抗原検出法は現在の感染を証明する方法である．
抗体検査によるフォローアップは予後や治癒判定に使える．
抗体検査用の抗原は，特異性や品質の向上，あるいは安定的供給のために，遺伝子組換え抗原も開発されている．

ある．本法の最大の利点は手技の簡便さと検査時間の迅速性(数分から10数分)にある．イムノクロマトキットは世界的に見ると，マラリア診断用キットが最も多く開発，市販されており，国内でもNow® Malaria(Binax社)やOptiMAL-IT(DiaMed社)が入手しやすい．トキソカラ症キットも含めたこれらのイムノクロマトキットは体外診断薬としては国内未承認であるが，総合的に診断するうえでの判断材料としては利用することができる．

4. 遺伝子検査

寄生虫の遺伝子検査は，原因となった寄生虫種を同定して確定診断する目的，あるいは病原性や薬剤耐性との関連性を調べるなどの目的で重要な検査法である．基本的にはすべての寄生虫に適用できる．遺伝子検査の対象となる検体は，糞便内の寄生虫卵や原虫の嚢子やオーシスト，自然排出，あるいは駆虫された虫体，内視鏡的，あるいは外科的に摘出された虫体や病巣，ホルマリン固定病理組織標本や血液塗抹標本などさまざまである．遺伝子検査法の基本原理は標的遺伝子をpolymerase chain reaction(PCR)によって増幅し，増幅産物の塩基配列を直接解析する方法，増幅産物の制限酵素切断パターン解析(Restriction Fragment Length Polymorphism：RFLP)，鑑別すべき寄生虫種に特異的な複数のプライマーを混ぜて1本のチューブ内でPCRを行うmultiplex PCR[3]，あるいは鎖置換反応を利用して一定温度(60～65℃)でPCRを行うloop-mediated isothermal amplification(LAMP)[4]などがある．標的遺伝子はミトコンドリアゲノムでコードされるcytochrome *c* oxidase subunit 1遺伝子(以下，*cox1*)や核DNAのリボソームRNA遺伝子のinternal transcribed spacer(ITS-1, ITS-2)領域が用いられることが多い．図4は互いに形態が酷似し，駆虫薬による治療にあたっては事前に鑑別が必要とされる無鉤条虫と有鉤条虫のmultiplex PCRによる鑑別例を示した．このmultiplex PCRでは，無鉤条虫の場合は827 bp，有鉤条虫の場合は984 bpの*cox1*遺伝子が増幅されるようにプライマーが設計されているので，増幅産物の大きさで両者を正確に鑑別することができる[3]．また，全長1,620 bpの*cox1*遺伝子の723番目の塩基は種間で高度に保存されているので，アデニンであれば無鉤条虫，シトシンであれば有鉤条虫と同定できる[3]．

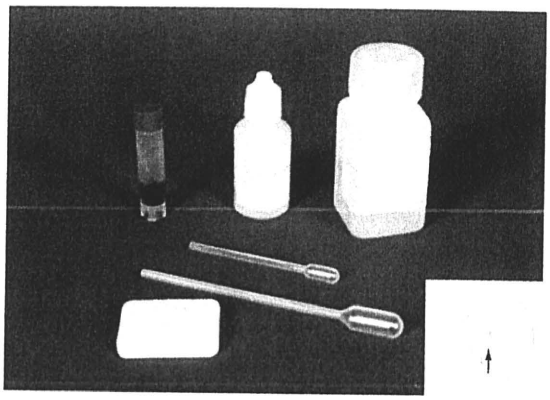

図3 イムノクロマト法によるトキソカラ症検査キット
キットに含まれるデバイスと試薬．陽性であれば，小孔中央部に赤色スポット(矢印)が出現する．
(東京医科歯科大学大学院国際環境寄生虫学分野　赤尾信明准教授提供)

遺伝子検査は，ホルマリン浸漬標本や病理組織切片内に検出される寄生虫の同定にも適用できるので，遺伝子検査の利用価値は高い．条虫類が中心ではあるが，遺伝子検査に関する総説があるので，詳細はそれを参照願いたい[5]．病理組織切片内に見出される寄生虫断端は，虫体の変性や石灰化，あるいは切片作製などによって寄生虫に特徴的な形態がいつも観察できるとは限らないので，そのような場合に遺伝子検査が威力を発揮する．

遺伝子増幅を中心とする遺伝子検査は高感度であり，寄生虫の虫卵1個からでもDNAは検出可能である．

遺伝子検査は染色標本(HE，PAS，酢酸カーミン，ギムザなど)から抽出したDNAで検査可能である．

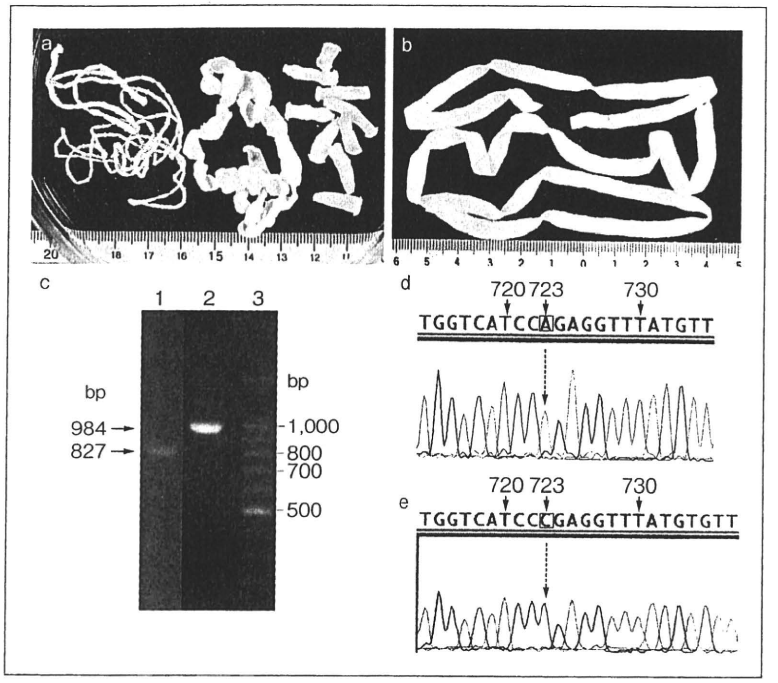

図4　形態的に類似した寄生虫の遺伝子鑑別

駆虫された無鉤条虫(a)と有鉤条虫の成虫(b)．c　multiplex PCRによって増幅された*cox1*遺伝子断片．無鉤条虫は827 bp(lane 1)，有鉤条虫は984 bp(lane 2)．また，塩基配列解析によって*cox1*遺伝子の723番目の塩基がアデニンであれば無鉤条虫(d)，シトシンであれば有鉤条虫(e)と同定できる．

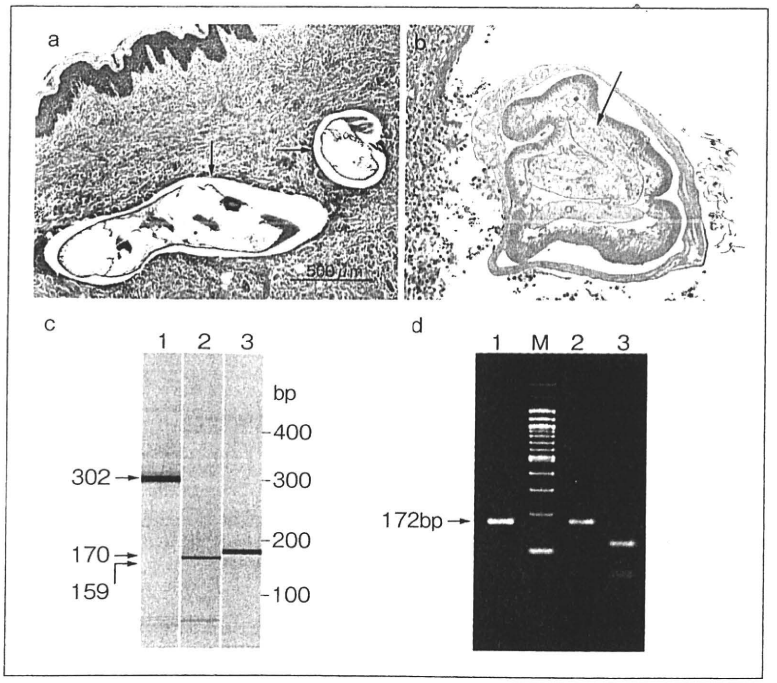

図5　ホルマリン固定された病理組織標本内に検出された寄生虫の分子同定

a　皮下組織内に見出された顎口虫の幼虫(矢印)．b　小腸組織内に検出されたアニサキス幼虫(矢印)．c　aの顎口虫由来DNAを用いてPCR増幅された*cox1*遺伝子断片(lanes 1～3)．d　bのアニサキス由来DNAを用いてPCR増幅されたITS-1の制限酵素*Hinf* I切断パターン(lane 1)．lane 2と3はそれぞれ比較に用いた*Anisakis simplex*と*Anisakis pegreffii*.

(文献6)，7)を改変引用)

◎遺伝子検査は日常検査のみならず，アーカイブ標本を用いた retrospective な研究にも使える．

図5はパラフィン包埋切片内の虫体部から抽出したDNAを用いて感染研で検査を行った例である．ホルマリン固定標本ではDNAが分解され，300bp以下の短いDNA断片しか増幅されないことが多いが[5]，増幅されたPCR産物の塩基配列解析から，aの虫体は中南米に分布する二核顎口虫(*Gnathostoma binucleatum*)[6]，bの虫体はアニサキスI型幼虫(*Anisakis simplex*)と同定された例である[7]．ホルマリン固定パラフィン切片標本を用いて遺伝子検査を行う場合には，ホルマリンによるDNA分解を考慮して，増幅産物の大きさが300bp以下になるようにプライマーを設計することが肝要であり，初回のPCRで増幅したDNAを鋳型として2回目のPCRを行うnested PCRが有効な場合が多い．

5. 検査依頼はどのように行うか

臨床家が寄生虫感染の疑いのある患者に遭遇した場合，どのような手順で寄生虫検査を依頼すればいいのか．抗体検出を目的とした血清検査を依頼する場合には，数mlの血清が必要である．また，肺吸虫症では胸水，眼部トキソカラ症では硝子体液や前房水，脳有鉤嚢虫症や広東住血線虫症の場合には髄液が用いられることもある．最近，マラリアや一部の寄生虫症で尿を用いた抗体検査が試験的に行われている．遺伝子検査依頼の場合，検体は70～80%エタノール固定，あるいは凍結保存が推奨される．ホルマリン固定標本も遺伝子検査は可能であるが，前述のとおり，ホルマリンはDNAを分解し，遺伝子増幅を困難にするので，ホルマリン固定は病理検査以外では極力避けていただきたい．抗体，遺伝子検査とも検体はクール宅配便で送付可能．検査依頼の方式は検査研究機関で異なるが，参考までに感染研の例を示す．事前に電話(03-5285-1111，ext. 2200)もしくは電子メールによって連絡を受けると，感染研様式の検査依頼書が依頼者に送付(メール添付)されるので，それに必要事項を記入のうえ，その検査依頼書とともに，検体，病歴経過(各種検査所見，画像所見，病巣部の写真など)を感染研に送付いただいている．検査に要する日数は，血清検査で通常1～2日，遺伝子検査は1週間程度．検査費用は無料．寄生虫検査は感染研(http://www.nih.go.jp/)以外でも，大学医学部寄生虫学関連講座(医動物学・熱帯医学・感染症学を含む)や一部の地方自治体衛生研究所，あるいは民間の検査会社などで実施可能である．

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2009年に経験した日本海裂頭条虫症の5例

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Key Words: 食物媒介性寄生虫症，プラジカンテル，小児

はじめに

日本海裂頭条虫症の診断や治療に関する相談は毎年数例ある．平成21年度には7件の相談があった．そのうち，遠方他県からの相談事例を除き，5例（成人男性3例，女児2例）について診断・治療に関与し，自宅駆虫や小児を含む家族例を経験したので報告する．

症例

症例1:67歳男性,維持人工透析を行っている．3月末デパートの北海道フェアーにて「サケ」を購入し，妻・息子と食した．5月初め軽度腹痛の後，約50cmの虫体排泄あり，持参して来院．3カ月にわたり数回の便虫卵検査を行ったが陰性，家族の便虫卵検査も陰性であった．

症例2：67歳男性，会社CEOにて渡航時々，

Five Cases of Diphyllobothliasis by *Diphyllobothrium Nihonkaiense* Encountered in 2009

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国内外問わず週一回は鮨店で食事をする．2 月に約 5cm の虫体排泄．5 月 14 日健診目的の大腸内視鏡前処置の洗腸時に約 30cm の虫体排泄あり，31 日排便時にも 3 度目の虫体排泄を認め，6 月 1 日に来院．自宅での駆虫を希望されたため，駆虫の説明書きを手渡し，自宅で駆虫を行った．駆虫法は，前日の夕食は低残渣食とし就寝前にクエン酸マグネシウム・センノシドを服用，翌朝は絶食としてプラジカンテル（PZQ）600mg を内服，その 2 時間後に硫酸マグネシウム 30g と水 300ml を服用したのちは，便意を感じてもできるだけ我慢していただき限界点で一気に排泄するように指導した．駆虫は成功し，全長約 2.4m の虫体排泄を認め頭節も確認した．以後虫体・虫卵排泄は認めていない．

症例 3：47 歳男性，7 日前に排泄した約 50cm の虫体を泡盛に保存し，11 月 30 日に来院．虫体の保存状態は良好であった．翌年 2 月まで数回便虫卵検査を行うも陰性であり，以後虫体排泄も認めていない．

症例 4：6 歳女児．父が鮨職人で鮨店を営む．3 人姉妹の末っ子．H20 年秋に一度虫体排泄を認めたが放置．H21 年 11 月再び虫体排泄があり近医を受診．医師とともに約 3.5m の虫体を引き出した．ホルマリン保存虫体を持参して 11 月 12 日に来院．下剤による前処置ののち，PZQ（15mg/kg）の内服にて駆虫を試みたが，虫体排泄は認めなかった．その後も，虫卵・虫体の排泄は認めていない．

症例 5：11 歳女児，症例 4 の姉（3 人姉妹の長女）．H21 年春，排便時に紐状物の排泄があったが放置，末妹の治療を機に来院した．妹と同様の方法で駆虫を行ったところ，約 4.5m の虫体排泄を認めた．頭節は確認できなかったが，以後虫卵・虫体の排泄は認めていない．症例 4・5 は家族例であり父親にも虫体排泄歴があった．一方，生魚摂取を好まない母親と次女には虫体排泄のエピソードは無く，便虫卵も陰性であった．

5 例をまとめて表 1 に示す．全例，虫体の排泄を契機に受診している．受診時およびその後数ヶ

表 1　日本海裂頭条虫症 5 例のまとめ

症例No	年齢・性別	症状（排便時排虫）	便虫卵	駆虫治療（PZQ）			特記事項
			初診 → 2ヶ月後	施行有無	排虫	便虫卵（1ヶ月後）	
1	67歳、男性	(+)	(-) (-) (-) (-) (-)	施行せず			北海道フェアーで「サケ」購入
2	67歳、男性	(+)	行わず	施行	有	(-)	鮨好き 自宅駆虫
3	47歳、男性	(+)	(-) (-) (-) (-) (-)	施行せず			虫体を泡盛にて保存
4*	6歳、女児	(+)	(+)	施行	無	(-)	実家が鮨屋 鮨好き
5*	11歳、女児	(+)	(+)	施行	有	(−)	実家が鮨屋 鮨好き

* 症例4,5は3人姉妹の長姉と末妹。
父親は鮨職人で鮨店を営む。父親も虫体排泄歴あり、便虫卵も陽性であった。
母親および次女は、生魚摂取を好まず、虫体排泄のエピソードも無く、便虫卵も陰性であった。

月の経過観察期間に虫体や虫卵排泄を認めなかった症例は寄生無しと判断した．PZQによる駆虫を行った3症例では，2例で排虫があり，1例で頭節を確認した．排虫の得られなかった症例および頭節の確認できなかった症例でも，以後の虫体および虫卵排泄は認めなかった．なお，5例とも虫体試料を用いたミトコンドリアチトクロームCオキシデースサブユニット1（cox1）遺伝子の塩基配列から *Diphyllobothrium nihonkaiense* と診断した．

考察

日本海裂頭条虫症は生息地域や感染経路が広節裂頭条虫症と異なり，顕微鏡的構造差異，さらに近年ではcox 1遺伝子などの塩基配列解析により，それらの鑑別がなされている[1-3]．感染源は主としてサケ・マス類，とくにサクラマスが知られていたが近年では，4〜7月に流通するトキシラズ（シロザケ）にも日本海裂頭条虫症のプレロセルコイドが高率に寄生していることも明らかにされた[3, 4]．治療は，低残渣食と下剤を使用した前処置後にPZQを投与し，さらにその後に再度下剤の服用により虫体排泄を得る方法が現在は一般に用いられ[5]，特に十二指腸ゾンデ挿入や被爆侵襲を避けたい小児では，ガストログラフィンによる駆虫より優れている．日本海裂頭条虫症は生魚の摂食習慣のある本邦では小児例もめずらしくはなく[5-9]，家族例も散見される[4]．日本海裂頭条虫症が疑われた際には，生活習慣を含む食習慣に対する詳細な問診が必要であり，時に同じ習慣を持つ家族に対する検査も必要であると考える．

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Molecular identification of *Anisakis* type I larvae isolated from hairtail fish off the coasts of Taiwan and Japan

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ABSTRACT

Anisakid nematodes are known to cause the zoonotic disease, anisakiasis, through the consumption of raw or undercooked fish. The parasites most frequently associated with the disease in humans are categorized as *Anisakis* type I, which comprise several species of the genus *Anisakis*. The larvae show primitive forms and lack the detailed morphological characteristics required for precise species identification. Thus, molecular characterization is necessary for determining the species of *Anisakis* type I larvae and acquiring important clinical and epidemiological information. In this study, we isolated *Anisakis* type I larvae from hairtail fish caught off the coasts of Taiwan and Japan. The ribosomal DNA (rDNA) internal transcribed spacer (ITS) region was sequenced, and restriction fragment length polymorphism (RFLP) analyses using *Hinf*I and *Hha*I was carried out for species identification. Most larvae isolated from hairtail caught in Taiwan were *Anisakis typica* (84%), while those isolated from hairtail caught in Japan were almost exclusively identified either as *Anisakis simplex* sensu stricto (65%) or *Anisakis pegreffii* (33%). This is the first report of *A. typica* in fish obtained from Taiwan. Our results shed the light on the epidemiology of *Anisakis* type I larvae, which is a potential cause of human anisakiasis in Taiwan and Japan.

1. Introduction

Larvae of anisakid nematodes are commonly found in marine fish and squid. Many cases of human anisakiasis caused by larvae belonging to the family Anisakidae have been reported in Japan and other countries due to the increasing popularity of eating raw fish (Lymbery and Cheah, 2007). Berland (1961) classified the larvae of anisakid nematodes into only two types, namely *Anisakis* types I and II, based on morphological characteristics such as the length of the ventriculus and presence/absence of mucron at the tip of the tail. The type I larvae were recognized as the parasite most frequently associated with human anisakiasis (Oshima, 1972; Smith and Wootten, 1978). Controversy regarding the species names of *Anisakis* type I and II larvae still remains because the larvae lack the detailed morphological characteristics required for precise species identification. The use of allozyme markers made it possible for the type I morphotypes to be identified as *Anisakis pegreffii*, *Anisakis simplex* sensu stricto, *Anisakis simplex* C, *Anisakis typica*, and *Anisakis ziphidarum* (Mattiucci et al., 1997, 2002; Paggi et al., 1998). The former three species have been recognized as sibling species belonging to *Anisakis simplex* sensu lato. The species comprising *Anisakis* types I and type II have also been characterized by sequencing and restriction fragment length polymorphism (RFLP) analyses of the ribosomal DNA (rDNA) internal transcribed spacer region (ITS region), namely the 5.8S rDNA and flanking ITS regions, ITS1 and ITS2 (Mattiucci and Nascetti, 2006, 2008). The classifications based on allozyme markers were fully consistent with the results obtained by these molecular methods.

It is imperative to identify *Anisakis* type I larvae detected in food fish in order to improve food safety. Hairtail is one of the most common and economically important food fish in the East and South China Seas. In Japan, Taiwan, and Korea, hairtail is often sold in markets and is favored for raw consumption as sashimi or sushi. However, few studies have investigated the molecular identification of anisakid larvae isolated from hairtail (Shih, 2004). Therefore, in this study, we detected *Anisakis* type I larvae in hairtail collected from Taiwan and Japan and applied these molecular methods for species identification.

2. Materials and methods

2.1. Parasite materials

Seven hairtail (*Trichiurus* spp.) specimens caught off the coast of each of the following 5 localities were examined for anisakid larvae:

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Taichung, Taiwan, and Nagasaki, Kochi, Wakayama and Shizuoka prefectures, Japan. We purchased the fish at retail fish markets in the respective localities, except for fish from Nagasaki, which were purchased from a retail fish market in Tokyo. Fish were examined immediately after transfer to our laboratory in Taichung (samples from Taiwan) or in Tokyo (samples from Japan).

Anisakid larvae were isolated from the visceral surface and body cavity of the fish. The larvae were observed under light and dissection microscopes for morphological identification (Ishii et al., 1989), and third-stage larvae of *Anisakis* type I were used for further molecular investigation.

2.2. DNA amplification

DNA samples were extracted from individual worms using a DNeasy Blood and Tissue Kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's instructions. The entire ITS region (ITS1, 5.8S rDNA and ITS2) was amplified by PCR using primers NC5 (forward; 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′) (Abe et al., 2005). PCR was conducted using a mixed solution (5 μl) of extracted DNA as a template, with a reaction mixture (45 μl) containing 1.25 units of *TaKaRa EX Taq*, 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP (Takara Bio Inc., Shiga, Japan) and 0.5 μM of each primer. PCR was performed using a LittleGene PCR machine (Bioer, Hangzhou, China) with 35 cycles as follows: denaturation at 98 °C for 5 s, annealing at 52 °C for 30 s, and extension at 72 °C for 60 s. A final extension was carried out at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.0% Seakem GTG agarose gels (Lonza Rockland, Inc., Rockland, ME, USA) in Tris-borate-EDTA buffer and visualized by illumination with short-wave ultraviolet light after ethidium bromide staining.

2.3. RFLP analysis

Restriction enzymes *Hin*fI and *Hha*I (New England Biolabs, Ipswithch, MA, USA) were used in the RFLP analysis for identifying the species of *Anisakis* type I, according to the genetic key of D'Amelio et al. (2000). The PCR products were digested according to the manufacturer's recommendations. The digested samples were then separated by electrophoresis on 2.0% Seakem GTG agarose gels (Lonza Rockland, Inc.) and visualized as previously described.

2.4. Sequencing

The PCR products were excised from the agarose gels and sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) on an automated sequencer (ABI3100, Applied Biosystems). Sequence similarities were determined by a BLAST search of the DNA Data Bank of Japan (DDBJ) (http://blast.ddbj.nig.ac.jp/top-j.html). Sequence alignment and comparison was facilitated by the GENETYX-WIN program (ver.7.0, Software Development Co, Tokyo, Japan).

3. Results

3.1. Detection of Anisakis type I larvae from Taiwan and Japan

Six of the 7 hairtails from Taiwan and 15 of the 28 hairtails from Japan were positive for anisakid nematodes (Table 1), and 110 and 61 larvae were identified as *Anisakis* type I from these locations, respectively. The identification was based on the presence of a long ventriculus with an oblique ventricular-intestinal junction and a rounded tail possessing a mucron.

Table 1
Molecular identification of the *Anisakis* type I larvae.

Locality	Date of collection	No. of fish	No. of parasites			
		Infected/examined	Collected	Identified as		
				At[a]	Ap[b]	As[c]
Taiwan (Taichung)	14 Oct 2008	6/7	110	93	15	2
Japan (Nagasaki)	3 Dec 2008	5/7	20	0	20	0
Japan (Kochi)	13 Jul 2010	1/7	2	0	0	2
Japan (Wakayama)	18 Jul 2010	2/7	6	0	0	6
Japan (Shizuoka)	12 Jul 2010	7/7	33	1	0	32

[a] *A. typica*.
[b] *A. pegreffii*.
[c] *A. simplex* sensu stricto.

3.2. PCR-RFLP analysis

Amplification of the entire ITS region produced a single band of about 950 bp for all specimens. In RFLP, digestion of the PCR products with *Hin*fI produced three different RFLP patterns, corresponding to *A. typica* (ca. 590 and 330 bp), *A. pegreffii* (ca. 330, 280 and 240 bp), or *A. simplex* s. str. and *A. simplex* C (ca. 620 and 240 bp) (Fig. 1). For specimens showing the *A. simplex* s. str. and *A. simplex* C patterns, we then digested their respective PCR products only with *Hha*I and confirmed bands of ca. 530 and 420 bp for the *A. simplex* s. str. pattern (data not shown). Based on RFLP analyses of the 110 larvae from Taiwan, 93 (84%) were identified as *A. typica*, 15 (14%) were *A. pegreffii*, and 2 (2%) were *A. simplex* s. str. However, among 61 specimens from Japan, 40 (65%) were identified as *A. simplex* s. str., 20 (33%) were *A. pegreffii*, and 1 (2%) was *A. typica* (Table 1, Fig. 2).

3.3. Sequence of the entire ITS region

Entire ITS region sequences were determined for 5 larvae of *A. typica*, 5 larvae of *A. pegreffii*, and 1 larva of *A. simplex* s. str. from Taiwan as well as 1 larva of *A. typica* from Japan. No intraspecific differences were found in the sequences of these specimens. Similarity searches of the GenBank/EMBL/DDBJ nucleotide database revealed that the sequence of *A. typica* was identical to deposited sequences of the same species obtained at the larval stage from chub mackerel (*Scomber japonicus*, accession number AB432908) and at the

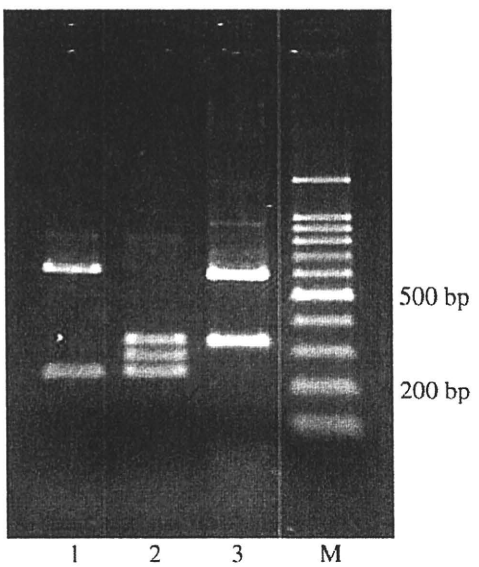

Fig. 1. RFLP analysis with *Hin*fI of the ITS PCR products amplified from the *Anisakis* type I larvae. Lane 1: *A. simplex* sensu stricto; Lane 2: *A. pegreffii*; Lane 3: *A. typica*. The 100-bp DNA ladder marker was used to estimate the size of the bands (lane M).

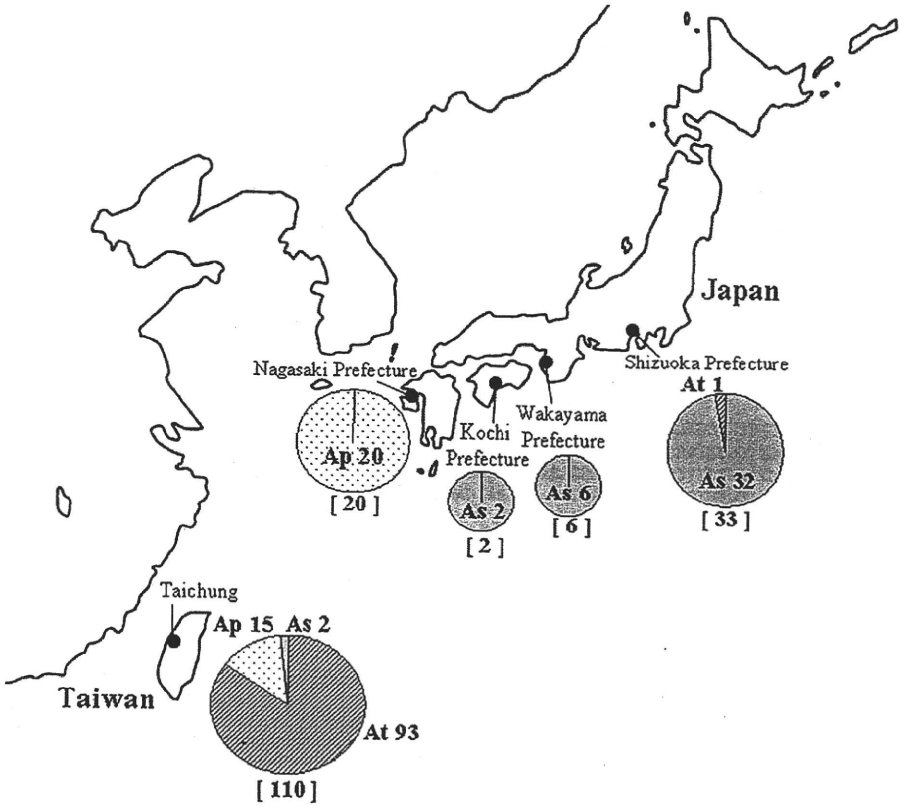

Fig. 2. Distribution of *A. typica* (At), *A. pegreffii* (Ap) and *A. simplex* sensu stricto (As) isolated from hairtail caught in coastal areas of Taiwan and Japan. Pie charts show the distribution of *Anisakis* type I larvae based on analysis of the rDNA ITS region. The numbers in square brackets and after the abbreviations of the species (At, Ap and As) represent the number of larvae examined and identified, respectively.

adult stage from rough-toothed dolphin (*Steno bredanensis*, AB479120). The nucleotide sequence of *A. typica* from Taiwan determined in this study has been deposited in the DDBJ/EMBL/GenBank database under the accession number AB551660. The sequences of *A. pegreffii* and *A. simplex* s. str. were also found to be identical to deposited sequences of each species obtained from chub mackerel (*S. japonicus*, AB277823) and arabesque greenling (*Pleurogrammus azonus*, AB277822), respectively.

The ITS sequence of *A. simplex* reported by Shih (2004) was not available from the GenBank/EMBL/DDBJ (shown in Fig. 3). Shih also isolated larvae for sequencing analysis from hairtail from Taiwan. Comparison with the sequence obtained in this study revealed that the sequence reported by Shih was 99% and 77% similar to those of *A. typica* and *A. simplex* s. str., respectively (Fig. 3).

4. Discussion

Anisakiasis is a zoonotic disease caused by the ingestion of anisakid nematodes in raw or undercooked fish. In previous reports, almost all the larvae recovered from humans have been identified as *Anisakis* type I based on their morphological characteristics (Oshima, 1972; Smith and Wootten, 1978). Since *Anisakis* type I is a morphotype comprising several species of the genus *Anisakis*, molecular characterization of *Anisakis* type I larvae is necessary for identifying each clinically and epidemiologically important species.

In this study, we observed that most of the larvae isolated from hairtail specimens from Taiwan were that of *A. typica*. To the best of our knowledge, this is the first report of *A. typica* in fish from Taiwan. In addition, a larva of *A. typica* was first detected in a hairtail specimen from Japan, though this species has been isolated from mackerel in Japan (Umehara et al., 2008a; Suzuki et al., 2010). In Asian countries besides Taiwan and Japan, *A. typica* has been reported in China, Korea, Indonesia and Thailand (Zhu et al., 2007; Chen et al., 2008; Palm et al., 2008; Lee et al., 2009; Du et al., 2010).

Shih (2004) reported the isolation of anisakid larvae from hairtail from Taiwan and sequenced the rDNA ITS region. Surprisingly, the so-called *A. simplex* sequence determined by Shih had a 99% similarity to the sequence of *A. typica* in this study. This implies that the sequence of anisakid larvae reported by Shih was probably that of *A. typica*, and not of *A. simplex*. According to the review by Mattiucci and Nascetti (2006, 2008), *A. typica* is widely distributed between 30°S and 35°N in warm and tropical waters. Nevertheless, there have been few reports of *A. typica* detected in both paratenic host fish and definitive host marine mammals. Kagei (2003) reported that the number of worms of *A. typica* in the definitive host was less than that for *A. simplex*. The lack of reports of *A. typica* might also be due to insufficient recognition of the species and hence misidentification of *A. typica* as *A. simplex*.

The sequence of *A. typica* obtained in this study was identical to the two sequences already deposited in the GenBank/EMBL/DDBJ (AB432908 and AB479120). However, when compared with the sequence reported by Shih as *A. simplex*, the *A. typica* sequence differed by 4 nucleotides in the ITS1 region and 1 nucleotide in the ITS2 region. Previous studies have reported geographical intraspecific variations in the entire ITS region of *A. typica* from Indonesia and Brazil (Palm et al., 2008, Iñiguez et al., 2009). This may indicate that some genotypes of *A. typica* are globally distributed in the ocean.

Japanese people traditionally eat fish raw as sushi and sashimi, and over 2000 cases of human anisakiasis are estimated to occur annually (Chai et al., 2005). In Taiwan, consumption of raw fish is also a common practice, and anisakiasis is therefore an important consideration. However, only one case has been reported in Taiwan to date, and the causative pathogen was not identified (Ishikura, 2003).

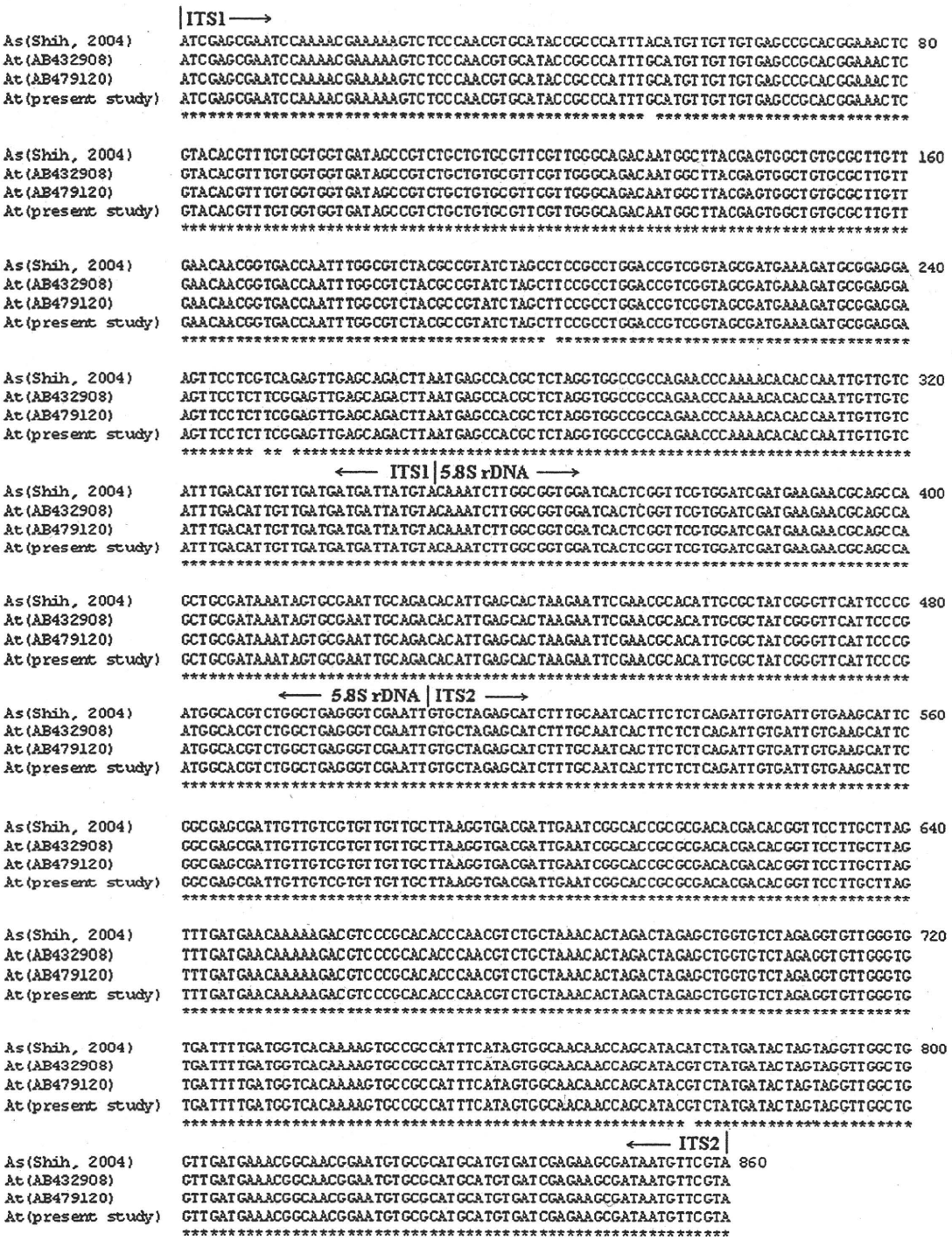

Fig. 3. Alignment of the entire ITS region sequences of *A. typica*. As (Shih, 2004), the sequence incorrectly reported as *A. simplex*; At (AB432908), the sequence obtained from larva isolated from chub mackerel; At (AB479120), the sequence obtained from adults isolated from rough-toothed dolphin; At (present study), the sequence obtained from larva isolated from hairtail in Taiwan in this study (AB551660). Asterisk (*) indicates identity to the reference sequence. GenBank/EMBL/DDBJ accession numbers are each shown in parentheses, where applicable.

We recently identified the species of the *Anisakis* type I larvae isolated from fish and from anisakiasis patients in Japan (Umehara et al., 2006, 2007, 2008b). Although both *A. simplex* s. str. and *A. pegreffii* were detected in fish, almost all larvae (99%) from patients were identified as *A. simplex* s. str. In other words, there is a striking discrepancy in the predominant species between fish and humans. Suzuki et al. (2010) explained that this was due to the higher penetration rate of *A. simplex* s. str. into the muscle tissue of the fish. *A. simplex* s. str. larvae were therefore ingested by humans together with the fish muscles, while *A. pegreffii* larvae were usually removed from the fish along with the internal organs prior to ingestion.

To date, although *A. typica* has not been recorded as a cause of human anisakiasis and the risk of human infection by *A. typica* has been assumed to be low, Palm et al. (2008) reported the isolation of a single larva of *A. typica* from the musculature of the bullet tuna (*Auxis rochei rochei*). Since *A. typica* is the predominant species in the hairtail

found in the coastal waters of Taiwan, there is always a possibility of human cases outbreak in that country where people have recently begun to eat raw hairtail. As it is not possible to distinguish *A. typica* from the other *Anisakis* type I larvae through morphology, molecular methods are indispensable for determining the incidence of *A. typica* infection in humans. This is important not only in Taiwan, but also in other countries where the presence of *A. typica* and other species comprising the *Anisakis* type I has been noted.

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Paragonimus westermani possesses aerobic and anaerobic mitochondria in different tissues, adapting to fluctuating oxygen tension in microaerobic habitats

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ABSTRACT

We previously showed that adult *Paragonimus westermani*, the causative agent of paragonimiasis and whose habitat is the host lung, possesses both aerobic and anaerobic respiratory chains, i.e., cyanide-sensitive succinate oxidase and NADH-fumarate reductase systems, in isolated mitochondria (Takamiya et al., 1994). This finding raises the intriguing question as to whether adult *Paragonimus* worms possess two different populations of mitochondria, one having an aerobic succinate oxidase system and the other an anaerobic fumarate reductase system, or whether the worms possess a single population of mitochondria possessing both respiratory chains (i.e., mixed-functional mitochondria). Staining of trematode tissues for cytochrome *c* oxidase activity showed three types of mitochondrial populations: small, strongly stained mitochondria with many cristae, localised in the tegument and tegumental cells; and two larger parenchymal cell mitochondria, one with developed cristae and the other with few cristae. The tegumental and parenchymal mitochondria could be separated by isopycnic density-gradient centrifugation and showed different morphological characteristics and respiratory activities, with low-density tegumental mitochondria having cytochrome *c* oxidase activity and high-density parenchymal mitochondria having fumarate reductase activity. These results indicate that *Paragonimus* worms possess three different populations of mitochondria, which are distributed throughout trematode tissues and function facultatively, rather than having mixed-functional mitochondria.

1. Introduction

Parasitic helminths including nematodes, trematodes and cestodes exhibit a variety of metabolic pathways (Tielens and van den Bergh, 1993; Komuniecki and Komuniecki, 1995; Behm, 2002; Kita and Takamiya, 2002). Their life cycle consists of two stages, a free-living larval stage that lives in an aerobic environment and a parasitic stage inside a host, in which oxygen tension depends on the tissues penetrated by migrating larvae or dwelt in by adult parasites. For example, the adult stage of *Ascaris suum*, a nematode that parasitises swine, dwells in the lumen of host small intestine, where oxygen tension is very low, while its fertilised eggs develop aerobically to form infective L3s. Therefore, during development parasitic helminths undergo aerobic–anaerobic transitions in energy metabolism resulting from adaptation to their environments. During its aerobic larval stage, *A. suum* possesses a functional tricarboxylic acid (TCA) cycle and a cyanide-sensitive respiratory chain similar to those in mammals (Takamiya et al., 1993; Amino et al., 2003; Iwata et al., 2008); both of these are localised in mitochondria and produce ATP by oxidative phosphorylation. That is, electrons derived from substrate oxidation are transported via NADH or flavin adenine dinucleotide (FADH) to oxygen by the proton-pumping electron-transfer complexes of the respiratory chain, and a proton gradient formed across the inner membrane drives ATP-synthase, resulting in ATP production with a backflow of protons. The respiratory chain consists of four electron-transfer complexes, i.e., complexes I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase, SQR), III (ubiquinol-cytochrome *c* oxidoreductase), and IV (cytochrome *c* oxidase, CCO), and two low-molecular weight electron carriers, cytochrome *c* and ubiquinone. Complexes I, III and IV have proton-pumping activity. Thus, mitochondria that produce ATP at the expense of oxygen can be defined as aerobic mitochondria, with 38 mol of ATP produced during the complete oxidation of each mol of glucose. Adult *Ascaris* nematodes, however, possess anaerobic mitochondria that yield ATP in the absence of oxygen.

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Mitochondria from adult *Ascaris* muscle have reduced levels of complex III and cytochrome *c*, and almost completely lack complex IV. These nematode mitochondria catalyze the NADH-dependent reduction of fumarate, which is endogenously derived from malate dismutation and serves as a terminal electron acceptor. NADH-linked fumarate reduction is mediated by complex I and by complex II, an isoform of the larval complex II (Takamiya et al., 1986). The latter, together with rhodoquinone, catalyzes rhodoquinol-fumarate reduction, the reverse of the succinate oxidation mediated by the larval enzyme, a reaction coupled to site 1 phosphorylation of ADP. Using this pathway, approximately 5 mol of ATP are produced per mol of glucose; a value higher than that for lactate fermentation (2 mol ATP per mol glucose) but lower than that observed using the aerobic pathway. Since adult *Ascaris* mitochondria can produce ATP by oxidative phosphorylation in the absence of oxygen, they are termed "anaerobic" mitochondria or more precisely, "anaerobically functioning mitochondria". Similar anaerobic mitochondria are present not only in parasitic helminths but also in other hypoxically functioning eukaryotes that live in microaerobic environments such as marine or freshwater sediments (Van Hellemond et al., 1995; Tielens et al., 2002). Therefore, oxygen tension where anaerobic mitochondria function is quite varied from extremely anoxic to less hypoxic or microaerobic. Although the molecular properties of anaerobic mitochondria, especially of their respiratory chains, have been elucidated in detail, the mechanisms of aerobic–anaerobic transition still remain to be determined with respect to mitochondrial biogenesis.

Paragonimus spp., lung flukes that infect mammals including humans, may provide appropriate material to investigate aerobic–anaerobic transitions in mitochondria. Adult *Paragonimus westermani* and *Paragonimus ohirai* inhabit mammalian lungs by forming cysts, in which oxygen tension fluctuates much more than in the lumen of the small intestine. We previously showed that the mitochondria of adult flukes possess both aerobic and anaerobic respiratory chains, i.e., mammalian-type, cyanide-sensitive respiratory chains and the NADH-fumarate reductase system (Ma et al., 1987; Takamiya et al., 1994). This finding raises the interesting question as to whether the adult *Paragonimus* spp. possess separate populations of aerobic and anaerobic mitochondria or a single mitochondrial population containing both the succinate oxidase and NADH-fumarate reductase systems. Our cytochemical study using *P. ohirai* supported the former view but we did not have sufficient biochemical evidence (Fujino et al., 1996). We have therefore attempted to separate the aerobic and anaerobic mitochondria, and to further characterise each individually.

2. Materials and methods

2.1. Isolation of P. westermani mitochondria

Adult *P. westermani* (3*n* type, 62 or 88 worms) were obtained from worm cysts from the lungs of dogs 8 or 12 months after inoculation with metacercariae. After removal of ovary tissue, the mitochondria were prepared as described (Takamiya et al., 1994). All procedures performed on laboratory animals were approved by the institutional animal care and use committee of the National Defense Medical College, Japan (the approval No. 09097) and all the animal experiments were carried out in compliance with the guidelines for animal experimentation of National Defense Medical College.

2.2. Isopycnic density-gradient centrifugation

Continuous 26 ml-sucrose density gradients, 32–52% (w/w) or 35–55% (w/w) containing 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 0.1% BSA were prepared using a density gradient fractionator (DGF-U, Hitachi, Japan). A mitochondrial suspension (0.9–1.2 ml) was loaded onto each gradient, which was centrifuged at 108,000*g* for 2 h in a Hitachi RP70T rotor at 4 °C. The contents of each tube were fractionated through the fractionator and CCO (Takamiya et al., 1994), fumarate reductase (FRD) (Kita et al., 1988) and SQR (Takamiya et al., 1994) activities, and the protein contents of these fractions, were measured. Using one drop of each fraction, the buoyant density was measured by measuring the sucrose concentration of each fraction with Abbe's refractometer and comparing each with a curve relating sucrose concentration to density. For protein assays, a 0.1 ml aliquot of each fraction was suspended in 0.9 ml of 0.025 M Tris–HCl buffer, pH 7.5, containing 0.21 M mannitol, 0.07 M sucrose and 0.1 mM EDTA. The suspensions were centrifuged at 14,000*g* for 15 min (Tomy MRX 150) to remove BSA and recover the mitochondria in the pellet. The supernatant was thoroughly removed and the protein content of each pellet was assayed (Markwell et al., 1978). For quinone determination, mitochondrial fractions were diluted with 10 vol. of 10 mM Tris–HCl, pH 7.4, containing 0.2 mM EDTA and the mixtures were centrifuged at 100,000*g* for 1 h to recover the mitochondrial particles. Each pellet was suspended in a small volume of the same buffer and used for quinone determination (Takamiya et al., 1994).

2.3. Electron microscopy

For cytochemical studies of CCO, worm tissues were fixed for 20 min at 4 °C in phosphate-buffered 2% glutaraldehyde, which had been purified overnight using active carbon. Small blocks of tissue were rinsed for several hours in the same buffer with 7% sucrose and sectioned at 40 μm on a Sorvall TC-2 tissue sectioner. The sections were collected in the same buffer and incubated for 2 h in medium (Seligman et al., 1968) at 37 °C. Control sections were incubated with the inhibitor, 10 mM KCN. The tissues were post-fixed for 45 min at 4 °C in 1% osmium tetroxide buffered to pH 7.4 with 0.05 M Veronal buffer and washed for 30 min with 0.1 M sodium acetate. Before dehydration with ethanol, the tissues were stained for 30 min in 0.5% uranyl acetate at 4 °C and examined using a JEOL JEM 100c electron microscope. Electron micrographs at magnifications of 5,000–10,000 were prepared to measure the maximum cross-sectional length and width and areas of mitochondria in trematode cells. The size of mitochondria in the micrographs were recorded using analysis Five software (Soft Imaging System GmbH, Münster, Germany).

Isolated mitochondria were fixed in 4% glutaraldehyde buffered with 10 mM Tris–HCl (pH 7.4), 0.21 M mannitol, 0.07 M sucrose and 0.1 mM EDTA, post-fixed in 1% osmium tetroxide in the same solution, dehydrated in ethanol, embedded in Quetal 812, and cut into sections of thickness 300–500 Å. These sections were stained with 4% uranyl acetate, treated with lead citrate hydroxide and examined under a Hitachi H-7,000 transmission electron microscope.

Electron micrographs at magnification 7,000 were prepared to measure the areas (μm^2) and brightness of cross-sections of individual fractionated mitochondria. The micrographs were examined using a computer-controlled image analyzer (KS400, Zeiss, Germany) equipped with a scanner (Epson GT-9100). Typically, for each fraction, 476–502 mitochondrial cross-sections were analyzed.

2.4. Statistical analyses

The areas and brightness of mitochondrial cross-sections were analyzed by Kaleida Graph ver. 4. For in situ observation, statistical analyses were performed using Prism 4 software (GraphPad Software, Inc., San Diego, CA, USA). Differences between means were

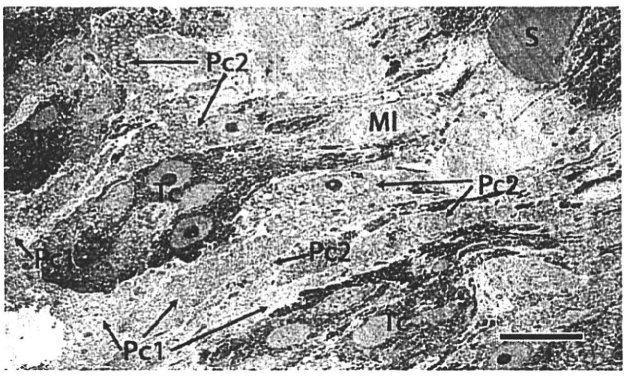

Fig. 1. Low magnification electron micrograph of the *Paragonimus* body wall. Ml, muscle layer; Pc1, parenchymal type-1 cells; Pc2, parenchymal type-2 cells; S, spine; T, tegument; Tc, tegumental cells. The magnification is 1000×. Scale bar = 20 μm.

analyzed using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. In situ morphological features

Examination of the body wall sections of *P. westermani* by transmission electron microscopy showed that body wall cells could be categorised into two types, tegumental and parenchymal cells, based on their shape, the density of their cytoplasm and the number and morphology of mitochondria (Fig. 1). The morphological features of the two cell types were similar to those described for *P. ohirai*; the parenchymal cells could be further divided into two types, parenchymal cells types-1 and -2 (Pc1 and Pc2, respectively) (Fujino et al., 1996). Pc1, with cytoplasm less dense than that of Pc2, contain a few round or oval mitochondria with several long cristae in rather dense matrices (Fig. 2A). In contrast, Pc2 possess

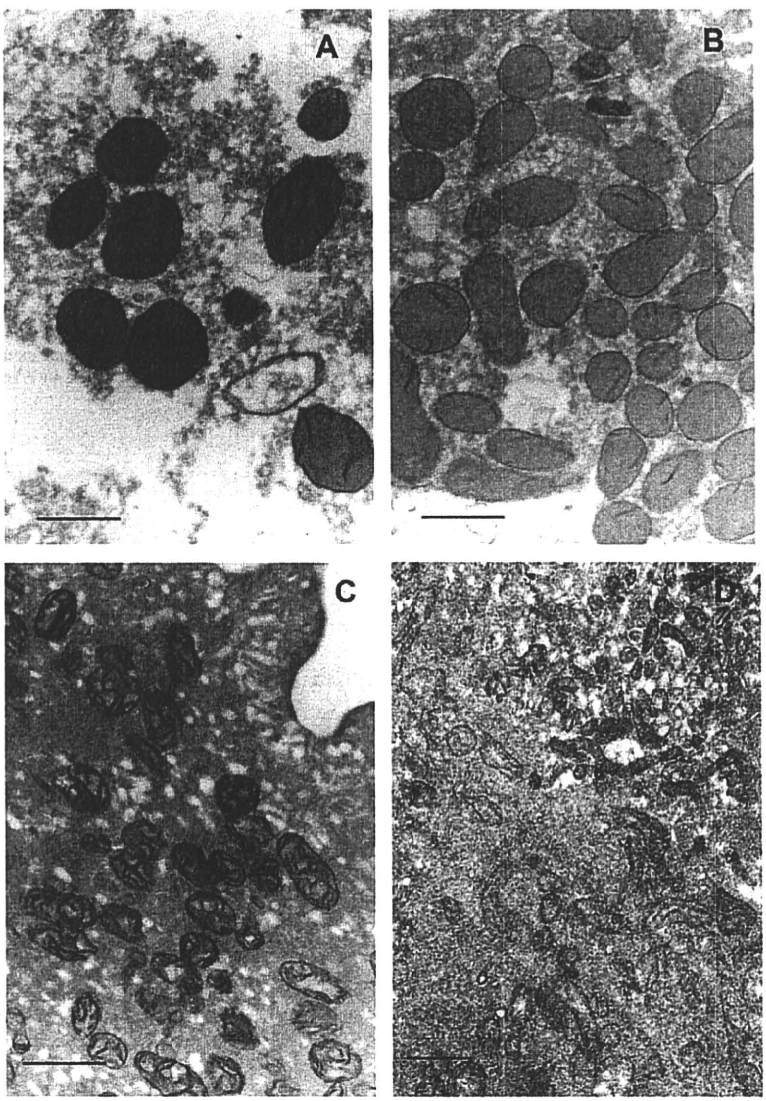

Fig. 2. Cytochrome c oxidase (CCO) staining of *Paragonimus* mitochondria in the (A) parenchymal type-1 cells; (B) parenchymal type-2 cells; and (C) tegument. (D) Tegumental cells for control. No significant staining was observed in the presence of KCN, a potent inhibitor of CCO. The magnification for A–C and D is 10,000× and 2,000×, respectively. Inserted bar represents 1 μm.

Table 1
In situ morphology of *Paragonimus westermani* mitochondria.

Sourc	Cross-section area (μm²)	Shape	Crista development	Matrix density	CCO staining
T or Tc	0.130 ± 0.0819	Oval	Well	Dense	Heavy
Pc1	0.330 ± 0.193	Round or oval	Moderate	Dense	Moderate
Pc2	0.350 ± 0.197	Round or oval	Poor	Less dense	Little

Cross-section areas (n = 190) were measured as described in Section 2.
T, tegument; Tc, tegumental cells; Pc1, parenchymal type-1cells; Pc2, parenchymal type-2 cells; CCO, cytochrome *c* oxidase.

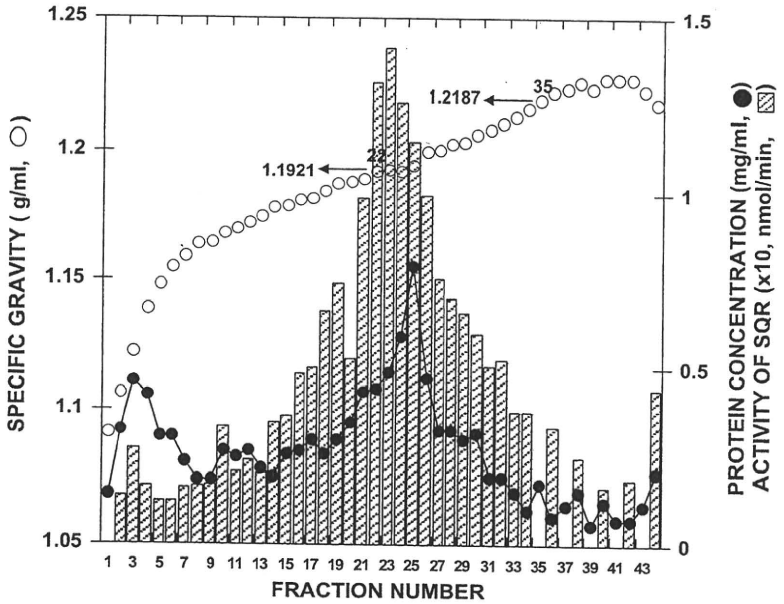

Fig. 3. Distribution of isolated *Paragonimus* mitochondria on isopycnic density gradient (32–52% (w/w) sucrose) centrifugation. Trematode mitochondria (10.0 mg protein) were centrifuged and the specific gravity (open circles), protein concentrations (closed circles), and activity of succinate-ubiquinone oxidoreductase (SQR) (shaded bars) were determined in each fraction. Mitochondria with high fumarate reductase (FRD)/cytochrome *c* oxidase ratios were present in Fractions 22–35. Detailed experimental procedures are described in Section 2.

numerous mitochondria, round or oval in shape, with less dense matrices than Pc1 and poorly developed cristae (Fig. 2B). Since the cristae of Pc1 mitochondria were positive for CCO, the terminal oxidase that reduces molecular oxygen, Pc1 mitochondria appear more aerobic than Pc2 mitochondria. In contrast to the parenchymal cells, the tegumental cells contain many small mitochondria with well-developed cristae and heavily positive for CCO activity (Fig. 2C). Therefore, among the three types of body wall mitochondria, the tegumental mitochondria appear most aerobic. Morphological features of *P. westermani* body wall mitochondria are summarised in Table 1.

3.2. Buoyant density and SQR activity of mitochondria fractionated by isopycnic density-gradient centrifugation

Paragonimus westermani mitochondria were analyzed by sucrose density-gradient centrifugation between 32% (w/w) and 50% (w/w) sucrose, with two gradient ranges, a low-density gradient range (1.0914–1.1546 g/ml) of Fractions 1–6 and a high density gradient range (1.1546–1.2267 g/ml) of Fractions 6–42. Since the density of 32% (w/w) sucrose solution is 1.1426 g/ml, higher than those for Fractions 1–4 (1.0914–1.1390 g/ml), the steep initial gradient was formed by dilution with sucrose–mannitol medium used to suspend mitochondria. The mitochondria were layered on top of the gradient.

Using SQR activity as the mitochondrial marker enzyme, *P. westermani* mitochondria were distributed at sucrose densities of 1.123–1.2267 g/ml (Fractions 2–42) with a main protein peak at 1.1946 g/ml (Fraction 25) (Fig. 3). The low-density peak at Fractions 2–4 contained small mitochondria with well-developed cristae, although cross-contaminating microsomal vesicles were

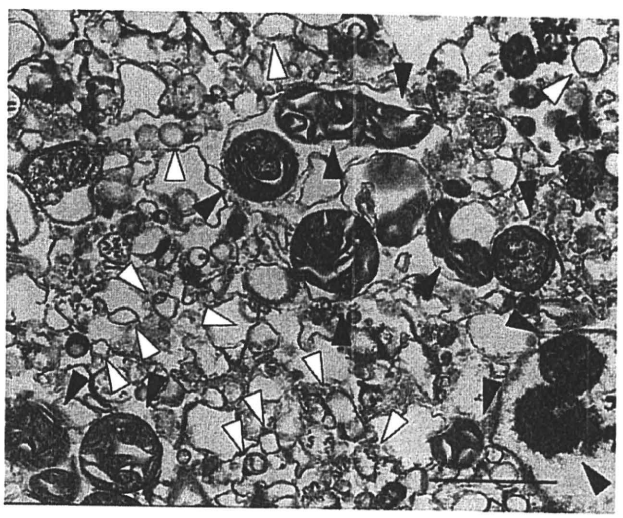

Fig. 4. Electron micrograph of *Paragonimus* mitochondrial preparation with lower densities (Fraction 2, 1.1065 g/ml) fractionated by isopycnic density-gradient (32–52% (w/w) sucrose) centrifugation. Black and white arrowheads indicate mitochondria and microsomal vesicles, respectively. Inserted bar represents 1 μm.

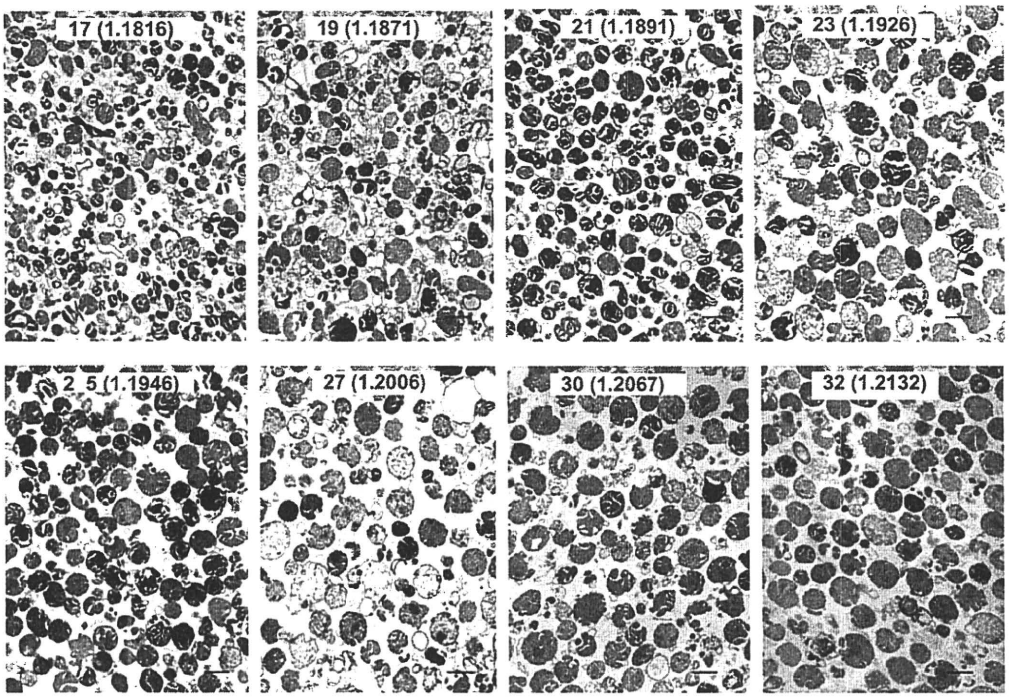

Fig. 5. Electron micrographs of *Paragonimus* mitochondria with higher densities (Fractions 17, 19, 21, 23, 25, 27, 30 and 32) fractionated by isopycnic density-gradient (32–52% (w/w) sucrose) centrifugation. Numbers and numerals in brackets represent fraction numbers and densities, respectively. Inserted bar represents 1 μm.

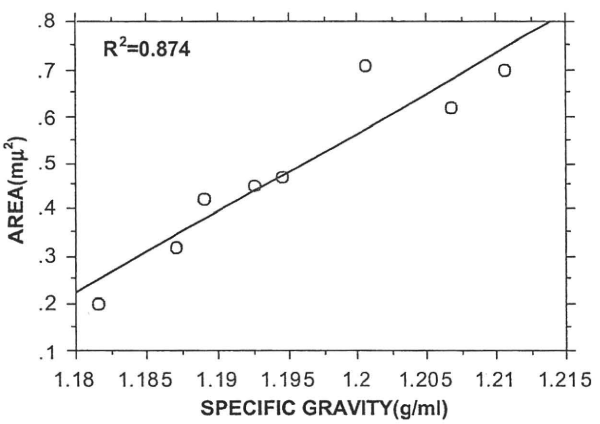

Fig. 6. Relationship between specific gravities and cross-sectional areas of *Paragonimus* mitochondria fractionated by isopycnic density-gradient (32–52% (w/w) sucrose) centrifugation. Medians of mitochondrial cross-sectional areas, as determined by a computer-controlled image analyzer, were plotted against the specific gravities of Fractions 17, 19, 21, 23, 25, 27, 30 and 32. Detailed experimental procedures are described in Section 2.

also detected (Fig. 4). Thus, the specific activity of SQR was lower in Fractions 1–7 than in mitochondria before centrifugation. The specific activity of this mitochondrial marker enzyme in the rest of fractions (Fractions 8–42) ranged from 0.177 (Fraction 8) to 0.595 μmol/min/mg protein (Fraction 22). This variation also suggested heterologous mitochondrial populations that differed in energy metabolism. The average specific activity was calculated to be 0.405 μmol/min/mg, indicating a 2.7-fold purification after centrifugation. Intact rat liver mitochondria have been fractionated by sucrose density gradient ultracentrifugation into two bands, with mean densities of 1.184 and 1.216 g/ml (Pollak and Munn, 1970) and human liver mitochondria exhibited an equilibrium density of 1.20 g/ml (Peters and Seymour, 1978). Thus, the buoyant densities of most fluke mitochondria were similar to those of mammalian aerobic mitochondria.

3.3. Morphological features of fractionated P. westermani mitochondria

Since we found that trematode mitochondria were heterologous in situ, we analyzed the morphology of mitochondria fractionated by isopycnic-density-gradient centrifugation. Electron micrographs showed mitochondria that differed in size and staining density; the small mitochondria appeared to be fractionated at lower densities and the large mitochondria at higher densities (Fig. 5). The size (area, μm^2) and staining density (brightness) of individual mitochondrial cross-sections in each fraction were determined one by one using a computer-controlled image analyzer. When we plotted the sizes and brightness of mitochondria versus their densities, we observed a linear relationship (Fig. 6), with small mitochondria present at low sucrose densities and large mitochondria at higher sucrose densities. However, we observed no relationship between the brightness of mitochondria and their density (data not shown). Rather, there were two populations of mitochondria, bright and dark stained, irrespective of their sizes (Figs. 7 and 8). The relative fraction of bright mitochondria appeared to be higher in the high sucrose density fractions, indicating morphological heterogeneity of the fractionated mitochondria.

3.4. Distribution of CCO and FRD activities on separated mitochondria

To further analyze *P. westermani* mitochondria, they were centrifuged on a higher density gradient, i.e., from 35% (w/w) to 55% (w/w) sucrose (Fig. 9). Two linear density gradients were observed, one from Fractions 1 to 6 (1.0865–1.163 g/ml) and the other from Fractions 6 to 37 (1.163–1.253 g/ml). Activities of CCO and FRD, the marker enzymes of the aerobic and anaerobic respiratory chains, were determined in each fraction, and relative ratios of their specific activities were calculated. Mitochondria with high CCO/FRD

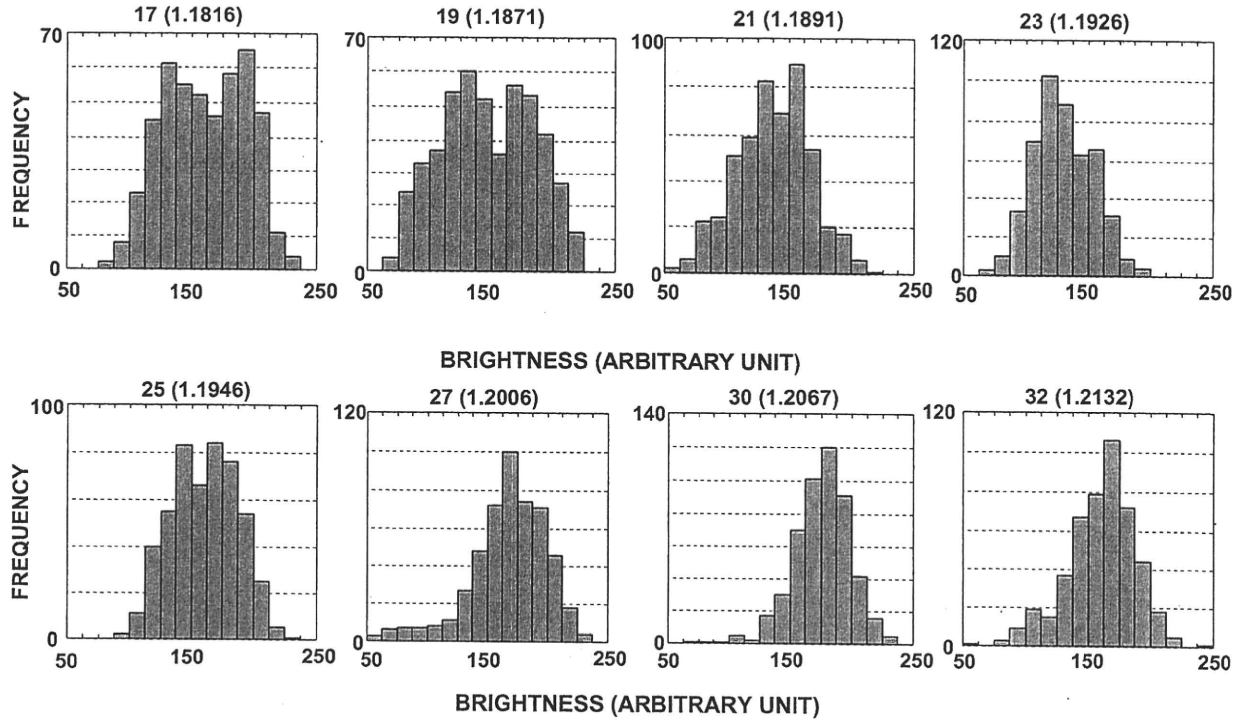

Fig. 7. Histograms of the brightness of *Paragonimus* mitochondria fractionated by isopycnic density-gradient (32–52% (w/w) sucrose) centrifugation. Numbers and numerals in brackets represent fraction numbers and densities, respectively. Brightness of individual mitochondria was determined by a computer-controlled image analyzer. Sample numbers for Fractions 17, 19, 21, 23, 25, 27, 30 and 32 were 477, 491, 502, 479, 502, 502, 502 and 476, respectively.

ratios were mainly recovered at low densities (Fractions 1–7, 1.0865–1.168 g/ml), and those with high FRD/CCO ratios at high densities (Fractions 13–23, 1.192–1.1285 g/ml), indicating that aerobic mitochondria are lighter than anaerobic mitochondria. We also determined the ubiquinone-10 and rhodoquinone-10 contents of fractionated mitochondria to compare the ubiquinone/rhodoquinone ratio in low- and high-density mitochondria. The ubiquinone/rhodoquinone molar ratio was 8.13 and 3.29 in low- and high-density mitochondria, respectively, indicating that the low-density mitochondria are also aerobic in terms of quinone contents.

4. Discussion

Although there have been several studies on the morphological and functional heterogeneity of *Paragonimus* mitochondria (Hamajima et al., 1982; Yamakami et al., 1984; Fujino et al., 1995, 1996), substantial evidence for the occurrence of aerobic and anaerobic mitochondria has been limited. We previously reported that there were three types of mitochondria, which differed in morphology and cytochemistry, in *P. ohirai* tissues (Fujino et al., 1996), but the relationships between morphology and function could not be determined due to limited amounts of material. No direct evidence was presented for Pc2 mitochondria with high FRD activity. By using *P. westermani*, which are larger, for mitochondrial preparation, we were able to further characterise trematode mitochondria. We found that adult *P. westermani* possess three types of mitochondria, small aerobic mitochondria localised in the tegument and tegumental cells and two types of large mitochondria, one localised in Pc1 and highly stained for CCO activity, and the other derived from Pc2 and poorly stained for CCO activity. Fractionation of these trematode mitochondria by isopycnic density-gradient centrifugation based on their buoyant densities, which

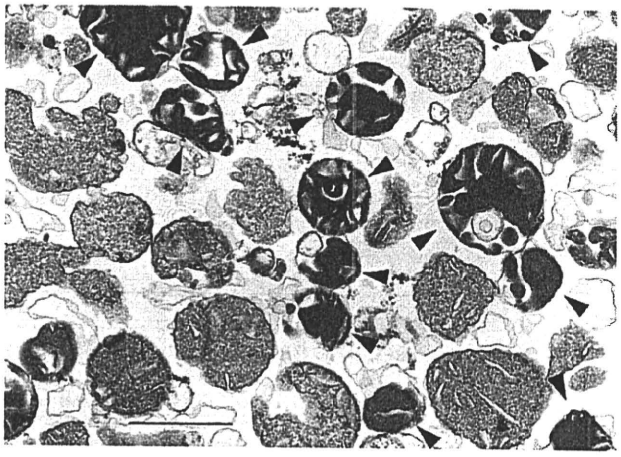

Fig. 8. Electron micrograph of *Paragonimus* mitochondria (Fraction 21) exhibiting different staining densities. Arrowheads show heavily stained mitochondria, with low brightness scores, with the remainder being lightly stained mitochondria, with high brightness scores. Inserted bar represents 1 μm.

are directly proportional to their cross-section areas or sizes, showed that the small mitochondria localised in the tegumental cells were present in the low-density fractions. These mitochondria, of average size $0.180 \pm 0.102\ \mu m^2$, comparable to their in situ average size of $0.130 \pm 0.0819\ \mu m^2$ (Table 1), had high CCO/FRD ratios and well-developed cristae. The parenchymal mitochondria were larger, with in situ sizes of $0.33 \pm 0.193\ \mu m^2$ for Pc1 mitochondria and $0.35 \pm 0.197\ \mu m^2$ for Pc2 mitochondria (Table 1), and were of average size $0.619 \pm 0.314\ \mu m^2$ after gradient fractionation. The larger cross-sectional areas of isolated compared with in situ mitochondria may have been due to unavoidable swelling during isolation.

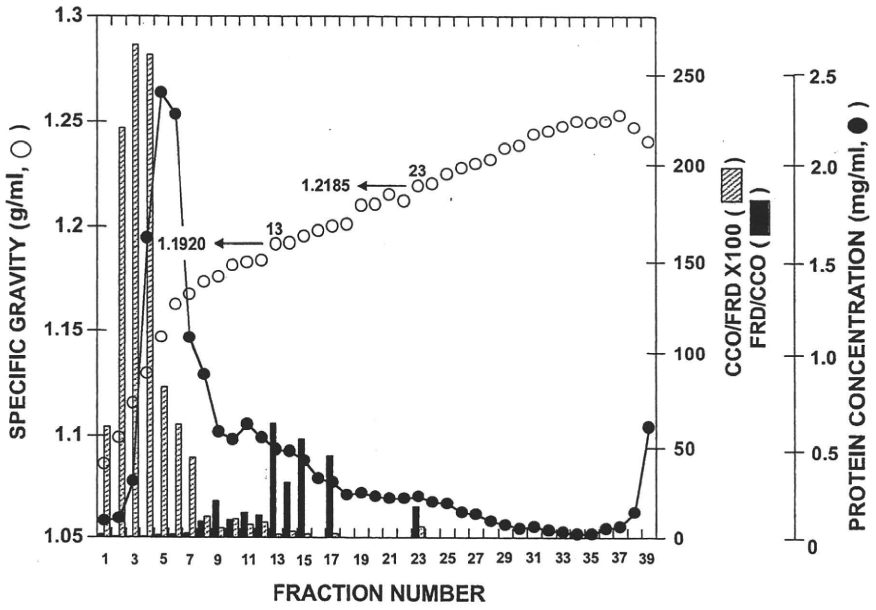

Fig. 9. Distribution of isolated *Paragonimus* mitochondria on isopycnic density gradient (35–55% (w/w) sucrose) centrifugation. Trematode mitochondria (24.9 mg protein) were centrifuged and the specific gravity (open circles), protein concentration (closed circles), and ratios of specific activity of cytochrome *c* oxidase (CCO) to fumarate reductase (FRD) (shaded bars) and of FRD to CCO (closed bars) were determined for each fraction. Specific activity of CCO and FRD for Fraction 3, giving the highest CCO/FRD × 100 value, is 38.7 s^{-1}/ml/mg and 14.6 μmol/min/mg, respectively. Specific activity of CCO and FRD for Fraction 13, giving the highest FRD/CCO value is 0.225 s^{-1}/ml/mg and 14.1 μmol/min/mg, respectively. Mitochondria with high FRD/CCO ratios were distributed between Fractions 13 and 23, with densities of 1.1920 and 1.2185 g/ml, respectively. Detailed experimental procedures are described in Section 2.

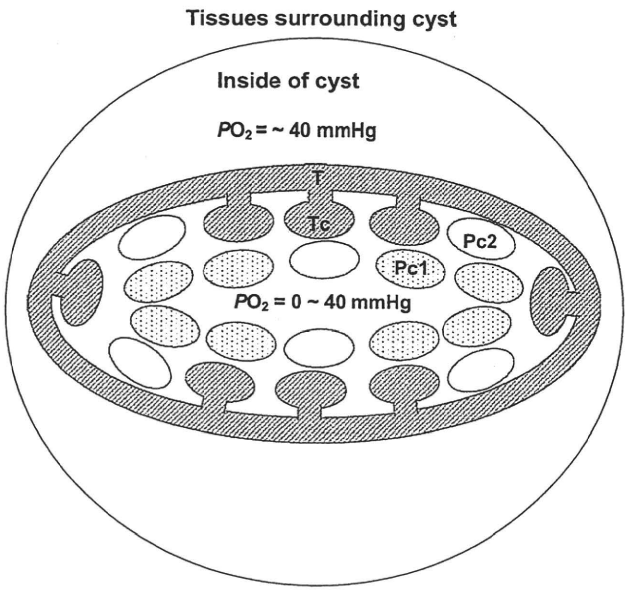

Fig. 10. Schematic cross-section of a cyst inhabited by adult *Paragonimus*. T and Tc represent the tegument and tegumental cells with aerobic mitochondria, and Pc1 and Pc2 represent types-1 and -2 parenchymal cells, respectively, the latter of which contain anaerobic mitochondria. PO_2 represents oxygen tension.

At present, it is difficult to separate Pc1 and Pc2 mitochondria because they do not differ significantly in size. Mitochondria present in Fractions 8–12 (1.174–1.1845 g/ml), with low CCO/FRD and FRD/CCO ratios, may be intermediate between aerobic and anaerobic mitochondria. Alternatively, their higher CCO activity and larger size might suggest that they consist primarily of Pc1 mitochondria. Although we previously reported that Pc1 mitochondria in *P. ohirai* are aerobic (Fujino et al., 1996), the staining intensity for CCO activity of *P. westermani* Pc1 mitochondria was significantly lower than that of tegument/tegumental cell mitochondria. Interestingly, we observed two types of mitochondria, one heavily stained with clear cristae and the other lightly stained with few cristae, which appear to correspond to our in situ observations, although artifacts may have been introduced during staining. The Pc2 mitochondria can be safely categorised as anaerobic with high FRD/CCO ratios. To summarise the results, the distribution of the three types of mitochondria is schematized in Fig. 10.

The presence in adult *P. westermani* of mosaic cells containing different types of respiratory chains, with varied energy efficiency, is intriguing. This situation may be due to the environmental oxygen tension surrounding the trematodes. In contrast to adult *A. suum*, which dwells in the host small intestinal lumen with low oxygen tension (PO_2 = 0–10 mm Hg; Minning et al., 1999), oxygen tension inside the cysts in which adult *P. westermani* live is likely higher than in the intestinal lumen, although this has not been demonstrated to date. Since the PO_2 of mammalian lung tissues through which blood circulates is approximately 40 mm Hg (Guyton, 1986), the PO_2 inside cysts may be similarly high enough to drive the aerobic respiratory chain of mitochondria localised in the outermost tegument and tegumental cells. In contrast, the parenchymal cells, which are located under the tegument and in the innermost part of the body, are in an environment of much lower PO_2. Therefore, the PO_2 of some parenchymal cells may be close to zero. Under these anaerobic conditions, as for adult *A. suum* (Kita and Takamiya, 2002; Yokota et al., 2006; Hashimoto et al., 2008; Takamiya et al., 2009), the parenchymal cells must depend on anaerobic respiration, in which fumarate functions as the electron acceptor instead of oxygen and oxygen homeostasis is maintained using haemoglobin and myoglobin. Thus, adult *P. westermani* adapt to their environmental conditions by optimising the mosaicism of their own tissues. Further studies are required to elucidate the mechanisms underlying aerobic–anaerobic transitions and the mosaic formation of tissues in these parasitic trematodes.

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総 説

食品媒介寄生虫による食中毒

Food-borne Parasitic Infection as Food Poisoning

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はじめに

食の安全・安心は，我々にとって大きな関心事となっている．この食の安全を担保する法律として，我が国では「食品衛生法」が重要な役割を果たしてきた．本法は「食品の安全性の確保のために公衆衛生の見地から必要な規制・措置を講じることにより，飲食に起因する衛生上の危害の発生を防止し，もって国民の健康の保護を図ることを目的とする（第1条）」として，1947年に制定された法律である．この条文に書かれている「飲食に起因する衛生上の危害」を，「飲食に起因する健康被害」と読み替え，さらにこれを「食中毒」としてとらえるのが，最近の行政上の，すなわち厚生労働省の方針となっている[16, 20] (http://www1.mhlw.go.jp/topics/syokueihou/tp1228-1_13.html).

食中毒の原因として対策を取る寄生虫

寄生虫も「飲食に起因する衛生上の危害」を引き起こすことから，「食中毒」の原因物質としてとらえる必要がある．我が国では，生鮮魚介類などの生食が嗜好され，食文化としても定着していることから，食品に媒介される寄生虫症の発生を認めることは，経験的にも知られている．この食品媒介寄生虫による疾患への対策を検討するため，1997年9月に当時の厚生省において，食品衛生調査会食中毒部会食中毒サーベイランス分科会が開催された．そのときにまず，我が国において特に対策が必要な寄生虫が指定された．検討に際しては，

イ）全国的に発生が多いもの，あるいは近年増加傾向にあるもの．

ロ）海外では発生が多く日本でも増加が懸念されるもの．

ハ）発生は多くなくとも重篤な被害が出る恐れのあるもの．

という三つの条件が考慮され，以下の14種類の寄生虫が対象として指定されることになった．

1) 原虫類（4種類）：単細胞の寄生虫（著者注）

クリプトスポリジウム，サイクロスポーラ，ジアルジア，赤痢アメーバ

2) 蠕虫類（計10種類）：多細胞の寄生虫，「ぜんちゅう」（著者注）

(1) 生鮮魚介類により感染するもの（6種類）

アニサキス，旋尾線虫，裂頭条虫，大複殖門条虫，横川吸虫，顎口虫

(2) その他の食品（獣生肉等）により感染するもの（4種類）

肺吸虫，マンソン孤虫，有鉤嚢虫，旋毛虫

これら寄生虫の感染を予防するために当面取るべき対策として，まず「国民及び関係者への安全な摂食方法等についての普及啓発」が挙げられた．また「国内外での食品の寄生虫汚染の実態及び当該疾患の発生状況についての情報把握」などの事項も取るべき対策とされた[17] (http://www1.mhlw.go.jp/houdou/0909/h0917-1.html).

食品衛生法に則した食中毒の届出

寄生虫疾患の発生状況についての情報を把握するには，法に則した届出を確実とすることが有効な手段となる．寄生虫も「食中毒」の原因物質としてとらえることから，飲食に起因した寄生虫症が発生すれば，これを食中毒として届け出ることになる．

「食中毒」が発生した場合の届出に関しては，食品衛生法の第58条に規定がある．その第1項には「食品，添加物，器具もしくは容器包装に起因して中毒した患者もしくはその疑いのある者を診断し，またはその死体を検案した医師は，直ちに最寄りの保健所長にその旨を届け出なければならない」と記載されている（医師の届出は文書，電話または口頭により24時間以内に行わなければ

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