# Molecular characterization of *Clinostomum* spp. infecting cultured Snakeskin gourami (*Trichopodus pectoralis*) in Thailand

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# การระบุชนิดพยาธิ *Clinostomum* spp. ที่ตรวจพบในฟาร์มปลาสลิด (*Trichopodus pectoralis*) ด้วยวิธีทางอณูชีววิทยาในประเทศไทย

นายเอสเค อินจามามุล อิสลาม



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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เอสเค อินจามามุล อิสลาม : การระบุชนิดพยาธิ *Clinostomum* spp. ที่ตรวจพบในฟาร์มปลาสลิด (*Trichopodus pectoralis*) ด้วยวิธีทางอณูชีววิทยาในประเทศไทย. ( Molecular characterization of *Clinostomum* spp. infecting cultured Snakeskin gourami (*Trichopodus pectoralis*) in Thailand) อ.ที่ปรึกษาหลัก : ปิยนันท์ ทวีถาวรสวัสดิ์, อ.ที่ปรึกษาร่วม : ชาญณรงค์ รอดคำ

พยาธิใบไม้ชนิด Clinostomum spp. เป็นปรสิตที่สำคัญในการนำโรคในปลาน้ำจืดทั่วไป ซึ่งมีพยาธินี้ความเฉพาะเจาะจงต่อโฮสต์ต่ำและพบว่ามีกระจายทางภูมิศาสตร์อย่างกว้างขวาง การวิเคราะห์ข้อมูลทางสัณฐานวิทยาและในระดับอณูชีวโมเลกุลของพยาธิ *Clinostomum* พบว่ามีความหลากหลายในพยาธิชนิดนี้ อย่างไรก็ตามการรายงานในระดับโมเลกุลของพยาธิ *Clinostomum* spp. ในการเพาะเลี้ยงสัตว์น้ำในประเทศไทยยังไม่ค่อยมีรายงานมากนัก ปลาสลิด ( *Trichopodus pectoralis* ) เป็นปลาน้ำจืดที่เพาะเลี้ยงในประเทศไทย และมีความสำคัญอย่างมากในทางเศรษฐกิจ สำหรับการศึกษาครั้งนี้ มุ่งเน้นไปที่การอธิบายการพบพยาธิใบไม้ *Clinostomum* ในช่องท้องของปลาสลิด และรายงานความชุกของปรสิตชนิดนี้จากเขตภาคกลางของประเทศไทย ปลาสลิดในช่วงอายุ 4 ถึง 8 เดือน จำนวน 260 ตัว ได้มาจากจังหวัด สมุทรสาคร สมุทรสงคราม สมุทรปราการ และกาญจนบุรีในภาคกลางของประเทศไทย ทำการวัดความยาวโดยรวมและ น้ำหนักตัวของปลาสลิด ตรวจดูภายในตัวปลาสลิด และพบระยะเมตร้าเซอร์คาร์เรียของพยาธิใบไม้ชนิด *C. piscidium* ภายในในช่องท้องของปลาสลิดที่ติดเชื้อ พยาธิถูกตรวจพบไม่ว่าจะอยู่เป็นอิสระหรือที่เกาะติดกับเนื้อเยื่อไขมันและ เยื่อหุ้มชั้นนอกของอวัยวะภายใน พบว่ามีความชุกรวมอยู่ที่ 13.35 % และพบสัดส่วนของการพบพยาธิใบไม้ชนิดนี้ ในปลาสลิดตัวเมียที่นำมาศึกษาจากฟาร์มสูงกว่าในปลาสลิดตัวผู้อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) และเมื่อ เปรียบเทียบกับปลาที่ไม่เป็นโรค ปลาที่ติดเชื้อจะมีน้ำหนักตัวน้อยกว่าปลาปกติมาก ทำให้เห็นลักษณะปลาที่ติดพยาธิผอมกว่าอย่างเห็นได้ชัด ($P<0.05$) การตรวจทางจุลพยาธิวิทยาพบรูปแบบการร่องรอยของการเคลื่อนที่สีขาว บนเซลล์ตับและม้าม และเมื่อตรวจสอบร่องรอยของการเคลื่อนที่ในเนื้อเยื่อส่วนของเนื้อตับเป็นลักษณะเป็นเนื้อตาย เกิดขึ้นที่บริเวณ hepatocentralell และมีลักษณะของเลือดออกที่ล้อมรอบด้วยชั้นของมาโครฟาจและเซลล์เยื่อบุผิว ร่วมด้วย การติดเมตาเซอร์คาเรียนี้ทำให้เนื้อเยื่อตับของโฮสต์เสียหาย ส่งผลให้เมแทบอลิซึมของตับถูกทำลาย เป็นผลให้ปลามีอัตราการเจริญเติบโตที่ช้าลงและน้ำหนักตัวลดลงนอกจากนี้การจำแนกลักษณะทางสัณฐานวิทยาและทางอณูชีวโมเลกุลของพยาธินี้ได้ดำเนินการโดยใช้ข้อมูลในส่วนของ 18S rDNA และ internal-transcribed spacer (ITS1 และ ITS2) โดยนำผลที่ได้มาเทียบเคียงในส่วนของ 18S rDNA ในโปรแกรม BLAST แสดงให้เห็นว่าพยาธิชนิดนี้ มีความคล้ายคลึงกันกับ *C. piscidium* (FJ970655) 100% ที่พบในปลาสลิด (*Colisa fasciata*) จากอินเดีย และมีความคล้ายคลึงกันกับพยาธิกลุ่ม *Clinostomum* อื่นๆ ในประเทศออสเตรเลีย สหรัฐอเมริกา จีน อิสราเอล และอิตาลี ที่ 90–98% เป็นต้น นอกจากนี้ข้อมูลลำดับนิวคลีโอไทด์ของ ITSยังเผยให้เห็นความคล้ายคลึงกันของลำดับ 100% กับ *C. pisicidium* ที่แยกได้จากปลาสลิดจากอินเดียเช่นเดียวกัน และจากการวิเคราะห์แผนภูมิวิวัฒนาการใน การศึกษานี้จึงเป็นการรายงานการจำแนกในระดับอณูชีวโมเลกุลของพยาธิใบไม้ชนิด *C. piscidium* เป็นครั้งแรก ที่มีการเพาะเลี้ยงปลาสลิดในประเทศไทย



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# # 6378506331 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY
KEYWORD: 18S rDNA, Clinostomum piscidium, Histopathology, ITS, Trichopodus pectoralis

Sk Injamamul Islam : Molecular characterization of *Clinostomum* spp. infecting cultured Snakeskin gourami (*Trichopodus pectoralis*) in Thailand. Advisor: Assoc. Prof. Dr. PIYANAN TAWEETHAVONSAWAT, D.V.M, Ph.D., D.T.B.V.P Co-advisor: Assoc. Prof. Dr. CHANNARONG RODKHUM, D.V.M, Ph.D., D.T.B.V.P.

The digeanean *Clinostomum* spp. is a significant parasitic pathogen of freshwater fish. It has low host specificity and a wide distribution geographically. Morphological and molecular data analyses have been performed on *Clinostomum* to assess its diversity. However, the molecular occurrence of *Clinostomum* spp. in aquaculture in Thailand has yet to be reported. Snakeskin gourami (*Trichopodus pectoralis*), a freshwater fish cultured in Thailand, has significant economic importance. Herein, the study focuses on describing a digenean *Clinostomum* in the abdominal cavity of *T. pectoralis* and reports the prevalence of this parasite from central Thailand. Two hundred and sixty-two 4 to 8- month-old *T. pectoralis* were obtained from Samut Sakorn, Samut Songkhram, Samut Prakarn, and Kanchanaburi provinces of central Thailand. We measured their overall length as well as their body weight. The body cavity of *T. pectoralis* was examined, and metacercariae of *C. piscidium* were discovered inside. In the abdominal cavity of infected fish, the parasites were discovered either free or adhered to adipose tissue and the outer membrane of the visceral organs. It was found that the total prevalence was 13.35 %, and the parasite intensities were found to be higher in the females taken from the farms than in the males ($P<0.05$). Pathological examination showed a few white migratory patterns on liver and spleen cells. The track presented histologically as the main central hepatic necrosis and hemorrhage surrounded by layers of macrophages and epithelioid cells. This metacercaria infection caused damage to the fish hosts' hepatic tissue, which disrupted their hepatic metabolism. As a result, the fish hosts experienced a slowdown in their rate of development and a reduction in their total body mass. In addition, morphological and molecular characterization of species was conducted using 18S rDNA and inter-transcribed spacer (ITS1 and ITS2) sequence data. A BLAST search of 18S rDNA sequence revealed 100% sequence similarity with *C. piscidium* (FJ970655) infecting banded gourami (*Colisa fasciata*) from India and 90–98% sequence similarity with other clinostomid in Australia, USA, China, Israel, and Italy. ITS sequence data also revealed 100% sequence homology with the *C. pisicidium* isolated from *C. fasciata* from India. Based on phylogenetic analysis, this study reports the first molecular identification of *C. piscidium* species in cultured *T. pectoralis* in Thailand.



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| | |
|---|---|
| AFA | Alcohol Formalin Acetic Acid |
| ANOVA | Analysis of variance |
| BLAST | Basic Local Alignment Search Tool |
| CI | Confidence Level |
| DoF | Department of Fisheries |
| DNA | Deoxyribonucleic Acid |
| FAO | Food Aid Organization |
| ITS | Internal Transcribed Spacer |
| MCL | Maximum Composite Likelihood |
| MEGA | Molecular Evolutionary Genetics Analysis |
| PCR | Polymerase Chain Reaction |
| PH | Potential for Hydrogen |
| SEM | Scanning Electron Microscope |
| SE | Standard Error |

# Chapter 1: Introduction

## 1.1. Importance and rationale

The snakeskin gourami (*Trichopodus pectoralis*, Regan 1910; Anabantoidei: Osphronemidae) is among the most widespread species of air-breathing fish from freshwater in the Indo-China Peninsula (Eegan, 2009; Morioka et al., 2013). It is native to the river systems of Cambodia, Indonesia, Laos, Vietnam, and Thailand. According to the observations of a researcher, this fish is an excellent species for aquaculture (Phong, 2014). This fish produces a lot of meat and is a popular dried fish in Southeast Asian countries. It is among the most important freshwater fish species because of its excellent flavor, taste and high export demand (Ahmadi, 2021). Most snakeskin gourami production in Thailand previously came from large, converted rice fields, with the greatest concentration in central Thailand. The Thai Department of Fisheries (DoF) extended *Trichopodus* sp. culture as a type of rice-fish farming in the 1950s. The capacity to increase production quickly became evident. The farmers extended the growing season of the fish, resulting in the modern aquaculture production of the species in Central Thailand (Yoonpundh & Little, 1997). The provinces of Samutprakarn, Suphanburi, Ubon Ratchathani, and Samutsakorn, the primary sources of supplies to the market, are abundant with snakeskin gourami fish farms. It is a highly economic species and has become one of the five most important freshwater species in the aquaculture of Thailand (Table 1) (DoF Thailand, 2018; FAO, 2019). The production of snakeskin gourami in Thailand ranked fourth among freshwater resources in 2016 (with a total of 26,077 metric tons) and accounted for 5.7% of the total annual production of freshwater in the nation (FAO, 2019). However, in the last decade, production has been on the decrease. The Mekong River basin and Chao Phraya basins of Thailand are home to this species (Kottelat et al., 1993) and later recorded in the Mekong and Southeast Thailand river systems.

Table 1 Lists of economically important fish species of Thailand (DoF, Thailand, 2018).

| **Freshwater fish species** | **Production (%)** |
|---|---|
| **Nile Tilapia** | 45.8 |
| **Catfish** | 29.8 |
| **Silver barb** | 11.2 |
| **Snakeskin gourami** | 5.7 |
| **Striped Catfish** | 4.6 |

One of the most significant barriers to snakeskin gourami cultivation is the increased occurrence of parasitic infections (Kanchan *et al.,* 2020). Parasite-infected fish developed ascites, with exudate fluids in the body cavity (Kanchan *et al*., 2020). Infection by *Clinostomum* spp. had previously been identified as the most serious problem with snakeskin gourami in aquaculture, leading to weight loss and decreased development of the fish (Paperna, 1991; Tansatit *et al*., 2014). The parasites can be found in the abdominal cavity of infected fish, either free or adhered to adipose tissue and the surface of visceral organs. It is well known that encysted and non-encysted forms of metacercariae of clinostomids may be found inside the host's tissue. These two forms of metacercariae can be distinguished by the presence or absence of an encysted form (Tansatit et al., 2014). As the non-cysted metacercaria of the species may migrate freely across the internal organs of their host, they have the potential to damage infected fish to a greater extent (Echi et al., 2012). Fish in the aquaculture environment may have growth retardation as a result of a severe *Clinostomum* spp. infestation. Thus, the objective of the present study was to investigate *Clinostomum* spp. by molecular techniques following statistical analysis to know the level of *Clinostomum* spp. prevalence in farmed snakeskin gourami in Thailand.

## 1.2 Statement of the problem

Fishes serve as hosts for parasites in the vast majority of aquatic habitats; sometimes, the biology and health of fish may be negatively impacted by these parasites (Lafferty, 2008). Fish serve as intermediate hosts for a variety of parasites, which may result in some of the most spectacular instances. One of the challenges that the fishing community is now experiencing in the aquatic population is the prevalence of parasites and diseases among fish. In the event that parasitic diseases are not kept under control, they may lead to widespread death tolls, decrease fish productivity, and, in certain instances, serve as a vector for the transmission of infections to people and other animals that consume diseased fish. An increase in fish parasites may be because of many events, including heavy stocking density, environmental changes, and external animals, such as birds, who are the definitive host for *Clinostomum* spp. *Clinostomum* spp. has the potential to transmit zoonotic diseases to humans as well as function as a source of secondary infections in fish species. Previously, there has been no research carried out in Thailand to determine the genetic identity of the *Clinostomum* spp. species found in snakeskin gourami. This study therefore investigated the molecular identification of *Clinostomum* parasites in snakeskin gourami culturing central part of Thailand, and by analyzing the level of prevalence of the parasite infestation, this research emphasis the information of overall scenario of parasites burden in the farmed snakeskin gourami.



## 1.3 Justification of the study

Since almost every population in the world consumes fish as a major source of protein, research is necessary to guarantee the production of healthy fish (FAO, 2009). It is imperative that research be conducted on fish parasites since it has been shown that parasites decrease fish productivity, negatively impact fish health, make fish more susceptible to other diseases, and even lead to the death of fish. Fish parasites cause a decrease in economic return and a reduction in the quantity of fish that may be consumed as sources of protein-rich foods (Barber, 2005). In order to use a strategy that is either successful in management or prevention, proper identification of parasites down to the level of the genus and species is required. This

is because the etiology of parasitic diseases, which in turn determines the choice of potential control. Furthermore, the prevalence and incidence of disease are two of the most basic indicators in epidemiology. Prevalence quantifies the prevalence of a disease in a population in a certain area and period by counting the number of affected individuals (Ward, 2013). Thai aquaculture relies significantly on the snakeskin gourami, thus the study of parasites is crucial to the aquaculture sector's sustainable future. Previously, *Clinostomum* spp. have been reported to cause both farmed Snakeskin gourami and human in Thailand (Tansatit et al., 2014; Tiewchaloern et al., 1999; Yooyen et al., 2006). Despite the relevance of farmed snakeskin gourami, the molecular identification of *Clinostomum* spp., as well as parasite prevalence studies, had not been addressed, prompting the present study.

**1.4 Objectives of the study**

- To identify and characterize *Clinostomum* spp. of snakeskin gourami cultivated in Thailand using morphological and molecular characteristics.
- To investigate the prevalence, intensity, and pathological change in snakeskin gourami because of *Clinostomum* spp. infection since this parasite critically affects production.

**1.5 Research Questions**

To address our hypothesis, the question needs to be answered.

- Which species of Clinostomum spp. and what level of prevalence can be found on snakeskin gourami farms in central Thailand?
- What is the relationship between fish size and sexes with parasite’s burden, and what will be the pathological changes that may affect the fish species?

**1.6 Significance of the study**

The findings of this study will improve our ability to identify parasites, monitor their behavior, link their expansion to environmental conditions, and comprehend their contribution to disease etiology. It will also help the fisheries community, government agencies, and other interested individuals comprehend how prevalent *Clinostomum* parasites are in snakeskin gourami aquaculture. The study addresses an important element of food safety and aquaculture management since snakeskin

gourami is an important source of nutrition for Thais. Knowing about the interactions of humans, animals, and aquatic species with their surroundings and spreading diseases between them is essential for effective molecular evidence-based parasite identification, which is necessary for protecting animal health. As a result, this will help to control parasites and boost aquaculture production.

# Chapter 2: Literature Review

## 2.1 Fish

"Fish" refers to an animal with a backbone, gills that grow throughout life, and fin-shaped limbs. Fish are a significant source of nourishment for people all around the globe. Various people in many nations rely on fishing for a livelihood (FAO, 2009). Most fish species prefer to inhabit saltwater, although freshwater is home to roughly 40% of all fish species. Fish may get a wide range of diseases and be infected with parasites (Yimer and Enyew, 2003).

### 2.1.2 Snakeskin gourami

The snakeskin gourami (*Trichopodus pectoralis*) has a high meat output and is one of Thailand's top five freshwater-farmed fishes (Panthum et al., 2021). They can live in environments with very little dissolved oxygen and high organic loads and are often found in densely vegetated rice paddies, ditches, and streams (Froese, 2006). The snakeskin gourami consumes zooplankton, crustaceans, and the larvae of insects as its primary food sources (Tacon et al., 2011). It is also one of the most well-known and beautiful kinds of fish that can be kept in aquariums, and it is a fascinating species that can be farmed for commercial purposes (Minh et al., 2019). Snakeskin gourami produces a lot of meat, and traditional dried snakeskin gourami is a popular food (Figures 1 and 2) (Froese, 2006). Dried snakeskin gourami is popular in Southeast Asia, and new advancements have resulted in higher prices for snakeskin gourami in the aquaculture market (Needham et al. 2015).



Figure 1 Picture of *Trichopodus pectoralis* (snakeskin gourami). (Source: Internet, Accessed on 22 March 2023)

Figure 2 Sun-dried snakeskin gourami fish. (Source: Internet, Accessed on 22 March, 2023)

People in Southeast Asian nations are familiar with this species under several distinct names (Berra, 2011), i.e., sepat siam in Malaysia (Ambak et al., 2010), pla salid in Thailand, *cà sac ran* in Vietnam, trey kawnthor in Myanmar, and pa salid in Laos (Boonsom, 1986). It is native to Thailand's central plain, which runs from the Chao Phraya Basin to Cambodia, Vietnam, and Laos (Boonsom, 1986); however, owing to several initial introductions, it is currently widespread across Southeast Asian nations. Numerous research has shown that it is endemic to the Chao Phraya and Mekong basins of Laos, Thailand, Cambodia, and Vietnam (Kottelat, 2001). and that it has been widely introduced to Malaysia (Boonsom, 1986), India, Bangladesh, Sri Lanka, Indonesia, and the Philippines (Boonsom, 1986). This fish is both a food source and an attractive fish. Nonetheless, because of habitat loss and degradation, notably in Thailand, its natural population has declined, although more aquaculture of this species has been developed (Vidthayanon, 2012).



**2.1.3 Physical characteristics of snakeskin gourami**

There are multiple species in the genus *Trichopodus*, but the snakeskin gourami is the most often traded species (Prianto et al., 2021). Before they combined, the snakeskin gourami was supposed to be the largest member of the Osphronemidae and Belontidae families. It is still the largest species in its genus and subfamily (Frose,

2006). When sexually mature, *Trichopodus* species are substantially bigger and have a shorter dorsal fin base. The snakeskin gourami (*T. pectoralis*) is the largest, reaching lengths of over 20 cm (Pinter, 1986). The snakeskin gourami is an elongated fish that is compressed and has a small dorsal fin.

Additionally, the fish has a tight body. It has a pelvic fin that is very long and thread-like, in addition to an anal fin that is almost as long as the body itself. The back is more of an olive tint, while the flanks are more of a greenish gray with a silvery shine to them. It is possible to make out a clear and curved black band going from the snout to the eye and then all the caudal peduncle (Frose, 2004). In addition, the fins have a color that may be described as grayish-green, and the iris of the eye can sometimes take on an amber hue when the conditions in the water are just right. In addition to being slenderer than its female counterpart, the male snakeskin gourami also has dorsal and anal fins that are longer and pointier than those of its female partner.

In contrast, the female has a considerably less vibrant coloration than the male. In specific individuals, an orange-red fringe will also grow around the outside edge of the male's pelvic fin. In juvenile snakeskin gouramis, the lines that extend from the eye to the base of the tail have a prominent zigzag pattern that is very spectacular. This pattern can be seen throughout the body.

**2.2 Parasites in snakeskin gourami**



Parasitic diseases of fish have consequences not only for human health but also for the economy, and these repercussions are felt equally in devloped and developing nations (Poulin, 2006). Each and every parasite consumes energy that would otherwise be available for the maintenance, expansion, establishment, and reproduction of its host, which may result in a variety of negative effects for the host (Barber, 2005) and affects the host's production (Marcogliese, 2005). In addition to inflicting harm to their hosts, parasites may occasionally cause large mortalities, which can result in considerable financial losses for commercial fisheries and aquaculture operations (Roberts, 1986). Parasites have the potential to behave as serious infections, either directly leading to the death of the fish or making it more susceptible to other pathogens. Castration or mechanical interference with spawning,

weight loss, and gross bodily deformation are some of the effects of parasites in fish. Muscle degeneration, liver dysfunction, interference with nutrition, cardiac disruption, nervous system impairment, and nutritional disruption are all caused by parasites. Other severe pathological conditions include visceral inflammation and atrophy brought on by the parasites' pressing of the organs, which often also results in an accumulation of ascetic fluid tinged with blood (Poulin, 2006). In natural water bodies, parasitic infection is widespread and inhibits fish growth, development, and reproduction. A continuous reduction in fishing resources is brought on by these diseases in addition to other causes. Additionally, parasites are held responsible for the severe pathological problems in the afflicted fish that devalue them economically and nutritionally.

**2.2.1 External parasites in snakeskin gourami**

Among the six genera of the family Trichodinidae, *Trichodina* represents over 30 species and causes trichodiniasis (Valladão et al., 2016). *Trichodina* is saucer-shaped and uses its cilia to travel around the surface of fish skin, fins, and gills (Wellborn, 1967). It uses tooth-like structures called denticles to feed on detritus and other particles present on the surface of the fish. The parasite scrapes the debris from the surface of the fish into its mouth with these denticles. When these organisms are plentiful, their scraping and movement irritate the skin and gill surfaces, causing epithelial hyperplasia (Bruno et al., 2006). In severe cases of hyperplasia, the fish host may experience decreased gas exchange or osmoregulation. More severe infestations may arise when environmental circumstances are unfavorable or when fish tissues are manually injured. *Trichodina* is transmitted to fish by direct contact with them or by water organisms from a subclinically infected reservoir host. Binary fission is a method of reproduction in which daughter organisms either adhere to the original host or seek a new host in the water column (Bruno et al., 2006). Compared to other external protozoans that infest fish, *Trichodina* protozoa are relatively mild pathogens. When parasite counts are modest, and the fish are not stressed, the prognosis for parasitized fish is favorable (MacMillan, 1991).

On the other hand, some of these protozoa are dangerous diseases that cause substantial fish mortality, particularly in hatchery-cultured species. This family of

parasitic protozoa can be found parasitizing freshwater and marine fish species worldwide. These parasites are more common in young fish (yearlings and younger). Trichodinosis parasitic infestations in *Trichopodus pectoralis* were first discovered in the Philippines years ago (Arthur and Lumanlan-Mayo, 1997). Researchers found infestations occur most commonly in the gills and skin of the fish species. After that, no records of *Trichodina* spp. have been reported on snakeskin fish species.

Another parasitic disease previously identified in snakeskin gourami is ichthyobodiasis or costiasis (Bassleer, 1997). However, the researcher provided little evidence regarding costiasis infection in snakeskin gourami and the presence of the parasite genus Ichthyobodo in farmed or aquarium conditions. Moreover, in 2006 Froese mentioned costiasis in *T. pectoralis* (Froese, 2006). These parasites are quite minute (5-10 um) and have free-swimming and attached stages, making them easy to miss during a physical examination. It is an obligatory parasite on fish's skin and/or gills. Ichthyobodo, which resides on the gills of juvenile fish, impairs their ability to adapt to their environment. Furthermore, this organism is transmitted from fish to fish horizontally. In the environment, subclinically parasitized fish act as reservoirs for parasites.

A novel Myxozoa parasite, *Henneguya* spp., was simultaneously found with a chlamydia-like organism in snakeskin gourami (*T. pectoralis)* in Thailand (Dinh et al., 2021). In the gill sections, typical myxospores of the species *Henneguya* with tails were found. Infection with *Henneguya* spp. caused significant gill filament damage, which most likely resulted in respiratory distress. *Henneguya* is one of the most widespread myxosporean genera in the family Myxobolidae, with more than 200 species (Eiras and Adriano, 2012). Several *Henneguya* taxa are known to be responsible for diseases that cause significant death rates, while most species are thought to have little or no harmful impact on fish health (Kent et al., 2001; Lom and Dyková, 2006). *Henneguya* spp. infection is characterized by cyst-like formations on the gill filaments and usually occurs in the gills (Kent et al., 2001; Molnár, 2002). Two equal polar caps, sporoplasm at the posterior pole, and two independent caudal processes distinguish *Henneguya* spp. from the other species of the Myxobolidae family (Eiras, 2002; Lom and Arthur, 1989; Molnár, 2002). The genus

*Henneguya* interacts with fish gill structures in various ways, resulting in differing degrees of damage (Dykova and Lom, 1978; Molnár and Eszterbauer, 2015). A farmer observed symptoms similar to those previously reported in fish infected with *Henneguya* species in the snakeskin gourami, including lethargy, gasping for oxygen, and loss of appetite (Dinh et al., 2021).

**2.2.2 Internal parasites in snakeskin gourami**

*Gnathostoma spinigerum* is a nematode in dogs and cat's native to Southeast Asia, Japan, China, Bangladesh, and India. Still, it has also been found in Mexico and parts of South America. Poelen et al., 2014 on the other hand, identified *G. spinigerum* as a pathogen of *T. pectoralis* (Poelen et al., 2014). *G. spinigerum* has a multi-host in the life cycle. The eggs hatch in freshwater, and water fleas of the genus Cyclops eat the larvae. Small fish eat the water fleas, the second intermediate host. Eventually, the larvae end up in the stomachs of carnivores, usually cats and dogs. Humans are accidental hosts; the only *G. spinigerum* forms discovered in humans are larvae or immature adults that never achieve reproductive maturity. Ingestion of intermediate hosts containing parasite larvae, such as raw or undercooked freshwater fish, chickens, snails, or frogs, causes infection. It has also been described that those larval forms penetrate the skin, and prenatal transmission occurs. Non-specific symptoms such as general malaise, fever, urticaria, anorexia, nausea, vomiting, diarrhea, and epigastric discomfort emerge 24-48 hours after eating larvae of *G. spinigerum* in humans. However, no symptoms in fish have yet been documented.

**2.2.3 *Clinostomum* spp.**

*Clinostomum* trematodes are worldwide parasites that infect fish, amphibians, reptiles, and snails as intermediate hosts. It has three hosts and various phases in its life cycle (McAllister et al., 2009). Despite the widespread geographic distribution of this genus, the question of how many species it contains and how their host preferences differ from one another has not been settled (Calhoun et al., 2019). Adult flukes are found in the digestive system, esophagus, throat, and/or mouth of fish-eating birds, most often Charadriiformes and Ciconiiformes (Kanev et al., 2002), or less commonly, within reptiles, mammals, or even humans (Hara et al., 2014). To date, various species of *Clinostomum* have been reported from over 40 species of

birds from six continents, from great blue herons (*Ardea herodias*) to African darters (*Anhinga rufa*). Eggs are laid by definitive hosts in an aquatic environment, where they hatch into miracidia and mature into sporocysts after being ingested by rams horn snails (usually of the genus *Helisoma*) (Smyth, 1980). There have been reports of individuals belonging to other snail genera and families (such as *Lymnaea*, *Radix*, *Bulinus*, and Biomphalaria) serving as hosts, but on a far less frequent basis (Pinto et al., 2014; Rosser et al., 2016). Sporocysts are responsible for the release of free-swimming cercariae, which are capable of infecting a wide variety of freshwater fish and amphibian species (McAllister et al., 2009), within which they form loosely encysted metacercariae (Kanev et al., 2002). Once an amphibian or fish infected with the parasite is consumed by an appropriate definitive host, the metacercariae may survive in the second intermediate host for up to four years before maturing into adults (Bruni and Angelini, 2016).

There have been reports of clinostome metacercaria in freshwater fish all over the globe. Clinostome metacercaria are large and visible to the unaided eye. Several genera and species of fish use the excysted progenetic larval metacercaria of *Clinostomum piscidium* as an infecting stage. Additionally, the digenetic trematode of the genus *Clinostomum* is found in a variety of freshwater fish species and spreads zoonotic infections. Consuming raw fish allows *Clinostomum* sp., an accidental host, to enter people. In Japan, at least 19 of these incidents have been documented (Hara et al., 2014). Human infection with clinostome has also been reported in Israel, India, and Korea (Chung et al., 1995; Yamashita, 1938). In Thailand and Korea, cases of pharyngitis and lacramalitis attributed to *Clinostomum* sp. have been documented (Chauhan et al., 2021). An ophthalmological examination of a man with discomfort in the frontal sinus region for two to three months found a white spot at the right lower inner eyelid, moderate inflammation, and conjunctivitis in one case report from Thailand that demonstrated how this worm affected the eye. The patient fully recovered after the worm was removed from the lacrimal opening (Tiewchaloern et al., 1999). In addition, metacercariae have also been reported in the stools of humans and cause serious digestion problems, respiratory disorders upon entry into the lungs, and many other severe consequences (Park et al., 2009; Zimik et al., 2019).

In Thailand, *C. piscidium* metacercariae were discovered in the body cavity of *T. pectoralis* (see(Tansatit et al., 2014)). The parasites were identified in the abdominal cavity, either free or adhered to adipose tissue and the exterior surface of the viscera of affected fish. The infected fish seemed starving and had a much lower body weight than the uninfected fish. On the hepatic surface, gross pathology results indicated a few white migratory trails. The track exhibited histologically core necrosis and hemorrhage of hepatic and pancreatic cells surrounded by a layer of macrophages and epithelioid cells, encircled by a rim of lymphocytes, eosinophilic granular cells, and fibroblasts (Tansatit et al., 2014). *Clinostomum piscidium* is one of the most severe issues in pond fish culture, causing weight loss, reduced growth, and loss of productivity (Paperna, 1991). The fish become susceptible to additional health problems because of parasitism, which can lead to death. *Clinostomum* sp. requires a molluscan host, a fish (as the second intermediate host), and a definitive host, which is often a piscivorous bird, in order to complete its life cycle (Dabrowski, 2012). The first intermediate host of this parasite, *Lymnaea lutiola*, is a snail, and the second intermediate host, *Ophiscephalus punctatus*, is a serpent-head fish, where the metacercariae move actively inside its body cavity. Fish-eating birds like the *Bulbulcus ibis* and *Egretta* sp. are the ultimate hosts (Tansatit et al., 2014).



Figure 3 Picture of *Clinostomum* spp. (Source: Internet, Accessed on 2 April, 2023)

Clinostomids are one of the digenean that are found the most often. During their life cycle (Figure 4), gastropods serve as the first invertebrate intermediate host, while various fish species are second intermediate hosts (Laimgruber et al., 2005). When the trematode eggs hatch, free-swimming miracidia are released; these miracidia

aggressively infect snails and then proliferate inside a sac-like sporocyst to create many rediae. These stages mature into cercariae released from the snails and either actively infect new definitive hosts or form encysted metacercariae (Dias et al., 2003). It may be found encysted in fish muscle with a thin cyst wall, or it can move freely throughout the fish body cavity with no cyst wall (Waikagul and Thaenkham, 2014). Due to this thin cyst wall, it is recommended not to use pepsin-HCl artificial digestion techniques; thus, it may damage the metacercariae of *Clinostomum* spp. (Sohn, 2009). Metacercariae are readily released when the cyst wall is extremely thin and just a little amount of pressure is applied to the coverslip with muscle compression (Sohn, 2009). Advantages of the compression method include knowing metacercariae's exact location and infection site in fish examined and economical.

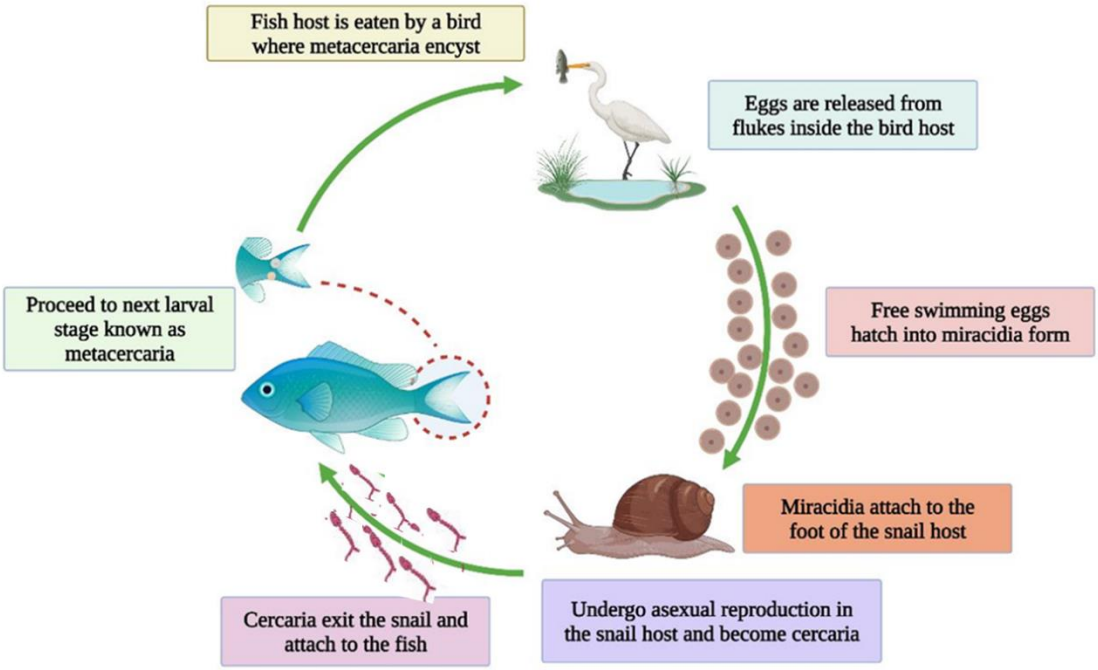


Figure 4 Life cycle of *Clinostomum* spp.

**2.3 Diagnosis of fish parasites**

The classification of fish parasites often uses a wide variety of diagnostic approaches. Among them are molecular diagnosis, histological diagnosis, and microscopic diagnosis is most common (Eissa et al., 2017).

### 2.3.1 Microscopic diagnosis

It is essential to promptly diagnose illnesses that affect fish to stop the further spread of the disease and maintain the overall health of the fish population. Microscopy is a crucial instrument for determining the nature of fish illnesses. The equipment's quality, the expert's experience, and the speed with which the procedure may be carried out all impact the accuracy of the results (Bruno et al., 2006). The light microscope is the most commonly used in illness diagnosis. Scrapings from the fish may be prepared for the examinations. Analyses of stained microorganisms and slides of histology specimens may also be performed using light microscopy.

### 2.3.2 Histological diagnosis

The symptoms of infected fish are not readily evident based on the fish's external appearance or their behavior in their natural environment. The first step of the test consists of a visual inspection of the fish's outsides for any signs of sickness (Khan, 2009). To receive nourishment, pathogens decompose the body's tissues or absorb food already digested from the intestines. The hyperplasia that occurs in the gills takes place between the gill lamellae. These parasites consume the freshly generated cells and cause harm to the gills. In histopathology, tissue sections are often examined to screen for damaged areas. However, the tissue is fixed for subsequent tests. Investigations of hematology have been gaining an ever-increasing amount of relevance in the field of applied fish pathology. Examining smears is the foundation of any hematological analysis; nevertheless, estimates of hemoglobin and total protein, serum electrophoresis, and several other biochemical measurements are also performed. Fish pathogens may also be identified using immunohistochemistry techniques applied to sections of tissue (Duraiyan, 2012).

### 2.3.3 Molecular diagnosis

Parasites in otherwise asymptomatic fish have been detected using molecular diagnostic methods in an effort to stave off epidemics. Two "primers" designate the site at which a thermostable DNA polymerase will begin replicating a DNA sequence, resulting in an amplification of a specified portion of the genome. Detection and quantification of *Clinostomum* spp. an internal parasite affecting fish worldwide has

utilized PCR (Aohagi et al., 1992). However, molecular analysis of *Clinostomum* spp. In Thailand is lacking.

# Chapter 3: Materials and Methods

## 3.1 Ethical approvement

The protocols for this study were designed in accordance with Chulalongkorn University's animal ethical standards, protocol numbers IBC 223103 and IACUC 2231043.

## 3.2 Study area

From February to October 2022, a total of 4- to 8-month-old 262 live specimens of *T. pectoralis* were collected from aquaculture farms located in Samut Prakan, Samut Songkhram, Samut Sakhon, and Kanchanaburi province in Thailand (Figure 5). These provinces are the primary sources of supplies to the market and are abundant with snakeskin gourami fish farms. These areas are most productive with snakeskin fish farming, and previous cases of *Clinostomum* spp. have been reported previously.

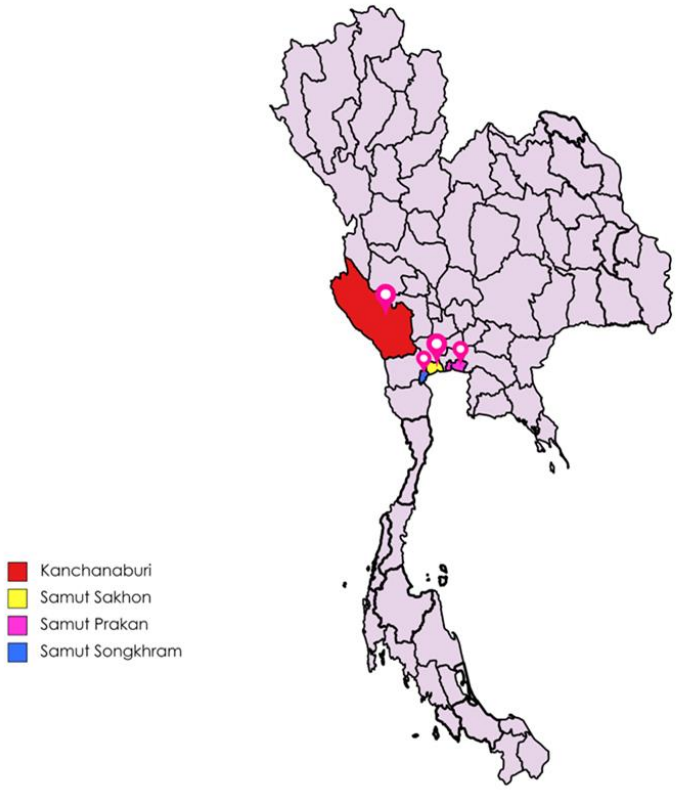


Figure 5 Sample collection areas located in different provinces of Thailand.

### 3.3 Sample size estimation

In this present study, we used Epitools (https://epitools.ausvet.com.au) online server for calculating the sample size for the prevalence study. These tools may be used to estimate population means and proportions, discover significant differences between two means or proportions, and calculate sample sizes needed to do so, as well as to determine the real prevalence of an animal at the herd level (Reiczigel et al., 2010).

### 3.4 Collection of parasites

The fish were transported in aerated polyethylene to the Parasitology Unit of the Faculty of Veterinary Science at Chulalongkorn University, Thailand, where they were kept in aquariums with the appropriate aeration. Tricaine methane sulphonate (250 mg/l) was used for euthanizing the fish to die. The fish were examined for parasites using an established methodology (Fernando, 1972). First, each fish will be measured in terms of overall length and weight. The external characteristics will determine the sexes of the fish. Necropsy was done, all internal organs were present, and the exposed body cavity was visually inspected for parasites. After counting the parasites, specific morphological and molecular examination samples were processed.

### 3.5 Prevalence study of the parasites

Analysis of parasite prevalence is not simple. Parasites are generally not distributed, either in the fish or in the population. Negative binomials are often found to describe the distribution of parasites, so non-parametric statistics are essential. Further, fish populations are not randomly distributed, so the fish sample may not represent the population. In the present study, the prevalence was determined as a percentage based on the ratio of the number of infected fish to the total number of fish investigated. By dividing the total number of parasites collected by the total number of infected fish, the mean intensity of parasite infection was calculated.

### 3.6 Muscle compression techniques for metacercariae collection

The flesh from the muscles and fins of the representative fish samples was collected to check the presence of the metacercariae of *Clinostomum* spp. After that, each

sample was compressed between two glass slides. This step was repeated 2-3 times to increase the detection rate of the metacercariae. Afterward, each sample was placed under a stereomicroscope to observe and identify the metacercariae.

**3.7 Morphological identification**

**3.7.1 Semichon's carmine staining**

Necropsy was done, all internal organs were present, and the exposed body cavity was visually inspected for parasites. Their visceral organs were dissected and placed on a petri plate, where they were rinsed with 0.85 % NaCl (NSS) and checked for parasites. After counting the parasites, specific samples were processed for light microscopic examination. The metacercariae flattened between two glass slides for carmine compression. At first, the parasite specimens were fixed in alcohol-formalin acetic acid (AFA) overnight before being rinsed in distilled water. After that, they were stained with Semichon's carmine for 24 hours or until overstain, and the specimens were destained in a mixture of 70% ethanol and 1% HCL solution 2-3 times. The samples were then dehydrated in graded series of ethanol (50%, 70%, 80%, 90%, and 95%) for 30 mins and after that with 100% for 30 mins two times. Followed by counterstain with a fast green, cleaned in xylene, mounted in permouth, then viewed and photographed using an Olympus stereomicroscope CX31.

**3.7.2 Scanning electron microscopic examination**

Parasites were post-fixed in 1% $OsO_4$ (Ted Pella Inc., USA) for two hours after being fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (Servicebio, China) in pH 7.2 for scanning electron microscopy. After that, specimens were rinsed twice with phosphate buffer and once with distilled water for 10 minutes each. Metacercaria was dehydrated with graded series of ethanol (30%, 50%, 70%, 95%, and 100% for 15 minutes each and three changes at 100%). Then it dried with $CO_2$ using the critical point technique (critical point dryer, Leica model EM CPD300, Austria). After that, mounting and gold (sputter coater, Balzers model SCD 040, Germany) coating was applied to the samples, and a scanning electron microscope (JSM-IT300, Japan) was used.

### 3.8 Histopathological analysis

Tissues from the liver, spleen, and intestine that had been infected with metacercaria were thoroughly cleaned with water before being preserved in buffered formalin for around 24 hours for histological analysis. The specimens were thoroughly cleaned, then dehydrated in a series of ethyl alcohols (50, 70, 90, and 100%) and acetone before being cleaned with methyl benzoate (Jithila and Prasadan, 2019). Samples were dehydrated in a graded ethanol series, and then they were paraffin-embedded. Wax was melted and stored in blocks called paraffins at 58℃. The Microtec rotatory microtome (Germany) was used to cut serial slices of 5 m thickness, which were subsequently stained with Heidenhain's Hematoxylin and Eosin to study the histopathology and cellular infiltration at the attachment site. An Olympus CX31 light microscope was used to view the DPX-mounted sections, and a Nikon Y-TV55 camera linked to the microscope was used to capture pictures.

### 3.9 Molecular identification

#### 3.9.1 Deoxyribonucleic acid extraction and polymerase chain reaction

Prior to being kept in 70% ethanol until DNA extraction, all of the metacercarial specimens were washed in saline. Using a Nucleospin DNA extraction kit and according to the manufacturer's instructions, genomic DNA was isolated from samples. The ITS1-5.8S rDNA-ITS2 and 18S rDNA sequences were amplified using the primer pairs specified in Table 4, respectively. A total of 25 µl were used for the PCR reaction, which included 1 µl of each primer, 4 µl of genomic DNA, 12.5 µl of the Go Taq® Master Mix (Madison, USA), and 6.5 µl of distilled water. The early steps of the PCR cycle condition were carried out with denaturation at 94 ℃ for 5 minutes, followed by 40 cycles of 94 ℃ for 30 seconds, annealing at a specific primer 65 ℃/60℃ (65 ℃ for 18s rDNA and 60℃ for ITS1-5.8S rDNA-ITS2) for 30 seconds, and 72℃ for 1 minute; with a final step of 72℃ for 7 minutes. The purification of PCR products was conducted through the utilization of a 1.5% agarose gel. The anticipated PCR product was purified using a commercially available kit in accordance with the manufacturer's protocol (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany). Subsequently, sequencing of the purified product was performed in both directions by a commercial DNA sequencing service provider

(Celemics, Korea). The primer set was devised utilizing the Pimer3plus server to enhance the amplification of the 18S rDNA coding and ITS1-5.8S rDNA-ITS2 non-coding region of the parasites (Kumar and Chordia, 2015). Initially, newly acquired sequences of the 18S rDNA (Table 2) and ITS1-5.8S rDNA-ITS2 (Table 3) genes of *Clinostomum* spp. were aligned with sequences sourced from the Genbank database using MEGA 11. The final comparisons were then edited and evaluated by Bioedit v.7.0.5.3 (Hall, 1999) and TBtools (Chen et al., 2020).

Table 2 List of 18S rDNA gene used for primer design.

| **Scientific Name** | **Description** | **Accession No.** |
|---|---|---|
| ***Clinostomum* sp.** | 18S small subunit ribosomal RNA gene | MW539004.1 |
| **Clinostomid sp.** | 18S small subunit ribosomal RNA gene | AY829252.1 |
| ***Clinostomum* sp.** | 18S small subunit ribosomal RNA gene | AY222094.1 |
| ***Clinostomum piscidium*** | 18S small subunit ribosomal RNA gene | FJ970655.1 |
| ***Clinostomum brieni*** | 18S small subunit ribosomal RNA gene | MH606189.1 |
| ***Clinostomum complanatum*** | 18S small subunit ribosomal RNA gene | KF811012.1 |
| ***Clinostomum sinensis*** | 18S small subunit ribosomal RNA gene | MK490986.1 |
| ***Clinostomum giganticum*** | 18S small subunit ribosomal RNA gene | FJ970654.1 |

| | | |
|---|---|---|
| ***Clinostomum marginatum*** | 18S small subunit ribosomal RNA gene | MF398350.1 |

Table 3 List of ITS1–5.8S rDNA-ITS2 gene used for primer design.

| **Scientific Name** | **Description** | **Accession No.** |
|---|---|---|
| ***Clinostomum piscidium*** | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY312848.1 |
| ***Clinostomum piscidium*** | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY290511.1 |
| ***Clinostomum tilapiae*** | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY649356.1 |
| ***Clinostomum complanatum*** | internal transcribed spacer 1 and internal transcribed spacer 2 region | MH845235.1 |
| ***Clinostomum complanatum*** | internal transcribed spacer 1 and internal transcribed spacer 2 region | MK796829.1 |
| ***Clinostomum piscidium*** | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY304779.1 |

Table 4 List of primers used in this research.

| Gene | Forward Primer | Reverse Primer | Product size (bp) | Reference |
|---|---|---|---|---|
| **18S rRNA** | ATTCCGGAGGGAGCCCTG | ATCAACCCAGTCAGCACCC | 395 | Present study |
| **ITS** | CACCGCCCTGGCGTAATA | CGACACTTCGAACGATTTCTAGA | 801 | Present study |

### 3.9.2 DNA sequencing and phylogenetic tree analysis

The PCR product was subjected to purification using a commercially available kit, following the manufacturer's instructions (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany), prior to being sent to a professional DNA sequencing service (Celemics, Korea). The alignment of sequences was performed through the utilization of ClustalW multiple alignments, which was executed on BioEdit version 7.0.5.3. The identity of the aligned sequences was assessed by comparing them with available sequences in the GenBank database, utilizing the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/). The construction of phylogenetic trees was carried out through the utilization of the maximum likelihood method, accompanied by a bootstrap method test of phylogeny consisting of 1000 bootstrap replications. The nucleotides substitution type and the Poisson substitution model were employed, and the computation of pairwise distance homology was executed. The software program utilized was the MEGA X software program (Newman et al., 2016).

### 3.10 Statistical analysis

The independent sample t-test was utilized to compare the data obtained from the infected and non-infected fish. A significance level of 0.05 was adopted, which a P-value was deemed statistically significant. The statistical analysis was conducted using Microsoft Excel.

# Chapter 4: Results

## 4.1 Study area

All the fish were collected from Samut Prakan, Samut Songkhram, Samut Sakhon, and Kanchanaburi provinces in Thailand. The fish were cultured in the earthen ponds (Figure 6), and the population density was 10,000-15,625 fingerlings per rai (1 rai = 0.395 acres). Water used for the fish farms was supplied from a nearby irrigation canal. The age of the fish during stocking in the grow-out pond was mainly between 3-4 months. In most cases, the farmers provided in-house produced fry to culture the fish and culture approximately 8-9 months marketable size. Farmers often collect the fish when they reach a size of 5 to 6 fish/kg to sell in the market.

Figure 6 Pictures of culture pond of snakeskin gourami from different locations of Thailand.

## 4.2 Sample size estimation

The fish's sample size was estimated using an online statistical prediction tool Epitools (https://epitools.ausvet.com.au). Here, we set the different parameters in the study to estimate the total sample size. At first, we selected the section “Sample

size to estimate a true prevalence with an imperfect test" and set the parameters as follows Assumed true prevalence = 0.4; Assumed sensitivity = 1; Assumed specificity = 1; Desired precision = 0.05 and Confidence level = 0.90 (Figure 7). The tool after that calculating the sample size required for our study was 260 (Figure 7). The aforementioned software application generates the necessary sample size for accurately approximating the actual prevalence, while maintaining the desired level of precision and confidence. Tables containing sample sizes for various prevalence, precision, sensitivity, and specificity values are also generated.

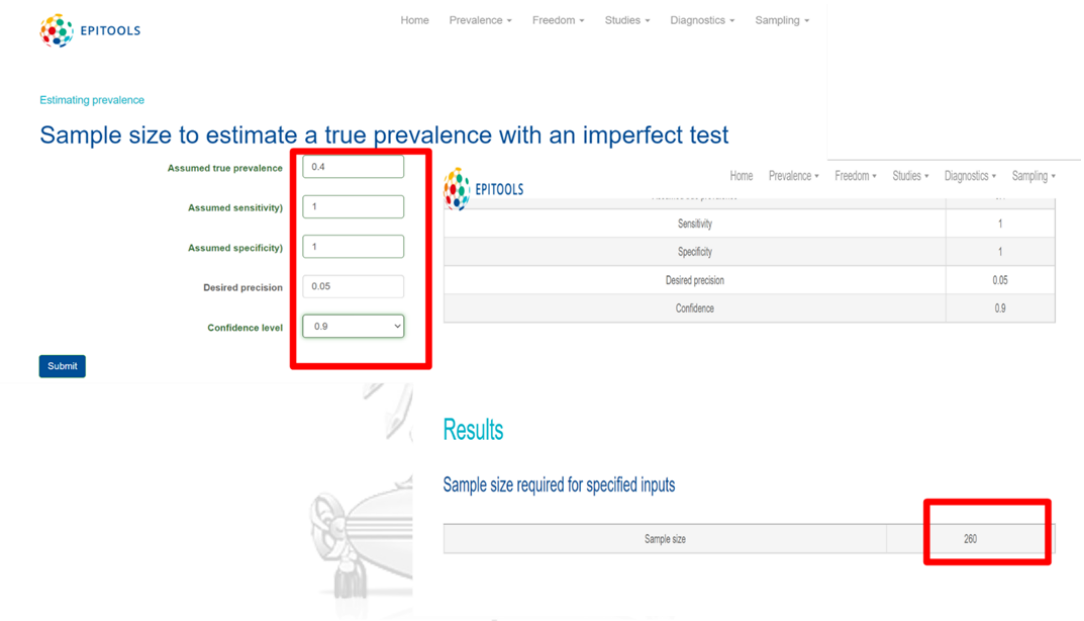


Figure 7 Estimation of the sample size of the study by Epitools.

**4.3 Collection and prevalence study of the parasites**

At first, a necropsy of the fish was done to collect the parasites. Each fish species' body weight and length were estimated, and sexes were determined before the necropsy procedure. Figure 8 provides illustrations of both the male and female snakeskin gourami. The metacercaria of the parasite was retrieved from the intra-abdominal cavity of the fish, specifically from the exterior of the intestinal and hepatic regions (Figure 9). In this study, 35 out of the 262 *T. pectoralis* were infected with metacercaria of *C. piscidium*. The total number of male and female fish was 46 and 216, respectively. The overall prevalence of infection was 13.35%, out of which 42.85% infection was recorded in farm No. 12 in Kanchanaburi was the highest, followed by Farm 2 in Samut Songkhram, where the prevalence was 41.66% (Table

5). Whereas, in some farms in Samut Songkhram (Farm 5 and Farm 8) and Samut Sakhon (Farm 9) the prevalence was the lowest (0.00%) (Table 5). The total number of infected and non-infected male were 5 and 41, respectively. Whereas a total number of 30 females were found infected, and 186 females were found non-infected. Significant ($P<0.05$) differences exist between infected and non-infected males and female's snakeskin gourami. The infection intensities were highest at 3 ± 0.00 in females in Samut Prakarn (Farm 11) and 4 ± 0.00 in the males of *T. pectoralis* collected from farm No. 4 in Samut Songkhram (Table 5). The infection and intensity percentage were significantly higher in the female fish than in the male collected from all the farms. The between body weight and body length of infected and non-infected females of *T. pectoralis* were highest 194 ± 0.00 gm and 252.33±23.41 gm and 20±0.00 cm and 18.50±0.86 cm, respectively (Table 6). The relationship between body weight and body length of infected and non-infected male fish from the study was statistically significant ($P<0.05$). However, in females, the relationship between body weight and body length between infected and non-infected fish was found non-significant ($P>0.05$) in this study. The number of males and females infected and non-infected is given in Figure 10 and Figure 11, respectively.

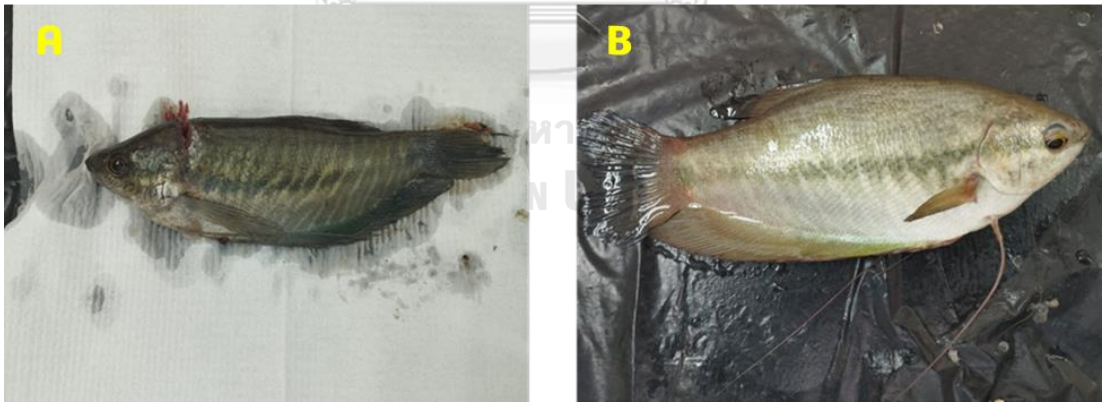


Figure 8 Pictures of *Trichopodus pectoralis* (A) Male and (B) Female fish.


Figure 9 (A-F) Collection of the metacercariae of *Clinostomum piscidium* in different organs of the fish.

Table 5 Prevalence and intensities of infection with metacercariae of *Clinostomum piscidium* in male and female *Trichopodus pectoralis*.

| Localities | Sex of Fish | No. of examined fish | Total number of recovered parasites | No. of infected fish | Prevalence (%) | Mean intensity of parasite (mean ± SD) | Significance |
|---|---|---|---|---|---|---|---|
| Farm No: 1 | Female | 27 | 2 | 2 | 7.40 | 1 ± 0.00 | $p < 0.001$ |
| | Male | 3 | 1 | 1 | 33.33 | 1 ± 0.00 | |
| Farm No: | Female | 24 | 25 | 10 | 41.66 | 2.5 ± 1.2 | $p < 0.001$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2 | Male | 5 | 1 | 1 | 20 | 1 ± 0.00 | |
| Farm No: 3 | Female | 22 | - | - | - | - | - |
| | Male | 3 | - | - | - | - | |
| Farm No: 4 | Female | 23 | 5 | 4 | 17.39 | 1.25 ± 0.43 | p < 0.001 |
| | Male | 5 | 4 | 1 | 20 | 4 ± 0.00 | |
| Farm No: 5 | Female | 9 | - | - | - | - | - |
| | Male | 6 | - | - | - | - | |
| Farm No: 6 | Female | 17 | 5 | 4 | 23.52 | 1.25 ± 0.4 | p < 0.001 |
| | Male | 5 | 1 | 1 | 20 | 1 ± 0.00 | |
| Farm No: 7 | Female | 13 | 1 | 1 | 7.69 | 1 ± 0.00 | p < 0.001 |
| | Male | 2 | - | - | - | - | |
| Farm No: 8 | Female | 14 | - | - | - | - | - |
| | Male | 3 | - | - | - | - | |
| Farm No: 9 | Female | 26 | - | - | - | - | - |
| | Male | 5 | - | - | - | - | |
| Farm No: 10 | Female | 26 | 1 | 1 | 3.84 | 1 ± 0.00 | p < 0.001 |
| | Male | 5 | - | - | - | - | |
| Farm No: 11 | Female | 8 | 3 | 1 | 12.5 | 3 ± 0.00 | p < 0.001 |
| | Male | 3 | - | - | - | - | |
| Farm No: 12 | Female | 7 | 5 | 3 | 42.85 | 1.66 ± 0.94 | p < 0.001 |
| | Male | 1 | 1 | 1 | 100 | 1 ± 0.00 | |

Table 6 Relationship between body weight and total length of *Trichopodus pectoralis* infected with *Clinostomum piscidium*.

| **Locality** | **Sex** | **Status of infection** | **No. of fish** | **Body weight (mean ± S.D., gm)** | **Total length (mean ± S.D., cm)** |
|---|---|---|---|---|---|
| Farm No. 1 | Female | Non-infected | 25 | 143.84±14.16 | 18.36±1.76 |
| | | Infected | 2 | 110 | 14.5±0.5 |
| | Male | Non-infected | 2 | 153±4.00 | 18±2 |
| | | Infected | 1 | 141 | 19 |
| Farm No. 2 | Female | Non-infected | 14 | 102.93±19.38 | 16.07±1.43 |
| | | Infected | 10 | 113.60±30.63 | 15.80±1.77 |
| | Male | Non-infected | 4 | 104.25±10.15 | 16.50±0.86 |
| | | Infected | 1 | 84 | 14 |
| Farm No. 3 | Female | Non-infected | 22 | 46.50±7.42 | 12.18±1.52 |
| | | Infected | - | - | - |
| | Male | Non-infected | 3 | 38.67±3.39 | 10.66±1.24 |
| | | Infected | - | - | - |
| Farm No. 4 | Female | Non-infected | 19 | 164.15±31.30 | 18.05±1.87 |
| | | Infected | 4 | 142.25±36.88 | 16.75±3.11 |
| | Male | Non-infected | 4 | 104.75±17.65 | 14.50±2.17 |
| | | Infected | 1 | 104 | 14 |

| | | | | | |
|---|---|---|---|---|---|
| Farm No. 5 | Female | Non-infected | 9 | 252.33±23.41 | 18.50±3.09 |
| | | Infected | - | - | - |
| | Male | Non-infected | 6 | 21.44±1.34 | 19±1.15 |
| | | Infected | - | - | - |
| Farm No. 6 | Female | Non-infected | 13 | 119.23±8.65 | 15.92±1.97 |
| | | Infected | 4 | 115.5±8.07 | 14.50±2.06 |
| | Male | Non-infected | 4 | 115.25±8.61 | 15.25±2.48 |
| | | Infected | 1 | 114 | 14 |
| Farm No. 7 | Female | Non-infected | 12 | 162.75±7.27 | 18.50±0.86 |
| | | Infected | 1 | 137 | 15 |
| | Male | Non-infected | 2 | 165.50±0.5 | 17.50±0.5 |
| | | Infected | - | - | - |
| Farm No. 8 | Female | Non-infected | 14 | 107.42±5.47 | 13.28±1.03 |
| | | Infected | - | - | - |
| | Male | Non-infected | 3 | 101.33±1.69 | 12.66±0.47 |
| | | Infected | - | - | - |
| Farm No. 9 | Female | Non-infected | 26 | 202.73±33.81 | 19.53±1.82 |
| | | Infected | - | - | - |
| | Male | Non-infected | 5 | 184±13.46 | 19±1.67 |
| | | Infected | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| Farm No. 10 | Female | Non-infected | 25 | 201.04±36.61 | 19.24±1.06 |
| | | Infected | 1 | 194 | 20 |
| | Male | Non-infected | 5 | 200.20±45.19 | 20±2.44 |
| | | Infected | - | - | - |
| Farm No. 11 | Female | Non-infected | 7 | 60.14±11.55 | 13.85±1.80 |
| | | Infected | 1 | 65 | 12 |
| | Male | Non-infected | 3 | 60.66±11.58 | 15±2.44 |
| | | Infected | - | - | - |
| Farm No. 12 | Female | Non-infected | 4 | 73.75±11.98 | 14.25±1.92 |
| | | Infected | 3 | 81±5.56 | 14.33±1.69 |
| | Male | Non-infected | - | - | - |
| | | Infected | 1 | 58 | 14 |

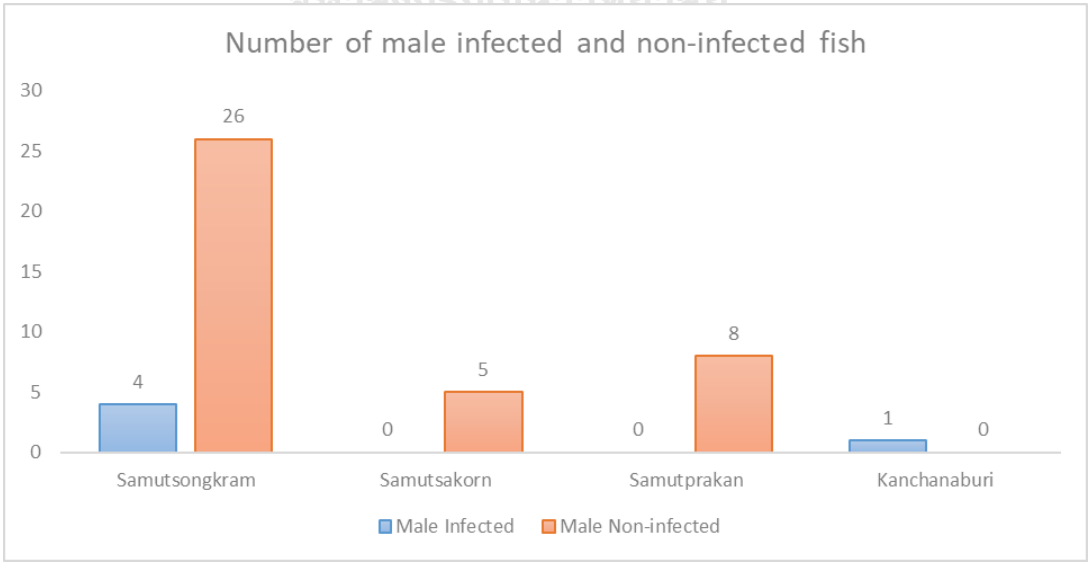

Figure 10 Number of male infected fish with metacercaria and non-infected fish.

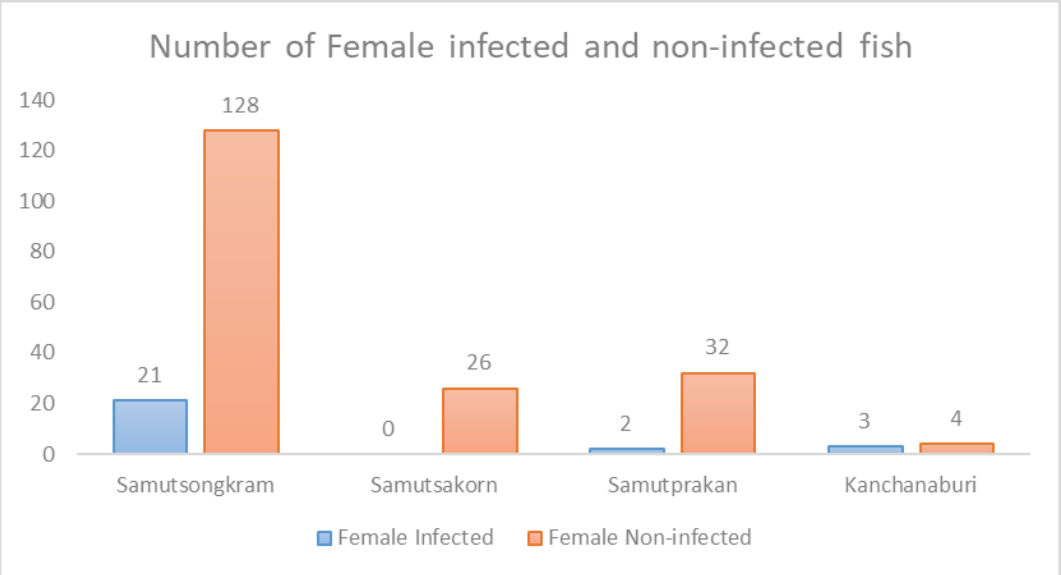


Figure 11 Number of infected female fish with metacercaria and non-infected fish.

**4.4 Morphological identification**

The parasite was slightly yellowish in color; its body was linguliform and dorsoventrally flattened with round anterior and posterior ends (Figure 12 A). A total of ten parasite was checked for morphological analysis where the minimum and maximum length of the fluke was determined 0.086 and 0.63 mm, respectively. While the minimum and maximum width at the middle and posterior one-third of the body was about 0.025 and 0.035 mm, respectively. The oral sucker was at the anterior tip, slightly oval, measured (0.014-0.024) × (0.011-0.025) mm in diameter. The ventral sucker was situated at the anterior one-third of the body, more significant than the oral sucker, oval-shaped, and measured (0.053-0.148) × (0.042-0.074) mm in diameter. The pharyngeal structure exhibited a limited length and expanded into the pharyngeal bulb. The paired testes were positioned in a tandem arrangement along the median longitudinal axis, specifically located at the posterior one-third of the body. The testis located anteriorly exhibited a triangular shape with lobulated margins, positioned slightly to the left of the median axis. The testis located posteriorly exhibited a symmetrical Y-shape and was positioned along the median

axis. The anatomical structures observed in Figure 12 B include the cirrus pouch and the ovary, which are in proximity to each other with the oval cirrus pouch positioned anteriorly to the ovary. The oval-shaped ovary was situated in a posterior position relative to the cirrus pouch on its left. The elongated sac of the uterus was situated towards the posterior aspect of the margin of the ventral sucker. The caudal region of the specimen exhibited the excretory orifice, serving as the aperture of the excretory vesicle (as depicted in Figure 12). Table 7 presents a comparison of the primary morphological characteristics of the metacercaria studied with those reported in other studies on *C. piscidium* metacercariae.

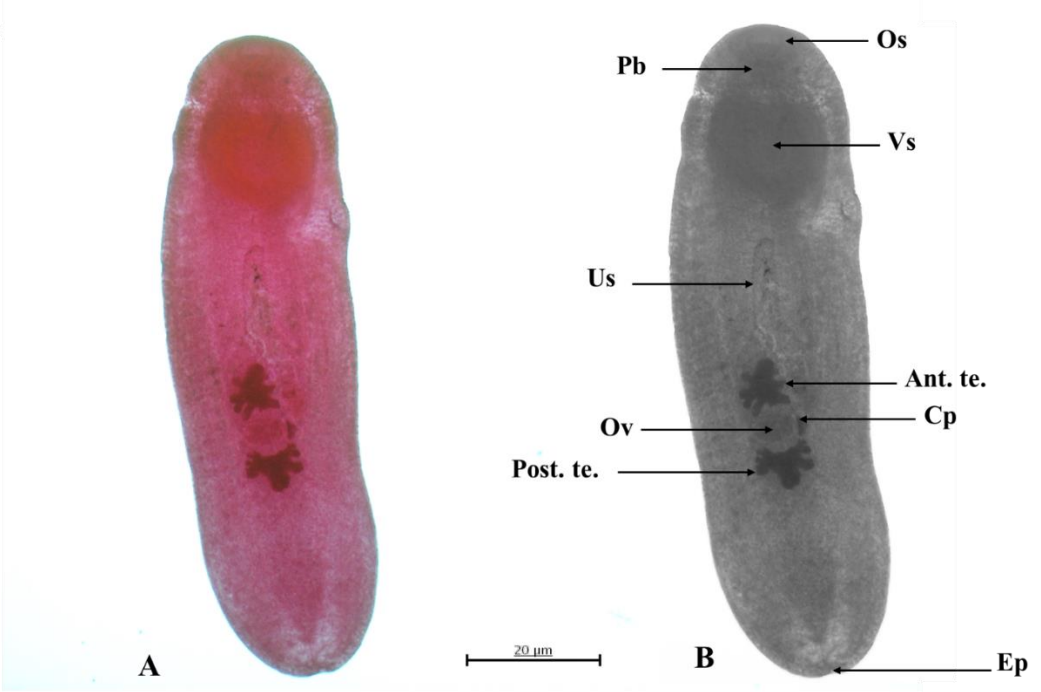


Figure 12 Light micrographs of the same metacercaria of *Clinostomum piscidium*. A) The whole mount (Semichon's carmine stained). B) Details of a whole mount: Oral sucker (Os), Ventral sucker (Vs), Pharyngeal bulb (Pb), Anterior testis (Ant. te.), Posterior testis (Post te.), Uterine sac (Us), Ovary (Ov), Cirrus pouch (Cp), and Excretory pore (Ep).

The host-isolated metacercaria was characterized by their oval shape with a body length of 500 µm (Figure 13A). The metacercaria taken from the hosts had a tiny oral sucker encircled by a conspicuous oral collar, as shown by scanning electron microscopy (Figure 13 (B-D)). Located in the anterior portion of the body, the

rectangular ventral sucker is much bigger than the oral sucker (Figure 13E). The cirrus pouch is curved and overlaps the anterior testis's right border; the ejaculatory duct is narrow, and the cirrus is noticeable (Figure 13F). The excretory pores, also known as the aperture of the excretory bladder, were located at the end of the posterior region (Figure 13G).

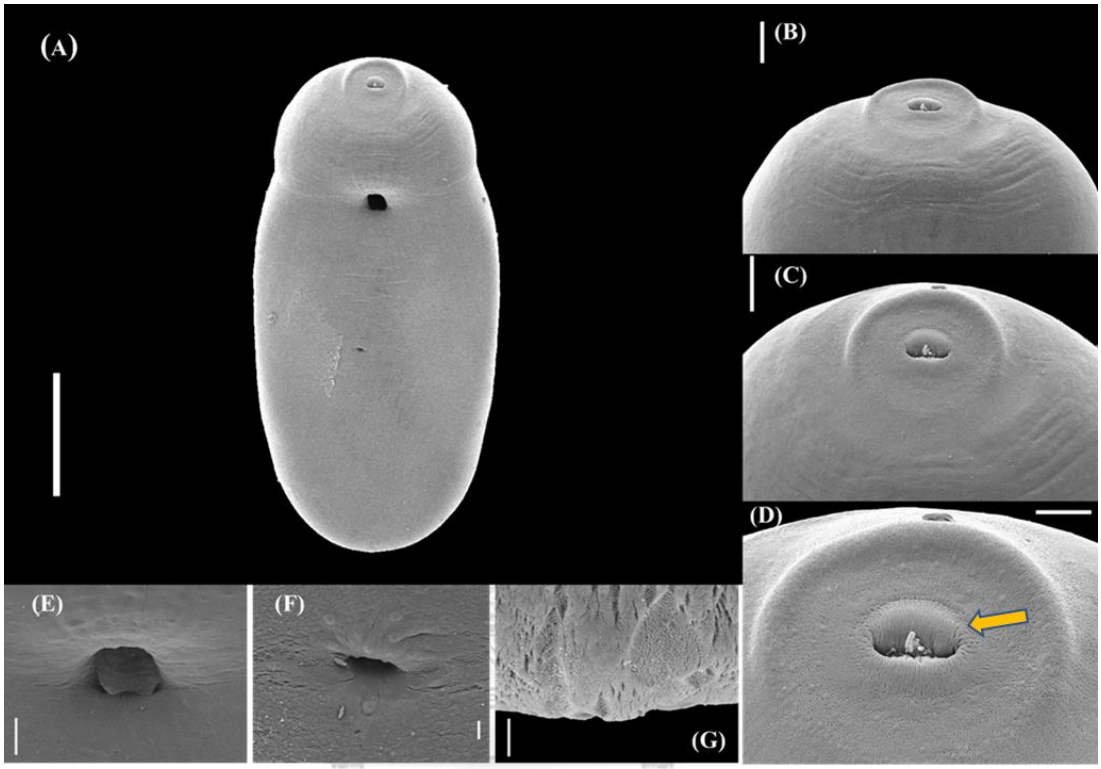


Figure 13 *Clinostomum piscidium* images from scanning electron microscopy. (A) Entire metacercariae body; scale bar = 500 µm. (B-C) The oral sucker collar is seen on metacercariae from the anterior end; scale bar = 100 µm. (D) Oral collar, showing ventral constriction; scale bar = 50 µm, sensilla (arrow) (E) Ventral sucker of metacercariae; scale bar = 50 µm, (F) Cirrus pouch of the worm; scale bar = 10 µm, and (G) Excretory pore of the metacercariae; scale bar = 10 µm.

Table 7 Comparison of morphological characteristics of *Clinostomum piscidium* metacercariae found in the present study and described previously.

| **Morphological characteristics** | **Southwell & Prashad, 1918** | **Singh et al., 2010** | **Tansatit et al., 2014** | **Present study** |
|---|---|---|---|---|
| Body length (mm) | 2.8-5.2 | 2.27-3.36 | 2.4-4.2 | 0.086-0.63 |
| Body width (mm) | 1.4-1.8 | 1.17-1.26 | 1.2-1.7 | 0.025-0.035 |
| Size of oral sucker (mm) | 0.18 in diameter | (0.14-0.18) × (0.23-0.3) | (0.09-0.20) × (0.25-0.35) | (0.014-0.024) × (0.011-0.025) |
| Size of ventral sucker (mm) | 0.6 × 0.48 | (0.60-0.68) × (0.7 -0.8) | (0.48-0.7) × (0.62-0.85) | (0.053-0.148) × (0.042-0.074) |
| Shape of ovary | Oval or bean | Globular | Oval | Oval, Globular |
| Shape of ant. testis | Lobed | Lobed | Triangle, Lobed | Triangle, Lobed |
| Shape of post. testis | Y-shaped | Y-shaped | Y-shaped | Y-shaped |
| Host species | *Nandus nandus*, *T. fasciatus* | *Colisa fasciata* | *T. pectoralis* | *T. pectoralis* |
| Microhabitat | Body cavity | Body cavity | Body cavity | Body cavity |
| Metacercariae form | Non encysted | Non encysted | Non encysted | Non encysted |

**4.5 Histopathological analysis**

The histopathology of Metacercaria infected liver, spleen, and intestine tissues was examined. In this study, gross pathological findings were observed on the liver. The hepatic tissue exhibited a slightly yellowish color, and uninfected piscine specimens demonstrated a typical configuration of hepatocytes arranged in cords or plates, as depicted in Figure 14A. The observation of the multiplication of bile ducts in a conventional arrangement encircling the portal vein of the liver was made (refer to Figure 14B). The non-encysted form of metacercariae of *C. piscidium* was observed to

be either freely moving or attaching to the adipose tissue. The migratory track of the metacercaria was observed in the liver and spleen of the infected fish (Figure 14 (C-D)). The liver of the non-infected fish exhibited no notable histopathological alterations and exhibited the presence of hepatopancreatic tissue surrounding a portal vein (Figure 14A). The hepatic tissue of *T. pectoralis* was observed to contain metacercariae of C. piscidium, which were characterized by a migratory path and successive layers of macrophages and epithelioid cells near the migratory route (Figure 14C). Furthermore, the liver exhibited the presence of inflammatory cells, as depicted in Figure 14E. Additionally, eosinophilic cells were observed in the cytoplasm surrounding the bile duct of the liver cell (Figure 14F). The metacercariae of C. piscidium were observed to be affixed to the serosal surface of the intestine. Additionally, the presence of eosinophilic granular cells in close proximity to the epithelial cells within the infected intestine was noted (Figure 14G). The infected spleen of *T. pectoralis* exhibited the presence of numerous melano-macrophage centers (Figure 14H).

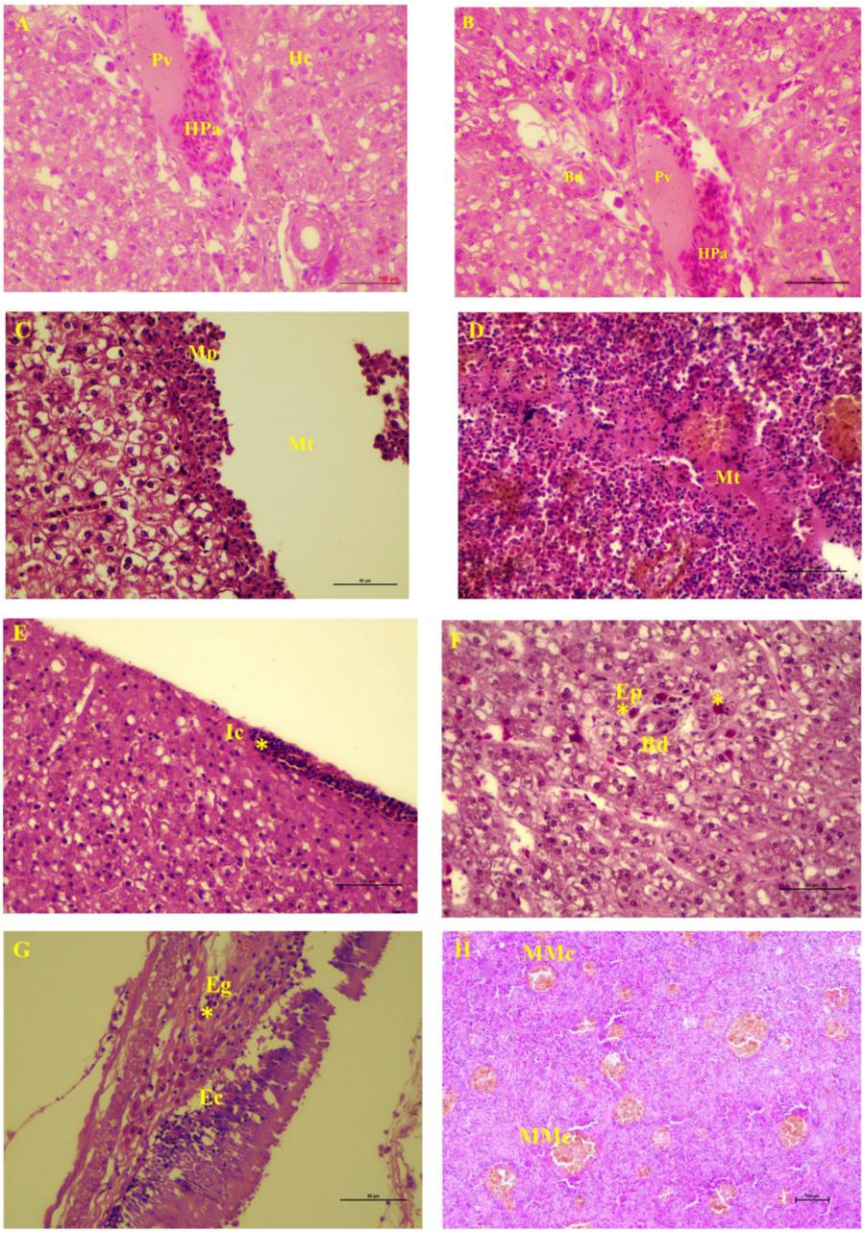


Figure 14 Light micrographs of visceral organs of *Trichopodus pectoralis* non-infected (A-B) and infected (C-H) with *C. piscidium* (H&E stained). A) Liver of non-infected fish shows normal arrangement of hepatocytes (Hc) into cords or plates. Hepatopancreas (Pa) lies around a portal vein (Pv). (B) Normal arrangement of bile duct (Bd) proliferation around the portal vein of the liver cell, (C) Liver of infected fish shows migratory track (Mt), macrophages (Mp) near the migratory track, (D) Spleen of infected fish shows migratory track where the red blood cells are absent and changes in the necrotic tissue, (E) Inflammatory cells (Ic) in the periphery (asterisk) of the liver cells, (F) Eosinophilic cells (asterisk) in the cytoplasm around the bile duct of the

liver cell, (G) Eosinophilic granular cells (Eg) (asterisk) near the epithelial cells (Ec) in the infected intestine, and (H) Many melano-macrophage centers (MMc) in the infected spleen.

**4.6 Molecular identification**

A partial fragment of the 18S rDNA gene was generated using PCR amplification (Figure 15). After sequencing, a contig of 395 bp lengths was formed, for which GenBank Accession No. OP793985, OP793986, OP793987, OP793988, OP793989, OP793990, OP793991, OP793992, OP793993, and OP793994 were obtained. A phylogenetic tree was created using *Schistosoma spindale* as an outgroup and various clinostomid trematode isolates from diverse geographic locations. (Figure 16). To determine the evolutionary history, the Kimura 2 parameter model and the Maximum Likelihood method were used (Mahadani et al., 2022). The branches indicate the percentage of trees in which the linked taxa are grouped (Figure 16). Initial tree(s) for the heuristic search were produced automatically by performing the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Maximum Composite Likelihood (MCL) approach, then selecting the topology with the maximum log-likelihood value. The branch lengths are expressed in terms of substitutions per site, and the tree is shown to scale.

The phylogenetic tree indicated that our isolate had 99% similarity with *Clinostomum piscidium* (FJ970655), *Clinostomum* sp. (AY222094), *Clinostomum* sp. (MW539004), and *Clinostomid* sp. (AY829252) from India, Australia, and the United States, respectively (Figure 16) (Table 8). Another group containing *C. complanatum* (MK490986) from China, *C. complanatum* (MK811210) from the USA, and *C. complanatum* (AY245701) of Israel origin under the same clade showed 97% similarity (Figure 16). According to the evolutionary tree, *C. breini* (KF811009) may have developed earlier. Additionally, it should be highlighted that *S. spindale* (Z11979) fell within the out-group, while *C. marginatum* (MF398350) and *C. tataxumui* (MF398349) are branched under the distinct clade.

Table 8 Sequence identity matrix (%) amongst the *Clinostomum* spp. based on the partial sequence of the 18S rDNA gene.

| Species Name | Accession No. | Identity (%) |
|---|---|---|
| ***Clinostomum* sp.** | MW539004 | 100 |
| ***Clinostomid* sp.** | AY829252 | 100 |
| ***Clinostomum* sp.** | AY222094 | 100 |
| ***Clinostomum piscidium*** | FJ970655 | 98.93 |
| ***Clinostomum brieni*** | MH606189 | 98.12 |
| ***Clinostomum brieni*** | MH606188 | 98.12 |
| ***Clinostomum complanatum*** | KF811012 | 98.12 |
| ***Clinostomum brieni*** | MH606187 | 97.86 |
| ***Clinostomum complanatum*** | FJ609420 | 97.59 |
| ***Clinostomum complanatum*** | AY245701 | 97.59 |
| ***Clinostomum brieni*** | KF811009.1 | 97.59 |
| ***Clinostomum giganticum*** | FJ970654 | 97.05 |
| ***Clinostomum marginatum*** | MF398350 | 96.51 |
| ***Clinostomum tataxumui*** | MF398349 | 96.51 |
| ***Clinostomum marginatum*** | AY245760 | 96.51 |
| ***Clinostomum* sp.** | AY222095 | 96.51 |
| ***Trematoda* sp.** | KX172121 | 96.60 |
| ***Schistosoma spindale*** | Z11979 | 90.74 |

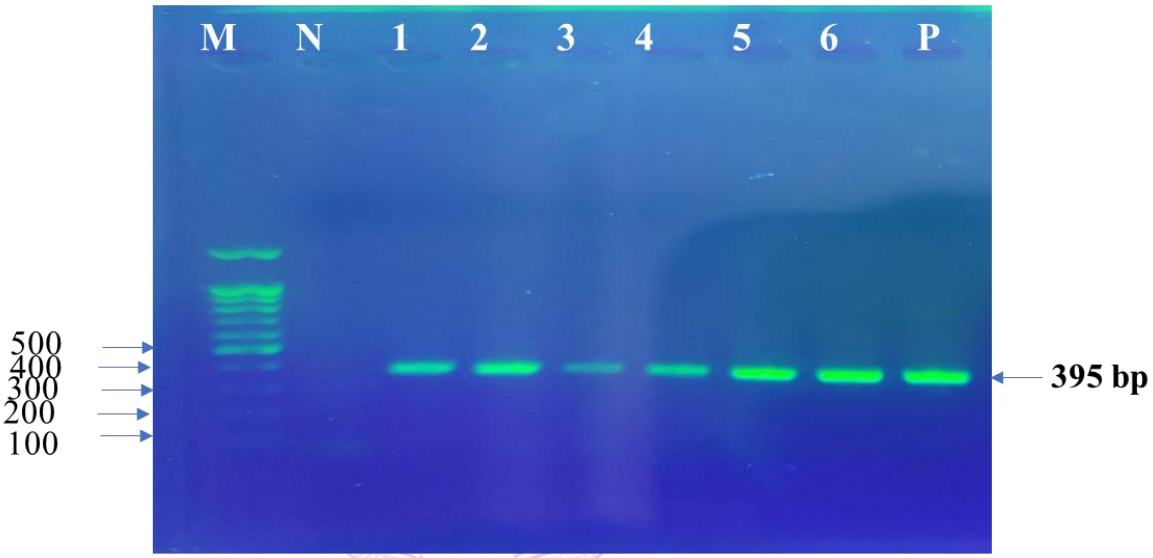


Figure 15 PCR Amplification of 18S rRNA of *Clinostomum* sp. Here, Lane M – DNA Ladder; Lane N - Negative Control; Lane 1 to 6 – Samples and Lane P – Positive control.

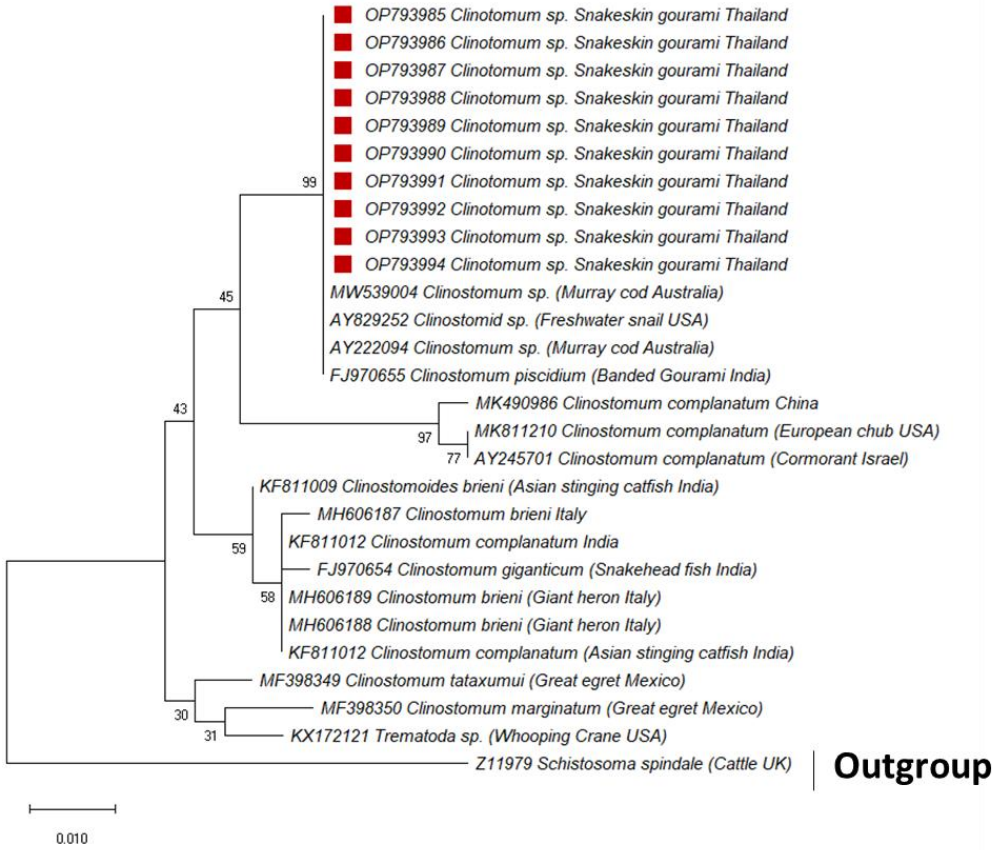

Figure 16 Based on a partial 18S rDNA sequence, the evolutionary relationships between clinostomids were determined using the maximum likelihood method. The percentage of replicate trees in which the related taxa grouped in the bootstrap test (1000 replicates) is displayed above the branches.

Furthermore, with the accession number OP782661, an 801 bp fragment of the *C. piscidium* ITS1-5.8S rDNA-ITS2 gene was uploaded to GenBank to facilitate molecular research. The partial sequence showed 100% homology with *C. piscidium* (KY312848) isolated from India (Table 9). In addition, for the phylogenetic analysis, additional sequences with lower similarity (96-99%) were employed (Table 9). The isolated *C. piscidium* from *Colisa fasciata* and the current species were closely linked in phylogenetic analysis, putting them in the same clade with a high bootstrap value (Figure 17). In most cases, the comparison that constitutes the rate of evolution study is one in which the evolutionary change corresponds to a change in the relative position of a species. In addition, the results of this research imply that the ITS-1 and ITS-2 regions may be more accurate for the interspecific distinction of *Clinostomum* species than initially assumed.

Table 9 Sequence identity matrix (%) amongst the *Clinostomum* spp. based on the partial sequence of ITS gene.

| **Species Name** | **Accession No.** | **Identity (%)** |
|---|---|---|
| ***Clinostomum piscidium*** | KY312848 | 100 |
| ***Clinostomum philippinense*** | KP110570 | 99.52 |
| ***Clinostomum* sp.** | MT446431 | 99.52 |
| ***Clinostomum* sp.** | KY865653 | 98.25 |
| ***Clinostomum tilapiae*** | KY649356 | 98.25% |
| ***Clinostomum phalacrocoracis*** | KP110569 | 98.09% |
| ***Clinostomum cutaneum*** | KP110564 | 97.93% |
| ***Euclinostomum heterostomum*** | KY312847 | 97.46% |
| ***Clinostomum chabaudi*** | MW528863 | 96.98% |

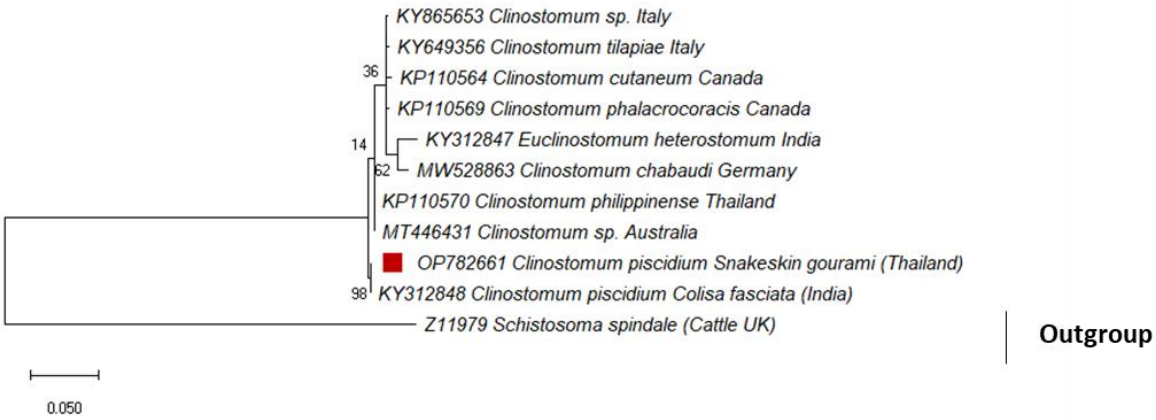


Figure 17 The phylogenetic tree of ITS gene constructed by maximum likelihood displays *C. piscidium* (OP782661) from *Trichopodus pectoralis* clustering next to the Indian group of *C. piscidium*. GenBank accession numbers are provided before species names, and the numbers on the nodes reflect bootstrap confidence levels.

# Chapter 5: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Discussion

In the present study, the overall prevalence of *C. piscidium* infection in *T. pectoralis* was rather high (13.35%) compared to the previous research by Tansatit et al. (2014) which was 43.95 %. It was discovered that there was a connection between the sex of the hosts and the level of parasite infection and intensity. Female fish were more prone to contracting the disease than male fish. From all the farms, it was found that the rate of infection and the severity of worms were higher in the females than in the males. Records on greater infectivity in female hosts are also reported by Tansatit et al. (2014), who observed that the prevalence of *C. piscidium* in *T. pectoralis* was higher in females than males. Both the prevalence and the degree of endoparasite infections were higher in female *Channa punctatus* than in males (Alam et al., 2011). Gholami et al. (2011) also reported a higher rate of *C. complanatum* infection was seen in female *Aphanius dispar* (1.33%) compared to male *A. dispar* (1%), who had a lower rate (Gholami et al., 2011). On the contrary, there have been reports of men having stronger infectivity than females. Ochieng et al. (2012) reported a more significant *C. tilapia* infection in males *Oreochromis niloticus* than female fish (Ochieng, 2012). The factors that contribute to differences in parasite infectivity between sexes are unclear. Female hosts may have a higher incidence and severity of parasite infection owing to physiological and biological characteristics that make females more sensitive and enable parasites to live longer. On the other hand, male fish could be better able to fight off parasites and infection (Siddiqui and Nizami, 1982). Possible behavioral variations between sexes contribute to the disparity in infection rates (Thompson & Kavaliers, 1994).

The parasites recovered from the body cavity of *T. pectoralis* showed morphological traits that differed from the metacercaria of *C. piscidium* previously reported by Southwell and Prashad (1918), Singh et al. (2010), and Tansatit et al. (2014). The form of the anterior and y-shaped posterior testis distinguishes this species. The anterior testis, ovary, and posterior testis shape were identical to the earlier research published by Tansatit et al. (2014) in *T. pectoralis*. However, the size of the body length, body width, oral sucker and the ventral sucker were found different from the

previous study. The metacercariae of *C. piscidium* were described for the first time in *Nandus nandus* in India (Southwell and Prashad, 1918). In Thailand, Charoenpornsook (1985) reported the finding of this metacercariae from the body cavity of *T. pectoralis* and *T. micropterus*. In this investigation, *C. piscidium* metacercariae were exclusively found in the fishes' abdominal cavities. Similar findings were also found in *N. nandus* (see(Southwell and Prashad, 1918)), *Colisa fasciata* (see(Singh et al., 2010)), and *M. aculeatus* (see(Khanum et al., 2011)). These indicate the highly specific microhabitat of the parasite in their hosts.

Morphological examination by scanning electron microscope showed that the metacercariae of the *C. piscidium* were found to be oval. However, the previous study described it as a rod-shaped structure (Tansatit et al., 2014). Moreover, the cuticular spines were seen in this work; nonetheless, the Clinostomidae family is distinguished by its lack of a cuticle protective mechanism (Woodyard et al., 2017). Notably, most cuticular spines might be destroyed during the staining and experimental process and not be seen on a permanent slide (Caffara et al., 2014).

The current study reveals significant histopathological impairment in the liver of *T. pectoralis* due to the presence of migratory tracks caused by the fluke and host response through chronic inflammation. The manifestation of granuloma formation was identified through the infiltration of macrophages and epithelioid cells along the outer perimeter of the track. This action was taken to isolate the affected area. The histopathological examination of liver damage in *Channa punctata*, following parasitization with encysted metacercaria of *Euclinostomum heterostomum*, exhibited a broad expanse of metacercariae cysts and impaired hepatic tissue (Kaur et al., 2012). The formation of tracks in the hepatic parenchyma may be attributed to two primary mechanisms. The first mechanism involves the prehensile action of the oral sucker, which causes mechanical damage. The second mechanism involves the mechanical abrasion caused by spines on the fluke's tegument after they have burrowed and migrated in the liver. Furthermore, it is plausible that the detrimental impact on the hepatic parenchyma could be attributed to the noxious properties of the excretory-secretory (ES) substances produced by the parasite. The cysteine protease present in the ES products released by the metacercaria of clinostomids

has been identified as the agent responsible for the degradation of host proteins (Shareef and Abidi, 2013; Shareef et al., 2010). These products comprise an enzyme that is indispensable for the advancement of the nascent flukes' locomotion and maturation. These compounds are utilized to infiltrate the host tissue and acquire nutrients for their own sustenance (Kasny et al., 2009). Increased eosinophils often accompany infection with metacercariae of different digenea (Adeyemo and Agbede, 2010; Prasanna and Chikkam, 2013). *Oreochromis niloticus*, which was parasitized by C. tilapia metacercariae, had an increase in eosinophilic granular cells in the gills, according to Adeyemo & Agbede (2008). *Tetracotyle* metacercariae infection of *Mastacembelus armatus* resulted in an increase in eosinophilic granular cells in the area between the host tissue and the parasite (Prasanna and Chikkam, 2013). According to Milbourne and Howell (1993), the ES product from the fluke has similar biological properties to interleukin-5 (IL-5), a cytokine that stimulates myeloid precursor cells to differentiate and activate eosinophils. According to this study, eosinophilic granular cells in the liver and intestine's periphery represent the host immune system's reaction to the ES antigen on the metacercariae. Due to the mechanical harm and toxicity brought on by ES products, infection with *C. piscidium* metacercariae may impair the host's ability to function their liver and pancreas. This may occur because ES product causes toxicity. The liver is thought to be the most significant digestive gland in fish since it performs various functions that are crucial to their survival. It significantly affects the metabolism of carbohydrates, proteins, and lipids as well as the glycogen store. Furthermore, here is where the detoxification process is carried out (Akiyoshi and Inoue, 2004). The extensive necrosis of the hepatic and pancreatic tissues may potentially interfere with metabolic processes, leading to a disruption of the host's overall metabolic function and a subsequent reduction in growth potential. The presence of melano-macrophages was detected in the spleen of the affected fish. There may be a correlation between heightened levels of pigmented macrophages and stress and a lack of food (Shinya et al., 2002).

Clinostomidae parasites are cosmopolitan, possibly zoonotic flukes that have been little researched in Thailand using molecular taxonomic methods. Mainly, morphological distinctions across the several phases of parasite growth have made

their identification more complex (Choudhary et al., 2022). Thus, to ensure more accuracy in identifying *Clinostomum* species, morphological identification has been advised to be paired with DNA evidence (Briosio-Aguilar et al., 2019); this is what the current research has done. We presented comprehensive morphological descriptions for identifying the metacercaria of *C. piscidium* and related sequencing data, which may be utilized in future research on diagnostics, freshwater conservation management plans, and public health concerns in Thailand. Although *Clinostomum* spp. is commonly recognized as a zoonotic parasite, it should be highlighted that many medical reports are predicated on this assumption rather than presenting proof of the parasite's identification (Park et al., 2009; Rahmati et al., 2020). As a result, the presence of infectious metacercariae in snakeskin gourami in the current research should be regarded as a potential risk.

This study also aimed to find the cercaria of *Clinostomum* sp. in their first intermediate host, freshwater snail (Figure 18). However, this study recovered no cercaria from the snail for further experiments. In addition, 20 representative samples from both infected and non-infected were processed for the muscle compression experiment to recover the parasite from the muscle and skin of the fish. As a result of the low parasite infestation, this study did not find any parasites in the muscle of the fish.



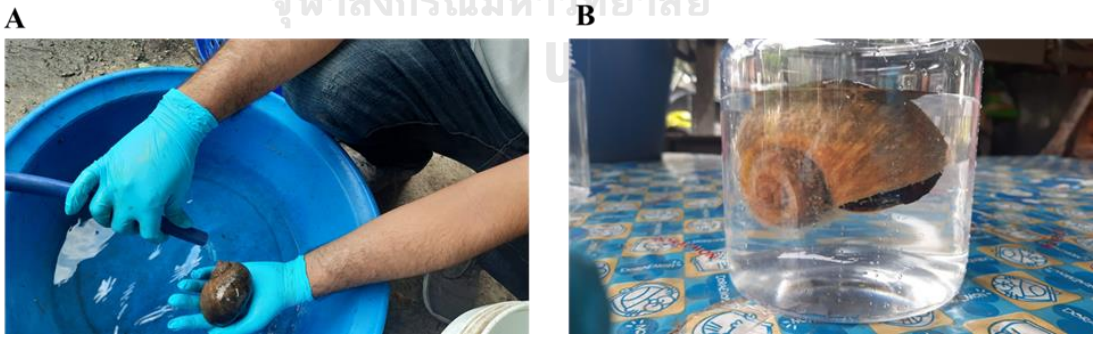


Figure 18 Collection of the snail (A) and checking for the presence of cercariae in the first intermediate host (B).

Clinostomid parasites have low host specificity and may infect a wide range of hosts, causing damage and impairment and, in extreme instances, death (Aghlmandi et al.,

2018; Dang, 2021; Montes et al., 2020; Sutili et al., 2014). Even though just a single species was studied in this work, it is evident that additional research into the prevalence of this parasite and its effects on the health of fish hosts is required. Fish-eating birds serve as definitive hosts in nature, but morphological evidence alone is often insufficient to connect the metacercaria and adult stages (Jousson et al., 1998; Rosser et al., 2018; Shamsi et al., 2013). For future research purposes, the current work established a relationship between metacercariae of *Clinostomum* spp. and *Clinostomum piscidium,* which are often indistinguishable. Body size and shape, sucker ratio, and, most noticeably, genital complex structure are only some morphological changes that occur throughout the metacercariae's metamorphosis into an adult (Choudhary et al., 2022). Notably, most of the cuticular spines might be destroyed during the staining and experimental process and not be seen on a permanent slide (Caffara et al., 2014).

The fish were confined in an intensive aquaculture environment in the present investigation. The snails noticed inside the pond could be another source of infection (Bera et al., 2021). The co-habituating cercariae of the snail may be able to identify the second intermediate host in the aquaculture system. The metacercariae of *Clinostomum* spp. show broad host specificity, according to fish from various zoogeographical regions and families, which makes the current research results relevant owing to the possible effects on key farmed fish and public health. Moreover, due to severe infection with *Clinostomum* metacercariae, amphibians have also been linked to muscular dystrophy and scoliosis (Belló et al., 2000; Perpiñán et al., 2010).

Previous studies have documented the occurrence of localized hemorrhage resulting from *C. piscidium* infection in *T. pectoralis* (Tansatit et al., 2014). The study reported that fish that were infected with clinostomatid metacercaria exhibited a delayed development (Roberts, 1986). In host tissue, a distinction has been made between encysted and non-encysted metacercariae of clinostomids. The metacercariae of the *C. piscidium* species possess the unique ability to endure within the cavity of the second intermediate host without encystment (Tansatit et al., 2014). The non-cysted metacercaria of this species possesses the capability to cause significantly greater

harm to the host fish due to its unrestricted mobility within the visceral organs (Echi et al., 2012). The present study reveals that *C. piscidium* metacercariae were solely retrieved from the abdominal cavity of the fish that were infected. Similar observations were found in both Nandus nandus and Colisa fasciata ("Notes from the Bengal Fisheries Laboratory. Parasites of Indian fishes, with a note on careinoma in the Climbing Perch," 1918; Singh et al., 2010). These findings offer proof of the distinct microenvironment that the parasite inhabits within its host organisms. The hepatic parenchyma of the host may suffer damage due to the toxic impact of the excretory-secretory (ES) secretions of the parasite, *C. piscidium*, in the event of an infection. The cysteine protease, which has the ability to degrade host proteins, has been identified as the ES product secreted by the metacercaria of clinostomids (Shareef et al., 2010; Shareef and Abidi, 2013). The mentioned products consist of enzymes that are essential for the advancement of undeveloped flukes during their migration and growth. The compounds are utilized to infiltrate the host tissue and acquire nutrients for self-sustenance (Kasny et al., 2009). Infection with metacercariae of various digenean is often accompanied by increased eosinophils (Adeyemo and Agbede, 2010). The infection with metacercariae of *C. piscidium* may compromise the host's liver and pancreas function because of the mechanical damage and toxicity caused by ES products. Consequently, severe necrosis of hepatic and pancreatic tissues may disrupt metabolic processes, disrupting the metabolism and leading to stunted development of the fish (Tansatit et al., 2014).

For the molecular characterization of trematodes from the piscine (second intermediate host), including clinostomids and allocreadiids, PCR amplification utilizing rDNA markers has proven effective (Olson et al., 2003). Molecular markers of mitochondrial and ribosomal origins may also help recognize a new species, such as *C. tataxumui*, and verifying *C. complanatum* in Mexico's freshwater fish and fish-eating birds (Sereno-Uribe et al., 2013). Recently, scientists used an internal transcribed spacer 1 (ITS1) region to validate *C. marginatum* and matched it to adult and larval specimens from various hosts and geographic areas (Calhoun et al., 2019). Our *Clinostomum* sp. isolates from *T. pectoralis* showed the most genetic resemblance to *C. piscidium* from banded gourami (*Colisa fasciata*) (FJ970655) of

Indian origin in a phylogenetic tree based on the 18S rDNA sequence. In addition, the *Clinostomum sp.* (AY222094) isolated from *Hypseleotris galii* (Murray cod) from Australia demonstrated a powerful association with the 99% sequence similarity matrix. Previous findings on *C. complanatum* from China, the United States of America, and Israel were all placed in a different group within the same clade as our isolate, which shared 97% of their sequence similarities. In conclusion, the 18S rDNA gene-based phylogenetic tree (Figure 3) and sequence identity matrix (Table 3) would be able to identify *C. breini*, *C. margunatum,* and *C. tataxumui* and placed them in a different clade, which corroborated the conclusions of the earlier research (Gustinelli et al., 2010; Caffara et al., 2014; Wang et al., 2017).

In addition, our findings imply that ITS-1 and ITS-2 sequences, rather than 28S sequences, may be more effective for discriminating between closely related species that belong to the family Clinostomidae. Previous research has supported this hypothesis (Curran et al., 2006; Lotfy et al., 2010; Phalee and Wongsawad, 2014). Figure 4 makes it very evident that our *C. piscidium* isolate from the snakeskin gourami grouped into the same cluster as an Indian isolate of *C. piscidium*. In this work, we report the first genetic identification of *C. piscidium* metacercariae from one of Thailand's most important commercial species, *T. pectoralis*. Regarding the solid genetic relationship between *Clinostomum* species, the phylogenetic analysis led us to recognize *C. piscidium* as a distinct parasite for snakeskin gourami based on a comparison of molecular data.

## 5.2 Conclusion and Recommendations

Snakeskin gourami (*Trichopodus pectoralis*) is a popular and economically valuable species in the commercial aquaculture industry in Thailand. However, the intensive culture practices employed in this industry make the snakeskin gourami highly susceptible to various parasitic diseases, particularly digenean trematodes. Among these parasites, *Clinostomum* spp. are economically important parasites that affect freshwater fishes, snails, and birds worldwide. Efficient control and preventative strategies for these parasitic infections require accurate identification of the parasites down to the genus and species level. However, before the available information, no studies have been conducted to establish the molecular identification of *Clinostomum* spp. in snakeskin gourami in Thailand. This knowledge gap underscores the need for further research to enhance our understanding of the prevalence, distribution, and genetic diversity of *Clinostomum* parasites in snakeskin gourami populations. To reduce the economic losses caused by *Clinostomum* infection in fish, preventive measures must be taken through the culmination of snails to prevent the disease in fish, or research on vaccine development and chemotherapeutics needs special attention since snakeskin gourami is a highly valued economic fish of Thailand.

In addition, it is crucial to encourage education and training among aquaculture farmers, industry participants, and relevant authorities. By educating farmers about the dangers of *Clinostomum* spp. infections and providing them with information and tools on disease prevention, detection, and management, we may help to reduce the incidence and spread of these diseases.

# REFERENCES

Adeyemo AO and Agbede SA 2010. Histopathology of tilapia tissues harbouring *Clinostomum tilapiae* parasites. African Journal of Biomedical Research. 11(1):122-128 https://doi.org/10.4314/ajbr.v11i1.50677

Aghlmandi F, Habibi F, Afraii MA, Abdoli A, and Shamsi S 2018. Infection with metacercaria of *Clinostomum complanatum* (Trematoda: Clinostomidae) in freshwater fishes from Southern Caspian Sea Basin. Revue De Medecine Veterinaire. 169:147-151

Ahmadi A 2021. Morphometric characteristic and condition factor of snakeskin gourami (*Trichogaster pectoralis*) from Sungai Batang Swamp, Indonesia. Aquaculture Research. 7(1):351-353

Akiyoshi H and Inoue A 2004. Comparative histological study of teleost livers in relation to phylogeny. Zoological science. 21:841-850. https://doi.org/10.2108/zsj.21.841

Alam M, Rakibuzzaman M and Hasan M 2011. Comparative study of endoparasitic infestation in *Channa punctatus* (Bloch, 1793) collected from hatchery and Sewage Lagoon. Daffodil International University Journal of Science and Technology. 6. https://doi.org/10.3329/diujst.v6i2.9339

Ambak MA, Isa MM, Zakaria MZ, Abd Ghaffar M. 2010. Fishes of Malaysia. Terengganu (Malaysia): Universiti Malaysia; p. 334

Aohagi Y, Shibahara T, Machida N, Yamaga Y and Kagota K 1992. *Clinostomum complanatum* (Trematoda: Clinostomatidae) in five new fish hosts in Japan. Journal of wildlife diseases. 28:467-469. https://doi.org/10.7589/0090-3558-28.3.467

Arthur JR and Lumanlan-Mayo S 1997. Checklist of the parasites of fishes of the Philippines. FAO Fisheries Technical Paper. 369:102p

Arya R, Antonisamy B and Kumar S 2012. Sample size estimation in prevalence studies. Indian J Pediatr. 79(11):1482-1488. https://doi.org/10.1007/s12098-012-0763-3

Barber I 2005. Parasites grow larger in faster growing fish hosts. Int J Parasitol. 35(2):137-143. https://doi.org/10.1016/j.ijpara.2004.11.010

Bassleer G 1997. Color guide of tropical fish diseases: on freshwater fish. Bassleer Biofish, Westmeerbeek, Belgium.

Belló AR, Fortes E, Belló-Klein A, Belló AA, Llesuy SF, Robaldo RB and Bianchini A 2000. Lipid peroxidation induced by *Clinostomum detruncatum* in muscle of the freshwater fish Rhamdia quelen. Dis Aquat Organ. 42(3):233-236. https://doi.org/10.3354/dao042233

Bera AK, Das N, Bhattacharya S, Malick RC, Swain HS, Chowdhury H, Sinha A, Manna S, Sarkar U and Das B 2021. Molecular confirmation of metacercaria of *Clinostomum complanatum* recovered from one-stripe spiny eel *Macrognathus aral*. Aquaculture Research. *52*:52(p) https://doi.org/10.1111/are.15273

Boonsom J, 1986. Pla-salid (*Trichogaster pectoralis* Regan). A life history and manual for culture. Thai Fishery Gazette, 39:589-601.

Briosio-Aguilar R, García-Varela M, Hernández-Mena DI, Rubio-Godoy M and Pérez-Ponce de León G 2019. Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America. J Helminthol 93(4):461-474. https://doi.org/10.1017/s0022149x18000445

Bruni G and Angelini C 2016. No short term effect of *Clinostomum complanatum* (Trematoda: Digenea: Clinostomatidae) on survival of *Triturus carnifex* (Amphibia: Urodela: Salamandridae). Acta Herpetologica. 11:75-79. https://doi.org/10.13128/Acta_Herpetol-17774

Bruno D, Nowak BF and Elliott D 2006. Guide to the identification of fish protozoan and metazoan parasites in stained tissue sections. Diseases of Aquatic Organisms. 70:1-36. https://doi.org/10.3354/dao070001

Caffara M, Bruni G, Paoletti C, Gustinelli A and Fioravanti ML 2014a. Metacercariae of *Clinostomum complanatum* (Trematoda: Digenea) in European newts *Triturus carnifex* and *Lissotriton vulgaris* (Caudata: Salamandridae). J Helminthol. 88(3):278-285. https://doi.org/10.1017/s0022149x13000151

Caffara M, Davidovich N, Falk R, Smirnov M, Ofek T, Cummings D, Gustinelli A and Fioravanti ML 2014b. Redescription of *Clinostomum phalacrocoracis* metacercariae (Digenea: Clinostomidae) in cichlids from Lake Kinneret, Israel. Parasite (Paris, France). 21:32. https://doi.org/10.1051/parasite/2014034

Calhoun DM, Leslie KL, Riepe TB, Achatz TJ, McDevitt-Galles T, Tkach VV and Johnson PT J 2019. Patterns of *Clinostomum marginatum* infection in fishes and amphibians: integration of field, genetic, and experimental approaches. J Helminthol. 94:e44. https://doi.org/10.1017/s0022149x18001244

Charoenpornsook K 1985. *Clinostomum piscidium* (Southwell & Phashad, 1918) the digenetic trematode in the body cavity of pla-salid (*Trichogaster pectoralis*, Regan, 1910). (M.S.Thesis in Microbiology). Bangkok: Faculty of Graduate Studies, Mahidol University.

Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y and Xia R 2020. TBtools: An integrative toolkit developed for interactive analyses of big biological data. Molecular Plant. 13(8):1194-1202. https://doi.org/https://doi.org/10.1016/j.molp.2020.06.009

Choudhary K, Ray S, Shamsi S and Agrawal N 2022. Characterization of *Clinostomum* (Digenea: Clinostomidae) spp. in India. Parasitol Res 121(11):3083-3089. https://doi.org/10.1007/s00436-022-07644-y

Chung, D. I., Moon, C. H., Kong, H. H., Choi, D. W., & Lim, D. K. (1995). The first human case of *Clinostomum complanatum* (Trematoda: Clinostomidae) infection in Korea. Korean J Parasitol, 33(3), 219-223. https://doi.org/10.3347/kjp.1995.33.3.219

Curran S, Tkach V and Overstreet R 2006. A review of polylekithum Arnold 1934 and its familial affinities using morphological and molecular data, with description of *Polylekithum catahoulensis* sp. nov. Acta Parasitologica. 51:238-248. https://doi.org/10.2478/s11686-006-0037-1

Dabrowsk J 2012. Water quality, metal bioaccumulation and parasite communities of *Oreochromis mossambicus* in Loskop dam, Mpumalanga, South Africa. [Online]. Available: https://repository.up.ac.za.

Dang M 2021. Wild fish as reservoirs of parasites on Australian Murray Cod farms. Aquaculture. 539. https://doi.org/10.1016/j.aquaculture.2021.736584

Dias M, Eiras J, Machado M, Souza G and Pavanelli G 2003. The life cycle of *Clinostomum complanatum* Rudolphi, 1814 (Digenea, Clinostomidae) on the floodplain of the high Paraná river, Brazil. Parasitology research. 89:506-508. https://doi.org/10.1007/s00436-002-0796-z

Dinh-Hung N, Dong HT, Rodkhum C, Soontara C, Nimitkul S, Chatchaiphan S, Srisapoome P and Kayansamruaj P 2021. Co-infection of a novel Chlamydia-like organism and *Henneguya sp*. (Myxosporea, Myxobolidae) in snakeskin gourami *Trichopodus pectoralis* (Regan 1910), in Thailand. bioRxiv. 2021.2006.2001.446678. https://doi.org/10.1101/2021.06.01.446678

DOF. Statistics of Freshwater Aquaculture Production. Fisheries Development Policy and Strategy Division No. 8/2018. (2016). Bangkok: Department of Fisheries, Ministry of Agriculture and Cooperatives (2018)

Duraiyan, J., Govindarajan, R., Kaliyappan, K., & Palanisamy, M. (2012). Applications of immunohistochemistry. J Pharm Bioallied Sci, 4(Suppl 2), S307-309. https://doi.org/10.4103/0975-7406.100281

Dykova I and Lom J 1978. Histopathological changes in fish gills infected with myxosporidian parasites of the genus *Henneguya*. Journal of Fish Biology.12(3):197-202. https://doi.org/https://doi.org/10.1111/j.1095-8649.1978.tb04165.x

Echi P, Eyo J, Okafor F, Onyishi G and Ivoke N 2012. First Record of Co – infection of three clinostomatid parasites in cichlids (Osteichthyes: Cichlidae) in a tropical freshwater lake. Iranian journal of public health. 41:86-90.

Eegan C 2009. The Asiatic fishes of the family Anabantidne. Journal of Zoology.79:767-787. https://doi.org/10.1111/j.1469-7998.1910.tb06972.x

Eiras J and Adriano E 2012. A checklist of new species of *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea, Myxobolidae) described between 2002 and 2012. Systematic parasitology. 83:95-104. https://doi.org/10.1007/s11230-012-9374-7

Eiras JC 2002. Synopsis of the species of the genus *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea: Myxobolidae. Syst Parasitol. 52(1):43-54. https://doi.org/10.1023/a:1015016312195

Eissa IAE, Elamie M, Aly S and Sallam N 2017. Studies on the prevailing parasitic diseases in some marine fishes. Egyptian Veterinary Medical Society of Parasitology Journal. 13:64-77. https://doi.org/10.21608/evmspj.2017.37769

FAO. Fishery and aquaculture statistics. Global Aquaculture Production 1950-2019 (FishstatJ). Rome: FAO Fisheries Division. Available online: http://www.fao.org/fishery/countrysector/naso_thailand/en

Fernando CH 1972. Methods for the study of freshwater fish parasites. [publisher not identified].

Froese R, Pauly D, 2004. FishBase DVD. Penang, Malaysia: Worldfish Center. Online at www.fishbase.org.

Froese R 2006. Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. Journal of Applied Ichthyology. 22(4):241-253. https://doi.org/https://doi.org/10.1111/j.1439-0426.2006.00805.x

Gholami Z, Mobedi I, Esmaeili H and Kia E 2011. Occurrence of *Clinostomum complanatum* in *Aphanius dispar* (Actinoptrygii: Cyprinodontidae) collected from Mehran River, Hormuzgan Province, South of Iran. Asian Pacific journal of tropical biomedicine. 1: 189-192. https://doi.org/10.1016/S2221-1691(11)60025-7

Gustinelli A, Caffara M, Florio D, Otachi EO, Wathuta EM and Fioravanti ML 2010. First description of the adult stage of *Clinostomum cutaneum* Paperna, 1964 (Digenea: Clinostomidae) from grey herons *Ardea cinerea* L. and a redescription of the metacercaria from the Nile tilapia *Oreochromis niloticus niloticus* (L.) in Kenya. Syst Parasitol. 76(1):39-51. https://doi.org/10.1007/s11230-010-9231-5

Hall TA 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series,

Hara H, Miyauchi Y, Tahara S and Yamashita H 2014. Human Laryngitis caused by *Clinostomum complanatum*. Nagoya journal of medical science. 76:109-113.

Jithila, P., and Prasadan, P 2019. Histopathology and other aspects of the *Clinostomum complanatum* infection in the freshwater fish, *Pseudosphromenus cupanus* from the south western ghats. Pakistan J. Parasitol. 68:33-38.

Jousson O, Bartoli P, Zaninetti L and Pawlowski J 1998. Use of the ITS rDNA for elucidation of some life-cycles of Mesometridae (Trematoda, Digenea). Int J Parasitol. 28(9):1403-1411. https://doi.org/10.1016/s0020-7519(98)00117-9

Kanev Radev V and Fried B 2002. Family Clinostomidae Luehe. 1901. In (Vol. 1, pp. 113-120). https://doi.org/10.1079/9780851995472.0113

Kasny M, Mikeš L, Hampl V, Dvořák J, Caffrey C, Dalton J and Horák P 2009. Peptidases of trematodes. Advances in parasitology. 69:205-297. https://doi.org/10.1016/S0065-308X(09)69004-7

Kaur P, Qureshi TA and Shrivastav R 2012. Histo-architectural alterations induced by *Euclinostomum heterostomum* (metacercariae) infected liver of *Channa punctata* (Bloch). Journal of parasitic diseases. 36:197-199. https://doi.org/10.1007/s12639-012-0102-7

Kent ML, Andree KB, Bartholomew JL, El-Matbouli M, Desser SS, Devlin RH, Feist SW, Hedrick RP, Hoffmann RW, Khattra JS, Hallett SL, Lester RJG, Longshaw M, Palenzeula O, Siddall ME and Xiao C 2001. Recent advances in our knowledge of the Myxozoa. The Journal of eukaryotic microbiology. 43(2).

Khan R 2009. Parasites causing disease in wild and cultured fish in Newfoundland. Icelandic Agricultural Sciences. 22.

Khanum H, Begum S and Begum A 2011. Seasonal prevalence, intensity and organal distribution of Helminth parasites in macrognathus aculeatus. Dhaka University Journal of Biological Sciences. 20. https://doi.org/10.3329/dujbs.v20i2.8971

Kottelat M. 2001. Fishes of Laos. Sri Lanka: WHT Publication Ltd; p. 198

Kumar A and Chordia N 2015. *In silico* PCR primer designing and validation. Methods in molecular biology (Clifton, N.J.). 1275:143-151. https://doi.org/10.1007/978-1-4939-2365-6_10

Lafferty KD 2008. Ecosystem consequences of fish parasites. Journal of Fish Biology.73(9): 2083-2093. https://doi.org/https://doi.org/10.1111/j.1095-8649.2008.02059.x

Laimgruber S, Schludermann C, Konecny R and Chovanec A 2005. Helminth communities of the barbel *Barbus barbus* from large river systems in Austria. Journal of helminthology. 79:143-149. https://doi.org/10.1079/JOH2005276

Lom J and Dyková I 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia parasitologica. 53(1):1-36.

Lom K and Arthur JR 1989. A guideline for the preparation of species descriptions in Myxosporea. Journal of Fish Diseases. 12:151-156.

Lotfy W, Brant S, Ashmawy K, Devkota R, Mkoji G and Loker E 2010. A molecular approach for identification of paramphistomes from Africa and Asia. Veterinary parasitology. 174: 234-240. https://doi.org/10.1016/j.vetpar.2010.08.027

Mahadani AK, Awasthi S, Sanyal G, Bhattacharjee P and Pippal S 2022. Indel-K2P: a modified Kimura 2 Parameters (K2P) model to incorporate insertion and deletion (Indel) information in phylogenetic analysis. Cyber-Physical Systems. 8(1):32-44. https://doi.org/10.1080/23335777.2021.1879274

Milbourne EA and Howell MJ 1993. Eosinophil differentiation in response to *Fasciola hepatica* and its excretory/secretory antigens. Int J Parasitol. 23(8): 1005-1009.

Marcogliese D 2005. Parasites of the superorganism: Are they indicators of ecosystem health? International Journal for Parasitology. 35:705-716. https://doi.org/10.1016/j.ijpara.2005.01.015

McAllister C, Bursey C, Crawford J, Kuhns A, Shaffer C and Trauth S 2009. Metacercariae of *Clinostomum* (Trematoda: Digenea) from three species of *Ambystoma* (Caudata: Ambystomatidae) from Arkansas and Illinois, U.S.A. Comparative Parasitology. 77:25-30. https://doi.org/10.1654/4385.1

Minh N, Mai P and Linh N 2019. Physical aspects influencing to the production of dry-salted snakeskin gourami (*Trichogaster Pectoralis*). Oriental Journal of Chemistry. 35:773-777. https://doi.org/10.13005/ojc/350238

Molnár K 2002. Site preference of fish myxosporeans in the gill. Diseases of Aquatic Organisms. 48 (3):197-207.

Molnár K and Eszterbauer E 2015. Specificity of infection sites in vertebrate hosts. In pp. 295-313). Springer International Publishing. https://doi.org/10.1007/978-3-319-14753-6_16

Montes MM, Plaul SE, Croci Y, Waldbillig M, Ferrari W, Topa E and Martorelli SR 2020. Pathology associated with three new *Clinostomum* metacercariae from Argentina with morphological and DNA barcode identification. J Helminthol. 94:e148. https://doi.org/10.1017/s0022149x20000292

Morioka S, Vongvichith B, Phommacha P and Chantasone P 2013. Growth and morphological development of laboratory-reared larval and juvenile giant gourami *Osphronemus goramy* (Perciformes: Osphronemidae). Ichthyological Research. 60(3):209-217. https://doi.org/10.1007/s10228-013-0334-2

Newman L, Duffus A and Lee C 2016. Using the free program MEGA to build phylogenetic trees from molecular data. The American Biology Teacher. 78:608-612. https://doi.org/10.1525/abt.2016.78.7.608

Ochieng VO 2012. A study of *Clinostomum* affecting *Oreochromis niloticus* in small water bodies in Eldoret-Kenya.

Olson PD, Cribb TH, Tkach VV, Bray RA and Littlewood DT 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int J Parasitol. 33(7):733-755. https://doi.org/10.1016/s0020-7519(03)00049-3

Panthum T, Laopichienpong N, Kraichak E, Singchat W, Ho My Nguyen D, Ariyaraphong N, Ahmad SF, Muangmai N, Duengkae P, Peyachoknagul S, Ezaz T and Srikulnath K 2021. The snakeskin gourami (*Trichopodus pectoralis*) tends to exhibit XX/XY Sex determination. Fishes.6(4):43. https://www.mdpi.com/2410-3888/6/4/43

Paperna I 1991. Diseases caused by parasites in the aquaculture of warm water fish. Annual Review of Fish Diseases. 1:155-194

Park CW, Kim JS, Joo HS and Kim J 2009. A human case of *Clinostomum complanatum* infection in Korea. Korean J Parasitol. 47(4):401-404. https://doi.org/10.3347/kjp.2009.47.4.401

Perpiñán D, Garner MM, Trupkiewicz JG, Malarchik J, Armstrong DL, Lucio-Forster A and Bowman DD 2010. Scoliosis in a tiger salamander (*Ambystoma tigrinum*) associated with encysted digenetic trematodes of the genus *Clinostomum*. J Wildl Dis. 46(2):579-584. https://doi.org/10.7589/0090-3558-46.2.579

Phalee A and Wongsawad C 2014. Prevalence of infection and molecular confirmation by using ITS-2 region of *Fasciola gigantica* found in domestic cattle from Chiang Mai province, Thailand. Asian Pacific Journal of Tropical Medicine. 7:207–211. https://doi.org/10.1016/S1995-7645(14)60022-5

Phong N 2014. Snakeskin gourami in the Mekong. [Online]. Available: https://doi.org/10.13140/RG.2.1.3762.5040

Pinter, H., 1986. Labyrinth Fish. (English edition). Barron's Educational Series, Inc., New York. 144 pp.

Pinto H, Caffara M, Fioravanti M and De Melo A 2014. Experimental and molecular study of cercariae of *Clinostomum* sp. (Trematoda: Clinostomidae) from

*Biomphalaria* spp. (Mollusca: Planorbidae) in Brazil. Journal of Parasitology, in press. https://doi.org/10.1645/14-497.1

Poulin R 2006. Variation in infection parameters among populations within parasite species: Intrinsic properties versus local factors. International Journal for Parasitology. 36:877-885. https://doi.org/10.1016/j.ijpara.2006.02.021

Poelen J, Simons J and Mungall C 2014. Global Biotic Interactions: An open infrastructure to share and analyze species-interaction datasets. Ecological Informatics. 24.

Prasanna A and Chikkam V 2013. Histopathology of heart of freshwater spiny eel, *Mastacembelus armatus* naturally infected with *Tetracotyle* metacercaria (Trematoda: Strigeidae). Research Journal of Parasitology. 8:45-54. https://doi.org/10.3923/jp.2013.45.54

Prianto E, Jhonnerie R and Kasim K 2021. Reproductive biology and dynamics of snakeskin gourami (*Trichopodus pectoralis*) larvae in Lubuk Lampam floodplain, Ogan Komering Ilir, Indonesia. AACL Bioflux. 14:284-286.

Rahmati AR, Kiani B, Afshari A, Moghaddas E, Williams M and Shamsi S 2020. World-wide prevalence of Anisakis larvae in fish and its relationship to human allergic anisakiasis: a systematic review. Parasitol Res. 119(11):3585-3594. https://doi.org/10.1007/s00436-020-06892-0

Randy MacMillan J 1991. Biological factors impinging upon control of external protozoan fish parasites. Annual Review of Fish Diseases. 1:119-131. https://doi.org/https://doi.org/10.1016/0959-8030(91)90026-G

Reiczigel J, Földi J and Ozsvári L 2010. Exact confidence limits for prevalence of a disease with an imperfect diagnostic test. Epidemiol Infect. 138(11):1674-1678. https://doi.org/10.1017/s0950268810000385

Roberts T 1986. Systematic review of the Mastacembelidae or spiny eels of Burma and Thailand, with description of two new species of Macrognathus. Japanese Journal of Ichthyology. 33:95-109. https://doi.org/10.1007/BF02905838

Rosser T, Baumgartner W, Alberson N, Woodyard E, Reichley S, Wise D, Pote L and Griffin M 2016. *Austrodiplostomum sp.*, *Bolbophorus sp.* (Digenea: Diplostomidae), and *Clinostomum marginatum* (Digenea: Clinostomidae) metacercariae in inland

silverside *Menidia beryllina* from catfish aquaculture ponds, with notes on the infectivity of *Austrodiplostomum sp.* cercariae in channel catfish *Ictalurus punctatus*. Parasitology research. 115. https://doi.org/10.1007/s00436-016-5222-z

Rosser TG, Baumgartner WA, Alberson NR, Noto TW, Woodyard ET, Tommy King D, Wise D J and Griffin MJ 2018. *Clinostomum poteae n. sp.* (Digenea: Clinostomidae), in the trachea of a double-crested cormorant *Phalacrocorax auritus* Lesson, 1831 and molecular data linking the life-cycle stages of *Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017 in Mississippi, USA. Syst Parasitol.95(6):543-566. https://doi.org/10.1007/s11230-018-9801-5

Sereno-Uribe AL, Pinacho-Pinacho CD, García-Varela M and de León GP 2013. Using mitochondrial and ribosomal DNA sequences to test the taxonomic validity of *Clinostomum complanatum* Rudolphi, 1814 in fish-eating birds and freshwater fishes in Mexico, with the description of a new species. Parasitol Res. 112(8):2855-2870. https://doi.org/10.1007/s00436-013-3457-5

Shamsi S, Halajian A, Tavakol S, Mortazavi P and Boulton J 2013. Pathogenicity of *Clinostomum complanatum* (Digenea: Clinostomidae) in piscivorous birds. Res Vet Sci. 95(2):537-539. https://doi.org/10.1016/j.rvsc.2013.06.018

Shareef P and Abidi S 2013. Cysteine protease is a major component in the excretory/secretory products of *Euclinostomum heterostomum* (Digenea: Clinostomidae). Parasitology research. 113. https://doi.org/10.1007/s00436-013-3627-5

Shareef P, Rizvi TF, Saifullah M and Abidi S 2010. Preliminary Analysis of *in vitro* released Excretory/ Secretory (E/S) Cysteine Proteases of the progenetic metacercariae of *Clinostomum complanatum*. XII international congress of parasitology (ICOPA).

Shinya M, Misaka N, Miyakoshi Y, Takeuchi K and Kasahara N 2002. Effects of starvation on melano-macrophages in the kidney of masu salmon (*Oncorhynchus masou*). Aquaculture. 209:247-255. https://doi.org/10.1016/S0044-8486(01)00716-5

Siddiqui AA and Nizami WA 1982. Seasonal population dynamics of the metacercariae of *Clinostomum complanatum* (Trematoda: Digenea) in relation to sex of the host. Rev Parasitol. 43:275-279.

Singh HS, Chaudhary A and Halajian A 2010. Further observations on *Clinostomum piscidium* Southwell and Prashad, 1918 recovered from the body cavity of *Colisa faciata* (Bloch and Schneider, 1801) in India. Sci Parasitol. 11:55-60.

Smyth J and Smyth M 1980. Frogs as host-parasite systems I. https://doi.org/10.1007/978-1-349-86094-4

Sohn WM 2009. Fish-borne zoonotic trematode metacercariae in the Republic of Korea. Korean J Parasitol. 47 Suppl:S103-113. https://doi.org/10.3347/kjp.2009.47.S.S103

Southwell T and Prashad B 1918. Notes from the Bengal fisheries laboratory 5. Parasites of Indian fishes, with a note on carcinoma in the climbing perch. Rec Ind Mus. 15: 341-355.

Sures B 2008. Environmental Parasitology. Interactions between parasites and pollutants in the aquatic environment. Parasite (Paris, France). 15:434-438. https://doi.org/10.1051/parasite/2008153434

Sutili F, Gressler L and Pelegrini L 2014. *Clinostomum complanatum* (Trematoda, Digenea): a parasite of birds and fishes with zoonotic potential in southern Brazil. A Review. Revista Brasileira de Higiene e Sanidade Animal. 8. https://doi.org/10.5935/1981-2965.20140007

Tacon, A.G.; Hasan, M.R.; Metian, M. Demand and supply of feed ingredients for farmed fish and Crustaceans: Trends and Prospects. 2011. Available online: https://www.proquest.com/docview/1328361199?pqorigsite=gscholar&fromopenview=true

Tansatit T, Sobhon P, Sahaphong S, Sangsuriya P and Klinsrithong S 2014. Prevalence and histopathology of *Trichogaster pectoralis* harbouring metacercaria of *Clinostomum piscidium* (Southwell and Prashad, 1918) in central Thailand. Thai Journal of Veterinary Medicine. 44:223-230.

Thompson S and Kavaliers M 1994. Physiological bases for parasite induced alterations of behaviour. Parasitology. 109:119-138. https://doi.org/10.1017/S0031182000085139

Tiewchaloern S, Udomkijdecha S, Suvouttho S, Chunchamsri K and Waikagul J 1999. *Clinostomum* trematode from human eye. Southeast Asian J Trop Med Public Health. 30(2):382-384.

Valladão MR, Alves LO and Pilarski F 2016. Trichodiniasis in Nile tilapia hatcheries: Diagnosis, parasite:host-stage relationship and treatment. Aquaculture. 451:444-450. https://doi.org/https://doi.org/10.1016/j.aquaculture.2015.09.030

Vidthayanon C. 2012. *Trichopodus pectoralis*. The IUCN Red List of Threatened Species 2012: e.T188087A1852593. Gland (Switzerland): IUCN. 10.2305/IUCN.UK.2012-1.RLTS.T188087A1852593.en. 11 October 2018

Waikagul J and Thaenkham U 2014. Medically important fish-borne zoonotic Trematodes. Approaches to Research on the Systematics of Fish-Borne Trematodes (pp. 1-16). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-407720-1.00001-7

Wang ML, Chen HY and Shih HH 2017. Occurrence and distribution of yellow grub trematodes (*Clinostomum complanatum*) infection in Taiwan. Parasitol Res. 116(6):1761-1771. https://doi.org/10.1007/s00436-017-5457-3

Ward MM 2013. Estimating disease prevalence and incidence using administrative data: some assembly required. J Rheumatol. 40(8):1241-1243. https://doi.org/10.3899/jrheum.130675

WELLBORN JR 1967. *Trichodina* (Ciliata: Urceolariidae) of freshwater fishes of the Southeastern United States. The Journal of Protozoology. 14(3):399-412. https://doi.org/https://doi.org/10.1111/j.1550-7408.1967.tb02017.x

Woodyard E, Rosser T and Rush S 2017. Alligator wrestling: morphological, molecular, and phylogenetic data on *Odhneriotrema incommodum* (Leidy, 1856) (Digenea: Clinostomidae) from *Alligator mississippiensis* Daudin, 1801 in Mississippi, USA. Parasitology research. 116. https://doi.org/10.1007/s00436-017-5607-7

Yamashita J 1938. *Clinostomum complanatum*, a trematode parasite new to man.

Yimer E and Enyew M 2003. Parasites of Fish at Lake Tana, Ethiopia. SINET: Ethiopian Journal of Science. 26:31-36. https://doi.org/10.4314/sinet.v26i1.18197

Yoonpundh R and Little D 1997. Trends in the farming of the snakeskin gourami (*Trichogaster pectoralis*) in Thailand. Naga WorldFish Center Quarterly. 20(3/4):18-20.

Yooyen T, Wongsawad C, Kumchoo K and Chaiyapo M 2006. A new record of *Clinostomum philippinensis* (Valasquez, 1959) in *Trichogaster microlepis* (Gunther, 1861) from Bung Borapet, Nakhon Sawan, Thailand. Southeast Asian J Trop Med Public Health. 37(3): 99-103.

## APPENDIX A

Table List of 18S rDNA gene used for primer design.

| Scientific Name | Description | Accession No. |
|---|---|---|
| ***Clinostomum* sp.** | 18S small subunit ribosomal RNA gene | MW539004 |
| ***Clinostomid* sp.** | 18S small subunit ribosomal RNA gene | AY829252 |
| ***Clinostomum* sp.** | 18S small subunit ribosomal RNA gene | AY222094 |
| ***Clinostomum piscidium*** | 18S small subunit ribosomal RNA gene | FJ970655 |
| ***Clinostomum brieni*** | 18S small subunit ribosomal RNA gene | MH606189 |
| ***Clinostomum complanatum*** | 18S small subunit ribosomal RNA gene | KF811012 |
| ***Clinostomum sinensis*** | 18S small subunit ribosomal RNA gene | MK490986 |
| ***Clinostomum giganticum*** | 18S small subunit ribosomal RNA gene | FJ970654 |
| ***Clinostomum marginatum*** | 18S small subunit ribosomal RNA gene | MF398350 |

## APPENDIX B

List of ITS1–5.8S rDNA-ITS2 gene used for primer design.

| Scientific Name | Description | Accession No. |
|---|---|---|
| *Clinostomum piscidium* | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY312848 |
| *Clinostomum piscidium* | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY290511 |
| *Clinostomum tilapiae* | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY649356 |
| *Clinostomum complanatum* | internal transcribed spacer 1 and internal transcribed spacer 2 region | MH845235 |
| *Clinostomum complanatum* | internal transcribed spacer 1 and internal transcribed spacer 2 region | MK796829 |
| *Clinostomum piscidium* | internal transcribed spacer 1 and internal transcribed spacer 2 region | KY304779 |

## APPENDIX C

Table List of primer set used in this study.

| Gene | Forward Primer | Reverse Primer | Product size (bp) | Reference |
|---|---|---|---|---|
| **18S rRNA** | ATTCCGGAGGGAGCCCTG | ATCAACCCAGTCAGCACCC | 395 | Present study |
| **ITS** | CACCGCCCTGGCGTAATA | CGACACTTCGAACGATTTCTAGA | 801 | Present study |

## APPENDIX D

Table Sequence identity matrix (%) amongst the clinostomids based on the partial sequence of 18S rDNA gene.

| Species Name | Accession No. | E-value | Identity (%) |
|---|---|---|---|
| *Clinostomum* sp. | MW539004 | 0.00 | 100 |
| *Clinostomid* sp. | AY829252 | 0.00 | 100 |
| *Clinostomum* sp. | AY222094 | 0.00 | 100 |
| *Clinostomum piscidium* | FJ970655 | 0.00 | 98.93 |
| *Clinostomum brieni* | MH606189 | 0.00 | 98.12 |
| *Clinostomum brieni* | MH606188 | 0.00 | 98.12 |
| *Clinostomum complanatum* | KF811012 | 0.00 | 98.12 |
| *Clinostomum brieni* | MH606187 | 5e-180 | 97.86 |
| *Clinostomum complanatum* | FJ609420 | 6e-179 | 97.59 |
| *Clinostomum complanatum* | AY245701 | 6e-179 | 97.59 |
| *Clinostomum brieni* | KF811009.1 | 2e-178 | 97.59 |
| *Clinostomum giganticum* | FJ970654 | 5e-175 | 97.05 |
| *Clinostomum marginatum* | MF398350 | 1e-171 | 96.51 |
| *Clinostomum tataxumui* | MF398349 | 1e-171 | 96.51 |
| *Clinostomum marginatum* | AY245760 | 1e-171 | 96.51 |
| *Clinostomum* sp. | AY222095 | 1e-171 | 96.51 |
| *Trematoda* sp. | KX172121 | 3e-147 | 96.60 |
| *Schistosoma spindale* | Z11979 | 3e-137 | 90.74 |

## APPENDIX E

Table Sequence identity matrix (%) amongst the clinostomids based on the partial sequence of ITS gene.

| Species Name | Accession No. | E-value | Identity (%) |
|---|---|---|---|
| *Clinostomum piscidium* | KY312848 | 0.00 | 100 |
| *Clinostomum philippinense* | KP110570 | 0.00 | 99.52 |
| *Clinostomum* sp. | MT446431 | 0.00 | 99.52 |
| *Clinostomum* sp. | KY865653 | 0.00 | 98.25 |
| *Clinostomum tilapiae* | KY649356 | 0.00 | 98.25% |
| *Clinostomum phalacrocoracis* | KP110569 | 0.00 | 98.09% |
| *Clinostomum cutaneum* | KP110564 | 0.00 | 97.93% |
| *Euclinostomum heterostomum* | KY312847 | 0.00 | 97.46% |
| *Clinostomum chabaudi* | MW528863 | 0.00 | 96.98% |
| *Schistosoma spindale* | Z11979 | | |

## APPENDIX F

Table Sex, weight and length of studied fishes

| Location | No. | Number of fish | | Weight (g) | | Total length (cm) | |
|---|---|---|---|---|---|---|---|
| | | Male | Female | Range (g) | Mean±S.D. | Range (cm) | Mean±S.D. |
| **Samutsongkram** | Farm-1 | 3 | 27 | 101-174 | 142.1±15.47 | 14-21 | 18.1±1.92 |
| **Samutsongkram** | Farm-2 | 5 | 24 | 74-184 | 106.13±23.28 | 13-19 | 15.96±1.51 |
| **Samutsongkram** | Farm-3 | 3 | 22 | 36-63 | 45.56±7.36 | 9-15 | 11.88±1.50 |
| **Samutsongkram** | Farm-4 | 5 | 23 | 76-230 | 150.39±36.98 | 12-20 | 17.21±2.48 |
| **Samutsongkram** | Farm-5 | 6 | 9 | 144-296 | 226±41.77 | 18-24 | 20.46±1.69 |
| **Samutsongkram** | Farm-6 | 5 | 17 | 101-131 | 117.59±8.39 | 11-18 | 15.45±2.1 |
| **Samutsongkram** | Farm-7 | 2 | 13 | 137-173 | 160.73±8.82 | 15-20 | 18.13±1.13 |
| **Samutsongkhram** | Farm-8 | 3 | 14 | 97-113 | 106.35±5.37 | 12-15 | 13.17±0.95 |
| **Samutsakorn** | Farm-9 | 5 | 26 | 153-223 | 199.70±31.67 | 17-25 | 19.45±1.78 |
| **Samut Prakarn** | Farm-10 | 5 | 26 | 140-298 | 200.67±36.99 | 17-22 | 19.38±1.38 |
| **Samut Prakarn** | Farm-11 | 3 | 8 | 48-87 | 60.72±10.63 | 10-18 | 14.00±2.00 |
| **Kanchanaburi** | Farm-12 | 1 | 7 | 57-89 | 74.5±10.91 | 11-16 | 14.25±1.61 |

# VITA

| | |
|---|---|
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| **AWARD RECEIVED** | ASEAN-NON ASEAN Scholarship, Awarded by Chulalongkorn University |