CHAPTER 5

Discussion

5.1 Study on the Prevalence of *Echinstoma revolutum* in Snails in Chiang Mai Province

Several studies on the infection of digenean larvae in snail hosts have been reported from Thailand (Woodruff and Upatham, 1992; Dechruksa *et al*., 2007; Sri-aroon *et al*., 2010). These studies noted many described and undescribed digenean species. In this study, metacercariae of *Echinostoma revolutum* were found in 7 snail species which act as the second intermediate host of this parasite. *Clea helena*, *E. revolutum* metacercariae were found for the first time in Thailand. For the experimental infection, metacercariae were force-fed to hamsters and domestic chicks and adult worms were successfully recovered from their small intestines. They were identified as of *E. revolutum* (Fröelich, 1802) Looss, 1899 based on the biological features of adult worms recovered; including the morphology, morphometrics, host-parasite relationships and geographic distribution.

However, some difficulty was reported in identifying the species, because many different species with similar morphologies were reported among the 37-collar spined echinostomes, the so-called '*revolutum*' group, and taxonomic problems exist among those species. Ten species of *Echinostoma* were listed as valid within the "*revolutum*" group by Fried and Graczyk (2004): *E. caproni* Richard, 1964; *E. trivolvis* (Cort, 1914); *E. paraensei* Lie & Basch, 1967; *E. revolutum* (Frölich, 1802); *E. friedi* Toledo *et al*., 2000; *E. miyagawai* Ishii, 1932; *E. echinatum* (Zeder, 1803); *E. parvocirrus* Nassy & Dupouy, 1988; *E. luisreyi* Maldonado *et al*., 2003 and *E. jurini* (Skvortzov, 1924). Reviews of the *Echinostoma* species in the "*revolutum*" group in terms of morphological features, morphometrics, host-parasite relationships and geographic distribution (Beaver, 1937; Lie and Kanev, 1983; Kanev, 1994; Kanev *et al*., 1995a; 1995b; Kostadinova, 1995; Kostadinova *et al*., 2000; Toledo *et al*., 2000; Fried and

Graczyk, 2004; Chai, 2009; Toledo *et al*., 2009; Chai *et al*., 2011) revealed that specimens most closely resembled *E. revolutum* and *E. jurini*. Of these, the specimens in this study were more compatible to *E. revolutum.*

E. revolutum differs from *E. jurini* by its definitive hosts. *E. jurini* have only mammals serve as the definitive hosts, whereas *E. revolutum* take both birds and mammals as the definitive host (Kanev *et al*., 1995a; Fried and Graczyk, 2004). Also in the present study, *E. revolutum* could be infected to both hamsters and domestic chicks. Kanev *et al*. (1995a) noted that *E. revolutum* has lymnaeid snails as the first intermediate host, and molluscs, tadpoles, fish, and freshwater turtles as the second intermediate host. *E. jurini* has viviparid snails as the first intermediate host, and molluscs, frogs, and freshwater turtles as the second intermediate host. *E. jurini* has been reported from Europe and possibly in Asia. It is suggested that *E. jurini* occur in Asia where viviparid snail hosts are distributed. In this study, hamsters and domestic chicks were both successfully infected, and the second intermediate hosts were *Filopaludina* spp. and *E. eryresi* (viviparid snails), *Bithynia* spp. and *C. helena*. The first intermediate host for this flukes may be the same or different species of these snails, although this was not confirmed in this study. These results were highly compatible with *E. revolutum* rather than *E. jurini*.

There were many reports of human infections with the Echinostomatidae in north and northeast Thailand (Nithikathkul *et al*., 2008). Six species were included; *E. malayanum*, *Hypoderaeum conoideum* (Bhaibulaya *et al*., 1964), *E. ilocanum*, *E. japonicus*, *E. revolutum*, and *Episthmium caninum* (Radomyos *et al.*, 1982; 1985; 1994). The intermediate hosts of these trematodes, including snail, fish, and tadpoles have been poorly studied. Several studies have been done on the occurrence of digenean larvae in snail hosts in northeast, central, and southern Thailand (Woodruff and Upatham, 1992; Dechruksa *et al*., 2007; Sri-aroon *et al*., 2010). These studies reported many described and undescribed digenean species which varied in their larval stages. Less is known in northern Thailand especially in Chiang Mai province.

This study is a progressive report of freshwater snail distribution and infection status with *E. revolutum* metacercariae in Chiang Mai province. A total of 10,692 snail samples comprising of 12 species were collected. They consisted of 5 families,

including Viviparidae, Bithyniidae, Buccinidae, Thiaridae and Lymnaeiidae. As shown by the results, 4 viviparid snails (*F. doliaris*, *F. sumatrensis polygramma*, *F. martensi martensi* and *E. eyriesi*), 2 bithyniid snails (*B. funiculata* and *B. siamensis siamensis*) and 1 buccinid snail (*C. helena*) were found to be infected with *E. revolutum* metacercariae. The highest prevalence was seen in viviparid snails, *Filopaludina* species (55.7% to 63.1%) and *E. eryresi* (16.4%). Meanwhile, 3 snail species showed low prevalences, *B. funiculata* (6.8%), *B. siamensis siamensis* (2.6%) and *C. helena* (1.0%). On the other hand, thiarid snails (*A. housei*, *Th. scabra*, *M. tuberculata* and *Ta. granifera*) and lymnaeid snail (*L. auricularia rubiginosa*) were not infected with *E. revolutum* metacercariae. The metacercarial were found chiefly in the pericardial sac of the snails. Esteban and Antoli (2009) similarly reported that the Echinostomatidae metacercariae were found in the pericardial sac and kidney region of the snail host.

According to Brandt (1974), who studied on non-marine aquatic mollusca of Thailand, many *Filopaludina* spp. were found to harbor several kinds of Echinostomatidae cercariae and metacercariae. The larvae were not identified to the genus and species level. Bithyniid snails, *B. funiculata* and *B. siamensis siamensis*, are important intermediate hosts of *Opisthorchis viverrini* (Brandt, 1974; Petney *et al*., 2012) while in this study they were found to harbor *E. revolutum* metacercariae. *C. helena* appeared to be infected with *E. revolutum* metacercariae, although they had never been considered to be of medical importance previously (Beaver, 1937; Brandt, 1974; Woodruff and Upatham, 1992; Chai *et al*. 2011; Sri-aroon, 2011; Petney *et al*., 2012). Detection of *E. revolutum* metacercariae in *C. helena* is reported for the first time. This study also confirmed that *Filopaludina* spp., *E. eyriesi*, and *Bithynia* spp. act as the second snail intermediate host of *E. revolutum* under natural conditions, which is the first report for Chiang Mai province.

E. revolutum metacercariae showed a broad range of host specificity for snails because they could infect various species of freshwater snails. Based on the high prevalence and intensity of metacercarial infections in the snail, the surveyed area is a high risk area. The finding of *E. revolutum* in Chiang Mai province also raises an interesting question about its distribution throughout northern Thailand, its infection ability to infect local snail species, and its ubiquity in this area. Freshwater snails in Chiang Mai province are heavily infected with *E. revolutum* metacercariae and their life

cycle is actively maintained in the study area. The discovery of this fluke in this area is important for public health control and for monitoring its dispersion because *E. revolutum* can be easily transmitted to humans if they consume raw or partially cooked snails. Further, it is hoped that this study will be helpful to design public health prevention and control strategies for human echinostomiasis. Further studies are needed to elucidate the biology of other larval trematode species previously reported and also of unidentified trematode species in snail host.

5.2 Study on the Life History of *Echinostoma revolutum*

It is probable that the first experimental work on the life history of *Echinostoma revolutum* was done by Pagenstecher in 1857 (Beaver, 1937). The life cycle of *E. revolutum* was completed experimentally in the laboratory by Kanev (1994). The experiment began with infected snails collected at the type locality, near Erlangen, Germany. The *E. revolutum* also it was fond in Thailand but the life cycle and general life history of this trematode are still not clear.

E. revolutum is a parasite that infects a large number of different warm-blooded hosts, both in nature and the laboratory. The study revealed that chicks were evaluated as a suitable laboratory host, with high incidence and high worm recovery rate up to 74%. The average of worm recovery was 27.1% and maintained thus in domestic chick until day 36 PI. Humphries *et al*. (1997) reported that domestic chick can serve as a suitable experimental definitive host for *E. revolutum* from snail, *L. elodes* in the U.S.A. The high worm recovery of *E. revolutum* were obtained for many weeks in chicks demonstrated were similar to those found in domestic chicks studied by Fried (1984) and Humphries *et al*. (1997). The worm recovery rate was relatively higher than previous reports in the domestic chick by Fried and Alenick (1981) and Fried *et al*. (1997). Based on the distribution of worm indicate that this worm will invade the jejunum and ileum, and some in the caecum. The mostly localization of worm it has been reported in the ileum, caeca, bursa of Fabricius, and rectum in the domestic chick (Fried and Alenick, 1981; Fried, 1984; Fried *et al*., 1997). The longevity of this worm in chicks demonstrated in this study was similar to that found in domestic chicks studied (Senger, 1954) and pigeon (Kanev, 1994). Senger (1954) reported a life span of 35 days

of this worm in chicks and they were live between 4 and 8 weeks under laboratory conditions in pigeons (Kanev, 1994), while in this study they were can survive in chick for 36 days (5 weeks).

The growth and development of *E. revolutum* in domestic chick has been reported (Beaver, 1937; Senger, 1954; Fried, 1984; Humphries *et al*., 1997). Beaver (1937) provided extensive growth data on this worm in naturally and experimentally infected mammalian and avian hosts. None of his work was directed specifically at the growth of this worm in experimentally infected day-old chicks. Also the studies of this worm by Humphries *et al*. (1997), they were regarded on the body areas, but its growth and development were not considered. Senger (1954) reported a rapid increase in length until day 23, after which there was no apparent increase in size, while Fried (1984) noted that increased in length until at least day 36, at which time growth measurements were ended. In the present study *E. revolutum* increased in length until at least day 30. *E. revolutum* can develop in different host species displaying different of growth, development and degrees of compatibility. The population density of worm probably influence on variation in growth and development (Fried and Freeborne, 1984; Franco *et al*., 1988; Toledo, 2009).

Studies on the surface ultrastructure of *E. revolutum* has been undertaken with various host i.e. birds and mammals (Smales and Blankespoor, 1984; Fried and Fujino, 1984; Chai *et al*., 2011). However, the surface ultrastructure of this trematode was observed in this study, according development stages. The surface ultrastructure of collar was found to be similar to that of *E. trivolvis* and *E. caproni* described by Smales and Blankespoor (1984), Fried and Fujino (1984), Kruse *et al*. (1992), Ursone and Fried (1995) and Fujino *et al.* (1995). In the aforementioned papers surface ultrastructure information and details of the collar region were incomplete. For this reason, detailed comparisons between this study and the afore-mentioned studies were not possible. However, it is well known that *E. revolutum* and the 37-collar-spined allies of the *'revolutum'* group and their avian and mammalian hosts, including humans, are considered relatively new in an evolutionary sense. The tegumental ultrastructure of *E. revolutum* was generally similar to that of other echinostomes in the shape of tegumental spines and cytoplasmic processes, and the distribution pattern of tegumental spines and sensory papillae (Lee *et. al*., 1986, 1992; Torii *et. al*., 1989). However, some

features such as the morphological change of tegumental spines and the appearance of sensory papillae on the ventral sucker according to development, and number, shape and arrangement of collar spines were characteristic, which may be of taxomic and bioecological significance. The shape and distribution of tegumental spines of digeneans is closely related to worm maturation and parasitic niche. Some intestinal digeneans, tegumental spines are enlarge and more pointed as the worm maturates (Lee *et al.*, 1985; Hong *et al.*, 1991; Chai *et al.*, 1998, 2000).

Eggs of *E. revolutum* usually appeared in feces on days 10 PI of young pigeons (Kanev, 1994) and days 11 or 12 PI in hamster (Franco *et al*., 1986). Humphries *et al*. (1997) noted that worms from the domestic chick were ovigerous on day 12 PI, while the worms in this study were ovigerous and began to produce eggs on days 10 PI. This finding agrees with those on young pigeons (Kanev, 1994). Several studies have used quantitative methods for measuring fecundity of echinostomes (Toledo, 2009). The quantitative measurements for fecundity of *E. revolutum* are not well understood and have not been reported. The fecundity of *E. revolutum* in this study was measured by the UEC and EPG. The patterns of EPG/worm is similar to *E. malayanum* in mice (Srisawangwong *et al*., 2004), *E. friedi* in rat (Toledo *et al*., 2006) and *E. caproni* in mice (Odaibo *et al*., 1988), hamster and rat (Toledo *et al*., 2004), that gradually increases and high egg output was observed on day 22. The UEC of *E. revolutum* was not constant during this experiment. The UEC rapidly increased during the first two weeks of infection, probably in relation to the progressive maturation of the adult worms. The UEC/worm in *E. revolutum* infection in domestic chick in my study is similar to that observed for the closely related *E. caproni* in NMRI mice (Christensen *et al*., 1990). Recovered worm dependent constraint on worm fecundity was observed in these studies. The correlation coefficient indicates that the worm recovery and EPG/worm were highly correlated. The result also showed that positive correlation between worm recovery and UEC/worm. An increase of recovered worm may reveal that egg production and egg output in feces was high. In contrast, the correlation between EPG/worm and UEC/worm are not correlated. The numbers of egg in the uterus may not reflect the number of egg released. According to Odaibo *et al.* (1988), fecundity of *E. caproni* expressed by uterine egg counts and EPG/worm. This study prefers both parameters, UEC/worm and EPG/worm can be used to determine the

fecundity of *E. revolutum* in experimental chick. Egg production on the basis of UEC/worm and EPG/worm constitute only partial measurement of fecundity, because the egg output of echinostomes depends on a number of factors. Toledo (2009) suggests that these factors include echinostome species, population density, age of infection and host species. Uterine egg counts and number of egg released as measurements of the fecundity in *E. revolutum* infections in other host species needs to be re-evaluated.

LM investigations revealed the opercular and abopercular region in eggs of *E. revolutum*, agrees with Chai *et al*. (2011). Eggs of *E. revolutum* are similar morphologically to other echinostome including *E. paraensei, E. caproni,* and *E. trivolvis* (Fujino *et al*., 2000). The SEM of *E. revolutum* eggs has not been documented previously. The results reveal that the surface ultrastructure is similar to other echinostomes (Krejci and Fried, 1994; Fujino *et al*., 2000). The opercular junction is not conspicuous as with the eggs of other trematodes, heterophyid and opisthorchiid (Ditrich *et al*., 1992; Lee *et al*., 2012). The abopercular knob has winkles, deep invagination and infolding of the shell, while that of other echinostomes (i.e. *E. paraensei*, *E. caproni* and *E. trivolvis*) has superficial winkles and shallow infolding (Krejci and Fried, 1994; Fujino *et al*., 2000). This distinguishes this worm from other echinostomes. SEM finding of this worm egg was newly added for study and significant finding of abopercular knob were obtained.

Egg development of *E. revolutum* was reported by Davis (2005). Direct comparison between that study and mine cannot be made because of differences in the methods of egg preparation and incubation. Davis (2005) determines the effect of cold storage duration on the incubation success of *E. revolutum* eggs. The eggs were cold stored (4-8°C) for up to 72 weeks before being examined, whereas this study were used fresh eggs isolated from host feces which were undeveloped (unembryonated) when laid. This was done by Huffman and Fried (1990), and took about 10 days to reach the fully developed miracidial stage after their removal from adults grown in domestic chicks. The eggs contained clusters of vitellocytes at an early phase of development which has also been described in *E. caproni* eggs by Schmidt (1998). The vitellocystes in other trematode, *Fasciola hepatica*, have been termed as yolk cells (Hussein *et al*., 2010). During the late phase of egg development, balloon-like vesicles are formed and filled with a clear refrainment fluid. Schmidt (1998) suggested that these vesicles are

probably cell debris left from the vitellocytes which are dispersed by movements of the miracidia.

This study, miracidia become fully developed and hatch at as early as 10 days, but maximal miracidia hatching occurred in day 11. In comparison, initial hatching of *E. trivolvis* occurred after 11 days (Nollen, 1994) and hatching of *E. caproni* from hamster-source eggs in day 11 and from mouse-source eggs after 13 days (Behrens and Nollen, 1992). However, more details about egg viability needs to be included in the determination of the fecundity and parasite reproductive success.

Newly hatched miracidia swim rapidly and change direction frequently. They are live for 4-6 hours, as has been described by Kanev (1994). The morphological characteristics of the miracidia obtained from eggs from adult parasites of chicks, the average dimensions were in accord with the ranges of values of Kanev (1994). The eyespots are arranged in one pairs of structures in different sizes and are an important characteristic for the identification of this species. *E. trivolvis* has only one pair and *E. perfoliatus* three pairs of eyespots (Lie and Basch, 1966).

In this study, the attempt to infect *L. rubiginosa auricularia* failed. These results contradict those of Kanev (1994). *E. revolutum* uses only snails of the family Lymnaeidae including *L. auricularis, L. auricularis rufescens, L. luteola typica, L. luteola gracilor, L. ovata, L. palustris, L. peregra, L. rubiginosa, L. stagnalis, L. swinhoei, L. truncatula and L. viridis*, as the first intermediate host. *E. revolutum* does not develop in some 100 other prosobranch and pulmonate snails, notably planorbid, physid and viviparid snails (Kanev, 1994). In this study, *E. revolutum* can develop in viviparid snails (*Filopaludina* spp.). Although more recent studies show that the first intermediate host species for each echinostome species may be broader than previously expected (Coustau *et al*., 2009).

Under experimental conditions, miracidia of *E. revolutum* infected *Filopaludina* spp. most easily taking about 1-3 hours. The penetration time of miracidia according to Huffman and Fried (1990), *L. ollula* took about 1 hour. *Echinostoma* miracidia usually enter the head foot region of the snail and transform into sporocysts at the site of penetration, typically the mantle collar, the foot, and head covering (including velum and tentacles), the mantle cavity, and the oral cavity (Esteban and Antoli, 2009). In this

study *E. revolutum* miracidia penetrated the head covering especially tentacles of host snails and transformed into sporocysts. According to species, sporocysts may settle near the site of miracidia entry, or migrate through the host tissues and settle in the heart area within 1-2 days (Lo, 1995; Ataev *et al.*, 1998; Sapp *et al*., 1998; Kanev *et al*., 2000). In this study, the sporocysts settled in heart or aorta of the snail host, agreeing with Jeyarasasingam *et al.* (1972). After that, they undergo an asexual multiplication process resulting in the production of generations of mother rediae, daughter rediae, and finally cercariae that emerge from the host. Johnson (1920) suggested that *E. revolutum* sporocysts occurred infrequently because the miracidia of this species could develop directly into rediae. In contrast, my results showed that *E. revolutum* sporocysts occurred at 7 days PI. Experimental studies on *E. revolutum* showed that all miracidia develop into sporocysts (Beaver, 1937; Kanev, 1994). No evidence has been found to support direct development of miracidia into rediae in *E. revolutum*. The period of sporocysts in this study was similar to the period of development (5-8 days) of echinostomes in the genus *Echinoparyphium* (Kanev *et al*., 1994) and in *Echinostoma* (Kanev, 1994, Kanev *et al*., 1995a; 1995b).

The mother rediae and daughter rediae were present at 16 and 60 days PI, respectively. The mother rediae released from sporocysts migrate to the digestive gland-gonad complex and develop into daughter rediae. According to Esteban and Muñoz-Antoli (2009), echinostomes rediae migrate within the snail body and prefer particular habitats, mainly the digestive gland and gonads. After that, the larval stages of *E. revolutum* develop and mature into the cercarial stage. In this study, the echinostome cercariae were found 60 days after exposure. The echinostome cercaria is typically distomate with an oral collar of spines, not always visible, and a simple tail with fin-fold structure. The cercariae are swimming for 4-6 hours and die several hours later, while most echinostome cercariae are short lived and rarely survive beyond 48 hours at ambient temperatures (Esteban and Muñoz-Antoli, 2009). The growth and development of *E. revolutum* larval stages in the first intermediate host were not different among *Filopaludina* spp. under experimental conditions.

Cercariae of *E. revolutum* encyst in a broad range of second intermediate hosts, including freshwater pulmonate and prosobranch snails, mussels, frogs, and turtles (Kanev, 1994). The experimental infection of second intermediate host showed that

cercariae of *E. revolutum* penetrated and infected only viviparid snails (prosobranch), *F. martensi martensi* and *F. dorliaris*, which are their first intermediate host where they develop into metacercariae. Similarities in metacercariae of some echinostomes have also been found in freshwater snails harboring rediae and cercariae and serving as the first intermediate host (Esteban and Muñoz-Antoli, 2009). The attempt to infect cercariae in *L. auricularia rubiginosa* (lymnaeid snail) failed. The second intermediate hosts for *E. revolutum* are similar to that reported for this species by Chai *et al*. (2011) from Vietnam who reported that the life cycle of *E. revolutum* is maintained in Vietnam, using *Filopaludina* snails. Similar use of viviparid snails serving as the second intermediate host were reported in *E. jurini* (Kanev *et al*., 1995a) in Europe. They successfully infected *E. jurini* cercariae in the viviparid snails, *Viviparus viviparous* and *V. contectus*. The penetration and encystment of most *Echinostoma* cercariae occurred within 24 hours post-emerge from snails and probably cercariae are infective within the first 8 hours of release from snails (Huffman and Fried, 1990). While encystment of *E. revolutum* cercariae in this study was found in 2 days after exposure. The localization of echinostome metacercariae is variable. Echinostome cercariae typically encyst in the heart, pericardium, and kidney, some ectopic encystment does occur, mainly on snail mucus or *in vitro* in a tissue culture medium (Jeyarasasingam *et al*., 1972; Esteban and Muñoz-Antoli, 2009). Encysted metacercariae in this study were found clumped together in the pericardial sac of the snail hosts, with each cyst enveloped by thin connective tissues produced by the host origin.

The life cycle of *E. revolutum* was completed experimentally. Based on this study beginning with infected snails from epidemic areas, it is shown that the life cycle consists of eight stages, viz. adult, egg, miracidium, sporocyst, mother redia, daughter redia, cercaria, and metacercaria. The first and second intermediate hosts are viviparid snails, *F. doliaris* and *F. martensi martensi*. The final hosts are the domestic chick (*G. gallus domesticus*).

5.3 Molecular Identification and Phylogenetic Analysis

It is difficult to distinguish the "*revolutum*" group of *Echinostoma* species using only morphology or by conventional methods (Kostadinova and Gibson, 2000).

Molecular approaches, including PCR and sequencing based technology, are often applied for detection and discrimination of *E. revolutum*. Traditional molecular approaches are often laborious and time-consuming. In this study, the LAMP assay has been developed for the detection of *E. revolutum*.

The *E. revolutum* specific-LAMP assay was successful developed, which used a set of 6 specific designed LAMP primers that recognized 8 distinct sequences of the target DNA. One of the critical factors for efficient amplification of this assay depends on the *Bst* DNA polymerase, which is more resistant than other DNA polymerases (Poon *et al*., 2006), such as *Taq* DNA polymerase, which is easily inhibited by biological substances. In addition, this enzyme is active at relatively high temperatures (60-65°C), which helps reduction of non-specific reactions, and it is more resistant than other DNA polymerases (Notomi *et al*., 2000; Nagamine *et al*., 2002; Poon *et al*., 2006). This LAMP assay specifically reacted with *E. revolutum* (adult and metacercaria) when it was tested with other related trematodes. In this experiment amplification was not observed with any of the other trematodes investigated. The LAMP assay in this experiment was more sensitive than the conventional PCR, as demonstrated by the amplified results of the same serially diluted genomic DNA of *E. revolutum*. These investigations revealed that the LAMP assay is sensitive enough to detect DNA at a low level (Notomi *et al*., 2000; Alhassan *et al*., 2007; Parida *et al*., 2008; Kalvatchev *et al*., 2010). The applicability of this assay for detection of *E. revolutum* was evaluated by examining field samples of echinostome metacercariae from naturally infected snails. As expected, 7 samples from infected snails produced positive amplification in the LAMP assay. This result showed that the LAMP assay can be used to effectively amplify the target DNA of *E. revolutum* metacercariae.

The LAMP assay also has several advantages over conventional PCR assay. This method was easy to perform and inexpensive for diagnosis, only a water bath or a heat block is required for amplification. Another feature is the loop primers that allow the amplification to be done within 1 hour (Nagamine *et al*., 2002). The newly developed LAMP assay is even more sensitive than the conventional PCR for detecting DNA that is present at a low level. The sensitivity of LAMP and PCR has been examined for detecting various pathogens, such as *Trypanosoma* spp. (Thekisoe *et al*., 2005; Laohasinnarong *et al*., 2011), *Theileria equi* and *Babesia caballi* (Alhassan *et al*.,

2007). The LAMP assay has been successfully developed for the diagnosis of pathogenic organisms such as viruses (Parida *et al*., 2008; Kalvatchev *et al*., 2010), bacteria (Enosawa *et al*., 2003; Maruyama *et al*., 2003) and protozoa (Kuboki *et al*., 2003; Han *et al*., 2007; Liang *et al*., 2009). Because of all these reasons, the LAMP assay has great potential for use in detection and epidemiological surveys of this species in the definitive and second intermediate hosts. This method has been applied for detection of trematodes including *Schistosoma* spp. (Abbasi *et al*., 2010; Xu *et al*., 2010), *Fasciola* spp. (Ai *et al*., 2010), *Clonorchis sinensis* (Cai *et al*., 2010), *Paragonimus westermani* (Chen *et al*., 2011), and *Opisthorchis viverrini* (Arimatsu *et al*., 2012; Le *et al*., 2012). This study reported successful application of LAMP method for *E. revolutum* detection for the first time.

DNA sequencing of the ITS2 and ND1 gene were used to determine the phylogenetic relationships of *E. revolutum*, which largely agree with previous results using nuclear rDNA internal transcribed spacers and mitochondrial gene which have been used for the characterization of a number of echinostome species that were difficult to compare using traditional methods (Morgan and Blair, 1995; Morgan and Blair, 1997a; 1997b). The results of the phylogenetic analyses of the ITS2 sequence data based on NJ analysis revealed that the 37 collar-spine group form a monophyletic clade, viz. *E. revolutum*, *E. trivolvis*, *E. caproni*, *E. paraensei* and *E. liei*; whereas *E. malayanum* (43 collar spines) did not cluster as a monophyletic clade and/or sister taxa of this group. These results were similar to Morgan and Blair (1995) and Kostadinova *et al*. (2003), which confirmed that all species within the 37-collar spined group represent a monophyletic group.

The phylogenetic tree inferred from ND1 sequences shows that two major clusters of *Echinostoma* contained a monophyletic clade of 37-collar spined group and monophyletic clade of *E. malayanum*. This agrees with Morgan and Blair (1995) and Kostadinova *et al*. (2003) who revealed that *Echinostoma* species of 37-collar spined group were aligned as a monophyletic group based on nuclear rDNA and mitochondrial DNA.

The first cluster contained a sister group forming a monophyletic clade, including *E. revolutum* and 37-collar spined group. Isolates of *E. revolutum* from Southeast Asia,

Australia, and Europe (1 sequence) were aligned as a monophyletic clade and were closely related to the monophyletic clades of the isolates from America and Europe. These results were similar to ITS1 data (Saijuntha *et al*., 2011a), which showed that isolates of *E. revolutum* from Thailand were more closely related to *E. revolutum* from Australia than to other isolates and isolates from the USA (America) and Bulgaria (Europe). They were aligned as a monophyletic clade and were closely related to a monophyletic group of the isolates from Thailand and Australia with strong bootstrap support. The phylogenetic relationships of *E. revolutum* in this study suggest the geographic origin of the isolates is related to the genetic clustering, i.e., the Southeast Asian isolates are more closely aligned to Australian isolates, whereas the American isolates are more closely aligned to the European isolates. Support for this was given by the analysis of an *E. revolutum* isolate using of ITS sequences (Sorensen *et al*., 1998), which indicated that American isolates were more closely related to European (Germany) isolate. Investigations of genetic variation between isolates of this *E. revolutum* in Southeast Asia and other isolates need to be carried out by using additional molecular markers (e.g. other mitochondrial genes) may provide evidence to support this hypothesis. The first cluster *E. revolutum* from various isolates were closely aligned as a sister taxon with the "*revolutum*" group. The genetic distances within Southeast Asian isolates were almost parallel to the geographic distances of sampling locations, although they were not calculated or valid values within isolates for Lao PDR and Vietnam isolates because of the small sample size (one sample each).

The other cluster, containing the monophyletic clade of *E. malayanum* (43-collar spines), this species is represented as a paraphyletic taxon with 37-collar spined group as reported based on ND1 sequences (Kostadinova *et al*., 2003). Although *E. malayanum* is closely aligned as a sister taxon with the *Hypoderaeum* (41-45 collar spines), has been reported by Saijuntha *et al*. (2010; 2011b).

Although the phylogenetic study of *E. revolutum* from the ITS1 and CO1 sequences in Thailand and Lao PDR has been reported by Saijuntha *et al*. (2011a; 2011b), my work is the first that has used sequence data of the ITS2 and ND1 to investigate phylogenetic relationship in this species in Southeast Asia. I have provided molecular evidence from partial ITS2 and ND1 sequences to assess the phylogenetic relationships of *E. revolutum* from Southeast Asia, including Thailand, Lao PDR,

Cambodia, and Vietnam. Genetic characterizations of *E. revolutu*m are useful to achieve basic information necessary for the systematics of this parasite. Further investigations using this method are needed for further genetic analysis of a wider range of isolates from different host species in order to better understand the genetic structure of *E. revolutum* populations and their transmission dynamics.