CHAPTER IV

RESULTS

1. PCR-RFLP of ITS1 for filarial nematodes identification

The semi-nested PCR using FL1-F, FL2-R, and Di5.8S 660-R primers could amplify ITS1 region of *B. malayi*, *B. pahangi*, and *D. immitis* that give PCR product size approximately 580 bp, 590, and 670, respectively (**Figure 4**). However, these primers were limited in the range of species they could detect. They could not detect *D. repens*, another filarial parasite in cats. Moreover, these PCR need semi-nested PCR for detection that are tedious and time consuming. In order to design new diagnotic primers for amplified all filarial nematodes, the ITS1 and flanking regions of *B. malayi* and *B. pahangi* were cloned and sequenced.

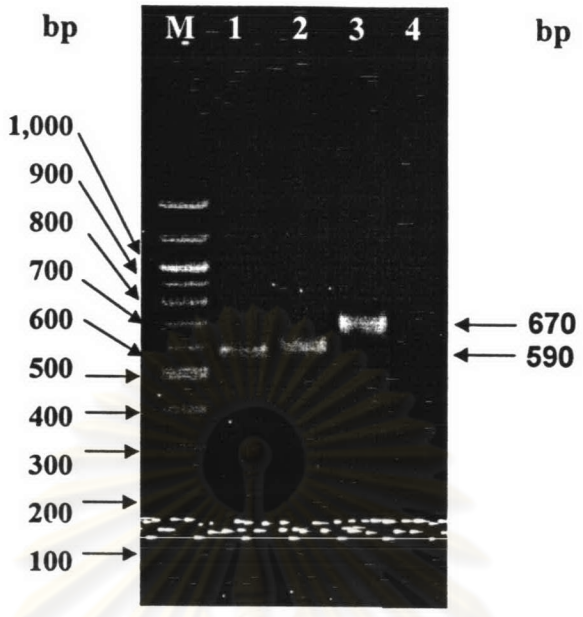

Figure 4 An ethidium bromide stained agarose gel showing semi-nested PCR of ITS1 of filarial parasites. Lane M, 100 bp DNA ladder; lane1, *B. malayi*; lanes 2, *B. pahangi*; lanes 3, *D. immitis*; lanes 4, *D. repens*.

1.1 Cloning and sequencing of filarial ITS1

After purification the ITS1 PCR product either *B. malayi* or *B. pahangi* and pGem®-T vector were ligated and transformed to the *E. coli* competent cells. After plasmid DNA extraction, the selected clones were digested with *Eco*R I, and then fractionated on agarose gel electrophoresis to screen the clones which containing of approximately 580 bp insertions (**Figure 5**).

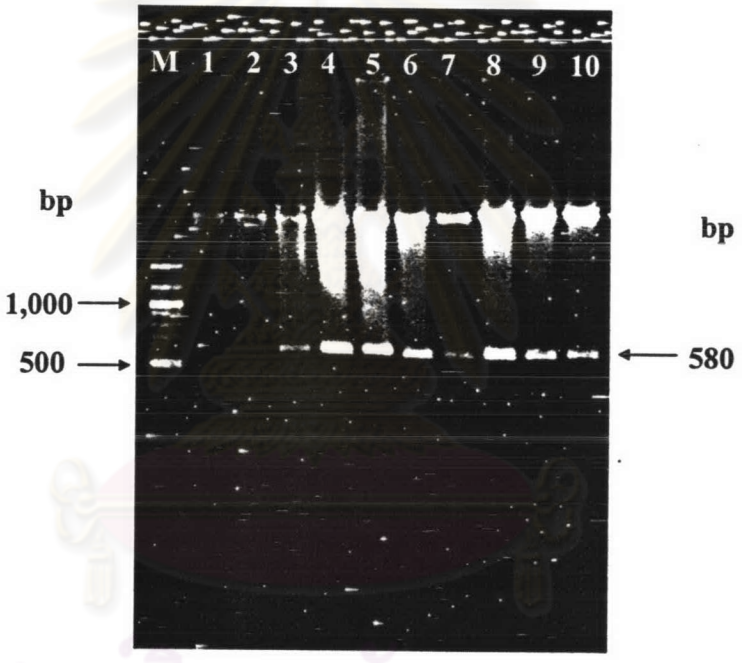

Figure 5 An ethidium bromide stained agarose gel showing digested recombinant ITS1/pGem®-T. Lane M; 100 bp DNA ladder, lane 1-10; digested ITS1 of *B. malayi* in pGem®-T.

1.2 Nucleotide sequence alignment and primers design

The nucleotide sequence of 18S rDNA, ITS1, and 5.8S rDNA of *B. malayi*, *B. pahangi*, and *W. bancrofti* from DNA sequencing of ITS1 cloned and other filarial parasites, *D. immitis* (AF217800), *Onchocerca volvulus* (AF228565), *Mansonella ozzardi* (AY228560), *Dipetalonema reconditum* (AF217801) from Genbank were align using CLUSTAL X. The result showed that 18S rDNA and 5.8S rDNA of all filarial nematode are nearly identical. This made it suitable to design new primers for ITS1 amplification. The new ITS1 forward primer (ITS1-F) and reverse primers (ITS1-R), located in the conserved region of 18S rDNA and 5.8S rDNA, respectively (**Figure 6**).

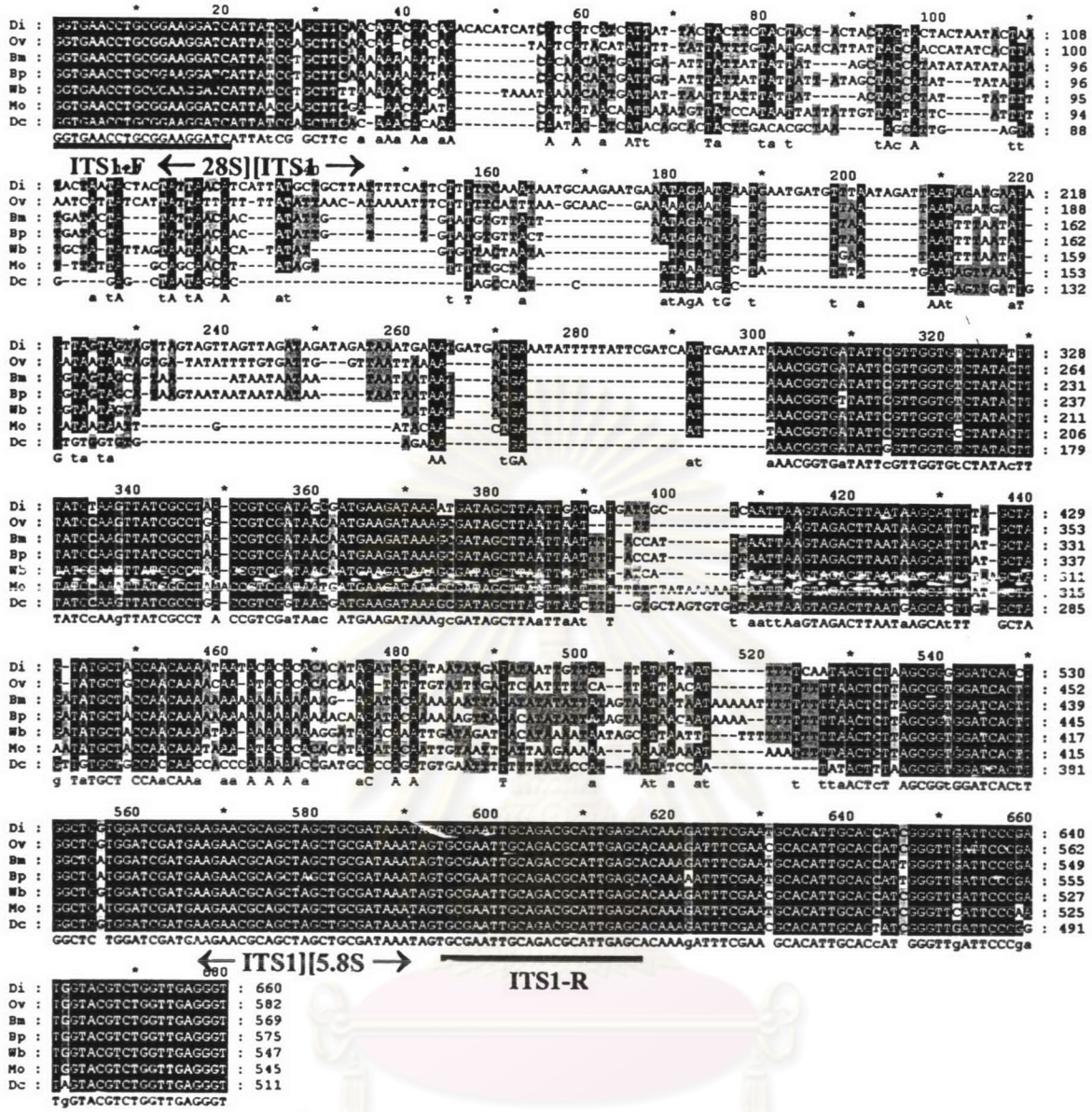

Figure 6 Alignment of rDNA from *B. malayi* (Bm), *B. pahangi* (Bp), *W. bancrofti* (Wb), *D. immitis* (Di), *O. volvulus* (Ov), *M. ozzardi* (Mo), and *D. reconditum* (Dc). Identical nucleotides were highlighted. The numbers refer to alignment position. Underlined indicate the regions (ITS1-F, ITS2-R), which were designed as the primers.

1.3 PCR-RFLP of filarial ITS1

1.3.1 PCR Amplification by ITS1-F and ITS1-R primers

After using ITS1-F and ITS1-R, *B. malayi*, *B. pahangi*, *D. immitis*, and *D. repens* could amplify in a single PCR. The ITS1 PCR products amplified by ITS1-F and ITS1-R shows in **Figure 7**. This primer set yielded 504, 510, 595, and 602 bp PCR products of *B. malayi*, *B. pahangi*, *D. immitis*, and *D. repens*, respectively. Comparison of undigested ITS1 PCR products revealed that filarial nematodes could be identified to genus level based on a product size.

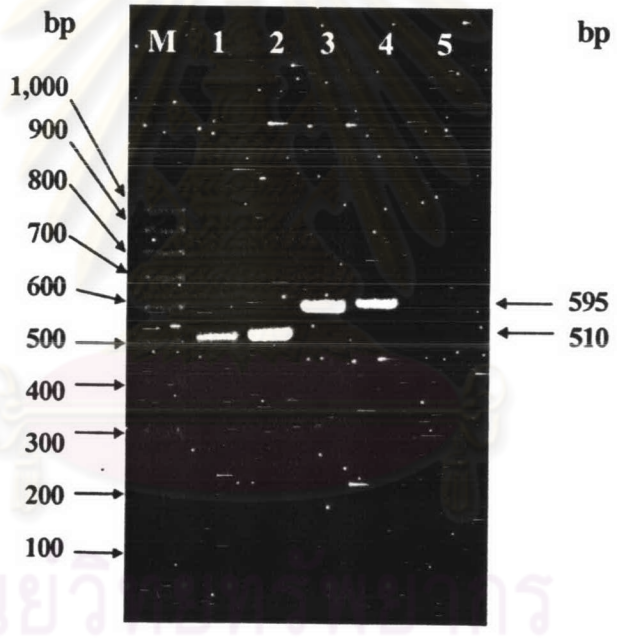

Figure 7 An ethidium bromide stained agarose gel showing the PCR product of filarial ITS1 using ITS1-F and ITS1-R primers in single PCR. Lane M, 100 bp DNA ladder; lane1, *B. malayi*; lanes 2, *B. pahangi*; lane 3, *D. immitis*; lane 4, *D. repens*; ; lane 5, negative control.

1.3.2 Sensivity of the PCR for detection of *B. malayi* ITS1

The PCR product of *B. malayi* using ITS1-F and ITS2-R primers could detect as little as 10 pg of filarial nematode (**Figure 8**).

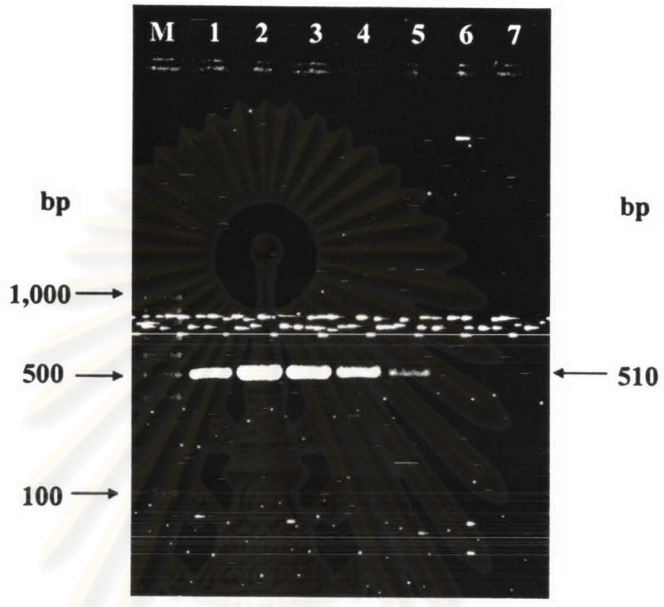

Figure 8 Sensivity of the PCR for detection of *B. malayi* ITS1 (510 bp) by using ITS1-F and ITS2-R. Lane M, 100 bp ladder; lane1, positive control (*B. malayi* genomic DNA 100 pg); lanes 2-6, *B. malayi* genomic DNA 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg, respectively; lane 7, negative control.

1.3.3 PCR-RFLP analysis of filarial ITS1 digested with *Ase* I

Restriction map analysis of ITS1 digested with *Ase* I allowed the unequivocal delineation of filarial nematodes. Digestion with *Ase* I produced three fragments of 218 bp, 153 bp and 133 bp for *B. malayi*, while two fragments of 292 bp and 218 bp were detected for *B. pahangi*. Two fragments of 380 bp and 215 bp were detected for *D. immitis*, while the 602 bp of *D. repens* PCR product remained undigested

The different band patterns generated after digested with *Ase* I were used to differentiate species of filarial nematodes as shown in **Figure 9**.

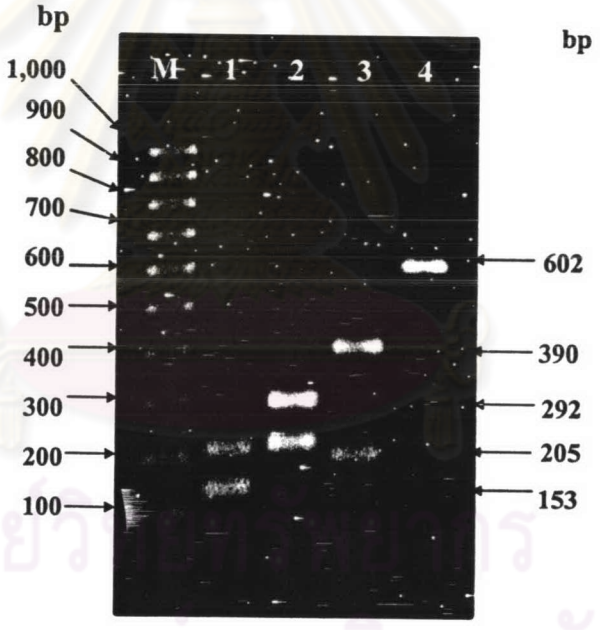

Figure 9 RFLP analysis of filarial ITS1 digested with *Ase* I.

Lane M, 100 bp DNA ladder; lane 1, *B. malayi* (133/ 153 / 218 bp); lane 2, *B. pahangi* (218/ 292 bp); lane 3, *D. immitis* (205/ 390 bp); lane 4, *D. repens* (602 bp).

2. Epidemiology studies

2.1 Description of the study area and specimen collection

Pra-sang district, Surat-thani province, the southern part of Thailand is an endemic area of filariasis. Most of the area is cultivated land consisting of rubber plantations, palm oil plantations and other agricultural crops. There is also a permanent large fresh water swamp with a various kinds of aquatic plants, grasses and weeds in it which exist throughout the year. This swamp is a suitable breeding site for *Mansonia* mosquito, a common vector of *B. malayi* (Filariasis Division, 2002). The domestic cats is served as an important hosehold pet, in this area.

The field blood specimens were collected from all domestic cats from villages No. 10 of Tri-khung subdistricts and villages No. 10 of E-pun subdistricts in Pra-sang district, Surat Thani Province **(Figure 10)**. Totals of 52 cats were included in this study. Twenty-five cats were male and 27 cats were female. The mean age of study cats was 4 years. The species of filarial nematodes in each sample was determined by two techniques; morphology and PCR-RFLP.

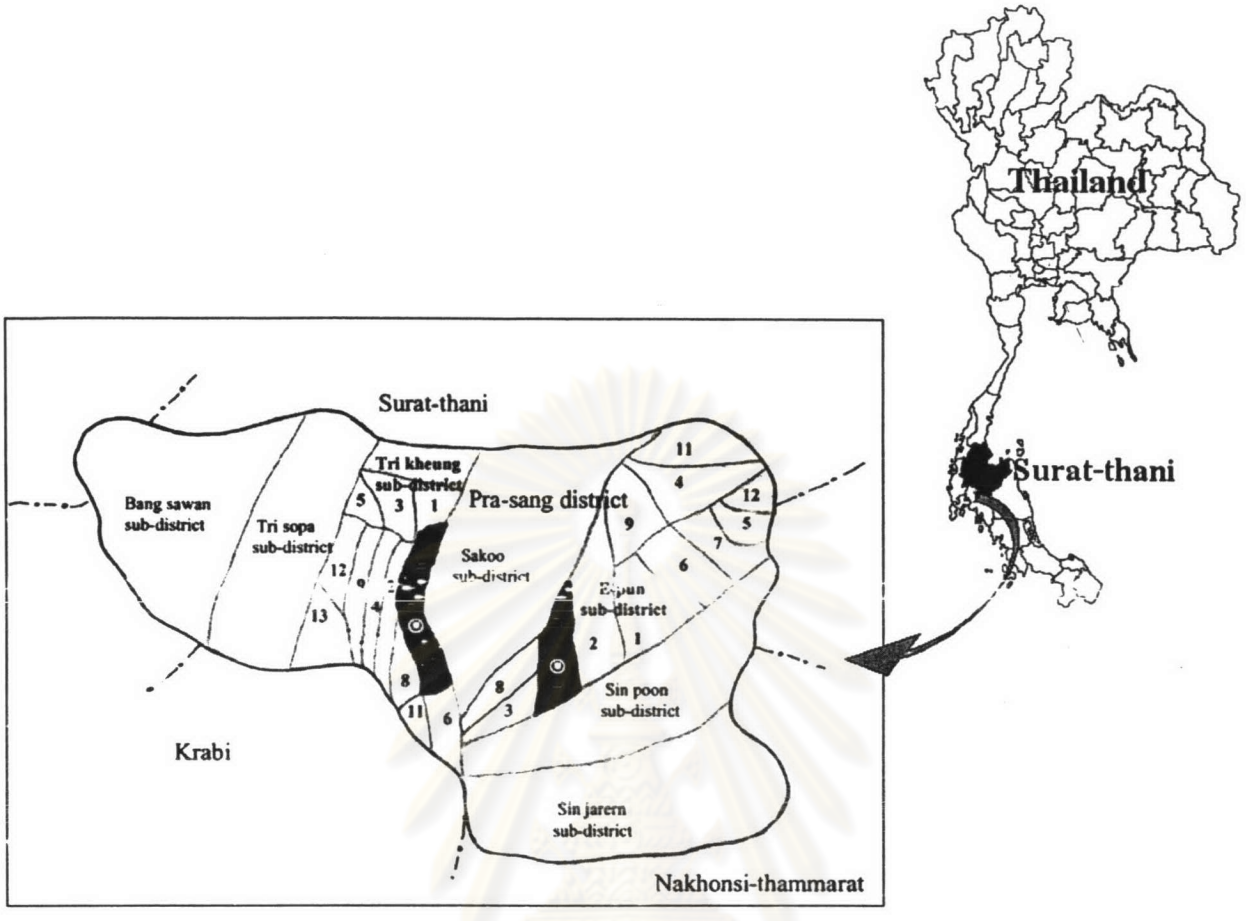

Figure 10 The study area (◉) at Pra-sang district, Surat-thani province.

2.2 Species identification by morphology

The conventional microscopic examination by Giemsa and acid phosphatase stained blood films for microfilariae were used for identification of the filarial nematodes.

Of the 52 cats examined, 3 had microfilariae: 2 (3.8%) with *B. pahangi* and 1 (1.9%) with *D. immitis* as shown in **Table 1**. Two cats were female and one cat was male. The mean age of infected cats was 4 years.

For *B. pahangi*, Giemsa staining showed the clear sheath covering microfilariae with 2 terminal nuclei (TN) (**Figure 11A**). Acid phosphatase staining showed heavy and diffuse acid phosphatase activity along entire body (**Figure 11B**).

For *D. imimitis*, Giemsa staining showed unsheathed microfilaria with single nucleus (N) at the beginning of nuclear column (**Figure 12A**). Acid phosphatase staining showed the phosphatase activity at the excretory vesicle (EV) and the anal vesicle (AV), while the rest of the body remained unstained (**Figure 12B**).

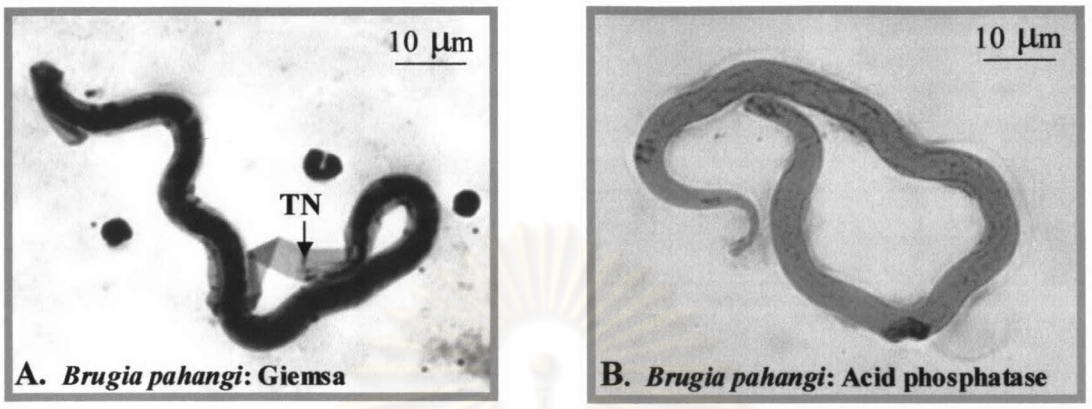

Figure 11 Microscopic examination of *B. pahangi* microfilariae from cats.

(A) Geimsa stained showing the sheathed microfilaria and 2 terminal nuclei (TN).

(B) Acid phosphatase stained showing the strong red color along entire body.

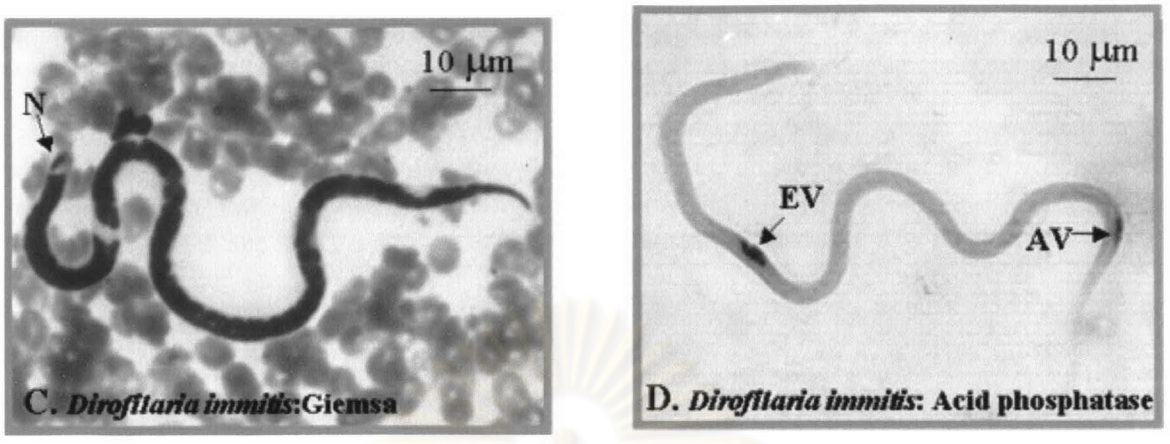

Figure 12 Microscopic examination of *D. immitis* microfilariae from cats.

(A) Geimsa stain showing unsheathed microfilaria with a single nucleus (N) at the beginning of nuclear column.

(B) Acid phosphatase stain showing strong red color at the excretory vesicle (EV) and the anal vesicle (AV), while the rest of the body remained unstained.

2.3 Species identification by PCR-RFLP of ITS1 digested with *Ase* I

The 52 blood samples were examined using PCR-RFLP digested with *Ase* I. The PCR-RFLP could detect all microfilariae positive cats. The data revealed that 4 cats were infected by *B. pahangi* and 1 cat was infected by *D. immitis* with the prevalence rates of infection at 7.6 and 1.9%, respectively. The species identification by PCR-RFLP technique provided the concordant result with the microscopic technique. Moreover, the PCR-RFLP technique could detect two more blood samples with *B. pahangi*, increasing the prevalence from 5.7% to 9.5% **(Table 1)**

Table 1 Detection of filarial nematodes infections in domestic cats by microscopic methods and PCR-RFLP in 2 villages of Pra-sang district, Surat-thani province, Thailand.

Technique	No. examined	No. positive (%)				
		B. malayi	*B. pahangi*	*D. immitis*	*D. repens*	Total
Microscopic Methods	52	0	2 (3.8)	1 (1.9)	0	3 (5.7)
PCR-RFLP	52	0	4 (7.6)	1(1.9)	0	5(9.5)

Note: Microscopic methods: Giemsa stain and acid phosphatase activity stain.

PCR-RFLP: digested with *Ase* I.