CHAPTER 2

Literature reviews

2.1 Taxonomic information (Jones *et al*., 2005)

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Trematoda
Subclass: Digenea
Order: Echinostomida
Suborder: Echinostomata
Superfamily: Echinostomatoidea
Family: Fasciolidae
Subfamily: Fasciolinae
Genus: *Fasciola*
Species: *F. gigantica*

2.2 Morphology of adult worm

Fasciola gigantica is body large and leaf-like shaped, dorso-ventrally flattened, usually relatively thin occasionally thick. The cephalic cone is presented. Oral and ventral suckers are rounded and well developed, usually close together on the cephalic cone. Oral sucker is opened on sub-terminal of body, usually smaller than ventral sucker. The ventral sucker is located at the base of the cephalic cone. Prepharynx is presented and shorted, pharynx is well developed. Esophagus is usually shorted. Genital pore is opened at the base of cephalic cone and nearly ventral sucker. Intestines are absolutely

branched and extended in lateral of body. Two highly dendritic testes are arranged tandem and occupied post-ovarian area between vitelline fields. One branched ovary is situated in sub-median of body. Vitelline follicles are diffusely branched and well developed, are extended in lateral of body. Uterus is shorted, convoluted, and situated between ventral sucker and ovary (Neva and Brown, 1994).

The tegument is covered with numerous scale-like spines, which the SEM photographs of the spines at higher magnification showing finger-like protrusions at their tips. It can be used to discrimination species among *F. gigantica* and *F. hepatica* (Degheidy and Shalaby, 2010). Based on the presence of various organelles and density of the tegument cytoskeleton are divided 4 layers (Sobhon *et al*., 2000).

The general morphology of *F. gigantica* was similar with the closely related-species, *F. hepatica* (Fig. 2.1). This fluke are greater length, shorter cephalic cone, larger ventral sucker, more anterior position of the reproductive organs, and larger eggs (Neva and Brown, 1994). The comparative morphometry between *F. gigantica* and *F. hepatica* revealed that the body length of *F. gigantica* was longer than *F. hepatica*, with ranges 16-39 mm and 12-29 mm, respectively, and the body width was narrower, with ranges 4-10 mm and 7-11 mm, respectively (Narva *et al*., 2011). All measurements of *F. gigantica* and *F. hepatica* are overlapped in the distance between the ventral sucker and the posterior end of the body, body roundness and body length/body width ratio (Periago *et al.*, 2006).

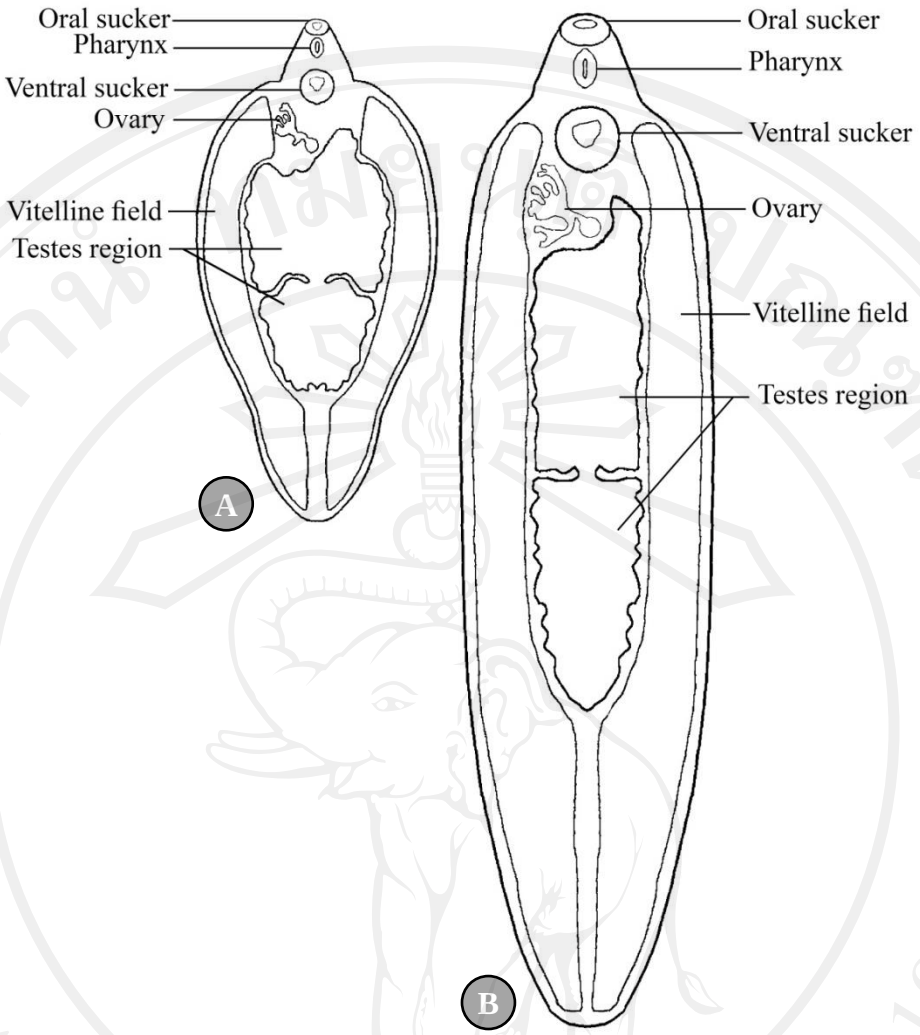

Figure 2.1 Illustration demonstrated the morphological comparison between (A) *Fasciola hepatica* and (B) *F. gigantica* (modified from Periago *et al.*, 2006)

2.3 Morphology of larval stages

The illustration of *F. gigantica* larval stages has not been presented in previous reported, but found only the closely related-species, *F. hepatica* which represented in figure 2.2. The larval stages of both species are similar morphology. Very few studies of larval stages demonstrated that the eggs are large yellowish and operculated with thin shell. It has a distinct, flat operculum and an umbilicus-like invagination at the posterior end of the shell (Hussein *et al*., 2010a). The eggs of *Fasciola* are consisted with fertilized ovum and vitelline cell surrounded by proteinous shell (Beaver *et al*., 1984). The outer

surface of the egg shell was smoothly and highly conspicuous umbilicus-like invagination at the posterior end of the eggs shell (Hussein *et al*., 2010a). The egg size of *F. gigantica* are bigger than *F. hepatica*, with range 150-190 µm by 70-90 µm and 130-150 µm by 63-90 µm, respectively (Neva and Brown, 1994) (Fig. 2.2a-b). Velero *et al*. (2009) indicated that the morphological traits of eggs from human feces are differenced the eggs from animals feces and *F. hepatica* eggs in humans are bigger than animals, whereas *F. gigantica* eggs are smaller than reports from animals. There are variable sizes of the eggs from the same fluke (Hussein *et al*., 2010a).

The miracidium are elongated conical body and a broad in anterior and tapering posterior end. The one pair of eye spots is presented at the right side of midline of anterior part. Germ cells are scattered in the posterior part and the apical papilla is shown in the middle of the anterior part. The tegument is covered with numerous cilia and the cilia on apical part of the anterior end are longer than on the posterior extremity of the body (Fig. 2.2c) (Hussein *et al*., 2010a). The size of miracidium is measured with 110 x 70 µm (Hussein *et al*., 2010b).

The rediae (mother and daughter redia) are a caudal papilliform and has an elongated, flat body with an anterior projecting circular ridge or collar and ended with caudal papilliform process. It has a muscular pharynx followed by a simple sac-like intestine. The mother redia is contained with undifferentiated structures and germ cells. Daughter redia visible is long and cylindrical body, but has not collar and the caudal papilliform process. It is contained the developing cercariae and germ cells. The birth pore is opened at anterior end (Figs. 2.2g-h) (Hussein *et al*., 2010a).

The cercaria has a large heart shaped body and the tail is longer than body, simple, not forked and provided with two lateral folds. The oral sucker is smaller than ventral sucker. It is circular and situated on extremely of the body. The ventral sucker is circular in shape and situated on middle of the body (Mohamed *et al*., 1999). The wall of body is thick and surrounded by minute spines. The intestines are bifurcated into two simple branches. The body is fully numerous cystogenous glands and it is concave ventrally (Hussein *et al*., 2010a) (Figs. 2.2I-J).

Metacercaria is circular in shape. The outer layer is roughened with irregular particulate material covering the whole surface. The inner cyst wall has a double thick cyst wall and the surface of cyst is smooth and lacks any furrows or tubercles (Mohamed *et al*., 1999). The diameter of cyst is ranging between 0.224-0.272 mm (Fig. 2.2k) (Hussein *et al*., 2010a).

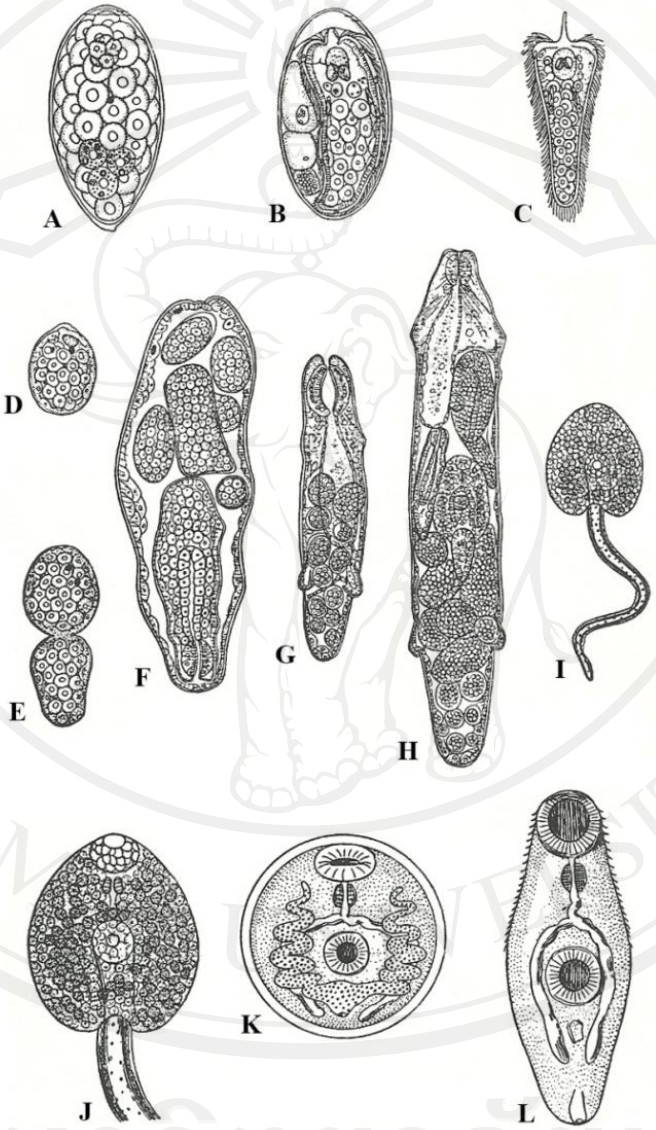

Figure 2.2 Illustration demonstrated the larval forms of the closely related-species, *Fasciola hepatica*; (A) immature egg, (B) fully miracidium egg, (C) miracidium, (D-F) sporocyst, (G-H) redia, (I-J) miracidium, (K) metacercaria, (L) excysted metacercaria (modified from Neva and Brown, 1994)

2.4 Epidemiology

Fascioliasis is important parasitic disease caused by two species of genus *Fasciola*; *F. hepatica* and *F. gigantica* (Valero *et al*., 2009). *Fasciola hepatica* has a cosmopolitan distributed, mainly in temperate region, except Oceania. *F. gigantica* is distributed in tropical regions of Africa, the Middle East, and Asia (Mas-Coma and Bargues, 1997; Soliman, 2008). These parasites are causative agent of hepatic damage in ruminants and have an economic impact on the growth, development and productivity of ruminants (Dargie, 1987). Hence, there are economically important helminthic diseases of farm livestock in each country. Although several information are reported the fascioliasis mainly found on ruminants, but in many countries have been reported in human, which human are an accidental host of parasites. Many ruminants and human acquired this parasite by consuming undercooked food prepared from aquatic plants containing metacercaria. The transmission for fascioliasis is mainly through the ingestion of raw aquatic vegetables, or the occasional consuming grass, on which larval parasites are encysted (Haswell-Elkins and Elkins, 1996). Encapsulated metacercaria of *F. gigantica* can be deposited in liver and gall bladder of hosts (Africa *et al*., 1936).

Several reports have been completed on the fascioliasis that is mainly found in ruminants. However, many countries have also conducted studies on humans, as humans can be an accidental host of these parasites. Countries such as India have reported fascioliasis in domestic ruminants, and this has been determined to have been directly caused by *F. gigantica* (Garg *et al*., 2009). Maqbool *et al*. (2002) reported that the epidemiology of *F. gigantica* at slaughter-houses, livestock farms, veterinary hospitals and in household buffalos in Punjab Province, were 25.59%, 26.16%, 13.7% and 10.5%, respectively. In Nigeria, the prevalence of *F. gigantica* infection in slaughterous cattle was 47.55% (Opera, 2005). Ulayi *et al*. (2007) reported that the prevalence (2.1%) of infection with *F. gigantica* was higher in the bulls (1.3%) than in the cows from slaughtered animals at Zaria abattoir, Nigeria.

In recent years, Abunna *et al*. (2010) found that the mixed-infection between *F. hepatica* and *F. gigantica* in indigenous adult cattle of Southern Ethiopia, found to contain fluke infection during post mortem inspection, 13 (3.2%) harbored F. *hepatica*, 37 (9.1%) and *F. gigantica*, 3 (0.7%) had mixed infections and 4 (1%) contained unidentified immature fluke. Tsegaye *et al*. (2011) reported that 42.25% of 400 cattle were found to be positive for fascioliasis and that the prevalence of bovine fascioliasis was higher in male cattle than in females. The infections of F. *gigantica* in cattle of Mount Elgon, Uganda depended on the altitude sites, at low (<1500 m) and high (>1500 m) altitude sites was 43.7% (95% CI 35.4-52.2) and 1.1% (95% CI 0.0-6.0). This is likely due to a growing paucity of intermediate hosts, specifically populations of *L. natalensis* for which a natural boundary of 1800 m appeared (Howell *et al*., 2012). In Thailand, the fascioliasis was rarely reported and the prevalence of *F. gigantica* in cattle and water buffalo ranges between 4-24%, with the highest incidences in the north and north east, and the lowest in the south (Sukhapesna *et al*., 1994; Sobhon *et al*., 1998).

Human fascioliasis has been reported in South America, Europe, Africa, Australia and the Far East with an estimated 2.4 million cases worldwide (Bayu *et al*., 2005). The diagnosis of human fascioliasis is based on the presence of *Fasciola* eggs in a stool or gallbladder sample, or on a positive serological test plus radiological findings indicating fascioliasis (Kabaalioğlu *et al*., 2000). Many cases reported that the high prevalence rates in several regions of Peru with 33.3% by fecal examinations and 51.9% by serology (Marcos *et al*., 2005) and 4 cases of human fascioliasis were found in Gondar town, northwest Ethiopia (Bayu *et al*., 2005). Human fascioliasis in 21,477 subjects of primary school children from endemic areas of the Nile Delta in Egypt was investigated by the Kato-Katz thick smear technique that revealed the 932 positive cases and higher prevalence of infection among girls than boys (Curtale *et al*., 2007), while human fascioliasis caused by *F. hepatica* in Peru was shown high prevalence in children linked to a man-made irrigation zone, with a 24.3% overall mean prevalence and local prevalence ranging between 18.8 and 31.3% (Esteban *et al*., 2002) and a rarely case of human fascioliasis in Japanese , which adult worms were recovered from the bile duct of Japanese man (Inoue *et al*., 2007). In Argentina, human fascioliasis has been literature search identifying 58 reports accounting for 619 cases, involving 13

provinces, their majority (97.7%) from high altitudes, in central mountainous areas and Andean valleys, concentrated in Cordoba (430 cases), Catamarca (73), San Luis (29) and Mendoza (28) (Sierra *et al*., 2011), and northern Iran was found two fasciolid adult specimens directly obtained from humans that both were *F. hepatica* (Sharifiyazdi, 2011). In Thailand, many cases of human fascioliasis were reported; including Sawangkit (1990) had reported 25 cases of fascioliasis in Thai patients that found 17 cases were identified as *F. gigantica* infection, 7 cases as *F. hepatica* infection, and 1 case of unidentified species. Aroonroch *et al*. (2006) was reported 2 additional cases of hepatic fascioliasis due to *F. hepatica* and Kanoksil *et al*. (2006) found that only one case in 67-year-old female patient from Angthong province, one live fluke was removed and identified as *F. gigantica*.

So, in the several literature reviews found that fascioliasis can be affected in Africa, Asia, many Pacific islands including Hawaii, the Middle East, Southern Europe, and the south of the USA (Hammond, 1974). Thus, this parasite is an important plant-borne parasitic diseases that affecting in livestock and human public health status. In Thailand, the most reports indicated that the fascioliasis in ruminants and human has mainly caused by *F. gigantica* and the adult stage has commonly recorded in cows and water buffaloes from different parts of country (Sukhapesna *et al*., 1994; Sobhon *et al*., 1998). But some reports revealed that human fascioliasis caused by *F. hepatica* (Sawangkit, 1990; Aroonroch *et al*., 2006) and Sawangkit (1990) reported that the *F. gigantica* and *F. hepatica* can be mixed-infection the same patient. From this, the previous studies of fascioliasis in Thailand were many confuse to identification, because these flukes are infected the same hosts. Moreover, morphology of larval stages is very similar. Hence, the accuracy methods for identification of *F. gigantica* need to be more study. This study used the molecular approach to identify the actually species of fasciolids in Thailand.

2.5 Life history

The two species of *Fasciola*; *F. hepatica* and *F. gigantica* are similar two hosts in life history as freshwater snails of the family Lymnaeidae act as intermediate hosts and transmitted to mammals including ruminants and human act as definitive host (Mas-Coma *et al*., 2005). The illustration of life cycle of *F. gigantica* was not found in the literature reviews, but only found *F. hepatica* was revealed in figure 2.3.

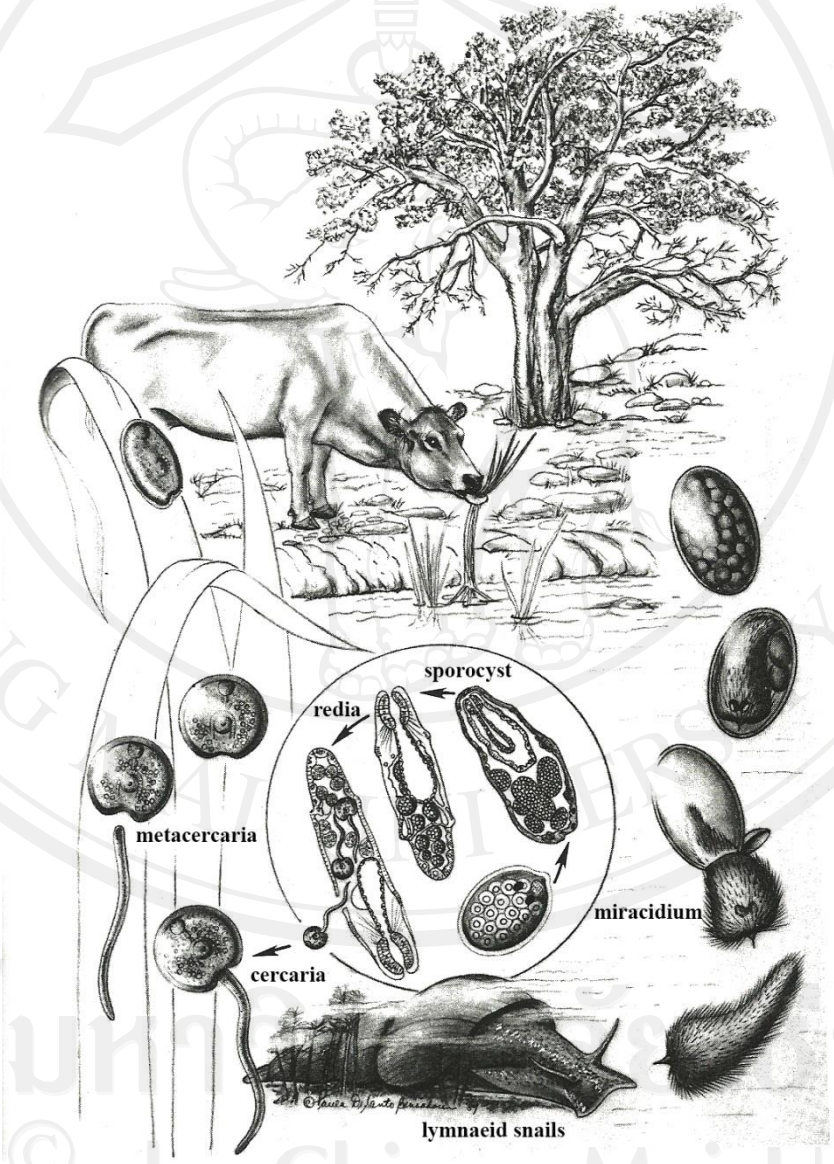

Figure 2.3 Illustration demonstrated the generally life cycle of closely related-species, *Fasciola hepatica* (Bowman, 1995)

In figure 2.3 demonstrated that adult worms live in bile ducts of definitive host, usually as cows and buffaloes. Their worms are shed eggs which evacuated in the feces of definitive hosts, deposited in water body and hatched to miracidium. The body of miracidium has an elongated conical body that has a broad anterior end and tapering posterior end. Surface of miracidium are found covered with cilia, excepted regions of lateral connection of epidermis plates (Hussein *et al*., 2010a). Free-swimming miracidium are punctured into snail intermediate hosts by using conical papillae at its anterior end, and then developed to sporocyst, radia, and ceracaria, respectively.

Many Lymnaeid snails were reported to serve as the intermediate hosts of *Fasciola gigantica* and *F. hepatica* in different parts of the world (Itagaki *et al*., 1989) such as *Lymnaea columella, L. cousini, L. natalensis, L. truncatula, L. viridis, L. ollula, L. columella* (Dinnik and Dinnik, 1957; Lee *et al*., 1995; Magalhães *et al*., 2004; Yoshihara and Ueno, 2004; Salazar *et al*., 2006). In Colombia, the snails *Lymnaea columella* and *Lymnaea cousini* have been reported as intermediate hosts of *Fasciola hepatica* and infection rates were 82.2 and 34% for *L. columella* and *L. cousini*, respectively (Salazar *et al*., 2006). Bargues and Mas-Coma (2005) reviewed that the intermediate snail hosts of *F. gigantica* in several countries are *Radix natalensis* in Africa, *R. auricularia* in the Near East, Middle East, Far East and southern states of the old USSR, *Fossaria cubensis* in the North American gulf coast, *R. rufescens* in Asia and the Indian subcontinent, *R. rubiginosa* in the Far East and Malaysia, *R. swinhoei* in South East Asia and the Philippines, and *Austropeplea ollula* in Hawaii and Japan, *Galba truncatula* in Africa, *R. peregra* in the Near East, Middle East, and southern states of the old USSR, *L. columella* in the North American gulf coast, and *A. ollula* in the Far East (Mas-Coma and Bargues, 1997). *Radix caillaudi* in Egypt, *R. gedrosiana* in Iran, *R. euphratica* in Iraq, *R. luteola* in Nepal, and *R. bactriana, R. tenera* and *R. subdisjuncta* in Turkmenia (Mas-Coma and Bargues, 1997). The experiment of *L. viridis* infected with 3 or 5 miracidium of *F. hepatica* were produced more metacercariae than those infected with a single miracidium (Lee *et al*., 1995). Rondelaud *et al*. (2009) was investigated the influence factor on radial generations and the results shown that the environmental and biotic factors were not modified the succession of radial generations, but most act by limiting the number of rediae, while Shalaby *et al*.

(2004) reported the prepatent period of *F. gigantica* inside the snails host was inversely related to temperature and markedly affected by age rather than the number of miracidia inoculated. In Thailand, only one species of Lymnaeid snails as *L. auricularia rubiginosa* has been reported serving as intermediate host of *F. gigantica* (Charoenchai *et al.*, 1997).

Cercariae are penetrated snails and encysted as metacercaria on vegetation, such as rice straw and stubble are important sources of fodder for cows and water buffalo in Indonesia (Suhardono *et al.*, 2006a) but in Japan found on Japanese parsley and water lilies (Yoshihara and Ueno, 2004). This stage is an infective stage and developed to adults in definitive hosts by ingestion and completely life cycle (Souza *et al*., 2002). The entire life cycle of *Fasciola*, from the time an egg is shed onto vegetation pasture until a newly infected animal reinfects the vegetation pasture with the next generation of fluke eggs, generally requires 18 to 24 weeks (Kaplan, 2001). Generally, abundance of snail intermediate hosts has been recognized as associative factor to facilitate the dynamic of infection including the development of parasites during developmental stages in their life cycle.

Although, no detail studies on the life history of *F. gigantica* but have some reported in the any countries, which those studies are did not covered all stages on life history traits such as, Dreyfuss and Rondeland (1995) compared the productivity of infected *F. gigantica* and *F. hepatica* in *Lymnaea tomentosa* found the numbers of rediae of *F. gigantica* were substantially greater than *F. hepatica*. While the patent period and number of cercariae of both fasciolids was closely correlated with the particular *L. truncatula* population and trematode species (Dreyfuss and Rondeland, 1997). Moreover, Yadav and Gupta (1988) reported the infection of *F. gigantica* in 2 rabbits by 50 metacercariae derived from feces of the snail (*L. truncatula*), both rabbits were died at 83 and 87 days post-infection, and recovered with 8 and 10 immature adults from the liver.

The above mentioned studies have not determined the life history traits of *F. gigantica* in Thailand which no detail studies not only morphology but also growth and development of sporocyst, mother and daughter redia. The most studies are focused on the epidemiological study and molecular detection. But the life history traits are neglected study. Therefore, this study is designed to investigate the all life history traits, morphology, and development of the larval stages, as well as the adult worms that are found in the experimental hosts. The results can be applied for treatments, management and the controlling programs of this parasite.

2.6 Clinical symptoms

Fascioliasis is a highly pathogenic disease. The pathogenesis in human was depends on the number of infecting flukes, and appears to be similar to the reported in animals (Mas-Coma and Bargues, 1997). The patients have clinical symptoms with anorexia, weight loss, and jaundice for one month. Intra-operative retrograded cholangiopancreatography revealed a filling defect considered as a stone and bile sludge (Kanoksil *et al*., 2006; Inoue *et al*., 2007).

2.7 Diagnosis

The diagnosis of fascioliasis in hosts is demonstrated of the large operculated eggs in feces of hosts. Saturated sucrose floats but distorts the eggs, or sedimentation techniques are preferred (Bowman, 1995). However, the qualitative examination of *Fasciola gigantica* eggs in faeces and bile were compared with the detection of precipitating antibodies in sera by agar gel precipitation test (AGPT) in 1000 cattle slaughtered at the Bodija municipal abattoir in Ibadan, Nigeria. Faecal and bile examination methods detected (196) 33.5% and (389) 38.9% of the animals as positive for fascioliasis, while (474) 47.4% were positive by AGPT. The results revealed that the AGPT could become a better test for the herd diagnosis of bovine fascioliasis for veterinarians and other investigators in Nigeria (Adedokun *et al*., 2008). In human fascioliasis can be diagnosed by directly identification of flukes obtained from surgical

removal, or detection of eggs in the bile from duodenal tube and by stool examination (Kanoksil *et al*., 2006).

2.8 Treatments

Clorsulon (Curatrem) and albendazole are used for treatment in ruminants; clorsulon is administered to ruminants orally as 8.5 per cent suspension at a dosage rate of mg per kg for treatment of immature and adult infections (Malone *et al*., 1984; Yazwinski *et al*., 1985). Albendazole is indicated for the removal of flukes in cattle at a dosage rate of 10 mg per kg of body weight and sheep at 7.5 mg per kg Albendazole (15 mg per kg) was effective in eliminating adult of *F. hepatica* and reducing the death rate among naturally infected goat in Montana (Leathers *et al*., 1982). For the medical treatments of human fascioliasis were used several drugs such as praziquantel 25 mg per kg per day three times daily for 3-7 days is ineffective (Price *et al*., 1993). Bithionol, 30-50 mg per kg per day on alternate days for 10-15 days, was recommended with a very high rate (Price *et al*., 1993; Noyer *et al*., 2002). Recently, a highly effective fasciolicidal drug, triclabendazole in the dose of 10 mg per kg in a single dose after an overnight fast was described (Mas-Coma *et al*., 1999; Inoue *et al*., 2007).

2.9 Prevention and control

The controls of flukes are dependent upon the eradication of disease in ruminants. Treatment is possible for domestic ruminants, but not for wild ruminants. The destruction of snail intermediate hosts in around livestock is necessary. The decreased snails are inhabited of transmits the larval stage to the other intermediate hosts. Infection in humans may be prevented by eliminating raw aquatic plants. Biological control is important method to the success of completely control. Infected duck and bovine faeces are enter the rice field at the same place, as miracidia of *F. gigantica* and *Echinostoma revolutum* will then hatch in close proximity to each other, missing the opportunity for competition between them in *L. rubiginosa* in the vicinity (Suhardono *et al*., 2006b).

2.10 Molecular parasitology

The identification of genus *Fasciola* can generally be distinguished on the basis of their adult morphology (Ashrafi *et al*., 2006). However this method has limitations when used to identify the closely-related species, *F. hepatica* and larval stages in snail intermediate hosts. The morphological characters of these parasites were confused and it is often difficult to distinguish between such morphologically similar trematode species, especially the larval stage in snails. Both fasciolids are similar hosts in life cycle, which freshwater snails of the family Lymnaeidae act as intermediate or vector host and a broad spectrum of mammals including humans, herbivorous, act as definitive host (Mas-Coma *et al*., 2005). The development of a reliable and sensitive method for parasite diagnosis and characterization is necessary for proper understanding of the epidemiology. Recently, a number of sensitive and specific techniques have been applied in the investigations of *F. gigantica* epidemiology. The conventional techniques were used for the detection of *F. gigantica*.

Molecular approaches using PCR methods have been used to resolve and support taxonomic issues, related to various trematode species such as Morozova *et al*. (2002) determined that random DNA amplification by PCR with arbitrary primers (RAPD–PCR) was used for the description and estimation of genetic variation in two trematode species, *F. hepatica* and *Dicrocoelium dendriticum.* The results revealed that polymorphism level (*P*) varied within a range of 35.5 to 83.2% in the studied *F. hepatica* population and reached 95.1% in the studied *D. dendriticum* population. Ramadan *et al*. (2010) used the molecular characterization of *F. gigantica* and *F. hepatica* isolates collected from cows and sheep, using the random amplified polymorphic DNA fragments-polymerase chain reaction (RAPDs-PCR) technique. The results showed genetic variations (polymorphisms) of *F. gigantica* and *F. hepatica* with amplification fragment based on a 400-500 bp. Furthermore, Random amplified polymorphic DNA (RAPD) analysis is an approach for DNA fingerprinting and for designing specific primers as well as Sequence Characterized Amplifying Region (SCAR-marker) which no prior DNA sequence information is needed. This method has been used in numerous studies such as genetic variation of *O. viverrini* from northeast

Thailand and Laos PDR (Sithithaworn *et al*., 2007), and to identify human pathogenic heterophyid trematode metacercariae by PCR-RFLP (Thaenkham *et al*., 2011) and specific DNA probes/primers have been developed to detect *O.viverrini* in hamsters and human stool (Wongratanacheewin *et al*., 2001, 2002), and fish (Parvathi *et al*., 2008).

High-annealing temperature random amplified polymorphic DNA PCR (HAT-RAPD PCR) technique was reported by Anuntalabhochai *et al*. (2007). This techinique was increased the annealing temperature to 46-48°C results in greater polymorphism, higher reproducibility and higher resolution. HAT-RAPD PCR was used to identification of 3 paramphistome flukes fromThailand, genomic DNA was amplified by polymerase chain reaction based on the HAT-RAPD technique. Five random 10-mer oligonucleotide primers (OPA2, OPA4, OPB18, OPC9, and OPH11) produced distinct banding patterns in three species (Sripalwit *et al*., 2007). HAT-RAPD profile confirmed that pleurolophocercus cercariae found in *Melanoides tuberculata* from Mae Taeng District belonged to *H. taichui* and in *Tarebia granifera* from Mueang District were *S. falcatus* (Chuboon and Wongsawad, 2009), A HAT-RAPD marker, generated from the OPP-11 primer, was found to be *H. taichui*-specific, which was shown to have a positive result, only for *H. taichui* DNA. It revealed no cross-reaction with any of the other tested parasite species (Wongsawad *et al*., 2009), and PCR amplification with *H. taichui* specific primers showed that *H. taichui* specific amplicon 260 bp was generated in all FEST-positive specimens, and also in some FEST negative specimens (Wongsawad *et al*., 2009). Moreover, HAT-RAPD method was used to compare their DNA fingerprints of *Stellantchasmus falcatus* and several others related species. It was found that OPA-09, OPN-03, and OPAD-01 were able to generate *S. falcatus* specific fragments in mullets which consisted of 200, 760, and 280 bp, respectively (Wongsawad and Wongsawad, 2010), and also was performed to compare DNA profiles and analyze phylogenetic relationships among adult flukes and metacercariae in the Fang-Mae Ai Agricultural Basin, the pattern of DNA profiles of metacercariae were compared with those of adult stages which were correctly identified as to species (Wongsawad *et al*., 2013).

Many molecular approaches was used to the species identify of flukes, but the popularly approaches were used to species identify of flukes as ribosomal DNA (rDNA), which codes for structural components of ribosomes is particularly useful for genetic studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Dixon, 1991). The internal transcribed spacers (ITS1 and ITS2) occurred between the 18S, 5.8S, and 28S coding regions (Fig. 2.4), have proven useful for diagnostic purposes at the level of species (Morgan and Blair, 1995; Kostadinova *et al*., 2003; Scholz *et al*., 2004; Prasad *et al*., 2007). Sequences of these regions are often assumed to be homogenized within population of the same species by concerting evolution (Dover, 1982; Hillis and Davis, 1988). ITS region is widely popular for used to discrimination and phylogenetic study of many trematodes such as the PCR-RFLP of ITS2 was used to systematic of 7 taeniid cestodes; *Echinococcus granulosus*, *E. multilocularis*, *Taenia hydatigena*, *T. ovis*, *T. pisiformis*, *T. multiceps* and *T. serialis*. The present study indicates that PCR-RFLP produced characteristic patterns for each taeniid species examined (Gasser and Chilton, 1995). Subsequently, the complete internal transcribed spacer region of the ribosomal DNA (ITS 1+5.8S+ITS 2) was sequenced for 29 specimens of the digenean family Opecoelidae occurring in various marine host organisms. This work also revealed that 6 cercariae and 3 metacercariae were found to match their corresponding adult form (Jousson *et al*., 1999) and ITS2 region of the ribosomal gene complex (rDNA) of *S. haematobium* and *S. bovis* isolates from Kenyan was sequenced and found to be a 98% match. The *S. bovis* sequences were nearly identical (99%) to conspecific sequences from Niger; the *S. haematobium* sequences were nearly identical (99%) to conspecific sequences from Egypt, Mali, and Niger (Barber *et al*., 2000). In recently year, PCR-RFLP analysis of the 18S-ITS1-5.8S nuclear ribosomal DNA region was used to identification of human-associated liver fluke species (*Opisthorchis viverrini*, *O. felineus*, and *Clonorchis sinensis*). These results indicate that PCR-linked restriction analysis of the ITS1region allows for the rapid and reliable molecular identification among these opisthorchid taxa. (Kang *et al*., 2008) and the ITS1-region amplicon sizes successfully differentiated 4 species; *O. viverrini*, *Clonorchis sinensis*, *Haplorchis pumilio*, and *H. taichui*, while only *H. taichui* were significantly different from the other 3 species in the ITS2 region (Sato *et al*., 2009).

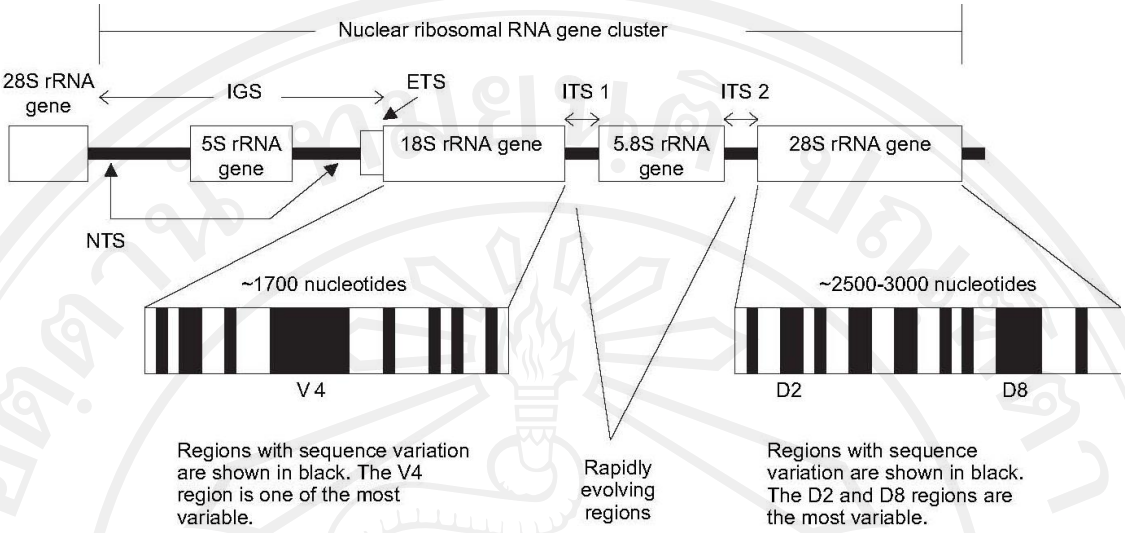

Figure 2.4 Diagram demonstrated the structure of ITS region in ribosomal gene complex (Mitchell and Zaccaro, 2006)

For, the PCR based methods targeting ITS regions have been developed for phylogenetic study of *F. gigantica* and closely related-species (*F. hepatica*) in several countries. For example, the development of restriction fragment length polymorphism of amplified DNA (PCR–RFLP) of the nuclear ribosomal internal transcribed spacer 1 (ITS1) region in *Fasciola* species. Amplicons with the sequences of both *Fasciola* species yielded fragments of 360, 170, 100, and 60 bp. The results of PCR–RFLP completely coincided with those of sequence analysis, and thus PCR–RFLP is a useful technique for determining the ITS1 type in *Fasciola* species (Ichikawa and Itagaki, 2010) and to detect *Fasciola gigantica* infection in snails (Velusamy *et al*., 2004) and *F. hepatica* in field-collected *L. columella* and *L. viatrix* snails (Cucher *et al*., 2006). Multiplex-PCR has been successfully developed for detection of *F. hepatica* in *L. columella* snail (Magalhães *et al*., 2008).

ITS2 sequences have demonstrated the presence in Vietnam of hybrid populations of liver flukes bearing genetic material from both *F. hepatica* and *F. gigantica* (Le *et al*., 2008). While, the phylogenetic trees constructed based upon the ITS (1 and 2)

sequences of the Indian liver fluke, *Fasciol*a revealed a close relationship with isolates of *F. gigantica* from China, Indonesia, Japan, Egypt, and Zambia, the isolate from China with significant bootstrap values being the closest (Prasad *et al*., 2008) and he first demonstration of *F. hepatica* in sheep in Turkey by the genetic approach using ITS-2 rDNA as genetic marker (Erensoy *et al*., 2009). Ribosomal ITS markers are promising tool for molecular discrimination of morphologically hardly distinguishable *F. hepatica*, *F. magna* and *P. cervi* eggs after coprological examinations (Bazsalovicsova *et al*., 2010) and the sequence analysis of genomic (ITS1 and ITS2) and mitochondrial (NDI and COI) gene markers was used to identification of *Fasciola* species isolated from Egypt. The results indicated that sheep were prone to *F. hepatica* (8 out of 10 animals) more than *F. gigantica* infection (Amer *et al*., 2011). However, ITS 2 sequences have been shown to be a sensitive marker at the species level of trematodes more than ITS 1 which it might be less conserved than those in the ITS 2 (Luton *et al*., 1992). For this reason, ITS 2 sequence was used to study the phylogeny of helminthes widely than ITS 1 sequence.

So, this study is aiming to investigate life history and to design species-specific molecular markers using SCAR-marker based on fragments generated by HAT-RAPD technique for the identification of larval stage of *F. gigantica* as miracidium (free living), sporocyst, redia, cercariae (in *L. auricularia rubiginosa*) and metacercaria (attached on plants). Furthermore, ITS2 sequences are also determined which usefulness to demonstrate the phylogenetic relationships among *F. gigantica* available in Genbank and the other related-trematodes.