MORPHOLOGICAL IDENTIFICATION AND MOLECULAR CHARACTERIZATIONS OF LUNG FLUKES (*PARAGONIMUS WESTERMANI* AND RELATED SPECIES) IN CENTRAL AND SOUTHERN THAILAND

A THESIS

BY

SUTHEEWAN BINCHAI

Presented in Partial Fulfillment of the Requirements for the

Master of Science degree in Biology

at Srinakharinwirot University

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AN ABSTRACT

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Sutheewan Binchai. (2006). *Morphological Identification and Molecular Characterizations of Lung Flukes (Paragonimus westermani and Related Species) in Central and Southern Thailand*. Master thesis, M.Sc. (Biology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Dr. Achariya Rangsiruji, Dr. Hiromu Sugiyama.

Paragonimus westermani is widely distributed in Asia and forms a complex of cryptic species. In Thailand, *P. westermani* metacercariae (infective stage) were recorded in the central and southern regions. In the present study, another form of *P. westermani* metacercariae was discovered. With the shape almost identical to *P. westermani* but the size was smaller, these metacercariae were called *P. westermani*-like. Morphological examination revealed that the adult flukes of *P. westermani* from the central and southern parts as well as *P. westermani*-like were identical. However, the susceptibility of feline hosts to *P. westermani* was found to be different from that of *P. westermani*-like. To clarify evolutionary relationships of *P. westermani* and *P. westermani*-like with other members of *Paragonimus* in Asia, parsimony method was employed in molecular analyses of the ITS2 and partial COI regions. The phylogenetic tree inferred from the ITS2 region contained two important clades. Clade I (BS = 79%) consists of the *P. westermani* complex and *P. siamensis* and clade II (BS = 84%) contains other *Paragonimus* species found in Thailand. Within the *P. westermani* complex, two groups of organism were revealed based on geographical origins. The first group comprises *P. westermani* from South East Asia (Thailand, Malaysia and the Philippines) and the second group composes of *P. westermani* from East Asia (Japan, China, Korea and Taiwan) plus *P. westermani*-like from Thailand. The strict consensus tree obtained from the partial COI region showed a single clade (BS = 98%) of the *P. westermani* complex from South East and East Asia. In contrast to the ITS2 tree, this tree revealed that *P. westermani*-like is excluded from the complex and placed as a sister group to it. From this study, it is evident that *P. westermani*-like is either placed well within the *P. westermani* complex (ITS2 data) or is located close to the complex (COI data). Since the protein-coding gene (COI) is under selective constraint while the ITS region is not, this suggests that the spacer is free to evolve with a rate that is close to the neutral rate of sequence evolution. In addition, due to a higher level of homoplasy present in the COI data, the tree inferred from the ITS2 data would be more reliable. The result of *P. westermani*-like being classified as one of the members of the *P. westermani* complex was strongly supported by the morphological characters of the adult worms.

ลักษณะทางสัณฐานวิทยา และชีววิทยาระดับโมเลกุลของพยาธิใบไม้ปอด (*Paragonimus westermani* และสปีชีส์ใกล้เคียง) ในภาคกลางและภาคใต้ของประเทศไทย

บทคัดย่อ

ของ

สุธีวรรณ บินชัย

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
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มีนาคม 2550

สุธีวรรณ บินชัย. (2549). *ลักษณะทางสัณฐานวิทยา และชีววิทยาระดับโมเลกุลของพยาธิใบไม้ปอด (Paragonimus westermani และสปีชีส์ใกล้เคียง) ในภาคกลางและภาคใต้ของประเทศไทย*. ปริญญานิพนธ์ วท.ม. (ชีววิทยา). กรุงเทพฯ: บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ. คณะกรรมการควบคุม: ดร.อัจฉริยา รังษิรุจิ ดร.ฮิโรมุ ซุกิยามา

พยาธิใบไม้ปอด *Paragonimus westermani* พบแพร่กระจายอย่างกว้างขวางในทวีปเอเชีย และประกอบด้วยกลุ่มของสปีชีส์ที่ซับซ้อน ในประเทศไทยมีรายงานพบ *P. westermani* ระยะเมตาเซอร์คาเรีย (พยาธิระยะติดต่อ) ในภาคกลางและภาคใต้ของประเทศ การศึกษาครั้งนี้พบเมตาเซอร์คาเรียซึ่งมีลักษณะคล้ายคลึงกับพยาธิสปีชีส์นี้แต่มีขนาดเล็กกว่า จึงเรียกว่า *P. westermani*-like จากการศึกษาด้านสัณฐานวิทยาของพยาธิตัวเต็มวัยพบว่า *P. westermani* ที่พบในภาคกลางและภาคใต้ และ *P. westermani*-like มีลักษณะเหมือนกันทุกประการ อย่างไรก็ตามยังพบความแตกต่างในเรื่อง susceptibility ของสัตว์จำพวกแมวต่อพยาธิ *P. westermani* และ *P. westermani*-like ในการศึกษาความสัมพันธ์เชิงวิวัฒนาการของ *P. westermani*, *P. westermani*-like และ *Paragonimus* สปีชีส์อื่นๆ ใน เอเชีย โดยวิธี parsimony ของบริเวณ ITS2 และ COI ผลของ phylogenetic tree จากบริเวณ ITS2แสดง 2 clade ที่สำคัญได้แก่ clade I (BS = 79%) ประกอบด้วย *P. westermani* complex และ *P. siamensis* และ clade II (BS = 84%) ประกอบด้วย *Paragonimus* สปีชีส์อื่นๆ ที่พบในประเทศไทย สำหรับ *P. westermani* complex สามารถแบ่งได้เป็น 2 กลุ่มตามแหล่งกำเนิดทางภูมิศาสตร์ กลุ่มแรกประกอบด้วย *P. westermani* จากเอเชียตะวันออกเฉียงใต้ (ไทย มาเลเซีย และ ฟิลิปปินส์) กลุ่มที่สองประกอบด้วย *P. westermani* จากเอเชียตะวันออก (ญี่ปุ่น จีน เกาหลี และ ไต้หวัน) และ *P. westermani*-like phylogenetic tree จากบริเวณ COI พบเพียง 1 clade (BS = 98%) ของ *P. westermani* complex จากเอเชียตะวันออกเฉียงใต้ และเอเชียตะวันออก ซึ่งต่างจากบริเวณ ITS2 โดยที่ *P. westermani*-like แยกออกเป็น sister group ของ *P. westermani* complex ผลจากการศึกษาในครั้งนี้เห็นได้ชัดว่า *P. westermani*-like ถูกจัดอยู่ใน *P. westermani* complex (ITS2) หรือใกล้ชิดกับ complex (COI) เนื่องจาก COI เป็นยีนที่กำหนดการแสดงออกของโปรตีน และอยู่ภายใต้ selective constraint ในขณะที่บริเวณ ITS เป็น spacer ซึ่งไม่ได้อยู่ภายใต้ selective constraint บริเวณ ITS จึงมีอิสระกว่าในการวิวัฒนาการในอัตราที่ใกล้เคียงกับ neutral rate นอกจากนี้เนื่องด้วยระดับ homoplasy ที่สูงในชุดข้อมูล COI ทำให้ phylogenetic tree ที่ได้จากบริเวณ ITS2 น่าเชื่อถือกว่า ซึ่งผลการทดลองที่จัดกลุ่ม *P. westermani*-like อยู่ใน *P. westermani* complex นั้นสอดคล้องกับลักษณะทางสัณฐานวิทยาของพยาธิตัวเต็มวัย

The thesis titled

"Morphological Identification and Molecular Characterizations of Lung Flukes (*Paragonimus westermani* and Related Species) in Central and Southern Thailand"

by

Sutheewan Binchai

Has approved by the Graduate School as partial fulfillment of the requirements for the Master of Science degree in Biology of Srinakharinwirot University.

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March 2007

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Sutheewan Binchai

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CHAPTER ONE

INTRODUCTION

General introduction

Lung flukes of the genus *Paragonimus* are most important zoonotic parasites, causing paragonimiasis in mammalian hosts which feed on crabs or crayfish that harbor *Paragonimus* metacercariae. At present more than 40 species of *Paragonimus* have been described mainly in Asia, Africa and the Americas.[reviewed in 1] Geographical distribution of Paragonimiasis is illustrated in Figure 1. Six species of *Paragonimus* which are *P. westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai* have been reported in Thailand. However, only two species, *P. westermani* and *P. heterotremus*, are known to be infective to humans.[2-3]

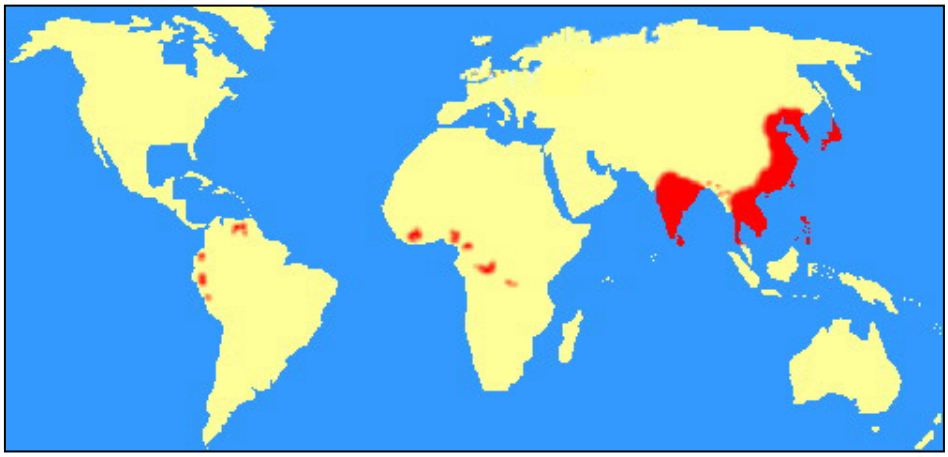

Figure 1. Geographical distribution of Paragonimiasis

Source: http://www.cdfound.to.it/HTML/pw2.htm

In Thailand, the first case of human paragonimiasis was reported in Phetchabun province by Prommas in 1928.[4] From epidemiological surveys carried out by Vajrasthira *et al.* in 1959 and 1966,[5-6] it was revealed that Saraburi and Nakhon Nayok provinces were endemic areas of this disease. The diagnosis in all cases was based on the finding of *Paragonimus* eggs in the sputum. The lung flukes were not found until in 1964 when Daengsvang *et al.*[7] isolated adult *P. westermani* from two leopards caught in Chumphon province, southern Thailand.

In 1965, *P. siamensis* was originally reported by Miyazaki and Wykoff[8] in Udon Thani province, northeastern Thailand. In 1967, Miyazaki and Vajrasthira[9-11] reported the occurrence of *P. heterotremus*, *P. bangkokensis* and *P. macrorchis* and in 1968 they described a new species, *P. harinasutai*.[12] For all the species mentioned, *P. heterotremus* is one of the most important species because it is a causative agent of paragonimiasis in humans which occurs widely in various parts of Thailand.[13]

Although human infection with *P. westermani* has not been reported in Thailand, this species has been found to infect millions of people in Asia. It was first described by Kerbert in 1878 from adult worms obtained from the lungs of a Bengal tiger that died in Amsterdam Zoo in 1877. The flukes had been named *Distoma westermani* after the zoo's director. However, in 1899, Braun suggested a new genus, *Paragonimus* (from Greek means having gonads side by side), for the mammalian lung flukes. Members of the genus are hermaphroditic, digenetic trematodes whose adults live in pairs in the lungs of carnivorous mammals.[reviewed in 14] Classification of the lung flukes is as follows.

Phylum: Platyhelminthes
Class: Trematoda
Subclass: Digenea
Order: Prosostomata
Superfamily: Plagiorchioidea
Family: Troglotrematidae
Genus: *Paragonimus*

Paragonimus westermani is the type species of the genus. It is widely distributed in Asia, including Taiwan, Japan, Korea, China, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Philippines, Indonesia, Papua New Guinea, India, Pakistan, Nepal, and Far East of Russia. Various studies on *P. westermani*, including morphology, cytology, immunology, ecology and molecular biology have been carried out. Based on chromosomal studies *P. westermani* in eastern Asia was found in three forms of diploid, triploid and tetraploid whereas those occurring elsewhere, including Thailand appeared to be diploid only.[reviewed in 1]

In Thailand, studies on *P. westermani* have been focused on metacercariae.[15-17] The metacercariae were reported only in the central part (Nakhon Nayok and Saraburi provinces)

but not in the other parts of the country. To study the adult stage of *P. westermani*, its definitive host is required. Adult worms were only reported from leopards in southern Thailand by Daengsvang *et al.* (1964). Experimental infection of *P. westermani* in mammals such as cats, dogs, rats, hamsters and raccoons to obtain adult worms were mostly unsuccessful. Only immature worms from two immunosuppressed kittens have been reported. The diploid chromosome number of these immature worms was 22.[18] Until now, only the leopard was found to be the natural host for *P. westermani* in Thailand. At present, little is known about adult Thai *P. westermani*.

Paragonimus siamensis was reported in Thailand[8] and Sri Lanka.[reviewed in 19] This species was shown to be closely resembled to *P. westermani* in morphological features of adult flukes. Only cuticular spines were different such that, in *P. siamensis* the spines were arranged in groups,[8] whereas in *P. westermani* they were singly spaced. Results obtained from molecular phylogenetic studies also showed a close relationship between these two species.[19 20]

Morphology of *Paragonimus*

General morphology of adult *Paragonimus* is illustrated in Figure 2. The living adult fluke is reddish brown, fleshy and oval with a rounded anterior end. The integument is covered with spines. Oral sucker is located at the anterior end and the ventral sucker is positioned in the middle of the body. The sizes of both suckers vary depending on the species. The intestine extends to the posterior end of the body. The excretory bladder is long, starting from the pharynx to the posterior end. The branched, paired testes are side by side in the posterior half of the worm. The uterus is on the opposite side of the branched ovary which is located anterior to the testes. The ovary is joined with short oviduct and connected to the seminal receptacle. Grapelike clusters of vitellaria are distributed throughout the body.[21]

The encysted metacercariae are round or oval in shape and their sizes vary from 200-900 μm depending on the species. Each metacercaria consists of one or two cyst walls, the outer cyst wall is usually fragile while the inner one is strong and thick. The thickness of the inner cyst wall also varies from species to species. The larva possesses a large convoluted intestine on both sides of a large excretory bladder.

Eggs are golden yellow in color, ovoidal with a thick shell and often asymmetrical. At the large end, the operculum is clearly visible.

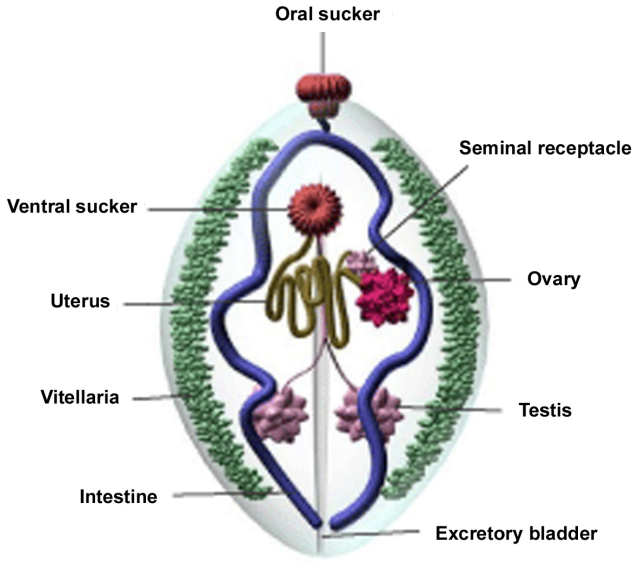

Figure 2. Morphology of *Paragonimus*

Source: http://www.atlas.or.kr/atlas/alphabet_view.php?my_codeName=Paragonimus%20westermani

Species identification

General morphological characters of *Paragonimus* species are similar. For species identification, many criteria are used in the investigation of features of metacercariae and adult flukes. For the metacercariae, the important features are number of cyst wall, shape and size of inner cyst, thickness of inner cyst wall, body size of excysted larvae, comparison of transverse diameters between oral and ventral suckers of excysted larvae, length of stylet in oral sucker, extent of excretory bladder and pinkish granules in larval body. For the adult flukes, the features to be examined are shape of the whole body, arrangement of cuticular spines, shape and size of ovary and testes, comparison of transverse diameters between oral and ventral suckers.[22]

Paragonimus westermani (Kerbert, 1878) Braun, 1899

The adult fluke of *P. westermani* (Figure 3A) has body stout in shape. Its integument is covered with singly spaced cuticular spines (Figure 3B). The oral sucker is usually slightly larger than the ventral sucker. For the sexual organs, the testes are divided into five or six lobes, and are in parallel in the posterior portion of the body, while the ovary is simply

branched into six lobes, located above the testes and opposite a tightly coiled uterus. The vitellaria are located laterally, running from the anterior to the posterior of the parasite. The metacercaria (Figure 3C) is spherical, approximately 300 - 400 μm in size. It has two layers of thick inner cyst wall and thin outer cyst wall. Larval body is expanded to the entire cyst. It has the excretory bladder which is filled with waste granules and the intestine which is wound on both sides of the bladder.

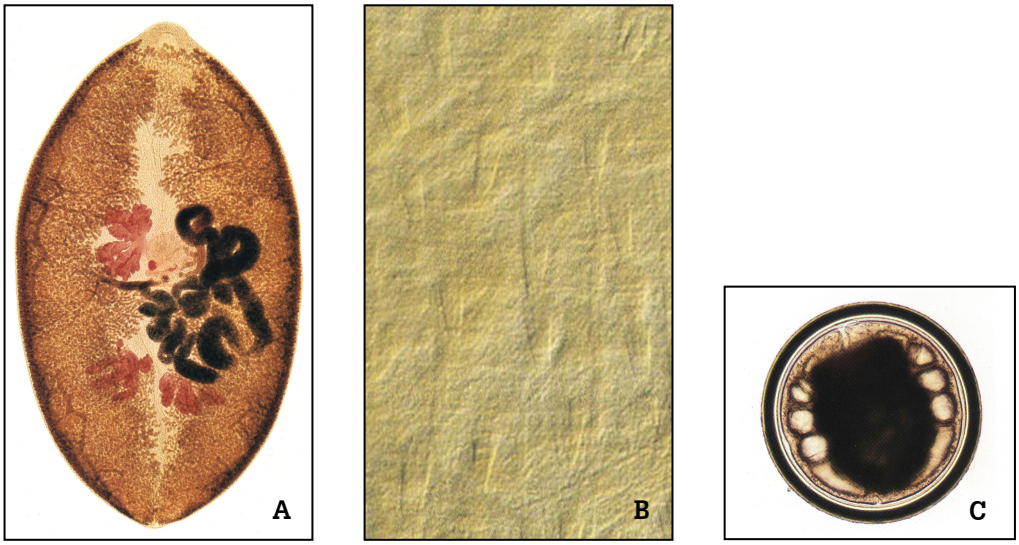

Figure 3. Morphology of *Paragonimus westermani* **A**: mounted adult specimen, **B**: cuticular spines and **C**: metacercaria

Source: Kenjiro Kawashima, editor. (1989). *Paragonimus in Asia: biology, genetic variation and speciation (II)*. unpaged.

***Paragonimus macrorchis* (Chen, 1962)**

The adult fluke of *P. macrorchis* (Figure 4A) has delicately branched ovary. The testes are extraordinarily large and divided profusely. Cuticular spines are originally single. The ventral sucker is almost the same size or slightly larger than the oral sucker. The metacercaria (Figure 4B) is provided with two layers of cyst wall. Both outer and inner cyst walls are so thin and fragile that their form and size are changeable and easily broken by the movement of the larva inside. The average diameter of the inner cyst is 337 x 292 μm.

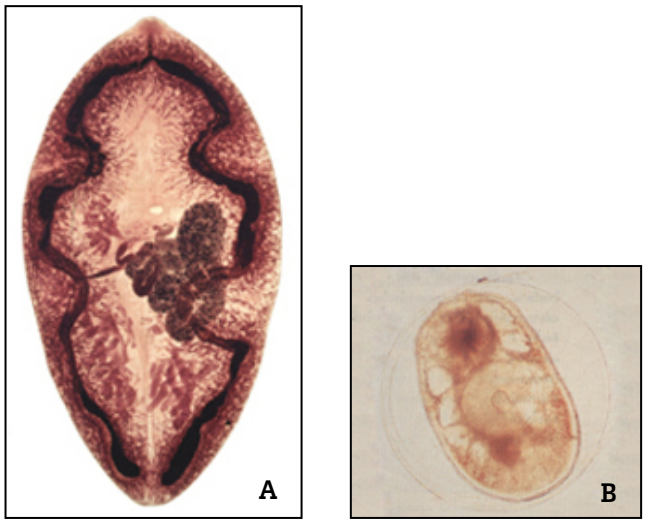

Figure 4. Morphology of *Paragonimus macrorchis* **A**: mounted adult specimen and **B**: metacercaria

Source: Decha Srison; Jitra Waikagul; & Sanan Yaemput. (1997). *Lung fluke*. p. 74.

Paragonimus heterotremus **(Chen et Hsia, 1964)**

The adult fluke of *P. heterotremus* (Figure 5A) has the oral sucker which is about twice as large as the ventral sucker. The surface of the body is covered with singly spaced cuticular spines (Figure 5B). The testes and ovary are delicately branched and the former usually larger than the latter. The metacercaria (Figure 5C) is oval in shape and provided with thin outer and thick inner cyst walls. The inner cyst wall is gradually thickened at both poles. This kind of thickening is the characteristic feature of this species. The average diameter of the inner cyst is 292 x 237 μm.

Paragonimus siamensis **(Miyazaki et Wykoff, 1965)**

The adult fluke of *P. siamensis* (Figure 6A) is very similar to *P. westermani*, but the cuticular spines of *P. siamensis* are always arranged in groups (Figure 6B). The metacercaria (Figure 6C) is oval in shape and possesses two layers of cyst wall. The outer wall is thin and fragile, while the inner wall is more elastic and stronger. The average diameter of the inner cyst is 480 x 340 μm. There are minute pinkish globules scattered throughout the larval body.

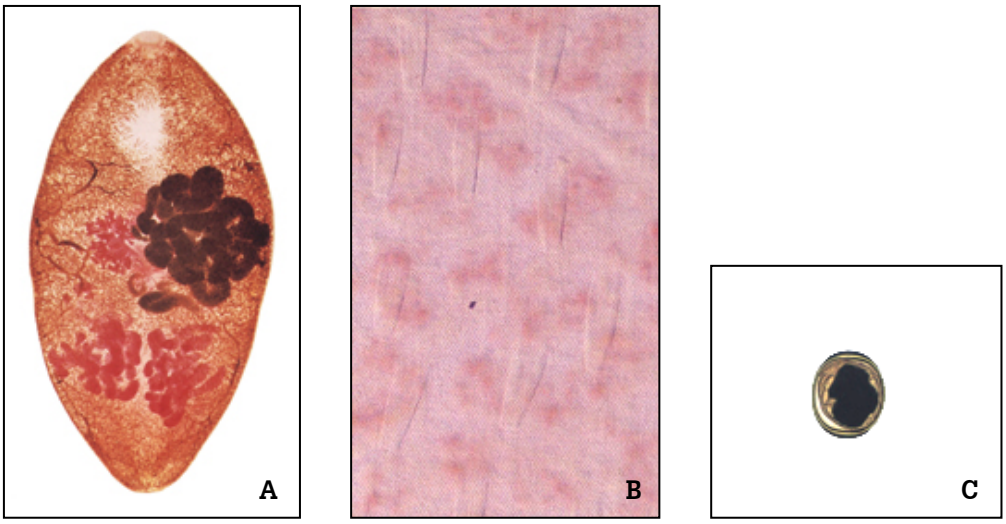

Figure 5. Morphology of *Paragonimus heterotremus* **A**: mounted adult specimen, **B**: cuticular spines and **C**: metacercaria

Source: Kenjiro Kawashima, editor. (1989). *Paragonimus in Asia: biology, genetic variation and speciation (II)*. unpaged.

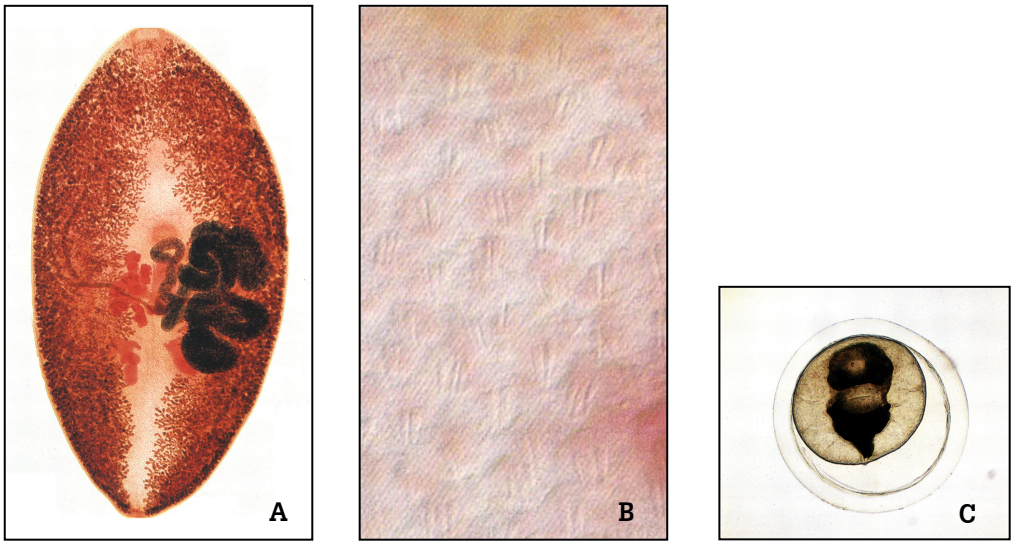

Figure 6. Morphology of *Paragonimus siamensis* **A**: mounted adult specimen, **B**: cuticular spines and **C**: metacercaria

Source: Kenjiro Kawashima, editor. (1989). *Paragonimus in Asia: biology, genetic variation and speciation (II)*. unpaged.

***Paragonimus bangkokensis* (Miyazaki et Vajrasthira, 1967)**

The body of *P. bangkokensis* adult fluke (Figure 7A) is somewhat elongated and covered with grouped cuticular spines (Figure 7B). The oral sucker is slightly smaller than the ventral sucker. The ovary is moderately branched while testes are divided into about four broad lobes without remarkable subdividing. Both ovary and testes are almost similar in size. The metacercaria (Figure 7C) is spherical or suboval in shape and possesses two layers of cyst wall. The wall of the outer cyst is thin and fragile, and that of the inner cyst is also thin but more elastic and stronger. The average diameter of the inner cyst is 435 x 398 μm. There is a large space between larva and the inner cyst. The larva usually contains numerous pinkish globules within the body and pale yellowish granules are usually observed within the inner cyst.

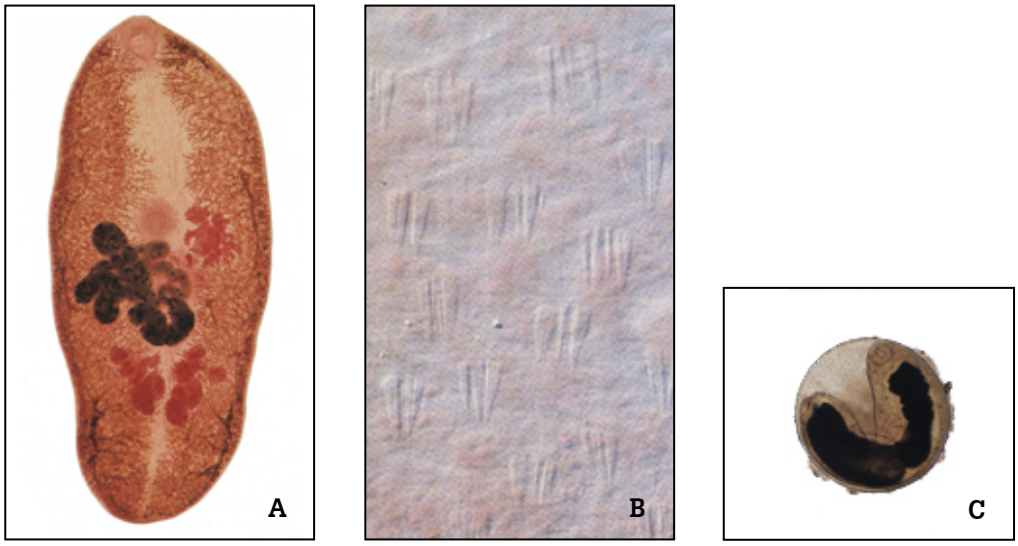

Figure 7. Morphology of *Paragonimus bangkokensis* **A**: mounted adult specimen, **B**: cuticular spines and **C**: metacercaria

Source: Kenjiro Kawashima, editor. (1989). *Paragonimus in Asia: biology, genetic variation and speciation (II)*. unpaged.

***Paragonimus harinasutai* (Miyazaki et Vajrasthira, 1968)**

The adult fluke of *P. harinasutai* (Figure 8A) has singly spaced cuticular spines (Figure 8B). The ventral sucker is slightly larger than the oral sucker. The ovary is moderately branched but appears compact as a whole because its lobes are short. Testes are much more simply branched than the ovary. Both ovary and testes are almost similar in size. The metacercaria (Figure 8C) is spherical in shape and provided with outer and inner cyst walls.

The wall of the outer cyst is easily broken while the inner cyst is thicker and much stronger. The average diameter of the inner cyst is 601 x 579 μm. The larva usually contains many pinkish globules within the body. There are different sizes of numerous pale yellowish granules in the space between the larval body and the inner cyst.

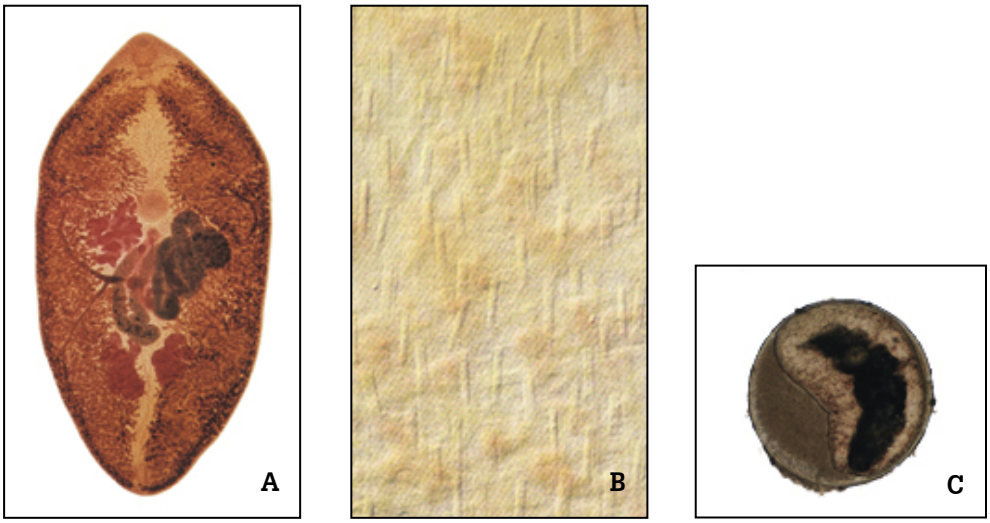

Figure 8. Morphology of *Paragonimus harinasutai* **A**: mounted adult specimen, **B**: cuticular spines and **C**: metacercaria

Source: Kenjiro Kawashima, editor. (1989). *Paragonimus in Asia: biology, genetic variation and speciation (II)*. unpaged.

Life cycle of *Paragonimus*

Paragonimus species require two intermediate hosts, aquatic snails and crustaceans, to complete their life cycles. Definitive host mammals, including humans, become infected by ingesting raw crustaceans containing metacercariae. The life cycle of *Paragonimus* species is illustrated in Figure 9.

Adult lung flukes are located in pairs in granulomatous capsules in the lungs. Eggs are asymmetrically ovoid, thick-shelled and golden brown in color. The operculum is flattened, with a thickened opercular rim. These operculated eggs are discharged from the flukes, through the cyst into the bronchi, where they are coughed up in the sputum or swallowed and passed in the feces. Eggs develop and hatch in about 3 weeks in freshwater, releasing a ciliated miracidium that swims to a suitable snail which acts as the first intermediate host (Figure 10).

The miracidium, covered with ciliated ectodermal plates, penetrates the tissue of the

snail, localizing in the hemocoel where it becomes a mother sporocyst. The process of polyembryony produces rediae and enormous numbers of cercariae.

Cercariae emerging from rediae possess stylets anteriorly and short, stumpy tails. They leave the snail and crawl to a suitable crustacean which acts as the second intermediate host (Figure 11). They then encyst into infective metacercariae in gills, muscles, legs and viscera.

When the crustacean which contains metacercariae is ingested by a definitive host, the metacercariae excyst in the intestine, migrate to the lungs and become adult worms which live in pairs. In paratenic hosts, the juvenile worms persist in the tissues but cannot mature into adult worms. Only when these hosts are eaten by a more suitable host, the juvenile worms can then mature.[1, 21]

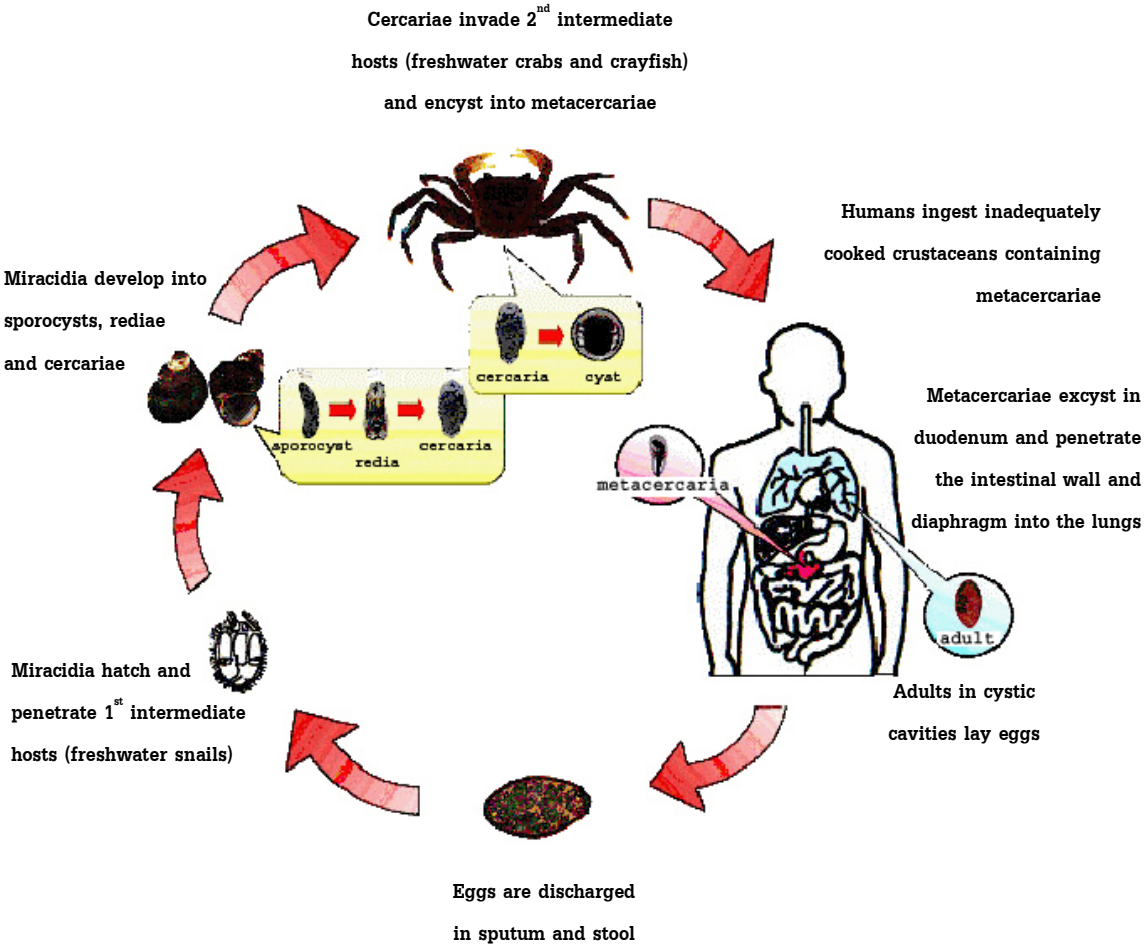

Figure 9. Life cycle of *Paragonimus*

Source: modified from Achariya Rangsiruji; et al. (2004, September-December). Genetic diversty of lung flukes in central and southern Thailand. *Journal of Scientific Research Chulalongkorn University* (section T). 3(3): 198.

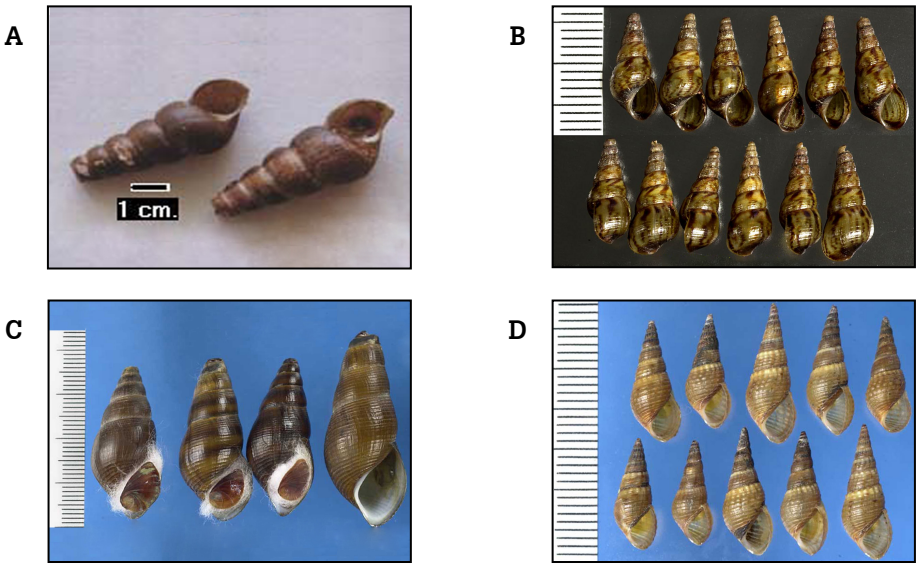

Figure 10. Aquatic snails as the first intermediate hosts of *Paragonimus* species **A**: *Brotia costula* (http://www.champa.kku.ac.th/somphong/snail/snail.htm), **B**: *Melanoides tuberculata* (http://shell.kwansei.ac.jp/~shell/pic_book/data31/r003078.html), **C**: *Semisulcospira libertina* (http://shell.kwansei.ac.jp/~shell/pic_book/data01/a0860.html), **D**: *Tarebia granifera* (http://shell.kwansei.ac.jp/~shell/pic_book/data29/r002808.html)

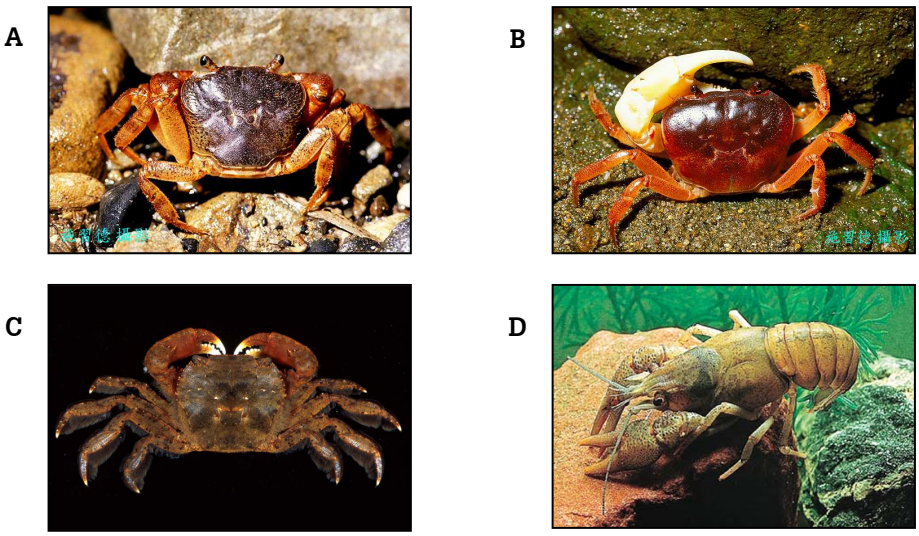

Figure 11. Freshwater crabs and crayfish as the second intermediate hosts of *Paragonimus* species **A**: *Candidiopotamon ratbuni* (http://crusta10.de/index.php?sideid=galerie&showpicture=1216&galerie_id=313), **B**: *Geothelphusa dehaani* (http://crusta10.de/index.php?sideid=galerie&showpicture=1227&galerie_id=69), **C**: *Varuna litterata* (http://decapoda.free.fr/illustration.php?n=2&sp=846), **D**: *Cambaroides schrenkii* (http://crusta10.de/index.php?sideid=galerie&showpicture=1143&galerie_id=272)

Clinical manifestation and pathology

Paragonimiasis is a chronic disease, persisting in some cases for many years. Pathological effects are due to the presence of adult worms and their eggs in the lungs and other organs, the movement of worms through tissues and the metabolites produced by worms. During their association with the human host, lung flukes penetrate the diaphragm, pass through the pleural cavity and then invade the lung parenchyma. The common symptoms are chest pain, cough with rust-colored sputum, fatigue, fever, focal hemorrhagic pneumonia and fibrotic encapsulation in the lung parenchyma. Extrapulmonary paragonimiasis can occur when the flukes migrate to ectopic sites, such as brain, spinal cord, liver, intestinal wall, peritoneum and heart. Cerebral paragonimiasis is most frequently encountered and is fatal.[reviewed in 23]

Diagnosis

Paragonimiasis may conceal the presence of tuberculosis and is regularly responsible for misdiagnosis of this killer disease. Hemoptysis (rust-colored sputum) is a characteristic of paragonimiasis and the cardinal sign of tuberculosis. Diagnosis of *Paragonimus* infection usually depends on the finding of eggs in sputum or stool samples and chest x-ray. However, egg production and transport from the lungs to the mouth is often erratic so that eggs may not be present if only one sputum sample is collected and examined or if a mild infection is present. Radiological findings of paragonimiasis are often indistinguishable from those of pulmonary tuberculosis and, on occasion, lung cancer. Alternative methods for the diagnosis of paragonimiasis include immunological assays, such as ELISA, intradermal tests and western blotting.[reviewed in 23]

CHAPTER TWO

LITERATURE REVIEW

The lung fluke was first reported in the otter from Brazil in 1850. Later, in 1878, the flukes were described by Kerbert from adult worms obtained in the lungs of a Bengal tiger died in Amsterdam Zoo. In 1879, Ringer reported the first case of human infection in Taiwan in which the patient was a Portuguese sailor who had lived in Taiwan for many years and died from a ruptured aortic aneurysm. In 1880, Manson in China and Baelz in Japan independently described parasite eggs in the sputum of patients. Both came to the conclusion that the eggs belonged to the lung flukes. At first, the lung flukes from the Bengal tiger were named *Distoma westermani* (Kerbert, 1878) and the flukes from man were named *D. ringeri* (Cobbold, 1880) and *D. pulmonalis* (Baelz, 1880). In 1889, Leuckart and Nakahama observed specimens from various hosts and realized that the parasites from man were identical to the parasites from the tiger. Later, Kerbert's lung flukes were classified into the genus *Paragonimus* by Braun in 1899 and are known as *Paragonimus westermani*.[reviewed in 24]

Paragonimus westermani (Kerbert, 1878) Braun, 1899 is the best known human pathogen which infects millions of people in Asia. It consists of a complex of cryptic species. Based on chromosomal studies *P. westermani* was found in three forms of diploid, triploid and tetraploid. The diploid form (chromosome number 2n = 22)[25] has bisexual reproduction and produces numerous normal sperm. The triploid form (3n = 33) has parthenogenetic reproduction and exhibits aberrant spermatogenesis, producing a few or none of normal sperm.[26] Triploid worms are larger and more pathogenic in humans. While the diploid form is distributed throughout Asia the triploid form is restricted only to eastern Asia, including Japan, Korea, Taiwan and China.[reviewed in 1] The tetraploid form (4n = 44) produces diploid sperm, but it remains unknown whether these sperm are biologically active.[27] This form of polyploidy was reported only in northeastern China where all three forms of ploidy (diploid, triploid and tetraploid) occur.[28] Terasaki *et al*. (1995) considered that these tetraploids were autotetraploids and probably produced by the fertilization of diploid and triploid individuals.[27]

From the isozyme studies of *P. westermani* in East and South East Asia carried out by Agatsuma *et al*. (1988, 1993)[29-30] it was observed that genetic distances of diploid populations in East Asia (Japan and Taiwan) were different form those in South East Asia (Malaysia and the Philippines). The diploid populations from East Asia were relatively similar to one another and

to the triploid populations, but in South East Asia the diploid populations were genetically distant from each other.

During the past several decades, molecular biology has been rapidly developed. Applications of molecular biology tools such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequencing techniques have become widespread. These techniques have been employed to resolve biological problems.

Molecular markers for phylogenetic studies

In situations where morphological variation is limited or the homology of morphological features is unclear, molecular approaches may provide new insights and solutions for some of the organism systematics. Ideal molecular markers for studies which involve closely related organisms should have rapid evolutionary rates and provide unambiguous data. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) and cytochrome *c* oxidase subunit I (COI or *cox*1) in mitochondrial DNA (mtDNA) are widely used as molecular markers in characterizations and phylogenetic studies.

Nuclear rDNA, which encodes rRNAs, has been most commonly applied in phylogenetic approaches. Eukaryotic nuclear rDNA is tandemly organized with high copy numbers up to approximately 5,000. Each repeat unit consists of genes coding for the nuclear small subunit (18S), large subunit (28S) and 5.8S rDNAs. These coding regions are separated from each other by a spacer called an intergenic spacer (IGS). The 5.8S rDNA is embedded in the two internal transcribed spacers (ITS1 and ITS2)[31] as shown in Figure 12.

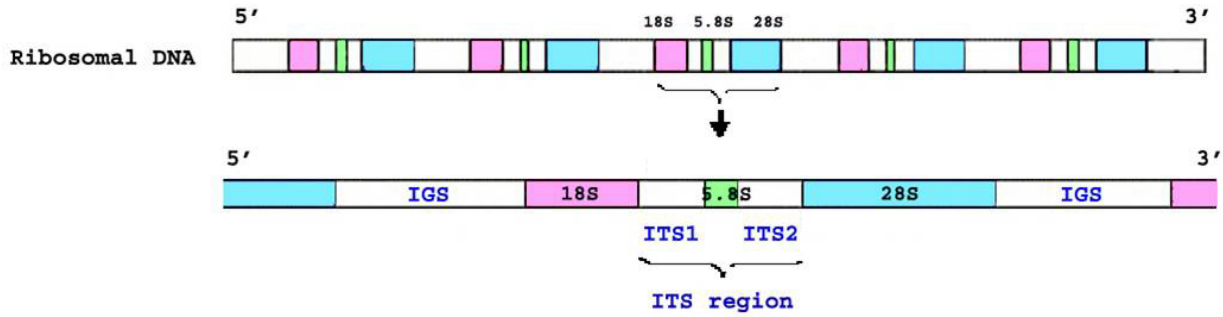

Figure 12. Repeat units of the nuclear ribosomal DNA and the organization of the ITS region.

Source: modified from Achariya Rangsiruji; et al. (2004, September-December). Genetic diversity of lung flukes in central and southern Thailand. *Journal of Scientific Research Chulalongkorn University* (section T). 3(3): 199.

The nuclear rDNA spacer regions evolve much faster than the nuclear rRNA coding regions and substitutions occurring in the spacer regions do not show lethal effects on the organisms. In contrast, mutations of rRNA coding regions can prevent the successful ribosome formation, which could have negative affects on the protein synthesis machinery. Thus, the rRNA coding regions are more conserved compared to the spacer regions.[31] Due to the high variability, the ITS region (ITS1, 5.8S and ITS2) has been employed to resolve phylogenetic problems in lower taxonomic levels among genera, species or populations.[32 33]

The majority of genetic information of eukaryotic cells is located in the nucleus. However, these cells also contain mitochondrial genomes. Mitochondrial genomes are circular, double stranded, non-recombining, maternally inherited and present in high copy numbers. In animals, mitochondrial genomes ordinarily contain 36-37 genes: two for rRNAs, 12-13 for proteins which are vital components of the respiratory chain enzyme complexes and 22 for tRNAs.[34 35] Mitochondrial DNA sequences of animals evolve at faster rates than nuclear sequences. Consequently, most of the mitochondrial protein coding genes such as COI and NADH dehydrogenase 1 (ND1), have been used to examine phylogenetic relationships in the levels of genera, species or populations.[36 37]

Complete mitochondrial genome of *Paragonimus westermani* was first submitted to the GenBank by Agatsuma and Iwagami (1999) (unpublished; GenBank accession number NC002354). The genome size is approximately 15 kb. It contains 37 genes: two for rRNAs, 12 for proteins (including COI with approximately 1.5 kb in size) and 23 for tRNAs (Figure 13).

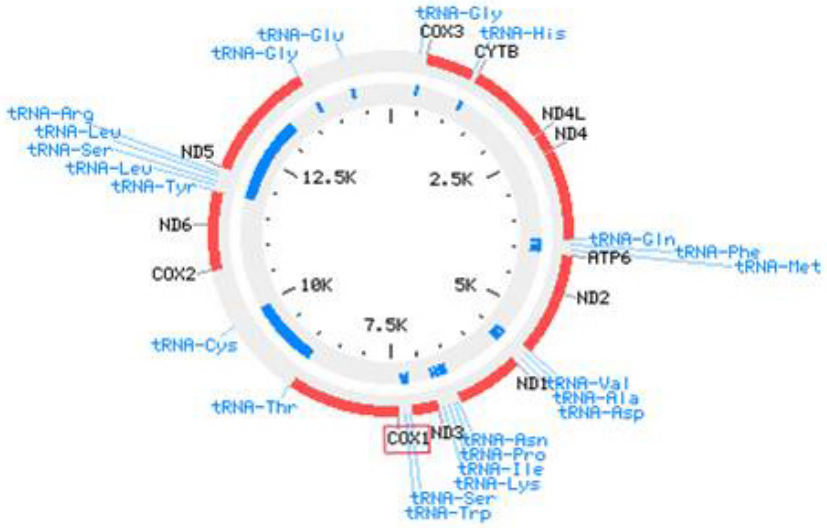

Figure 13. Mitochondrial DNA map of *P. westermani* and the position of COI (*cox*1) gene.

Source: http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=15422

Molecular phylogenies of the genus *Paragonimus*, using the nuclear rDNA ITS region and mtDNA COI region, have made many insights into the identities and relationships of the lung flukes. Nucleotide sequence analyses of the ITS2 and COI regions have shown that *P. westermani* is partitioned into at least 2 groups (Figure 14). One group from East Asia (China, Japan, Korea, Taiwan), which is relatively uniform and includes both diploid and triploid forms.[20, 36] Park *et al*. (2003) reported that this group exhibited relatively small molecular variation and postulated that the triploid form could have arisen somewhere in the East Asia group.[20] The second group from South East Asia (Malaysia, Thailand, the Philippines) includes members which are genetically distant from one another.[20,36] These results were in accordance with previous isozyme studies by Agatsuma *et al*. (1988, 1993).[29 30]

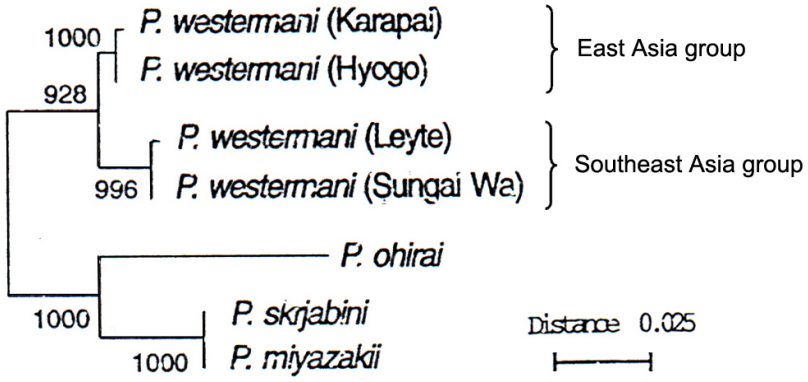

Figure 14. Phylogenetic relationships among *Paragonimus* species inferred from the ITS2 data based on the neighbor-joining approach. The data set was bootstrapped 1,000 times and the bootstrap values were placed on branches. Note: Identical ITS2 sequences were obtained from all Japanese, Korean and Chinese *P. westermani* isolates, whether diploid or triploid. Only the diploid from Hyogo is included as a representative of these isolates. The isolates of *P. westermani* included in the tree were from Karapai (Taiwan), Hyogo (Japan), Leyte (the Philippines), Sungai Wa (Malaysia).[36]

Blair *et al*. (1998)[33] used DNA sequences from the ITS2 and COI regions to investigate relationships among *Paragonimus* species from Thailand and elsewhere. Trees inferred from nucleotide sequences from both regions were all broadly congruent with one another (Figure 15). The tree suggests the existence of two main groups in the genus. One consists of *P. westermani* and *P. siamensis*. The other contains all other species from Asia and the Americas. Therefore, molecular data have confirmed a close relationship between *P. westermani* and *P. siamensis*.

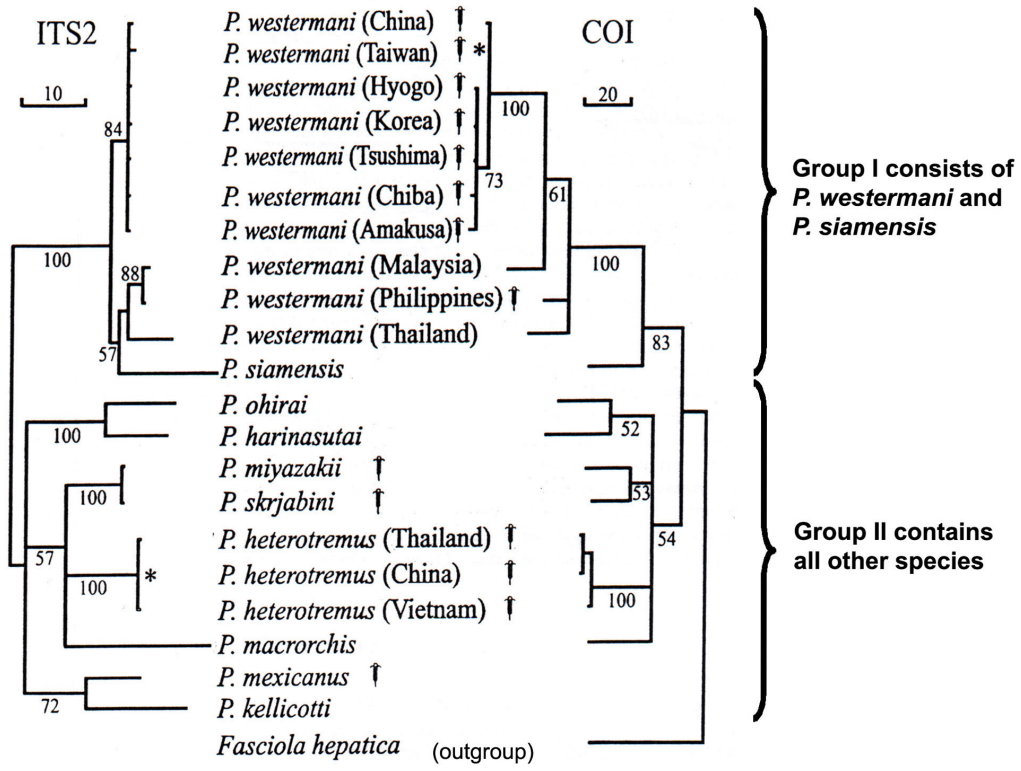

Figure 15. Phylogenetic relationships among *Paragonimus* species from Thailand and elsewhere based on the ITS2 and COI regions. Numbers on branches indicate bootstrap values. Isolates of *P. westermani* were from Fujian (China), Karapai (Taiwan), Hyogo, Chiba, Tsushima and Amakusa (Japan), Bogil Island (Korea), Peninsular Malaysia, Leyte (the Phillipines) and Thailand. Isolates from Tsushima, Amakusa and Korea were all triploids. Species or populations known to infect humans are indicated by a syringe symbol. Isolates not sequenced for one of the markers are indicated by an asterisk (*).[33]

In Thailand, many reports concerning about *P. westermani* including morphological, isozyme and molecular studies were confined to the metacercariael stage.[15-17] In all studies the metacercariae of this species were collected from the central part of Thailand. There was no report of *P. westermani* in other parts of the country except in Chumphon province where adult worms of *P. westermani* were obtained from the naturally infected leopards. The finding of these adult worms had led Kawashima and colleagues to start searching for *P. westermani* in the south.[38] Their surveys in Surat Thani province in 1987 were proved to be successful because *P. westermani* metacercariae were discovered. Later they found another type of metacercariae which resembled *P. westermani* but had a smaller size (personal communication). These metacercariae were called *P. westermani*-like. At present, there was no further study of both *P. westermani* and *P. westermani*-like from southern Thailand. Therefore, detailed studies should be carried out to confirm identities of both organisms and their relationships with related species.

Objectives of the study

1. To clarify morphological characteristics at the adult stage of Thai *P. westermani*, *P. westermani*-like and *P. siamensis*.
2. To investigate the susceptibility of mammalian hosts to Thai *P. westermani*, *P. westermani*-like and *P. siamensis*.
3. To study phylogenetic relationships of Thai *P. westermani*, *P. westermani*-like and *P. siamensis* with other *Paragonimus*.

CHAPTER THREE

MATERIALS AND METHODS

Materials

Chemicals and reagents used in this study are listed below.

Chemicals and reagents	Brands
10X z-*Taq* buffer	Takara
5X loading buffer	Bioline
Agar powder	Himedia
Agarose	GibcoBRL
Ampicillin	Roche
Bacto™ Yeast Extract	Difco
Boric acid	Promega
Canada balsam	Merck
Carmine	Fluka
DNeasy® Tissue Kit	Qiagen
dNTP mixture	Takara
*Eco*RI	New England Biolabs
EDTA (ethylenediaminetetraacetic acid)	Promega
Ethanol	Merck
Ether	J.T.Baker
Ethidium bromide	BIO-RAD
Formaline	Carlo Erba
Glacial acetic acid	Merck
Glucose	GibcoBRL
Glycogen	Nippon Gene
Hydrochloric acid	Merck
Hyper ladder I	Bioline
IPTG (isopropylthiogalactoside)	Promega
Isopropanol	Merck

Chemicals and reagents	**Brands**
Nuclease-free water	GibcoBRL
Pepsin	Nacalai tesque
pGEM®-T Easy Vector System I	Promega
Potassium acetate	Merck
Proteinase K	Qiagen
QIAGEN® Plasmid Mini Kit	Qiagen
QIAquick™ Gel Extraction Kit	Qiagen
QIAquick™ PCR Purification Kit	Qiagen
RNase A	Qiagen
Sodium acetate	Carlo Erba
Sodium chloride	Univar
SDS (sodium dodecyl sulfate)	Promega
Sodium hydroxide	Merck
Sodium tetraborate	Carlo Erba
Tris base	Promega
Tryptone Type I	Himedia
X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside)	Promega
Xylene	Merck
z-*Taq* DNA polymerase	Takara

Methods

1. Source of parasitic materials

1.1 Collection of crab intermediate hosts

The second intermediate hosts, including waterfall and rice field crabs, used in this study are listed in table 1.

Table 1. Species and locations of crabs used in this study.

Species of crabs		Locations	
Larnaudia larnaudii	(waterfall crab)	Saraburi province	(central Thailand)
Larnaudia larnaudii	(waterfall crab)	Prachin Buri province	(central Thailand)
Ranguna smalleyi	(waterfall crab)	Surat Thani province	(southern Thailand)
Phricotelphusa aedes	(waterfall crab)	Surat Thani province	(southern Thailand)
Sayamia germaini	(rice field crab)	Prachin Buri province	(central Thailand)

1.2 Collection of metacercariae

Metacercariae of *P. westermani* and *P. westermani*-like were collected from waterfall crabs whereas those of *P. siamensis* were harvested from rice field crabs. Separate species of crabs were ground using blender and digested with pepsin (1%, w/v) and concentrated HCl (0.7%, v/v). The preparation was stirred for 1-2 hours at 37°C, filtered and 0.9% saline was added. It was allowed to stand for approximately 1 hour at room temperature and the saline was changed 5-6 times or until the supernatant becomes clear. The sediment was examined under a stereomicroscope for *Paragonimus* metacercariae. The metacercariae recovered were washed several times with the saline and kept at 4°C. They were used for experimental infection and DNA extraction.

1.3 Experimental infection and recovery of *Paragonimus* adult worms

Adult worms of *P. westermani*, *P. westemani*-like and *P. siamensis* were obtained from the lungs of specific pathogen free cats which were peritoneally inoculated with 20 to 60 metacercariae of Thai *P. westermani*, *P. westemani*-like and *P. siamensis*, isolated from naturally infected crabs. The cats were kept at the

Department of Parasitology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan. The adult flukes were provided by Dr. Hiromu Sugiyama.

Adult worms of *P. westermani* and *P. siamensis* were obtained from the lungs of Wistar rats (National Laboratory Animal Centre, Mahidol University) which were fed with 20 to 120 metacercariae isolated from waterfall and rice field crabs. Two months after infection, feces of the rats were examined for *Paragonimus* eggs. The feces were softened by soaking in distilled water overnight and 10% formaline was added. The sample was then filtered through gauze and the flow-through was collected and centrifuged at 740 xg for 10 minutes. The sediment was added with 10% formaline:ether (4:1), mixed vigorously and centrifuged at 740 xg for 10 minutes. The sediment was examined under a light microscope for *Paragonimus* eggs. The infected rats were sacrificed and the lungs were washed with the saline. The lung cysts containing adult flukes were obtained. The active worms were washed with the saline and kept until used for whole mount preparations and DNA extraction.

2. Morphological studies (whole mount preparations)

2.1 Fixation

All adhering mucus and debris were removed from the worms by shaking gently with 0.9% saline which was changed several times. Each worm was placed on a glass slide and covered with another glass slide. These two glass slides were separated with cardboards (about 1 mm thick) before applying pressure to flatten the worm. They were tied together with rubber bands and the entire preparation was soaked overnight in 70% ethanol.

2.2 Staining and mounting

The worm was untied, then transferred into Borax Carmine solution (Carmine (2%, w/v), Sodium tetraborate (4%, w/v)), and left overnight. The specimen was destained in acid alcohol (1% conc. HCl in 70% ethanol) until it becomes light pink in color. It was washed in 70% ethanol to remove acid and dehydrated by placing in 80% ethanol, 95% ethanol, absolute ethanol and xylene, respectively for 3 hours each. The worm was mounted with Canada balsam for further examination.

3. Molecular studies

3.1 Extraction of genomic DNA from single metacercaria or adult worm

Genomic DNA from an individual metacercaria was prepared. The metacercaria was pipetted into 1.5 ml microcentrifuge tube under a stereomicroscope. It was lysed with lysis buffer containing SDS/proteinase K (99:1), then centrifuged at 13,800 xg for 15-20 seconds, and incubated at 60°C for 1-3 hours. The 0.5 μl of RNase A (100 mg/ml) was added, followed by heating at 95°C for 10 minutes. The DNA was precipitated by adding 0.23 μl of glycogen, 0.8 μl of 3M sodium acetate and 19.8 μl of absolute ethanol, then allowed to stand for 10-15 minutes at −70°C. The suspension was centrifuged at 13,800 xg for 15 minutes and the supernatant was discarded. The DNA pellet was washed with 70% ethanol and centrifuged at 13,800 xg for 5 minutes. The resultant pellet was resuspended in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and kept at −20°C until used.

In the case of adult worms, the genomic DNA from each individual was extracted using DNeasy® Tissue Kit. The procedure was modified from the instruction manual provided by the company. The adult worm was ground in 180 μl of lysis buffer (50 mM Tris-HCl, pH 9.0, 100 mM EDTA, 50 mM Sodium chloride, 2% SDS) and 20 μl of Proteinase K was added and mixed gently. The tube containing the tissue was incubated at 55°C until the tissue was completely lysed. Four microliters of RNase A was added and the tube was allowed to stand for 2 minutes at room temperature. Two hundred microliters of buffer AL was added, mixed immediately and incubated at 70°C for 10 minutes. Two hundred microliters of absolute ethanol was added into the mixture and mixed gently. The mixture was applied to the DNeasy spin column, centrifuged for 1 minute at 5,220 xg and the flow-through was discarded. To wash the column, 500 μl of buffer AW1 was added and centrifuged at 5,220 xg for 1 minute. The column was washed again with 500 μl of buffer AW2 and centrifuged at 16,000 xg for 3 minutes. The genomic DNA was eluted by adding 50 μl of buffer AE. The tube was allowed to stand at room temperature for 1 minute and centrifuged at 5,220 xg for 1 minute. The DNA will be kept at −20°C until used.

3.2 Polymerase Chain Reaction (PCR) for amplifications of the internal transcribed spacer (ITS) region and partial cytochrome oxidase subunit I (COI) region

Synthetic oligonucleotide primers purchased from Operon Biotechnologies which were used in PCR and sequencing are listed in table 2. While Pwfor and A28 primers were used to amplify the ITS region, JB3 and JB4.5 primers were used to amplify a portion of the COI region. The PCR amplifications were performed using MiniCycler™ (MJ Research, PTC-150).

Table 2. PCR and sequencing primers.

Primers	Sequences of primers from 5' to 3'	Directions	References
Pwfor	5'-TTA TAC TTG CAG CAG GGT GCC-3'	forward	Van Herwerden *et al*., 1999 [39]
A28	5'-GGG ATC CTG GTT AGT TTC TTT TCC TCC GC-3'	reverse	Blair *et al*., 1997b [36]
JB3	5'-TTT TTT GGG CAT CCT GAG GTT TAT-3'	forward	Bowles *et al*., 1995 [40]
JB4.5	5'-TAA AGA AAG AAC ATA ATG AAA ATG-3'	reverse	Bowles *et al*., 1995 [40]

For amplifications of the ITS and partial COI regions, each reaction was performed in 25 μl containing 50 ng genomic DNA template, 1X z-*Taq* buffer, 0.2 mM of each dNTP, 0.5 μM of each primer and 1 unit of z-*Taq* DNA polymerase. The PCR cycle consisted of three major steps: 98°C for 5 seconds to denature DNA, 55°C for 10 seconds for annealing and 72°C for 10 seconds for extension. The cycle was repeated 30 times, followed by a final extension at 72°C for 10 minutes.

PCR products were examined using electrophoresis through 1% (w/v) agarose gel in 1X TBE buffer (diluted from 10X TBE stock, 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA) at 100 volts for approximately 50 minutes. To confirm the correct size of the PCR products Hyper ladder I marker was employed. The gel was stained with ethidium bromide, transilluminated under ultraviolet light and then photographed.

3.3 Purification of PCR products

3.3.1 Using QIAquick PCR Purification Kit

The procedure of purification was performed according to the instruction manual provided by the company. Five volumes of buffer PB were added to 1 volume of the PCR reaction and mixed. A QIAquick spin column was placed in a provided

2-ml collection tube and the sample was applied to the QIAquick column and centrifuged for 30-60 seconds at 13,800 xg. The flow-through was discarded and the column was placed back into the same tube. To wash the product, 750 μl of buffer PE was added to the column which was then centrifuged for 30-60 seconds at 13,800 xg. The flow-through was again discarded and the column was placed back into the same tube and centrifuged for an additional 1 minute at 13,800 xg. The column was transferred to a clean 1.5 ml microcentrifuge tube, and 40 μl of sterile nuclease-free water was added to the center of the QIAquick membrane. The column was allowed to stand for 1 minute, and then centrifuged for 1 minute at a maximum speed. The products were analyzed using electrophoresis through 1% (w/v) agarose gel.

3.3.2 Using QIAquick™ Gel Extraction Kit

For the PCR products which consisted of multiple bands, the method of gel extraction was used in order to purify and obtain the required band product from the gel. The PCR products were separated using electrophoresis through 1.5% (w/v) agarose gel. The required DNA fragment was excised from the gel and purified using QIAquick™ Gel Extraction Kit. The procedure of purification was modified from the instruction manual provided by the company. The gel slices were weighed in microcentrifuge tubes and 3 volumes of buffer QG were added to 1 volume of the gel. The samples were incubated at 50°C for 10 minutes or until the gel was completely dissolved. The mixture was added with 10 μl of 3M sodium acetate and 1 gel volume of isopropanol, and the contents were well mixed. It was then applied into the QIAquick column and centrifuged at 13,800 xg for 2 minutes. After discarding the flow-through the column was placed back in the same collection tube. Five hundred microliters of buffer QG was added into the column and centrifuged at 13,800 xg for 1 minute. To wash the DNA, 750 μl of buffer PE was added, then centrifuged at 13,800 xg for 1 minute and the column was placed into a clean 1.5 ml microcentrifuge tube. The DNA fragments were eluted with 35 μl of sterile nuclease-free water to the center of the membrane. The column was allowed to stand for 1 minute, and then centrifuged for 1 minute at a maximum speed. The products were analyzed using electrophoresis through 1% (w/v) agarose gel.

3.4 Molecular cloning

3.4.1 DNA ligation condition

pGEM®-T Easy Vector was used for cloning. The physical map of this plasmid is shown in Figure 16. The plasmid and DNA fragment (ITS region) were ligated together using T_4 DNA ligase. The ligation was performed in 15 μl reaction by using the appropriate molar ratio of plasmid vector to insert, 3 units of T_4 DNA ligase and 1X rapid ligation buffer. Sterile nuclease-free water was used to make a final volume to 15 μl. The contents were mixed gently and kept at approximately 4°C for 16 hours.

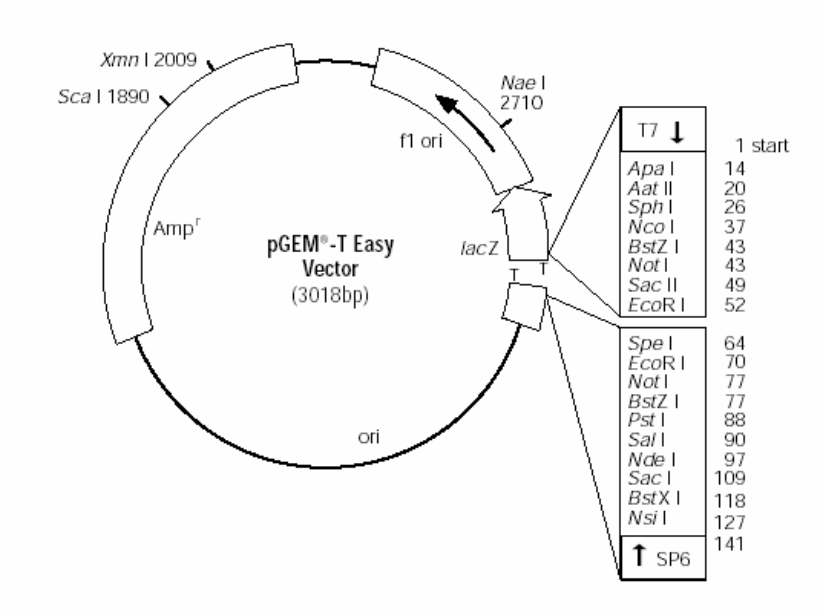

Figure 16. pGEM-T Easy Vector map and sequence reference points.

Source: Promega, pGEM-T and pGEM-T Easy Vector Systems Promega technical manual

3.4.2 Transformation and selection

The recombinant plasmids were transformed into *E. coli* strain XL1-Blue competent cells. The 15 μl of ligation mixture was added into 150 μl competent cells, mixed and incubated on ice for 30 minutes. The plasmids (both recombinant and non-recombinant types) were introduced into the competent cells by heat shock at 42°C for 2 minutes and the cells were put on ice immediately for 20 minutes. One milliliter of LB broth (tryptone (1%, w/v), Bacto™ Yeast Extract (0.5%, w/v), Sodium chloride (1%,

w/v)) was added to the mixture and incubated at 37°C for 2 hours. After incubation the transformed cells were spread on indicator LB agar plates (containing 100 μg/ml ampicillin, 25 μl of 40 mg/μl X-gal and 6 μl of 200 ng/ml of IPTG), and incubated at 37°C overnight.

Transformants were differentiated by blue/white screening using pGEM-T Easy Vector which contains a multiple cloning region within the *lac* Z gene. This gene codes for β-galactosidase enzyme that can hydrolyze chromogenic substrate (x-gal) and result in blue color product. Insertional inactivation of the *lac* Z gene allows recombinant clones to be identified on the indicator plates. Clones that contain PCR products are incapable of producing β-galactosidase enzyme, and result in white colonies. Ten to fifteen white colonies obtained was individually streaked on selective plates containing 100 μg/ml of ampicillin and incubated at 37°C overnight. Each clone was used for verification of the insert.

3.4.3 Plasmid extraction

3.4.3.1 Using Alkaline lysis method

Bacterial cultures for plasmid extraction were prepared by inoculating a single white colony into 1.5 ml LB broth containing 100 μg/ml of ampicillin and incubated at 37°C overnight. Each culture was centrifuged at 13,800 xg for 3 minutes and the supernatant was discarded. The bacterial pellet was resuspended in 100 μl of buffer GTE (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), then, allowed to stand for 5 minutes at room temperature. A mixture of 200 μl of 1% (w/v) SDS and 0.2 N sodium hydroxide was added, mixed gently and incubated on ice for 5 minutes. One hundred and fifty microliters of potassium acetate (60% (v/v) 5 M Potassium acetate, 11.5% (v/v) Glacial acetic acid) was added, mixed gently and incubated on ice for 7 minutes. The sample was then centrifuged at 13,800 xg for 3 minutes. The supernatant was transferred to a new microcentrifuge tube. Nine hundred microliters of absolute ethanol was added, mixed gently and incubated at -70°C for 5 minutes. The solution was then centrifuged at 13,800 xg for 10 minutes and the supernatant was discarded. The DNA pellet was washed with 500 μl of 70% ethanol, air dried for 5 minutes, and dissolved in 20 μl of TE buffer. The plasmid was kept in −20°C for analysis of recombinant plasmids.

3.4.3.2 Using QIAGEN® Plasmid Mini Kit

The procedure of plasmid extraction and purification was performed according to the instruction manual provided by QIAGEN Plasmid Mini Purification Kit. To prepare bacterial cultures for plasmid extraction, a single white colony was inoculated into 3 ml LB broth containing 100 μg/ml of ampicillin and incubated at 37°C overnight. Each culture was centrifuged at 13,800 xg for 3 minutes and the supernatant was discarded. The bacterial pellet was resuspended in 300 μl of buffer P1, leaving no cell clumps. To lyse the bacterial cells, 300 μl of buffer P2 was was added, mixed gently and incubated at room temperature for 5 minutes. The lysate was neutralized by the addition of 300 μl chilled buffer P3, mixed and incubated on ice for 5 minutes. It was then centrifuged at 13,800 xg for 10 minutes and the supernatant was removed promptly. QIAGEN-tips were set by placing in vials using tip holders provided with the kit. To equilibrate a QIAGEN-tip, 1 ml of buffer QBT was applied to the column and allowed to empty by gravity flow. The supernatant was then applied to the QIAGEN-tip. To wash the QIAGEN-tip, two times of 2 ml buffer QC were added. The QIAGEN-tip was placed over a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 800 μl of buffer QF. The DNA was precipitated by adding 560 μl of room temperature isopropanol, then centrifuged at 13,800 xg for 30 minutes and the supernatant was carefuly decanted. The DNA pellet was washed with 1 ml 70% ethanol, air dried for 5 minutes, and dissolved in 35 μl of sterile nuclease-free water. The purified plasmid was kept in – 20°C for sequencing.

3.4.4 Analysis of recombinant plasmids containing the ITS region

3.4.4.1 Using PCR reaction

The PCR reactions of the bacterial white colonies were used to screen the recombinant plasmids which contained the required DNA fragments. The bacterial cells were used directly for the amplification instead of the DNA template. The PCR components and condition were the same as previously described except for an additional preheating step at 98°C for 3 minutes used to lyse the bacterial cells. The PCR products were then analyzed using 1% (w/v) agarose gel electrophoresis.

3.4.4.2 Using endonuclease restriction (*Eco*RI)

*Eco*RI endonuclease was used to analyze the recombinant plasmids which contained the required DNA fragments. The reaction was performed in 20 μl using 8 μl of plasmid (from alkaline lysis method), 1X *Eco*RI buffer, 1 unit of *Eco*RI endonuclease and 10 μg of RNase A. Sterile nuclease-free water was used to make volume to 20 μl. The mixture was mixed gently and incubated at 37°C for 2-3 hours. The resultant product was analyzed using 1% (w/v) agarose gel electrophoresis.

3.5 DNA sequencing

DNA sequencing was performed by the Division of Genetic Resources, National Institute of Infectious Diseases, Japan and Macrogen Inc., Korea.

3.6 Phylogenetic analysis

The nucleotide sequences obtained were compared with other nucleotide sequences in the GenBank database using BLAST program from http://www.ncbi.nlm.nih.gov/BLAST. All sequence data from this study were aligned using Clustal X program[41] with additional sequences of *Paragonimus* species and *Fasciola hepatica* (outgroup) from the GenBank database. The alignments were corrected by eye and edited using GENEDOC program.[42] Phylogenetic trees were reconstructed using maximum parsimony analysis with branch-and-bound algorithm to infer the shortest tree. The analyses included the following setting: addition sequence (simple); branch-swapping algorithm (tree-bisection-reconnection or TBR); steepest descent option not in effect; branches collapsed (creating polytomies) if maximum branch length is zero; 'MulTrees' option in effect and topological constraints not enforced. Alignment gaps were treated as missing data, all characters were assigned equal weight. The reliability of internal branches of the trees was assessed using the bootstrap method,[43] with 1,000 replicates. Numbers of transitions, transversions, nucleotide differences and sequence divergence were computed. All phylogenetic analyses were performed using PAUP* version 4.0b.[44]

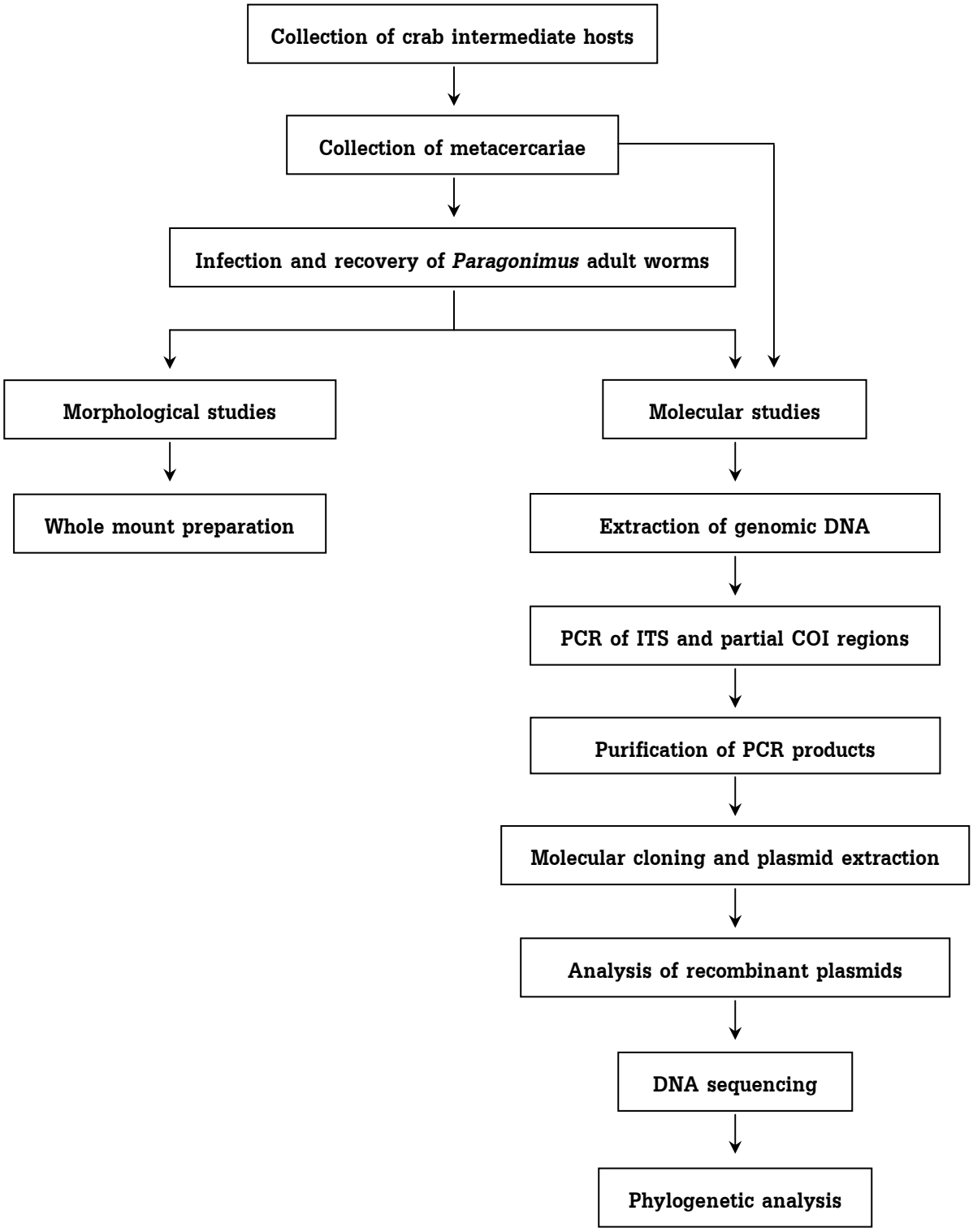

Figure 17. Summary of the methods used in this study.

CHAPTER FOUR

RESULTS

1. Collection of crab intermediate hosts

During the period of May 2003 to November 2005, field surveys on *Paragonimus* were carried out in the central and southern parts of Thailand. In the central part waterfall crabs, *Larnaudia larnaudii* (Figure 18A), the second intermediate host of *Paragonimus* species were collected in two localities, including Kaeng Khoi district, Saraburi province and Hin-tung sub-district, Nakhon Nayok province. Rice field crabs, *Sayamia germaini* (Figure 18B), were obtained in Na Di district, Prachin Buri province. In the southern part, two species of freshwater crabs, *Ranguna smalleyi* (Figure 18C) and *Phricotelphusa aedes* (Figure 18D), were caught from mountainous streams in Phanom district, Surat Thani province. All crabs obtained were further examined for *Paragonimus* metacercariae. The results of crab examination were shown in Table 3.

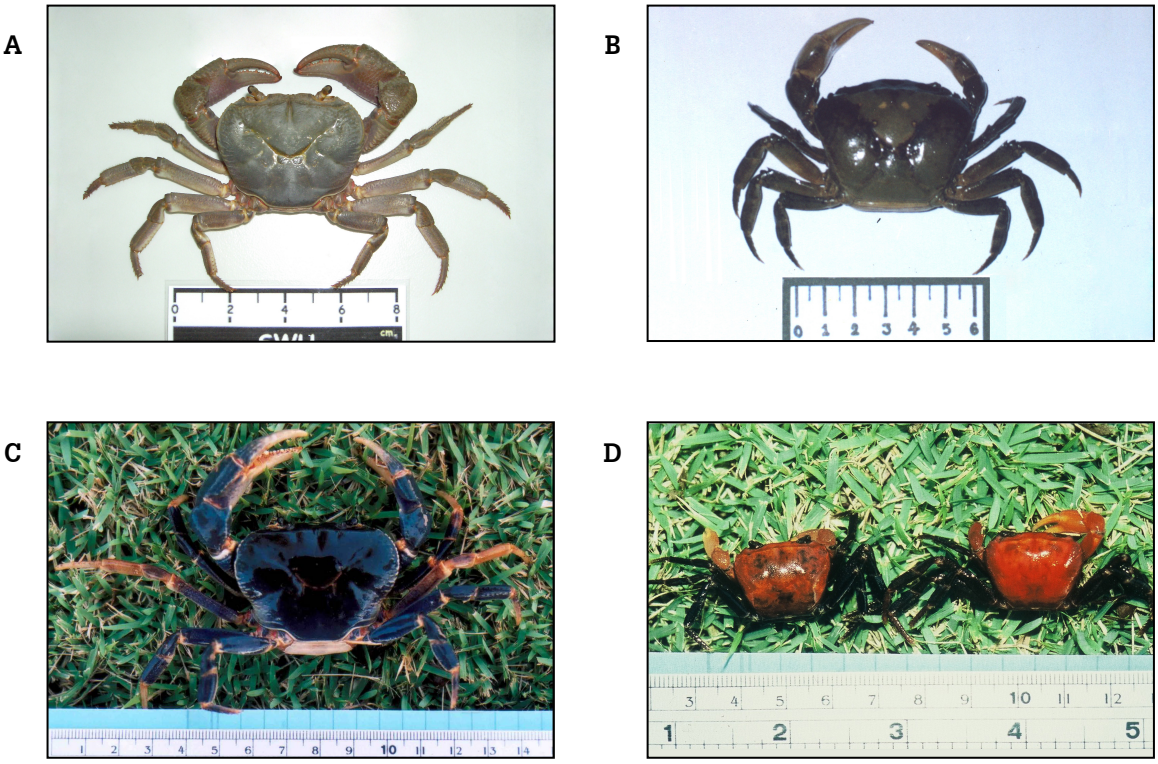

Figure 18. Freshwater crabs collected in field surveys **A**: *Larnaudia larnaudii*, **B**: *Sayamia germaini*, **C**: *Ranguna smalleyi*, **D**: *Phricotelphusa aedes*.

Table 3. Results of crab examination for *Paragonimus* infection.

Localities	Crab hosts	No. of crabs examined	No. of metacercariae collected			
			P. westermani	*P. westermani*-like	*P. siamensis*	Total
Central part						
Kaeng Khoi district Saraburi province	*Larnaudia larnaudii*	237	86	-	-	86
Hin-tung sub-district Nakhon Nayok province	*Larnaudia larnaudii*	107	19	-	-	19
Na Di district Prachin Buri province	*Sayamia germaini*	743	-	-	1738	1738
Southern part						
Phanom district Surat Thani province	*Ranguna smalleyi*	483	60	-	-	60
Phanom district Surat Thani province	*Phricotelphusa aedes*	1261	21	112	-	133

A total of 344 waterfall crabs, *L. larnaudii*, were collected from two different localities in the central part which were Kaeng Khoi district, Saraburi province and Hin-tung sub-district, Nakhon Nayok province. Average numbers of metacercariae per crab examined were 0.36 and 0.18, respectively. For the rice field crabs, *S. germaini*, 743 individuals were examined and the average number of metacercariae per crab was 2.34. In the southern part, two species of waterfall crabs, *R. smalleyi* and *P. aedes* were examined. The average numbers of metacercariae per crab were 0.12 and 0.11, respectively. All metacercariae recovered were differentiated into three types based on their morphological features. They were *P. westermani*, *P. westermani*-like and *P. siamensis*. The metacercariae of *P. westermani* were harvested from the waterfall crabs in both the central and southern parts while those of *P. westermani*-like were collected only in the southern part. The metacercariae of *P. siamensis* were isolated only from the rice field crabs in the central part.

2. Morphological observation on metacercariae recovered

The morphological features of *P. westermani* metacercariae (Figure 19A-C) from three localities, including Kaeng Khoi district (Saraburi province), Hin-tung sub-district (Nakhon Nayok province) and Phanom district (Surat Thani province) were identical. The encysted metacercariae were spherical in shape and provided with two layers of cyst wall. The inner cyst wall was fairly thick and measured approximately 29.5 μm. The outer cyst wall was thin and usually broken. In 69 fresh specimens measured, the long diameter of the metacercariae, excluding the outer membrane varied from 394 to 528 μm (average 473 μm), and the short diameter varied from 383 to 522 μm (average 463 μm) (Figure 20). The larva was fully extended to the entire cyst. It had a conspicuous excretory bladder in the center and the intestine wound on both sides of the bladder.

The characteristics of *P. westermani*-like metacercariae (Figure 19D) resembled *P. westermani* except the size was smaller. The metacercariae were enveloped with two layers of cyst wall. The inner cyst was spherical in shape with the thickness of the inner membrane measured about 9 μm. In 30 fresh specimens measured, the long diameter of the inner cyst varied from 212 to 252 μm (average 227 μm), and the short diameter varied from 204 to 240 μm (average 221 μm) (Figure 20). The larval body was expanded to the entire cyst. It had a conspicuous excretory bladder in the center and the transparent intestine wound on both sides of the bladder.

The metacercariae of *P. siamensis* (Figure 19E) were spherical or suboval in shape and provided with outer and inner cyst walls. The outer cyst wall was thin and had a dome shape on one side which was used to adhere to the host tissues. The inner cyst wall was approximately 7 μm in thickness. The inner cyst was slightly larger than that of *P. westermani* with an average of 517 x 492 μm in longitudinal and transverse diameters. The larval body did not expand to the entire cyst so there was a large space between the larva and the inner cyst. The encysted larva had excretory bladder in the center and the intestine wound on both sides.

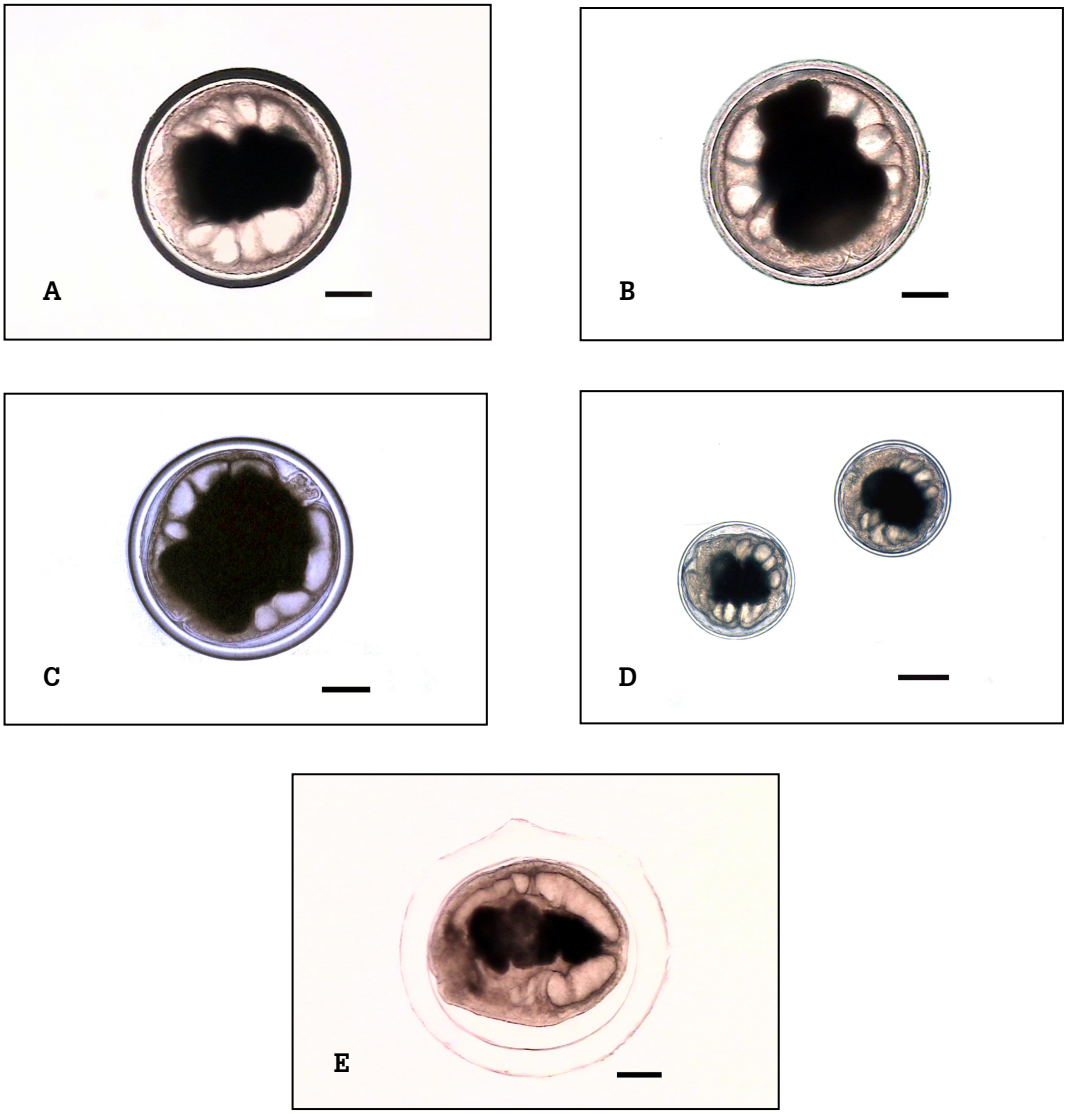

Figure 19. Metacercariae of *Paragonimus* species **A**: *P. westermani* from Saraburi province, **B**: *P. westermani* from Hin-tung sub-district, Nakhon Nayok province, **C**: *P. westermani* from Surat Thani province, **D**: *P. westermani*-like from Surat Thani province, **E**: *P. siamensis* from Prachin Buri province. Scale bar indicates 100 μm.

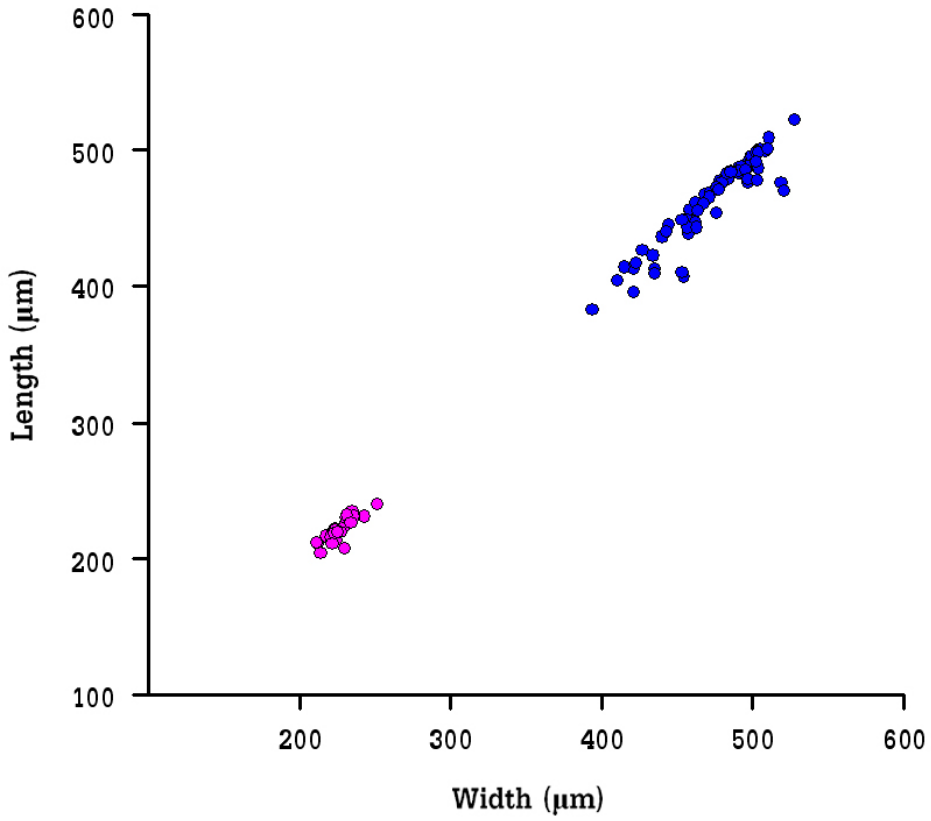

Figure 20. Measurements of encysted metacercariae of *P. westermani* from central and sounthern Thailand (blue spots) and *P. westermani*-like (pink spots).

3. Experimental infection and recovery of *Paragonimus* adult worms

Six specific pathogen free cats and nine Wistar rats were used for experimental infection. Five cats were inoculated intraperitoneally with separate types of metacercariae, namely *P. westermani* (central) (cat No.1), *P. westermani* (southern) (cat No.2), *P. westermani*-like (cat Nos.3 and 4) and *P. siamensis* (cat No.5). Three rats were inoculated orally with *P. westermani* metacercariae from Saraburi province while six rats were inoculated with *P. siamensis* metacercariae. Infected animals were necropsied 59 to 321 days after inoculation for the recovery of the worms. All cats were infected while only two *P. siamensis*-inoculated rats were infected. The locality, type and number of metacercariae, duration of infection and number of worms recovered at necropsy were summarized in Table 4.

In cat No.1, the recovery rate was 35%. From a total of seven worms recovered, six were found in the lungs and one was found in the pleural cavity. From a total of 24 worms recovered from cat No.2, 18 were found in the lungs while six were found in the pleural cavities. The recovery rate was 66.7%. In cat No.3, out of four worms recovered, two were found in the lungs and the rest were found in the pleural cavities. Nine juvenile worms (slightly developed stage) were obtained from the liver. The recovery rate was 21.7%. In cat No.4, 10 juvenile worms were collected and transferred to a new specific pathogen free cat (cat No.6). No worm was detected from the lungs. The recovery rate was 32.3% In cat No.5, out of 13 worms recovered, 11 were found in the lungs and two were found in the pleural cavities. The recovery rate was 29.6%. In cat No.6, four worms were obtained from the lungs and pleural cavities while one juvenile worm was found in the muscle tissues. The recovery rate was 50%. In experimentally infected rats Nos.1 and 2 which were inoculated orally with *P. siamensis*, the recovery rates were 3.3% and 4.2%, respectively. All of the flukes recovered were found encysted in the lungs.

Table 4. Results of experimental infection of cats and rats with *Paragonimus* metacercariae.

Locality	Host No.	Method of inoculation	Type of metacercariae inoculated	No. of metacercariae/ juvenile worms given	Duration of infection	No. of worms recovered						
						lung	pleural cavity	diaphragm	liver	peritoneal cavity	muscle tissue	Total (%)
Kaeng Khoi district Saraburi province	Cat No.1	intraperitoneal	*P. westermani* (central part)	20	161	6 (4A+2PA)	1 (PA)	-	-	-	-	7 (35.0)
Pa Nom district Surat Thani province	Cat No.2	intraperitoneal	*P. westermani* (southern part)	36	138	18 (A)	6 (PA)	-	-	-	-	24 (66.7)
	Cat No.3	intraperitoneal	*P. westermani* -like	60	148	2 (A+PA)	2 (A+PA)	-	9 (J)	-	-	13 (21.7)
	Cat No.4	intraperitoneal	*P. westermani -like*	31	321	-	-	1 (J)	8 (J)	1 (J)	-	10 (32.2)
	Cat No.6	intraperitoneal	*P. westermani -like*	10 (J)	163	2 (A)	2 (PA)	-	-	-	1 (PA)	5 (50)
Na Dee district Prachin Buri province	Cat No.5	intraperitoneal	*P. siamensis*	44	218	11 (10A+PA)	2 (PA)	-	-	-	-	13 (29.6)
	Rat No.1	oral	*P. siamensis*	120	59	4 (PA)	-	-	-	-	-	4 (3.3)
	Rat No.2	oral	*P. siamensis*	120	91	5 (PA)	-	-	-	-	-	5 (4.2)

Note: J = Juvenile worms, A = Adult, PA = Preadult

4. Morphological observation on flukes recovered

The characteristics of living worms were similar among *Paragonimus* species. The flukes were reddish brown in color and their bodies were fleshy and broadly fusiform. The adult and preadult flukes of *P. westermani* from the central part were obtained from cat number one. In four mounted specimens, two worms were identified as adult (with eggs in the uterus) while the other two were identified as preadult (without eggs in the uterus). The morphological characteristics of the adult flukes were shown in Figure 21A-B. The average size of four mounted specimens was 4.79 mm in length varying from 5.65 to 4.42 mm and 3.05 mm in width varying from 3.36 to 2.46 mm. The oral sucker was slightly larger than the ventral sucker. The transverse diameters of the oral and ventral suckers measured about 711 μm and 692 μm, respectively. The ovary was situated somewhat anterior of the body and located at the opposite side of the uterus. The ovary was divided into six lobes (Figure 21C-D) while the testes were simply branched into five or six lobes. The vitellaria were distributed bilaterally from the anterior to the end of the body and the intestine wound on both sides. The integument was covered with single cuticular spines (Figure 21E).

In eight mounted specimens of *P. westermani* from the southern part, four flukes were recognized as adult while the rest were preadult. The morphology of adult flukes was illustrated in Figure 22A-B. The average size of the specimens were 5.62 x 3.05 mm, with the length varying from 7.43 to 3.05 mm and its width varying from 3.79 to 1.75 mm. The oral and ventral suckers were almost the same size, the average of transverse diameters measured 634 μm and 631 μm, respectively. The ovary was divided into six lobes (Figure 22C-D) and located at the opposite side of the uterus. The testes were seen in parallel in the posterior end of the body and simply branched into five or six lobes. The vitelline glands were widely distributed along both sides of the body. The cuticular spines were originally single (Figure 22E).

Five mounted specimens of *P. westermani*-like adult (Figure 23A-B) and preadult flukes obtained from the cat Nos.3 and 6 resembled *P. westermani*. The average body size was 3.90 mm in length and 2.71 mm in width. The average transverse diameters of the oral and ventral suckers measured 523 μm and 518 μm, respectively. The ovary was divided into six lobes (Figure 23C-D) and the testes were simply branched into five or six lobes. The cuticular spines were singly spaced (Figure 23E).

In five mounted specimens of *P. siamensis* adult flukes from the cat No.5, the characteristics of the flukes resembled *P. westermani*, except the cuticular spines were arranged in groups (Figure 24E). The morphology of adult flukes was illustrated in Figure 24A-B. The average size was 6.7 x 3.7 mm, ranging from 8.33 to 5.38 mm in length and from 4.70 to 3.11 mm in width. The ventral sucker was slightly smaller than the oral sucker. The transverse diameters of the ventral and oral suckers measured approximately 639 μm and 672 μm, respectively. The ovary was branched into six lobes (Figure 24C-D) and situated on the opposite side of the uterus. The testes were simply branched and located in parallel in the posterior end of the body.

Three mounted specimens of *P. siamensis* flukes from the rat Nos.1 and 2 (Figure 25A-B) were identified as preadult. The average size of the body was 4.42 mm in length and 3.04 mm in width. The oral sucker was slightly larger than the ventral sucker. The average transverse diameters of the oral and ventral suckers measured 579 μm and 560 μm, respectively. The ovary was divided into six lobes (Figure 25C-D) while the testes were much more simply branched. The vitelline glands were not well developed. The surface of the body was covered with grouped cuticular spines (Figure 25E).

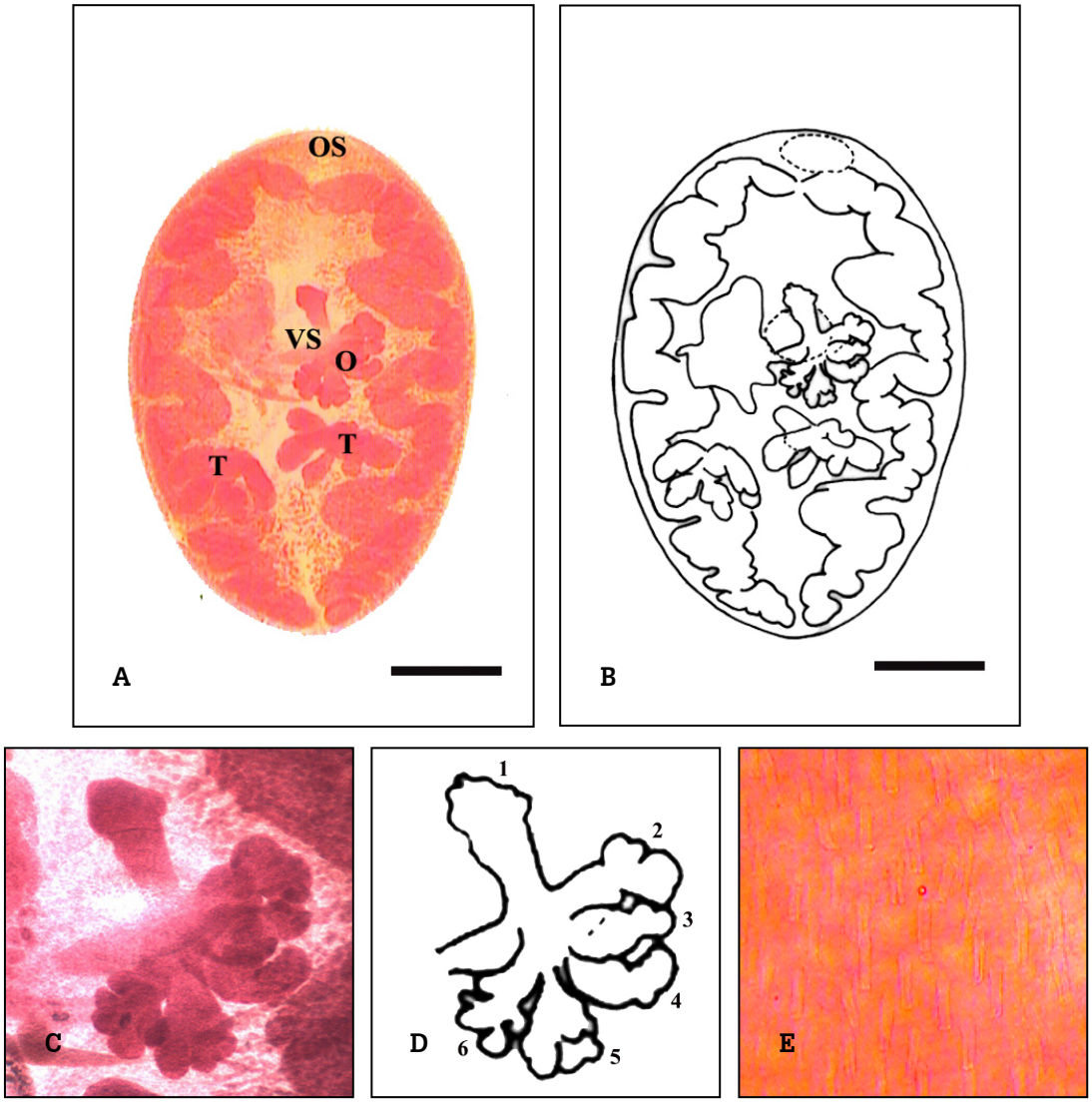

Figure 21. Adult worm of *P. westermani* (central Thailand) obtained from the cat No.1 **A**: Photomicrograph of the adult worm (**OS**: oral sucker, **VS**: ventral sucker, **O**: ovary, **T**: testes), **B**: Drawing of the whole body, **C**: Photomicrograph of the ovary, **D**: Drawing of the ovary, **E**: Photomicrograph of cuticular spines. Scale bar indicates 1 mm.

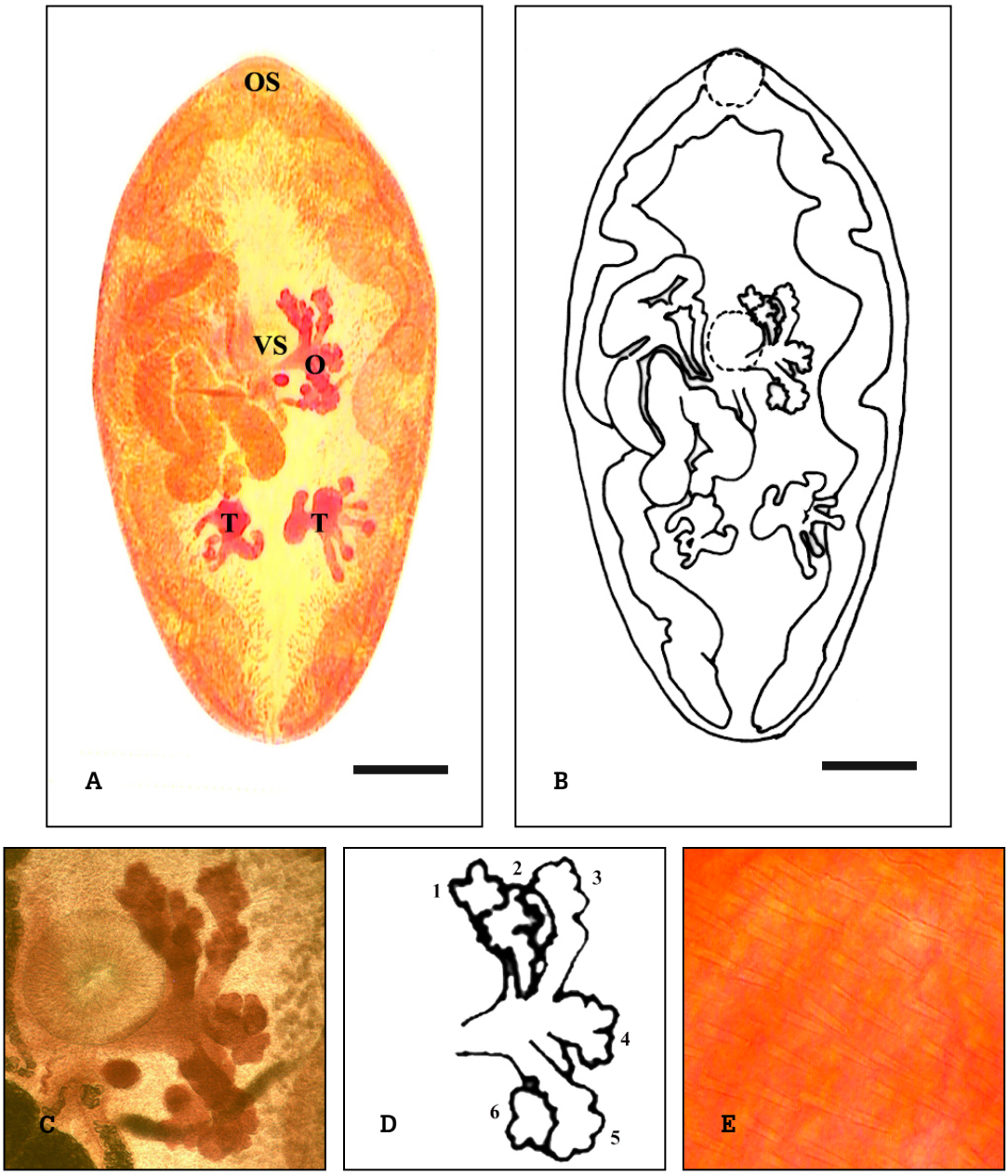

Figure 22. Adult worm of *P. westermani* (southern Thailand) obtained from the cat No.2 **A**: Photomicrograph of the adult worm (**OS**: oral sucker, **VS**: ventral sucker, **O**: ovary, **T**: testes), **B**: Drawing of the whole body, **C**: Photomicrograph of the ovary, **D**: Drawing of the ovary, **E**: Photomicrograph of cuticular spines. Scale bar indicates 1 mm.

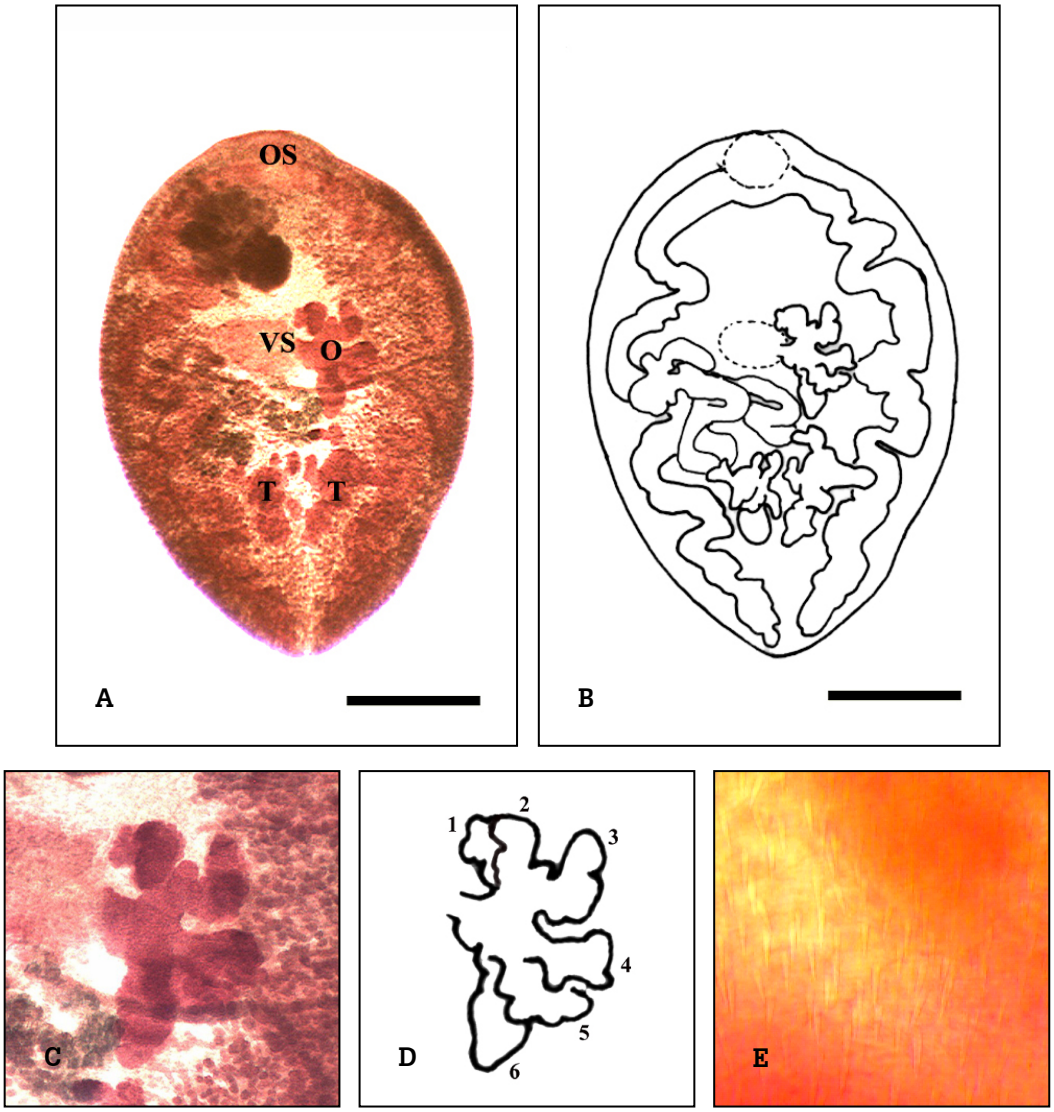

Figure 23. Adult worm of *P. westermani*-like (southern Thailand) obtained from the cat No.3 **A**: Photomicrograph of the adult worm (**OS**: oral sucker, **VS**: ventral sucker, **O**: ovary, **T**: testes), **B**: Drawing of the whole body, **C**: Photomicrograph of the ovary, **D**: Drawing of the ovary, **E**: Photomicrograph of cuticular spines. Scale bar indicates 1 mm.

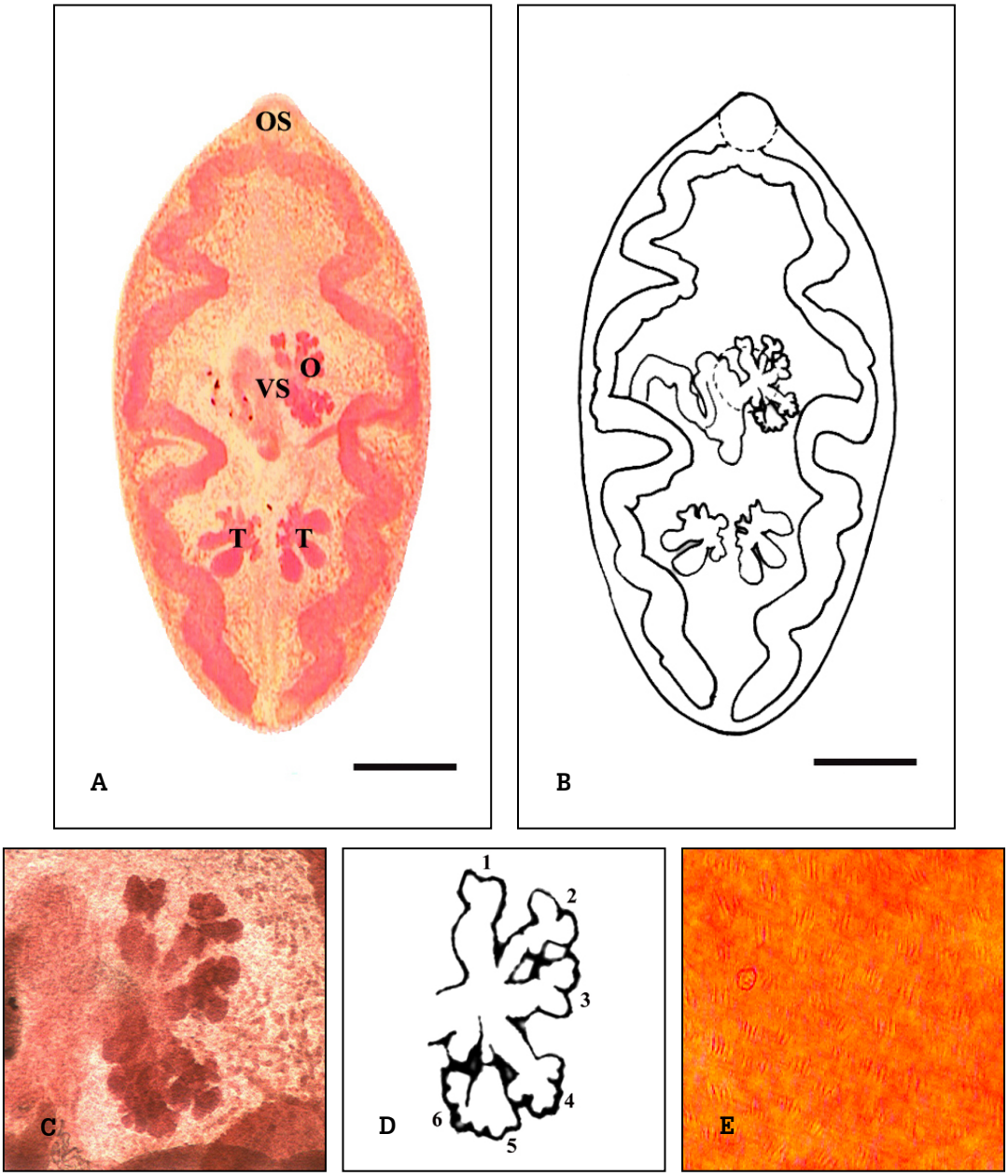

Figure 24. Adult worm of *P. siamensis* obtained from the cat No.5 **A**: Photomicrograph of the adult worm (**OS**: oral sucker, **VS**: ventral sucker, **O**: ovary, **T**: testes), **B**: Drawing of the whole body, **C**: Photomicrograph of the ovary, **D**: Drawing of the ovary, **E**: Photomicrograph of cuticular spines. Scale bar indicates 1 mm.

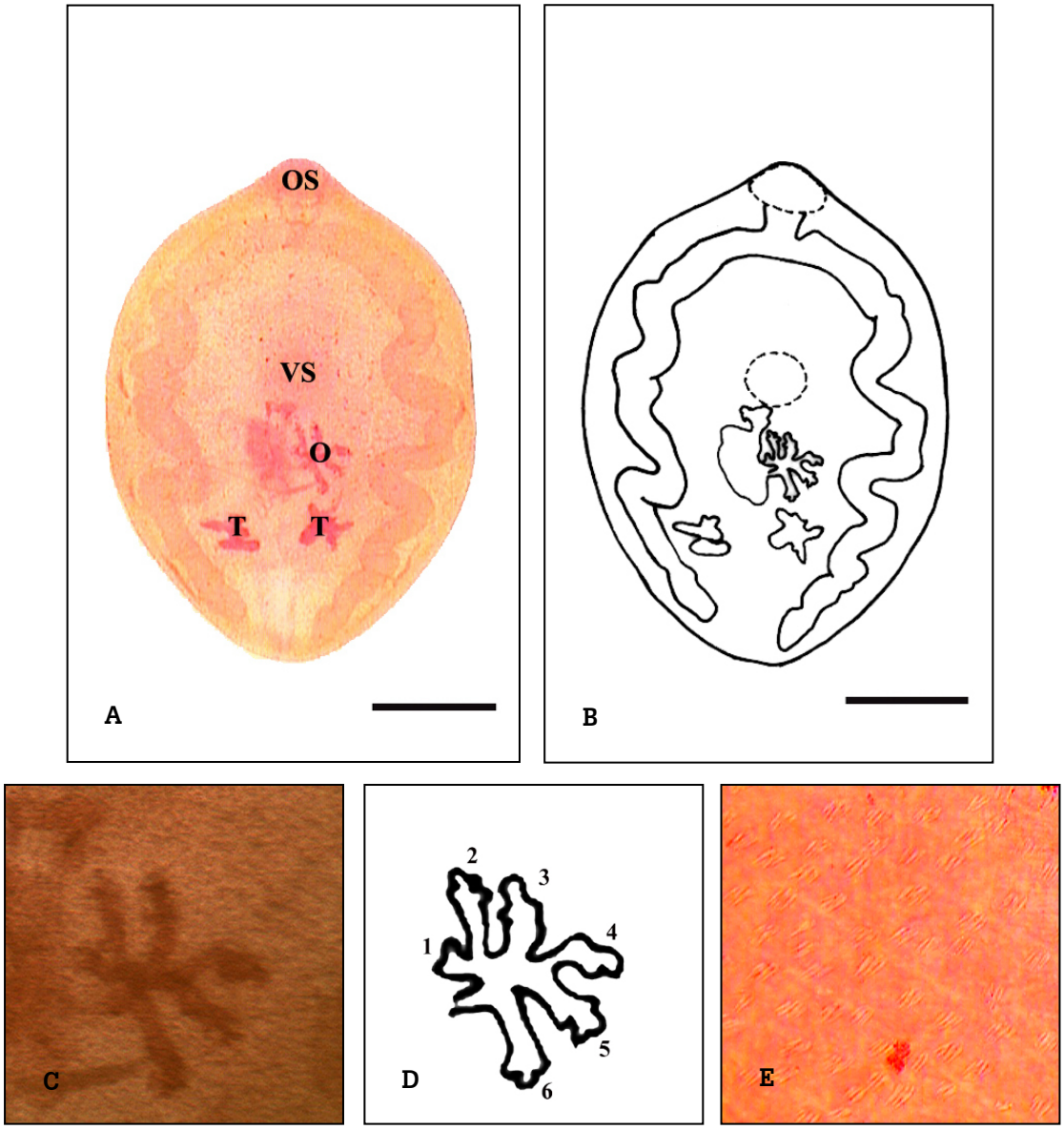

Figure 25. Adult worm of *P. siamensis* obtained from the rat No.1 **A**: Photomicrograph of the adult worm (**OS**: oral sucker, **VS**: ventral sucker, **O**: ovary, **T**: testes), **B**: Drawing of the whole body, **C**: Photomicrograph of the ovary, **D**: Drawing of the ovary, **E**: Photomicrograph of cuticular spines. Scale bar indicates 1 mm.

5. Total genomic DNA extraction

Total genomic DNA of *Paragonimus* species was extracted from both metacercariae and adult worms. Figure 26 shows the result of the agarose gel electrophoresis using DNA sample extracted from individual adult worms and metacercariae. Only DNA bands from the adult worms could be observed due to large amount of DNA extracts present.

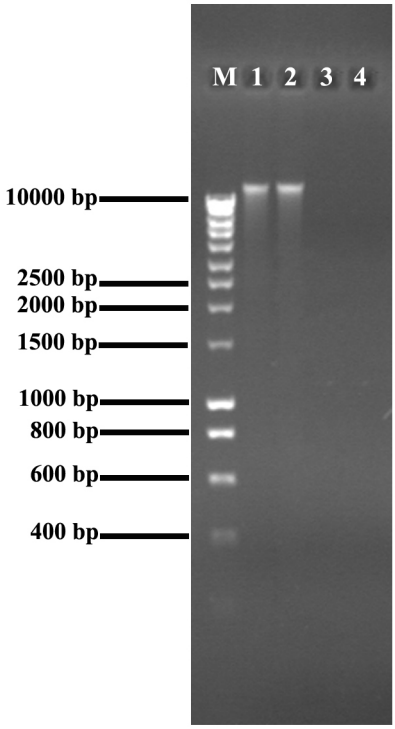

Figure 26. Genomic DNA extracted from adult worms and metacercariae. Lane M: Hyper Ladder I marker, Lanes 1-2: Genomic DNA extracted from adult worms, Lanes 3-4: Genomic DNA extracted from metacercariae.

6. PCR amplifications of the ITS and partial COI regions

DNA extracted from both adult worms and metacercariae were used for PCR amplifications of the entire ITS region (ITS1, 5.8S and ITS2) and a portion of the COI region. Although a low concentration of the genomic DNA was extracted from the individual metacercariae, PCR products were still amplified. Figure 27 shows the results of PCR amplifications of the entire ITS region using primers "Pwfor" and "A28". The size of PCR products obtained were approximately 900 base pairs (bp). Results of PCR amplifications of the partial COI region using primers "JB3" and "JB4.5" were shown in Figure 28. The size of PCR products obtained were approximately 450 bp.

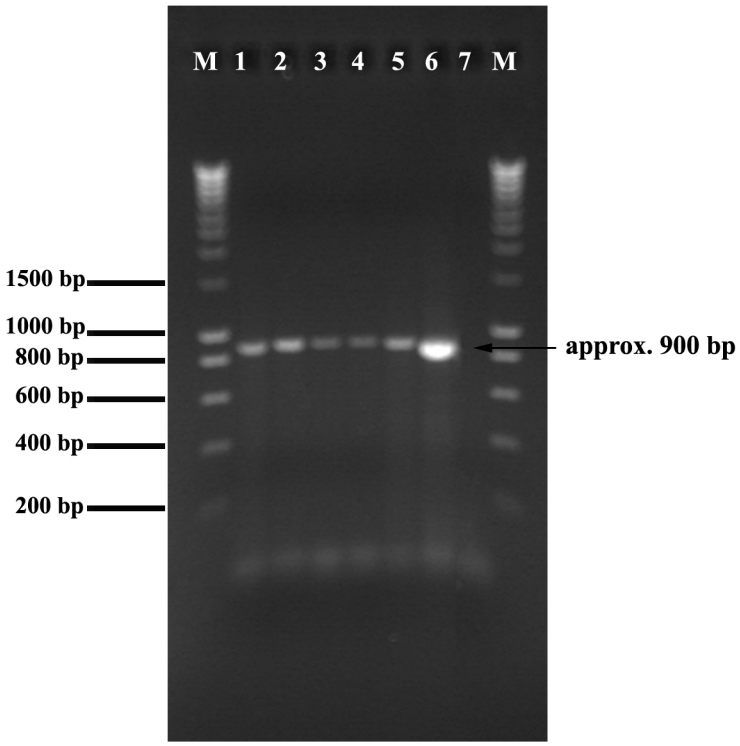

Figure 27. PCR amplification products of the entire ITS region (including 5.8S rDNA). Lane M: Hyper Ladder I marker, Lane 1: *P. westermani* from Saraburi province, Lane 2: *P. westermani* from Hin-tung sub-district, Nakhon Nayok province, Lane 3: *P. westermani* from Surat Thani province, Lane 4: *P. westermani*-like from Surat Thani province, Lane 5: *P. siamensis* from Prachin Buri province, Lane 6: Positive control (*P. westermani* from Japan), Lane 7: Negative control.

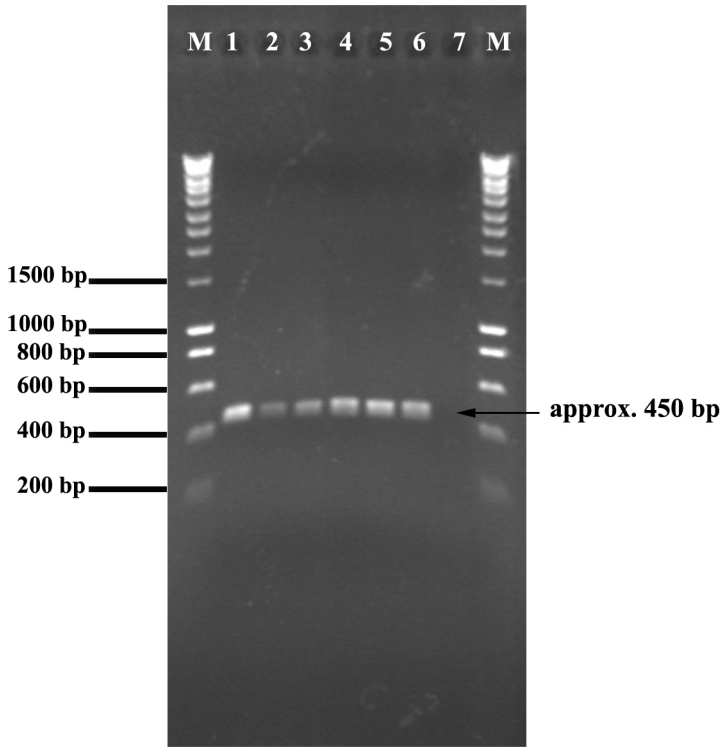

Figure 28. PCR amplification products of a portion of the COI region. Lane M: Hyper Ladder I marker, Lane 1: *P. westermani* from Saraburi province, Lane 2: *P. westermani* from Hin-tung sub-district, Nakhon Nayok province, Lane 3: *P. westermani* from Surat Thani province, Lane 4: *P. westermani*-like from Surat Thani province, Lane 5: *P. siamensis* from Prachin Buri province, Lane 6: positive control (*P. westermani* from Japan), Lane 7: negative control.

7. Molecular cloning

The amplified DNA fragments of the entire ITS region were purified using QIAquick™ Gel Extraction Kit. After cloning the entire ITS region white colonies were selected and screened for recombinant plasmids containing the required inserts using PCR reaction (Figure 29) and *Eco*RI restriction (Figure 30). Ten to thirty five positive clones per taxon were employed for sequencing. In the case of the partial COI region the PCR products were purified and directly sequenced.

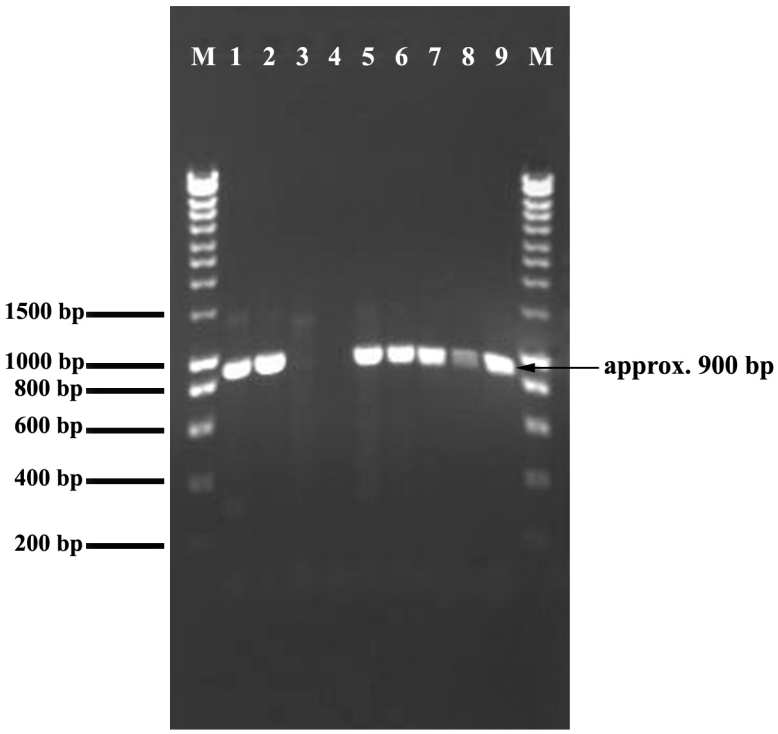

Figure 29. Results of PCR amplification from bacterial white colonies for analyses of recombinant plasmids containing the entire ITS region. Lane M: Hyper Ladder I marker, Lanes 1-2 and 5-9: Positive recombinant plasmids containing the entire ITS region (approximately 900 bp), Lanes 3-4: Negative clones.

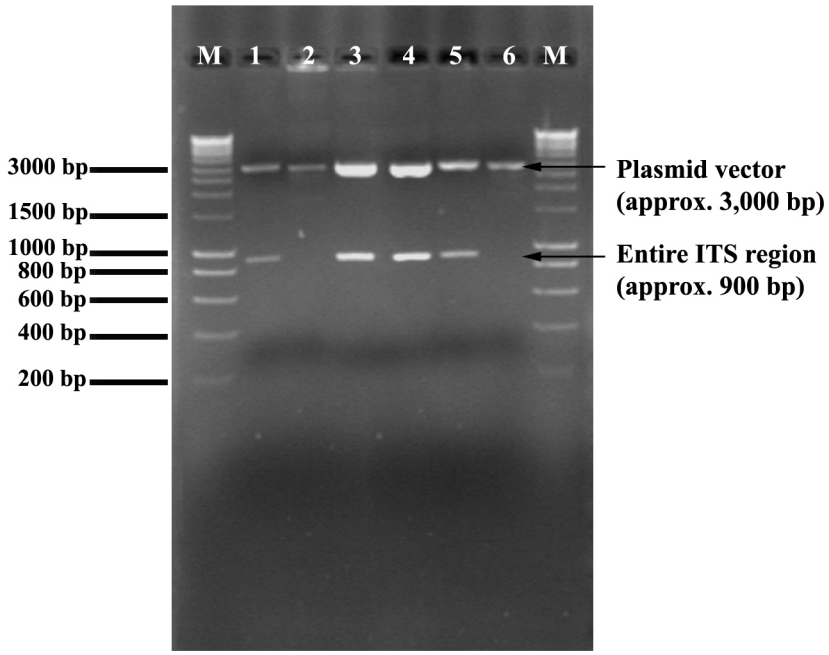

Figure 30. Results of *Eco*RI restriction for analyses of recombinant plasmids containing the entire ITS region. Lane M: Hyper Ladder I marker, Lanes 1 and 3-5: Positive recombinant plasmids produced two bands of the linearized form of the plasmid vector (approximately 3,000 bp) and the entire ITS region (approximately 900 bp), Lanes 2 and 6: Negative clones.

8. Sequence analyses

For each taxon the ITS sequence data from 10 to 35 positive clones were obtained. Genetic polymorphism was observed among the sequence data (0 to 4 base differences). Consensus sequence data of the entire ITS region (803 bp) of *Paragonimus* species were shown in Tables 5. On the other hand, within each taxon no base differences was found among the COI sequence data. The length of the partial COI region studied was 381 bp (Table 6).

Table 5. Consensus sequence data of the entire ITS region of five *Paragonimus* under study.

Species	Consensus sequence data
P. westermani **Saraburi (803 bp: no base difference)**	TACCTGTCTGATGCCCTACGTTTTGCTTGCCATTTTCGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGT CTTCACGGACAGTGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCTGTTTGTTTCA ATTTATGCCATTTTACACTGTTCAAGTGGTTCAGGTCAGCTCGTCTGGGCTGTTCCACTGCCCGACATGCACCCGG CCTCGTGCTGGACTGCATGTACAGTCGCCTGGCGGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCG TCCGGGTGACTGGATGTTCGATGTGAGTACAACTCTGTGCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGC AGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGG TTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTT GCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTC GCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTAAATGATTTATGT GCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCTGACCTACGTATGTG CCATGTGGTCCATTCTTCTGACCTCGGATTAGACGTGAGTACC
P. westermani **Nakhon Nayok (803 bp: 2 base differences)**	TACCTGTCTGATGCCCTACGTTTTGCTTGCCATTTTCGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGT CTTCACGGACAGTGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCTGTTTGTTTCA ATTTATGCCATTTTACACTGTTCAAGTGGTTCAGGTCAGCTCGTCTGGGCTGTTCCACTGCCCGACATGCACCCGG CCTCGTGCTGGACTGCATGTACAGTCGCCTGGCGGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCG TCCGGGTGACTGGWTGTTCGATGTGAGTACAACTCTGTGCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGC AGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGG TTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTT GCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTC GCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTAAATGATTTATGT GCGCGTTTCGCTGTCCTGTCYTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCTGACCTACGTATGTG CCATGTGGTCCATTCTTCTGACCTCGGATTAGACGTGAGTACC
P. westermani **Surat Thani (803 bp: 4 base differences)**	TACCTGTCTGATGCCCTACGTTTTGCTTGCCATTTTCGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGT CTTCACGGACAGTGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCTGTTTGTTTCA ATTTATGCCATTTTACACTGTTCAAGTGGTTCAGGTCAGCTCGTCTGGGCTGTTCCACTGCCCGACATGCACCCGG CCTCGTGCTGGACTGCATGTACAGTCGCCTGGCGGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCG TCCGGGTGACTGGATGTTCGATGTGAGTACAACTCTGTGCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGC AGCCAACTGTGTGAATTAATGCGWACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGG TTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTT GCCAGCTGGCGTGRTCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGRTCTATGGCGTTTCCCTAACATACTC GCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCYCAGTAAATGATTTATGT GCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCTGACCTACGTATGTG CCATGTGGTCCATTCTTCTGACCTCGGATTAGACGTGAGTACC
***P. westermani*-like** **Surat Thani (803 bp: 3 base differences)**	TACCTGTCTGATGCTCTACGTGTTGCTTGCCATTTTCGGATGGCCAGTCCATCTCGTGGGTGACGGGTTGTGCTGT CTTCACCGACAGTGCTAGGCTTAATGAGTGGTATGGCATATTGAGCTACGGCTCGGCCACCGCCCTGTTTGTTTCA ATTTATGCCATTTTACRCTGTTCAAGTGgTTCAGGTCAGCTCGTCTGGGCTGTTCCGCTGCCCGACATGCACCCGG TCTCGTGCTGGACTGCATGTACAGTCGCCTGGCGGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCG TCTGGGTGACTGGATGTTCGATGTGAGTACAACTCTGTRCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGC AGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGG TTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATGAAYTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTT GCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTCTCCCTAACATACTC GGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTGATTGGTTTATGT GCGCGTTCCGCTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGTtCGATGCTGACCTACGTATGTG CCATGTGGTTCATTCTCCTGACCTCGGATCAGACGTGAGTACC

Table 5. (continue)

Species	Consensus sequence data
P. siamensis **Prachin Buri (803 bp: no base difference)**	TACCTGTCTGATGCCCTTCGTTTTGCTTGCCATTTTCGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGT CTTCATGGACAGTGCTAGGCTTAATGAGTGGTACGGCATATTGAGCCACGGCTCGGCCACCGCCCTGTTTTTTTCA ATTTATGCCATTTTACACTGTTCAAGTGGTTCAGATCAGCTCGTCTGGACTGTTCCATTGCCCGACATGCACCCGG TCTCGTGCTGGACTGCATGTACAGTCGCCTGGCGGTGCCTTATCCCGGGCTAGACTGGTAAACCATACGTCGTTCG TCTGGGTGACTGGATGTTCGATGTGAGTACAACTCTGTGCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGC AGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCACGGG TTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTCTT GCCAGCTGGCGTGATCTCCCCAATCAGGTCTCGTGCCTGTGGGGTGTCAGATCTATGGCGTTTCCCTAACATACTC GGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTAGATGAATTATGT GCGCGTTCCGTTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGTGTTCGACGTTGACCTATCTATGTG CCATATGGTTCATTCTCCTGACCTCGGATCAGACGTGAGTACC

Table 6. Nucleotide sequence data of the partial COI region of five *Paragonimus* under study.

Species	Nucleotide sequence data
P. westermani **Saraburi (381 bp)**	TTGCCGGGGTTTGGTATTGTGAGTCACATCTGCATGACTTTGACTAACAATGATTCCTTGTTTGGGTATTACGGGT TAGTGTTTGCGATGGGGGCCATCGTGTGCTTGGGCAGTGTTGTGTGGGCGCACCACATGTTTATGGTTGGTTTGGA TGTTAAGACTGCTGTCTTTTTTAGCTCTGTCACTGGGGTGATTGGGATACCTACGGGGATTAAGGTCTTCTCTTGG TTGTTCATGTTGGGTGGGTCTCGTTTACGATTTTGAGATCCTGTTTTGTGGTGGATCCTTGGGTTCATTTTTCTGT TCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCTTCCATCCTGGATAGTCTGTTGCATGACACGTGGTTTGTT
P. westermani **Nakhon Nayok (381 bp)**	TTGCCGGGGTTTGGTATTGTGAGTCACATCTGCATGACTTTGACTAACAATGATTCCTTGTTTGGGTATTACGGGT TAGTGTTTGCGATGGGGGCCATCGTGTGCTTGGGCAGTGTTGTGTGGGCGCACCACATGTTTATGGTTGGTTTGGA TGTTAAGACTGCTGTCTTTTTTAGCTCTGTCACTGGGGTGATTGGGATACCTACGGGGATTAAGGTCTTCTCTTGG TTGTTCATGTTGGGTGGGTCTCGTTTACGATTTTGAGATCCTGTTTTGTGGTGGATCCTTGGGTTCATTTTTCTGT TCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCTTCCATCCTGGATAGTCTGTTGCATGACACGTGGTTTGTT
P. westermani **Surat Thani (381 bp)**	TTGCCGGGGTTTGGTATCGTGAGTCACATCTGCATGACTTTGACTAACAATGATTCCTTGTTTGGGTATTACGGGT TAGTGTTTGCGATGGGGGCCATCGTGTGCTTGGGCAGTGTTGTGTGGGCGCACCACATGTTTATGGTTGGTTTGGA TGTTAAGACTGCTGTCTTTTTTAGCTCTGTCACTGGGGTGATTGGGATACCTACGGGGATTAAGGTCTTCTCTTGG TTGTTCATGTTGGGTGGATCTCGTTTACGATTTTGAGATCCTGTTTTGTGGTGGATCCTTGGGTTCATTTTTCTGT TCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCTTCCATCCTGGATAGTCTGTTGCATGACACGTGGTTTGTT
***P. westermani*-like** **Surat Thani (381 bp)**	TTGCCAGGGTTTGGGATTGTGAGTCACATCTGCATGACTTTAACTAACAATGATTCCTTGTTTGGGTATTATGGGT TGGTGTTCGCGATGGGGGCTATCGTATGTTTGGGTAGTGTTGTGTGAGCGCACCATATGTTCATGGTTGGTTTGGA TGTTAAGACTGCTGTTTTTTTTTAGTTCTGTTACGGGGGTGATTGGGATACCTACGGGGATTAAGGTTTTTTCCTGG TTGTTTATGTTGGGTGGAACTCGTTTGCGGTTTTGAGATCCTGTTTTGTGGTGGATACTCGGGTTTATTTTTCTGT TCACCATAGGTGGTGTGACTGGCATTATTTTGTCTTCTTCCATATTGGATAGCCTGTTGCATGACACGTGGTTTGTT
P. siamensis **Prachin Buri (381 bp)**	TTGCCTGGTTTTGGGATTGTGAGACATATTTGTATGACTTTGACGAATAATGATTCTCTGTTTGGTTATTATGGGT TAGTGTTTGCGATGGGAGCTATTGTGTGTTTGGGAAGTGTTGTGTGGGCGCATCATATGTTTATGGTTGGTTTGGA TGTTAAGACTGCTGTGTTTTTTAGTTCTGTCACCGGGGTTATTGGGATACCTACGGGTATTAAGGTTTTTTCTTGA TTATTCATGTTAGGTGGGGCTCGTTTGCGTCTTTGGGATCCTGTCTTGTGGTGGATTCTTGGTTTCATTTTCTTGT TTACTATAGGTGGTGTGACCGGTATTGTTTTTGTCCTCTTCAATATTGGATAGTTTGTTGCATGATACGTGGTTTGTG

9. Sequence comparison and phylogenetic analyses

Sequence alignments were carried out using Clustal X program[41] with additional sequences of *Paragonimus* species and *Fasciola hepatica* (outgroup) from GenBank database. The GenBank accession numbers of the additional sequences were shown in Table 7.

Table 7. The GenBank accession numbers of *Paragonimus* species and *Fasciola hepatica***.**

Species	Origin	ITS1	ITS2	COI
P. westermani	Hyogo, Japan	AF040941	U96907	U97205
	Minchin, China	AF040940	U96907*	AY140681
	Haenam, Korea	-	AF333278	AF333281
	Karapai, Taiwan	-	U96908	AY140673
	Philippines	AF040943	U96910	U97213
	Malaysia	AF071426	U96909	U97211
P. bangkokensis	Thailand	**	**	**
P. harinasutai	Thailand	**	**	**
P. heterotremus	Thailand	**	**	**
P. macrorchis	Thailand	AF040936	AF159608	AF159598
Fasciola hepatica	Australia	AB207140	AB207148	AF216697

Note: - = no sequence data reported

* = sequence identical to *P. westermani* from Hyogo, Japan[36]

** = sequences obtained from specimens collected in field surveys from this study

For populations of *P. westermani* collected in Saraburi, Nakhon Nayok and Surat Thani provinces, *P. westermani*-like and *P. siamensis* sequence variation of the ITS2 region was examined. Identical sequences were observed among *P. westermani* from Saraburi population as well as *P. siamensis* from Prachin Buri population. Based on a few base differences found in each population among five taxa under study a phylogenetic tree (Figure 31) reconstructed from the ITS2 data of all clones showed five clusters. Three clusters of *P. westermani* (Bootstrap value (BS) = 100%) from three different localities were grouped together while *P. westermani*-like and *P. siamensis* were separated.

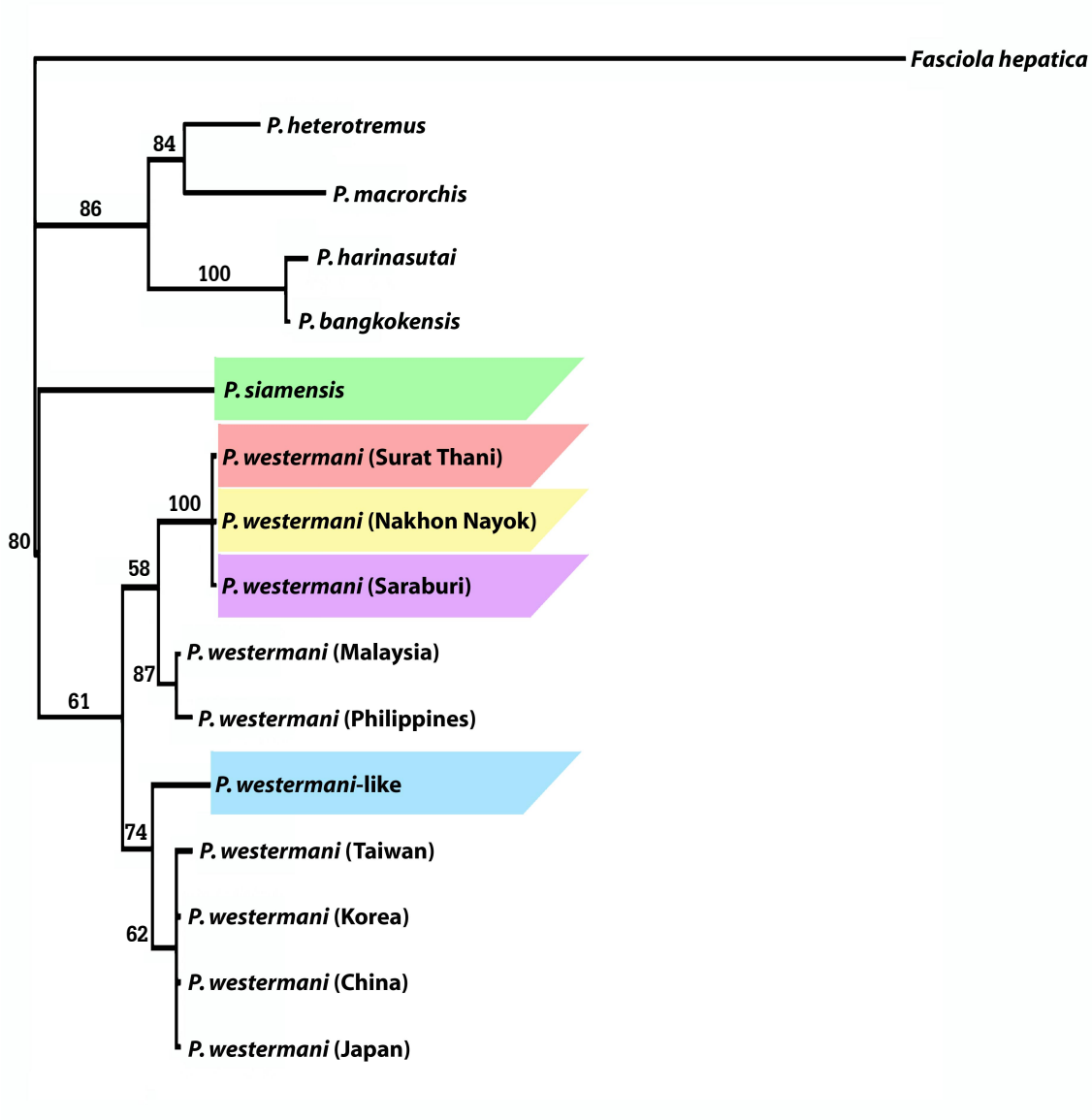

Figure 31. Single most parsimonious tree depicting relationships among five *Paragonimus* populations under study inferred from the ITS2 region using *Fasciola hepatica* as outgroup. Pink box indicates *P. westermani* from Surat Thani population, yellow box indicates *P. westermani* from Nakhon Nayok population, purple box indicates *P. westermani* from Saraburi population, blue box indicates *P. westermani*-like population and green box indicates *P. siamensis* population. Numbers above the branches are bootstrap values (%) of 1,000 replicates.

9.1 Sequence and phylogenetic analyses of the ITS2 region

The ITS2 region has been commonly used in many studies of *Digeneans*. In this study it was employed for phylogenetic analyses to infer relationships among *Paragonimus* taxa. The alignment of the ITS2 region of 16 taxa of *Paragonimus* species and its outgroup was 378 bp in length with 10 sites of indels (indicated by "p-y") (Figure 32). The actual length of the ITS2 region of each taxon was shown in parentheses at the end of the sequence. The length of the ITS2 region of 16 taxa under study varied from 359-372 bp. Out of 378 total characters of the alignment, 231 (61.1%) characters were constant, 91 (24.1%) were parsimony-uninformative and 56 (14.8%) characters were parsimony-informative. Sequence divergence between ingroup and outgroup taxa obtained from pairwise distance analysis ranged from 37.6-41.9%. The G+C content of all taxa ranged from 50.5-57.0% and transition/transversion ratio was 2.65. The sequence characteristics of the ITS2 region were summarized in Table 8.

A single most parsimonious tree (Figure 33) and phylogram (Figure 34) of length 190 steps were obtained based on parsimony analysis (branch-and-bound search) of the informative characters using PAUP. Tree reliability was estimated using bootstrapping of 1,000 replicates. Fit measures of the tree were as follows: Consistency index (CI) = 0.9053, Homoplasy index (HI) = 0.0947, Retention index (RI) = 0.8500 and Rescaled consistency index (RC) = 0.7695. Based on the phylogenetic analyses two important clades were revealed. Clade I consists of *P. westermani* from Thailand, Malaysia, the Philippines, Taiwan, Korea, China, Japan, *P. westermani*-like and *P. siamensis* (BS = 79%). Within this clade, there is *P. westermani* complex which is partitioned into two groups. The first group (South East Asian group) comprises *P. westermani* from Thailand, Malaysia and the Philippines (BS = 58%) and the second group (East Asian group) contains *P. westermani* from Taiwan, Korea, China and Japan plus *P. westermani*-like from Thailand (BS = 69%). *Paragonimus siamensis* is a sister group to the *P. westermani* complex. Clade II composes of other *Paragonimus* species found in Thailand (*P. heterotremus*, *P. macrorchis, P. harinasutai* and *P. bangkokensis*, BS = 84%).

Table 8. Sequence characteristics of the ITS2 region.

Sequence characteristics	**ITS2**
Length range (total) (bp)	359-372
Length mean (total) (bp)	363.2
Length range (ingroup) (bp)	359-363
Length mean (ingroup) (bp)	362.6
Length range (outgroup) (bp)	372
Aligned length (bp)	378
G+C content range (%)	50.5-57.0
G+C content mean (%)	55.5
Sequence divergence range (ingroup) (%)	0-13.7
Sequence divergence range (total) (%)	37.6-41.9
Number of indels (total)	10
Size of indels (total) (bp)	1-11
Number of constant sites (%)	231 (61.1)
Number of variable sites (%)	147 (38.9)
Number of informative sites (%)	56 (14.8)
Number of uninformative sites (%)	91 (24.1)
Transition/transversion ratio (ts/tv ratio)	2.65

Figure 32. Sequence data matrix of the aligned ITS2 region of nuclear ribosomal DNA for 16 taxa of *Paragonimus* and its outgroup. Uncertain nucleotide states are coded as follows: R = A/G, Y = C/T; hyphens "-" denote alignment gaps; "**p-y**" above the nucleotide matrix indicate the positions of alignment gaps (indels); numbers in parentheses at the end of sequences indicate the actual length of the ITS2 region.

```
                                  10        20        30        40        50        60        70        80        90
Taxon

P. westermani (Japan)             ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (China)             ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Korea)             ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Taiwan)            ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Philippines)       ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Malaysia)          ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Saraburi)          ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Nakhon Nayok)      ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani (Surat Thani)       ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. westermani-like                ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATGAAYTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. siamensis                      ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTC
P. bangkokensis                   ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. harinasutai                    ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
P. macrorchis                     ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCAACGCCCAAAAAGTTGCGGCTTGGGTT
P. heterotremus                   ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTT
Fasciola hepatica                 ATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTT
```

Figure 32. (continued)

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                                100       110       120       130       140       150       160       170       180
Taxon
                                                              | p |
P. westermani (Japan)           TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. westermani (China)           TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. westermani (Korea)           TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. westermani (Taiwan)          TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. westermani (Philippines)     TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. westermani (Malaysia)        TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. westermani (Saraburi)        TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTCGCGCGCACCCAC
P. westermani (Nakhon Nayok)    TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACATACTCGCGCGCACCCAC
P. westermani (Surat Thani)     TTGCCAGCTGGCGTGRTCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGRTCTATGGCGTTTCCCTAACATACTCGCGCGCACCCAC
P. westermani-like              TTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTCTCCCTAACATACTCGGGCGCACCCAC
P. siamensis                    TTGCCAGCTGGCGTGATCTCCCCAATCAGGTCTCGTGCCTGTGGGGTGTCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCCAC
P. bangkokensis                 TTGCCAGCTGGCGTGATTTCCCCGATCTGACCTTGTGTCGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCTGTCCGGGCGTACCCAT
P. harinasutai                  TTGCCAGCTGGCGTGATTTCCCCGGTCTGACCTTGTGTCGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCTGTCCGGGCGTACCCAT
P. macrorchis                   CTGCCAGCTGGCGTGATTTCCCCAACCTGGCCTCGTGTCTGTGGGGTGCCGGATCTGTGGCGTTTCCCTAAAAAATCCGGACGTACCCGT
P. heterotremus                 TTGCCAGCTGGCGTGATTTCCCCAACGTGGCCTTGTGTCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAAATCCGGGCGTATCCAT
Fasciola hepatica               TTGCCAGCTGGCGTGATCTCCTCTATGAGTAAT----CATGTGAGGTGCCAGATCTATGGCGTTTCCCTAATGTATCCGGATGCACCCTT

                                190       200       210       220       230       240       250       260       270
Taxon
                                                    q                                 |       r     |
P. westermani (Japan)           GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTGAA-----------TGATTTATGTGCGCGTTCCGCTGTC
P. westermani (China)           GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTGAA-----------TGATTTATGTGCGCGTTCCGCTGTC
P. westermani (Korea)           GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTGAA-----------TGATTTATGTGCGCGTTCCGCTGTC
P. westermani (Taiwan)          GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAATGAA-----------TGATTTATGTGCGCGTTCCGCTGTC
P. westermani (Philippines)     GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGAAATCGTGGCTCAGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTC
P. westermani (Malaysia)        GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGAAATCGTGGCTCAGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTC
P. westermani (Saraburi)        GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTC
P. westermani (Nakhon Nayok)    GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTC
P. westermani (Surat Thani)     GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCYCAGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTC
P. westermani-like              GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTGAT-----------TGGTTTATGTGCGCGTTCCGCTGTC
P. siamensis                    GTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTCAGTAGA-----------TGAATTATGTGCGCGTTCCGTTGTC
P. bangkokensis                 GTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTGGCTCAGTGAA-----------TTATTTATGTGCGCGTTCCGTCGTC
P. harinasutai                  GTTGTGGCTGAAGGCCTTGGTGGGGAAGTGGCAACGGAATCGTGGCTCAGTGAA-----------TTATTTATGTGCGCGTTCCGTCGTC
P. macrorchis                   GTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTGGCTCAGTGAA-----------AAATTTATGTGCGCGTTCCGCTGTC
P. heterotremus                 GTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTGGCTCAGTGAA-----------TGATTTATGTGCACGTTCCGCTGTC
Fasciola hepatica               GTCTTGGCAGAAAGCCGTGGTGAGG-TGCAGTGGCGGAATCGTGGTTTAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTTGGCGATC
```

Figure 32. (continued)

```
                                  280        290        300        310        320        330        340        350        360
Taxon
                                                                  |s|t|u|        v                  w          x
P. westermani (Japan)             CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCTTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. westermani (China)             CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCTTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. westermani (Korea)             CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCTTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. westermani (Taiwan)            CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCTTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. westermani (Philippines)       CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGATCGATGCT-GACCTACGTTTGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. westermani (Malaysia)          CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGATCGATGCT-GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. westermani (Saraburi)          CTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTCCATTC--TTCTGACCTC
P. westermani (Nakhon Nayok)      CTGTCYTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTCCATTC--TTCTGACCTC
P. westermani (Surat Thani)       CTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTCCATTC--TTCTGACCTC
P. westermani-like                CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGTTCGATGCT-GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTC
P. siamensis                      CTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGTGTTCGACGTT-GACCTATCTATGTGCCAT-ATGGTTCATTC--TCCTGACCTC
P. bangkokensis                   CTATCATCATCTATGGTTGATGCTGCGTGTGGTGTGCGTCTGATGCT-GACCTGAGTATGTGCCAT-GTGGCTCATTC--TCCTGACCTC
P. harinasutai                    CTATCATCATCTATGGTTGATGCTGCGTGTGGTGTGCGTCTGATGCT-GACCTGAGTATGTGCCAT-GTGGCTCATTC--TCCTGACCTC
P. macrorchis                     CTGTCATCATCTTTGGTTGATGTTGCATGTGGTGT----CCGATGCT-GACCTATGTTTGTGCCGT-GCGGCTCATTC--TCCTGACCTC
P. heterotremus                   CCGTCATCATCTATGGTTGAAGTTGCGCGTGGTGTG--TCCGATGCT-GACCTATATATGTGCCAT-GTGGCTCATTT--TCCTGACCTC
Fasciola hepatica                 CCCTAGTCGGCACACTTATGATTTCTGGGATAATTCCATACCAGGCACGTTCCGTCACTGTCACTTTGTTATTGGTTTGATGCTGAACTT

                                  370
Taxon
                                  y
P. westermani (Japan)             GGATCAGACGTGAGTACC   [363]
P. westermani (China)             GGATCAGACGTGAGTACC   [363]
P. westermani (Korea)             GGATCAGACGTGAGTACC   [363]
P. westermani (Taiwan)            GGATCAGACGTGAGTACC   [363]
P. westermani (Philippines)       GGATCAGACGTGAGTACC   [363]
P. westermani (Malaysia)          GGATCAGACGTGAGTACC   [363]
P. westermani (Saraburi)          GGATTAGACGTGAGTACC   [363]
P. westermani (Nakhon Nayok)      GGATTAGACGTGAGTACC   [363]
P. westermani (Surat Thani)       GGATTAGACGTGAGTACC   [363]
P. westermani-like                GGATCAGACGTGAGTACC   [363]
P. siamensis                      GGATCAGACGTGAGTACC   [363]
P. bangkokensis                   GGATCAGACGTGAGTACC   [363]
P. harinasutai                    GGATCAGACGTGAGTACC   [363]
P. macrorchis                     GGATCAGACGTGAGTACC   [359]
P. heterotremus                   GGATCAGACGTGAGTACC   [361]
Fasciola hepatica                 GG-TCATGTGTCTGATGC   [372]
```

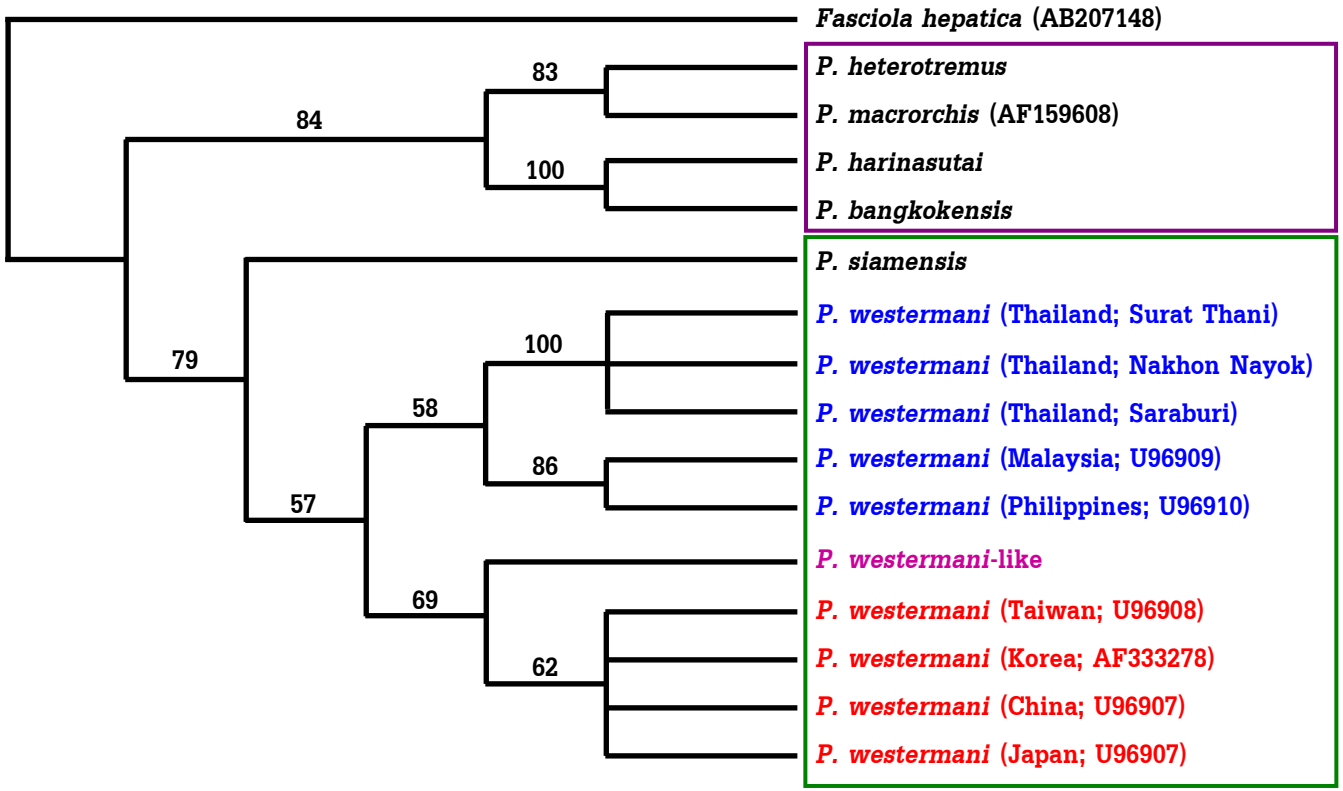

Figure 33. Single most parsimonious tree of length 190 steps based on parsimony analysis of the informative characters of the ITS2 region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. Clade I (green box) includes *P. westermani* complex and *P. siamensis*. Within the *P. westermani* complex, taxa in blue indicate *P. westermani* from South East Asia while taxa in red indicate *P. westermani* from East Asia and a taxon in pink denotes *P. westermani*-like from Thailand. Clade II (purple box) contains other *Paragonimus* species found in Thailand.

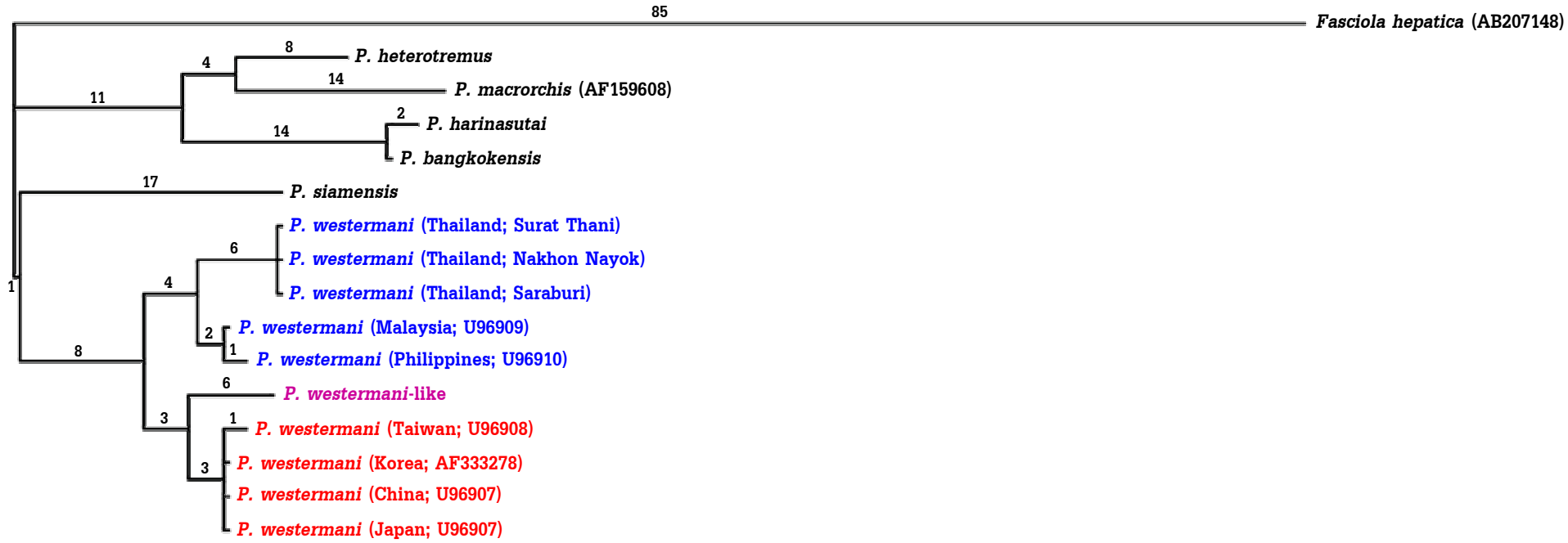

Figure 34. Phylogram of most parsimonious tree of length 190 steps based on parsimony analysis of the ITS2 region. Numbers above the branches indicate branch length. The tree was rooted using *Fasciola hepatica*. Taxa in blue indicate *P. westermani* from South East Asia while taxa in red indicate *P. westermani* from East Asia.

9.2 Sequence and phylogenetic analyses of the ITS region

In order to obtain more informative characters for resolving phylogenetic relationships within *Paragonimus* taxa the ITS region (ITS1 + ITS2) was employed. The alignment of the ITS region excluding 137 bp of highly conserved region, 5.8S rDNA, of 14 taxa of *Paragonimus* species and its outgroup was 690 bp in length. This alignment possessed 25 sites of insertions and deletions or indels (indicated by "a-y") (Figure 35). Fifteen indels ("a-o", size 1-6 bp) were found in the ITS1 region while 10 indels were found in the ITS2 region ("p-y", size 1-11 bp). The actual length of the combined ITS1 and ITS2 regions of each taxon was given in parentheses at the end of the sequence. The partial ITS1 sequence of *P. westermani* was obtained from the GenBank database. The incomplete sites of the ITS1 region (identified by "?") were treated as missing data and included as constant sites.

Sequence characteristics and analyses of the ITS region are summarized in Table 9. The length of the ITS region excluding 5.8S rDNA of 14 taxa under study varied from 630-668 bp. The average nucleotide frequencies were A = 16.3%, C = 26.4%, G = 28.3% and T = 29.0%. Thus, the mean of G+C content was 54.6%. Sequence divergence between ingroup and outgroup ranged from 36.0-40.3%. The transition/transversion ratio was 2.63.

Regarding the ITS data matrix (Figure 35), there were 690 characters for analyses, of which 425 (61.6%) characters were constant and 98 (14.2%) characters were informative under parsimony. Only one most parsimonious tree (Figure 36) of length 340 steps was obtained based on parsimony analysis of the informative characters with 1,000 bootstrap replicates. Fit measures of the tree were as follows: CI = 0.8941, HI = 0.1059, RI = 0.8414 and RC = 0.7523.

Figures 36 and 37 illustrate the most parsimonious tree and phylogram obtained from PAUP analysis of the ITS region using parsimony method. Phylogenetic tree inferred from the ITS region was identical to that from the ITS2 region. The tree composed of two clades which are clade I, including the *P. westermani* complex and *P. siamensis* (BS = 98%) and clade II, including other Thai *Paragonimus* species (BS = 91%). Within the *P. westermani* complex, two groups of organism can be obtained based on geographical distribution. The first group contains *P. westermani* from South East Asia (BS = 76%) whereas the second group contains *P. westermani* from East Asia and *P. westermani*-like from Thailand (BS = 70%).

Table 9. Sequence characteristics of the ITS1 and ITS2 regions.

Sequence characteristics	ITS1	ITS2	ITS1 and ITS2
Length range (total) (bp)	267-307	359-372	630-668
Length mean (total) (bp)	292.4	363.2	655.6
Length range (ingroup) (bp)	267-307	359-363	630-666
Length mean (ingroup) (bp)	292.2	362.5	654.7
Length range (outgroup) (bp)	296	372	668
Aligned length (bp)	312	378	690
G+C content range (%)	52.1-54.3	50.5-57.0	52.1-55.7
G+C content mean (%)	53.6	55.5	54.6
Sequence divergence range (ingroup) (%)	0-16.8	0-13.7	0-14.4
Sequence divergence range (total) (%)	33.7-38.5	37.6-41.9	36.0-40.3
Number of indels (total)	15	10	25
Size of indels (total) (bp)	1-6	1-11	1-11
Number of constant sites (%)	194 (62.2)	231 (61.1)	425 (61.6)
Number of variable sites (%)	118 (37.8)	147 (38.9)	265 (38.4)
Number of informative sites (%)	43 (13.8)	55 (14.6)	98(14.2)
Number of uninformative sites (%)	75 (24.0)	92 (24.3)	167 (24.2)
Transition/transversion ratio (ts/tv ratio)	2.42	2.72	2.63

Figure 35. Sequence data matrix of the aligned ITS1 (site 1 to 312) and ITS2 (site 313 to 690) regions of nuclear ribosomal DNA for 14 taxa of *Paragonimus* and its outgroup. Uncertain nucleotide states are coded as follows: N = A/C/G/T, R = A/G, W = A/T, Y = C/T; hyphens "-" denote alignment gaps; "**a-y**" above the nucleotide matrix indicate the positions of alignment gaps (indels); question marks "?" indicate missing data; numbers in parentheses at the end of sequences indicate the actual length of the ITS region.

```
                                    10        20        30        40        50        60        70        80        90
Taxon                               ITS1
                                                                                          a
P. westermani (Japan)               ??????????????????????????????????????CGGATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACCGACAG
P. westermani (China)               ??????????????????????????????????????CGGATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACCGACAG
P. westermani (Philippines)         ??????????????????????????????????????CGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCAGTCTTCACGGACAG
P. westermani (Malaysia)            ??????????????????????????????????????CGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACGGACAG
P. westermani (Saraburi)            TACCTGTCTGATGCCCT-ACGTTTTGCTTGCCATTTT-CGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACGGACAG
P. westermani (Nakhon Nayok)        TACCTGTCTGATGCCCT-ACGTTTTGCTTGCCATTTT-CGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACGGACAG
P. westermani (Surat Thani)         TACCTGTCTGATGCCCT-ACGTTTTGCTTGCCATTTT-CGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACGGACAG
P. westermani-like                  TACCTGTCTGATGCTCT-ACGTGTTGCTTGCCATTTT-CGGATGGCCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCACCGACAG
P. siamensis                        TACCTGTCTGATGCCCT-TCGTTTTGCTTGCCATTTT-CGAATGGTCAGTCCATCTCGTGGGTGACGGGTTGTGCTGTCTTCATGGACAG
P. bangkokensis                     TACCCGTCTGATGCTCT-TTGTTTTGCTTGCCATCTT-CGGATGGTCAGTCCATCTCGGGAGTGACGGGTTGTACTGTTCTCATAGACAG
P. harinasutai                      TACCCGTCTGATGCTCT-TTGTTTTGCTTGCCATCTT-CGGATGGTCAGTCCATCTCGGGAGTGACGGGTTGTACTGTTCTCATAGACAG
P. macrorchis                       TGCCCGTCTGATGCTCT-TTGATTTGCTTGCCATTTTCCGGATGGTCAGTCCATCTCGGGAGTGACGGGTTGTGCTGTCCACGTAGGCAG
P. heterotremus                     TACCCGTCTGATGCTCT-TTGATTTGCTTGCCATTTT-CGGATGGTCAGTCCATTTCGGGAGTGACGGGTTGTGCTGTCCTCATAGACAG
Fasciola hepatica                   TACCTGTATGATACTCCGATGGTATGCTTGCGTCTCT-CGGGGCGCTTGTCCAAGCCAGGAGA-ACGGGTTGTACTGCCACGATTGGTAG
```

Figure 35. (continued)

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                                100       110       120       130       140       150       160       170       180

Taxon
                                        |   b   | c                             d  e         f          g
P. westermani (Japan)           TGCTATGCTTAATGAGTGGTATGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani (China)           TGCTAGGCTTAATGAGTGGTATGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani (Philippines)     TGCTATGCTTAATGAGTGGTACGGCATATTGARCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani (Malaysia)        TGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani (Saraburi)        TGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani (Nakhon Nayok)    TGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani (Surat Thani)     TGCTAGGCTTAATGAGTGGTACGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
P. westermani-like              TGCTAGGCTTAATGAGTGGTATGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACRCTGTTC
P. siamensis                    TGCTAGGCTTAATGAGTGGTACGGCATATTGAGCCACGGCTCGGCCACCGCCCT--G-TTTTTTTCAATTTATGCCATTTTACACTGTTC
P. bangkokensis                 TGCTAGGCTTAATGAGTGGTAAGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCGTTCTACACTGTTC
P. harinasutai                  TGCTAGGCTTAATGAGTGGTAAGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCGTTCTACACTGTTC
P. macrorchis                   CGCTTGGCTTAATGAGTGGTGAGGTATATTGAGCTACGGCTCGACCACCGCCCT--GCTTTGTTTCAATTTATTCCATTTTACACTGTTC
P. heterotremus                 CGCTAGGCTTAATGAGTGGTAAGGCATATTGAGCTACGGCTCGGCCACCGCCCT--G-TTTGTTTCAATTTATGCCATTTTACACTGTTC
Fasciola hepatica               TGCTAGGCTTAAAGAGGAG------AT-TTGGGCTACGGCCCTGCTCCCGCCCTATGAACTGTTTCA--TTACTACATT-TACACTGTTA


                                190       200       210       220       230       240       250       260       270

Taxon
                                      hi    j                       k               l   m               n
P. westermani (Japan)           AAGTGGTTCAGGTCAG-CTCGTCTGAACTGTTCCACTGCCCGA-CATGCACCCGGTCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani (China)           AAGTGGTTCAGGTCAG-CTCGTCTGAACTGTTCCACTGCCCGA-CATGCACCCGGTCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani (Philippines)     AAGTGGTTCAGGTCAG-CTCGTCTGGGCTGTTCCACTGCCCGA-CATGCACCCGGTCTC-GCG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani (Malaysia)        AAGTGGTTCAGGTCAG-CTCGCCTGGGCTGTTCCACTGCCCGA-CATGCACCCGGTCTTTGTG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani (Saraburi)        AAGTGGTTCAGGTCAG-CTCGTCTGGGCTGTTCCACTGCCCGA-CATGCACCCGGCCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani (Nakhon Nayok)    AAGTGGTTCAGGTCAG-CTCGTCTGGGCTGTTCCACTGCCCGA-CATGCACCCGGCCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani (Surat Thani)     AAGTGGTTCAGGTCAG-CTCGTCTGGGCTGTTCCACTGCCCGA-CATGCACCCGGCCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. westermani-like              AAGTGGTTCAGGTCAG-CTCGTCTGGGCTGTTCCGCTGCCCGA-CATGCACCCGGTCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. siamensis                    AAGTGGTTCAGATCAG-CTCGTCTGGACTGTTCCATTGCCCGA-CATGCACCCGGTCTC-GTG-CTGGACTGCATGTACAGTCGCCTGGC
P. bangkokensis                 AAGTGGTTCAGGTCG--CCTGTTCGACCTGTTCCGTTGCCCGA-CATGCACCCGGTCCC-GTA-CTGGACTGCATGTACAGTCGCCTGGC
P. harinasutai                  AAGTGGTTCAGGTCG--CCTGTTCGACCTGTTCCGTTGCCCGA-CATGCACCCGGTCCC-GTA-CTGGACTGCATGTACAGTCGCCTGGC
P. macrorchis                   AAGTGGCTCAGGTCGGCCCTGTTCGACCTGGTCCATTGCCCGA-CATGCACCTGGTCCCTGTA-CCGGACTGTATGTACAGTCGCCTGGC
P. heterotremus                 AAGTGGTTCAGGTCGG-CCTGTCTGACTTTGTCCATTGCCCGA-CATGCACCCGGTCCC-GTA-CTGGACTGCATGTACAGTCGCCTGGC
Fasciola hepatica               AAGTGGTACTGAATGG-CTTG-CCATTCTTTGCCATTGCCCTCGCATGCACCCGGTCCTTGTGGCTGGACTGCACGTAC-GTCGCCCGGC
```

Figure 35. (continued)

```
                                 280        290        300        310        320        330        340        350        360
Taxon                                                                ITS2
                                 o
P. westermani (Japan)            GGTGCCTTATCCCGGGCTACACTGGTTAACCATACATCGTGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani (China)            GGTGCCTTATCCCGGGCTANACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani (Philippines)      GGTGCCTTATCCCGGGCTARACTGGTTAACCATACATCGTGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani (Malaysia)         GGTGCCTTATCCCGGGCTAAACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani (Saraburi)         GGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani (Nakhon Nayok)     GGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani (Surat Thani)      GGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. westermani-like               GGTGCCTTATCCCGGGCTAGACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. siamensis                     GGTGCCTTATCCCGGGCTAGACTGGTAAACCATACGTCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. bangkokensis                  GGTGCCTTATCCTGGGCTTGACTGGTTAACCATACGTCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. harinasutai                   GGTGCCTTATCCTGGGCTTGACTGGTTAACCATACGTCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. macrorchis                    GGTGCCTTATCCCGGGCTTGACCGGTTAACCATACGACGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
P. heterotremus                  GGTGCCTTATCCCGGGCTTGACTGGTTAACCATACATCGTTCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG
Fasciola hepatica                GGTGCCT-ATCCCGGGTTGGACTGATAACCTGGTCTTTGACCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG

                                 370        380        390        400        410        420        430        440        450
Taxon
                                                                                                      |  p  |
P. westermani (Japan)            CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani (China)            CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani (Philippines)      CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani (Malaysia)         CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani (Saraburi)         CTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani (Nakhon Nayok)     CTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani (Surat Thani)      CTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGRTCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. westermani-like               CTTATGAAYTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTG
P. siamensis                     CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTCTTGCCAGCTGGCGTGATCTCCCCAATCAGGTCTCGTGCCTGTGGGGTG
P. bangkokensis                  CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATTTCCCCGATCTGACCTTGTGTCGGTGGGGTG
P. harinasutai                   CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATTTCCCCGGTCTGACCTTGTGTCGGTGGGGTG
P. macrorchis                    CTTATAAACTATCGCAACGCCCAAAAAGTTGCGGCTTGGGTTCTGCCAGCTGGCGTGATTTCCCCAACCTGGCCTCGTGTCTGTGGGGTG
P. heterotremus                  CTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATTTCCCCAACGTGGCCTTGTGTCTGTGGGGTG
Fasciola hepatica                CTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCTCTATGAGTAAT----CATGTGAGGTG
```

Figure 35. (continued)

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                                460       470       480       490       500       510       520       530       540

Taxon
                                                                                                q
P. westermani (Japan)           CCAGATCTGTGGCGTTTCCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTC
P. westermani (China)           CCAGATCTGTGGCGTTTCCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTC
P. westermani (Philippines)     CCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGAAATCGTGGCTC
P. westermani (Malaysia)        CCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGAAATCGTGGCTC
P. westermani (Saraburi)        CCAGATCTATGGCGTTTCCCTAACATACTCGCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTC
P. westermani (Nakhon Nayok)    CCAGATCTATGGCGTTTCCCTAACATACTCGCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTC
P. westermani (Surat Thani)     CCAGRTCTATGGCGTTTCCCTAACATACTCGCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCYC
P. westermani-like              CCAGATCTGTGGCGTCTCCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTC
P. siamensis                    TCAGATCTATGGCGTTTCCCTAACATACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTGGCTC
P. bangkokensis                 CCAGATCTATGGCGTTTCCCTAACCTGTCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTGGCTC
P. harinasutai                  CCAGATCTATGGCGTTTCCCTAACCTGTCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGAAGTGGCAACGGAATCGTGGCTC
P. macrorchis                   CCGGATCTGTGGCGTTTCCCTAAAAAATCCGGACGTACCCGTGTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTGGCTC
P. heterotremus                 CCAGATCTGTGGCGTTTCCCTAACAAATCCGGGCGTATCCATGTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTGGCTC
Fasciola hepatica               CCAGATCTATGGCGTTTCCCTAATGTATCCGGATGCACCCTTGTCTTGGCAGAAAGCCGTGGTGAGG-TGCAGTGGCGGAATCGTGGTTT

                                550       560       570       580       590       600       610       620       630

Taxon
                                     |     r     |                                                           |s|t|u|      v
P. westermani (Japan)           AGTGAA-----------TGATTTATGTGCGCGTTCCGCTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCTTTCGATGCT-
P. westermani (China)           AGTGAA-----------TGATTTATGTGCGCGTTCCGCTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCTTTCGATGCT-
P. westermani (Philippines)     AGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGATCGATGCT-
P. westermani (Malaysia)        AGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGATCGATGCT-
P. westermani (Saraburi)        AGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCT-
P. westermani (Nakhon Nayok)    AGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTCCTGTCYTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCT-
P. westermani (Surat Thani)     AGTAAA-----------TGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTGCGCGTGGTCTGCGTTCGATGCT-
P. westermani-like              AGTGAT-----------TGGTTTATGTGCGCGTTCCGCTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGCGTTCGATGCT-
P. siamensis                    AGTAGA-----------TGAATTATGTGCGCGTTCCGTTGTCCTGTCTTCATCTGTGGTTTATGTTGCGCGTGGTCTGTGTTCGACGTT-
P. bangkokensis                 AGTGAA-----------TTATTTATGTGCGCGTTCCGTCGTCCTATCATCATCTATGGTTGATGCTGCGTGTGGTGTGCGTCTGATGCT-
P. harinasutai                  AGTGAA-----------TTATTTATGTGCGCGTTCCGTCGTCCTATCATCATCTATGGTTGATGCTGCGTGTGGTGTGCGTCTGATGCT-
P. macrorchis                   AGTGAA-----------AAATTTATGTGCGCGTTCCGCTGTCCTGTCATCATCTTTGGTTGATGTTGCATGTGGTGT----CCGATGCT-
P. heterotremus                 AGTGAA-----------TGATTTATGTGCACGTTCCGCTGTCCCGTCATCATCTATGGTTGAAGTTGCGCGTGGTGTG--TCCGATGCT-
Fasciola hepatica               AATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTTGGCGATCCCCTAGTCGGCACACTTATGATTTCTGGGATAATTCCATACCAGGCAC
```

Figure 35. (continued)

```
                                    640        650        660        670        680        690

Taxon
                                                w          x          y
P. westermani (Japan)               GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [630]
P. westermani (China)               GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [630]
P. westermani (Philippines)         GACCTACGTTTGTGCCAT-GTGGTTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [630]
P. westermani (Malaysia)            GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [631]
P. westermani (Saraburi)            GACCTACGTATGTGCCAT-GTGGTCCATTC--TTCTGACCTCGGATTAGACGTGAGTACC   [666]
P. westermani (Nakhon Nayok)        GACCTACGTATGTGCCAT-GTGGTCCATTC--TTCTGACCTCGGATTAGACGTGAGTACC   [666]
P. westermani (Surat Thani)         GACCTACGTATGTGCCAT-GTGGTCCATTC--TTCTGACCTCGGATTAGACGTGAGTACC   [666]
P. westermani-like                  GACCTACGTATGTGCCAT-GTGGTTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [666]
P. siamensis                        GACCTATCTATGTGCCAT-ATGGTTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [666]
P. bangkokensis                     GACCTGAGTATGTGCCAT-GTGGCTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [665]
P. harinasutai                      GACCTGAGTATGTGCCAT-GTGGCTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [665]
P. macrorchis                       GACCTATGTTTGTGCCGT-GCGGCTCATTC--TCCTGACCTCGGATCAGACGTGAGTACC   [666]
P. heterotremus                     GACCTATATATGTGCCAT-GTGGCTCATTT--TCCTGACCTCGGATCAGACGTGAGTACC   [664]
Fasciola hepatica                   GTTCCGTCACTGTCACTTTGTTATTGGTTTGATGCTGAACTTGG-TCATGTGTCTGATGC   [668]
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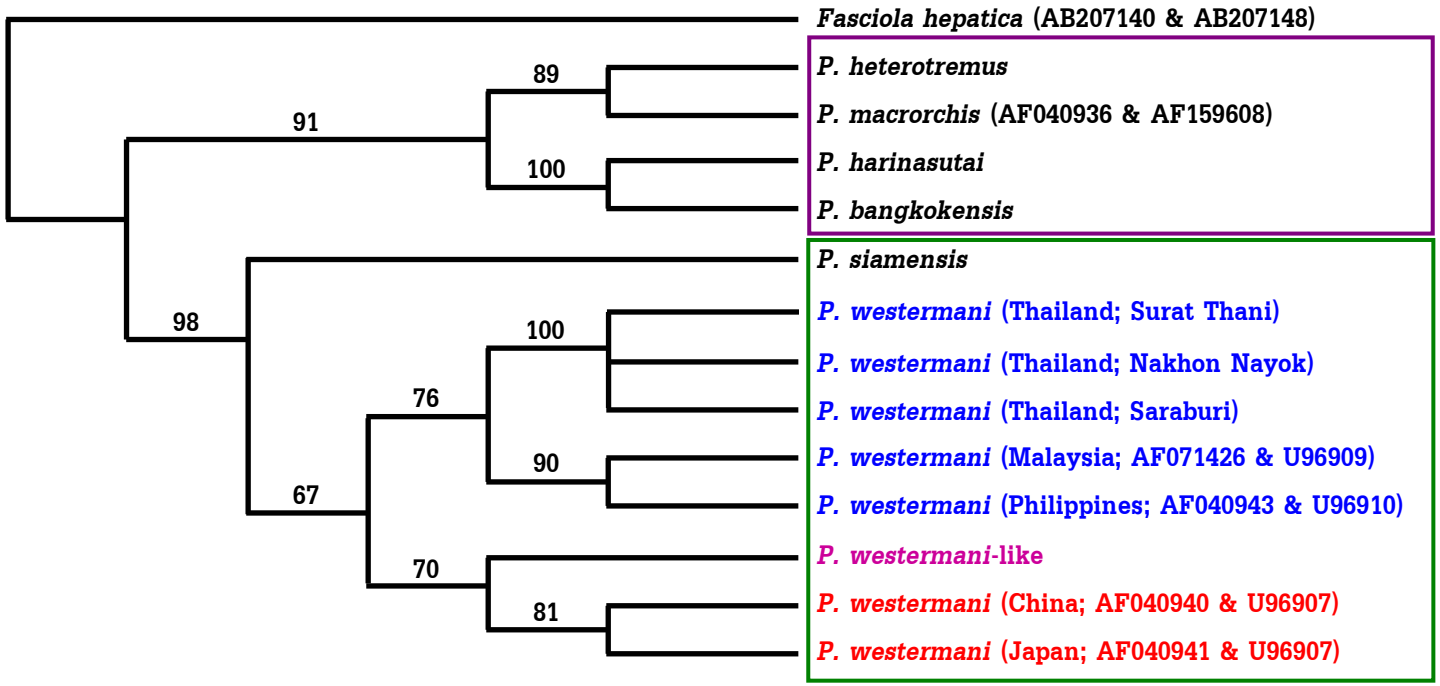

Figure 36. Single most parsimonious tree of length 340 steps based on parsimony analysis of the informative characters of the combined ITS1 and ITS2 regions. Numbers above the branches are bootstrap values (%) of 1,000 replicates. Clade I (green box) includes *P. westermani* complex and *P. siamensis*. Within the *P. westermani* complex, taxa in blue indicate *P. westermani* from South East Asia while taxa in red indicate *P. westermani* from East Asia and a taxon in pink denotes *P. westermani*-like from Thailand. Clade II (purple box) contains other *Paragonimus* species found in Thailand.

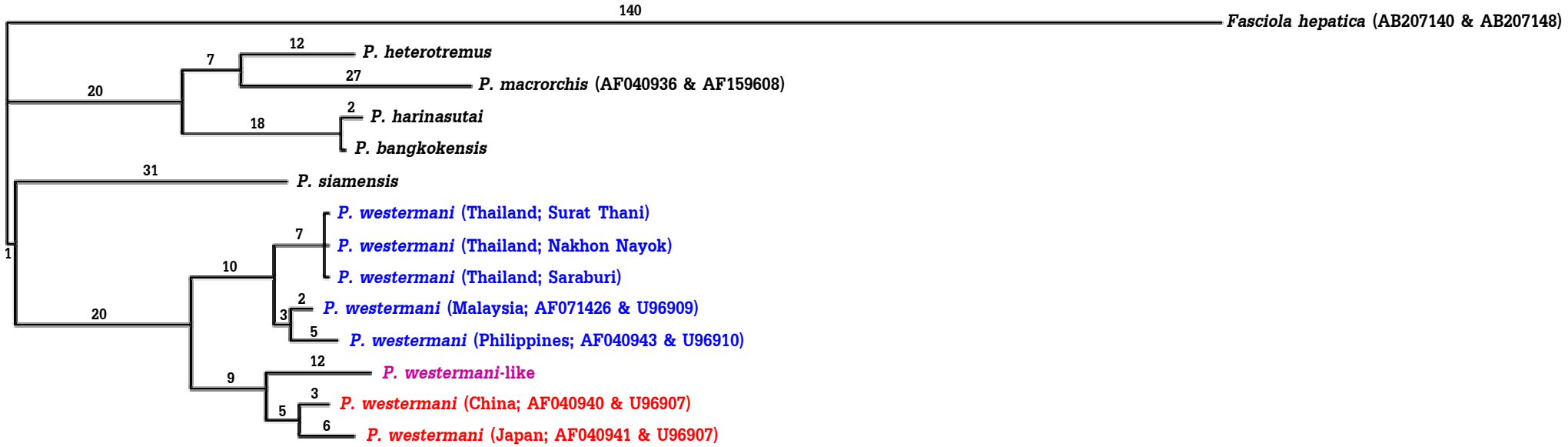

Figure 37. Phylogram of most parsimonious tree of length 340 steps based on parsimony analysis of the combined ITS1 and ITS2 regions. Numbers above the branches indicate branch length. The tree was rooted using *Fasciola hepatica*. Taxa in blue indicate *P. westermani* from South East Asia while taxa in red indicate *P. westermani* from East Asia.

9.3 Sequence and phylogenetic analyses of the partial COI region

The alignment of the partial COI region was 384 bases long with only one site of indels (3 bases long, indicated by "z") (Figure 38). The length of the sequence of 16 taxa under study varied from 381-384 bp. Within 384 characters, 241 (62.8%) were constant, 38 (9.9%) were parsimony-uninformative and 105 (27.3%) were parsimony-informative. Sequence divergence between ingroup and outgroup taxa computed using pairwise distance analysis ranged from 22.2-34.7%. The G+C content ranged from 36.5-49.3% and transition/transversion ratio was 3.86. The sequence characteristics of the partial COI region were summarized in Table 10.

Strict consensus tree (Figure 39) was derived from 10 equally parsimonious trees and a phylogram (Figure 40) showed one of ten most parsimonious trees of length 319 steps based on parsimony analysis with 1,000 bootstrap replicates. Fit measures of the tree were as follows: CI = 0.6364, HI = 0.3636, RI = 0.7010 and RC = 0.4461. The tree inferred from the partial COI region showed a single clade with strong bootstrap support of 98%. This clade forms a complex of *P. westermani* from South East Asia and East Asia (BS = 76%) and *P. westermani*-like. *Paragonimus westermani*-like, however, is excluded from the complex and placed as a sister group to it. In addition, the position of *P. siamensis* confirms a close relationship with *P. westermani* and *P. westermani*-like while other *Paragonimus* species (*P. heterotremus*, *P. macrorchis, P. harinasutai* and *P. bangkokensis*) are more distantly related.

Table 10. Sequence characteristics of the partial COI region.

Sequence characteristics	Partial COI region
Length range (total) (bp)	381-384
Length mean (total) (bp)	381.2
Length range (ingroup) (bp)	381
Length range (outgroup) (bp)	384
Aligned length (bp)	384
G+C content range (%)	36.5-49.3
G+C content mean (%)	44.2
Sequence divergence range (ingroup) (%)	0-25.3
Sequence divergence range (total) (%)	22.2-34.7
Number of indels (total)	1
Size of indels (total) (bp)	3
Number of constant sites (%)	241 (62.8)
Number of variable sites (%)	143 (37.2)
Number of informative sites (%)	105 (27.3)
Number of uninformative sites (%)	38 (9.9)
Transition/transversion ratio (ts/tv ratio)	3.86

Figure 38. Sequence data matrix of the aligned partial COI region of mitochondrial DNA for 16 taxa of *Paragonimus* and its outgroup. Hyphens "-" denote alignment gaps; "**z**" above the nucleotide matrix indicates the position of alignment gap (indels); numbers in parentheses at the end of sequences indicate the actual length of the partial COI region.

```
                                  10        20        30        40        50        60        70        80        90
Taxon
P. westermani (Japan)             TTGCCTGGGTTTGGTATCGTGAGCCACATTTGCATGACCCTGACCAACAACGATTCCTTGTTCGGGTACTACGGGCTGGTGTTTGCGATG
P. westermani (China)             TTGCCTGGGTTTGGTATCGTGAGCCACATTTGCATGACCCTGACCAACAATGATTCCTTGTTCGGGTACTACGGGCTGGTGTTTGCGATG
P. westermani (Korea)             TTGCCTGGGTTTGGTATCGTGAGCCACATTTGCATGACCCTGACCAACAATGATTCCTTGTTCGGGTACTACGGGCTGGTGTTTGCGATG
P. westermani (Taiwan)            TTGCCTGGGTTTGGTATCGTGAGCCACATTTGCATGACCCTGACCAACAATGATTCCTTGTTCGGGTACTACGGGCTGGTGTTTGCGATG
P. westermani (Philippines)       TTGCCTGGGTTTGGCATCGTGAGTCACATCTGCATGACTTTGACTAATAATGATTCCTTGTTTGGGTATTATGGGTTGGTGTTTGCGATG
P. westermani (Malaysia)          TTGCCAGGGTTTGGTATCGTGAGTCACATCTGCATGACTTTAACCAACAATGATTCCTTGTTTGGGTATTACGGGTTGGTGTTTGCGATG
P. westermani (Saraburi)          TTGCCGGGGTTTGGTATTGTGAGTCACATCTGCATGACTTTGACTAACAATGATTCCTTGTTTGGGTATTACGGGTTAGTGTTTGCGATG
P. westermani (Nakhon Nayok)      TTGCCGGGGTTTGGTATTGTGAGTCACATCTGCATGACTTTGACTAACAATGATTCCTTGTTTGGGTATTACGGGTTAGTGTTTGCGATG
P. westermani (Surat Thani)       TTGCCGGGGTTTGGTATCGTGAGTCACATCTGCATGACTTTGACTAACAATGATTCCTTGTTTGGGTATTACGGGTTAGTGTTTGCGATG
P. westermani-like                TTGCCAGGGTTTGGGATTGTGAGTCACATCTGCATGACTTTAACTAACAATGATTCCTTGTTTGGGTATTATGGGTTGGTGTTCGCGATG
P. siamensis                      TTGCCTGGTTTTGGGATTGTGAGACATATTTGTATGACTTTGACGAATAATGATTCTCTGTTTGGTTATTATGGGTTAGTGTTTGCGATG
P. bangkokensis                   TTGCCGGGATTTGGGGTTGTGAGCCATATTTGCATGACTTTAACTAATAATGATTCGCTGTTTGGTTATTATGGGTTGGTGTTCGCCATG
P. harinasutai                    TTACCGGGATTTGGAGTTGTAAGCCATATTTGCATGACTTTGACTAATAAAGATTCGCTGTTTGGTTACTATGGGTTGGTGTTCGCTATG
P. macrorchis                     TTGCCAGGGTTTGGTGTTGTGAGACATATTTGTATGACGTTAACTAATAACGACTCTTTGTTTGGTTATTATGGTTTAGTTTTTGCCATG
P. heterotremus                   TTGCCTGGATTTGGTGTTGTGAGACATATTTGCATGACTTTGACTAATAAAGATTCTTTGTTTGGTTATTATGGCTTGGTTTTTGCCATG
Fasciola hepatica                 TTACCCGGGTTTGGGGTTATTAGTCATATTTGTGTGACTTTAACTAATAATGATTCTTTGTTTGGTTATTATGGTCTTATTTTAGCTATG
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Figure 38. (continued)

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                                  100        110        120        130        140        150        160        170        180
Taxon

P. westermani (Japan)             GGGGCCATTGTGTGTCTGGGTAGTGTTGTGTGGGCGCATCACATGTTCATGGTTGGTTTGGATGTCAAGACTGCTGTTTTCTTTAGTTCT
P. westermani (China)             GGGGCCATTGTGTGTCTGGGTAGTGTTGTGTGGGCGCATCACATGTTCATGGTTGGTTTGGATGTCAAGACTGCTGTTTTCTTTAGTTCT
P. westermani (Korea)             GGGGCCATTGTGTGTCTGGGTAGTGTTGTGTGGGCGCATCACATGTTCATGGTTGGTTTGGATGTCAAGACTGCTGTTTTCTTTAGTTCT
P. westermani (Taiwan)            GGGGCCATTGTGTGTCTGGGTAGTGTTGTGTGGGCGCATCACATGTTCATGGTTGGTTTGGATGTCAAGACTGCTGTTTTCTTTAGTTCT
P. westermani (Philippines)       GGGGCTATTGTGTGTCTGGGCAGTGTTGTGTGGGCGCATCACATGTTCATGGTTGGTTTGGATGTTAAGACTGCTGTTTTTTTTAGTTCT
P. westermani (Malaysia)          GGGGCCATTGTGTGCTTGGGCAGTGTTGTGTGGGCGCATCATATGTTCATGGTTGGTCTAGATGTCAAGACCGCTGTCTTTTTCAGTTCT
P. westermani (Saraburi)          GGGGCCATCGTGTGCTTGGGCAGTGTTGTGTGGGCGCACCACATGTTTATGGTTGGTTTGGATGTTAAGACTGCTGTCTTTTTTAGCTCT
P. westermani (Nakhon Nayok)      GGGGCCATCGTGTGCTTGGGCAGTGTTGTGTGGGCGCACCACATGTTTATGGTTGGTTTGGATGTTAAGACTGCTGTCTTTTTTAGCTCT
P. westermani (Surat Thani)       GGGGCCATCGTGTGCTTGGGCAGTGTTGTGTGGGCGCACCACATGTTTATGGTTGGTTTGGATGTTAAGACTGCTGTCTTTTTTAGCTCT
P. westermani-like                GGGGCTATCGTATGTTTGGGTAGTGTTGTGTGAGCGCACCATATGTTCATGGTTGGTTTGGATGTTAAGACTGCTGTTTTTTTTAGTTCT
P. siamensis                      GGAGCTATTGTGTGTTTGGGAAGTGTTGTGTGGGCGCATCATATGTTTATGGTTGGTTTGGATGTTAAGACTGCTGTGTTTTTTAGTTCT
P. bangkokensis                   GGGGCGATTGTTTGTTTGGGCAGTGTTGTATGGGCTCATCACATGTTTATGGTTGGTCTGGATGTTAAGACCGCGGTGTTTTTTAGATCT
P. harinasutai                    GGGGCGATTGTTTGTTTGGGTAGTGTTGTGTGAGCCCATCATATGTTTATGGTTGGTTTAGATGTTAAGACCGCGGTGTTTTTTAGATCT
P. macrorchis                     GGGGCTATTGTTTGTTTGGGGAGTGTGGTTTGGGCGCATCATATGTTTATGGTTGGTCTTGATGTTAAGACTGCTGTTTTTTTTAGCTCT
P. heterotremus                   GGGGCTATTGTGTGTTTGGGGAGGGTTGTTTGAGCGCACCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGTTCT
Fasciola hepatica                 GCTGCTATAGTATGTTTAGGTAGTGTTGTTTGGGCTCATCATATGTTTATGGTGGGTTTGGATGTGCATACTGCTGTTTTTTTTAGTTCT

                                  190        200        210        220        230        240        250        260        270
Taxon
                                                                                                      z
P. westermani (Japan)             GTCACGGGAGTGATTGGGATACCCACGGGGATTAAGGTTTTCTCTTGGTTGTTCATGCTGGGTGGAACTCGTT---TGCGGTTTTGGGAT
P. westermani (China)             GTCACGGGAGTGATTGGGATACCCACGGGGATTAAGGTTTTCTCCTGGTTGTTCATGCTGGGTGGAACTCGTT---TGCGGTTTTGGGAT
P. westermani (Korea)             GTCACGGGAGTGATTGGGATACCCACAGGGATTAAGGTTTTCCCTTGGTTGTTCATGCTGGGTGGAACTCGTT---TGCGGTTTTGGGAT
P. westermani (Taiwan)            GTCACTGGAGTGATTGGGATACCCACGGGGATTAAGGTTTTCTCCTGGTTGTTCATGCTGGGTGGAACTCGTT---TGCGGTTTTGGGAT
P. westermani (Philippines)       GTCACGGGGGTGATTGGGATACCTACGGGGATTAAGGTTTTTTCTTGGTTGTTTATGTTGGGTGGATCTCGCT---TGCGATTTTGAGAT
P. westermani (Malaysia)          GTTACGGGGGTGATTGGTATACCTACGGGGATTAAGGTTTTTTCCTGGTTGTTTATGCTGGGTGGAGCTCGTT---TACGGTTTTGGGAT
P. westermani (Saraburi)          GTCACTGGGGTGATTGGGATACCTACGGGGATTAAGGTCTTCTCTTGGTTGTTCATGTTGGGTGGGTCTCGTT---TACGATTTTGAGAT
P. westermani (Nakhon Nayok)      GTCACTGGGGTGATTGGGATACCTACGGGGATTAAGGTCTTCTCTTGGTTGTTCATGTTGGGTGGGTCTCGTT---TACGATTTTGAGAT
P. westermani (Surat Thani)       GTCACTGGGGTGATTGGGATACCTACGGGGATTAAGGTCTTCTCTTGGTTGTTCATGTTGGGTGGATCTCGTT---TACGATTTTGAGAT
P. westermani-like                GTTACGGGGGTGATTGGGATACCTACGGGGATTAAGGTTTTTTCCTGGTTGTTTATGTTGGGTGGAACTCGTT---TGCGGTTTTGAGAT
P. siamensis                      GTCACCGGGGTTATTGGGATACCTACGGGTATTAAGGTTTTTTTCTTGATTATTCATGTTAGGTGGGGCTCGTT---TGCGTCTTTGGGAT
P. bangkokensis                   GTTACTGGGGTGATAGGTATCCCGACAGGGATTAAGGTTTTTTTCTTGGTTGTTTATGTTGGGGGGCACTCGTT---TACGGTTTTGAGAT
P. harinasutai                    GTTACTGGGGTGATAGGTATCCCGACAGGGATTAAGGTTTTTTTCCTGGTTGTTTATGTTGGGTGGCACTCGTT---TGCGGTTTTGAGAT
P. macrorchis                     GTTACGGGGGTGATTGGTATTCCTACGGGTATTAAGGTTTTTTTCTTGGTTGTTTATGCTAGGGGGAACTCGCT---TACGGTTTTGGGAT
P. heterotremus                   GTTACTGGGGTGATTGGGATTCCCACAGGGATTAAGGTTTTTTTCTTGGTTGTTTATGTTGGGGGGGACTCGTT---TACGGTTTTGAGAT
Fasciola hepatica                 GTTACTATGGTTATTGGTATTCCTACAGGTATTAAGGTCTTTTCCTGGTTGATAATGTTGGGGGGGGGTAGTTCTGTTCGTATATGGGAT
```

Figure 38. (continued)

Taxon	280 290 300 310 320 330 340 350 360
P. westermani (Japan)	CCTGTTTTGTGGTGAATTCTTGGGTTTATCTTCCTGTTCACCATAGGTGGTGTGACCGGCATCATTCTGTCCTCCTCCATACTGGATAGT
P. westermani (China)	CCTGTTTTGTGGTGAATTCTTGGGTTTATCTTCCTGTTCACCATAGGTGGTGTGACCGGCATCATTCTGTCCTCCTCCATACTGGATAGT
P. westermani (Korea)	CCTGTTTTGTGGTGAATTCTTGGGTTTATCTTCCTGTTCACCATAGGTGGTGTGACCGGCATCATTCTGTCCTCCTCCATACTGGATAGT
P. westermani (Taiwan)	CCTGTTTTGTGGTGAATTCTTGGGTTTATCTTCCTGTTCACCATAGGTGGTGTGACCGGCATCATTCTGTCCTCCTCCATACTGGATAGT
P. westermani (Philippines)	CCTGTTTTGTGGTGGATCCTTGGGTTTATCTTTCTGTTCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCCTCTATTCTGGACAGT
P. westermani (Malaysia)	CCTGTTTTGTGGTGGATCCTTGGGTTTATCTTTTTGTTCACCATAGGGGGCGTGACTGGCATCATTTTGTCTTCCTCCATACTGGATAGT
P. westermani (Saraburi)	CCTGTTTTGTGGTGGATCCTTGGGTTCATTTTTCTGTTCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCTTCCATCCTGGATAGT
P. westermani (Nakhon Nayok)	CCTGTTTTGTGGTGGATCCTTGGGTTCATTTTTCTGTTCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCTTCCATCCTGGATAGT
P. westermani (Surat Thani)	CCTGTTTTGTGGTGGATCCTTGGGTTCATTTTTCTGTTCACCATAGGGGGTGTGACTGGCATCATTTTGTCTTCTTCCATCCTGGATAGT
P. westermani-like	CCTGTTTTGTGGTGGATACTCGGGTTTATTTTTCTGTTCACCATAGGTGGTGTGACTGGCATTATTTTGTCTTCTTCCATATTGGATAGC
P. siamensis	CCTGTCTTGTGGTGGATTCTTGGTTTCATTTTCTTGTTTACTATAGGTGGTGTGACCGGTATTGTTTTGTCCTCTTCAATATTGGATAGT
P. bangkokensis	CCTGTAATATGGTGAATTTTGGGGTTTATCTTTCTGTTTACCATTGGGGGTGTAACTGGGATTATCTTGTCTTCTTCTATCTTGGACAGT
P. harinasutai	CCTGTAATATGGTGAATTTTGGGGTTTATTTTTCTGTTTACTATTGGGGGTGTGACTGGAATTATTTTGTCTTCTTCTATTTTGGATAGT
P. macrorchis	CCCGTAATTTGATGAATTTTGGGATTTATTTTTCTTTTTACTATAGGTGGGGTGACGGGGATTATTTTGTCTTCTTCTATTTTAGATAGT
P. heterotremus	CCGGTGGTTTGGTGAATTTTAGGCTTTATTTTTCTTTTTACTATTGGTGGTGTAACTGGGATTATTTTGTCTTCTTCTATTTTGGATAGT
Fasciola hepatica	CCTGTTGTGTGGTGAATTATAGGGTTTATTGTTTTATTTACTATTGGTGGGGTTACTGGTATTATGCTTTCTGCTTCTCTTTTGGATACT

Taxon	370 380	
P. westermani (Japan)	CTGTTGCATGATACGTGGTTCGTC	[381]
P. westermani (China)	CTGTTGCATGATACGTGGTTCGTC	[381]
P. westermani (Korea)	CTGTTGCATGATACGTGGTTCGTC	[381]
P. westermani (Taiwan)	CTGTTGCATGATACGTGGTTCGTC	[381]
P. westermani (Philippines)	CTGTTGCATGACACGTGGTTTGTT	[381]
P. westermani (Malaysia)	CTGTTGCATGATACGTGGTTTGTT	[381]
P. westermani (Saraburi)	CTGTTGCATGACACGTGGTTTGTT	[381]
P. westermani (Nakhon Nayok)	CTGTTGCATGACACGTGGTTTGTT	[381]
P. westermani (Surat Thani)	CTGTTGCATGACACGTGGTTTGTT	[381]
P. westermani-like	CTGTTGCATGACACGTGGTTTGTT	[381]
P. siamensis	TTGTTGCATGATACGTGGTTTGTG	[381]
P. bangkokensis	TTGTTGCATGACACTTGATTTGTT	[381]
P. harinasutai	TTGTTGCATGATACCTGATTTGTT	[381]
P. macrorchis	TTGTTGCATGATACTTGGTTTGTT	[381]
P. heterotremus	CTGTTACATGATACCTGGTTTGTT	[381]
Fasciola hepatica	TTGCTTCATGATACATGGTTTGTG	[384]

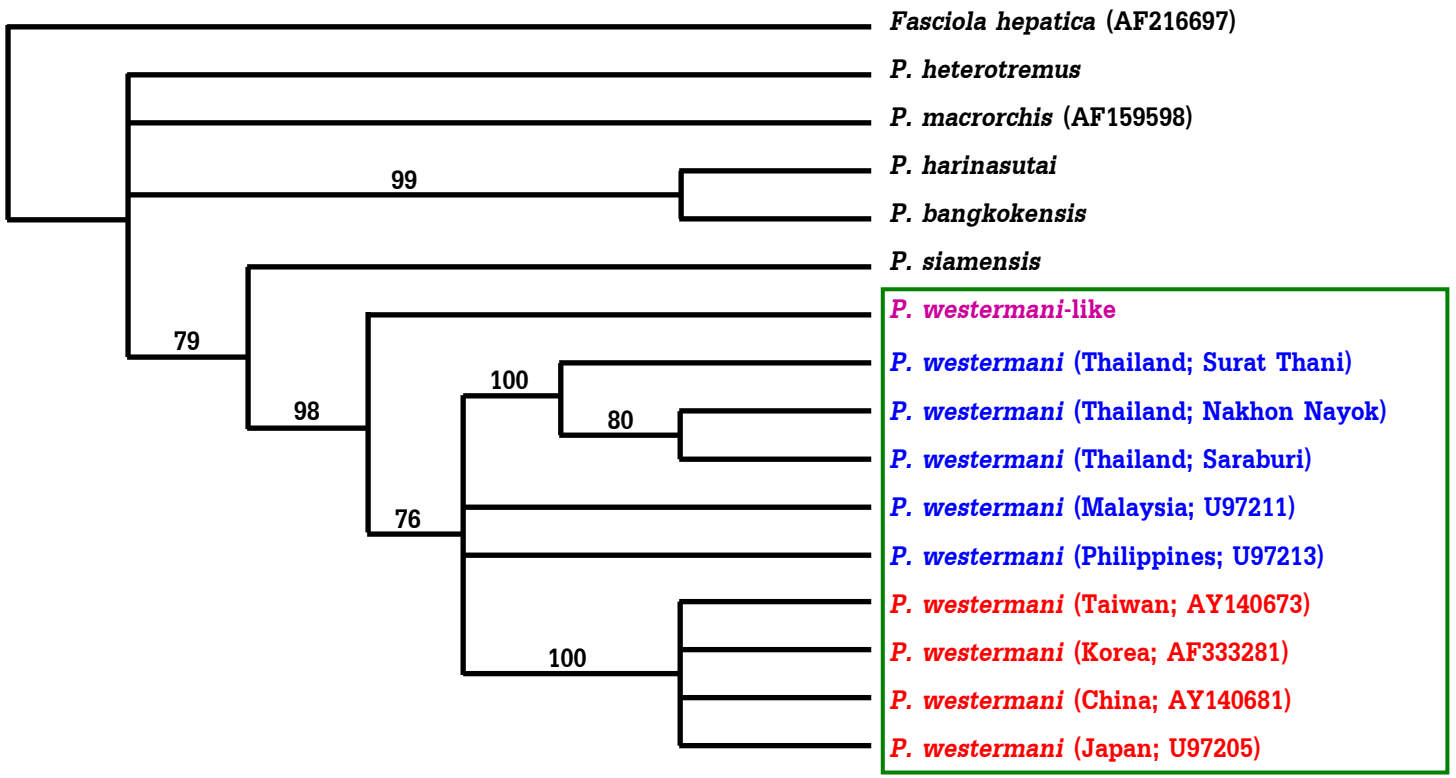

Figure 39. Strict consensus tree derived from 10 equally parsimonious trees of length 319 steps based on parsimony analysis of the informative characters of the partial COI region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. Green box indicates a single clade of *P. westermani* complex plus *P. westermani*-like from Thailand. Taxa in blue indicate *P. westermani* from South East Asia while taxa in red indicate *P. westermani* from East Asia.

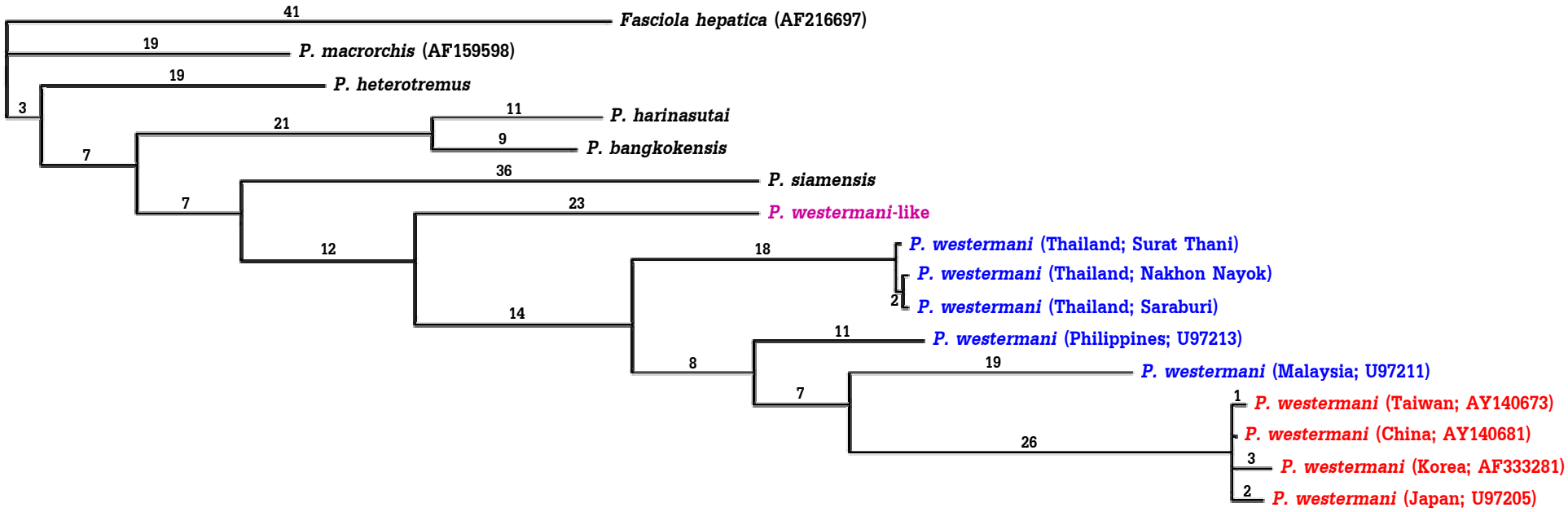

Figure 40. Phylogram of one of ten most parsimonious trees of length 319 steps based on parsimony analysis of the informative characters of the partial COI region. Numbers above the branches indicate branch length. The tree was rooted using *Fasciola hepatica*. Taxa in blue indicate *P. westermani* from South East Asia while taxa in red indicate *P. westermani* from East Asia.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

Morphology of *Paragonimus westermani*, *P. westermani*-like and *P. siamensis*

Paragonimus westermani is an important causative agent of human paragonimiasis in Asia. In Thailand, however, there is no report of human infection at present. Previous studies of *P. westermani* metacercariae were carried out only in the central part[15 17] and adult worms were reported from natural infected leopards in the southern part of the country.[7] In this study, following the field survey by Kawashima and colleagues in the southern part the metacercariae of *P. westermani* were obtained from the new crab intermediate hosts, *Ranguna smalleyi* and *Phricotelphusa aedes*. In addition, the metacercariae of *P. westermani*-like were also found in the same crab host, *Phricotelphusa aedes*, with *P. westermani*. For *P. westermani*, the morphological features of the metacercariae collected in the central part (Saraburi and Nakhon Nayok provinces) and the southern part (Surat Thani province) were identical. The characteristics of these metacercariae were in agreement with the description of *P. westermani* by Miyazaki (1974).[22] The metacercariae of *P. westermani*-like were similar to that of *P. westermani* except for the smaller size. Size variation of *P. westermani* metacercariae has been reported in other countries such as China,[27] Japan,[45] Taiwan,[46] Korea[47] and the Philippines.[48] Among them, *P. westermani*-like metacercariae obtained in this study were the smallest. The metacercariae from China were separated into three groups of small, medium and large sizes.[27] The flukes obtained from the medium and large metacercariae were a mixture of diploid, triploid and tetraploid while only diploid flukes were obtained from small metacercariae. It is probable that the size variation of the metacercariae is a polymorphic characteristic in the *P. westermani* complex. In addition to this characteristic metacercarial polymorphism also occurs in *P. ohirai* which includes characteristics such as the number of cyst walls and shape of the metacercariae.[49] Thus, *P. westermani*-like should merely be another form of *P. westermani* based on the metacercarial morphology.

For the morphological features of the adult flukes, *P. westermani* from the central and southern parts and *P. westermani*-like were identical to each other based on important characteristics described by Miyazaki (1974).[22] These characteristics included six-lobed ovary, simply branched testes and singly spaced cuticular spines. These flukes were morphologically similar to *P. westermani* diploid type detected in the leopards captured in southern Thailand

(described by Sugiyama *et al*. (2001)).[50] The adult flukes of *P. westermani* were closely resembled *P. siamensis* in morphological features except that the latter had the cuticular spines arranged in groups. Therefore, based on their morphological features of the adult flukes *P. westermani* and *P. westermani*-like could not be distinguished. This strongly confirms a very close relationship of *P. westermani* and *P. westermani*-like.

Susceptibility of *Paragonimus westermani*, *P. westermani*-like and *P. siamensis*

In many Asian countries cats have been used as excellent experimental animals in order to obtain *P. westermani* adult flukes,[48, 51-55] but in Thailand cats have been regarded as poor hosts for Thai *P. westermani*.[18] In the present study, cats were used for experimental infection to obtain the adult flukes of *P. westermani* (central and southern parts), *P. westermani*-like and *P. siamensis*. For *P. westermani* from the central and southern parts, both adult and preadult flukes were found in the lungs and pleural cavities of the cats. For *P. westermani* obtained from these regions, the recovery rates were 35% and 66.7%, respectively. These rates were close to that from Malaysia (57%), but were rather low compared to those from Japan, Korea, China and the Philippines (>90%).[reviewed in 55] This was the first report of Thai *P. westermani* adults being recovered from the experimental cats. This result confirmed that *P. westermani* strain Thailand had the potential to mature into adults in feline hosts. However, the susceptibility of feline hosts to *P. westermani*-like was found to be different from that of *P. westermani*. As shown in this study, juvenile worms of *P. westermani*-like lodged predominantly in the liver, while some matured into adults in the pleural cavities or lungs (cat No.3). In cat No.4, *P. westermani*-like was unable to develop into sexually mature adult flukes but remained in liver, diaphragm and peritoneal cavity. However, the juvenile worms were capable of developing into adult worms when transferred to a new cat (cat No.6). From this result, it was suggested that cats could serve as experimental definitive hosts as well as paratenic hosts of *P. westermani*-like. Habe *et al*. (1996)[55] reported the susceptibility of feline hosts to Malaysian *P. westermani*, they found about half of the worms recovered were identified as juvenile worms, and the principal domicile of the juveniles was the skeletal muscles. When transferred the juvenile worms to the new cat hosts, adult flukes were found in the lungs but some juvenile worms were still remained in the muscles of the cats. This susceptibility to Malaysian *P. westermani* was different from that of *P. westermani* in other countries but quite similar to *P. westermani*-like.

Rats have been employed as experimental paratenic hosts for *P. westermani*.[56-58] However, only small number of mature worms were obtained from experimental rats in the Philippines and Japan.[48, 59] In Thailand, Waikagul (1992)[18] reported failures of using rats and some rodents for experimental infection with *P. westermani* metacercariae. In this study, no worm was detected from experimentally infected rats with *P. westermani* metacercariae. This result confirmed that the rats were not susceptible for Thai *P. westermani*.

Miyazaki and Wykoff (1965),[8] Yaemput *et al*. (1994)[60] and Waikagul *et al*. (1986)[61] reported that cats and rats played a role as definitive hosts for *P. siamensis* in Thailand. In the present study, the cat was also found to be a suitable host for *P. siamensis* while rats showed a lower susceptibility compared to previous reports.

Sequence characteristics of the ITS and partial COI regions

As reviewed by Nolan and Cribb (2005), the length of the entire ITS region including 5.8S of digenean species varied from 820 to 1120 bp depending on the families.[reviewed in 62] However, the entire length of the ITS region of *Paragonimus* species obtained in this study was slightly shorter (803 bp). In this study, although the number of parsimony informative characters from the combined ITS1 and ITS2 regions (98 bp) were more than that of the ITS2 region (56 bp) the overall percentages of the informative sites were similar (ITS1 + ITS2: 14.2%, ITS2: 14.8%). Thus, this result supported other authors' preference for the used of the ITS2 region alone. This region exhibited intra- and interspecific variations with numbers of base difference range from 1 to 10 and 6 to 48 bases, respectively.[19 20, 32-33, 36, 63-66] For most studies, the partial COI region was usually used in parallel with the ITS2 region. This region exhibited far more variation than the ITS2 region. It varied from 383 to 393 bp in length and exhibited intra- and interspecific variations ranging from 1 to 46 and 30 to 81 bases, respectively.[19, 32, 36, 64-65]

In the present study, the alignment of the ITS2 region of *Paragonimus* species and its outgroup was 378 bp in length which was similar to those of other digeneans such as *Schistosoma* (398 bp)[40] and *Fasciola*(364 bp).[67] The level of sequence variation between *P. westermani*-like and *P. westermani* (1.39-3.72%; 5 – 13 bp) was close to the intraspecific variation within *P. westermani* from different geographical origins (0-3.13%; 0 – 11 bp). The intraspecific variation in the ITS2 region was also observed in other digeneans, including *Schistosoma*[68] and *Fasciola*[69] For the partial COI region the aligned length was 384 bp. The

sequence variation among *P. westermani* from different geographical regions was 0-14.25% (0 – 47 bp) while the variation between *P. westermani* and *P. westermani*-like was 8.25-14.75% (29 – 48 bp). From all characters analyzed, the numbers of the variable characters of the partial COI (37.2%; 143 characters) and the ITS2 (38.9%; 147 characters) sequences were almost equal. However, this region of the COI gene exhibited approximately two times more informative characters (27.3%) than the ITS2 region (14.8%). Nonetheless, a remarkably large amount of homoplasy was observed in the COI data (HI = 0.3636) compared to the ITS2 data (HI = 0.0947). This level of homoplasy was also reported in *Schistosoma*.[40]

Phylogenetic analyses of the ITS and partial COI regions

From this study, the overall tree resolution and topologies obtained from both the ITS2 and the ITS (ITS1 and ITS2) regions were highly congruent. The trees contained two important clades. Clade I consists of the *P. westermani* complex and *P. siamensis* and clade II contains other *Paragonimus* species found in Thailand. For the *P. westermani* complex, two groups of organism were revealed based on geographical distribution. The first group comprises *P. westermani* from South East Asia (Thailand, Malaysia and the Philippines) and the second group composes of *P. westermani* from East Asia (Japan, China, Korea and Taiwan) plus *P. westermani*-like from Thailand. These phylogenetic trees were similar to the resulting trees reconstructed by Blair *et al*. (1997),[36] (1998),[33] Iwagami *et al*. (2000)[64] and *Park et a*l. (2003).[20]

The strict consensus tree obtained from the partial COI region showed a single clade of the *P. westermani* complex from South East and East Asia plus *P. westermani*-like. In contrast to the ITS2 tree, this tree revealed that *P. westermani*-like is excluded from the complex and placed as a sister group to it. Thus, it is evident that *P. westermani*-like is either placed well within the *P. westermani* complex (ITS2 data) or is located close to the complex (COI data). Since the protein-coding gene (COI) is under selective constraint while the non-coding ITS region is not, this suggests that the spacer is free to diverge and evolve with a rate that is close to the neutral rate of sequence evolution. In addition, due to a higher level of homoplasious characters present in the COI data, the tree inferred from the ITS2 data would be more reliable. This result of *P. westermani*-like being classified as one of the members of the *P. westermani* complex was strongly supported by the morphological characters of the adult worms.

In both analyses based on the ITS2 and partial COI data, *P. siamensis* is placed as a sister group to the *P. westermani* complex and *P. westermani*-like. This study confirmed the position of *P. siamensis* as being closely related to the *P. westermani* complex and *P. westermani*-like, but not nested within them. Thus, based on the morphological, ITS2 and COI data it is convincing that *P. siamensis* is regarded as a distinct species.

Concerning the utilization of the first intermediate hosts, *P. westermani* populations were divided into two groups. This type of division was well correlated with the grouping of *P. westermani* based on different geographical distribution. *Paragonimus westermani* from East Asia (Japan, China, Korea and Taiwan) utilized the snail host family Pleuroceridae whereas *P. westermani* from South East Asia (Malaysia and the Philippines) utilized the family Thiaridae.[reviewed in 1] For *P. westermani* and *P. westermani*-like in Thailand the use of snail hosts is still unknown.

Since the susceptibility of feline hosts to *P. westermani*-like was found to be different from that of Thai *P. westermani* and a significant genetic variation was also observed between them, further investigation on the specificity of first intermediate hosts and molecular approaches such as RAPD, RFLP and microsatellite markers should be carried out to determine a proper taxonomic status of *P. westermani*-like.

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APPENDIX

Table 1. Sequence divergence matrix for the ITS2 sequences.

Species	origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
P. westermani	1 Japan	-															
	2 China	0.00000	-														
	3 Korea	0.00000	0.00000	-													
	4 Taiwan	0.00276	0.00276	0.00276	-												
	5 Philippines	0.01956	0.01956	0.01956	0.02241	-											
	6 Malaysia	0.01674	0.01674	0.01674	0.01959	0.00276	-										
	7 Thailand (Sa)	0.02822	0.02822	0.02822	0.03114	0.02528	0.02245	-									
	8 Thailand (N)	0.02831	0.02831	0.02831	0.03123	0.02537	0.02253	0.00000	-								
	9 Thailand (Su)	0.02831	0.02831	0.02831	0.03133	0.02547	0.02263	0.00000	0.00000	-							
P. westermani-like	10 Thailand	0.01392	0.01392	0.01392	0.01674	0.02817	0.02533	0.03712	0.03721	0.03721	-						
P. siamensis	11 Thailand	0.04576	0.04576	0.04576	0.04877	0.04867	0.04576	0.05789	0.05813	0.05824	0.05494	-					
P. bangkokensis	12 Thailand	0.09865	0.09865	0.09865	0.10195	0.10838	0.10526	0.11545	0.11544	0.11624	0.10895	0.12867	-				
P. harinasutai	13 Thailand	0.10507	0.10507	0.10507	0.10841	0.11490	0.11175	0.12204	0.12204	0.12286	0.11548	0.13540	0.00553	-			
P. macrorchis	14 Thailand	0.10328	0.10328	0.10328	0.10661	0.11340	0.11657	0.13034	0.13031	0.13130	0.11683	0.13671	0.09941	0.10589	-		
P. heterotremus	15 Thailand	0.07703	0.07703	0.07703	0.08021	0.09579	0.09272	0.10277	0.10277	0.10352	0.08982	0.11222	0.07978	0.08605	0.06761	-	
Fasciola hepatica	16 Australia	0.38099	0.38099	0.38099	0.37597	0.37652	0.37607	0.39084	0.39056	0.39445	0.39262	0.39364	0.39524	0.40509	0.41907	0.37998	-

Note: Thailand (Sa) is Saraburi province, Thailand (N) is Nakhon Nayok province and Thailand (Su) is Surat Thani province.

Table 2. Pairwise differences among the ITS2 nucleotide sequences.

Species	origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
P. westermani	1 Japan	-	0	0	1	7	6	10	10	10	5	16	33	35	34	26	106
	2 China	0/0	-	0	1	7	6	10	10	10	5	16	33	35	34	26	106
	3 Korea	0/0	0/0	-	1	7	6	10	10	10	5	16	33	35	34	26	106
	4 Taiwan	1/0	1/0	1/0	-	8	7	11	11	11	6	17	34	36	35	27	105
	5 Philippines	4/3	4/3	4/3	5/3	-	1	9	9	9	10	17	36	38	37	32	105
	6 Malaysia	4/2	4/2	4/2	5/2	0/1	-	8	8	8	9	16	35	37	38	31	105
	7 Thailand (Sa)	8/2	8/2	8/2	9/2	6/3	6/2	-	0	0	13	20	38	40	42	34	108
	8 Thailand (N)	8/2	8/2	8/2	9/2	6/3	6/2	0/0	-	0	13	20	37	39	41	33	107
	9 Thailand (Su)	8/2	8/2	8/2	9/2	6/3	6/2	0/0	0/0	-	13	20	38	40	42	34	108
P. westermani-like	10 Thailand	3/2	3/2	3/2	4/2	7/3	7/2	11/2	11/2	11/2	-	19	36	38	38	30	108
P. siamensis	11 Thailand	12/4	12/4	12/4	13/4	12/5	12/4	16/4	16/4	16/4	15/4	-	42	44	44	37	108
P. bangkokensis	12 Thailand	25/8	25/8	25/8	26/8	27/9	27/8	30/8	30/7	30/8	28/8	32/10	-	2	33	27	109
P. harinasutai	13 Thailand	26/9	26/9	26/9	27/9	28/10	28/9	31/9	31/8	31/9	29/9	33/11	1/1	-	35	29	111
P. macrorchis	14 Thailand	26/8	26/8	26/8	27/8	30/7	30/8	33/9	33/8	33/9	29/9	33/11	24/9	25/10	-	23	112
P. heterotremus	15 Thailand	20/6	20/6	20/6	21/6	24/8	24/7	27/7	27/6	27/7	23/7	28/9	20/7	21/8	17/6	-	105
Fasciola hepatica	16 Australia	48/58	48/58	48/58	47/58	48/57	47/58	50/58	50/57	50/58	52/56	54/54	49/60	50/61	54/58	48/57	-

Note: Values above the diagonal are nucleotide differences. Those below the diagonal are transitions/transversions. Thailand (Sa) is Saraburi province, Thailand (N) is Nakhon Nayok province and Thailand (Su) is Surat Thani province.

Table 3. Sequence divergence matrix for the ITS (ITS1 and ITS2) sequences.

Species	origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14
P. westermani	1 Japan	-													
	2 China	0.00318	-												
	3 Philippines	0.02427	0.02597	-											
	4 Malaysia	0.02588	0.02106	0.01121	-										
	5 Thailand (Sa)	0.03081	0.02595	0.02283	0.01954	-									
	6 Thailand (N)	0.03086	0.02600	0.02287	0.01959	0.00000	-								
	7 Thailand (Su)	0.03092	0.02606	0.0229	0.01965	0.00000	0.00000	-							
P. westermani-like	8 Thailand	0.01924	0.01445	0.03113	0.02771	0.03258	0.03263	0.03263	-						
P. siamensis	9 Thailand	0.0494	0.04444	0.04807	0.04471	0.04683	0.04692	0.04702	0.05516	-					
P. bangkokensis	10 Thailand	0.10186	0.09668	0.11382	0.11018	0.11445	0.11444	0.11495	0.10715	0.11957	-				
P. harinasutai	11 Thailand	0.10548	0.10029	0.11754	0.11386	0.11803	0.11803	0.11854	0.11070	0.12317	0.00301	-			
P. macrorchis	12 Thailand	0.1235	0.11814	0.13595	0.13569	0.14257	0.14256	0.14317	0.13490	0.14390	0.09453	0.09801	-		
P. heterotremus	13 Thailand	0.08188	0.07673	0.09857	0.09504	0.09968	0.09968	0.10011	0.09261	0.10650	0.06863	0.07197	0.07038	-	
Fasciola hepatica	14 Australia	0.37264	0.36834	0.37643	0.36654	0.37952	0.37936	0.38150	0.37610	0.37494	0.37405	0.37941	0.40307	0.36036	-

Note: Thailand (Sa) is Saraburi province, Thailand (N) is Nakhon Nayok province and Thailand (Su) is Surat Thani province.

Table 4. Pairwise differences among the ITS (ITS1 and ITS2) nucleotide sequences.

Species	origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14
P. westermani	1 Japan	-	2	14	16	19	19	19	12	30	59	61	70	48	177
	2 China	0/2	-	16	13	16	16	16	9	27	56	58	67	45	175
	3 Philippines	9/5	9/7	-	7	14	14	14	19	29	65	67	76	57	178
	4 Malaysia	10/6	10/3	3/4	-	12	12	12	17	27	63	65	76	55	175
	5 Thailand (Sa)	13/6	13/3	8/6	10/2	-	0	0	21	30	69	71	84	61	190
	6 Thailand (N)	13/6	13/3	8/6	10/2	0/0	-	0	21	30	68	70	83	60	189
	7 Thailand (Su)	13/6	13/3	8/6	10/2	0/0	0/0	-	21	30	69	71	84	61	190
P. westermani-like	8 Thailand	7/5	7/2	12/7	14/3	17/4	17/4	17/4	-	35	65	67	80	57	188
P. siamensis	9 Thailand	20/10	20/7	19/10	21/6	23/7	23/7	23/7	26/9	-	72	74	85	65	188
P. bangkokensis	10 Thailand	43/16	43/13	49/16	51/12	56/13	56/12	56/13	50/15	56/16	-	2	58	43	188
P. harinasutai	11 Thailand	44/17	44/14	50/17	52/13	57/14	57/13	57/14	51/16	57/17	1/1	-	60	45	190
P. macrorchis	12 Thailand	49/21	49/18	57/19	59/17	64/20	64/19	64/20	58/22	62/23	43/15	44/16	-	44	198
P. heterotremus	13 Thailand	32/16	32/13	40/17	42/13	46/15	46/14	46/15	40/17	47/18	33/10	34/11	33/11	-	182
Fasciola hepatica	14 Australia	84/93	85/90	85/93	84/91	94/96	94/95	94/96	94/94	95/93	90/98	91/99	99/99	88/94	-

Note: Values above the diagonal are nucleotide differences. Those below the diagonal are transitions/transversions. Thailand (Sa) is Saraburi province, Thailand (N) is Nakhon Nayok province and Thailand (Su) is Surat Thani province.

Table 5. Sequence divergence matrix for the partial COI sequences.

Species	origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	15
P. westermani	1 Japan	-															
	2 China	0.00528	-														
	3 Korea	0.00795	0.00795	-													
	4 Taiwan	0.00792	0.00263	0.01060	-												
	5 Philippines	0.11239	0.11239	0.11575	0.11537	-											
	6 Malaysia	0.11888	0.11209	0.12227	0.11507	0.08923	-										
	7 Thailand (Sa)	0.13897	0.13897	0.14250	0.13592	0.07718	0.09879	-									
	8 Thailand (N)	0.13897	0.13897	0.14250	0.13592	0.07718	0.09879	0.00000	-								
	9 Thailand (Su)	0.13183	0.13183	0.13531	0.12881	0.07091	0.09226	0.00528	0.00528	-							
P. westermani-like	10 Thailand	0.14393	0.13671	0.14753	0.13978	0.08248	0.09896	0.09509	0.09509	0.09505	-						
P. siamensis	11 Thailand	0.18719	0.18719	0.19105	0.18785	0.16414	0.19414	0.16367	0.16367	0.17099	0.16525	-					
P. bangkokensis	12 Thailand	0.24743	0.24743	0.24313	0.24391	0.17106	0.19754	0.18623	0.18623	0.18990	0.17109	0.21424	-				
P. harinasutai	13 Thailand	0.23528	0.23115	0.23521	0.22769	0.18986	0.20571	0.20587	0.20587	0.20970	0.16059	0.19913	0.07426	-			
P. macrorchis	14 Thailand	0.24019	0.24873	0.25298	0.25234	0.20096	0.20126	0.22429	0.22429	0.22412	0.18573	0.18458	0.16060	0.17459	-		
P. heterotremus	15 Thailand	0.22157	0.22569	0.22151	0.22227	0.17213	0.20872	0.18284	0.18284	0.19030	0.15362	0.18287	0.14017	0.12701	0.11966	-	
Fasciola hepatica	16 Australia	0.34722	0.33732	0.34221	0.33334	0.30272	0.30582	0.30605	0.30605	0.31499	0.25982	0.24510	0.26373	0.25159	0.23743	0.22236	-

Note: Thailand (Sa) is Saraburi province, Thailand (N) is Nakhon Nayok province and Thailand (Su) is Surat Thani province.

Table 6. Pairwise differences among the partial COI nucleotide sequences.

Species	origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
P. westermani	1 Japan	-	2	3	3	38	40	46	46	44	47	60	77	74	75	70	102
	2 China	2/0	-	3	1	38	38	46	46	44	45	60	77	73	77	71	100
	3 Korea	3/0	3/0	-	4	39	41	47	47	45	48	61	76	74	78	70	101
	4 Taiwan	2/1	0/1	3/1	-	39	39	45	45	43	46	60	76	72	78	70	99
	5 Philippines	35/3	35/3	36/3	35/4	-	31	27	27	25	29	54	57	62	65	57	92
	6 Malaysia	37/3	35/3	38/3	35/4	27/4	-	34	34	32	34	62	64	66	65	67	93
	7 Thailand (Sa)	41/5	41/5	42/5	41/4	25/2	30/4	-	0	2	33	54	61	66	71	60	93
	8 Thailand (N)	41/5	41/5	42/5	41/4	25/2	30/4	0/0	-	2	33	54	61	66	71	60	93
	9 Thailand (Su)	39/5	39/5	40/5	39/4	23/2	28/4	2/0	2/0	-	33	56	62	67	71	62	95
P. westermani-like	10 Thailand	44/3	42/3	45/3	42/4	23/6	30/4	27/6	27/6	27/6	-	54	57	54	61	52	82
P. siamensis	11 Thailand	46/14	46/14	47/14	47/13	37/17	45/17	37/17	37/17	39/17	39/15	-	69	65	61	60	78
P. bangkokensis	12 Thailand	51/26	51/26	50/26	51/25	32/25	41/23	38/23	38/23	39/23	32/25	37/32	-	26	54	48	83
P. harinasutai	13 Thailand	48/26	47/26	48/26	47/25	37/25	43/23	43/23	43/23	44/23	29/25	33/32	24/2	-	58	44	80
P. macrorchis	14 Thailand	49/26	51/26	52/26	51/27	38/27	40/25	44/27	44/27	44/27	34/27	29/32	28/26	30/28	-	41	76
P. heterotremus	15 Thailand	47/23	48/23	47/23	48/22	33/24	41/26	36/24	36/24	38/24	26/26	33/27	23/25	19/25	26/15	-	72
Fasciola hepatica	16 Australia	55/47	53/47	54/47	53/46	44/48	45/48	45/48	45/48	47/48	34/48	31/47	34/49	29/51	29/47	26/46	-

Note: Values above the diagonal are nucleotide differences. Those below the diagonal are transitions/transversions. Thailand (Sa) is Saraburi province, Thailand (N) is Nakhon Nayok province and Thailand (Su) is Surat Thani province.

GLOSSARY

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Bootstrap : A statistical procedure for achieving a better estimate of the parametric variance of a distribution than the observed sample variance by averaging pseudoreplicate variances. The original data set is sampled with replacement to produce a pseudoreplicate of the same dimensions as the original.

Branch-and-bound : An **exact algorithm** for cladogram construction. The method begins by constructing a cladogram by means of a **heuristic method**. The length of this cladogram is used as the initial upper bound for an **exhaustive search**. The number of topologies to be examined is then restricted by discarding all partial cladograms whose length exceeds the upper bound. If a complete cladogram is found that is shorter than the upper bound, then the upper bound is reset to this length in order to increase efficiency further.

Clade : A group that includes a most recent common ancestor plus all of its descendants. **Syn. monophyletic group.**

Consistency index (CI) : A measure of the amount of **homoplasy** in a character relative to a given cladogram. The consistency index is calculated as the ratio of **m**, the minimum number of steps a character can exhibit on any cladogram, to **s**, the minimum number of steps the same character can exhibit on the cladogram in question.

Exact algorithm : An algorithm for constructing cladograms that is guaranteed to find one or all of the most parsimonious cladogram.

Exhaustive search : An exact algorithm that examines every possible fully-resolved, unrooted cladogram for the taxa included in that data set in order to find the most parsimonious solution(s).

Homoplasy : Similarities found in two taxa which are not due to common ancestry. These include convergence, parallelism and reversal. The **homoplasy index (HI)** is calculated as 1 – CI = HI

Heuristic method : An algorithm for constructing cladograms that is not guaranteed to find the most parsimonious solution.

Ingroup : The group under investigation in a cladistic analysis in order to resolve the relationships of its members.

Outgroup : A taxon used in a cladistic analysis for comparative purposes, usually with respect to character polarity determination.

Parsimony : A criterion for estimating a parameter from observed data based on the principle of minimizing the number of events needed to explain the data. In phylogenetic analysis, the optimal tree under the maximum parsimony criterion is the tree that requires the fewest number of character state changes (which may be differentially weighted across characters and/or character states).

Phylogenetics : A method of classification that utilizes hypotheses of character transformation to group taxa hierarchically into nested sets and then interprets these relationships as a phylogenetic tree.

Phylogram : A phylogenetic tree that explicitly represents number of character changes through its branch lengths.

Rescaled consistency index (RC) : The product of the consistency index and the retention index of a character.

Retention index (RI) : A measure of the amount of similarity in a character that can be interpreted as **synapomorphy** on a given cladogram. The retention index is calculated as the ratio of (**g** - **s**) to (**g** - **m**), where **g** is the greatest number of steps a character can exhibit on any cladogram, **m** is the minimum number of steps a character can exhibit on any cladogram and **s** is the minimum number of steps the same character can exhibit on the cladogram in question.

Sisiter group : (1) Two taxa that are more closely related to each other than either is to a third taxon. (2) The taxon that is genealogically most closely related to the **ingroup**.

Synapomorphy : A derived character or character state that unites two or more taxa into a monophyletic group (clade).

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