SPACER» DU 5S RIBOSOMAL

Cette première étude sur la phylogénie moléculaire des filaires (famille des Onchocercidae) – Nématodes chez lesquels les phénomènes de convergence sont particulièrement importants en raison de leur vie tissulaire – inclut 16 espèces appartenant à 6 genres différents : Brugia beaveri Ash et Little, 1964 ; B. buckleyi Dissanaike et Paramananthan, 1961 ; B. malayi (Brug, 1927) Buckley, 1960 ; B. pahangi (Buckley et Edeson, 1956) Buckley, 1960 ; B. patei (Buckley, Nelson et Heisch, 1958) Buckley, 1960 ; B. timori Partono et al, 1977 ; Wuchereria bancrofti (Cobbold, 1877) Seurat, 1921 ; W. kalimantani Palmieri, Purnomo, Dennis et Marwoto, 1980 ; Mansonella perstans (Manson, 1891) Eberhard et Orihel, 1984 ; Loa loa Stiles, 1905 ; Onchocerca volvulus (Leuckart, 1983) Railliet et Henry, 1910 ; O. ochengi Bwangamoi, 1969 ; O. gutturosa Neumann, 1910 ; Dirofilaria immitis (Leidy, 1856) Railliet et Henry, 1911 ; Acanthocheilonema viteae (Krepkogorskaya, 1933) Bain, Baker, Chabaud, 1982 et Litomosoides sigmodontis Chandler, 1931. Le gène «spacer» du 5S rRNA a été collecté, cloné et séquencé par PCR. Les séquences des différentes espèces ont ensuite 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 résultats indiquent que cette séquence est fortement conservée dans une même espèce alors qu'elle diffère significativement d'une espèce à l'autre. La séquence d'épissure est présente sans variation dans toutes les espèces tandis que les régions flanquantes présentent une hétérogénéité même au niveau intra-spécifique. Les différentes méthodes de reconstruction d'arbre présentent quelques contradictions mais elles sont semblables sur plusieurs points et l'étude, très partielle il est vrai comparée à l'abondance des genres et espèces chez les filaires, permet quelques conclusions. 1- le bootstrap soutient fortement quatre clades: le clade Brugia, le clade Wuchereria, le clade Brugia-Wuchereria, le clade Onchocerca ; ces clades correspondent aux genres, ou à des genres très proches,définis par la morphologie classique. 2- Les analyses suggèrent que L. sigmodontis et A. viteae pourraient être les plus primitives des 16 espèces étudiées; cette notion n'est pas en contradiction avec les hypothèses faites antérieurement. 3- Les analyses ne rapprochent pas L. loa et D. immitis, actuellement placés dans la même sous-famille, et la question de la composition des Dirofilariinae se pose.

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INTRODUCTION

Filarioid nematodes are a large group of parasites with species of medical or veterinary importance. Numerous detailed morphological and biological studies, pioneered by Wehr (1935), have been conducted in an attempt to clarify the phylogenetic relationships of these parasites (Chabaud and Choquet, 1953; Anderson and Bain, 1976 ; Chabaud and Bain, 1976). Since convergence phenomena are common in these tissue dwelling parasites, phylogenies derived solely from morphological data may be misleading. DNA sequence data collected by molecular biological methods provide a new approach in phylogenetic studies. This paper will focus on these molecular data and the results of this study will be compared to those obtained by the classical methods. There were sixteen species of six genera in the Onchocercidae family included in this study : subfamily Onchocercinae : *Brugia beaveri* Ash et Little, 1964 ; *B. buckleyi* Dissanaike et Paramananthan, 1961 ; *B. malayi* (Brug, 1927) Buckley, 1960 ; *B. pahangi* (Buckley et Edeson, 1956) Buckley, 1960 ; *B. patei* (Buckley, Nelson et Heisch, 1958) Buckley, 1960 ; *B. timori* Partono *et al.*, 1977 ; *Wuchereria bancrofti* (Cobbold, 1877) Seurat, 1921 ; *W. kalimantani* Palmieri, Purnomo, Dennis and Marwoto, 1980 ; *Mansonella perstans* (Manson, 1891) Eberhard and Orihel, 1984 ; *Onchocerca volvulus* (Leuckart, 1983) Railliet et Henry, 1910 ; *O. ochengi* Bwangamoi, 1969 ; *O. gutturosa* Neumann, 1910 ; *Acanthocheilonema viteae* (Krepkogorskaya, 1933) Bain, Baker et Chabaud, 1982 and *Litomosoides sigmodontis* Chandler, 1931 ; subfamily Dirofilariinae : *Loa loa*, Stiles, 1905 and *Dirofilaria immitis* (Leidy, 1856) Railliet et Henry, 1911.

The ribosomal RNA gene spacer regions can be used to infer phylogeny among closely related taxa (<50 million years old) (Verbeet *et al.*, 1984 ; McIntyre *et al.*, 1988 ; Yokota *et al.*, 1989 ; Gonzalez *et al.*, 1990 ; Kjems and Garrett, 1990). The 5S rDNA gene spacer region was chosen for this study based on the following three observations : 1. Filarial parasites may be recently derived (less than 65 million years old, Maggenti, 1983). Therefore, the 5S rDNA spacer region sequences were likely to possess more phylogenetic information than the ribosomal DNA coding region sequences. 2. The 5S rDNA gene organization was already established for *B. malayi*. Genomic titration experiments indicated that the *Brugia malayi* nuclear genome contains about 250 copies of the 5S rRNA genes (Ransohofff *et al.*, 1989). In addition, the detailed organization of 5S genes in nematodes has been studied in *C. elegans* (Nelson and Honda, 1985). 3. Preliminary data suggested that the coding regions of 5S rDNA genes were basically identical across all of the filarial species whereas the 5S rDNA spacer region sequences were very well conserved within species, but showed significant variation between species.

MATERIALS AND METHODS

FILARIAL PARASITE MATERIALS

Laboratory strains of *B. pahangi* and *B. malayi* were obtained from Dr. J. McCall (TRS Laboratory, Athens, GA, U.S.A.). *B. patei* adults were kindly provided by Drs. U.R. Rao and A.C. Vickery (University of Southern Florida, Gainsville, FL, U.S.A.). *B. beaveri* adults preserved in 100mM EDTA were kindly provided by Dr. T.C. Orihel (Tulane University Medical Center, New Orleans, LA, U.S.A.). *B. buckleyi* adults preserved in 70% ethanol were kindly provided by Dr. A.S. Dissanaike (Colombo, Sri Lanka). *B. timori* microfilariae and *W. kalimantani* adults preserved in 100mM EDTA were kindly provided by Dr. F. Partono (University of Indonesia, Jakarta). Genomic DNA samples of *M. perstans* preserved in ethanol were kindly provided by Dr. S.E.O. Meredith (Amsterdam, the Netherlands). Genomic DNA samples of *Loa loa, D. immitis, O. volvulus, A. viteae*, and *L. sigmodontis* as well as *Loa loa* microfilariae were kindly provided by Drs. T.B. Nutman and E.A. Ottesen (National Institute of Health, Bethesda, MD, U.S.A.). Genomic DNA samples of *O. volvulus, O. ochengi* and *O. gutturosa* were kindly provided by Dr. T.R. Unnasch (University of Alabama at Birmingham, Birmingham, AL, U.S.A). Morphological analyses were conducted to verify the identity of some specimens.

EXPERIMENTAL PROCEDURES

Extraction of parasite DNA

Individual adult filarial parasites were picked from 70% ethanol solution and evaporated to dryness. The worms were resuspended in 50 µl H_2O and boiled for 10 minutes. These preparations were used as the PCR templates. Similar treatment was performed for *B. timori* and *Loa loa* microfilariae, and for *W. kalimantani* and *B. beaveri* adult worms which were preserved in 100mM EDTA. This treatment was not necessary for those species whose genomic DNA was already available (except the DNA sample of *M. perstans* which was preserved in ethanol). These genomic DNA samples were used directly in the PCR reactions. The DNA sample of *M. perstans* was evaporated dry from an original volume of 1 ml, resuspended in 100 µl of dd H_2O and dialyzed against 0.05x TE buffer for four hours before it was used for PCR reactions.

Amplification of 5S rDNA sequences from parasite DNA

The polymerase chain reactions (PCR) were conducted using Perkin-Elmer-Cetus 480 and System 9600 thermal

cyclers. Two primers matching the coding region of the *B. malayi* 5S rDNA gene were custom synthesized by the DNA oligo nucleotide service of the Program in Molecular and Cellular Biology, University of Massachusetts (Amherst, MA. U.S.A.). The sequences of the two primers were :

Primer S2 : 5'-GTTAAGCAACGTTGGGGCCTGG-3' ;
Primer S16 : 5'-TTGACAGATCGGACGAGATG-3'.

All PCR reagents except primers, template and double distilled water (ddH$_2$O) were from the GeneAmp PCR kit purchased from Perkin-Elmer Cetus (Norwalk, CT). 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 ddH$_2$O 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) ; and 93°C, 30 seconds ; 55°C, 30 seconds ; 72°C, 30 seconds for a total of 30 cycles (System 9600 thermal cycler).

Cloning of the PCR amplified 5S rDNA spacer region sequences

The TA cloning system from Invitrogen Corporation (San Diego, CA) was used to clone the 5S rDNA PCR products. All of the reagents needed for ligation (except the amplified 5S rDNA products) were provided in the TA cloning kit. The manufacturer's protocols were strictly followed. Recombinant *Escherichia coli* colonies (white colonies) were picked with a sterilized platinum wire loop and screened by PCR using the same primer pairs as in the original PCR amplification. The loop of *E. coli* cells was placed directly into the PCR reaction tube. The PCR program was the same as for the original 5S rDNA amplification except the reactions were incubated in the thermal cycler at 95°C for five minutes to burst the cells before the initiation of the 30 cycles. The PCR products were run on 1.5% agarose gels and stained in ethidium bromide to identify positive clones.

Plasmid DNA preparation and purification

Individual *E. coli* clones with 5S rDNA inserts were inoculated into 20 ml of LB medium containing 50µg/ml of kanamycin and incubated at 37°C with vigorous shaking overnight. The cells were harvested the next morning by centrifugation at 5,000g for ten minutes. The cell pellets were resuspended in PI buffer from the Plasmid Midi Kit and plasmid DNA was isolated following the manufacturer's protocol [Qiagen Inc. (Chatsworth, CA, U.S.A.)]

DNA sequencing

The DNA sequencing primers used were primers S2 and S16 as described before and the M13 universal sequencing primers (primer 1201 and 1211 from New England Biolabs, Beverly, MA, U.S.A.). The CircumVent Thermal Cycle Sequencing Kit from New England Biolabs was used to sequence the plasmid DNA. The manufacturer's protocols for thermal cycle sequencing with labeled [35]S dATP incorporation were followed. [35]S dATP was purchased from Amersham Corp. (Arlington Heights, IL, USA). About 200

ng of plasmid DNA and 2 pmol of primer were used for each sequencing reaction. All of the other reagents were provided in the kit. The Perkin Elmer Cetus thermal cycler 480 was used with the following program : 95°C, 20 seconds ; 55°C, 20 seconds ; 72°C, 20 seconds for a total of 20 cycles.

SEQUENCE DATA ANALYSIS

Sequence alignment

The *Pileup* computer program from the GCG package (Genetics Computer Group, Inc. Madison, WI, USA) was used to align all of the 5S rDNA sequences. Because more than one 5S rDNA spacer region sequence was obtained for each species, *pileup* was used first to align the multiple sequences from the same species. The gap penalty (penalty for opening a gap) was set to two and gap length penalty (penalty for each character space in the gap) was set to zero. Another program in the GCG package, *Pretty*, was used to print out consensus sequences for each species. Any site that had more than 40% of a nucleotide other than the major nucleotide was considered polymorphic and IUPAC-IUB nucleotide notations were used. The final consensus sequences of all the sixteen species were then aligned by the *pileup* program. Only the portion of the sequences that were well aligned were selected for further analysis.

Phylogenetic reconstruction from sequence data

PAUP 3.1 (Phylogenetic Analysis Using Parsimony, Swofford, 1993) was used for phylogenetic tree reconstruction (Swofford and Olsen, 1990). Characters were treated as unordered (Fitch, 1971) and were equally weighted. Gaps were treated as missing data. The exhaustive search option was used on ten of the sixteen species. The branch-and-bound algorithm was used for the full set of 16 species. Bootstrap analysis was used with PAUP 3.1.

PHYLIP 3.5c (Phylogeny Inference Package, Felsenstein, 1993) was also used for data analysis. Maximum likelihood algorithms (Felsenstein, 1981) and distance methods (Neighbor-joining algorithm, Saitou and Nei, 1987) were used in the analyses. Bootstrapping analysis was also used with the PHYLIP package to estimate confidence intervals in the phylogenetic trees.

All of the PAUP computation and some of the PHYLIP distance method analyses were done using a Macintosh SE-30 and a Macintosh IIci computer. Sequence alignment, maximum likelihood analyses, and bootstrap analyses (except bootstrap with PAUP) were done on a Sun Sparc workstation running Sun OS 4.1.1 located in the University of Massachusetts at Amherst.

```
Constant :          *        *   **       * *          ******        *    *    *         *
  Uninf. :       *   *   * ***  **    *     * **    *  **     *********    *    *         *

B.malayi     AGCTGAATGTTGAATACACAACAACTATATGATA------ATGATGCAGGTATGATCGAC
B.timori     AGCTGAATGTTGAATACACAACAACTATATGATA------ATGATGCAGGTATGATCGAC
B.beaveri    AGCTGAATGTTGAATACACAACAACTATATGATA------ATGATGCAGGTATGATCGAC
B.patei      AGCTGAATGTTGAATGCACAACAACTATATGATA------ATGATGCAGGTATGATCGAC
B.pahangi    AGCTAAATGTTGAATGCACAACAACTATATGATA------ATGATGCAGGTATGATCGAC
B.buckleyi   AGCTGAATGTTGAATGCACAACAACTAGATGATA------ATGATGCAGGTATGATCGAC
Loa loa      CGATGATTATTGAATGCACGACAATGATATGAAA------ATGATGAAGTTAGATTTGAC
M.perstans   CGTTGATATTTGAATGCACAACAATGATATGTGA------ATGATGATGGTGTRCACGTC
O.ochengi    TAATTATTTTYGRATGYACAACAGTCATATGAGA------ATGAAGTAGTTACAAACATT
O.volvulus   TAATTATTTTTTGAATGTACAACAGTCATATGAGA------ATGAAGTAGTTACAAACATT
O.gutturos   TAATTATTTTTGAATGCACAACAGTCAGATGAGA------ATGGAGTAGTTRCAWACAKT
W.bancroft   TGATGAATGTTGAATGCACAACAACTATATGGGA------ATGGTGCAGGTAGGATCACC
W.kalimant   TGATGAATGTTGAATGCACAACAACTATATGGGA------ATGATGCAGGTAGAATCACC
D.immitis    AAGCCATTTTTCGATGCACTAAAATGATATATGA------ATGAAGCAATGATATTTGAC
A.viteae     CAACYATTGTTGAATGCAGCACAAACGTATCAGC------GTGTTGGGGTTGCATTCCAC
L.sigmodon   CACCGATCGATGGATGCAGCACAACCATAACTAC------ACGATGCGGATGAGGTCGTC
A.lumbrico   GGGAAATGATTGAATGTGATACTCGTTTTTGCACTTACCGATTGTGGGGATGATCTCGAT
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Constant : * * * * * * * *   *   * * * * * * * * * * * *   * * * *     * *  * * *   * *  * * *  * * * * * * *  *        *  *
  Uninf. : * * * * * * * * * * *   * * * * * * * * * * * * * * * *     * * * * * *   * * * * * *  * * * * * * * * *   *   * *  *

B.malayi     GGTTTAATTAYCCAAGTTTGAGRTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
B.timori     GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
B.beaveri    GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
B.patei      GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
B.pahangi    GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
B.buckleyi   GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
Loa loa      GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTCGGCTAC
M.perstans   GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGTG----TTTCAGCTAC
O.ochengi    GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCTGCCCAGAG----TTTCGACTGC
O.volvulus   GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCTGCCCAGAG----TTTCGACTGC
O.gutturos   GGTTTAAWTRCCCAAGTTTGAGGTAATYKAATGYTTCAGCCCAGAG----TTTMGAMTGC
W.bancroft   GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
W.kalimant   GGTTTAATTASCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTAGGCTAC
D.immitis    GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCAGCCCAGAA----TTATATCTGC
A.viteae     GGTTTAATTACCCAAGTTTGAGGTAATTGAATGTTTCGGCCCAGAG----TTTCGGCTGC
L.sigmodon   GGTTTAATTACCCAAGTTTGAGGTAATTGAGTGTTTCGGCCCAGAG----TTTCGGCTTC
A.lumbrico   GGTTTAATTACCCAAGTTTGAGGTAATTCCGTGTTTCAGCTCAGTGCTTCTATCGGCT-C
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22-nt spliced leader sequence

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Constant :   * * * * * * * * * *      * * * * * * * * * * *   *       *         *   *   *    * *
  Uninf. :   * * * * * * * * * * *     * * * * * * * * * * * * * * * * * *      * *    * * * * * * *   * *

B.malayi       TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATATTTGTGGGACAATAA
B.timori       TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATATTTGTGGGACAATAA
B.beaveri      TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATAATTGTGGGACAATAA
B.patei        TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATATTTGTGGGACAATAA
B.pahangi      TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATATTTGTGGGACAATAA
B.buckleyi     TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATATTTGTGGGACAATAA
Loa loa        TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCAATTTTGTGGGACAACAA
M.perstans     TGTGGCTTGAAGTAAAATTTTGGAACGTCCTACAAATTTGTGGGACAACAA
O.ochengi      TGTGGCTTGAAGCGAAATTTTGGAACGTCCTGCATG--AGCGGGACAACAA
O.volvulus     TGTGGCTTGAAGCGAAATTTTGGAACGTCCTGCATG--AGCGGGACAACAA
O.gutturosa    TGTGGCTTGAAGTGAAATTTTGGAAMGTCCTGYRYR--AGCGGGACAACAA
W.bancrofti    TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATGTTTGTGGGACAATAA
W.kalimantani  TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCATGTTTGTGGGACAATAA
D.immitis      TGTGGCTTGAAATGAAATTTTGGAAAGTCCTACATT--AGTGGGACAACAA
A.viteae       TGTGGCTTGAAGTAAAATTTTGGAACGTCCTGCTACTTAGCGGGACAACAA
L.sigmodontis  TGTGGCTTGAAGAAAAATTTTGGAACGTCCTGCTATATAGCGGGACAACA-
A.lumbricoides TGTGGCTTGAAAAATAAATTTTGGAACGCTTTGCCGTATGGCGAAGCACTTT
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Table I. — 5S rDNA sequence data matrix used for phylogenetic tree reconstruction. There are 161 nucleotide positions in this data matrix. Forty-eight of the 161 sites (30%) are informative. ("-" = gap ; Constant = invariable site ; Uninf. = uninformative site as calculated by PAUP 3.1. Underlined nucleotides are the spliced leader sequence).

RESULTS

DNA SEQUENCE COLLECTION AND ALIGNMENT

At least two 5S rDNA sequences were obtained for each species. Consensus sequences from each species were constructed and aligned to one another. The sequence data matrix which is the input for all of the tree reconstruction algorithms is shown in Table I. The *Ascaris lumbricoides* 5S rDNA spacer region sequence (Nelsen *et al.*, 1989) was included as the outgroup.

PHYLOGENETIC TREE RECONSTRUCTION

1. Analysis by maximum parsimony methods (Swofford, 1993)

Exhaustive search

Due to the relatively large size of this data set, ten species representing all six genera were selected to undergo exhaustive search in PAUP 3.1.

Exhaustive search settings :
Branches having a maximum length of zero collapsed to yield polytomies; topological constraints were not enforced ; trees were unrooted ; multi-state taxa were interpreted as polymorphism ; character-state optimization was by accelerated transformation (reversals preferred)

Search results :
One shortest tree of length 121was found (Fig. 1) The statistics of the exhaustive search tree are the following (Kluge and Farris, 1969 ; Farris, 1989 ; Sanderson and Donoghue, 1989) : consistency index (CI) = 0.777 ; CI excluding uninformative characters = 0.710; CI expected = 0.684; retention index (RI) = 0.690; rescaled consistency index (RC) = 0.536. The frequency distribution of tree lengths is shown in Figure 2.

Branch-and-bound search

Search settings :
An analysis of all sixteen ingroup species was undertaken with *Ascaris lumbricoides* as the outgroup species. Initial upper bound (upper limit of tree length) was unknown (to be computed via stepwise) ; addition sequence was set to furthest ; branches having a maximum length of zero were collapsed to yield polytomies ; topological constraints were not enforced ; multi-state taxa were interpreted as polymorphism ; character-state optimization was by accelerated transformation.

Search results :
One shortest tree with length 198 was found in the branch-and-bound search (Fig. 3). The tree statistics

are the following : consistency index (CI) = 0.758 ; CI excluding uninformative characters = 0.624 ; CI expected = 0.588 ; retention index (RI) = 0.727 ; rescaled consistency index (RC) = 0.551.

2. Analyses by the neighbor-joining method (Saitou and Nei, 1987) and by the maximum likelihood method (Felsenstein, 1981)

Ascaris lumbricoides was used as the outgroup species in these analyses. The branching pattern of the shortest trees obtained was very similar to that of Figure 3, except that *Loa loa* and *M. perstans* form a group which switches position with the *Onchocerca* and *D. immitis* group in these analyses.

3. Analysis by bootstrap methods (Felsenstein, 1985)

Bootstrapping with parsimony analysis

A total of 500 bootstrap replicates were analyzed using the parsimony algorithm. The strongly supported clades are the *Onchocerca* clade (100%), the *Brugia-Wuchereria* clade (97%), the *A. viteae* and *L. sigmodontis* clade (97%) and the *Brugia* clade (94%). This tree is unable to resolve the branching order of the *Brugia-Wuchereria* clade, *Loa loa*, *M. perstans*, *D. immitis* and the *Onchocerca* clade (Fig. 4 and Table II).

Bootstrapping with neighbor-joining analysis

A total of 485 bootstrap replicates were analyzed. This analysis demonstrated that the *Onchocerca* clade (100%), *Brugia* clade (99%), *Wuchereria* clade (98%) and *Brugia-Wuchereria* clade (99%) were strongly supported by the data set (Fig. 4 and Table II).

Bootstrapping with Maximum likelihood analysis

A total of 100 replicates were examined. The *Onchocerca* clade (100%) and the *Brugia* clade (94%) were strongly supported (Fig. 4 and Table II).

DISCUSSION

DNA SEQUENCE DATA COLLECTION AND SEQUENCE ALIGNMENT

Most of the parasite materials were available in only limited quantities for this study. It was not practical to use traditional methods for extracting genomic DNA, endonuclease digestion, and cloning. A very effective method was developed using PCR to amplify 5S rDNA spacers and then to directly clone the PCR products. This method proved very efficient and reliable since the *B. malayi* 5S
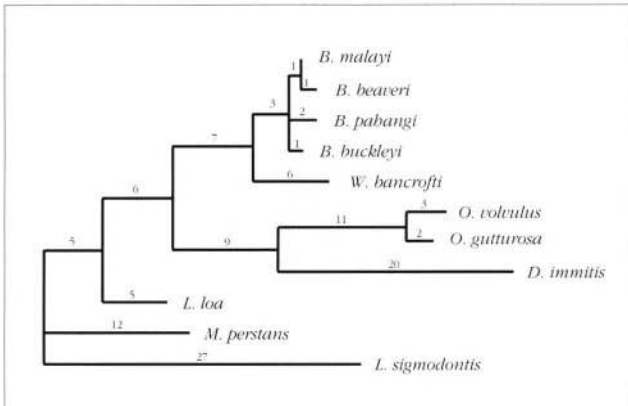
Fig. 1. – The tree found by exhaustive search. Tree length: 121. The numbers above each branch denote the branch length calculated by PAUP 3.1
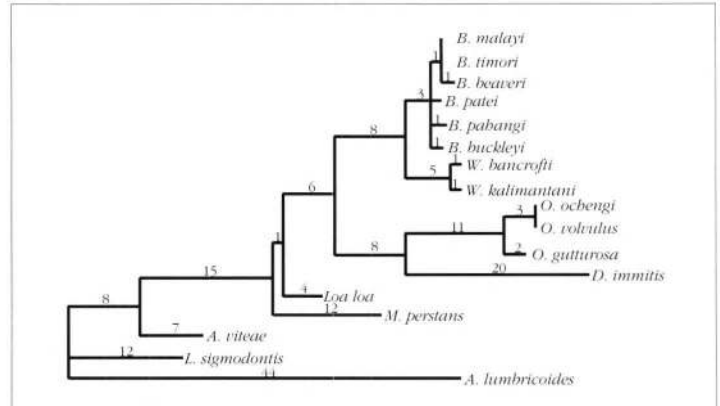

Fig. 3. – The branch-and-bound tree rooted by *A. lumbricoides*. Tree length: 198. This analysis showed that *A. lumbricoides* joins the tree at the *L. sigmodontis* branch.
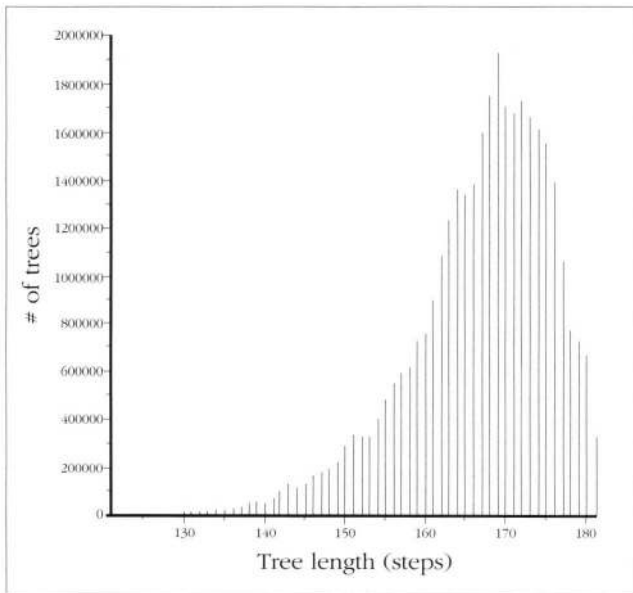

Fig. 2. – The frequency distribution of tree lengths of the 5S rDNA spacer sequences. A total of 34,459,425 trees were evaluated. The mean tree length is 167 steps.
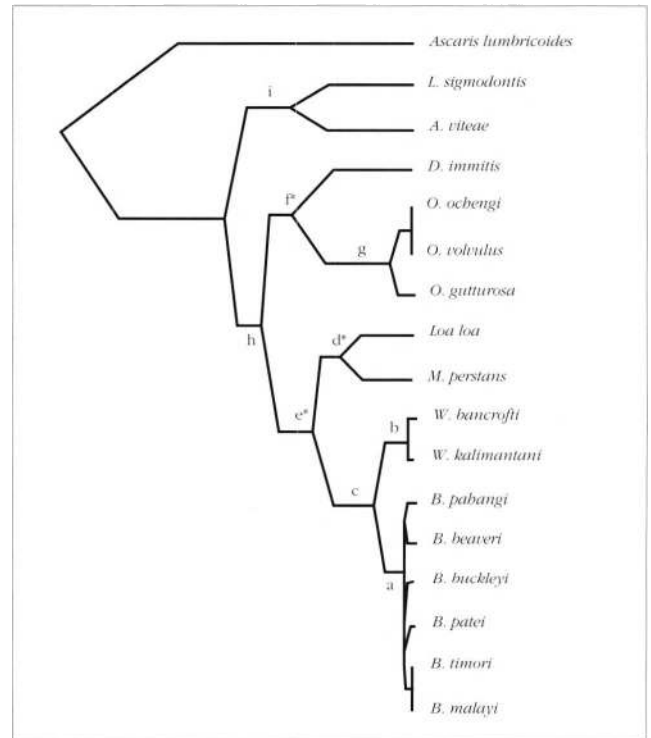

Fig. 4. – The basic bootstrapping pattern of the species studied in this paper. Small case letters denote the clades identified by the various bootstrap analyses. Clade names noted with an * indicate low bootstrap values for those clades.

Clade	a	b	c	d	e	f	g	h	i
PA	94%	87%	97%	<50%	<50%	<50%	100%	97%	97%
NJ	99%	98%	99%	<40%	40%	69%	100%	85%	85%
ML	94%	76%	91%	<40%	<40%	<40%	100%	<40%	92%

* PA : Parsimony; NJ: Neighbor-joining; ML: Maximum likelihood

Table II
Summary of the bootstrap values*

rDNA gene sequences obtained from PCR and cloning were identical to the sequences in the literature which were collected by traditional genomic DNA cloning and sequencing methods.

The coding region sequence is virtually identical across the 16 species studied here (data not shown). On the contrary, the spacer region of the 5S rDNA exhibited great length variations among the different species. They were as short as 400 nucleotides in *Onchocerca* species or as long as 600 nucleotides in some *Brugia* species. Even within the same species, there were sometimes different size classes of 5S spacers. The spacer length heterogeneity is probably due to deletion and insertion events that have occurred during the evolution of these parasites. This phenomenon was confirmed by sequence comparison analysis. Sequence alignment is the first step in sequence comparison and phylogenetic reconstruction analysis. The 22-nucleotide spliced leader region sequences were found in all of the filarial species studied (Table I). This conserved region was used as a focus for the sequence alignment.

TREE RECONSTRUCTION ALGORITHMS

Three main categories of methods of analysis were used in this study: parsimony, distance and maximum likelihood.

Parsimony analysis :

Branch-and-bound and exhaustive searches were performed in this analysis. Because the sequence region studied was the non-coding 5S rDNA spacer region, all of the characters were equally weighted. Different sequence addition methods were tried and all gave the same topologies. The consistency indices were high (except in the exhaustive search, where some taxa were excluded) for the 5S spacer region data set, indicating a low level of homoplasy in the spacer region. The 5S spacer region data set showed a frequency distribution of tree length that is heavily skewed towards the end of long trees (Fig. 2), which is an indication of the phylogenetic information content in the data sets (Hillis, 1991). All of the parsimony analyses (some not shown) indicate that the *Brugia* clade and the *Wuchereria* clade are close neighbors. *A. viteae* and *L. sigmodontis* also form a clade, while the *Onchocerca* species form their own clade. Although the two shortest trees (Fig.1 and Fig. 3) indicate that *D. immitis* and the *Onchocerca* clade are closer to the *Brugia-Wuchereria* clade than are *M. perstans* and *Loa loa*, this relationship can be reversed with a cost of less than five steps (data not shown). In other words, parsimony analysis was unable to resolve the branching order of *Loa loa, M.*

perstans, D. immitis and the *Onchocerca* clade (Table II). When *D. immitis*, which belongs to the Dirofilariinae subfamily and has a long branch length (this can cause problems in parsimony analysis), was excluded from the analysis, *M. perstans* and *Loa loa* appeared more closely related to the *Brugia-Wuchereria* clade (data not shown). Parsimony analysis is very sensitive to unequal evolution rates in the branches due to parallelisms and reversals. This is especially a problem with long branches (Felsenstein, 1978 ; Lake, 1987). For closely related sequences, such as those studied in this paper, parallelisms and reversals should be rare. Therefore, the parsimony method can be considered reliable.

Distance analysis :

Distance methods may not be accurate for distantly related sequences since the observed pairwise distances usually underestimate the true evolutionary distances due to homoplasy. Proper correction is needed in many cases to compensate for multiple-hits at a single site (Gojobori *et al.*, 1990). Nei's (1991) computer simulation studies have shown that the neighbor-joining method is superior to most of the other methods in retrieving the true tree under a variety of circumstances. Different distance methods were used in this study and they all gave similar branching patterns (data not shown). The neighbor-joining method also gave very high bootstrap values at some branches (Table II). It is interesting to note that *D. immitis* and the *Onchocerca* clade appeared in close proximity in most of the trees generated by different distance methods (data not shown). However, the bootstrap value for such groupings are low (Table II). The same is true for *Loa loa, M. perstans* and the *Wuchereria-Brugia* clade. This phenomenon was observed in parsimony and maximum likelihood analysis as well.

Maximum likelihood analysis :

Maximum likelihood methods are not used as frequently as parsimony or distance methods because of the computational complexities of the former. The PHYLIP 3.5c program used on a SUN Sparc workstation greatly facilitated the computations. Maximum likelihood methods are the only ones that assume stochastic models of sequence evolution in which the user can specify rates of evolution at individual sites of the sequences. These methods are considered potentially the most reliable because of their statistical properties (Sidow and Wilson, 1991). In this study, the maximum likelihood method gave very similar branching patterns to those seen with other methods. Different transition/transversion ratios were used in various tree reconstructions. All ratios yielded very similar results (data not shown).

CONFIDENCE LIMITS ON PHYLOGENIES

One of the statistical approaches used to assess confidence levels in phylogenetic hypotheses is the bootstrap analysis (Felsenstein, 1985). Bootstrap analysis is a method for the estimation of statistical error in situations where the underlying sampling distribution is difficult to assess (Efron, 1982 ; Efron and Gong, 1983). Instead of repeatedly sampling from the underlying distribution of taxonomic characters itself, which is often impractical, the bootstrap resamples the original data set to approximate the distribution.

In this study, algorithms from all three major categories were applied to the bootstrapped data sets (Table II). Taking all of the bootstrap results together, it is clear that the *Brugia* clade, the *Wuchereria* clade, the *Brugia-Wuchereria* clade and the *Onchocerca* clade are strongly supported by the original data set. It was shown that confidence levels estimated by bootstrap analysis usually underestimates the real relationships among taxa (Sanderson, 1989). Some of the groups in this study did not reach a 95% confidence level by bootstrap analysis, but that does not necessarily mean these groupings should be discarded (Sanderson, 1989). The *A. viteae-L. sigmodontis* group had confidence levels ranging from 85% to 97% in the different bootstrap analyses , indicating a close relationship. The *D.immitis-Onchocerca* group appeared in the majority of trees generated by various methods in this study, although it had only a confidence level of 69% in the bootstrap analysis with the neighbor-joining method (data not shown, see previous discussion).

THE HYPOTHESIS

It is very clear from analysis of the 5S rDNA spacer sequence data and the above discussion, that there are at least four strongly supported clades: the *Brugia* clade, the *Wuchereria* clade, the *Brugia-Wuchereria* clade and the *Onchocerca* clade. The *A. viteae* and *L. sigmodontis* group is also strongly supported (97%) in the bootstrap analysis using parsimony. *Litomosoides* has a long buccal capsule in the L3 and adult stages which is considered a very primitive character state (Anderson and Bain, 1976). The genus *Acanthocheilonema* has a developed buccal cavity and was considered the most primitive genus of the *Dipetalonema* line of the Onchocercinae subfamily (Chabaud and Bain, 1976). The outgroup *Ascaris lumbricoides* rooted most of the 5S rDNA trees at the *L. sigmodontis* branch (except those trees derived by algorithms which assume constant rates of evolution, data not shown). These observations are examples of congruence of molecular data with morphological data. The outgroup analyses also suggest that at least two subfamilies (Dirofilariinae and Onchocercinae) of the Onchocercidae family form a single natural group which does not exclude the suggestion of Bain (1981a).

In the *Onchocerca* clade, the 5S rDNA spacer sequence of *O. volvulus* is indistinguishable from that of *O. ochengi*. It was suggested that *O. volvulus* belongs to a small line of *Onchocerca* in African Bovidae of the Savanna which is morphologically highly evolved (musculature atrophied and hypodermis hypertrophied in the females, Bain, 1981b). Chabaud (1981) recognized the capture phenomenon in parasite evolution. According to Chabaud, a captured parasite is defined as a parasite which, after becoming isolated in a new host, undergoes speciation and becomes morphologically distinct from the original species. *O. volvulus* only infects humans while *O. ochengi* infects cattle. Morphological evolutionary studies indicated that *O. volvulus* and *O. ochengi* are the most closely related species in the genus *Onchocerca*. It is possible that *O. volvulus* evolved from the captured *O. ochengi* of the herbivore reservoir too recently for *O. volvulus* to show genetic distinctions from *O. ochengi*.

The two species in the *Wuchereria* clade are the only species that have been described for this genus. They form a natural group and are very closely related based on the DNA sequence data in this study. *W. bancrofti* has a world-wide distribution and its only host is humans, while *W. kalimantani* is limited to South Kalimantan in Indonesia and infects monkeys. There is no correlation between morphological characters and geographical origin in *W. bancrofti* except at the microfilarial level (Schacher and Geddawi, 1969 ; Bain *et al*, 1985). 5S rDNA spacer region sequences of *W. bancrofti* from India, Egypt, Indonesia and Tahiti were analyzed and were identical to one another in this study (data not shown). Laurence (1989) placed the early infection of humans by *W. bancrofti* in Southeast Asia about 3,000 years ago based on historical records. He suggested that only through mass migration of human beings did Bancroftian filariasis become widely spread. It is possible that both species of *Wuchereria* originated from the same place in Indonesia and the speciation was completed long enough ago to allow the observation of differences at the DNA level as revealed in this study.

The bootstrap analyses of the 5S rDNA spacer region sequence data demonstrated that *Brugia* and *Wuchereria* are very closely related. These results are in very good agreement with morphological data (Buckley, 1958). For the six species studied in the *Brugia* clade, the 5S rDNA spacer sequences alone

were not sufficient to resolve the branching order. This fact, combined with the observation that *Hha* I repeats are only present in *Brugia*, clearly indicates that *Brugia* is a natural group and that the species in this genus are very closely related (Xie, 1993).

The hypothesis for the phylogenetic relationships in the filarial parasite Onchocercidae family is illustrated in Figure 5. The use of 5S rDNA spacer region sequences seems to confirm that the filarial parasites in this study (filarial parasites of mammals) are recently derived, perhaps after the Spiruria went through a bottleneck in the Cretaceous disaster about 60 million years ago (Maggenti, 1983). In fact, the origin and speciation events of the *Onchocerca* genus in Africa may all be within the past 2.5 million years (during the Pleistocene epoch, Bain, 1981b). However, the filariae in the subfamily Oswaldofilariiinae have a Gondwanian distribution which suggests their existence possibly as early as the late Jurassic period (about 120 million years ago, Bain, 1981).
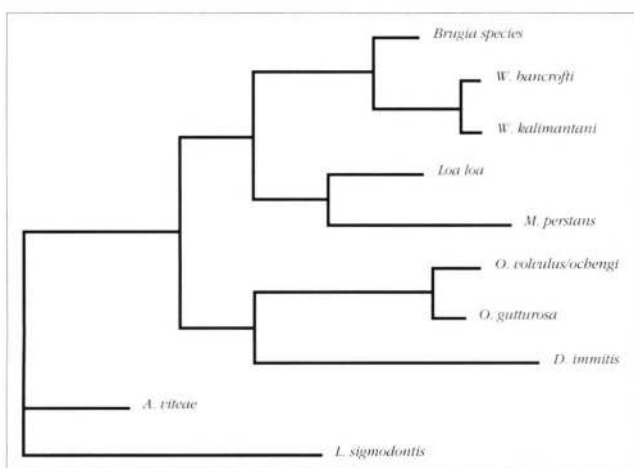

Fig. 5. – The proposed phylogenetic tree for the species studied in this paper. The six species in *Brugia* were lumped together in a single branch because their branching order is ambiguous based on 5S rRNA gene spacer region sequences alone.

The only serious conflict between the results of this molecular phylogenetic study and the traditional morphology-based classifications is in the positions of two species : *Loa loa* and *D. immitis*. Based on morphological studies, they have been placed in the subfamily Dirofilariinae, yet they are not very close to each other in any of the DNA sequence-based phylogenetic trees. Morphologically, the L3 (third-stage larvae) of *Loa loa* does not resemble that of *Dirofilaria*. In this study, although the bootstrap value is low (Table II), *D. immitis* appeared in most of the trees generated by different methods to be closest to the *Onchocerca* clade (data not shown). These results correspond to certain aspects of the morphology of their L3 larvae (Bain and Chabaud, 1986). Based on

these DNA sequence data, *Loa loa* appeared to be closest to *M. perstans* and the *Brugia-Wuchereria* clade in most of the trees generated by various methods (data not shown). The bootstrap value is low, however, and morphologically the L3 of *Loa loa* and *M. perstans* are totally different. The Dirofilariinae subfamily is characterized by highly developed caudal alae and papillae while the Onchocercinae subfamily is characterized by a long, non-alate tail. Although some trees were rooted at the *D. immitis* branch (data not shown) which might support *D. immitis* as a different subfamily, the position of *Loa loa* in all of the trees generated by all of the different methods suggest its close connection with the Onchocercinae subfamily. These molecular data question the constitution of the Dirofilariinae subfamily.

ACKNOWLEDGMENTS

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