CHAPTER V

DISCUSSION AND CONCLUSION

The conventional method used for detection and species differentiation of lymphatic filarial parasites is Giemsa stain. Although this method is not expensive, it requires skill and experience to differentiate among closely-related species. The histochemical stian of acid phosphatase activity is another useful technique for species identification. It clearly discriminates different filarial nematode species (Yen & Mak, 1978; Chungpivat *et al.*, 1990; Nithiuthai & Chungpivat, 1992; Chungpivat & Sucharit, 1993), but there was not a consistent feature for *W. bancrofti* (Yen & Mak, 1978). However, the enzymatic activity is rather sensitive to light and requires fresh specimens. As a result, PCR-RFLP of ITS regions maybe an alternative method to differentiate closely-related species, which are indistinguishable by morphology (Gasser *et al.*, 1998). Furthermore, the PCR-RFLP could be used to identify any parasite in any developmental stages (Gasser & Chilton, 1995; Newton *et al.*, 1998; Almeyda-Artigas RJ *et al.*, 2000) in different geographic distributions (Gasser & Chilton, 1995; Ramachandran *et al.*, 1997). In addition, it is a powerful method to diagnose zoonotic infection caused by *Cryptosporidium* (Morgan *et al.*, 1999). However, this technique is more expensive than those two staining methods described above.

The ITS regions of rDNA are useful to investigate some variations among closely-related species (Ramachandran *et al.*, 1997; Gasser *et al.*, 1996). The ITS regions are flanked by conserved rRNA genes, which the sequences can be used to design primers to amplify the intervening regions by PCR. Moreover, the presence of multiple copies provides a large number of target sequences for PCR in most organisms (Long & Dawid, 1980). The ITS regions of rDNA of filarial nematodes are suitable targets for the amplification and detection even in mild infected individuals. Although the first round PCR was inadequate to provide detectable amount of the products, a semi-nested PCR was utilized to increase the sensitivity for the detection of ITS1 and ITS2 regions. Recent study of Verweij and colleagues succeeded in

diagnosing *O. bifurcum* infection in human using this method (Verweij *et al.*, 2000). Since, filarial DNA was extracted from whole blood of infected individuals, host genomeic DNA might be possible to interfere with the PCR reaction. However, the predicted PCR product size of ITS1 and ITS2 regions of humans are much different from the filarial DNA. The sizes of human host ITS1 and ITS2 PCR products (Genbank Accession No. U13369) are over 1.1 Kb. The ITS1 and ITS2 of filarial nematode are around 0.5-0.7 Kb. In addition, the bases at the 3'end of both internal primers designed for semi-nested PCR are not complimentary to human rDNA. Human DNA was used as negative control to preclude the possibility of co-amplification with filarial DNA.

The ITS1 and ITS2 PCR products of examined filarial species ranged from 0.5 to 0.7 Kb in length. The ITS1 and ITS2 PCR product sizes of all tested species (*W. bancrofti, B. malayi* and *B. pahangi*), except *D. immitis* were approximately the same and could not be clearly distinguished by size of the PCR product on the agarose gel electrophoresis. There were no detectable variations in ITS1 and ITS2 RFLP patterns within the same filarial species tested, which suggested that they were genetically similar. Occasionally, the faint bands were observed in the ITS2 PCR products, suggesting that they probably represent non-specific amplification of PCR products (Gasser *et al.*, 1994; Ramachandran *et al.*, 1997; Morgan *et al.*, 1999) or might be true variants in a minority of rDNA copies (Gasser & Chilton, 1995; Gasser *et al.*, 1998). Enzymatic digestion of the ITS1 PCR products with *Ase* I could produce diagnostic patterns for differentiation among filarial nematode species: *W. bancrofti, B. malayi, B. pahangi* and *D. immitis*. The ITS1 PCR products were applied to diagnose *B. malayi* and *B. pahangi*, which were difficult to be identified morphologically. In addition, the PCR-RFLP patterns of ITS1 region using *Acc* I could delineate genera of filarial nematodes. However, the PCR-RFLP profiles of *Hinf* I could not differentiate filarial parasites in ITS1 region. For ITS2 region, *Ase* I could distinguish among filarial genus, but *Rsa* I could not be clearly characterized by RFLP patterns. The PCR-RFLP profiles between cat *B. malayi* and human *B. malayi* were similar for all restriction endonucleases used. There was no difference in restriction patterns observed in *W. bancrofti* between Thai-Karens and Myanmar workers, although previous reports demonstrated different morphology features among mosquito vectors

between nocturnal sub-periodic form (Thai-Karens) and nocturnal periodic form (Myanmar workers) (Sumethvanich *et al.*, 1996; WHO, 1992). Of four restriction endonucleases used only *Acc* I could be used for distinguish four filarial species: *W. bancrofti, B. malayi, B. pahangi* and *D. immitis*. However the species differentiation was possible if more enzymes were used in PCR-RFLP.

Studies have shown the applicability of the rDNA region in parasites for differentiation among distinct species (Gasser *et al.*, 1994; Newton *et al.*, 1998; Heise *et al.*, 1999). Evidence has showed ITS2 sequence is digested more than ITS1. However, study of *Schistosoma sp.* showed that a part of ITS2 sequences was identical in all the Schistosome species tested (Kane & Rollinson, 1994), as in study of *Fasciola sp.* (Adlard *et al.*, 1993). In our study, it is most likely that the variation in RFLP patterns among examined filarial nematodes is due to significant difference in the ITS1 region as in *Schistosoma sp.*(Kane & Rollinson, 1994) and *Ascaris sp.* (Zhu *et al.*, 1999).

However, recent report of filarial parasite diagnosis using glutathione peroxidase gene (GSHPx) demonstrated that the RFLP profiles of this gene was a valuable tool for diagnosis and differentiation of human lymphatic filarial parasites (*W. bancrofti* and *B. malayi*) and *B. pahangi* (Thanomsub *et al.*, 2000). Moreover, the study proved that this gene is not quite specific for blood parasite, *Plasmodium falciparum*, which always co-exist in the endemic areas of Thailand. However, this gene could not be employed in animal filarial parasites, *D. immitis*, that could lead to zoonotic infection (Theis *et al.*, 2001). In this study using the ITS regions could differentiate not only lymphatic filarial parasites but also animal filarial parasites that cause zoonotic infection. The primers used in this study are not specific for blood parasites: malaria, trypanosome and leishmania, which proved that this experiment could be applied in the field study for the detection of co-existent of these parasites.