CHAPTER 5

DISCUSSION

This study reveals a high variety of the freshwater snails in the North of Thailand. The freshwater snails are represented in the 8 families, consisting of 15 genera and 21 species/taxa, including, *Adamietta housei*, *Bithynia funiculata, B. siamensis siamensis, Brotia costula costula, Br. citrina*, *Br. wykoffi*, *Clea helena, Eyriesia eyriesi*, *Filopaludina doliaris, F. martensi martensi, F. sumatrensis polygramma, F. sumatrensis speciosa, Indoplanorbis exustus, Lymnaea auricularia rubiginosa, Makongia swainsoni, Melanoides tuberculata, Paludomus siamensis*, *Pomacea canaliculata*, *Tarebia granifera, Thiara scabra,* and *Sinotaia mandahlbarthi.* The difference the of various reported is Mard-arhin *et al*. (2001) which is conducted a survey of helminthes in the snails from 5 provinces of the North of Thailand including Lampang, Phrae, Phayao, Chiang Rai, and Chiang Mai province and they found 6 species of snails only (*Bithynia* sp., *Filopaludina* sp., *Lymnaea rubiginosa*, *Melanoides tuberculata*, *Planorbis* sp., and *Pila polita*), while it was reported there were only 5 families, 12 genera, and 16 species of the freshwater snails found in the Northeast of Thailand. (Sri-aroon *et al*., 2005).

Some species of snails are wildly distribution such as *Melanoides tuberculata* and *Tarebia granifera*. They are the most common and can be found in almost every kinds of the freshwater in all sampling areas, but some were not, such as *A. housei*, *Brotia* spp. *E. eyriesi F. s. speciosa*, *Ma swaisoni*, *P. siamensis*,

Po. canaliculata, and *S. mandahlbarthi*. Compared worked to the Sri-aroon *et al.* (2005), the species of freshwater snails were difference from this study.

Regarding the evaluation of biodiversity index of the freshwater snails in this study using Shanon-Wiener Index. The result shown that 64 sampling sites from 12 provinces in the North of Thailand (including Chiang Rai, Chiang Mai, Lamphun, Lampang, Phayao, Mae Hong Son, Phrae, Nan, Sukhothai, Tak, Uttaradit, and Phitsanulok) were considered to be a high diversity and species evenness. This occurrence are possibly from the cause that the North of Thailand has been known as the most important of irrigation areas such as, Sirikit dam (Uttaradit), Mae Ngad Somboonchon Dam and Mae Kuang (Chiang Mai), Bhumibol dam (Tak), Kil Lom dam (Lam pang), Kaew Noi Bamrung Dan dam (Phitsanulok), and etc. Therefore, these areas may also were supported by the peculiar ecological conditions of water resources, which represented a complex and diverse freshwater ecosystem. There are good conditions for development and maturation of snails (Chontananarth and Wongsawad, 2010a). This study adds a more new data of the freshwater snails diversity and this is a preliminary step to understand their distribution.

In accordance with the cercarial infections in intermediate host snails, total number of 4,533 snail individuals were examined, 12 snail species were infected by cercariae including. *A. housei*, *M. tuberculata*, *T. granifera*, *Th. scabra*, *B. funiculat*, *B. siamensis siamensis*, *F. doliaris*, *F. martensi martensi*, *F. sumatrensis polygramma*, *Br. citrina*, *L. auricularia rubiginosa*, and *Indoplanorbis exustus.*

Form the prevalence of infected snails, the thiarid snails were shown a high susceptibility for the cercarial infections, which is get along to the previous report

of Supian and Ikhwanuddin (2002). That concludes that the snail family Thiaridae which harbor the larvae of the intestinal and blood flukes. Furthermore, *M. tuberculata* and *T. granifera* (Thiaridae) are medically important because they can serve as the first of intermediate host for intestinal flukes (Bogéa, 2005).

From the results, a total of 11 morphology types (14 triads) of cercaria were found. They were divided into separate groups based on morphologically distinguishable differences according to Schell (internal organ arrangement, place and number of sucker etc.). Total prevalence of cercarial infections this study increased by 14.65% (664 / 4,533) (Mard- arhin *et al.*, 2001; Sri-aroon *et al*., 2005; Ngern-klun *et al.* 2006; Dechruksa *et al*., 2007; Ukong; 2007; Krailas *et al*., 2012). The higher prevalence of cercarial infection in the North of Thailand represents a complex and diverse freshwater ecosystem. The suitable conditions make cercariae able to develop in snails and fish which lead to perpetuation in their life cycle. Moreover, the generic composition of the obtained trematode larvae in the studied snails might be influenced by the anthropogenic factors and specific fauna in the observed regions, including microorganisms that could be the potential secondary or/and intermediate hosts of the larvae found in the freshwater snails (Chontananarth and Wongsawad, 2013). Furthermore, the difference in overall prevalence of cercarial infection may causes the involvement of many factors, including the difference of collection time, season, localities, *etc.*

From the study, the snail in Family Thiaridae had shown a high susceptibility for parapleurolophocercous cercaria Type I and pleurolophocercous cercaria infection. These types of cercarial infected many species of snails. It means that, there is the existing of very low specificity for infection in snail

intermediate hosts. The various reports indicated the parapleurophocercous cercaria as the cercarial stage of the intestinal trematode in the family Heterophyidae such as, *Haplorchis taichui, Haplorchis pumilio, Stellantchasmus falcatus, Centrocestus caninus* and *Procerovum* sp. were reported (Umadevi and Madhavi, 2006; Ukong *et al*., 2007; Pinto and Melo 2010; Chontananarth and Wongsawad 2010b; Chontananarth and Wongsawad, 2013). The identification of the morphology of cercariae were similar to those described by the previous reported (Ito, 1980; Krailas *et al*., 2011). Form the result, this type of cercarial are wildly distribution, may be it make the trematode in family Heterophyidae infection in second intermediate host and/or definitive host is trend to increase. Regarding to this relationship, the previous surveys revealed that metacercariae, most found in Mae Taeng district, Chiang Mai province, belong to the family Heterophyidae (Boonchot and Wongsawad, 2005; Kumchoo *et al*., 2005; Nithikathkul and Wongsawad, 2008).

The parapleurophocercous cercaria Type II was found in *M. tuberculata* from Chiang Mai and Uttaradit provinces, while parapleurophocercous cercaria Type III were infected in *A. housei* from Lamphun province only. Hence, These cercarial types are very specific for infected in snail hosts, which it might be hardly distribution their completely of life cycle. They were classified into the family of Heterophyidae because its tail has the lateral folded fin; also it has one pair of the eyespots pigment. Nevertheless, they could not be identified into the genus level.

Form the results, the *A. housei, M. tuberculata, T. granifera,* and *Th. scabra* were infected by megalurous cercariae, which have been morphologically characterized as belonging to the genus *Philophthalmus*. Because these cercariae have neither a collar-like thickening nor a crown of spines, they encysts on the surface of

the bottom of the container. The metacercarial cysts are flask-shaped. Moreover, the partial mtCOI sequence of this cercaria was similar to *Philophthalmus* sp. (JQ 675731) with 89.0%. Hence, it is possible that this cercariae are *Philophthalmus* as the morphological characteristic. In general, this cercarial types can be found in *M. tuberculata* (Kalatan *et al*., 1997; Pinto and Melo, 2010), but some investigations reported that there were also other snail namely *Fagotia* spp., *Amphimeania holandri and Melanopsis praemorsa (*Kanev *et al.,* 1993*), Semisulcospira libertine* (Urabe, 2005) and *Zeacumantus subcarinatus* (Martorelli *et al.,* 2008.) presented. However, this investigation demonstrated that the cercaria can exists in a *A. housei, T. granifera, and Th. scabra* which have not been reported in other study yet. In addition, this cercarial of *Philophthalmus* is the first to be reported in the northern Thailand.

This present work describes the furcocercous cercaria type I as *Transversotrema* cercaria, which was discovered in *Melanoides tuberculata.* The morphological characteristic of this cercaria from the same and other snail species was described in the literature. *Transversotrema* cercaria was found in *Melanoides tuberculata* (Ukong *et al*., 2007 Chontananarth and Wongsawad, 2010b;) which was contrast to Wongsawad and Kumchoo (2000) found this cercarial type in *Th. scabra* (Kumchoo and Wongsawad, 2000*).* This cercarial can develop into adult stage of trematode in genus *Transversotrema* because the body is very flatten, genital pore is located medially. Tail is longer than the body, and with arm-like processes at the anterior end of the tail-stem (Crusz and Raatnayake, 1964; Kumchoo and Wongsawad, 2000). This trematode are ecto-parasite of freshwater and marine fishes.

The furcocercous cercaria type II have been reported the strigea cercaria, which developed the trematode in the family Strigeaidae or Diplostomatidae. This cercariae appear in 4 snail species (*B. siamensis siamensis*, *B. funiculata*, *L. auricularia rubiginosa*, and *F. martensi martensi*). The cercaria presents in the ventral sucker, esophagus; that develop in sporocyst. These cercariae resemble as in the recent report (Bdir and Adwan, 2012). In addition, for the first time, the present study discovers the strigea cercaria infecting in 2 snail species including *B. siamensis siamensis* and *B. funiculata*.

In this study, the xiphidiocercaria was found in the 12 snail species, while the previous reported found the xiphidiocercaria presented in *Lymnaea caillaudi*, *Cleopatra bulinoides*, *Lonistes carnatus*, *Thiara tuberculata* and *Tarebia granifera*. The results demonstrated that cercariae are very low specificity for the infection in intermediated hosts that possibly can be wildly distributed in the North of Thailand. They were reported as the cercarial stage of the trematodes in Superfamily Plagiorchiidea (Dechruksa *et al.*, 2007; Yousif *et al.*, 2010). The previous report found the adult stage of trematode in similar group, *Encyclometra bungara* in reptile, *Xenochrophis piscator* from Doi Suthep-Pui National park, Chain Mai province (Wongsawad *et al*.,1998). This evidence can be supports to be this trematode family dispersal in northern Thailand, which developed from the xiphidiocercous cercaria.

Virgulate cercaria is found in the 4 snail species (*T. granifera*, *Th. scabra*, *B. siamensis siamensis* and *B. funiculata*). The cercaria is presented a stylet and it has a virgule organ, which is a major characteristic of virgulate cercaria (Shell, 1970). The morphological characteristics of this cercaria are recognized in the recent

report (Yousif *et al*., 2010) which suggested that it might be the larval stage of trematode in family Lecithodendriidae. However, they found only in *M. tuberculata*. On the other hand, Dechruksa *et al.* (2007) found them in *T. granifera* snails and identified as the *Acanthatrium hitaense.* Moreover, Yousif *et al*., (2010), also described the gymnocephalous cercaria in *M. tuberculata* while the study is the first time to be found in *B. siamensis siamensis*, *I. exustus*, and *F. martensi martensi*.

The cercariae and distome cercariae were infected only in bithynid snails from Chiang Mai and Lamphun provinces, which have not been reported in the other study in same area. Thus, it seems that these types of cercariae have a high specificity for the infection in snail hosts.

As can be seen in Figure 4-50, there is an interesting point that in area of Lampang and Sukhothai province. In generally no cercarial infections were observed. In my point of view, there might be some geological barrier or ecological conditions not optimal for first and second intermediate develop and disperse, which related to this issue. Any further investigations would be useful to answer this mystery.

Fish-borne trematodes are normally easy to diagnose with microscopy when adult samples are available. However, the identification of the cercarial stage morphological appearance is not sufficient to be identified in genus or species level. Particularly, the cercarial stage of *Haplorchis taichui* and *H. pumilio* are very similar. Moreover, are notoriously difficult to identify, especially when there are infections by multiple species in intermediate and definitive hosts. It is possible that this technique may also be applied to detect cercariae collected from contaminated water or rediae/sporocysts in infected snails (Magalhes *et al*., 2004). Therefore, an accurate identification of cercarial stage is very important for understanding the

epidemiological situation and for developing the effective control measures, the molecular biology has been developed for detection and diagnosis the trematode infection in intermediate hosts and definitive hosts.

These results showed the advantages of the mtCOI gene sequences. The gene has been reported as the barcoding region consisting of the conserve and the hyper variation area. In addition, high copy numbers per cell, which make it easy to increase the number of DNA in PCR reaction (McManus *et al*., 2004). For instance, mtCOI sequences of the several related to heterophyid species have been examined to detect and identify the larval of this trematodes (Lee *et al*., 2004; Thaenkham *et al*., 2007)

The partial mtCOI sequence of trematodes has a length of 393-425 bp of about 1700 bp. These sequences of eight trematode species including, *H. pumilio, Centrocestus caninus*, *Stellantchasmus falcatus*, *Haplorchoides* sp., *Ganeo trigrinus*, *Prostorchigenes majeedi*, *Orthocoelium streptocoelium*, *Fischoederius elongatus* are available on the NCBI database, which first data of mtCOI gene. This sequences will be applicable to further studies on biology and epidemiology of each trematode species. Moreover, these data will be applied for designed DNA specific primer for detection and/or investigated the biogeography, phylogenetic relationship, population genetic, *etc*. Especially, the partial mtCOI sequences will be applicable to further studies on biology and epidemiology of each trematode species. The results will also be applicable to further studies on infection of each freshwater snail species with trematode cercariae. This study will be applicable for prevention and control of zoonotic trematode infections of high public health significance.

To achieve the objective of the study, the specific primer was designed the specific detected *H. taichui* and *H. pumilio*. The sequence of both primers were demonstrated as follows; HT-F 5’ GTT-TGG-TTA-TGG-GGG-TTT-AGT-TCT-T 3’ and HT-R 5’AAC-CTT-TAT-ACC-TGT-GGG-GAC-T 3’ for *H. taichui* (anticipated product size 160 bp), HP-F 5’GGA-TGT-AAA-GAC-GGC-TGT-GTT-CTT-C3’ and HP-R 5’GAA-TAG-GAT-CTC-AAA-ATC-GTC-TA 3’ for *H. pumilio* (anticipated product size 125 bp). The sensitivity and specificity of both primers for PCR amplification was investigated. The *H. taichui* specific primer shown with the optimal concentration of DNA template was 7.5 ng/µl. But the lowest concentration of DNA template that cloud be amplified by species-specific PCR reaction was around at 0.45 ng/µl, which is very low compared with other reports (Chuboon, 2013). While optimal concentration odf *H. pumilio* specific primer was 12.5 ng/µl. Nevertheless the lowest concentration of DNA template that cloud be amplified by species-specific PCR reaction was around at 1.81 ng/µl. This result confirms the effective of two folds serial dilution procedure that suitable foe evaluate the optimal and lowest of DNA concentration for PCR amplification.

The specificity of PCR reaction was confirmed using combined primers together with DNA template of 10 other trematodes. Genomic DNA of snails hosts were also tested to make sure the specificity by lacking cross reaction.

For *H. taichui* and *H. pumilio* multiplex PCR for identifying and discriminating between *H. taichui* and *H. pumilio* infection in intermediate host snails was developed as a species – specific PCR, which is sensitive and effective tool for accurate diagnosis of *H. taichui* and/or *H. pumilio*. The difference size of PCR products is designed to obtain the clearly separate on agarose gel as indicated in

Le *et al*. (2006). The optimal PCR conditions for multiplex PCR was performed in a thermocycles with the initial denaturation step at 95 ºC for 5 minutes, then 35 cycles including denaturation at 95 ºC for 45 seconds, primer annealing at 55 ºC for 45 seconds, extension at 72 ºC for 1 minutes, and final extension at 72 ºC for 7 minutes.

The molecular identification of *H. taichui* and *H. pumilio* were performed in parapleurolophocercous cercaria Type I, II, and III form difference sampling sites. This result revealed that *H. taichui* and *H. pumilio* are develop from parapleurolophocercous cercaria Type I only. However, not all DNA samples of parapleurolophocercous cercaria Type I is *H. taichui* and *H. pumilio*. This provided the effective evidence by indicating that not only *H. taichui* and *H. pumilio* were developed from parapleurolophocercous cercariae, but also there were other trematode species which were generated from the same cercarial type like both of trematodes species (Olsen 1974; Malek, 1922; Umadevi and Madhavi, 2000).

The study found that, *M. tuberculata, T. granifera,* and *Th. scabra* serve as the first intermediate hosts of *H. taichui*. In Thailand, 5 species of snails have been reported as the sources of *H. taichui* infection in difference geographical habitat ; *M. tuberculata*, *M. jugicostis T. scabra* and *T. granifera* in the North and Central of Thailand (Dechruksa *et al*. ,2007; Ukong *et al*., 2007; Chontananarth and Wongsawad, 2010a, Chontananarth and wongsawad,2010b), *Neoradina prasongo* in the South of, Thailand (Krailas *et al*., 2011). Accordingly, a high prevalence of *H. taichui* infection in the freshwater fishes and human form some areas of the North of Thailand (Wongsawad and Wongsawad, 2009). Furthermore, Nithikakul and Wongsawad (2008) reported the high prevalence of metacercarial state of *H. taichui* and *Haplorchoides* sp. infecting in cyprinoid fishes.

The present geographic distribution of cercarial stage of *H. taichui* in northern Thailand now include Chiang Rai, Chiang Mai, Lamphun, Lampang, Phayao, Mae Hong Son, Phrae, Nan, Tak, Uttaradit, and Phitsanulok provinces. However, the occurrence of the parasite in other part of Thailand is highly probable and its absent is likely due to lack of investigations into the fish parasites in these area rather than actual absence of the parasite. Recently, *H. taichui* (cercarial and metacercarial stage) was found in Southern part of Thailand (Krailas *et al*., 2011). This finding indicates a high potential of the parasite colonize geographically very distance region. Hence, our findings agree with Radomyos *et al*. (1998) who reported that *H. taichui* are widely distributed and with high prevalence in the North of Thailand. While, the geographic distribution of *H. pumilio* was occurred in lower part of northern, Thailand including Tak and Phitsanulok provinces. The previous reports indicated this parasite are distribute in central (Ukong *et al*., 2007; Sato *et al*., 2009), and southern parts of Thailand (Krailas *et al*., 2011). However, there is only one previous report of this parasite in only found in Phitsanulok province, which corresponds to this study. From the result shown that Sukhothai province were not found of both trematode species may be the number of collecting sites are incommensurate and/or maybe this area has the barrier, which these trematode cannot disperse.

The present analysis has shown that snail and cercarial distribution data generally correspond to the heterophyid infection prevalence data to the forecast the endemic areas based on present data. The heterophyid trematode showed a wide range for host specificity because it is able to infect the several species of snails. Snail-borne trematode can be assessed by the prevalence of infection. The most areas of the North

of Thailand showed that the highest prevalence of trematode infection in the first and second intermediate hosts. It is possible that this area has a diverse ecosystem and farm forestry containing rice paddy, dam, irrigation canal and river, producing a high biodiversity that is suitable for life cycle of trematode for human and animal infections. Therefore, the study of cercarial state infection is very important. The necessary control measures, namely for species important to human health, require the identification of the several stages of trematode, the information of the life cycle, and the way distinguish the difference parasites which infect the same host. Hence, there is need to identify the cercarial stage of all the trematodes, even of those which are not so importance, because of the possibility of competition for the same intermediate host snails.

Regarding the intermediate host snail of *H. taichui* are similar with the other reports (Chontananarth and Wongsawad, 2010a, Chontananarth and Wongsawad, 2010b). The snail in family Thiaridae especially, *M. tuberculata*, *T. granifera* and *Th. scabra* are first intermediate host of this trematode . While there are only 2 species of snails (*M. tuberculata* and *T. granifera)* served as the intermediate host of *H. pumilio*, according to Diáz *et al*. (2008) and Krailas *et al*., (2011) the report stated that the *M. tuberculata* serve as the intermediate host of *H. pumilio*.

According to the phylogenetic analysis, the partial sequence of mtCOI of heterophyid trematodes are declared now know. Only a few studies have shown about the relationship among mtCOI of heterophyid species in Thailand (Thaenkham *et al*., 2010). The molecular approach and DNA sequencing technologies have been successfully developed for studying their phylogenetic relationship.

The analysis revealed invariably a monophyletic tree of trematode in Family Heterophyidae. This agrees with reports on, PCR-RFLP and RAPD patterns and phylogenetic relationship of heterophyid trematodes (Yu *et al*., 1997; Thaenkham *et al*., 2007; Van *et al*., 2009).

The mtCOI has been used to study the systematic of some heterophyid trematodes viz., *Haplorchis* (Dung *et al*., 2012), *Metagonimus* (Lee *et al*., 2004). In our opinion this work did not come from Heterophyidae species. Three species of trematodes; *Stellantchasmus, Centrocestus caninus*, and *Haplorchoides* the usually occurred with high prevalence in Thailand, but the systematic of these parasites has not been studies.

The sequence data of mtCOI can be used to investigate the phylogenetic relationships of trematodes at the genus level. Each clade of different genera of heterophyid trematodes was separated into sister groups that correlated with the morphological characteristic, kind of secondary intermediate host and geographic distribution.

Our hypothesis is that trematodes whose cycle ended in fishes are able to switch to definitive hosts. In the heterophyid groups, the *H. taichui* uses the cyprinoid fishes as second intermediate hosts in the genera *Henicorhynchus* and *Puntioplites* (Rim *et al*. 2008), *H. pumilio* used *Trichogaster microlepis* and *Rivulus harti* (Diáz *et al*., 2008), *Centrocestus* caninus uses *Carassius auratus* (Glurcevic *et al*. 2007), *Haplorchoides* sp. used *Henicorhynchus siamensis* (Wongsawad *et al.,* 2009). While the *Metagonimus* group used *Lisa, Mulgi, Tribolodon, Lateolabex, Cichlasoma, Plecoglossus*, and *Salmo* for serve as second intermediate hosts (Tomas and Guillermo, 2000; Chai *et al*., 2005; Shin *et al*., 2008).

Furthermore, morphological characteristic of the ventrogenital complex and the number of testes can be separated as a group. *H. taichui* and *H. pumilio* have single testis and a modified ventrogenital sac. While, *C. caninus*, *S. falcatus*, *Metagonimus* spp. have two testes and a simple ventrogenital sac. On the other hand, *Haplorchoides* has a single testis and a modified ventrogenital sac.

Surprisingly, the geographic distribution of *Metagonimus* spp. are found in Korean and Japan, which never been reported from Thailand. In this context, *Metagonimus* and other heterophyid trematodes have not similar geographic distribution and ecological condition. As same as the *H. taichui* and *H. pumilio* are separated sister group. Because the locations of second intermediate host are difference (*H. taichui* from Chiang Mai and *H. pumilio* from Bangkok). Hence, the identification accuracy of heterophyid trematodes should considering the king of intermediate host of parasites, morphological characteristic, and geographic distribution of each trematode species.

The NJ tree show that the group of multiples closely related to *H. taichui*. The Bootstrapping of sequences with both of trees were indicated significant support for this group. However, few genetic variation are observed among difference trematode species at nucleotide level. Since mtCOI sequences have been approved for studying the phylogenetic relationship of trematode by it was introduced in a number of other reported (Yu *et al*., 1997; Nakano *et al*., 2006; Saijuntha *et al*., 2008; Sato *et al*., 2008; Thaenkham *et al*., 2010)

The phylogenetic and systematic of heterophyid trematodes can be determined by a molecular approach using mtCOI for DNA barcoding. We have that established that species-level identifications can be achieved, and mtCOI analysis

actually provides a phylogenetic for these parasites. Further works will study the phylogenetic relationship of larval stages of heterophyid trematodes.

Hence, the study of geographic distribution of trematodes provides a dynamics easily updated mapping system that can be used by health managements officers to plan and monitor control programs. By virtue of its potential to “match” the relative suitability of various environments to the life cycle and transmission dynamics of host-parasite system. For GIS and molecular biology provides a new way to address classic concepts of landscape epidemiology.