CHAPTER 2

LITERRATURE REVIEW

Life cycles of heterophyid trematodes

The members of the family Heterophyidae especially those of in the genus *Haplorchis* are tiny intestinal trematode inhibiting the digestive systems of birds, and mammals including human (Pearson and Ow-Yang; 1982; Fried *et al*., 2004; Uthpala *et al*., 2010). These parasites are commonly found in East and Southeast Asia. The trematode has a complicated life cycle consisting of several larval stages within two hosts (Esch *et al.*, 2001; Jousson *et al*., 1999). In adult stage which observed in the definitive host , they produce eggs that pass to the diverse environments. Then, they develop to the larval stage as followed: miracidium, sporocyst, redia, and cercaria (Malek, 1922; Yamaguti, 1958; Cheng, 1964; Schell, 1970; Robert and Janovy, 2005) (Figure 2-1).

Miracidium

During the optimum conditions, the miracidium will be hatched. In this stage, their bodies are tiny, covered with cilia, and being free-living organism that could easily be mistaken with protozoa. It is a piriform shaped, with a retractable apical papilla at the anterior end of the body and gland ducts connect to their penetration gland inside the body. The free-living miracidia are very active so they have to find the suitable snail hosts. When the appropriate snail host is found, a miracidium attached to it by its apical papilla and then penetrated into the snail tissue.

Sporocyst

The larval stage of trematodes development have been studied in various group of trematode, such as echinostome, psilistome, fasciolids, heterophyid, etc. The sporocyst stage varies slightly in shape, size, structure and shape, but an oval or elongate with one end round and the other conical are the common forms. The germinal cells eventually multiply and give rise to embryos of a second generation of larvae which might be either daughter sporocyst or rediae, depending on the trematode species. Although daughter sporocysts are similar to the mother sporocyst, they can be distinguished by the inclusion of the radiae or cercariae.

Redia

Individuals in this stage can be found in the digestive gland of infected snails. In general, the morphology of redial stage is large and elongate. Moreover, trematodes found in this stage have an alimentary system, starting with the mouth which leads to the prominent muscular pharynx and an intestine sac. The redia is capable of motility through the movement of the ambulatory buds and body contractions, and the host cells were ingested. The radial stage of heterophyid trematodes are light brown in colour, encloses a number of mature and immature cercariae and few germ balls. Its birth pore is located in the first of third part of anterior end of body. The body of redia is full with germinal masses and few cercariae of different developmental stages. The extreme posterior end of redia is covered by a group of glandular cells, probably the adhesive cells. In body cavity, there are about 20-30 cercariae employs of various developmental stages.

Cercaria

The cercariae are found to be varied in morphological characteristics. Most of them have unique specialization enabling to survive as brief free-living existence, and make themselves available to their second intermediate host or definitive host. In adult stage, the organs for attachment (suckers), digestion, excretion and primordial of the adult reproductive organs are completely developed because these adult structures typically start to develop in cercarial stage.

Most cercariae have mouth near the anterior end of body. The mouth is usually surrounded by an oral sucker, pre-pharynx, muscular pharynx, and bifurcate intestine are normally present. Most cercariae have a long tail for swimming. However, some has very short or absent tail. The locomotion in the latter is limited to creep. Many cercarial types have various glands, near the anterior margin, often call “penetration gland” because of their assumed function. The cercariae of trematode are in various type, and their classification is usually based on their external morphology. Early morphological designations were base on the number and position of the sucker present in the cercarial body, shape, relative size of the tail, and tail structure. The following types are recognized: (Schell, 1970; Olsen, 1974; Ito, 1980; Robert and Janovy, 2005)

1. Amphistome cercaria: These cercariae possess a posterior sucker and eyespots. Adhesive glands are absent. This cercariae develop in radiae and

give rise to amphistome adult. In addition, adult stage are members of the family Paramphistomatidae (Figure 2-2.1).

2. Monostome cercaria: This cercaria presented only one sucker, and located anterior margin of the body. They possess two or three eyespot and long simple tail, usually with point locomotor, also processes directed posterior extremely of the body proper. This type of cercaria develops in the rediae and encyst in hemispherical membrane on aquatic plants, produced by trematode in the family Notocotylidae and Pronocephalidae (Figure 2-2.2).
3. Distome cercaria and gymnocephalous cercaria: The distome cercaria This cercaria possess two suckers (the anteriorly located oral sucker and mid-ventrally located the ventral sucker). This cercariae develop in rediae and give rise to distome adult. While gymnocephalous cercariae are usually develop in rediae, mature to be adults belonging Family Fasciolidae (Figure 2-2.3).
4. Pleurolophocercous cercaria and parapleurolophocercous cercaria: The tail of comes with dorso-ventral fin fold. There are pigment eyespots on their bodies. The cercaria develops in a redia prosobranch (operculate) snails and encysts in fishes. This type is represented in the Family Heterophyidae, Opisthorchiidae and Cryptogonimidae. While the parapleurolophocercous cercaria is similar to the pleurolophocercous cercaria except its tail has the lateral fin fold in addition to the dorso-ventral fin fold (Figure 2-2.4).
5. Cystophorous cercaria: This cercariae are characterized by a cavity or cyst at the base of the tail, into which the proper body can be withdrawn. These

are regular parasites of amphibians as adult to the Gorgoderidae (Figure 2-2.5).

6. Tricocercous cercaria: These cercariae are characterized by tail armed, with spines or bristles. Occasionally two pair of eyespots. These cercarial type are marine parasite in Family Lapocreadiidae (Figure 2-2.6).
7. Echinostome cercaria: This cercaria processes of large spines around the oral sucker. The cercaria develops in collared rediae, with stump appendages in aquatic snails and encysts in the open or requires as a second intermediate hosts (snail, amphibian and fish). Adult stage is members of the Family Echinostomatidae (Figure 2-2.7).
8. Microcercous cercaria: This cercarial are characterized by a small tail that may be knob-like or conical. The microcercous cercariae are not swimming and do not represent any specific taxonomic group (Figure 2-2.8).
9. Xiphidiocercaria and virgulate cercaria: The xiphidiocercaria posses an anteriorly located stylet. The penetration grand are exceptionally well develop. These cercaria develop in sporocysts. Certain xiphidiocercariae posses a bipartite, transparent, fluid-filled sac that overlaps the oral sucker. Mostly, if not all, of these cercariae belong to the family Lecithodendriidae. While virgulate cercariae are characterized by a virgulate organ located in the oral sucker area. The material secreted by the virgulate organ aids the cercaria in attachment to the host and may also provide some protection for the cercaria (Figure 2-2.9).

10. Ophthalmoxiphidiocercaria: This type of cercaria possess the stytet on the oral sucker and pigmented eye spots present. These cercaria encysts in the larvae of aquatic insects. With increased knowledge of life cycle, this might prove to be the type of cercaria produced by trematodes of the family Allocreadiidae (Figure 2-2.10).
11. Garterostome cercaria: This cercarial type possess a oral sucker located on the ventral surface of the body. They eventually develop into the gastreostome adults of the Family Buccephalidae (Figure 2-2.11).
12. Lophocercous-apharyngeate cercaria: The body possess dorso-median fin fold on the body. The ventral sucker is vestigial or absent. Pharynx absent. This type is represented in family Sanguinicilidae (Figure 2-2.12).
13. Brevifurcate-apharyngeate cercaria: This body of this cercaria does not possess a dorso-median fin fold and cercariae develop in simple sporocyst and they penetrates the final host directly. Adult stage are members of the Family Schistosomatidae and Spirorchiidae (Figure 2-2.13).
14. Strigea cercaria: The ventral sucker of this cercaria well develop. The body consist one pair of longitudinal collecting ducts connected to excretory pores located on sides of furcae. They develop in sporocysts in non-operculated snails. This type of cercaria produces by trematode in Family Strigeidae and Diplostomatidae (Figure 2-2.14).
15. Brevifurcate-pharyngeate clinostomatid cercaria: The body possess dorso-median fin fold on the body. The ventral sucker is vestigial or absent. Tail stem mush longer than furcae, and develop in rediae in aquatic snails. This

cercariae are produce by trematode of the family Clinostomatidae (Figure 2-2.15).

16. Cotylocercous cercaria: This cercarial type are possess stylet. Tail cup-shaped and apparently function as an adhesive gland. These cercariae develop in snails and encysts in aquatic arthropods. This is thought to be the cercaria of trematode of the Family Opecoelidae and Dicrocoeliidae (Figure 2-2.16).
17. Rhopalocercouscercaria: This cercarial type of family Gorgoderidae was found emerging from freshwater clams. This cercaria possess two suckers (the anteriorly located oral sucker and mid-ventrally located the ventral sucker. The tail is broad, even wider than the body (Figure 2-2.17).
18. Cercariae: This type of cercaria lack tail, it does not usually leave the snail host, and adult stage is a member of the family Brachylaemidae, Monorchiidae, Zoogonidae and Cyclocoelidae (Figure 2-2.18).
19. Rat-king cercaria: These marine cercariae occur in writhing masses with the tips of the tails being attached to a body of protoplasm. However, some literatures are call this type of cercaria that "zygocercous cercaria" (Galaktionov and Dobrovolskij, 2003) (Figure 2-2.19).

Metacercaria

This stage is the final larval stage of trematodes. When the free-swimming cercaria comes to rest on suitable plants or penetrates a compatible host, plant and other substrates, it loses its tail and encysts. The body of this stage usually resembles that the cercaria. However, some of the cercaria adaptive structures (such as stylet, spines, and penetration gland) are lost. Generally the excretory vesicle in metacercariae are greatly distended and close large quantity of waste material and appear as retractile granules. The metacercaria in some cases does not increase size, while the other expand and assume a characteristic shape. The metacercarial stage of heterophyid trematodes were embedded in the skeletal muscles of cyprinoid fishes, appearing as brown nodules in the body region. The cyst wall is transparent and the larva lies folded inside the cyst with fully developed digestive system with caeca filled by disc-like bodies, the genital complex fully formed with typical spine pattern. The single oval testis and globular excretory bladder were clearly visible.

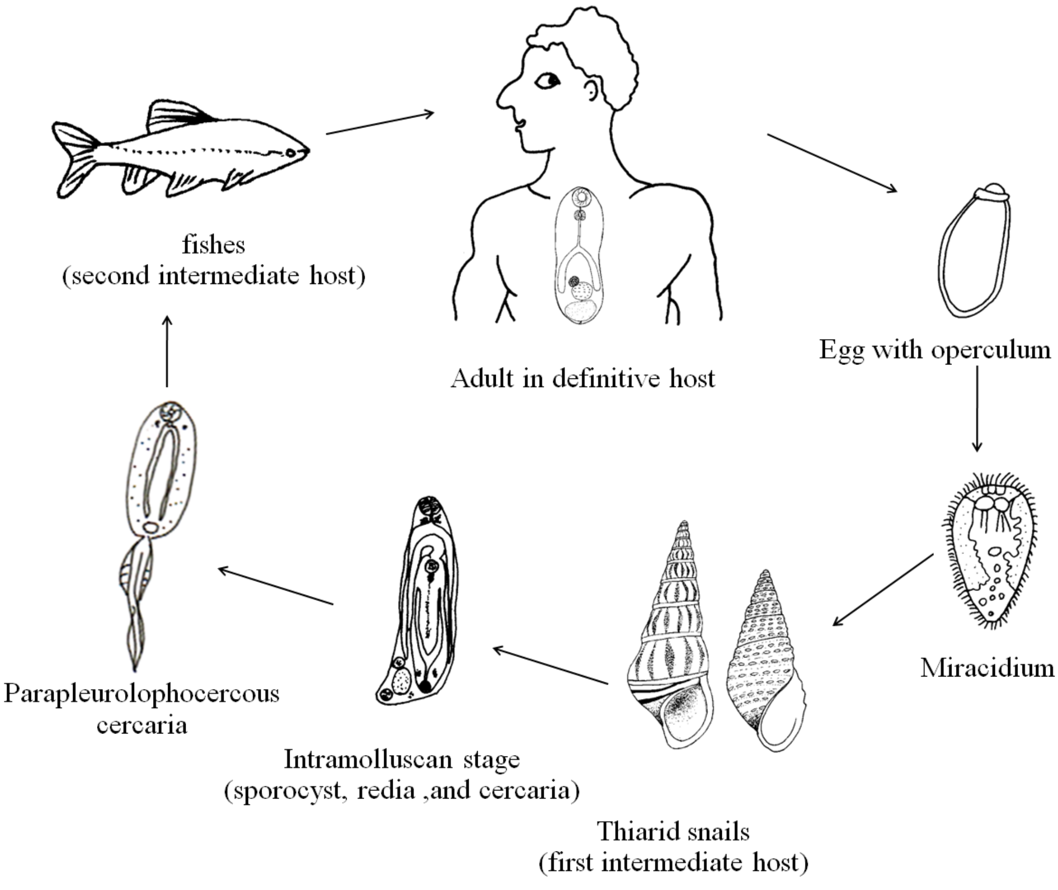

Figure 2-1. General pattern of heterophyid trematodes life cycle by using thiarid snails and cyprinoid fishes as a first and second intermediate host.

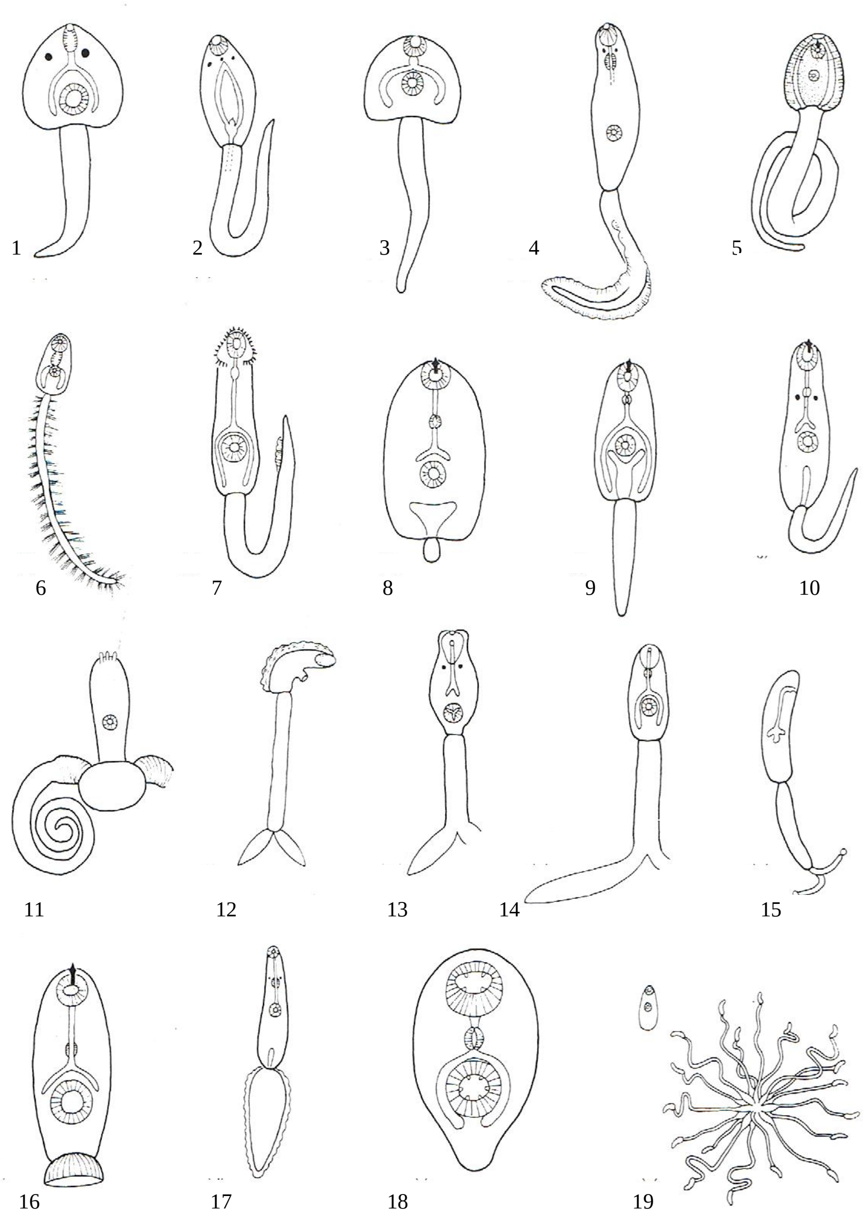

Figure 2-2. Type of cercaria. 1: amphistome cercaria, 2: monostome cercaria, 3: Distome cercaria, 4: pleurolophocercous cercaria, 5: cystophorous cercaria, 6: tricocercous cercaria, 7: echinostome cercaria, 8: microtome cercaria, 9: xiphidiocercaria, 10: ophthalmoxiphidiocercaria, 11: gasterostome cercaria, 12: lophocercous-appharyngeate cercaria, 13: Brevifurcate- apharyngeate cercaria, 14: strigea cercaria, 15: Brevifurcate-pharyngeate clinostomatid cercaria, 16: cotylocercous cercaria, 17: rhopalocercous cercaria, 18: cercariae, 19: rat-king cercaria (Olsen, 1974)

Cercarial infections and intermediate host snails

Many species of snails serve as intermediate hosts of trematode (Caron *et al*., 2008). Consequently, the distribution of freshwater snails accounts for the occurrence of difference trematode species in a particular region. As the trematodes are mostly host specific, higher heterogeneity of the host promotes higher heterogeneity of trematode. The life cycle of cercarial emergence are recognized as an adaptive mechanism to enhance the trematode transmission (Shostak and Each, 1990). The most accepted hypothesis suggests that the cercarial emergent is timed to coincide with the present of the second intermediate host. Particularly, for those cercarial species in which the target hosts do not regularly cohabit with the snail host producing the cercariae (Combes *et al*.,1994).

The prevalence of infection are major determinant factor of the rate of transmission of trematode from intermediate host snail to the second intermediates host in their life cycle (Margolis *et al*., 1982).

Several reported have been investigated the prevalence of cercarial infection in intermediate host snails. For example, Loy and Hass (2001) investigated the cercarial infection of 43,441 snail specimens from 174 natural water resources in Germany, and found that 8 type of cercariae (*Diplostomum spathaceum, Trichobilharzia ocellata, Apatemon minor, Echinostoma revolutum, Pseudoechinoparyphium echinatum, Hypoderaeum conoideum, Isthmiophora melis*, furcocercous cercaria, and xiphiocercaria) with overall prevalence of 44.90%. However, there are some reports concerning the potential of cercarial infection in Thailand. Ngern-klun *et al.* (2006) found 4 types of cercariae (virgulate cercaria, lophocercous cercaria, monostome cercaria and pleurolophocercous cercaria) were discovered in *Bithynia funiculata* with an overall prevalence of 9.60%. Wongsawad and Kumchoo (2000) reported the recovery of *Transversotrema patialensis* cercariae in snails, *Thiara scabra*. Moreover, Dechruksa *et al*. (2007) reported 2 types of cercaria (parapleurolophocercous cercaria and xiphidiocercaria) were infected in thiarid snails from the Khek river, Phitsanulok province with an overall prevalence of 0.90%. The previous report had been studied the epidemiology in freshwater animal (fish, snails, crab and shrimp) in 5 provinces including Chiang Mai, Chiang Rai, Phayao, Lampang and Phrae provinces found furcocercous and pleurolophocercous cercariae in *Melanoides tuberculata* snail from Phrae province only. Recently, Krailas *et al*. (2012) have been investigated cercarial infection in freshwater snails from Khao Yai National park, Thailand and reported 3 species of trematode namely, *Apatemon gracilis*, *Mesostephanus appendiculatus*, and *Loxogenoides bicolor*.

In Thailand, many species of freshwater snails were reported to be intermediate hosts. For example, *M. tuberculata*, *M. jujicostis* and *Neoradina prasongi* are the intermediate hosts of *Haplorchis taichui* and *Haplorchis pumilio* (Chontananarth and Wongsawad, 2010a; Krailas *et al*., 2011). Other snail species, *Filopaludina* spp., *Parafossarulus manchouricus*, and *Lymnaea* spp. were reported to act as the first and second most common intermediate hosts of trematodes in the family Echinostomatidae (Lee *et al*., 1983; Fried and Toledo, 2009; Chai *et al*., 2011). Furthermore, the planorbis snails are intermediate hosts of blood fluke in family Schistosomatidae (Isaac, 2009; Aldhun *et al*., 2012).

Many species of heterophyid trematodes have been reported from parapleurolophocercous cercaria such as *Haplorchis taichui, H. pumilio, Stellantchasmus falcatus,* and *Procerovum varium* (Schell, 1970; Brandt, 1974; Díaz *et al*, 2008). About 100 species of snails have been reported to act as intermediate hosts for the trematodes especially in thiarid snails which harbor the larvae of many species of trematode viz. Echinostomatidae, Allocreadiidae, Heterophyidae, etc. For instant, *Melanoides tuberculata*, *Thiara scabra* and *Tarebia granifera* are the first intermediate host for *H. taichui*, *H. pumilio*, *C. caninus* (Dias, 2002, Skov *et al.*, 2009), *Thiara granifera* and *Stenomelania newcombi* are first intermediate host for *Stellantchasmus falcatus* (Noda, 1959). The study of Bogéa *et al*. (2005) showed that *Melanoides tuberculata*, is a first intermediate host of heterophyid trematodes developing from pleurolophocercous cercariae, and Ukong *et al*. (2007) confirmed that *H. pumilio* developed from the same type of cercaria.

Geographic distribution and epidemiological status of heterophyid trematodes

The heterophyid trematodes are observed in broad geographical range. It has been reported that there have many species of them could be found in many countries, such as China, Japan, Korea, Laos, Vietnam, Philippines Egypt and Thailand (Vicente *et al*., 2004; Thu *et al.*, 2007; Rim *et al.*, 2008; Chai *et al.*, 2009; Chai *et al.*, 2011). The most of them are predominantly from bird, fish and occasionally from mammal, including human. Their distribution depends on the presence on the first and second intermediate host, including the ratting habit of the local people (Radomyos *et al*., 1998).

In Thailand, trematode in family Heterophyidae there are in five genera, viz. *Haplorchis, Centrocestus, Stellantchasmus, Procerovum,* and *Haplorchoides* (Waikagul *et al*., 1997; Seanphet *et al*., 2001; Chuboon and Wongsawad, 2009; Chontananarth and Wongsawad 2010b; Thaenkham *et al*., 2010). The outbreak of heterophyid trematodes infection have been continually reported in Thailand**.** This area is covered by the diverse ecosystem of farm forestry along with plenty of water resources, producing a high biodiversity to support the life cycle of trematodes to easily infect both humans and animals (Boonchot and Wongsawad, 2005). Epidemiological surveys throughout 1997-2011 have shown an increase of trematode infections in northern and central part of Thailand also**.** This region has been reported as epidemic for heterophyid trematodes with a high prevalence of infection in many kind of intermediate hosts and definitive hosts. In freshwater fish, the family Cyprinidae (cyprinoid fishes) is a common intermediate host of *Haplorchis taichui*. Many species of cyprinoid fishes collected from Chiang Mai, Phitsanulok, and Lamphun provinces were infected with *H. taichui* (Wongsawad *et al*., 2009a). For instant, Wongsawad *et al.* (2000) investigated for trematode infection in freshwater fish from Mae Sa stream, Chiang Mai province, 4 species of heterophyid trematodes found viz. *Haplorchis* sp., *Haplorchoides* sp., *Centrocestus caninus* and *Stellantchasmus falcatus*. Recently, a high prevalence of *H. taichui* metacercariae was reported in cyprinoid fishes collected from Mae Ngad Somboonchon reservoir, Chiang Mai, Thailand (Sukantason *et al*., 1999; Kumchoo *et al.*, 2005). In addition, Nithikathkul and Wongsawad (2008) reported a high infection rate of *H. taichui* and *Haplorchoides* sp. (83.90%) in the same area**.** While, *Haplorchis pumilio* and *Procerovum* sp. have been usually reported from Central and Southern, Thailand (Dechruksa *et al*, 2007; Ukong *et al*., 2007; Krailas *et al*., 2011; Noikong *et al*., 2011).

Morphology of intermediated host snails

The Mollusca are coelomate, bilateral, often secondarily altered to an asymmetrical condition, without (or rarely with) evidences of metamerism, with soft body usually protected by a calcareous shell. Typically a mollusk presents the following feature: a head that bears a terminal mouth, eyes, tentacle, and other sensory organ; a muscular for locomotion; a fold of body wall , mantle cavity. The classification of gastropods or snails usually based on their external morphology (Figure 2-3). Early morphology designations were based in the shell, shape, operculum, number of whorls and relative size of the shell. So, some general characteristics of the shell for identification to species level are as follows: (Malek, 1922; Mandahl-Barth, 1962; Morton, 1968; Purchon, 1977)

1. Shell sculpture: Apart from the basic functions of shell morphology, there is also a range of functional features occurring within the shell. These features are often adaptive in relation to habitat, locomotion, feeding and defense from predators However, a specific character can function in several different ways and it is difficult to make a strict division. There is reason for caution when making interpretations of morphological features, as the presence or absence of a character might be misleading. The whole organism should be regarded as a complete adaptive.
2. Body whorls: The body whorl or last whorls is the most recently formed and largest whorl of a spiral or helical shell, terminating in the aperture. It is called the "body whorl" because most of the body of the soft parts of the animal fits into this whorl. The proportional size of the body whorl in shells differs greatly according to the actual shell morphology.

3. Apex: The apex is most often used to mean the tip of the spire of the shell of a snails. The apex is the first-formed, and therefore the oldest, part of the shell.
4. Operculum: The operculum is a corneous or calcareous anatomical structure which exists in many groups of snails. The operculum is attached to the upper surface of the foot and in its most complete state, it serves as a sort of "trapdoor" to close the aperture of the shell when the soft parts of the animal are retracted. The shape of the operculum varies greatly from one family of snail to another. It is often circular, or more or less oval in shape. In species where the operculum fits snugly, its outline corresponds exactly to the shape of the aperture of the shell and it serves to seal the entrance of the shell. The structure of the operculum can be described as follows:
    - 4.1 Concentric: the nucleus is central or subcentral as in *Clea helena* and *Ampullaria*, and in other the nucleus is near the parietal margin of the shell.
    - 4.2 Paucispiral or oligogyrous: with few spirals as in *Adamietta Melanoides*, and *Thiara*
    - 4.3 Multispiral or polygyrous: having many closely spaced spirals as in *Paludomus* and, *Trochus* where they sometimes amount to twenty; the number of turns which the operculum makes is not determined by the number of whorls in the shell, but by the curvature of the aperture, and the necessity that the operculum should revolve fast enough to fit it constantly.

5. Spire: A spire is a part of the coiled shell of snails. The spire consists of all of the whorls except for the body whorl. Each spire whorl represents a rotation of 360°. The spire, when it is not damaged or eroded, includes the protoconch (also called the nuclear whorls or the larval shell), and most of the subsequent teleoconch whorls (also called the postnuclear whorls), which gradually increase in size as they are formed. Thus the spire in most snails is pointed, the tip being known as the "apex". The word "spire" is used, in an analogy to a church spire or rock spire, a high, thin, pinnacle. The "spire angle" is the angle, as seen from the apex, at which a spire increases in size. It is an angle formed by imaginary lines tangent to the spire.
6. Umbilicus: The umbilicus is clearly visible on the underside of the left shell of these three shells of *Xerolenta obvia*. The umbilicus of a shell is the axially aligned, hollow cone-shaped space within the whorls of a coiled snail shell. The term umbilicus is often used in descriptions of gastropod shells, i.e. it is a feature present on the ventral (or under) side of many snail shells.
7. Columella: The columella runs from the apex of the shell to the midpoint of the undersurface of the shell, or the tip of the siphonal canal in those shells which have a siphonal canal. If you visualize a snail shell as a cone of shelly material which is wrapped around a central axis, then the columella more or less coincides spatially with the central axis of the shell. In the case of shells that have an umbilicus, the columella is a hollow structure. The columella of some groups of gastropod shells can have a number of

plications or folds (the columellar fold, plaits or plicae), which are usually visible when looking to the inner lip into the aperture of the shell. These fold can be wide or narrow, prominent or subtle. These features of the columella are often useful in identifying the family, genus, or species of the gastropod.

8. Siphon: The siphonal canal is an anatomical feature of the shells of certain groups of freshwater snails and sea snails within the clade Neogastropoda. Some freshwater snails (*Clea helena*) have a soft tubular anterior extension of the mantle called a siphon through which water is drawn into the mantle cavity and over the gill and which serves as a chemoreceptor to locate food. In certain groups of carnivorous snails, where the siphon is particularly long, the structure of the shell has been modified in order to house and protect the soft structure of the siphon. Thus the siphonal canal is a semi-tubular extension of the aperture of the shell through which the siphon is extended when the animal is active.

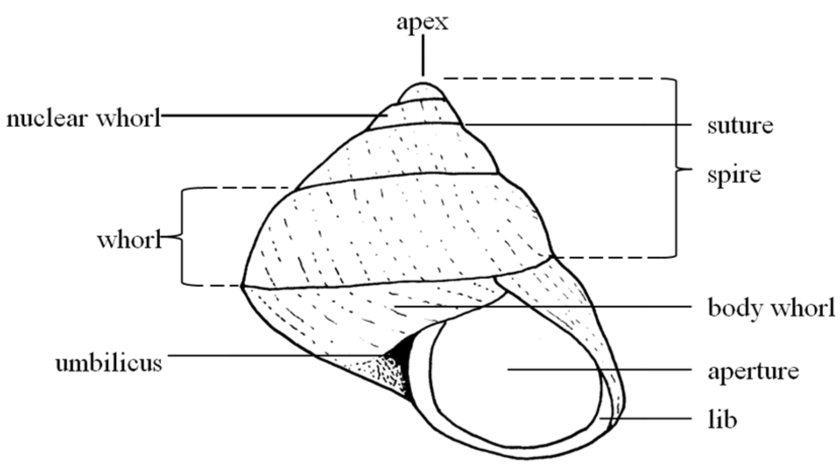

Figure 2-3. Important features of a dextral, or right-turned, spire of gastropod shell.

Molecular identification

From the parasitological surveys, mixed infections with heterophyid trematodes with liver fluke (*Opisthorchis viverrini*) are common (Radomyos *et al*., 1998). The morphological characteristic of adult stage of heterophyid and opisthorchid trematodes has been clearly describe, and their species can be identified by light microscope (Scholz, 1991). Nevertheless, the morphology of larval stage might not be distinguishable accurately other than by a specially trained for diagnostic. Moreover, difficulties arise because they are small and soft also have only few stable morphological characters and are subject to host-induced phenotypic variation (Graczyk, 1991). Thus, an identification of closely relate species of trematodes only based on the morphological characteristic can be confused or misidentified. Consequencely, the specific and accurate identification procedure is required for parasitic differentiation.

Several studies have been applied the Polymerase Chain Reaction (PCR) approach for identifying the trematode infections for understanding the epidemiological status of each parasite species. Various conventional PCR assays have been developed to detect parasite DNA in feces, definitive hosts and intermediate hosts. Polymerase Chain Reaction– Restriction Fragment Length Polymorphism (PCR-RFLP) have been introduced to detect various of parasites infection namely, *Schistosoma hematobium* and *S. bovis* (Barber *et al*. 2000), *Haplorchis taichui* and *H. pumilio* (Thaenkham *et al*., 2007) and *Fasciola* spp. (Lchikawa and Itagaki, 2010). The High Annealing Temperature–Random Amplify Polymorphic DNA method (HAT-RAPD) was used to analyze closely related species of trematode in family Heterophyidae, for demonstrated inter-specific polymorphism of DNA profile. (Wongsawad and Wongsawad, 2007). In addition, this method is providing the greater polymorphism , reproducibility, high resolution and can gave the SCAR (Sequence Characterized Amplified Regions) markers, that has a specific sequence of approximately 20 bases. This marker increased specificity for identification by the DNA specific primer. For instant, the DNA specific primer has been used to detect the *Echinostoma trivovis* and *E. caproni* infected in definitive host (Fujino *et al.*, 1997), *Haplorchis taichui* in intermediated hosts (Chuboon and Wongsawad , 2009), *Stellantchasmus falcatus* (Wongsawad and Wongsawad, 2010).

Several regions of nuclear ribosomal DNA (nu rDNA) were selected and used for identification of various stages and studying life cycles of trematodes (cercaria, metacercarial and adult stages) in freshwater fishes and snails because of highly accuracy, sensitivity and rapidity. Especially, the used of ITS2 (inter transcribe spacer 2) has been revealed to be a sensitive marker at the species level of trematode. The PCR based-method targeting ITS2 has been applied to used for phylogenetic study of various trematode, such as *H. taichui* and *H. pumilio* (Van *et al*., 2009; Skov *et al*., 2009), *Clonorchis sinensis* (Müller *et al*., 2007), *Fasciola hepatica* (Erensoy *et al*., 2009), and trematode in Family Paramphistomidae (Loffy *et al*., 2010)

The mitochondrial cytochrome c oxidase subunit I (mtCOI) gene is small with approximately 1,700 bp , circular and maternally inherited (Boore, 1999). High copy number per cell, made it attractive and more tractable gene, which is targeted for characterizations, genetic variation, population genetic, biogeography, and phylogenetic studies (Pakendorf and Stoneking, 2005). This region in the mtDNA has been demonstrated to be useful in biology, epidemiology, and diagnosis of several parasitic infection in intermediate hosts and definitive hosts. The cytochrome c oxidase gene is a 13 protein complexes located on the inner mitochondrial membrane that catalyzes electron transfer, production of up 95% of the energy of eukaryotic cells (Le *et al*., 2002; Johnton *et al*., 2005). Therefore, mtCOI is the most highly conserved among 3 gene coding for cytochrome c oxidase. Therefore, it has been used in molecular identification and phylogenetic studies. Hebert *et al*. (2003) stated that context that the mtCOI gene is a suitable candidate gene for DNA bercoding of trematode in family Fasciolidae.

Most studies on mtCOI of trematodes focused on the phylogenetic questions. This gene was used to analyze phylogenetic relationship of numerous species of trematodes including, 3 species of *Metagonimus* (*Metagonimus yokogawai, M. takahashii* and *M. miyatai*) in Korea (Lee *et al.*, 2004), *Clonorchis sinensis* and *Opisthorchis viverrini* in China (Park, 2007), *Opisthorchis viverrini* in Thailand and Laos (Saijuntha *et al.*, 2008), *Schistosoma nasale* in Sri lankan (Sato *et al*., 2008), *Haplorchis taichui* in Thailand and Vietnam (Dung *et al*., 2012).

The molecular diagnosis method using mtCOI gene has also been introduced to detect trematode infections. Le *et al.* (2006) designed mtCOI DNA specific primer and developed the multiplex PCR of *C. sinensis* and *O. viverrini* to discriminate between them. In addition, the mtCOI sequences have been applied to examine the polymorphism of cercarial stage of bird schistosomes from Moscow (Lopatkin *et al.*, 2010). The PCR-RFLP of mtCOI gene shown differently fragmented DNA pattern of trematode in family Heterophyidae (Lee *et al.*, 2004; Thaenkham *et al.*, 2007; Thaenkham *et al.*, 2011). However, Using the restriction enzymes to examine the DNA pattern is limited because of time consuming and expensive.

Hence, a main purpose of this work is to design DNA specific primers based on mtCOI sequence for *H. taichui* and *H. pumilio* to detect infections in snail intermediate hosts from northern Thailand. The designed primers will be taken to used in the multiplex PCR detection. This approach using the primers was expected to be very accurate, rapid and less time consuming with higher sensitivity than the previous reported methods such as RADP, AFLP and PCR-RFLP. The distribution of both trematode species using Geographic Information System (GIS) will be used to gain information on the distribution of trematode infections. In addition, the phylogenetic relationship of heterophyid trematodes based on mtCOI sequences will be also investigated to provide more systematic data on heterophyid trematodes. This is a preliminary studies to understand host-parasite relationships.