TABLE 2. *Number of goblet cells in the jejunum and ileum after* N. brasiliensis *infection.*

Days after infection	Jejunum	Ileum
0	13.1 ± 0.7	12.3 ± 0.5
7	19.1 ± 1.2*	14.9 ± 1.2
14	21.5 ± 1.2*	20.0 ± 0.9*
21	11.3 ± 1.0	11.3 ± 1.3

Each measurement was performed on paraffin-embedded tissue sections. Figures in the table represent numbers of goblet cells/100 villus epithelial cells. All data are mean ± SE of 4 rats. *Significantly different from day 0 ($P<0.05$).

Figure legends

Fig 1. Expression of goblet cell- and mucin glycosylation-related genes in the jejunal (closed columns) and ileal (open columns) villus epithelium of BN rats after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction, reverse transcribed, and relative quantification was carried out by RT-PCR. The quantified value for each sample was normalized with respect to that for β-action. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 2. Expression of mucin glycosylation-related genes in the jejunal villus epithelium of mast cell-deficient *Ws/Ws* rats after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction and semi-quantitative RT-PCR was performed as described in Fig. 1. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 3. Expression of goblet cell- and mucin glycosylation-related genes in the jejunal villus epithelium of BN rats in the early period after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction and semi-quantitative RT-PCR was performed as described in Fig. 1. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 4. Successive upregulation of goblet cell- and mucin glycosylation-related genes in the intestinal epithelial cells during the course of *N. brasiliensis* infection.

Fig. 1

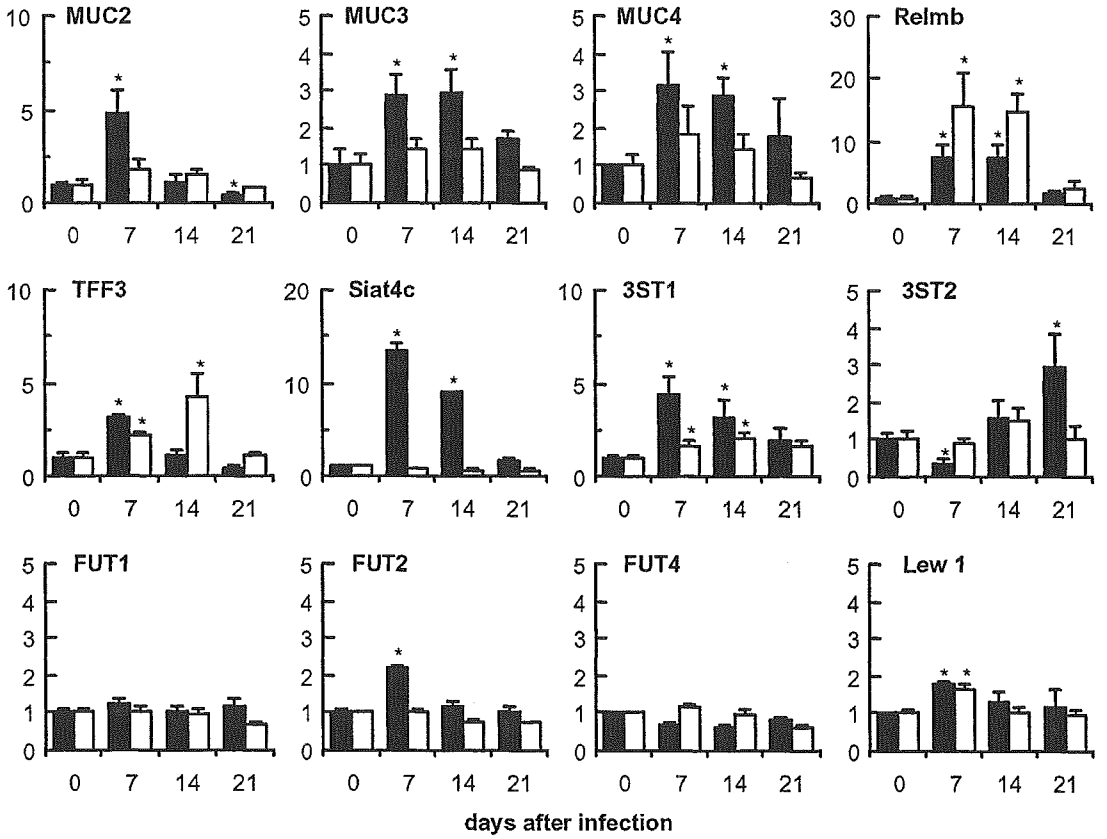

MUC2
MUC3
MUC4
Relmb
TFF3
Siat4c
3ST1
3ST2
FUT1
FUT2
FUT4
Lew 1
0 7 14 21
days after infection

Fig. 2.

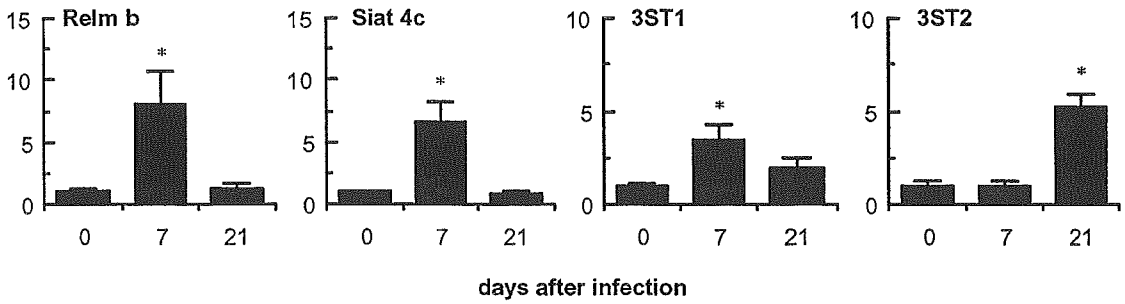


Fig. 3.

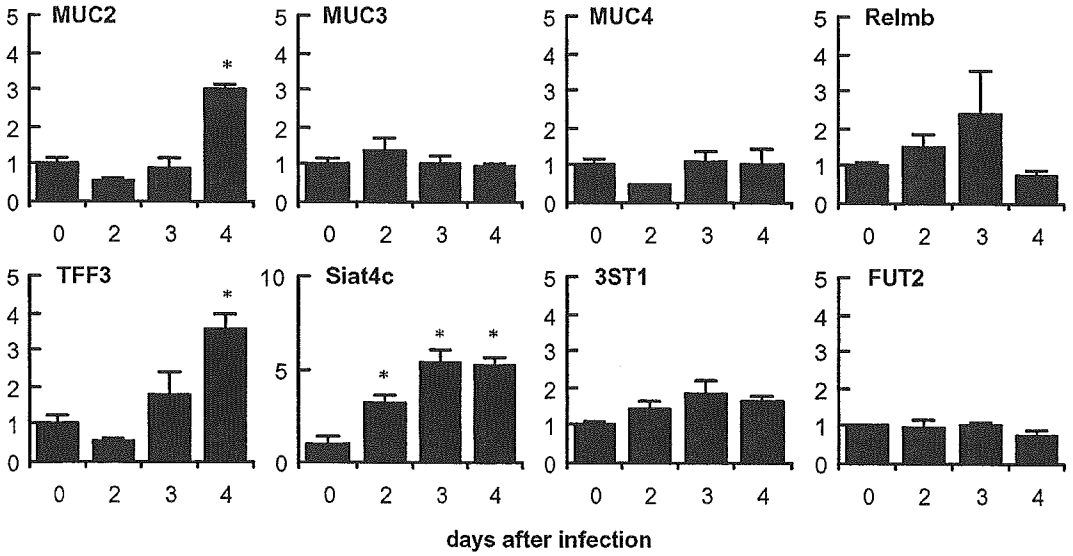
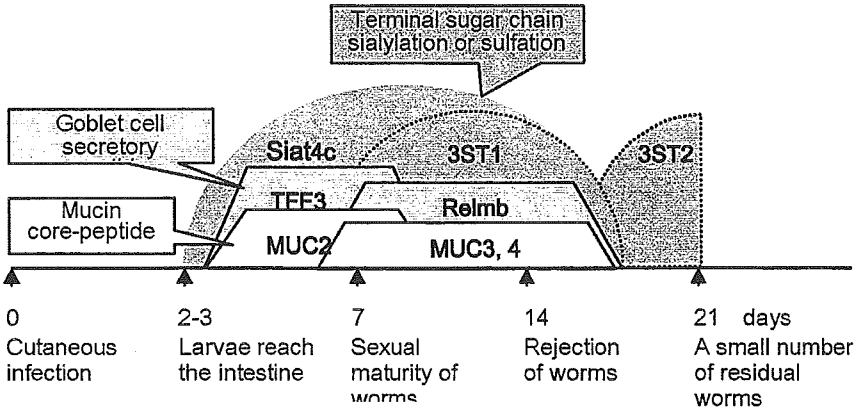

MUC2
MUC3
MUC4
Relmb
TFF3
Siat4c
3ST1
FUT2
days after infection

Fig. 4


Terminal sugar chain sialylation or sulfation
Goblet cell secretory
Mucin core-peptide
Siat4c
3ST1
3ST2
TFF3
Relmb
MUC2
MUC3, 4
0
Cutaneous infection
2-3
Larvae reach the intestine
7
Sexual maturity of worms
14
Rejection of worms
21 days
A small number of residual worms

原著

PET検査が鑑別の一助となった脳有鉤嚢虫症の1例*

松本ルミネ**1)　宇羽野　惠**　上坂義和**
國本雅也**　川中正憲***

Key Words : neurocysticercosis, ring-enhanced lesion, positron emission tomography, guideline, treatment

はじめに

脳有鉤嚢虫症は本邦では稀であるが，中南米・東欧・インドや中国などのアジア諸国ではよくみられ，成人発症のけいれんの鑑別診断に第一にあげるべき疾患となっている．本稿では，鑑別診断にPETを用いた点と，近年提唱された診断基準や治療ガイドラインに基づいた本例の治療を中心に報告する．

症　例

患者：21歳，中国人女性．

主訴：けいれん発作．

既往歴：特記事項はない．

生活歴：中国黒龍江省ハルビン出身の留学生で1年半前に来日した．

現病歴：2003年12月30日，就寝中に3～5分続くけいれん発作(右上肢伸展，左上肢屈曲，顔面は右を向く)が2回あり，当センター救急外来を受診した．来院時けいれんはなく，他の神経学的所見も異常なかったが，頭部CTで左前頭葉に低吸収域を認め，造影によりその一部がリング状に増強された．胸部CTで肺炎像もあり，肺炎および脳膿瘍を疑われ，加療目的で当科に入院した．

入院時現症：体温37.5℃，脈拍数93/分・整，血圧126/54mmHg．胸・腹部や四肢に異常なく，皮下結節もなかった．

神経学的所見：意識清明，けいれんはない．高次機能，脳神経領域，運動系，感覚系，平衡感覚，自律神経系に異常を認めなかった．

血液検査所見：WBC 12,640/μl (Neu 79.6%, Lym 14.1%, Mono 5.8%, Baso 0.1%, Eosino 0.4%), CK 180IU/l (正常値 0～165IU/l), CRP 0.27mg/dl, IgE 34IU/ml (0～199.9IU/ml)であり，白血球数とCKに軽度上昇がみられたほかは異常なかった．

髄液検査所見：初圧15.5cmH_2O, 細胞数 1.3/μl (多核球 0.3/μl, 単核球 1.0/μl), 糖 72mg/dl (同時血糖 80mg/dl), 蛋白 13mg/dl, IgG 1.06mg/dl, IgG index 0.44.

脳波：基礎律動 9～10Hz, 30～100μVのα波が

* A case of neurocysticercosis diagnosed with positron emission tomography (PET). (Accepted September 30, 2005).

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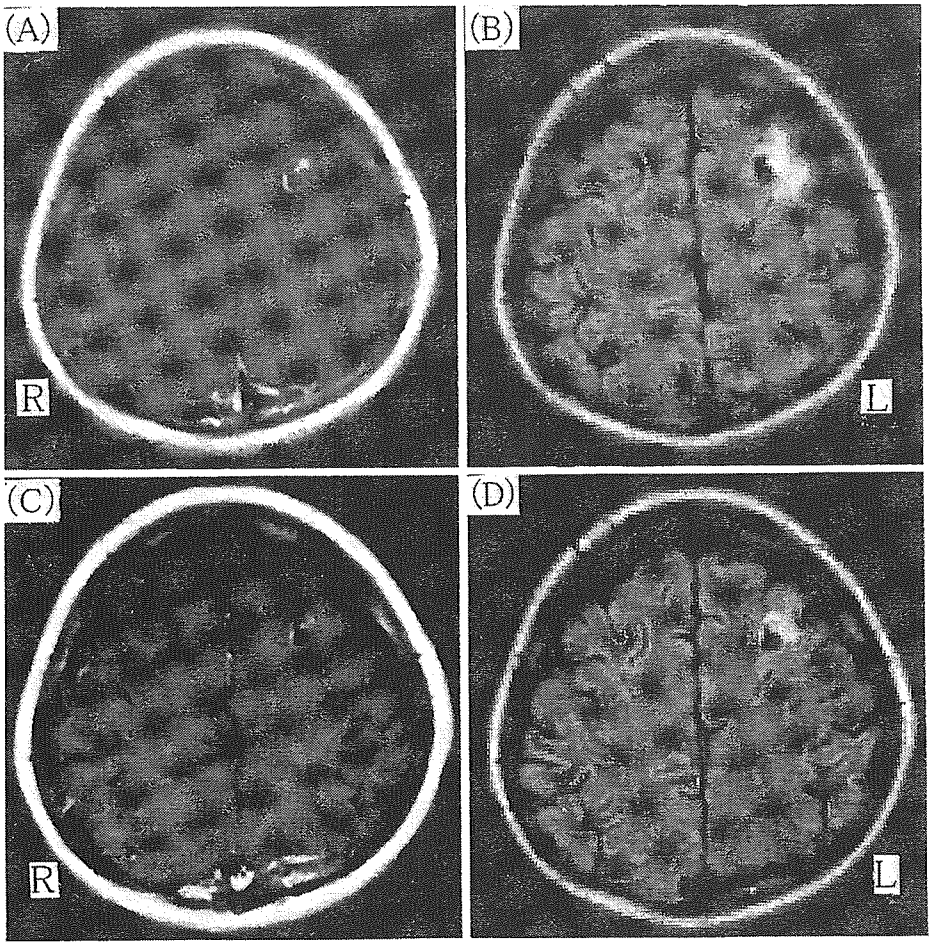

図1 Transition of the magnetic resonance imaging of the brain from January 8 (A, B) to January 23 (C, D)
(A) T1-weighted MR image after contrast administration showing a ring-like enhanced cyst with a scolex inside. (B) Edematous lesion around the cyst on FLAIR image. (C) T1-weighted image with gadolinium enhancement and (D) FLAIR image of fifteen days later. The enhancement of the cyst and surrounding edema decreased without any treatment.

中等量，後頭部優位に出現し持続は良好．左右差や突発性異常波を認めない．

胸部X線：右中肺野に透過性低下域がある．

胸部CT：右S2, S6に気道に沿って多発する斑状影と，その周囲にすりガラス影がみられる．

入院後経過：入院時，発熱，白血球数上昇，胸部X線において肺炎像を認めた．頭部CTでの異常陰影に関して，当初は脳膿瘍を疑いセフトリアキソン4g/日を投与した．また，抗けいれん薬としてフェニトイン250mg/日の投与を開始した．第9病日（2004年1月8日）に頭部MRIを撮影したところ，左前頭葉に周囲がリング状に，一部が結節状に増強される径約1cm強の円形嚢胞性病変を認めた（図1-A, B）．その周囲には浮腫を伴っており，拡散強調画像では病変部の信号は低下していた．脳有鉤嚢虫症（neurocysticercosis）の抗体測定を行ったところ，ウエスタンブロット法〔QualiCode Cysticercosis Kit®（Immunetics, Inc., USA）〕において，血清で陽性，髄液では陰性であった．小腸造影を行ったが腸管内に虫体はみられず，糞便検査も陰性であった．

脳の^{18}F-FDG-PETでは，左前頭葉のリング状病変部位に一致して集積低下を認め，^{11}C-コリン-PETでは，^{18}F-FDG-PETで集積が低下していた病巣内の一部で軽度のコリンの集積を認めた（図2）．第24病日（2004年1月23日）に再検したMRIでは結節の増強効果や周囲の浮腫は縮小しており，脳有鉤嚢虫症の経過として合致する所見であった

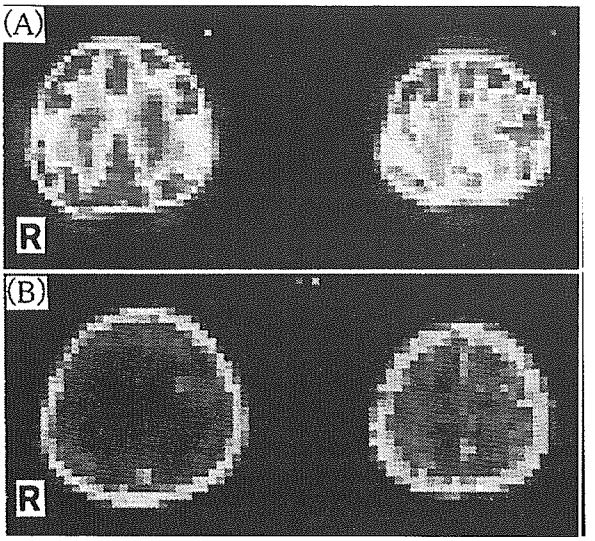

図2 PET images of the brain. (A) ^{18}F-FDG-PET shows a decreased uptake of frontal mass lesion. (B) ^{11}C-choline-PET shows a partial uptake.

(図1-C, D)．単発病変であったため，本疾患のガイドラインに従い駆虫薬を用いずに抗けいれん薬の投与のみを継続した．経過中けいれんを含め神経学的異常はみられず，2004年1月24日に退院した．

考　察

脳有鉤嚢虫症のMRI所見は病変のstageによって変化する．嚢虫の生育している間は増強効果を伴う結節病変を呈し，嚢胞内容液はT1, T2強調画像の両者で髄液と同じ信号強度を示す．虫体の死滅・変性に伴い結節の周囲がリング状増強効果をもち，周囲に浮腫が出現する．変性が進むにつれて嚢胞内容液の蛋白濃度が上昇し，T1強調画像で信号強度が上昇する[1]．本症例では，MRIで周囲が増強される嚢胞の内部に小結節を認め，嚢虫の結節と考えられた．2001年に発表されたBruttoらによる診断基準[2]では，CTやMRIで嚢虫の頭節を認める場合はそれのみで診断が確定するとされている．本症例ではさらに，出身が好発地域の中国であること，血清抗体陽性，自然経過で画像所見が改善したことも診断を裏づけた．しかし，無治療で経過をみるためにはできるかぎりの鑑別をする必要があると考え，とくに脳腫瘍・脳膿瘍との鑑別のためPETを施行した．

^{18}F-FDG-PETでは，脳膿瘍や悪性度の高い脳腫瘍において集積が上昇するが，本症例ではMRIでの腫瘤部位に一致して集積が低下していたことから，これらの疾患は否定的と考えられた．^{11}C-コリン-PETでは，リング状の病巣内の一部で軽度のコリンの集積を認めた．^{11}C-コリンは体内で細胞膜のリン脂質にとり込まれるため，細胞膜合成の速い場所で集積が上昇する．^{11}C-コリン-PETは一部の例外を除き，その集積の程度により腫瘍の悪性度を判定することができるが，低悪性度の腫瘍や膿瘍でも軽度の集積を示す[3]ために，本症例においてそれらを完全に除外することはできなかった．われわれの検索した限り，脳有鉤嚢虫症のPET所見に関してはNagayamaら[4]による^{18}F-FDG-PETの報告があるのみで，本症例と同様に集積低下を認めている．脳有鉤嚢虫症に対し^{11}C-コリン-PETを撮影した例は本症例が初めてであり，^{18}F-FDG-PETで集積低下，^{11}C-コリン-PETでは一部集積という所見の組み合わせは，悪性度の高い脳腫瘍や脳膿瘍ではみられない所見と考えられ鑑別に有用であった．ただし，脳有鉤嚢虫症が常にこのような所見になるか否かはさらなる症例の集積を要する．

2002年のGarciaら[5]の治療ガイドラインによると，本症例のように画像で虫体の死滅が示唆され病変が少数の場合には自然経過で病変が縮小するため，抗けいれん薬などの対症療法のみで画像の経過を追うことが推奨されている．脳有鉤嚢虫症患者のけいれんの再発率は40.3%であり，治療の有無はけいれんの再発には影響を与えないが，頭部CT上嚢胞病変が持続している症例ではけいれん再発率が有意に高いため，急性期変化が消えるまでは抗けいれん薬を継続することが推奨される[6]．本症例も石灰化を確認するまでは抗けいれん薬を継続することとした．

まとめ

けいれんで発症した脳有鉤嚢虫症の21歳中国人女性例を報告した．単発ですでに活動性を失っている病変であることから，ガイドラインに従いけいれんのコントロールのみで経過をみたところ病変は自然に縮小した．無治療で経過をみるためには病初期にリング状腫瘤を呈する疾患

との鑑別が重要である．そのための鑑別の一助として^{18}F-FDG-PETならびに^{11}C-コリン-PETの有用性を報告した．最近の治療ガイドラインに基づいた治療についても言及した．

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<Abstract>

A case of neurocysticercosis diagnosed with positron emission tomography(PET).

by

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We report a 21-year-old Chinese woman of neurocysticercosis starting with convulsive seizure. CT scan disclosed a mass lesion in her left frontal lobe and its margin was ring-enhanced by contrast media. The initial diagnosis was a brain abscess, but a characteristic finding of nodular tip in the enhanced circular lesion by magnetic resonance imaging (MRI) strongly indicated neurocysticercosis. The positive reaction to *Taenia solium* was detected through serological analysis using Western blot techniques. The lesion was surrounded by edema, which implied that the *Taenia solium* had already been dead and degenerated. ^{18}F-FDG-PET showed a decreased uptake lesion in which partial uptake was seen in ^{11}C-choline-PET. These findings with different radioisotope markers were useful for excluding the possibility of malignant tumor or brain abscess.

According to the newly proposed guideline, we observed her without any treatment against cysticercosis except anti-convulsants. Two weeks later, MRI showed reduction of the contrast enhancement of the mass lesion and the surrounding edema, which again confirmed the diagnosis of neurocysticercosis. Anti-convulsive therapy should be continued until the lesion becomes calcified because of the considerable risk of recurrent seizure.

This is the first report of neurocysticercosis with ^{11}C-choline-PET finding. The mild uptake of ^{11}C-choline suggested the synthesized membrane inside the lesion.

*　　*　　*

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分子生物学的手法によるタイ産肺吸虫の種鑑別・同定：メタセルカリアでの検討

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Molecular discrimination between individual metacercariae of *Paragonimus heterotremus* and *P. westermani* occurring in Thailand

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[1]Department of Parasitology and [2]Division of Genetic Resources, NIID and [3]Department of Biology, Faculty of Science, Srinakharinwirot University.

肺吸虫類の同定にあたっては、中間宿主（カニ類）に由来するメタセルカリアだけでなく、成虫についても形態を精査することが必須とされてきた。しかしながら、メタセルカリアを出発材料として成虫を得るには、終宿主動物への感染試験が必要となり、経費と時間を掛けた検討を相応の施設で実施すると云う困難さが伴う。一方で、各種肺吸虫のリボソームDNA（rDNA）やミトコンドリアDNAの配列が、成虫を主たる検索材料に解読・登録されてきた。得られた配列情報は、系統関係の解析だけでなく、種の判定にも適用されている。我々は、メタセルカリアから得た配列情報を利用することで、本邦産肺吸虫が種鑑別・同定できることを明らかにしてきた（Sugiyama *et al.*, 2002, 2004）が、今回はタイ産の肺吸虫を用いて検討を加えた。

タイには少なくとも６種類の肺吸虫が分布するが、人体寄生が証明されているのはヒロクチ肺吸虫 *Paragonimus heterotremus* だけである。我が国を含む東アジアでは、ウェステルマン肺吸虫 *P. westermani* が人体寄生種として重要視されているが、タイのウェステルマン肺吸虫は人には感染しないと考えられている。ヒロクチ肺吸虫とウェステルマン肺吸虫は、タイでは同一種の淡水産カニを第２中間宿主（終宿主への感染源）とする。このため、メタセルカリアでの種鑑別は、それが人体寄生性（ヒロクチ肺吸虫）であるのか、動物寄生性（ウェステルマン肺吸虫）であるのかを判定するためにも重要となる。そこで、これら２種のメタセルカリアからゲノムDNAを調整し、rDNAのためのコンセンサスなプライマー（3SおよびA28）を用いて、ITS2領域のPCR増幅を試みた。その結果、１個のメタセルカリアからでも配列解読に十分量のPCR産物（何れも約520bp）が得られた。その配列を解読したところ、メタセルカリア由来の配列は成虫由来のものと完全に一致すること、従って１個のメタセルカリアでもその種を正確に鑑別・同定できることが明らかとなった。

より簡便で迅速な鑑別方法として、ITS2領域のPCR産物を制限酵素で切断したパターンによる解析（PCR-RFLP）と、種に特異的なプライマーを用いたマルチプレックスPCRについて検討を加えた。PCR-RFLPには制限酵素*Apa*L1を選び、PCR産物を処理した。その結果、ウェステルマン肺吸虫のPCR産物は予想どおり切断されなかったのに対し、ヒロクチ肺吸虫では予想サイズ（約350bpおよび約170bp）に切断された。マルチプレックスPCRでは、種特異的プライマー（PhTF1およびPwTF1）とコンセンサスプライマー（3SおよびA28）とを総て混合してPCRを行った。その結果、共通バンド（約520bp）と同時に、DNAの由来種に一致する種特異バンド（ヒロクチ肺吸虫では約310bp、ウェステルマン肺吸虫では約140bp）が増幅された。PCR-RFLPおよびマルチプレックスPCRでも、両種を正確に鑑別・同定できることが明らかとなった。タイに分布する肺吸虫をメタセルカリアで正確かつ迅速・簡便に種分類することを目指して、他の４種の肺吸虫についても材料を集めて検討を進めている。

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Key Words: *Paragonimus*, species discrimination, Thailand

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MOLECULAR DISCRIMINATION BETWEEN INDIVIDUAL METACERCARIAE OF *PARAGONIMUS HETEROTREMUS* AND *P. WESTERMANI* OCCURRING IN THAILAND

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Abstract. To accurately discriminate between individual metacercariae of *Paragonimus heterotremus* and *P. westermani* occurring in Thailand, polymerase chain reaction (PCR)-based molecular methods were established and subjected to an evaluation. We first amplified and sequenced the second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA of the two species. Based on their nucleotide differences, *P. heterotremus* and *P. westermani* were unequivocally discriminated from each other. These nucleotide differences were further utilized to select the *Apa*L1 endonuclease site for PCR-restriction fragment length polymorphism (PCR-RFLP) analyses and to design species-specific primers for multiplex PCR reactions. Both PCR-RFLP and multiplex PCR methods allowed a more rapid and labor-effective species discrimination. Furthermore, the multiplex PCR method enabled the most efficient discrimination because species identification involved a single round of PCR in a single tube. In Thailand, *P. heterotremus* is the only species affecting humans. Thus, the methods established in the present study can be used as reliable tools to identify the lung fluke metacercariae that cause human disease.

INTRODUCTION

Six lung fluke species have been documented to date in Thailand: *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai* (Srisont *et al*, 1997; Blair *et al*, 1999). Identification of the metacercariae of these lung flukes to the species level requires careful morphological observation of not only the fresh metacercariae, but also the adult flukes from experimentally infected animals (Miyazaki, 1991). This process, however, can be laborious and time-consuming.

We recently reported the successful development of molecular methods for the unequivocal species discrimination between individual metacercariae of the Japanese lung flukes, *P. westermani* and *P. miyazakii*, which cause human infection (Sugiyama *et al*, 2002, 2004). Previously established methods included the direct cycle sequencing of polymerase chain reaction (PCR) products, PCR-restriction fragment length polymorphism (PCR-RFLP), and direct PCR-amplification using consensus and/or species-specific primers. All of these methods utilize nucleotide differences in the second internal transcribed spacer (ITS2) of the nuclear ribosomal DNA (rDNA) for dicrimination between the two species. In the present study, we focused on the lung flukes occurring in Thailand and applied the methods for species discrimination between individual metacercariae of *P. heterotremus* and *P. westermani*.

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MATERIALS AND METHODS

Parasite material and DNA isolation

The metacercariae of *P. heterotremus* and *P. westermani* (Figs 1 and 2) were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification, restriction digestion and sequencing

The rDNA region spanning the ITS2 from individual metacercariae of the two species was amplified by PCR using the primers, 3S (forward, 5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and A28 (reverse, 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'). These primers were designed on the basis of the conserved rDNA sequences of the *Schistosoma* species (Bowles *et al*, 1995) and were previously used as consensus primers for discriminating between the Japanese species of

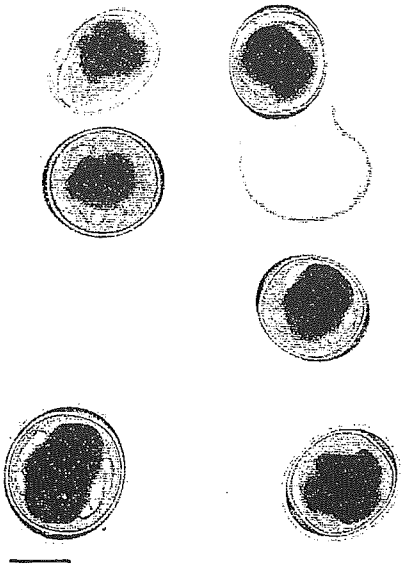

Fig 1- Photomicrograph of fresh *P. heterotremus* metacercariae. The metacercariae were encysted with a wall and exhibited a suboval shape. The thickness of the wall on the side (9.5 μm on average) gradually increased at both poles (21.6 μm on average). The longitudinal and transverse diameters of the cyst averaged 302 by 232 μm, respectively. Bar 150 μm.

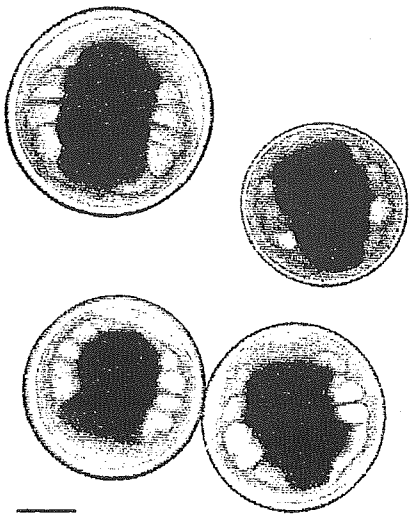

Fig 2- Photomicrograph of fresh *P. westermani* metacercariae. The metacercariae were encysted with a wall and exhibited a spherical shape. The wall thickness averaged 14.6 μm. The diameter of the cyst ranged from 410 to 570 μm with an average of 475 μm. Bar 150 μm.

Paragonimus (Sugiyama *et al*, 2002, 2004). In the present study, 0.5 μM of each primer, 2.5 units of DNA polymerase (TaKaRa Z-Taq, Takara Shuzo, Japan) and 10 ng of the DNA samples were added to each PCR reaction (final reaction volume, 100 μl). The resultant PCR products (10 μl) were restricted with five units of the *Apa*LI endonuclease (New England Biolabs, USA) at 37°C for 12 hours, then separated by electrophoresis through 2% (w/v) agarose gels. The intact PCR products were also electrophoresed and excised from the gels and sequenced using the corresponding primer and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems). Sequence alignment and comparison were completed using the GENETYX-WIN (version 5.0, Software Development, Japan) program.

Design of specific primers and amplification by (multiplex) PCR

To establish a more direct PCR procedure for species discrimination, we designed species-specific forward primers; PhTF1 for *P. heterotremus* (5'-TTCCCCAACGTGGCCTTGTGT-3', nucleotide positions 184 to 204 for the ITS2 region) and PwTF1 for *P. westermani* (5'-GTTCATGTTGCGCGTGGT CTGCGTTC-3', nucleotide positions 351 to 376) (Fig 3). The species-specific primer(s) as well as the consensus primer(s) were incorporated into single tubes. The multiplex PCR amplification was performed under the conditions described above using 0.5 μM of the consensus primers and 0.1 μM of the species-specific primers.

RESULTS

The ITS2-PCR products were amplified from DNA samples of individual *P. heterotremus*- and *P. westermani*-metacercariae using the consensus primers, 3S and A28. Agarose gel electrophoresis showed that the generated products were about 520 bp in size for both species. Sequence analysis of the products revealed that the aligned ITS2 region of *P. heterotremus* and *P. westermani* was 463 bp in length. A pairwise comparison of the sequences showed 36 (7.8%) nucleotide differences consisting of two deletions/insertions and 34 substitutions (Fig 3). Similarity searches of the nucleotide databases revealed that the ITS2 and flanking regions were identical to those deposited in the GenBank/EMBL/DDBJ nucleotide databases, accession numbers AF159603 for *P. heterotremus* and AF159604 for *P. westermani*.

The *Apa*LI endonuclease was selected for species discrimination by PCR-RFLP based on the theoretical

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Ph 001:TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA 060
Pw 001:.....C..... 060

Ph 061:TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw 061:..... 120

Ph 121:TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw 121:.....C..... 180

          PhTF1-->
Ph 181:GATTTCCCCAACGTGGCCTTGTGTCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAA 240
Pw 181:...C.......TC...T......C.....A.....T 240

Ph 241:ATCCGGGCGTATCCATGTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTG 300
Pw 241:.CT..C...C.C...C.....C.....A..... 300

                    ApaLI
Ph 301:GCTCAGTGAATGATTTATGTGCACGTTCCGCTGTCCCGTCATCATCTATGGTTGAAGTTG 360
Pw 301:.......A.....G....T........T...T.....G...C.T.... 360
                                                PwTF1-->

Ph 361:CGCGTGGTGTG--TCCGATGCTGACCTATATATGTGCCATGTGGCTCATTTTCCTGACCT 418
Pw 361:........C..CG.T.....CG.....TC....C.T..... 420

Ph 419:CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA 461
Pw 421:.....T..... 463
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Fig 3- Aligned nucleotide sequences of the ITS2 region from *P. heterotremus* (Ph) and *P. westermani* (Pw) metacercariae. A hyphen indicates an alignment gap. A dot in the *P. westermani* sequence indicates a nucleotide identical to that in *P. heterotremus*. The 5' and 3' ends of the sequences include the 5.8S rDNA and 28S rDNA, respectively. The recognition site of the *Apa*LI endonuclease (G/TGCAC) is boxed. The locations of the *P. heterotremus*-specific forward primer (PhTF1; 5'-TTCCCCAACGTGGCCTTGTGT-3') and *P. westermani*-specific forward primer (PwTF1; 5'-GTTCATGTTGCGCGTGGTCTGCGTTC-3') are underlined. Numbers refer to the actual length of the nucleotide sequences.

restriction maps generated from the ITS2 sequences of the two species (Fig 3). When the enzyme was applied, expected results were obtained. The PCR product of *P. heterotremus* (520 bp) was cleaved, thereby producing two fragments with sizes about 350 and 170 bp (Fig 4). In contrast, the PCR product of *P. westermani* remained unrestricted (Fig 4).

For species discrimination by direct PCR, the *P. heterotremus*-specific and *P. westermani*-specific primers (PhTF1 and PwTF1, respectively) were designed on the basis of the nucleotide differences in the ITS2 region. The specificity of these primers was evaluated as to whether they could amplify different sized species-specific fragments only from the respective DNA samples in combination with the primer A28. As expected, the PhTF1-A28 primer set amplified a PCR product of about 310 bp from *P. heterotremus* DNAs, but not from *P. westermani* DNAs. In contrast, PwTF1-A28 amplified a product of about 140 bp from *P. westermani* DNAs, but not from *P. heterotremus* DNAs (figure not shown).

Having demonstrated the species-specificity of the PhTF1 and PwTF1 primers, both were incorporated

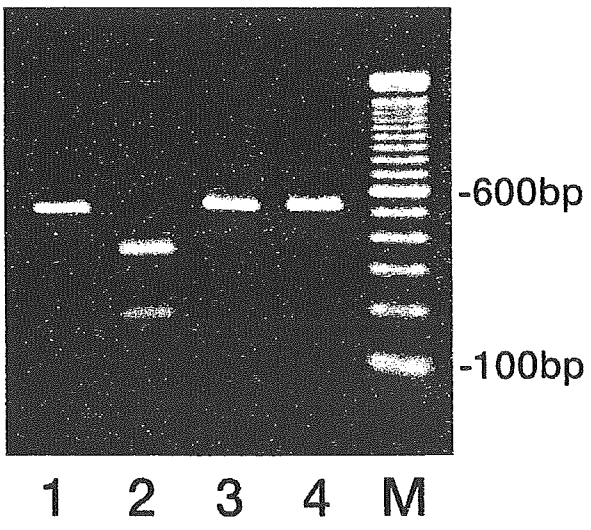

Fig 4- PCR amplification products of the ITS2 region from *P. heterotremus* (lanes 1 and 2) and *P. westermani* (lanes 3 and 4) metacercarial DNAs. The ITS2-PCR products were then restricted with the *Apa*LI endonuclease (lanes 2 and 4). The 100-bp DNA ladder marker was used to estimate the size of the fragments (lane M).

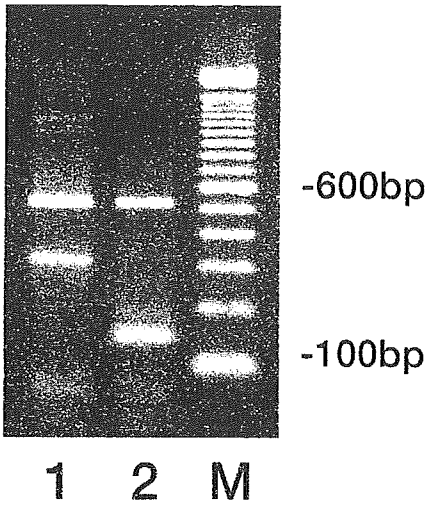

Fig 5- Results of multiplex PCR amplifications from *P. heterotremus* (lane 1) or *P. westermani* (lane 2) metacercarial DNAs. For amplification, two species-specific primers, PhTF1 and PwTF1, were incorporated into single tubes containing the two consensus primers, 3S and A28. A 100-bp DNA ladder was used to estimate the size of the fragments (lane M).

into single tubes with the two consensus primers, 3S and A28, and then a multiplex PCR amplification was carried out. As a result, two PCR products of about 520 bp and 310 bp were amplified from the *P. heterotremus* DNAs and products of about 520 bp and 140 bp were amplified from the *P. westermani* DNAs (Fig 5). The amplified PCR products were sequenced, which confirmed that they corresponded to the ITS2 region of the rDNA from their respective species.

DISCUSSION

The phylogenetic relationships of the *Paragonimus* species occurring in Thailand have been studied using genetic markers in the ITS2 region of rDNA (Blair *et al*, 1998; Iwagami *et al*, 2000). In these studies, the ITS2 sequences were generated from DNA samples prepared from adult worms using the consensus PCR primers, 3S and A28. Using these primers, we demonstrated that the ITS2 sequences were generated from the individual metacercariae of *P. heterotremus* and *P. westermani*. By pairwise comparison of the generated products, the two species were unequivocally discriminated from each other. These nucleotide differences were utilized to select the *Apa*LI endonuclease for the PCR-RFLP analyses and to design species-specific primers for the multiplex PCR reactions, both of which allowed the more rapid and labor-effective discrimination between *P. heterotremus* and *P. westermani* at the metacercarial stage.

In Thailand, the metacercariae of *P. heterotremus* and *P. westermani* have been detected in the same crab species (Kawashima *et al*, 1989; Blair *et al*, 1998) but only the former is known to affect humans (Srisont *et al*, 1997; Blair *et al*, 1998). Thus, the discrimination methods established in the present study can be used as reliable tools to identify the lung fluke metacercariae that cause human disease.

Of the molecular methods examined, we confirmed that the multiplex PCR method allowed the most efficient discrimination because only a single-round PCR was required. By this method, species-specific products of different sizes were produced from the *P. heterotremus* (310 bp) and *P. westermani* (140 bp) DNA samples. At the same time, 520 bp-products were also generated from the DNA samples of both species, which could serve as sets of internal controls to confirm the integrity of the PCR reactions and might function as diagnostic reagents for the (genus and) species identification(s) (Sugiyama *et al*, 2004). In fact, we found that the 520-bp product alone was generated from DNA samples of *P. harinasutai* metacercariae whose crab host is identical to that of *P. heterotremus* and *P. westermani* (data not shown). Further studies are in progress to evaluate the usefulness of the established multiplex PCR method for species discrimination and/or identification using DNA samples from the remaining three species of lung flukes occurring in Thailand (*P. siamensis*, *P. bangkokensis* and *P. macrorchis*).

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APPLICATION OF MULTIPLEX PCR FOR SPECIES DISCRIMINATION USING INDIVIDUAL METACERCARIAE OF *PARAGONIMUS* OCCURRING IN THAILAND

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SHORT TITLE: Molecular discrimination of Thai *Paragonimus* species

ABSTRACT. The number of lung fluke species documented in Thailand totals six. Of them, *P. heterotremus* is the most important, as it affects humans. Although *P. westermani* is found as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed. In order to accurately discriminate between individual metacercariae of these two species, we established a multiplex PCR method. Through this method, two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). In contrast, 520-bp products alone were found to be generated from the DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, three other species of lung flukes known to occur in Thailand. Digestion of these 520-bp products with the restriction enzyme *Scr*FI could unequivocally discriminate species by the number and size of the produced band(s): three bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, two bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for

P. siamensis. The established multiplex PCR used in combination with restriction enzyme digestion (PCR-RFLP with *Scr*FI) is effective for discriminating the five different species of the lung flukes occurring in Thailand, even at the metacercarial stage.

INTRODUCTION

The number of lung fluke species documented in Thailand totals six (Blair *et al*, 1999; Srisont *et al*, 1997): *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai*. Of them, *P. heterotremus* is the most important, as it affects humans. Although *P. westermani* occurs as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed (Blair *et al*, 1998). In order to accurately discriminate between individual metacercariae of these two species, we established a multiplex PCR method (Sugiyama *et al*, 2005). In this study, we further evaluated the usefulness of the previously established multiplex PCR for species-level discrimination among *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, three other species of lung flukes known to occur in Thailand.

MATERIALS AND METHODS

Parasite samples and DNA isolation

The metacercariae of *P. siamensis* (Fig. 1) were harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al*, 1997). The metacercariae of *P. bangkokensis* (Fig. 2) were harvested from the freshwater crab, *Ranguna smalleyi*, captured in a mountain stream in Surat Thani Province, Thailand (Rangsiruji *et al*, in press). The metacercariae of *P. heterotremus*, *P. westermani* (strain Thailand) and *P. harinasutai* (Fig. 3) were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification (Sugiyama *et al*, 2005), the *P.*

heterotremus-specific forward primer (PhTF1; 5'-TTCCCCAACGTGGCCTTGTGT-3', alignment positions 184 to 204 for the *P. heterotremus* second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA (rDNA)) and a newly designed *P. westermani*-specific forward primer (PwTF3; 5'-GTCTGCGTTCGATGCTGACCTACG-3', alignment positions 367 to 390 for the *P. westermani* ITS2 region) were used in combination with an interspecies-conserved primer pair, 3S (forward, 5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and A28 (reverse, 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3') (Bowles *et al*, 1995). These primers were all incorporated into a single-tube reaction. The multiplex PCR amplification was performed as previously described (Sugiyama *et al*, 2004) using 0.1 μM of PhTF1 and PwTF3 primers, 0.5 μM of 3S and A28 primers, 2.5 units of the Taq polymerase (Invitrogen, USA) and 10 ng of the DNA template. The resultant PCR products were separated by electrophoresis on 2% (w/v) agarose gels.

The amplified products were extracted from agarose gels and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems). The sequence alignment and comparison were completed using the GENETYX-WIN (ver. 7.0, Software Development Co., Japan) program.

Restriction enzyme digestion of the multiplex PCR products (PCR-linked restriction fragment length polymorphism (PCR-RFLP))

The amplified products (4 μl) were also treated with five units of the restriction enzyme *Hinc*II, *Stu*I or *Scr*FI (New England Biolabs, USA) at 37°C for 12 h. The treated samples were then separated by electrophoresis on 3% (w/v) agarose gels.

RESULTS

Based on the established multiplex PCR method (Sugiyama *et al*, 2005), we confirmed that two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp) (Fig. 4). On the other hand, 520-bp products alone were generated from the metacercarial DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig. 4). Sequence analysis of the amplified products revealed that the aligned ITS2 region was 463 bp in length in each of the latter three species (Fig. 5).