11. Lau HK, Li CH, Lee AC: **Acute massive haemolysis in children with glucose-6-phosphate dehydrogenase deficiency.** *Hong Kong Med J* 2006, **12**:149-151.
12. WHO Working Group: **Glucose-6-phosphate dehydrogenase deficiency.** *Bull World Health Organ* 1989, **67**:601-611.
13. Choudhry VP, Madan N, Food SK, Ghai OP: **Chloroquine induced haemolysis and acute renal failure in subjects with G6PD deficiency.** *Trop Geogr Med* 1978, **30**:331-335.
14. Choudhry VP, Ghafry A, Zaher M, Qureshi MA, Fazel I, Ghani R: **Drug-induced haemolysis and renal failure in children with G6PD in Afghanistan.** *Ann Trop Paediatric* 1990, **10**:335-338.
15. Price RN, Simpson JA, Nosten F, Luxemburger C, Hkirjaroen L, ter Kuile F, Chongsuphajaisidhi T, White NJ: **Factors contributing to anemia after uncomplicated malaria.** *Am J Trop Med Hyg* 2001, **65**:614-622.
16. Roestenberg M, McCall M, Mollness TE, van Deuren M, Sprong T, Klasen I, Hermsen CC, Sauerwein RW, van der Ven A: **Complement activation in experimental human malaria infection.** *Trans Roy Soc Trop Med Hyg* 2007. doi: 0.1016/j.trstmh.2007.02.023
17. Perlmann P, Perlmann H, Flyg BW, Hagstedt M, Elghazali G, Worku S: **Immunoglobulin E, a pathogenic factor in *Plasmodium falciparum* malaria.** *Inf Immunol* 1997, **65**:116-121.
18. Salmon D, Vilde JL, Andrieu B, Simonovic R, Lebras J: **Role of immune serum and complement in stimulation of the metabolic burst of human neutrophils by *Plasmodium falciparum*.** *Inf Immunol* 1986, **51**:801-806.
19. Abdalla SH: **Red cell associated IgG in patients suffering from Plasmodium malaria.** *Br J Haemtol* 1986, **62**:13-19.
20. Zuckerman A: **Recent studies in Malaria anaemia.** *Mil Med* 1966, **131**:1201-1216.
21. Goka BQ, Kwarko H, Kurtzhals JAL, Gyan B, Ofori-Adjei E, Ohene SA, Hviid L, Akanmori BD, Neequaye J: **Complement binding to erythrocytes is associated with macrophage activation and reduced haemoglobin in *Plasmodium falciparum* malaria.** *Trans Soc Trop Med Hyg* 2001, **95**:545-549.
22. Facer C, Bray RS, Brown J: **Direct Coombs antiglobulin reaction in Gambian children with *Plasmodium falciparum* malaria. I. Incidence and class specificity.** *Clin Exp Immunol* 1979, **35**:119-127.
23. Abdalla SH, Weatherall DJ: **The anaemia of *Plasmodium falciparum* malaria.** *Brit Med Bulletin* 1982, **38**:147-151.
24. Waitumbi JN, Opollo MO, Muga RO, Misore AO, Stoute JA: **Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anaemia.** *Blood* 2000, **95**:1481-1486.
25. Stoute JA, Odindo AO, Owuor BO, Mibei EK, Opollo MO, Waitumbi JN: **Loss of Red Blood Cell-Complement Regulatory Proteins and Increased levels of circulating Immune Complexes Are Associated with Severe Malaria Anaemia.** *J Inf Dis* 2003, **187**:522-525.
26. Stoute JA: **Complement-regulatory proteins in severe malaria: too little or too much of a good thing.** *Trends Parasitol* 2005, **21**:218-223.
27. Stoute JA, Odindo AO, Owuor BO, Mibei EK, Opollo MO, Waitumbi N: **Loss of red cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anaemia.** *J Infect Dis* 2003, **187**:522-525.
28. Waitumbi JN, Donvito B, Kisserli A, Cohan JH, Stoute JA: **Age-related changes in red blood cell complement regulatory proteins and susceptibility to severe malaria.** *J infect Dis* 2004, **190**:1183-1191.
29. Awandare GA, Goka B, Boeuf P, Tetteh JKA, Kurtzhals JAL, Behr C, Akanmori BD: **Increased levels of inflammatory Mediators in children with severe *Plasmodium falciparum* malaria with respiratory distress.** *J Inf Dis* 2006, **194**:1438-46.
30. Kurtzhals JA, Adabayeri V, Goka BQ, Akanmori BD, Oliver-Commey JOO, Nkrumah FK, Behr C, Hviid L: **Low plasma concentrations of interleukin-10 in severe malaria anaemia compared with cerebral and uncomplicated malaria.** *Lancet* 1998, **351**:1768-1772.
31. Afari EA, Appau M, Dunyo S, Baffoe-Wilmot A, Nkrumah FK: **Malaria infection, morbidity and transmission in two ecological zones in southern Ghana.** *Afr J Health Sci* 1995, **2**:312-316.
32. **Immunoflourescent Staining for Flow Cytometry** [http://www.ebioscience.com/ebioscience/appls/FCS.htm]
33. Wenisch C, Spitzaner S, Florris-Linau K, Rumpold H, Vanaphan S, Parschalk B, Graniger W, Looareesuwan S: **Complement activation in severe *Plasmodium falciparum*.** *Clin Immuno Immunolpathol* 1997, **85**:166-171.
34. Akanmori BD, Kurtzhals JA, Goka BQ, Adabayeri V, Ofori MF, Nkrumah FK, Behr C, Hviid L: **Distinct patterns of cytokine regulation in discrete clinical forms of *Plasmodium falciparum* malaria.** *Eur Cytokine Netw* 2000, **11**:113-118.
35. Wachter H, Fuchs D, Hausen A, Reibnegger G, Werenr ER: **Neopterin as marker for activation of cellular immunity:immunologic basis and clinical application.** *Adv Clin Chem* 1989, **27**:81-141.
36. Turrini F, Ginsberg H, Bussolino F, Pescarmona GP, Serra MV, Arese P: **Phagocytosis of *Plasmodium falciparum*-infected human red cells by human ertythrocytes: involvement of immune and non-immune determinants and dependence on parasite development stage.** *Blood* 1992, **80**:801-808.
37. Chau TN, Lai ST, Lai JY, Yuen : **Haemolysis complicating acute viral hepatitis in patients with normal or deficient glucose-6-phosphate dehydrogenase activity.** *Scan J Infect Dis* 1997, **29**:551-553.
38. Dhaliwal G, Cornett PA, Tierney LM Jr: **Haemolytic anaemia.** *Am Fam Physician* 2004, **69**:2599-2606.
39. Kurtzhals JA, Addae MM, Akanmori BD, Dunyo S, Koram KA, Appawu MA, Nkrumah FK, Hviid L: **Anaemia caused by asymptomatic Plasmodium falciparum infection in semi-immune African school children.** *Trans Soc Trop Med Hyg* 1999, **93**:623-627.
40. Coombs RRA, Mourant AE, Race RR: **Detection of weak and incomplete Rh agglutinins.** *Lancet* 1945:15-16.
41. Pawluczkowycz AW, Lindorfer MA, Waitumbi NJ, Taylor RP: **Hematin promotes complement alternative pathway-mediated deposition of C3 activation fragments on human erythrocytes: potential implications for the pathogenesis of anaemia in malaria.** *J Immunol* 2007, **179**:5543-5552.
42. Risner I, Ueland T, Lundbland R, Mollnes TE, Baksaas ST, Aukrust P, Svennevig JL: **Changes in the cytokine network and complement parameters during openheart surgery.** *Interact Cardiovasc Thorac Surg* 2003, **2**:19-24.
43. Bojang KA, Van Hensbroek MB, Palmer A, Banya WA, Jaffar S, Greenwood BM: **Predictors of mortality in Gambian children with severe malaria anaemia.** *Ann Trop Paediatr* 1997, **17**:355-359.

狩猟界 51(11), 88-91, 2007

危険情報

イノシシ肉を生で食べて感染する肺吸虫

国立感染症研究所寄生動物部 杉山 広

肺吸虫という寄生虫を知っていますか?

咳が続き、また痰が出てその中に血液が混じる。胸痛があり、呼吸に困難を感じ、また熱が出ることもある。このような症状の病気として、一般的には「肺結核」や「肺癌」が知られている。

ところが、肺に住み着く「肺吸虫」という寄生虫によっても、このような症状の病気になる。この寄生虫は、かつては「肺(臓)ジストマ」とも呼ばれていた。これらの病気、すなわち「肺吸虫症(あるいは肺ジストマ症)」と「肺結核」や「肺癌」とは、治療方法がそれぞれ違うので、医療機関において的確な診断を受け、治療する必要がある。

肺吸虫は食品を介して感染する食品由来寄生虫の一つである。主な感染源は、サワガニ(写真1)やモクズガニ(写真2)で、これを生(非加熱)や生煮え・生焼け(加熱不十分)で食べることが最も危険である。一方でカニを調理する時にカニの体内に潜んでいた幼虫が、包丁やまな板に飛び散ることがある。この幼虫が次に調理した野菜などに付着し、この野菜を生で食べて、肺吸虫に感染することもある。調理器具にも注意が必要だ。

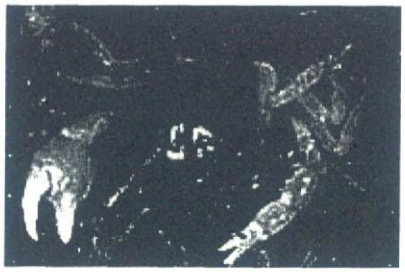


写真1 サワガニ

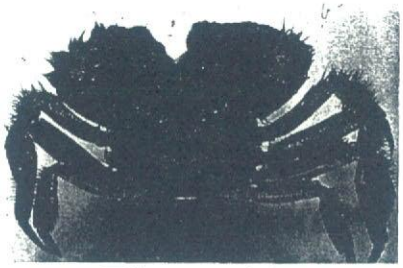


写真2 モクズガニ

イノシシ肉を生で食べて肺吸虫にかかる

本誌の読者の中には、イノシシ肉が肺吸虫の感染源として重要なことをご存知の方も多いと思う。イノシシはカニを好んで食べる。しかしカニに潜む肺吸虫の幼虫は、イノシシに食べられても肺には行かず、イノシシの筋肉に移動する。しかも生きたままで筋肉に長期間にわたって住み続け、人に食べられるのを待つ。

実際に九州南部で捕獲されたイノシシの筋肉から、肺吸虫の幼虫が見つかっている。この幼虫をイヌに食べさせると、カニに潜む幼虫を食べさせた時と同様に、虫は肺に移動して成虫となり、肺吸虫症が引き起こされた。人も同様に感染するので、肺吸虫の感染源としてイノシシ肉は重要となる(写真3および写真4)。

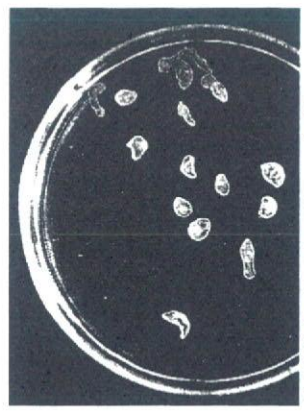


写真3 肺吸虫の成虫(生標本)

写真4
肺吸虫の成虫
(染色標本)

イノシシはどこにいるのか

イノシシを介して人が肺吸虫にかかるには、まずイノシシとの接点があり、しかもそのイノシシが肺吸虫にかかっていて、さらにそ

のイノシシを生(非加熱や加熱不十分)で食べるという食習慣が必要となる。肺吸虫感染に結びつくようなイノシシを取り巻く状況は、現実にはどうなっているのだろうか。まず最初にイノシシはどこで捕獲されているのか述べてみたい。

環境省の「鳥獣関係統計(平成16年度、狩猟者登録を受けた者による捕獲鳥獣数)」によれば、イノシシの捕獲数は全国で約17万頭を数える。捕獲頭数を地方別にまとめると、近畿が約27,000頭、中国が約38,000頭、四国が約18,000頭、九州・沖縄が約58,000頭であった。これらの合計は約14万頭で、イノシシの約8割が西日本で捕獲されたことになる。この傾向は長期にわたって変わっていない。イノシシとの接点は西日本に中心があることになる。

イノシシへの感染源となるカニはどこにいるのか

イノシシもサワガニやモクズガニを食べて肺吸虫に感染する。このサワガニやモクズガニは日本全国に分布している。ではどこのカニが肺吸虫を持っていて、イノシシへの感染源になっているのだろうか。

サワガニについては、1980年代から90年代に掛けて、全国各地で調査が行なわれ、肺吸虫の寄生状況が詳しく調べられた。その結果、寄生率に差はあるが、東北から九州に至る広範な地域で、肺吸虫の幼虫を持つサワガニが見つかった。

一方、モクズガニについての全国調査は近年は実施されていない。しかし1960年代には見つからなかった陽性のモクズガニが、ごく最近の調査で検出された河川がある。自然環境を保全する運動が全国で推進され、「川の水がきれいになった川や河原での生物の数や種類が増えた」などの成果が最近になって現れ始めた。陽性モクズガニが見つかった上述の河川では、他の生物と共にモクズガニが増えているようだ。このような河川は他にもある。

どこにいるイノシシが肺吸虫に感染しているのか

では肺吸虫に感染したイノシシはどこにいるのだろうか。従来の研究報告をまとめると、九州南部で捕獲された合計4頭の野生イノシシから、筋肉に寄生する肺吸虫の幼虫が検出されている(写真5)。

写真5 肺吸虫の幼虫
(生標本・イノシシの筋肉から検出)

4頭の内2頭(体重13・5kgおよび38kg)については全身が調べられたが、他の2頭は可食部の筋肉をごく少量(183gおよび150g)調べただけでこの成績が得られた。

肺吸虫を実験的に感染させたイノシシについても検討が行なわれている。その結果、投与した幼虫(各1,000匹)の約半数(494匹および540匹)という非常に多くの虫が、実験に用いたイノシシ(2頭)の筋肉から回収された。従って野生の成獣を対象として全身を詳しく検査すれば、当然ながらたくさんのイノシシから多数の肺吸虫幼虫が見つかるものと考えられる。つまり特別な地域だけでなく、カニに肺吸虫が見つかる地方では、イノシシはその筋肉に肺吸虫の幼虫を蓄えている、このように考えて良いと思われる。

誰がイノシシを生で食べているのか

イノシシに関する食習慣はどうなっているのだろうか。

我々が生で食べる獣肉の代表は馬肉で、「馬刺し」は既に日本人の食習慣に溶け込んでいる。さらに「牛刺し(ユッケ)」や「レバ刺し(牛肝)」を好む人もいる。これらの牛や馬の肉(や肝臓)を生食用食肉と呼ぶ。厚生労働省は生食用食肉に対する衛生基準を厳密に定めており、この基準に適合した食肉だけを生食用として取り扱うように通知している。

イノシシの生肉はこの基準の適用外になる。しかもイノシシの肉が一般家庭で生食されているという話は聞こえて来ない。生で食べているとすれば、やはりイノシシハンターということになる。

これを確認するために鹿児島、宮崎、山口の各県に住むイノシシハンターの方々44名にご協力をいただき、アンケート形式の実態調査を実施した。ここではその結果を紹介したい。

まず「イノシシ肉を生食するか」を尋ねた。その結果、生食する方は29名(66%)であった。次に「イノシシ肉の生食で肺吸虫にかかる

ことを知っているか」について質問した。結果は38名(86%)の方がご存知であった。しかも「肺吸虫にかかったことがあるか」の問いに対しては、各県1名ずつ合計3名(7%)の方がイエスであった。

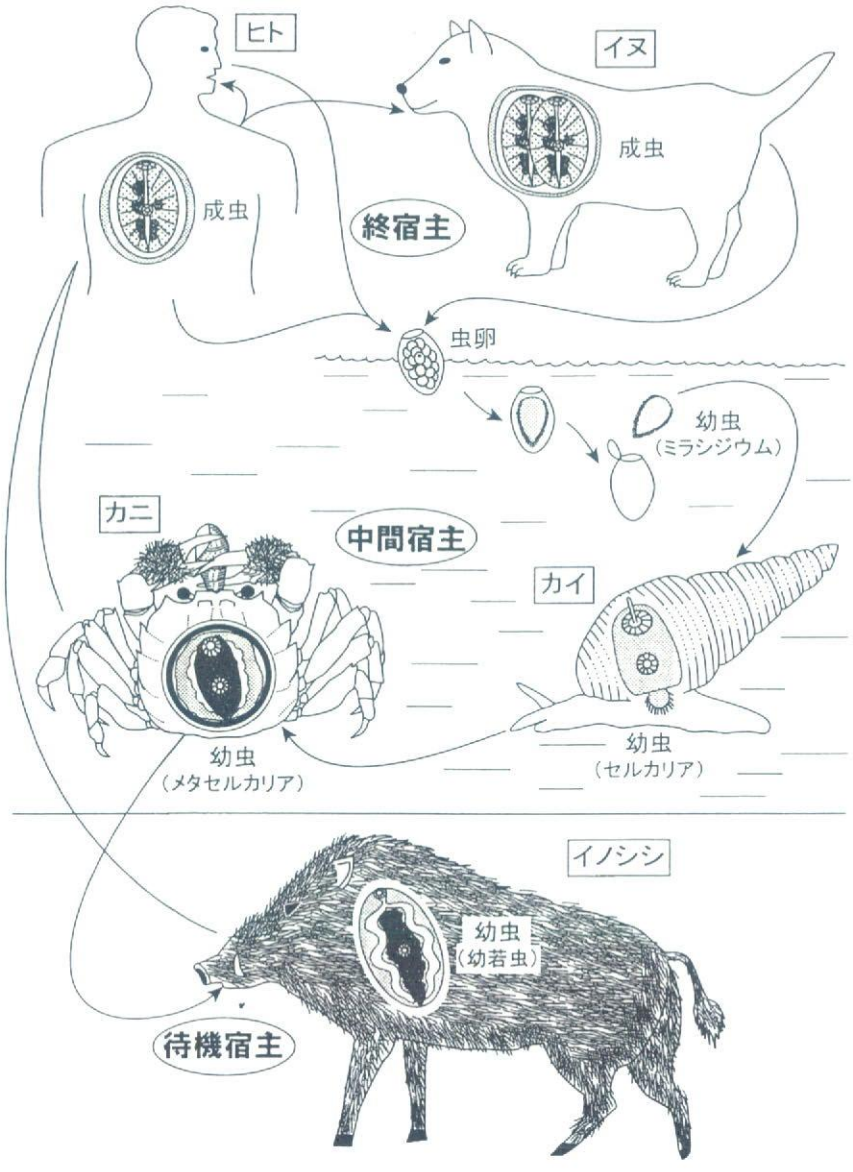
このアンケートの結果から、イノシシハンターの方々はやはりイノシシ肉を生で食べ、肺吸虫感染のリスクグループになっていることが分かった。食習慣と肺吸虫感染との因果関係についても知識があった。さらに「家族も生食している」とのコメントもあり、家族の方々もリスクを共有されていた。肺吸虫の危険性が過小評価されていたのである。

このようにイノシシハンターとその家族の方々を中心として、肺吸虫はその生活を続けるのであろう。イノシシハンターの方々に対して継続的な啓発活動を行ない、肺吸虫の危険性を知っていただく、このような必要性が痛感された。

イヌもイノシシ肉から肺吸虫に感染する

ハンターの方々へのアンケート調査で、「イノシシ肉を生で猟犬に与えたことがあるか」についても尋ねた。その結果、イヌを飼育されている34名の内25名(74%)の方が、生肉を与えたことがあると回答された。この結果は何を意味するのであろうか。

最近の九州南部での調査がその答えを明らかにしている。すなわち224頭のイノシシ猟犬が調べられた結果、83頭(37%)が肺吸虫に感染していたのである。感染していたイヌは咳が続き、激しい運動が出来ない状態であった。さらに呼吸困難に陥り、急死したイヌも見つかっている。イヌの体の中でも虫は肺に移動して成虫となり、人と同様に肺吸虫症を発症する。



図：肺吸虫の一生(生活環)と感染経路

猟芸を仕込んだ愛犬を守るためにも、不用意にイノシシの生肉を与えることは避けねばならない。

肺吸虫という病気とその恐ろしさ

「肺吸虫」という寄生虫が発育して子孫を残すには、発育の場として「中間宿主」のカニや「終宿主」の哺乳動物が必要となる。イノシシは中間宿主と終宿主との間に入り込んで、終宿主が肺吸虫に感染する機会を増やしている。このような役割を演じる動物を「待機宿主」と呼ぶ。イノシシは肺吸虫がより円滑に一生を過ごせるように、協力しているとも考えることができる(図)。

肺吸虫症という病気の治療方法は既に確立している。駆虫剤(虫下し)を飲めば、肺に住み着く虫は死んで病気は治る。しかし時として虫は肺に留まらず、脳や目に移動したり、また全身を動き回ることがある。その場合に予想もつかないような危険な事態が引き起こされる。肺吸虫という寄生虫が危険なのは、肺以外の場所に入り込むことがあるからである。

やはり危険なイノシシ肉の生食

ブタなどの家畜は、食用となる前に法律に基づく検査を受けることが義務づけられている。この検査には、と殺前の検査(生体検査)、と殺後の検査、さらに解体後の検査(肉や内臓の検査)が含まれる(と畜場法の定めによる)。一方、イノシシは野生動物として取り扱われるので、と畜場法に基づく検査は義務づけられていない。それに代わって、食肉処理業として営業許可を受けた施設で解体され、食肉として処理されることになる(食品衛生法の定めによる)。

捕獲したイノシシをハンターが食べる場合、そのイノシシ肉は、しかるべき施設での処理や法律に基づく検査を受けることがない。肉に病原体の汚染がなく、食べても安全であることを総て自分で判断することになる。どのようにして食べるかも、自己責任で決定することになる。

最近の傾向として、農作物被害をもたらすイノシシを捕獲し、その肉の有効利用をめざす動きが目立ってきた。この動きには自家消費の拡大だけではなく、食肉としての流通の拡大も含まれている。

これに対応してか、例えば島根県は平成18年に「猪肉に係る衛生管理ガイドライン」を独自に作成した。イノシシ肉が生食用食肉の適用外であることは既に述べたが、このガイドラインでも「(イノシシ肉は)生食用の出荷または販売を行なわないこと(第9条)」と明記されている。イノシシ肉を安全に、また安心して供給する立場からの重要な見解で評価される。

本稿ではイノシシ肉の生食が肺吸虫感染の原因となっていることを説明した。特にイノシシハンターの方々に対して注意を喚起したい。

コラム 我が国に分布する肺吸虫の種類

本稿では「肺吸虫」として一括したが、我が国にはウェステルマン肺吸虫と宮崎肺吸虫という2種類の人体寄生性肺吸虫が分布する。このうちウェステルマン肺吸虫は、虫の染色体構成から、2倍体型のものと3倍体型のものが見つかっている。両者には生物学的な特徴にも違いがある。一方の宮崎肺吸虫は総て2倍体で、3倍体型の虫体は見つかっていない。これら肺吸虫の主な中間宿主は、ウェステルマン肺吸虫の2倍体型と宮崎肺吸虫がサワガニ、ウェステルマン肺吸虫の3倍体型がモクズガニである。しかしながらいずれの肺吸虫も、イノシシ体内では幼虫が筋肉に移行し、そこに長期間住み続ける。このような結果が感染実験により証明されている。しかし野生イノシシの筋肉から検出された肺吸虫は、もっぱらウェステルマン肺吸虫の3倍体型であった。イノシシ肉の生食を原因とする患者も、主としてウェステルマン肺吸虫の3倍体型によるものと考えられる。なお我が国にはもう1種類、大平肺吸虫という肺吸虫も分布する。この肺吸虫は動物寄生性で、イノシシに感染すると肺に移行して成虫となる。人体感染の原因にはならない。

NEW FORM OF *PARAGONIMUS WESTERMANI* DISCOVERED IN THAILAND: MORPHOLOGICAL CHARACTERISTICS AND HOST SUSCEPTIBILITY

Hiromu Sugiyama[1], Yasuyuki Morishima[1], Sutheewan Binchai[2], Achariya Rangsiruji[2] and Punsin Ketudat[2]

[1]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; [2]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand

Abstract. During an intensive field survey for *P. westermani* in southern Thailand, a new form of *Paragonimus* metacercariae was isolated. In this study, we referred to this new form as *P. westermani*-like, as it was almost identical to *P. westermani* in shape. To investigate the susceptibility of feline host to *P. westermani*-like, as well as its morphology at the adult stage, we inoculated the peritoneal cavity of a cat with 60 *P. westermani*-like metacercariae. Morphological examination revealed that the adult *P. westermani*-like recovered from the lungs had a six-lobed ovary, a spermatozoa-filled seminal receptacle, and singly spaced cuticular spines. These findings indicated that the morphological features of *P. westermani*-like were fundamentally identical to those of *P. westermani* (diploid type) at the adult stage. The susceptibility of feline hosts to *P. westermani*-like was different from that of *P. westermani*. To determine the proper taxonomic status of *P. westermani*-like, we have been investigating the phylogenetic relationships between *P. westermani*-like and *P. westermani* in southern Thailand.

INTRODUCTION

Paragonimus westermani is widely distributed in Asia (Miyazaki, 1991). Individuals from different geographical regions (or countries) show variations in animal and/or human susceptibility, although they share almost identical morphological features at both the adult and metacercarial stages (Blair *et al*, 1998). This implies that they form a complex of cryptic species (Blair *et al*, 1997).

During an intensive field survey for *Paragonimus* in southern Thailand (Rangsiruji *et al*, 2005), we collected another form of *Paragonimus westermani* metacercariae from freshwater crabs, *Phricotelphusa aedes*. These crabs simultaneously acted as the second intermediate host of *P. westermani*. Metacercariae of newly isolated *Paragonimus* were almost identical to those of *P. westermani* in shape, but were much smaller. For descriptive purposes, we refer to this new form as *P. westermani*-like.

Correspondence: Hiromu Sugiyama, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel: +81-3-5285-1111; Fax: +81-3-5285-1173
E-mail: hsugi@nih.go.jp

In this study, we inoculated a cat with *P. westermani*-like metacercariae isolated from freshwater crabs, *Phricotelphusa aedes*, in order to identify the susceptibility of feline hosts. The morphological characteristics of *P. westermani*-like at the adult stage were also compared with those of *P. westermani*.

MATERIALS AND METHODS

Identification of freshwater crabs

The captured freshwater crabs, belonging to the family Potamidae, were identified as *Phricotelphusa aedes* according to the method of Naiyanetr (1988).

Isolation of *Paragonimus* metacercariae

Between January and May 2003, we collected 922 freshwater crabs, *Phricotelphusa aedes*, from mountain streams in the Phanom District of Surat Thani Province, Thailand. We examined the crabs for metacercariae, as described previously (Rangsiruji *et al*, 2005). Isolated metacercariae were placed on glass slides and gently pressed under a coverglass for morphological observation and measurement.

Worm recovery from test animal

We inoculated the peritoneal cavity of a cat

with 60 *P. westermani*-like metacercariae. The cat was then treated with prednisolone (20 mg/kg) at 7-day intervals and was necropsied 148 days after inoculation. We examined the whole body of the cat for worms, as described previously (Sugiyama *et al*, 1984). Recovered worms were pressed between two glass slides, fixed in 70% ethanol, stained with borax carmine, and mounted with Canada balsam for morphological observation and measurement.

DNA amplification and sequencing of ITS2 region

We prepared DNA samples from individual *P. westermani* and *P. westermani*-like metacercariae (five metacercariae each). The ITS2 region of the nuclear ribosomal DNA was amplified by PCR and sequenced, as described previously (Sugiyama *et al*, 2002). The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward: Bowels *et al*, 1995) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse: Blair *et al*, 1997). We aligned and compared sequences using GENETYX-WIN software (ver 7.0, Software Development, Tokyo, Japan).

RESULTS

New crab intermediate host of *Paragonimus* in southern Thailand

We captured 922 freshwater crabs (Fig 1) from mountain streams in the Phanom District of Surat Thani Province. The crabs were positive for *P. westermani* metacercariae; this is the first report of this crab species serving as a second intermediate host of *P. westermani*. *P. westermani*-like metacercariae were also isolated from the same crab species captured at the same sites.

Morphology of *P. westermani*-like metacercariae from crabs

We isolated 89 *P. westermani*-like metacercariae from the crabs. All were spherical in shape and had thin walls (Fig 2). The thickness of the cyst wall in 30 specimens ranged from 4-14 μm, with an average of 8.7 μm. The longitudinal and transverse diameters of the cyst ranged from

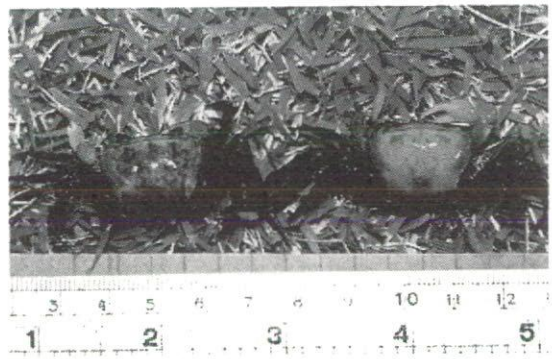


Fig 1- Freshwater crabs, *Phricotelphusa aedes*, which serve as the second intermediate host of both *P. westermani* and *P. westermani*-like in southern Thailand.

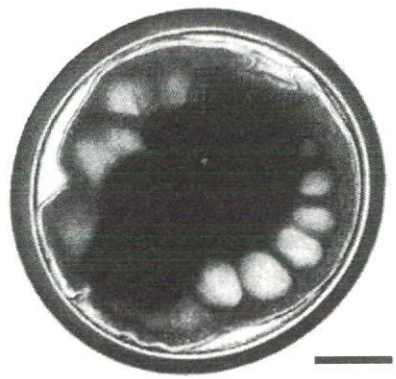


Fig 2- Photomicrograph of fresh *P. westermani* metacercaria. Bar is 100 μm.

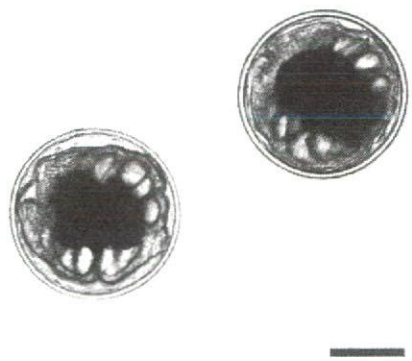


Fig 3- Photomicrograph of fresh *P. westermani*-like metacercariae. Bar is 100 μm.

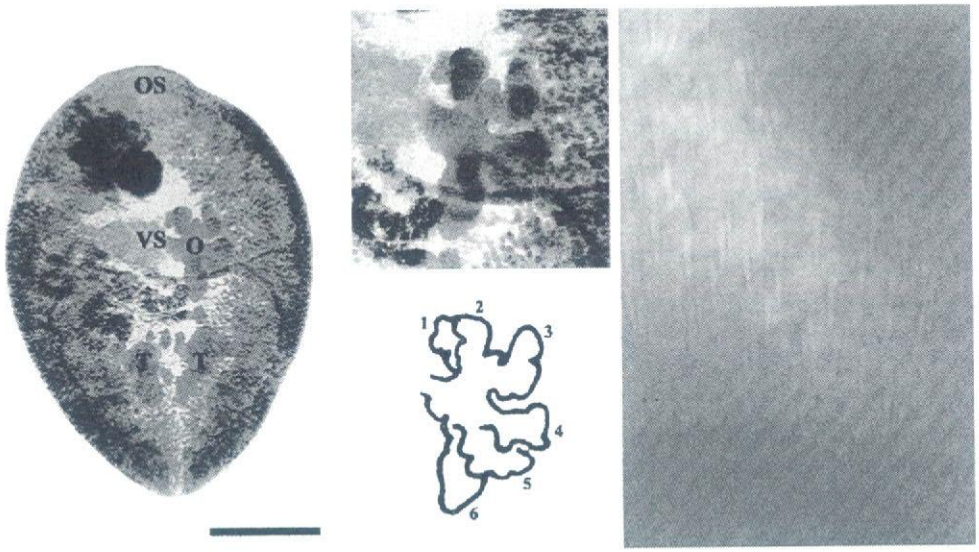


Fig 4- Adult worm of *P. westermani*-like from a cat inoculated with metacercariae. The worm had six-lobed ovary and singly spaced cuticular spines.

```
Pw1 ------------------------------------------------------------ 060
Pw2 TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA 060
PwL ............................................................ 060

Pw1 ----------------ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw2 TCGACATCTTGAACGC.............................................. 120
PwL ............................................................ 120

Pw1 TCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw2 ............................................................ 180
PwL .........G...T.............................................. 180

Pw1 GATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACAT 240
Pw2 ............................................................ 240
PwL ..............................................G......C..... 240

Pw1 ACTCGCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG 300
Pw2 ............................................................ 300
PwL .....G...................................................... 300

Pw1 GCTCAGTAAATGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTG 360
Pw2 ............................................................ 360
PwL .......G.T..G..............C.........................T....... 360

Pw1 CGCGTGGTCTGCGTTCGATGCTGACCTACGTATGTGCCATGTGGTCCATTCTTCTGACCT 420
Pw2 ............................................................ 420
PwL ..........................................................T......C....... 420

Pw1 CGGATTAGACGTGAGTACC---------------------- 463
Pw2 ...................CGCTGAACTTAAGCATATCACTAA 463
PwL .....C..................................... 463
```

Fig 5- Aligned sequences of the ITS2 region from *P. westermani* strain Thailand (AF159604, Pw1), *P. westermani* (Pw2) and *P. westermani*-like (PwL). Identical bases are represented by dots. Hyphen indicates missing data. Numbers refer to nucleotide sequence length.

212-252 μm and from 204-240 μm, respectively, with an average of 227 x 221 μm.

The metacercariae of *P. westermani*, also isolated from the same crab hosts, were spherical in shape and had thick walls (Fig 3). The thickness of the cyst wall in five specimens ranged from 19-37 μm, with an average of 28.2 μm. The longitudinal and transverse diameters of the cysts ranged from 458-510 μm and from 438-501 μm, respectively, with an average of 492 x 480 μm.

Morphology of an adult worm

On postmortem examination of the test cat, 148 days after inoculation, 13 worms were recovered; 2 from the lungs (being paired in the worm cyst), 2 from the pleural cavity, and 9 from the liver. The worms from the lungs and pleural cavity were identified as either adults (one each, with eggs in the uterus) or pre-adults (without eggs), while the worms from the liver remained in the juvenile stage.

The size of the adult worm from the lung was 3.95 mm in length and 2.83 mm in width. The transverse diameters of the oral and ventral suckers measured 504 μm and 500 μm, respectively. The adult worm had a six-lobed ovary and singly spaced cuticular spines (Fig 4). The seminal receptacle was filled with spermatozoa.

ITS2 sequence analysis

The ITS2 region was amplified from DNA samples of individual *P. westermani* and *P. westermani*-like metacercariae using the consensus primers 3S and A28. Sequence analysis of the PCR products revealed that the aligned ITS2 region was 463 bp in length for both *P. westermani* and *P. westermani*-like samples. Pairwise comparison of the sequences showed 13 (2.8%) nucleotide differences (Fig 5). Similarity searches of the nucleotide databases GenBank/EMBL/DDBJ revealed that the ITS2 sequences of *P. westermani* were identical to those found in the databases under the accession number AF159604 for the *P. westermani* strain Thailand. However, the sequences of *P. westermani*-like did not exhibit a striking similarity to any of those found in the databases.

DISCUSSION

In this study, we observed adult *P. westermani*-like samples obtained from a cat that was inoculated with the metacercariae. The adult had an ovary that was simply divided into six lobes, a seminal receptacle filled with spermatozoa, and cuticular spines arranged singly. These morphological features at the adult stage are in good agreement with the description of *P. westermani* (Thai strain) (Sugiyama *et al*, 2001; Binchai *et al*, 2005). With regard to the morphology of metacercariae, other than the size, the features of *P. westermani*-like were almost identical to those of P. *westermani*. Therefore, it can be concluded that *P. westermani*-like should be classified as *P. westermani*, or as one of the members (a cryptic species) of the *P. westermani* complex (Blair *et al*, 1997), based on the anatomical similarities.

We investigated the susceptibility of feline hosts to *P. westermani*-like by experimental infection, and compared the results with those of *P. westermani*. From the cat experimentally infected with *P. westermani*, worms were detected only in the lungs or pleural cavity. The worms recovered were identified as adults or at least pre-adults (Binchai *et al*, 2005). In contrast, as shown in this study, juvenile *P. westermani*-like lodged predominantly in the liver, while some matured into adults in the pleural cavity or lungs. These findings suggested that the susceptibility in cats differed between *P. westermani* and *P. westermani*-like. The susceptibility of feline hosts to *P. westermani* was also examined using worms from Malaysia (Habe *et al*, 1996). About half of the worms recovered were identified as juvenile worms, but the principal domicile of the juveniles was not the liver but the skeletal muscles.

Molecular comparison based on ITS2 sequences revealed that there were a few nucleotide differences (2.8%) between *P. westermani* (*P. westermani* strain Thailand) and *P. westermani*-like. Therefore, in order to determine the proper taxonomic status of *P. westermani*-like, we need to investigate the detailed phylogenetic relationships between *P. westermani*-like and *P. westermani*. In terms of the susceptibility of *P.*

westermani-like, information regarding host-parasite relationships, particularly relating to the first intermediate hosts, is required. Studies into these issues are currently underway (Binchai *et al*, 2007).

REFERENCES

Binchai S, Rangsiruji A, Ketudat P, Morishima Y, Sugiyama H. Morphological and genetic characterization of Thai *Paragonimus westermani* matured in cat. [Abstract]. The Joint International Tropical Medicine Meeting, 2005. Bangkok: Mahidol University, 2005:193.

Binchai S, Rangsiruji A, Ketudat P, Morishima Y, Sugiyama H. Molecular systematics of a new form of *Paragonimus westermani* discovered in Thailand. *Southeast Asian J Trop Med Public Health* 2007;38 (suppl 1):92-6.

Blair D, Agatsuma T, Watanobe T, Okamoto M, Ito A. Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. *Parasitology* 1997;115: 411-7.

Blair D, Waikagul J, Honzako Y, Agatsuma T. Phylogenetic relationships among the Thai species of *Paragonimus* inferred from DNA sequences. In: Tada I, Kojima S, Tsuji M, eds. Proceedings of the Ninth International Congress of Parasitology. Bologna: Monduzzi Editore, 1998:643-7.

Bowles J, Blair D, McManus DP. A molecular phylogeny of the human schistosomes. *Mol Phylogenet Evol* 1995;4:103-9.

Habe S, Lai KPF, Agatsuma T, Ow-Yang CK, Kawashima K. Growth of Malaysian *Paragonimus westermani* in mammals and the mode of transmission of the fluke among mammals. *Trop Med Health* 1996;24:225-32.

Miyazaki I. Paragonimiasis. In: Miyazaki I, ed. An illustrated book of helminthic zoonoses. Tokyo: International Medical Foundation of Japan, 1991:76-146.

Naiyanetr P. Freshwater crabs in Thailand. Bangkok: Phaisalsipa Press, Bangkok. 1988:15 (Book published in memory of the royal cremation of Associate Professor Dr Praphun Chitchumnong of Chulalongkorn University).

Rangsiruji A, Sugiyama H, Morishima Y, *et al*. A new record of *Paragonimus* other than *P. westermani* in southern Thailand. *Southeast Asian J Trop Med Public Health* 2006;37 (suppl 3):57-61.

Sugiyama H, Sonoda J, Okuda M, Tomimura T. The macaque monkey as an experimental paratenic host for *Paragonimus westermani* (Kerbert, 1878) Braun, 1899. *J Vet Med Sci* 1984;46:345-56.

Sugiyama H, Shibahara T, Ketudat P, Thaithong S, Kawashima K. Morphological re-examination of *Paragonimus westermani* described by Daengsvang and others in 1964. *Trop Med Health* 2001;29:371-4.

Sugiyama H, Morishima Y, Kameoka Y, Kawanaka M. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol Cell Probes* 2002;16:231-6.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF *PARAGONIMUS HETEROTREMUS*, THE CAUSATIVE AGENT OF HUMAN PARAGONIMIASIS IN INDIA

T Shantikumar Singh[1], Hiromu Sugiyama[2], Achariya Rangsiruji[3] and K Ranjana Devi[4]

[1]Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Sikkim, India; [2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; [3]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok Thailand; Department of Microbiology, Regional Institute of Medical Sciences, Imphal, Manipur, India

Abstract. In order to identify the causative species of human paragonimiasis, we performed a combined morphological and molecular investigation on the metacercariae and *Paragonimus* eggs isolated from the freshwater crab host, *Potamiscus manipurensis*, and sputum specimens of a patient, respectively. Experimental infection of laboratory animals with the metacercariae resulted in the isolation of adult worms that were morphologically identified as *P. heterotremus*. Molecular characterization based on polymerase chain reaction and DNA sequencing of the metacercariae and *Paragonimus* eggs from the sputum specimens yielded identical ITS2 sequences. Results of phylogenetic analyses of the ITS2 region suggested that Indian *P. heterotremus* is nested within the *P. heterotremus* clade; the Indian population is less closely related to other members within the clade.

INTRODUCTION

Paragonimus species hitherto reported in Asia number 17, of which *P. westermani* is the most common cause of human paragonimiasis (Miyazaki, 1974). *Paragonimus heterotremus* was first described in rats in Guangxi, China (Chen and Hsia, 1964). The first human paragonimiasis due to *P. heterotremus* in the world was reported by Miyazaki and Harinasuta (1964). This species is considered medically more important than other species in Thailand, Lao PDR, Vietnam, and some parts of China where man and mammals serve as naturally infected final hosts (Miyazaki and Harinasuta, 1964; Doanh *et al*, 2005). In Manipur in India, a recently recognized endemic area, *P. westermani* was presumed to be the etiological agent of human paragonimiasis (Singh *et al*, 1982;1993). However, no scientific study supported this speculation nor was able to determine which lung fluke species occurred in Manipur until recently. A joint Indo-Japan research on *Paragonimus* and paragonimiasis in Manipur resulted in the identification of *Potamiscus manipurensis*, a freshwater crab species, as the second intermediate host of at least three *Paragonimus* species, including *P. heterotremus*.

In this study, further investigation on the determination of etiological agents of human paragonimiasis was performed by nucleotide sequencing of the ITS2 region on *Paragonimus* (Sugiyama *et al*, 2002). The study also aimed to determine the phylogenetic relationships of the Indian species with other *Paragonimus* found in various geographical areas in Asia.

Corresponence: T Shantikumas Singh, Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Sikkim, India.
E-mail: shantikumar_singh@rediffmail.com

MATERIALS AND METHODS

Parasite material

Metacercariae harvested from freshwater crab host, *Potamiscus manipurensis*, which were collected from Luwangsangbam Matai in Imphal East and Motbung in Senapati Districts both in Manipur State were used for morphological study, laboratory animal infections, and molecular study. Adult worms as well as immature worms that were recovered from the experimentally infected puppies and albino rats were used for morphological identification. *Paragonimus* eggs were collected from sputum specimens of a patient in Senapati District. All materials, metacercariae, adult worms, and eggs were preserved in equal

proportions in 70% ethanol and 10% formalin until utilized. Morphological features of both fresh and preserved metacercariae and borax-carmine-stained worms were examined under microscope.

DNA isolation, amplification and sequencing

DNA samples were prepared from individual metacercariae and eggs. The ITS2 region of the nuclear ribosomal DNA was amplified by PCR and sequenced as described previously (Sugiyama *et al*, 2002). The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward: Bowels *et al*, 1995) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse: Blair *et al*, 1997).

Sequence and phylogenetic analyses

The Indian *Paragonimus* ITS2 sequences were aligned with other *Paragonimus* sequences obtained from the GenBank database and an outgroup (*Fasciola hepatica*; Table 1), using the Clustal X program (Jeanmougin *et al*, 1998). Maximum parsimony analysis was conducted with the branch-and-bound algorithm using PAUP* (version 4.0b) (Swofford, 1998). The robustness of tree(s) inferred from the analysis was evaluated using bootstrap analyses with heuristic searching (Felsenstein, 1985).

RESULTS

Characteristics of metacercariae, eggs, and adult worms

The metacercariae (Fig 1) were oval to suboval in shape. The inner cyst measured 163 to 215 μm (av = 196 μm) in the long axis and 133 to 188 μm (av = 162 μm) in the transverse axis. The thickness of the inner wall was 4.2 to 10.4 μm (av = 6.3 μm) on the side and 10.4 to 27.1 μm (av = 18.2 μm) at the pole. The oral sucker, provided with a stylet, was smaller than the ventral sucker.

Paragonimus eggs (Fig 2), golden-yellow in color, oval shaped, and operculated, measured 89-100 μm (av = 92 μm) in length and 47-58 μm (av = 50 μm) in width. The eggshell thickness was almost uniform in 22 (63%) and discernible at the nonoperculated end in 13 (37%). The

Table 1
GenBank accession numbers of *Paragonimus* species and *Fasciola hepatica*.

Species	Origin	Accession No.
P. heterotremus	Thailand	AF159603
P. heterotremus	China	AY618758
P. heterotremus	India	AB308377, AB308378
P. skrjabini	China	AY618752
P. miyazakii	China	AY618741
P. westermani	Thailand	AF159604
Fasciola hepatica	Australia	AB207148

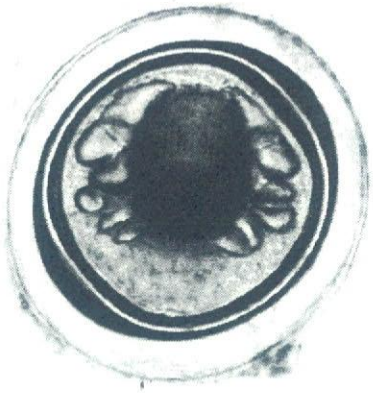


Fig 1- *P. heterotremus* metacercariae: average longitudinal diameter 196 μm and average transverse diameter 162 μm.

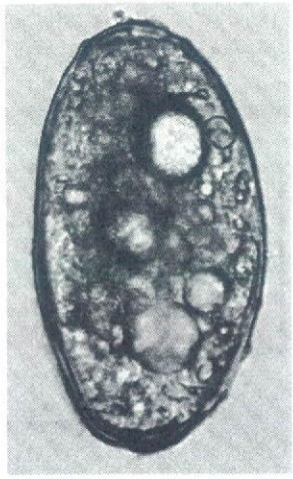


Fig 2- Morphological characteristics of eggs discharged from a patient. Size of eggs: av length = 92 μm, av width = 50 μm.

widest transverse diameter was located at the middle 28 (80%), at operculated half 6 (17%), and at nonoperculated half 1(3%).

The borax-carmine-stained worms (Fig 3) that were recovered from the experimentally infected puppies showed singly spaced cuticular spines, oral suckers (385-500 μm) that were much larger than the ventral suckers (260-300 μm), and the ovaries and testes that were delicately branched. The vitellaria were not seen in immature worms. The morphological features of metacercariae, eggs, and worms conform to the features of *P. heterotremus*.

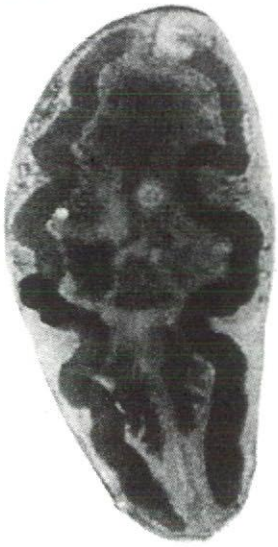


Fig 3- *P. heterotremus* adult worm recovered from the experimentally infected puppies showed delicately branched ovary and testes and the oral sucker was much larger than the ventral sucker.

Sequence and phylogenetic analyses

Molecular characterization, which is based on PCR and DNA sequencing of the metacercariae (accession No. AB308377) and eggs (AB308378), yielded identical ITS2 sequences. The alignment of the ITS2 region of six taxa of *Paragonimus* and its outgroup was 378 bp in length. Twenty-four characters (6.3%) were phylogenetically informative. A single most parsimonious tree (Fig 4), with a length of 144 steps, was obtained from a maximum parsimony analysis of the informative characters with 1,000 bootstrap (BS) replicates. Fit measures of the tree were as follows: consistency index (CI) = 0.951, retention index (RI) = 0.811, and rescaled consistency index (RC) = 0.771. The phylogenetic tree revealed that Indian *P. heterotremus* is nested within *P. heterotremus* clade (BS = 99%), which includes *P. heterotremus* from Thailand and China. The Indian population is however, less closely related to other members of the clade.

DISCUSSION

Although India is the first country from whence *P. westermani* was first described by Kerbert in 1878, from a Bengal tiger, very little attention has been given to this parasite because human paragonimiasis was never considered a public health problem. In India, there was no record of an autochthonous human case of paragonimiasis, although *P. westermani* infection was described in many mammals.

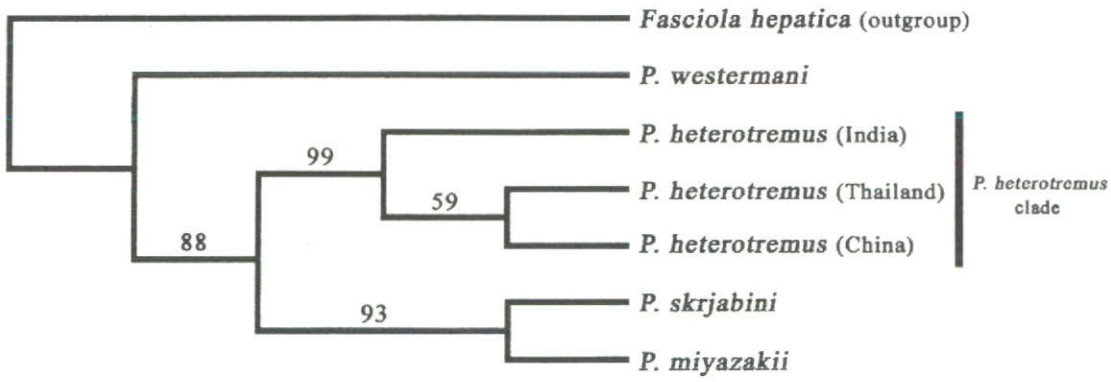


Fig 4- Single most parsimonious tree with a length of length 144 steps, based on parsimony analysis of the informative characters of the ITS2 region. Fit measures of the tree: CI = 0.951, RI = 0.811, RC = 0.771. Numbers above the branches indicate bootstrap values (%).

Evidence of infection with lung flukes of the genus *Paragonimus* in wild mammals has often been reported in India (Gaur *et al*, 1980; Rao, 1935; Srivastava, 1938; Singh and Somvanshi, 1978; Parihar and Shrivastava, 1988; Sano *et al*, 1994). The authors described *P. westermani* as the causative agent, based on the morphology of the eggs in the fecal specimens only or sections of worms and worm cysts in the lungs obtained on autopsy or postmortem examination of the animals. In the absence of detailed morphological descriptions of the adult worms, it was not possible to identify the species by examination of histopathological sections of the worm or worm cyst in the tissue and eggs in the feces. *P. westermani* was also reported to be the causative agent of human paragonimiasis in Manipur, based on the morphology of the eggs seen on microscopy examination of the sputum specimens of the patients (Singh *et al*, 1982). Therefore, doubts prevailed as to whether or not *P. westermani* was actually the only species infecting mammals and humans in India. Singh and Vashum (1994) first described the *P. heterotremus* adult worm from the biopsy specimen of a subcutaneous nodule in a 10-year-old boy in Imphal, Manipur. No other information on the *Paragonimus* species causing human paragonimiasis has been available in India.

The occurrence of *P. heterotremus* in freshwater crab, *Barytelphusa lugubris*, in an endemic area of paragonimiasis in Arunachal Pradesh was reported by Narain *et al* (2003). However, the morphological features of the metacercariae and adult worms, as described by these authors require further confirmation. In addition, it may not be safe to assume that this species is the causative agent of human paragonimiasis without morphological and molecular characterization of the parasite material recovered from the patient.

Recently, molecular analysis of any one of the developmental stages of the parasite has proved to be highly sensitive, and specific techniques are required to confirm the parasite species and its relationship with other species occurring elsewhere in the world. Technique is of importance in the identification of *Paragonimus* species, which can be made from the eggs in clinical specimens. Adult worms are rarely recovered from the patient, and hence not available for morphological identification and molecular characterization. The results of the present study confirmed that *P. heterotremus* was the causative agent of human paragonimiasis in Manipur, India.

Phylogenetic analysis indicated that all *P. heterotremus* species that originate from Vietnam, Thailand, and China form a distinct group (Le *et al*, 2006). However, our study revealed that the Indian species, although situated within the *P. heterotremus* group, is distantly related to the Chinese and Thai species.

This species has been identified as significant cause of human paragonimiasis in Southeast Asia, and endemic in South/Southwest China, Thailand, Lao PDR, and Vietnam (Blair *et al*, 1997; De *et al*, 2000; Doanh *et al*, 2005; Waikagul and Yoonuan, 2005). Morphometric and molecular characterization of the *Paragonimus* species are important for epidemiological, ecological, and taxonomic studies. This knowledge will also help in the control and treatment of paragonimiasis.

Potamiscus manipurensis, the natural second intermediate crustacean host of *P. heterotremus*, was found to contain metacercariae of *P. skrjabini* (Singh *et al*, 2006), and possibly two more species as well. The metacercariae of *P. skrjabini* were most frequently isolated from the freshwater crabs in some localities in Manipur State, where patients of pulmonary as well as cutaneous paragonimiasis have been reported. The possible relationship of *P. skrjabini* with human paragonimiasis in these localities is now under investigation.

REFERENCES

Blair D, Agatsuma T, Okamoto M, Ito A. Geographical genetic structure within the human lung fluke, *Paragonimus westermani* detected from DNA sequences. *Parasitology* 1997;115:411-7.

Bowles J, Blair D, McManus DP. A molecular phylogeny of the human Schistosomes. *Mol Phylogenet Evol* 1995;4:103-9.

Chen HT, Hsia TK. A preliminary report of

new species of *Paragonimus*. *Paragonimus heterotremus* sp. nov. *Zhongshan Daxue Xuebao* 1964;2:236-8.

Doanh PN, Le NT, Tat D. *Paragonimus* and paragonimiasis in Vietnam. In: Arizono N, Chai JY, Nawa Y, Takahashi Y, eds. Asian parasitology. Vol 1. Food-borne helminthiasis in Asia. Chiba, Japan: Federation of Asian Parasitologists, 2005:149-53.

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783-91.

Gaur SNS, Tewari HC, Sethi MS, Prakash O. Helminth parasites from tiger (*Panthera tigris*) in India. *Indian J Parasitol* 1980;4: 71-2.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. *Trends Biochem Sci* 1998; 23:403-5.

Le TH, De NV, Blair D, McManus DP, Kino H, Agatsuma T. *Paragonimus heterotremus* Chen and Hsia (1964), in Vietnam: a molecular identification and relationships of isolates from different hosts and geographical origins. *Acta Trop* 2006;98:25-33.

Miyazaki I. Lung fluke in the world: morphology and life history. In: Sasa M, ed. A symposium on epidemiology of parasitic diseases. Tokyo: International Medical Foundation of Japan, 1974:101-35.

Miyazaki I, Harinasuta T. The first case of human paragonimiasis caused by *Paragonimus heterotremus* Chen et Hsia, (1964). *Ann Trop Med Parasitol* 1964;60:509-14.

Narain K, Devi KR, Mahanta J. *Paragonimus* and paragonimiasis-A new focus in Arunachal Pradesh, India. *Curr Sci* 2003;84:985-7.

Parihar NS, Shrivastava SN. Bronchial hyperplasia in a tiger (*Panthera tigris*). *Indian J Anim Sci* 1988;58,230-3.

Rao MAN. Lung flukes in two dogs in the Madras presidency. *Indian J Vet Sci Anim Husb* 1935; 5:30-2.

Sano M, Agrawal MC, Kotwal PC, Gopal R. *Paragonimus* infection in tigers at Kanha National Park. *J Parasitol Appl Anim Biol* 1994;3:115-6.

Singh NP, Somvanshi R. *Paragonimus westermani* in tigers (*Panthera tigris*) in India. *J Wild Life Dis* 1978;14:322-4.

Singh TS, Mutum S, Razaque MA, Singh YI, Singh EY. Paragonimiasis in Manipur. *Indian J Med Res*, 1993;97:247-52.

Singh TS, Vashum H. Cutaneous paragonimiasis: a case report. *Indian J Pathol Microbiol* 1994; 37 (suppl): S33-4.

Singh YI, Singh NB, Devi SS, Singh YM, Razaque M. Pulmonary paragonimiasis in Manipur. *Indian J Chest Dis Allied Sci* 1982; 24:304-6.

Singh TS, Singh LD, Sugiyama H. Possible discovery of Chinese lung fluke, *Paragonimus skrjabini*, in Manipur, India. *Southeast Asian J Trop Med Public Health* 2006;37(suppl 3): 53-6.

Srivastava HD. The occurrence of *Paragonimus westermani* in the lungs of cats in India. *Indian J Vet Sci Anim Husb* 1938;8:255-7.

Sugiyama H, Morishima Y, Kameoka Y, Kawanaka M. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol Cell Probes* 2002;16:231-6.

MOLECULAR SYSTEMATICS OF A NEW FORM OF *PARAGONIMUS WESTERMANI* DISCOVERED IN THAILAND

Sutheewan Binchai[1], Achariya Rangsiruji[1], Punsin Ketudat[1], Yasuyuki Morishima[2] and Hiromu Sugiyama[2]

[1]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok Thailand; [2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan

Abstract. This study aimed to clarify evolutionary relationships of *P. westermani*-like with other members of *Paragonimus* in Asia. The parsimony method was employed in molecular analyses of the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA and the partial cytochrome c oxidase subunit I (COI) region of mitochondrial DNA. A single most parsimonious tree obtained from the ITS2 region revealed two important groups within *P. westermani* complex that is based on geographical origins. From this study, it is evident that *P. westermani*-like is either placed well within the *P. westermani* complex or is located close to the complex. Since a significant genetic variation was observed between Thai *P. westermani* and *P. westermani*-like, further investigation on the specificity of first intermediate hosts should be carried out to determine a proper taxonomic status of *P. westermani*-like.

INTRODUCTION

Paragonimus westermani is widely distributed in Asia (Miyazaki, 1991). In Thailand, *P. westermani* metacercariae were reported in the central and southern parts of the country (Miyazaki, 1982; Kawashima *et al*, 1989). During our field survey, a new form of *P. westermani* metacercariae was discovered. The metacercariae obtained were almost identical to *P. westermani* metacercariae, except the size was smaller; thus, they were provisionally named *P. westermani*-like. Studies concerning the morphology of adult worms and susceptibility of feline hosts to *P. westermani*-like carried out by Sugiyama *et al* (2007) indicated that the adult worms resembled a diploid-type *P. westermani*, but the susceptibility in cats differed between *P. westermani* and *P. westermani*-like. This present study aimed to characterize genetically *P. westermani*-like as well as to clarify its phylogenetic relationships with other members of *Paragonimus* in Asia using nucleotide sequences of the ITS2 region of nuclear ribosomal DNA and a portion of the mitochondrial COI gene.

Correspondence: Achariya Rangsiruji, Department of Biology, Faculty of Science, Srinakharinwirot University, Sukhumwit 23, Bangkok 10110, Thailand.
Tel: 66 (0) 2664 1000 ext 8403;
Fax: 66 (0) 2260 0128
E-mail: achariya@swu.ac.th

MATERIALS AND METHODS

Parasite materials

Metacercariae of *P. westermani* and *P. westermani*-like were obtained from the waterfall crab, *Phricotelphusa aedes*, which were collected in Phanom District, Surat Thani Province. The metacercariae of other Thai *Paragonimus* species were harvested as follows: P. bangkokensis from *Ranguna smalleyi* (Phanom District, Surat Thani Province); *P. harinasutai* and *P. heterotremus* from *Larnaudia larnaudii* (Kaeng Khoi District, Saraburi Province) and *P. siamensis* from *Sayamia germaini* (Na Di District, Prachin Buri Province).

DNA sequencing and amplification

Total genomic DNA was prepared from individual metacercariae following Sugiyama *et al* (2002). The ITS2 region of nuclear ribosomal DNA and a portion of the mitochondrial COI gene were amplified by PCR and sequenced using primers 3S (forward: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3'; Bowels *et al*, 1995), A28 (reverse: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCC GC-3'; Blair *et al*, 1997), JB3 (forward: 5'-TTTTTTGGGCATCCTGAGGTTTAT-3'; Bowels *et al*, 1995) and JB 4.5 (reverse: 5'-TAAAGAAAGAACATAATGAAAATG-3'; Bowels *et al*, 1995), respectively. The PCR cycle consisted of three major steps: 98° C for 5 seconds to denature DNA, 55° C for 10 seconds

for primer annealing, and 72° C for 10 seconds for primer extension. The cycle was repeated 30 times, followed by a final extension at 72° C for 1 minute.

Sequence and phylogenetic analyses

Sequence alignments were carried out using Clustal X program (Jeanmougin *et al*, 1998) with additional sequences of *Paragonimus* species and *Fasciola hepatica* (outgroup) from GenBank database. The GenBank accession numbers of all sequences employed are shown in Table 1. Phylogenetic trees were reconstructed using maximum parsimony analysis with a branch-and-bound algorithm. 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 (Felsenstein, 1985), with 1,000 replicates. All phylogenetic analyses were performed using PAUP* version 4.0b (Swofford, 1998).

RESULTS

Metacercariae of *Paragonimus* species employed in this study were shown in Fig 1.

Sequence characteristics

ITS2. The actual length range of the ITS2 region of the ingroup was 359-363 bp. The alignment of this region of 15 taxa of *Paragonimus* species and its outgroup was 378 bp in length, with 10 sites of insertion or deletion. Out of 378 total characters, 230 (60.8%) were constant, 92 (24.4%) were parsimony-uninformative and 56 (14.8%) were parsimony-informative. Sequence divergence between ingroup and outgroup taxa obtained from pairwise distance analysis ranged from 37.6-41.9% but within the ingroup the sequence divergence range was 0-13.7%. The mean G+C content of all taxa was 55.5%, and transition/transversion ratio was 2.60.

COI. The actual length of the partial COI region of the ingroup was 381 bp. The alignment of this region for all 15 taxa under study was 384-bases long, with only one site of deletion. From 384 characters, 241 (62.8%) were constant, 38 (9.9%) were parsimony-uninformative, and 105 (27.3%) were parsimony-informative. The sequence divergence between ingroup and outgroup taxa was computed using pairwise distance analysis, and ranged from 22.2-34.7%;

Table 1
GenBank accession numbers of *Paragonimus* and *Fasciola* used in this study.

Species	Origin	ITS2	COI
P. westermani	Hyogo, Japan	U96907	U97205
	Minchin, China	U96907[a]	AY140681
	Haenam, Korea	AF333278	AF333281
	Karapai, Taiwan	U96908	AY140673
	Philippines	U96910	U97213
	Malaysia	U96909	U97211
	Central Thailand	AF159604	U97212
	Southern Thailand	AB354216	AB354224
P. westermani-like	Thailand	AB354218	AB354225
P. macrorchis	Thailand	AF159608	AF159598
P. heterotremus	Thailand	AB354221	AB354229
P. harinasutai	Thailand	AB354220	AB354226
P. bangkokensis	Thailand	AB248091	Ab354228
P. siamensis	Thailand	AB354222	AB354231
Fasciola hepatica	Australia	AB207148	AF216697

[a]sequence identical to *P. westermani* from Hyogo, Japan (Blair *et al*, 1997).

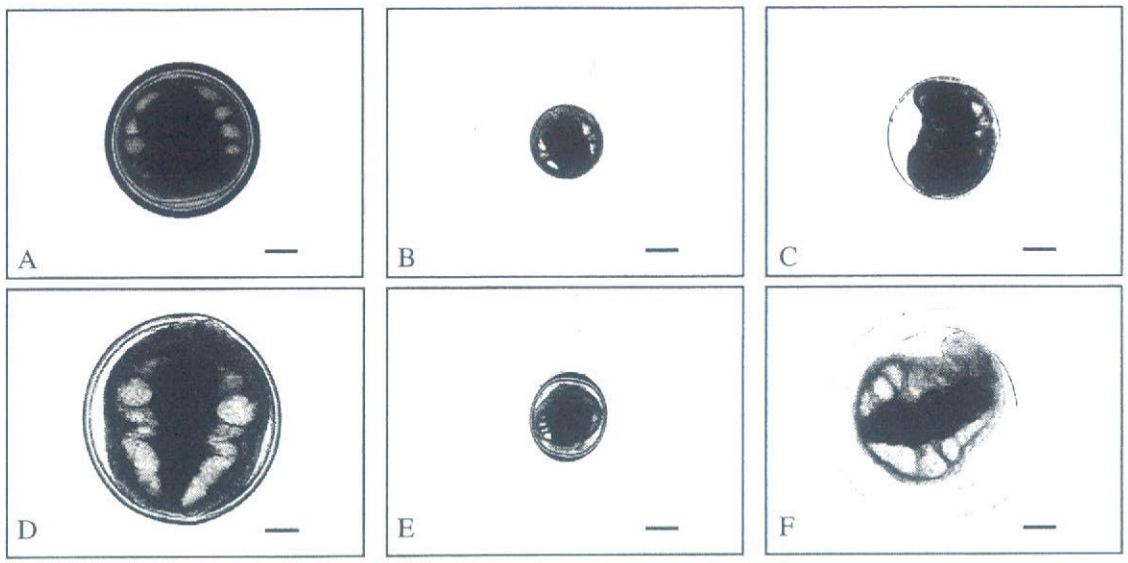


Fig 1- Metacercariae of *Paragonimus* species A: *P. westermani*, B: *P. westermani*-like, C: *P. bangkokensis*, D: *P. harinasutai*, E: *P. heterotremus* and F: *P. siamensis*. Scale bar indicates 100 µm.

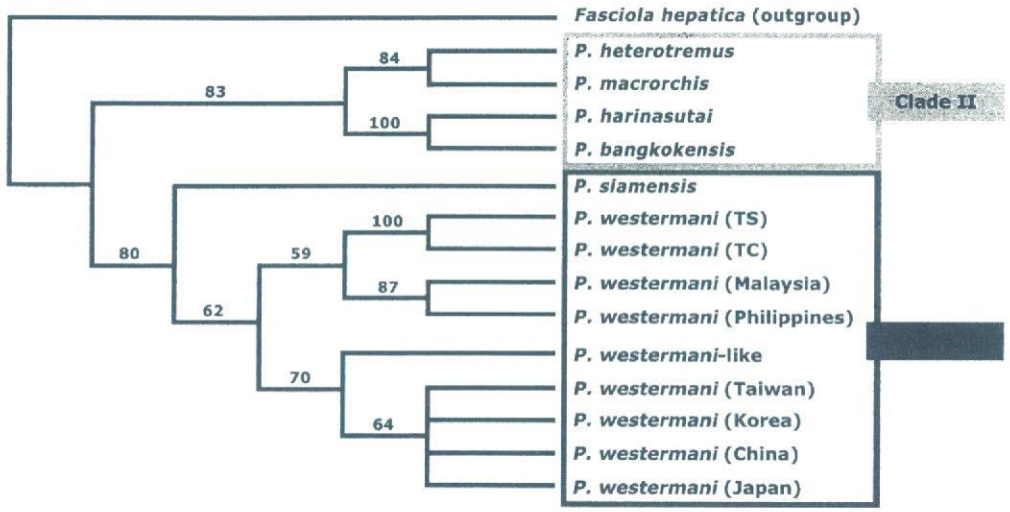


Fig 2- Single most parsimonious tree of length 191 steps based on parsimony analysis of the informative characters of the ITS2 region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. *P. westermani* (TS) = *P. westermani* from southern Thailand. *P. westermani* (TC) = *P. westermani* from central Thailand.

whereas, within the ingroup, it ranged from 0.3-25.3%. The mean G+C content was 44.0%, and transition/transversion ratio was 3.85.

Phylogenetic analyses

ITS2. A single most parsimonious tree (Fig 2) of length 191 steps was obtained based on parsimony analysis of the informative characters with 1,000 bootstrap replicates. Fit measures of the tree were as follows: consistency index (CI) = 0.9058, homoplasy index (HI) = 0.0942, retention index (RI) = 0.8393, and rescaled consistency index (RC) = 0.7602. The phylogenetic tree comprised two clades: clade I, including the *P. westermani* complex and *P. siamensis* (bootstrap value (BS) = 62%), and clade II, including other Thai *Paragonimus* species (BS = 83%). Within the *P. westermani* complex, two groups of organism can be obtained based on geographical distribution. The first group

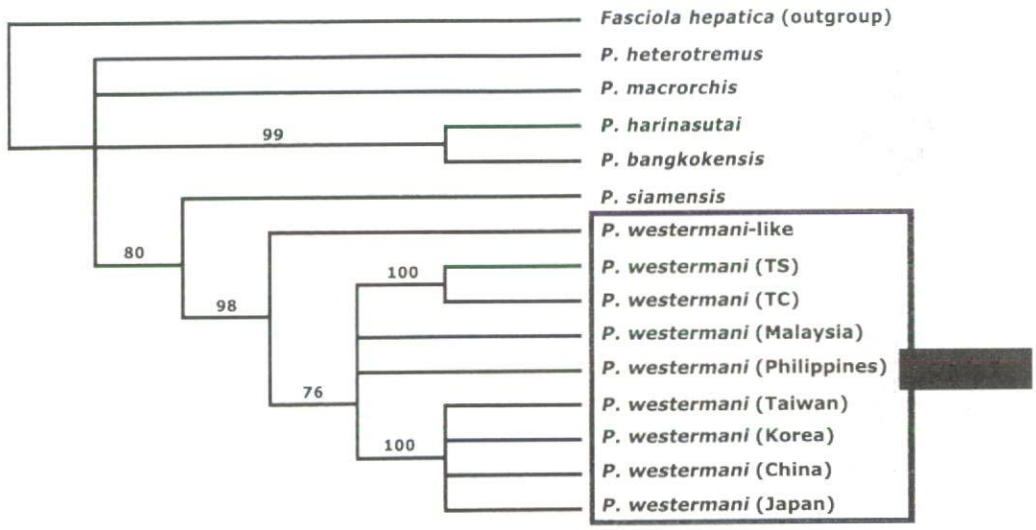


Fig 3- Strict consensus tree derived from 10 equally parsimonious trees of length 319 steps based on parsimony analysis of the informative characters of the partial COI region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. *P. westermani* (TS) = *P. westermani* from southern Thailand. *P. westermani* (TC) = *P. westermani* from central Thailand.

contains *P. westermani* from Southeast Asia (BS = 59%), while the second group contains *P. westermani* from East Asia and *P. westermani*-like from Thailand (BS = 70%).

COI. A strict consensus tree (Fig 3) was derived from 10 equally parsimonious trees of 319 steps long, based on parsimony analysis with 1,000 bootstrap replicates. Fit measures of the tree were as follows: CI = 0.6364, HI = 0.3636, RI = 0.6822, and RC = 0.4341. 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 Southeast and East Asia (BS = 76%). *Paragonimus westermani*-like is excluded from the complex and designated as a sister group.

DISCUSSION

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; Bowles *et al*, 1995) and *Fasciola* (364 bp; Mas-Coma et al, 2001). The level of sequence variation between *P. westermani*-like and *P. westermani* (1.39-4.0%) was close to the intraspecific variation within *P. westermani* from different geographical origins (0-3.41%). Intraspecific variation in the ITS2 region was also observed in other digeneans, including *Schistosoma* (Agatsuma *et al*, 2001) and *Fasciola* (Adlard *et al*, 1993).

The numbers of the variable characters of the partial COI (143 characters) and the ITS2 (148 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) as compared to the ITS2 data (HI = 0.0942).

From this study, the phylogenetic tree inferred from the ITS2 region showed that *P. westermani* formed a complex of cryptic species and could be divided into two groups as previously reported (Blair *et al*, 1997, 1998). The first group comprises *P. westermani* from Southeast Asia (Thailand, Malaysia, and the Philippines), and the second group composes of *P. westermani* from East Asia (Taiwan, Korea, China, and Japan), which was closely related to *P. westermani*-like. In contrast to the ITS2 tree, the phylogenetic tree reconstructed from the COI region revealed that *P. westermani*-like is excluded from the complex and

designated as a sister group. Thus, it is evident that *P. westermani*-like is either well placed within the *P. westermani* complex (ITS2 data), or it is located close to the complex (COI data). However, since the protein-coding gene (COI) is uder 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 such a high 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 (Sugiyama *et al*, 2007).

Since the susceptibility of feline hosts to *P. westermani*-like was found to be different from that of Thai *P. westermani* (Sugiyama *et al*, 2007) and a significant genetic variation was also observed between them, further investigation on the specificity of first intermediate hosts should be carried out to determine the proper taxonomic status of *P westermani*-like.

REFERENCES

Adlard RD, Barker SC, Blair D, Cribb TH. Comparisons of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). *Int J Parasitol* 1993;23:423-5.

Agatsuma T, Iwagami M, Liu CX, *et al.* Molecular phylogenetic position of *Schistosoma sinensium* in the genus *Schistosoma*. *J Helminthol* 2001;75:215-21.

Blair D, Agatsuma T, Watanobe T, Okamoto M, Ito A. Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. *Parasitology* 1997;115: 411-7.

Blair D, Waikagul J, Honzako Y, Agatsuma T. Phylogenetic relationships among the Thai species of *Paragonimus* inferred from DNA sequences. In: Tada I, Kojima S, Tsuji M, eds. Proceedings of the Ninth International Congress of Parasitology. Bologna: Monduzzi Editore, 1998:643-7.

Bowles J, Blair D, McManus DP. A molecular phylogeny of the human schistosomes. *Mol Phylogenet Evol* 1995;4:103-9.

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783-91.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. *Trends Biochem Sci* 1998; 23:403-5.

Kawashima K, Sugiyama H, Thaithong S, Ketudat P. *Paragonimus* in Thailand. In: Kawashima K, ed. *Paragonimus* in Asia: biology, genetic variation and speciation. Fukuoka: Shunpposha Photographic Printing, 1989:72-4.

Mas-Coma S, Funatsu IR, Bargues MD. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology* 2001;123:S115-27.

Miyazaki I. Geographical distribution of *Paragonimus westermani* and *P. pulmonaris* in Asia. *Med Bull Fukuoka Univ* 1982;9:11-22 (Japanese, English abstract).

Miyazaki I. Paragonimiasis. In: Miyazaki I, ed. An illustrated book of helminthic zoonoses. Tokyo: International Medical Foundation of Japan, 1991:76-146.

Sugiyama H, Morishima Y, Binchai S, Rangsiruji A, Ketudat P. New form of *Paragonimus westermani* discovered in Thailand: morphological characteristics and host susceptibility. *Southeast Asian J Trop Med Public Health* 2007;38 (suppl 1):87-91.

Sugiyama H, Morishima Y, Kameoka Y, Kawanaka M. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol Cell Probes* 2002;16:231-6.

Swofford DL. PAUP*: phylogenetic analysis using parasimony (*and other methods. Version 4. Sunderland MA: Sinauer Associates, 1998.