RESEARCH NOTE

MOLECULAR GENETICS ANALYSIS FOR CO-INFECTION OF *BRUGIA MALAYI* AND *BRUGIA PAHANGI* IN CAT RESERVOIRS BASED ON INTERNAL TRANSCRIBED SPACER REGION 1

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Abstract. This study described the diagnosis of a mixed infection of *Brugia malayi* and *Brugia pahangi* in a single domestic cat using the internal transcribed spacer 1 (ITS1) region. Following polymerase chain reaction amplification of the ITS1 region, the 580 bp amplicon was cloned, and 29 white colonies were randomly selected for DNA sequencing and phylogenetic tree construction. A DNA parsimony tree generated two groups of *Brugia* spp with one group containing 6 clones corresponding to *B. pahangi* and the other 23 clones corresponding to *B. malayi*. This indicated that mixed infection of the two *Brugia* spp, *B. pahangi* and *B. malayi*, had occurred in a single host.

INTRODUCTION

Lymphatic filaria, *Brugia malayi*, is mainly distributed in many Asian countries. It has been reported that *B. malayi* can infect not only humans but also animals such as cats, monkeys, and dogs (Laing *et al*, 1960; Mak *et al*, 1982; Mak, 1984). These animal reservoirs play an important role as disease carriers and lead to problems with eradication in endemic areas.

Basically, *B. malayi* and *B. pahangi* microfilaria cannot be distinguished morphologically by using traditional Giemsa staining. Although acid phosphatase staining is effective but it is not reproducible and the procedure is complicated (Yen and Mak, 1978). This makes it difficult for diagnosis of mixed infection of *B. malayi* and *B. pahangi* in a single cat reservoir. Molecular methods based on polymerase chain reaction (PCR) have been introduced for discrimination of *B. malayi* and *B. pahangi* (Thanomsub *et al*, 2000; Chansiri *et al*, 2002; Fischer *et al*, 2002; Nuchprayoon *et al*, 2005; Rishniw *et al*, 2006). However, because there is more than 95% homology in nucleotide sequences of various genes in the two species, it is difficult for their application in diagnosis of mixed infection of these two closely related species.

In this study, molecular phylogenetic analysis for identification of mixed *Brugia* spp infection based on internal transcribed spacer region 1 (ITS1) was explored in a single cat reservoir. ITS region has been routinely used for investigation of closely related species of

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a wide range of organisms, particularly within groups with controversial taxonomy (Chilton *et al*, 2001; Subbotin *et al*, 2001; Weekers *et al*, 2001; de Rojas *et al*, 2002). The phylogenetic trees inferred from ITS1 nucleotide sequences were analyzed and clearly indicated a mixed infection of *B. malayi* and *B. pahangi* in a single host.

MATERIALS AND METHODS

Blood samples

A blood sample was taken from a cat naturally infected with *Brugia* from *B. malayi* endemic area of Narathiwas, Thailand. The blood sample was screened by traditional blood smear technique prior to parasite isolation.

Parasite isolation

Five ml of microfilaria-infected blood were collected in an EDTA-containing (7 mg/ml) tube. Blood sample was diluted with an equal volume of phosphate buffered-saline, pH 7.0 (PBS) and filtered through a 5 μm polycarbonate membrane (Minipore). Microfilariae were then resuspended in PBS and centrifuged at 6,300*g* for 10 minutes at 4ºC. The pellet containing microfilariae was washed three times with PBS prior to storage at -70ºC until use.

PCR amplification

DNA was extracted and purified from the filarial parasites using a commercial DNA purification kit (Gentra) according to manufacture's instructions. PCR amplification of ITS1 was achieved using primers designed from 3´ end of 18S rDNA (AY621466), ITS1F (5´ GGT GAA CCT GCG GAA GGA TC 3´), and 5´ end of 5.8S rDNA (AY621466), ITS1R (5´ AAC CCA ATG GTG CAA TGT GC 3´). The reaction was conducted in 25 μl volume containing 50 ng of genomic DNA in 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 μM each of the primers, 100 μM each of dATP, dTTP, dCTP and dGTP, 1.5 mM $MgCl_2$ and 1.5 U of *Taq* DNA polymerase (Invitrogen). PCR was performed using a DNA thermal cycler (MJ Research PTC-200 Peltier thermal cycler) for 30 cycles. Each cycle consisted of 94ºC for 1 minute, 46ºC for 1 minute 72ºC for 1 minute. PCR amplicon was analyzed by electrophoresis in a 1.5% agarose gel at 110 V for approximately 30 minutes prior to staining in 0.5 μg/ml of ethidium bromide solution and observation under ultraviolet light.

Cloning and DNA sequencing

PCR fragment was eluted from the gel and purified using the QIAGEN® Purification system prior to cloning. The amplicon was inserted into pGEM® -T Easy Vector (Invitrogen, Promega) and transfected into *E. coli* strain DH5α competent cells using electroporation (BIO-RAD MicroPulser™). Transformants were selected on LB-ampicillin agar plates containing X-gal (Sigma) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) for 17 hours at 37ºC. Twenty-nine white colonies were randomly selected and recombinant DNA was extracted and purified using QIAGEN® QIAprep Spin Miniprep Kit prior to DNA sequencing at Macrogen (Seoul, Korea) using the Big Dye Terminator Cycle Sequencing procedure.

Phylogenetic tree construction

Complete nucleotide sequences of ITS1 (Table 1) were aligned against those of *B. malayi* (accession numbers AY499507, AY621464, AY621465, AY621466, AY621467 and AY621468), *B. pahangi* (accession numbers AY621469, AY621470, AY621471 and AY621472) and *D. immitis* (accession number AF 217800) using the Clustal X software version 1.83 (multiple sequence alignment) program (Thompson *et al*, 1997) and refined using the manual method. Alignment was further analyzed using PAUP version 4.0b10 for construction of phylogenetic trees based

on DNA parsimony (DNA PARS) (Swofford, 2002) using *D. immitis* as the outgroup. Trees were created via stepwise addition by means of maximum likelihood and maximum parsimony methods using a heuristic search setting. Bootstrap values were replicated 1,000 times and computed using PAUP program version 4.0b10.

RESULTS

Upon PCR amplification of the complete ITS1 region from *Brugia* genomic DNA, a 580 bp amplicon was obtained (Fig 1). The nucleotide sequences of 29 randomly selected clones containing the ITS1 region were identified using BLAST program, which revealed that they all were *Brugia* spp (Table 1). The ITS1 tree demonstrated that two groups of *Brugia* spp were clearly separated (Fig 2). Group 1 contained six clones of *Brugia* ITS, and *B. pahangi* (accession numbers AY621469, AY621470, AY621471 and AY621472) with a bootstrap value of 86%. Group 2 consisted of 23 clones of *Brugia* ITS,

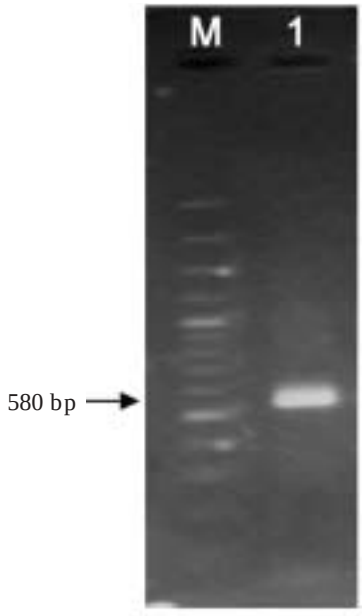

Fig 1–Agarose gel-electrophoresis profile of PCR amplification of ITS1 region from *Brugia* spp DNA using ITS1 specific primers, ITS1F and ITS1R. Lane M and lane 1 represent 100 bp DNA ladder marker and 580 bp PCR product amplified from *Brugia* spp ITS1 region, respectively.

Table1
GenBank accession numbers of ITS regions of *Brugia* spp.

Parasite name	Specific host/lab host	Regions	Origin	GenBank accession numbers
B. malayi clone No. 55, 53, 26, 22, 37, 55, 39, 63, 32, 62, 40, 49, 50, 57, 58, 64, 60, 66, 20, 51, 33, 65, 17	Cat	ITS1	Narathiwas,Thailand	EU419325- EU419347
B. pahangi No. 61, 43, 36, 46,38, 34	Cat	ITS1	Narathiwas,Thailand	EU419348- EU419353
B. malayi No. AY499507Bm	Unknown	ITS1	Thailand	AY499507
B. malayi No. AY621464Bm	Unknown	ITS1	Thailand	AY621464
B. malayi No. AY621465Bm	Unknown	ITS1	Thailand	AY621465
B. malayi No. AY621466Bm	Unknown	ITS1	Thailand	AY621466
B. malayi No. AY621467Bm	Unknown	ITS1	Thailand	AY621467
B. malayi No. AY621468Bm	Unknown	ITS1	Thailand	AY621468
B. pahangi No. AY621469Bp	Unknown	ITS1	Thailand	AY621469
B. pahangi No. AY621470Bp	Unknown	ITS1	Thailand	AY621470
B. pahangi No. AY621471Bp	Unknown	ITS1	Thailand	AY621471
B. pahangi No. AY621472Bp	Unknown	ITS1	Thailand	AY621472
D. immitis No. AF217800Di	Unknown	ITS	Taipei, Taiwan	AF217800

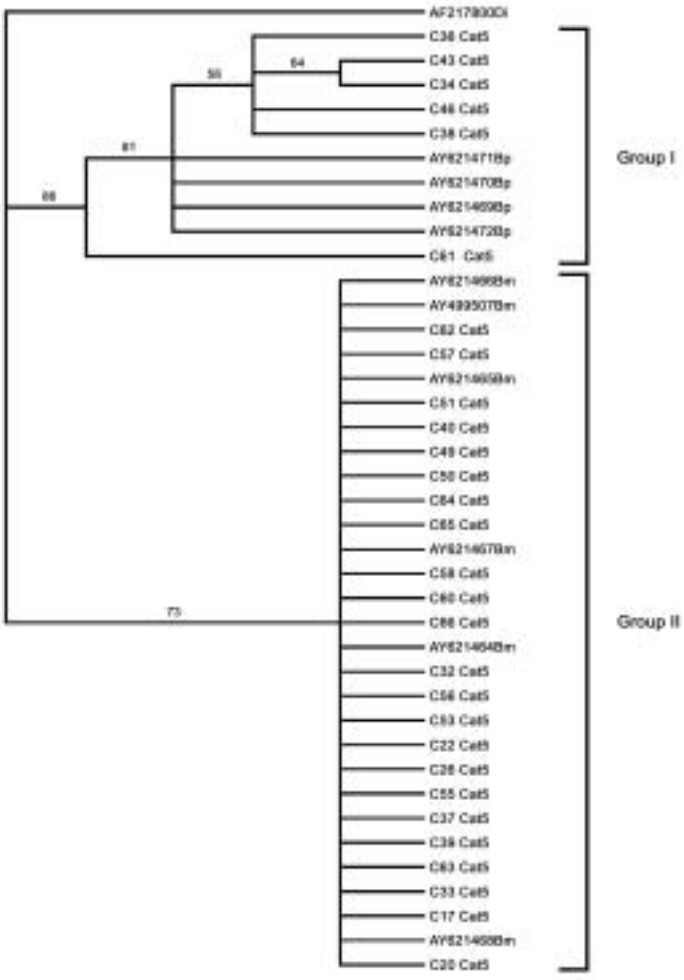

Fig 2–DNA PAR tree of ITS1 region from feline *Brugia* spp against *B. malayi*, *B. pahangi*, *B. timori*, and *D. immitis* (outgroup). Trees were produced by heuristic search using PAUP version 4.0b10 and inferred from the complete ITS1 region using a bootstrap re-sampling of the data set. Number nodes represent percentage of times the group occurred out of 100 trees. Branches with less than 50% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species.

and *B. malayi* (accession numbers AY499507, AY621464, AY621465, AY621466, AY621467 and AY621468) with a bootstrap value of 73%.

DISCUSSION

This study applied molecular phylogenetic analysis of ITS1 nucleotide sequences for differentiation of *Brugia* spp in a single cat reservoir from an area endemic for *B. malayi*. It is known that *B. malayi* and *B. pahangi* are very similar morphologically. Species identification by Giemsa staining is not sufficient (Yen and Mak, 1978). In addition, most of the coding and non-coding genes of the two species show a high degree of homology (Williams *et al*, 1988; Harasawa *et al*, 1997). Our preliminary study of the 18S rDNA sequences of both species showed that they were more than 95% homologous, and the data failed to generate a phylogenetic tree (data not shown). Similarly, other genes, such as those encoding the cuticle, sheath protein, heat shock protein, trans-spliced exon 1, H*ha*I repetitive region, and glutathione showed little differences in nucleotide sequences between the two *Brugia* species (data not shown).

This study exploited the ITS region because it has been reported to be informative for intra-species discrimination or differentiation of very closely related species. The tree inferred from the ITS1 data was able to differentiate between *B. malayi* and *B. pahangi* and indicated the presence of mixed infection in a single cat. This method could be applicable for study of the dynamics of these parasites as well as their

epidemiology, control, and eradication in endemic areas.

ACKNOWLEDGEMENTS

We thank Mr Sirichai Phantana, Ministry of Public Health, Bangkok, Thailand, for advice regarding sample collection. This project was supported by Srinakharinwirot University (grant number 042/2549).

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