Kamalambigeswari R et al /J. Pharm. Sci. & Res. Vol. 9(12), 2017, 2604-2608

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Evolutionary Study of Lymphatic Filarial Parasites through Amplification of ITS2 Region of Ribosomal RNA Cistron

Kamalambigeswari R[1*], Jeyanthi Rebecca L[2], Sharmila S[3], Kowsalya E[4].
Department of Industrial Biotechnology,
Bharath Institute of Higher Education and Research, Chennai, Tamil nadu, India.

Abstract

The present study was primarily aimed at the amplification of Internal transcribed Spacer (ITS), region of the filarial parasites and to depict the evolutionary significance of various nematodes that causes lymphatic filariasis. Lymphatic filariasis has been targeted by the World Health Organization (WHO) to be eliminated by the year 2020. Filarial nematode parasites are a serious cause of morbidity in humans and animals. The ITS along with the flanking 18S and 5.8S ribosomal DNA (rDNA) were amplified from the isolated DNA of *Wuchereria bancrofti*, *Brugia malayi*, and *Setaria cervi* which was found to be ~1200bp.The amplification of ITS2 region of these filarial parasites illustrated that it is of ~600bp. The ITS2 region was sequenced and the nucleotide sequences homology was studied using BLAST. Phylogenetic tree was constructed using UPGMA

Key words: Lymphatic filariasis, Nematodes, ITS sequences, Phylogenetic analysis

INTRODUCTION

Helminthic parasites cause several diseases such as filariasis, onchocerciasis, dracunculiasis and schistosomiasis. Filariasis is a group of human and animal infectious disease caused by nematode parasites of the order filariadae containing three important families, filariadae, stephanofilaridae and Dipetalonematidae. Among these families 39 genera and 229 species are parasitic on mammals.

Human lymphatic filariasis is caused by the filarial parasites, *W. bancrofti* , *B. malayi* [1] are of major importance due to their wide distribution and endemicity around the world. Approximately 90% of lymphatic filariasis (LF) worldwide is caused by *Wuchereria bancrofti*, with a majority of the remaining 10% caused by *Brugia malayi* [2]

Transmission of lymphatic filariasis occurs through an infective bite of a vector of the genera *Anopheles, Aedes, Culex or Mansonia*. The nocturnally periodic form of *W. bancrofti*, the predominant infecting agent is an urban parasite, and is transmitted very efficiently by *Culex quinquefaciatus*, the mosquito associated with poor urban sanitation, whereas the rural forms are transmited by *Anopheles* and *Aedes* mosquitoes. The subperiodic form mainly seen in eastern pacific islands, is transmitted by *Aedes* species.

The application of Internal transcribed spacer (ITS) to identify the organism has received the most attention by nematologist during the past decade [3]. The most spectacular symptom of lymphatic filariasis is elephantiasis (thickening of the skin and underlying tissues), which was the first disease discovered to be transmitted by insects. Elephantiasis is caused when the parasites lodge in the lymphatic system. Infection with lymphatic filariasis is not lethal, but can be seriously debilitating causing an economic burden on infected individuals and the communities they live in.

Cytoplasmic rRNA genes are highly repetitive because of huge demand of ribosomes for protein synthesis ('gene dosage') in the cell. Within the tandemnly repeated rRNA gene complex coding sequences for small (18S) and large (5.8S+28S) subunit rRNA components are flanked by non-transcribed and internal transcribed spacer region (ITS). W*uchereria bancrofti* is the most common filarial parasite in humans; there are an estimated 82 million infected individuals and more than 900 million people at risk of infection [4] studied the factors affecting development of *W. bancrofti* in *Anopheline* mosquitoes.

Since *W. bancrofti* and *B. malayi* co-exist in many places [5], their identification to species level is very important in diagnosis as well as epidemiological surveys.

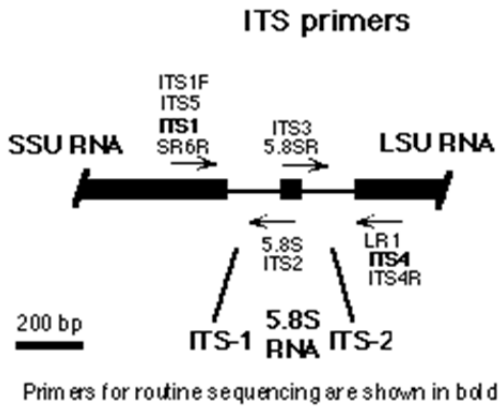
Markers for *W.bancrofti*:

Ribosomal DNA:

The rDNA sequence encodes for ribosomal DNA. It is typically clustered in one or a few regions of the genome called nucleoli. The rDNA is usually arranged in tandemnly linked genes, all arrayed in the same transcriptional orientation, with copy numbers ranging from 100- 1000 in insects [6]. Portions of the rDNA are highly conserved, being the same from bacteria to humans [7].

Within each transcriptional unit, there are two major coding regions, for 18S and 28S ribosomal RNA, and a region coding for the small 5.8S RNA which occurs within the internal transcribed spacer (ITS) region between the 18S and 28S regions. The ITS is divided into ITS1 lying between the 18S and 5.8S coding regions, and the ITS2, lying between the 18S and 5.8S coding regions. ITS1 and ITS2 are highly variable regions [8].

The function of ITS2 region is unclear but it is thought that they form a hairpin secondary structure that is important in the processing of transcribed rRNA The ITS2 regions seem to undergo a higher mutation rate than transcribed genes and have been used to distinguish closely related species [9].



Primers for routine sequencing are shown in bold

Phylogenetic Analysis:

Phylogenetic analysis programs are widely available. The main ones in use are PHYLIP (Phylogenetic Inference package) [10] and PAUP (Phylogenetic analysis using parsimony) available from Sinauer Associates. Current version of these programs provides three main methods for phylogentic analysis – parsimony, distance and maximum likelihood.

MATERIALS AND METHODS

Collection of blood samples:

Ten milliliters of venous blood from *W. bancrofti*–infected patients were collected under sterile technique and universal precautions between 8:00 PM and midnight as previously described [11].

Isolation of microfilariae:

Blood samples were diluted with an equal volume of normal saline(0.9%). The microfilariae are purified by percoll method.

Isolation of DNA from microfilariae:

DNA was extracted from microfilariae using the phenol chloroform method [12]. The microfilariae were digested with the cell lysis buffer and incubated for 2hrs at 60ºC. After digestion the total DNA was isolated from the lysate by Standard phenol/cholorofom extraction and by ethanol precipitation, RNA is precipitated by treating with $LiCl_2$. The pellet was dried, resuspended in 10µl.

Polymerase chain reaction for amplification and optimization of ITS:

The extracted DNA is used for the PCR reaction as a template. The ITS region was amplified by polymerase chain reaction from genomic DNA samples. The primers used for the amplification of ITS1-5.8S-ITS2 were FL1-F (forward) 5' TTCCGTAGGTGAACCTGC and FL2-R (reverse) 5' ATATGCTTAAATTCAGCGGG primers. These primers were designed from conserved stretches proximal to the 3' end of the 18S rDNA and 5' end of the 28S rDNA. The PCR was performed using 50µl reaction mixture which included 10 ng of template , 3mM $mgcl_2$, 2 µl f 10mM dNTP's, 10pmol of each of the primers, 5µl of 10X PCR buffer and 1 unit of taq polymerase. All PCR are followed under the controlled condition Using Initial denaturation at 94 C for 5 min followed by 35 cycles of actual denaturation 94°C for 1 min, annealing at 51° C for 1 min extension of about 72°C for 1 min. The amplified products undergo the final extension at 72° C for 10 mins.

Polymerase chain reaction for amplification and optimization of ITS2:

The DNA which was extracted from microfilariae using the phenol-chloroform method is used for the amplification of ITS2 region. The amplification was obtained by employing the primers ITS News2 (forward) 5' TGTGTCGATGAAGAACGCAG and FL2-R (reverse) 5' ATATGCTTAAATTCAGCGGG. They correspond to the conserved region of 3'end of 5.8s gene and the ITS2. These primers were designed from conserved stretches proximal to the 3' end of the 5.8S rDNA of *Gnathostoma* and 5' end of the 28S rDNA of *Dirofilaria immitis*. The PCR was performed using 50µl reaction mixture which included 10 ng of template , 2mM of $mgcl_2$, 2µl of 10mM dNTP's, 10pmol of each of the primers, 5µl of 10X PCR buffer and 1 unit of taq polymerase. The PCR conditions resembles the same as that used for the amplification of ITS. The amplicons were analyzed by gel electrophoresis using 1% agarose gel along with 100 bp ladder.

Analysis of nucleotide sequence of ITS

The complete nucleotide sequence of ITS were analyzed by using blast programs from https://blast.ncbi.nlm.nih.gov/. The nucleotide sequences were then compared with various organisms which have been previously submitted in gen bank which is an essential data for phylogenetic tree construction.

Phylogenetic tree construction:

The ITS2 sequences of both the free living and parasitic nematodes have been obtained from Genbank. These ITS2 sequences have been aligned and their evolutionary relationship has been observed through the phylogenetic tree construction. The tree was constructed based on distance method, UPGMA (unweighted pair group method with arithmetic mean).

RESULTS AND DISCUSSION

The genomic DNA was isolated from different species which causes filiariasis. The ITS1 and ITS2 regions of rDNA was useful for investigating some variations among closely related species and for organism evolving over a shorter time period [3]. Moreover, the presence of multiple copies provides a large number of target sequences for PCR in most organisms. Amplification of ITS region from *B. malayi, W. bancrofti,* and *Setaria cervi* were obtained by using the oligonucleotide primers designed based on the reported conserved sequence of the 18S, 5.8S and 28S sequence of rDNA of nematodes (Figure-1,2,3) respectively. The primers were designed from highly conserved regions of filarial 18S and 5.8S rDNAs [13]. The ITS region of *W. bancrofti*, which spans about 1.2kb, was amplified by primers (FLF-F and FL2-R). The FL1-F primer was designed from the conserved region of 3'end of *B. malayi, W. bancrofti* and *D.immitis* 18SrDNA. The FL2-R corresponds to the beginning of 5'end of *D.immitis* rDNA, which was also conserved among 28SrDNA form other nematodes. The optimization by $mgcl_2$ gradient was found to be essential to get rid of some non specific bands. After the PCR, very faint bands of ITS1-5.8S-ITS2 were obtained (Figure-4)

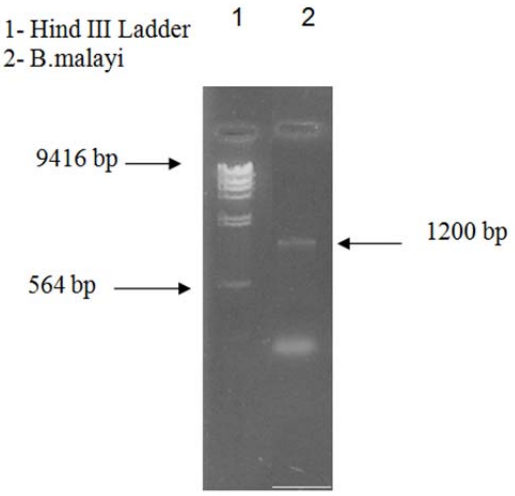


Figure-1 Amplification of ITS in *B.malayi*

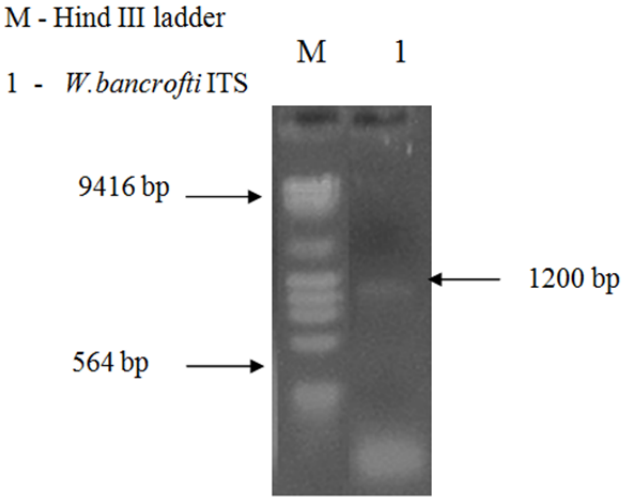


Figure-2 Amplification of ITS in *w.bancrofti*

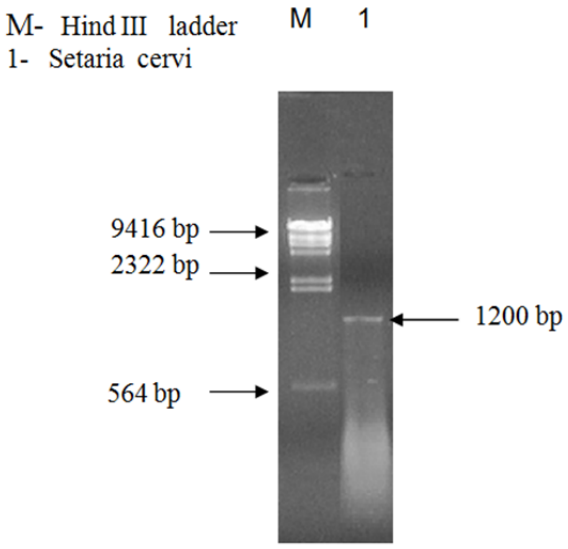


Figure- 3 Amplification of ITS in *Setaria cervi*

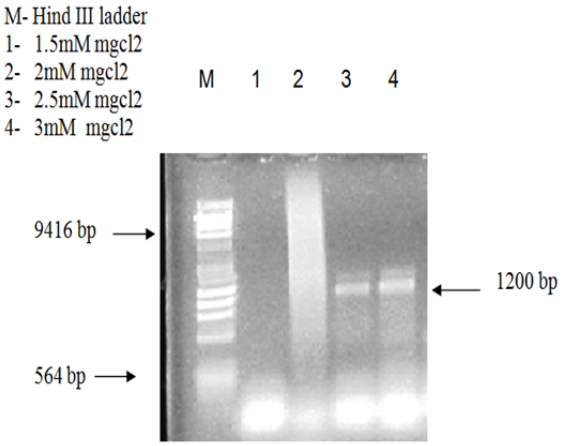


Figure-4 Optimisation of ITS in *W.bancrofti* using mgcl2

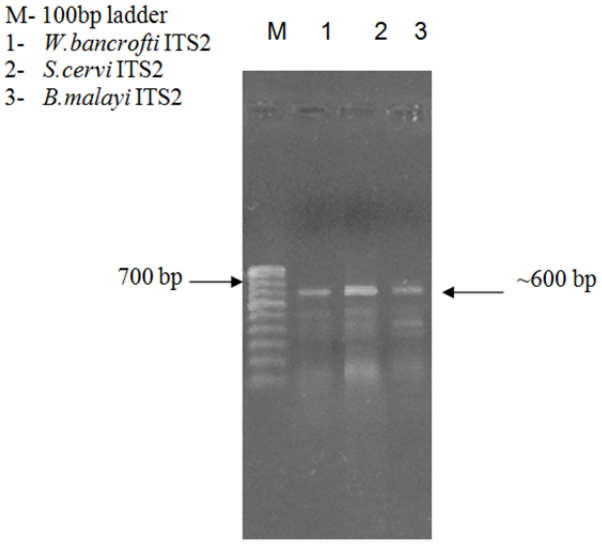


Figure-5 Amplification of ITS2 region of *W. bancrofti* and *B.malayi*

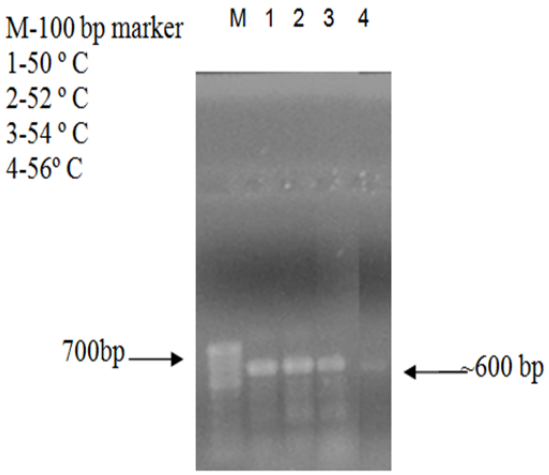


Figure-6 Optimisation of ITS2 in *W.bancrofti* using temperature gradient

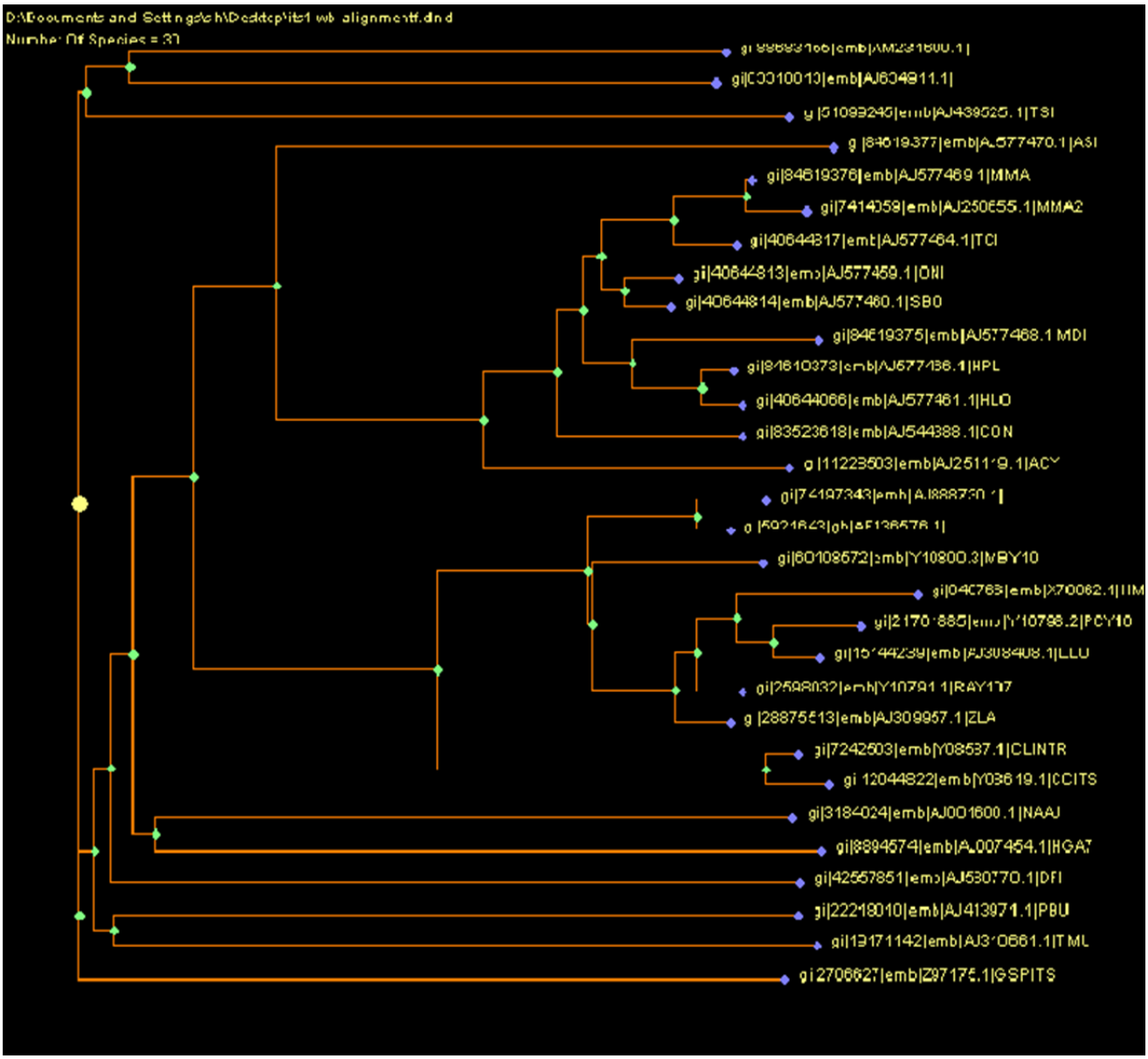


Figure-7 Phylogenetic analysis of Filarial nematodes using ITS2 sequences

Subsequently, another set of primers which possess the conserved region of 3' end of the 5.8S gene of *Gnathostoma* (ITS2-News2) (Forward) and the 5'end of the 28SrDNA of (FL2-R) (reverse) were used for the amplification of about 600bp of ITS-2region from *B. malayi, W. bancrofti, Setaria cervi* (Figure-5). The amplification of ITS-2 from W. *bancrofti* was done with different temperature and *Setaria* sample were sent for sequencing (Figure-6). The ITS2 sequences were used for phylogenetic analysis. The classification and organization of the lymphatic dwelling filariae clearly deserves to be reconsidered in the genomic era. A phylogenetic tree was constructed by compiling ITS2 sequences from free living and parasitic nematodes available from Genbank (Figure-7). This is in accordance with the outcome of [14] who estimated that *Brugia* and *Wuchereria* may have diverged some 675,000 years ago, a relatively recent split given the phylogenetic age of the superfamily Filarioidea.

CONCLUSION

The ITS region of W*uchereria bancrofti* which stretches about 1.2 kb was amplified by employing the specific primers. *Brugia malayi* and *Setaria* ITS region was also amplified by using the same primers. This reveals that the primers are based on the conserved regions in the 3'and 5'end of different filarial nematodes. The ITS2 segment of rDNA was also amplified in these filarial parasites and the size of the amplified fragment was found to be approximately 600bp. A more accurate classification of the lymphatic-dwelling filariae is not only interesting from a basic biological or taxonomical perspective. It could also have practical importance for the global effort to eliminate LF.

REFERENCES

1. David HL , Edeson JF. Filariasis in Portuguese Timer, with observations on a new microfilaria found in man. Ann Trop Med Parasitol. 1965;59:193–204.
2. World Health Organization, 1995. Bridging the Gaps. The World Health Report. Geneva: World Health Organization.
3. Gasser RB., Chilton NB., Hoste H. and Beveridge I. (1993) Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Res. 21, 2525-2526.
4. Bryan JH., Southgate BA. Factors affecting transmission of *Wuchereria bancrofti* by anopheline mosquitoes. 2. Damage to ingested microfilariae by mosquito foregut armatures and development of filarial larvae in mosquitoes. Trans Royal Soc Trop Med Hygiene. 1988;82:138–145
5. Raina VK, Joshi MC, Singh S, Joshi RD, Bhattacharjee KK, Kumar A, Verghese T (1990) Epidemiology of Brugia malayi infection and its co-existence with Wuchereria bancrofti in and around Sillaberia PHC, District Midnapur, West Bengal. J Commun Dis, 22:205–208
6. Beckingham, K. 1982. Insect rDNA. *Cell NucL*, **10**, 205–263.
7. Gerbi SA. 1985. Evolution of ribosomal DNA. In Maclntyre, R. (ed.) *Molecular Evolutionary Genetics*, Plenum, New York, pp. 419–517.
8. Musters W, Boon K, Sande Van Der CAFM, Heerikhuizan HV and Planta RJ 1990 Function analysis of transcribed spacers of yeast ribosomal DNA. EMBO 9: 3989-3996.
9. Porter CH, Collins FH 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *Am J Trop Med Hyg 45*: 271-279.
10. Nuchprayoon S, Sangprakarn S, Junpee A, Nithiuthai S, Chungpivat S, Poovorawan Y, 2003. Differentiation of Brugia malayi and Brugia pahangi by PCR-RFLP of ITS-1 and ITS-2. Southeast Asian J Trop Med Public Health 34: 67–73.
11. Joseph Felsenstein PHYLIP - Phylogeny Inference Package *Cladistics*, Vol. 5 (1989), pp. 164-166
12. Maniatis T., Fritsch EF. & Sambrook J (1982). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
13. Robin B. Gasser A, Louise E. Stewart B, Richard Speare B. Genetic markers in ribosomal DNA for hookworm identification ,Acta Tropica 62 (1996) 15-21.
14. Gasser RB, 2001. Identification of parasitic nematodes and study of genetic variability using PCR approaches. Kennedy MW, Harnett W, eds. Parasitic Nematodes. Wallingford, United Kingdom: CAB International, 53–82.