開発され報告されてきた．組換え抗原を用いた抗体検査法，モノクローナル抗体を用いた抗原検出法，種特異的塩基配列を標的にした PCR 法などである．これらは，それぞれが特定の寄生虫を検出するために開発されてきたものであるが，今ここでこれらの知見を総合しリソースを集中させ，寄生虫疾患総合診断システムの構築を目指してはどうだろうか（図 2）．

抗体検査では組換え抗原の開発に力を入れ，糞便抗原検出スクリーニング検査が困難なら糞便遺伝子検出の標準化を推進する．いずれにしても，検体数が膨大になっても結果にばらつきのない検査システムであることが重要である．そのような検査が保険適用になり気軽にオーダーできるようになれば，寄生虫疾患そのものもなじみのある疾患になり，いつか「苦手感染症」ではなくなるのではないだろうか．今が，具体的な動きを起こすときであると考えている．

文　献

1) 田辺清勝，他 (2000)：肺結核が疑われたウエステルマン肺吸虫症の 1 例．日本臨床寄生虫学雑誌，11, 49-51.
2) 杉山広，他 (2001)：肺切除により診断を得た肺吸虫症：患者肺組織標本の虫嚢内虫体を材料とした塩基配列解読による虫種同定の試み．日本臨床寄生虫学雑誌，12, 59-61.
3) 米満伸久，他 (2002)：全身播種性糞線虫症の 1 剖検例．日本臨床寄生虫学雑誌，13, 89-91.
4) 久我武弘，他 (2000)：免疫抑制剤投与後に重症化し胃粘膜生検により診断された糞線虫症の 1 例．日本臨床寄生虫学雑誌，11, 90-92.
5) 礒田幸太郎，他 (2004)：寄生部位に異型腺腫様過形成を認めた重症糞線虫症の 1 例．日本臨床寄生虫学雑誌，15, 62-67.

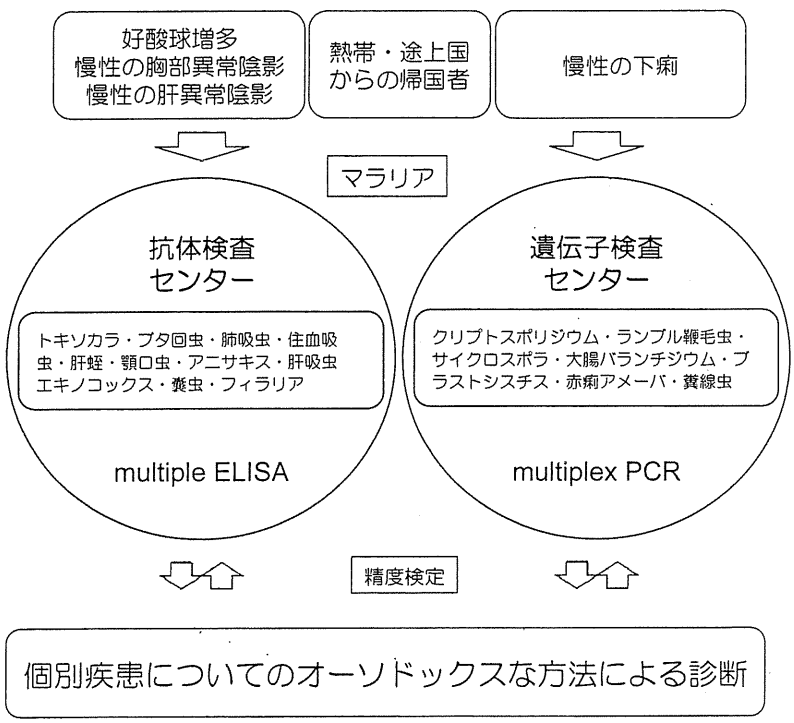


図 2　寄生虫検査体制構築に向けた多施設共同研究案

症例報告

好酸球増多を伴った糞線虫症の1例

1）栄和会泉川病院
2）長崎大学医学部　第二内科
3）宮崎大学医学部　寄生虫学
4）長崎大学医学部　第一病理

河野仁寿[1)]，近藤晃[1)]，金沢祐星[1)]
原耕平[1)]，泉川欣一[1)]，泉川公一[2)]
河野茂[2)]，丸山治彦[3)]，林洋子[4)]

要　旨

症例は78歳女性．2008年6月乾性咳嗽と38℃の発熱があったため，当院を受診した．CRP値は上昇し，白血球増多と好酸球増多，胸部X線上左下肺野に網状の浸潤影をみとめ，CTでは小葉中心性の粒状影や浸潤影をみとめた．メロペネムを投与したが反応せず，プレドニゾロンの投与でも症状や胸部陰影の増悪や寛解を繰り返した．約2ヵ月後から再び好酸球の増多とIgE値の上昇をみとめた．上部消化管の内視鏡検査で十二指腸に小白粉性隆起をみとめ，病理学的に同部に寄生虫体の構造をみとめ，また糞便中にラブジチス型の幼虫が認められ，糞線虫に対する血清抗体価も上昇していた．糞線虫症と診断し，ivermectinを2週間隔で2回投与したところ，症状も所見も全く消失した．幼少時にフィリピンにて感染し，老齢化により症状が顕性化したものと考えた．

キーワード：糞線虫症，小白粉性隆起，イベルメクチン

はじめに

糞線虫（通常ヒトへの感染は*Strongyloides stecoralis*）は熱帯・亜熱帯に分布し，日本では沖縄地方に多くみられる寄生虫で，フィラリア型幼虫が経皮的に侵入して感染が成立するとされている[1)]．私達は九州・長崎において，その発症初期に慢性の好酸球性肺炎の徴候を示し，十二指腸内視鏡では小白粉性隆起を認め，血清中に糞線虫抗体の上昇と，且つ糞便中に糞線虫の虫体を認めた一例を経験したので，文献的考察を加えて報告する．

症　例

患者：78歳　女性．

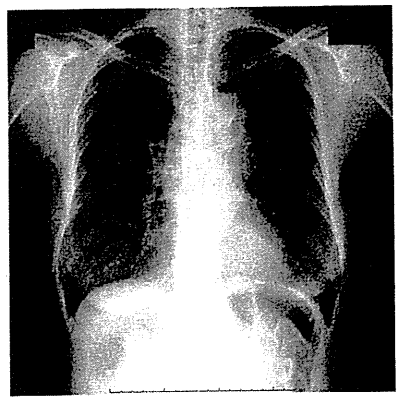
既往歴，家族歴：特記すべきものはない．

生活歴：1933年フィリピン・ミンダナオ島で出生．1945年終戦で長崎県口之津に引き揚げ（13歳時），その後成人になってからは農業に従事．豚堆肥を用いた有機野菜を摂取．生肉，川魚，沢蟹は食べていない．ペットの飼育歴はなく，喫煙歴や旅行歴もない．

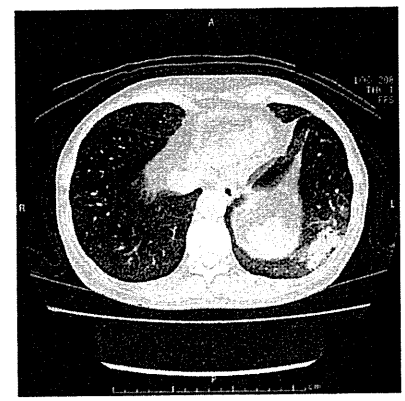
臨床経過：

第1回入院時：2008年5月中旬より乾性咳嗽が続き，前日より38℃台の発熱があった

a



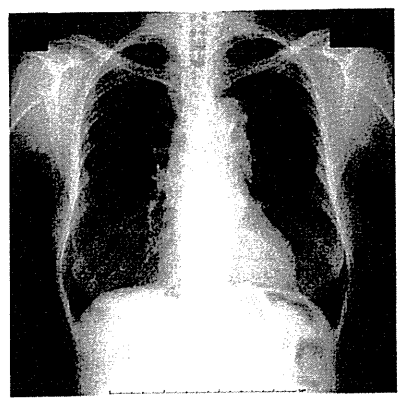
PA view



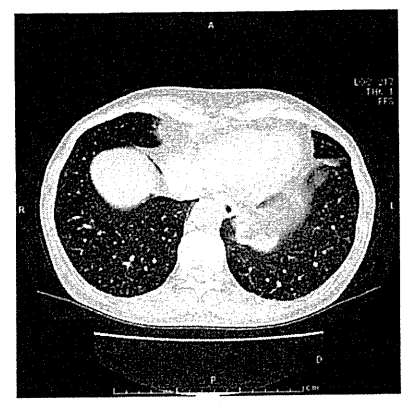
CT scan

(28th Jun, 2008)

b



PA view



CT scan

(8th Feb, 2010)

Fig. 1　Rontgenographic findings of chest

Dirofilaria immitis	+−	−	*Paragonimus westermani*
Toxocora canis	−	−	*P. miyazakii*
Ascaris suum	+−	+−	*Fasciola sp.*
Anisakis sp.	+−	−	*Clonorchis sinensis*
Gnathostoma doloresi	+−	−	*Spirometra erinacei*
Strongyloides sp.	++	−	*Cysticercus cellulosus*

Fig. 2　Multiple-dot ELISA of the patient's serum showing positive reaction against S. stercoralis.

ため，2008年6月下旬当院を受診，入院した．入院時，CRP値は上昇し，白血球増多と好中球増多，さらに好酸球増多(実数1436)をみとめ，胸部X線像では左下肺野に斑状の浸潤影をみとめ，CTでは小葉中心性の粒状影や淡い境界不明瞭な斑状の陰影を認めた(Fig 1a)．

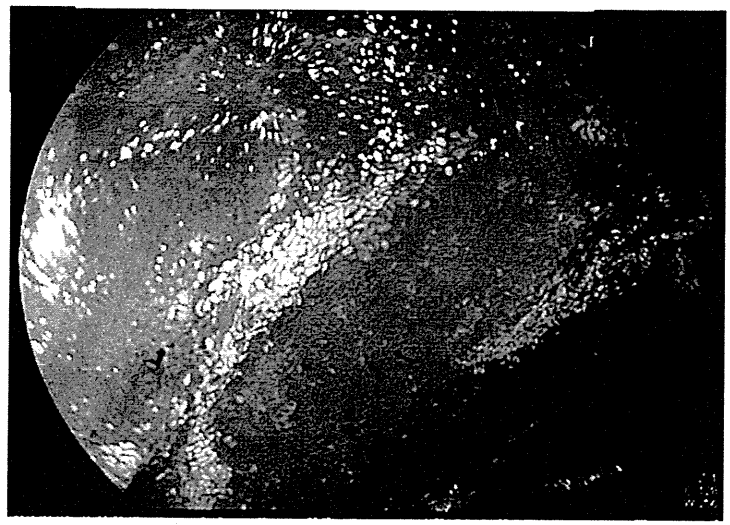


Fig. 3 Endoscopy findings in duodenum indicate a huge number of small white powder-like protrusions.

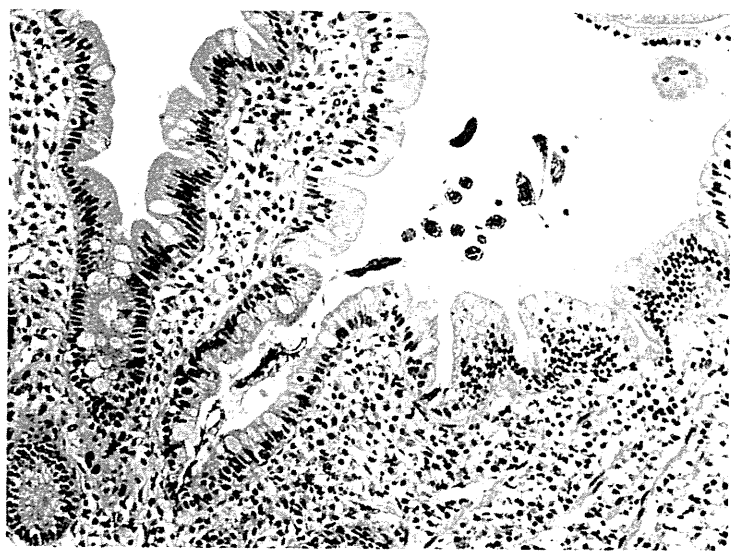


Fig. 4 In a duodenal biopsy specimen, eosinophilic inflammation of mucosa and parasites are recognized.

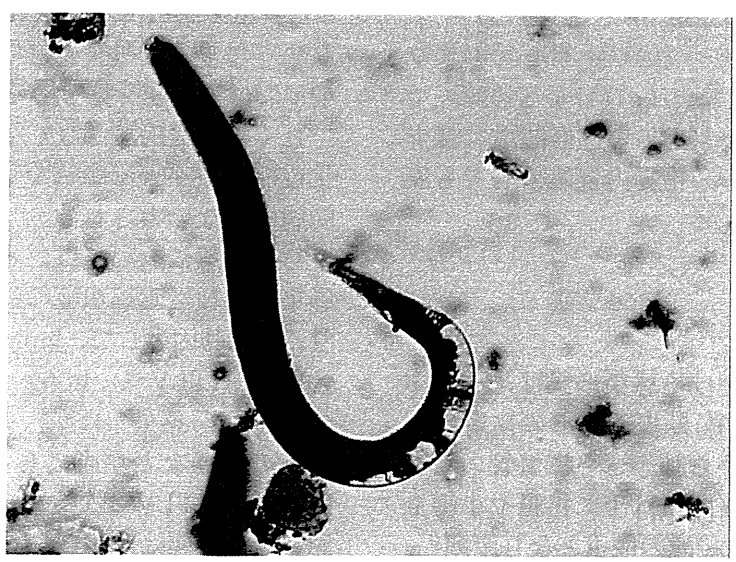


Fig. 5 Stool examination shows strongyloides larva.

細菌性肺炎の併発も考えてsulbactam/ampicillin(SBT/ABPC)で治療したが解熱せず，さらにmeropenem(MEPM)に変更したが，陰影は改善しなかった．好酸球性肺炎の診断のもとに，2008年7月上旬からpredonisolone 30mg/日より開始し，毎週5mgで漸減したが，症状も胸部の陰影も増悪や寛解を操り返した．さらに精査を進めようとしたが，同意が得られなかったので，外来で経過を観察することとした．その後も，慢性の咳嗽は持続したが，胸部の陰影は次第に消失した(Fig 1b)．2009年7月にPredonisoloneを中止したところ，同年9月頃から徐々に好酸球数の増加をみとめた．

第2回入院時：2010年2月上旬，咳嗽も続くため，精査のため第2回目の入院となった．入院時に貧血や黄疸はなく，口腔内は正常で，頚部や表在リンパ節は触知せず，心音は純，呼吸音も正常，腹部にも異常を認めなかった．検査では好酸球増多(実数1330)とIgEの上昇(1865，7 IU/ml)を認め，HTLV-1は陰性で，気管支肺胞洗浄液では，細胞数1130/μl，CD4/8 1.1，好中球42%，好酸球11%，リンパ球33%，肺胞マクロアージ14%であった．血清の寄生虫検査ではmultiple-dot ELISAにて糞線虫が陽性(Fig 2)で，十二指腸の内視鏡検査では小白粉性隆起(small white powder-like protrusion)を認め(Fig 3)，この部の生検では病理学的に十二指腸の腺窩に寄生虫(糞線虫に矛盾しない)と考えられる構造を認めた(Fig 4)．さらに糞便検査ではラブジチス型幼虫が確認された(Fig 5)．

糞線虫症と診断し，2010年3月上旬よりivermectin 6 mg(0.2mg/kg)/日を2週間隔で2回に分けて投与したところ，好酸球は減少し，咳嗽も消失，十二指腸内視鏡検査でも表層粘膜は正常となった．

考　　察

糞線虫のヒトにおける感染形式は，土の中のフィラリア型(F型)幼虫がヒトの皮膚から侵入し，リンパ系から肺，さらに気管・食道などを移動し，最終的には十二指腸や小腸上部に寄生する．そこで雌の成虫となり産卵し，孵化したラブジチス型幼虫は便と共に体外に排出されるが，一部の幼虫は排出される前に腸管内でフィラリア型幼虫に発育し，腸粘膜や肛門周囲の皮膚から再び体内に入ることがある．これを自家感染といい，糞線虫が長期に亘り持続感染する原因となっている[2]．

本患者においては，長期に亘って好酸球増多が認められ，肺の浸潤影と共に，気管支肺胞洗浄液中にも好酸球増多が認められた．糞線虫症による肺病変として，斉藤は虫体の直接的な機械的肺障害(direct destruction of vessels)すなわち肺内に寄生したラブジチス型幼虫が肺内毛細血管から遊出して血管の破綻をきたすものと，生体反応によるallergic reaction，および二次的な細菌感染によるaccompanied lesionの3つの型に分けている[3]．本症例では肺の組織学的所見が得られていないので明らかでないが，抗菌薬の効果が得られなかったことからaccompanied lesionは考え難く，前二者のいずれかであろうと考えられた．

消化器症状は本症にみられる一般的な症状で，腹痛・腹鳴，腹満感，軟便など，その程度は多様とされる．このような症例では，十二指腸や小腸上部の粘膜にカタル性ないしは浮腫性変化が強く認められ(small white powder-like protrusion)，潰瘍形成を伴うこともあるとされている[2]．糞線虫症の病理解剖学的検討を行った所見としては，十二指腸から回腸にかけて高度の浮腫状肥厚があり，組織的に粘膜に多数の虫や卵を認めたとしている[4]．私達の症例では，消化器症状は認められなかったが，これを裏付ける内視鏡所見と生検における病理所見を得ることができた．

とくに基礎疾患がない場合には，軽度の消化器症状や呼吸器症状を示す程度で，臨床上問題は少ないとされる．しかし，免疫能が低下した状態においては，本病原体による過剰感染や播種性糞線虫症の病態となることがあり，その誘因として，human T-lymphotropic virus 1(HTLV-1)，human immunodeficiency virus(HIV)感染のほか，免疫抑制剤やステロイドの投与などが考えられている[5),6]．本症例では，HTLV-1陰性で，とくに免疫不全を思わせる病態は考えられなかった．

本症の治療薬剤について，Zahaらはmebendazoleは高頻度の肝機能障害があり，albendazoleと共に，その駆虫効果は十分でなかったが，ivermectinは強い駆虫効果が認められ，毒性も少なかったことから，本症の駆虫における最も有用な薬剤として推奨している[1]．本症例においても，ivermectin 6 mg/日の2週間隔2回の投与によって，好酸球数の低下と共に諸症状の改善をみとめた．

最後に本症例の感染であるが，おそらく幼少時のミンダナオ島での生活で起こったと考えるのが妥当であろう．その後，誘因は明らかでないが，高齢化などの何らかの免疫能の低下がおこり，症状や所見が明らかになったものと推察された．しかし，長年農業に従事してきたことや，有機野菜を摂取してきたことなど，長崎県口之津での感染も全く否定はできない．日本においては最近になって，沖縄以外に宮崎[7]や福岡[8]，さらに長崎においても本症の報告がみられるようになってきた[9),10),11]．いずれにしろ，好酸球増多を伴って呼吸器系や消化器系の症状を示す感染性疾患においては，糞線虫症も念頭に入れるべきと思われた．

文　献

1) Zaha O, Hirata T, Kinjo F, Saito A: Strongyloidiasis -progress in diagnosis and treatment. Internal Medicine 39: 695-700, 2000.

2) Saito A: Strongyloidiasis: epidemiology, clinical manifestation and new methods for diagnosis and treatment. J Infect Chemother. 1: 98-106, 1995.

3) 斉藤　厚：寄生虫感染症-肺胞気道系への interaction-. Therapeutic Research 3: 1231-1236, 1992.

4) 松井克明，崎浜秀一，当山清美，伊藤悦男，長谷川英男，川平　稔，大浦　孝：糞線虫症の病理解剖学的検討．病理と臨床 1: 605-614, 1983.

5) Keiser PB and Nutman TB: Strongyloides stercoralis in the immunocompromised population. Clin Microbiol Rev. 17: 208-217, 2004.

6) Fardet L, Genereau T, Poirot JL, Guidet B, Kettaneh A, Cabane J: Severe stronlgyloidiasis in corticosteroid -treated patients: case series and literature review. J Infect. 54: 18-27, 2007.

7) Hidaka K, Ageta M, Maruyama H, Kobayashi T, Ishikawa N, Tomita M, Ide H, Itoh H, Nawa Y: Immunoserologically diagnosed strongyloides -pneumonitis with pleural effusion and eosinophilia. Jpn J parasitol. 44: 176-180, 1995.

8) 宮原道明，松木康真，三原哲文：好酸球増多を伴った糞線虫症の一例．九州大学医療技術短期大学部紀要 24: 43-48, 1997.

9) 山中淳子，中尾美紀子，松永マサ子，梅木公子：当院で経験した播種性糞線虫症の一例，五島中央紀要 11: 73-75, 2009.

10) 米満伸久，安部恵代，髙雄善則，福間利英：全身藩種性糞線虫症の1剖検例．Clinical parasitology 13: 89-91, 2002.

11) 東山康仁，坂田英雄，尾長谷靖，坂田慎吾，嶋藤登美子，森理比古，石野　徹，河野　茂，斎藤　厚，原　耕平：細菌性髄膜炎を併発した播種性糞線虫症の1例．感染症誌 71: 680-683, 1997.

（平成23年5月27日受付）

Mitochondrion 11 (2011) 273–278

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Concatenated mitochondrial DNA of the coccidian parasite *Eimeria tenella*

Kenji Hikosaka [a], Yutaka Nakai [b], Yoh-ichi Watanabe [c], Shin-Ichiro Tachibana [a], Nobuko Arisue [d], Nirianne Marie Q. Palacpac [d], Tomoko Toyama [a,d], Hajime Honma [a,e], Toshihiro Horii [d], Kiyoshi Kita [c], Kazuyuki Tanabe [a,*]

[a] *Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*
[b] *Laboratory of Sustainable Environmental Biology, Graduate School of Agricultural Science, Tohoku University, Osaki, Miyagi 989-6711, Japan*
[c] *Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan*
[d] *Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*
[e] *Japan Society for the Promotion of Science, Japan*

ARTICLE INFO

Article history:
Received 28 June 2010
Received in revised form 12 October 2010
Accepted 25 October 2010
Available online 31 October 2010

Keywords:
Mitochondrion
Mitochondrial genome
Eimeria
Plasmodium
Apicomplexa
Nuclear mitochondrial DNA

ABSTRACT

Apicomplexan parasites of the genus *Plasmodium*, pathogens causing malaria, and the genera *Babesia* and *Theileria*, aetiological agents of piroplasmosis, are closely related. However, their mitochondrial (mt) genome structures are highly divergent: *Plasmodium* has a concatemer of 6-kb unit and *Babesia/Theileria* a monomer of 6.6- to 8.2-kb with terminal inverted repeats. Fragmentation of ribosomal RNA (rRNA) genes and gene arrangements are remarkably distinctive. To elucidate the evolutionary origin of this structural divergence, we determined the mt genome of *Eimeria tenella*, pathogens of coccidiosis in domestic fowls. Analysis revealed that *E. tenella* mt genome was concatemeric with similar protein-coding genes and rRNA gene fragments to *Plasmodium*. Copy number was 50-fold of the nuclear genome. Evolution of structural divergence in the apicomplexan mt genomes is discussed.

1. Introduction

Mitochondria, organelles essential for a range of cellular processes and cellular signaling, are ubiquitous in all eukaryotes. Mitochondrial (mt) genomes exhibit remarkable variation in structure and size (Gray et al., 2004), from the 6-kb genome in the malaria parasite *Plasmodium* (Feagin, 1992) to the large (180 to 2400 kb) mt genome in land plants (Ward et al., 1981; Palmer et al., 1992). *Plasmodium* belongs to the phylum Apicomplexa, with more than 5000 species, all clinically and/or economically important pathogens (Levine, 1988): *Eimeria*, responsible for the diseases of intestinal coccidiosis in intensively reared livestock; *Toxoplasma*, etiological agent of toxoplasmosis in immune-compromised patients and congenitally infected fetuses; *Cryptosporidium*, pathogens for cryptosporidiosis in humans and animals; *Babesia*, causing babesiosis in ruminants and humans; and *Theileria*, causal agents of tropical theileriosis and East Coast fever in cattle.

Mt genomes of a few apicomplexan genera have been studied, and available data suggest that they are remarkably diverse in structure and genome organization. The minuscule 6-kb tandemly repeated linear or concatenated mtDNA of *Plasmodium* encodes only three protein-coding genes (cytochrome *c* oxidase subunits I [*cox1*] and III [*cox3*] and cytochrome *b* [*cob*]) in addition to large subunit (LSU) and small subunit (SSU) ribosomal RNA (rRNA) genes (Preiser et al., 1996). The two rRNA genes are highly fragmented with 19 identified rRNA pieces (Feagin et al., 1997). The arrangement of these mt genes is completely conserved in the genus (Perkins, 2008). In the genera of *Babesia* and *Theileria*, known as piroplasms, closely related to *Plasmodium* (Lau, 2009), the mt genomes are monomeric linear, from 6.6 kb to 8.2 kb, with terminal inverted repeats on both ends (Kairo et al., 1994; Hikosaka et al., 2010). Although the *Babesia/Theileria* mt genomes encode the same three protein-coding genes, gene array and transcriptional direction are different. Furthermore, only six fragmented LSU have been identified in the *Babesia/Theileria* mt genomes, with fragmentation different from that of *Plasmodium*. Thus, the mt genomes of *Plasmodium* and *Babesia/Theileria* are structurally highly divergent regardless of their close relatedness. Although the mt genome of *Toxoplasma gondii* has yet to be sequenced, multiple copies of partial mt genes (*cox1* and *cob*) were found to be scattered throughout the nuclear genome (Ossorio et al., 1991). In *Cryptosporidium*, the mitochondrion is reduced to mitosome and has no DNA (Mogi and Kita, 2010). The phylum Apicomplexa, therefore, encompasses a large number of interesting genera to further understand the evolution of mt genomes.

* Corresponding author. Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 4260; fax: +81 6 6879 4262.
E-mail address: kztanabe@biken.osaka-u.ac.jp (K. Tanabe).

1567-7249/$ – see front matter © 2010 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2010.10.003

In Apicomplexa, *Plasmodium* and *Babesia/Theileria* belong to the class Haematozoea, and *Eimeria* and other intestinal coccidian parasites including *Toxoplasma* belong to the class Coccidea (Hausmann and Hülsmann, 1996). The genus *Eimeria* undergoes all of its developmental stages in one host, whereas parasites belonging to the class Haematozoea require two hosts to complete their life cycles, namely, the sexual development in invertebrate vectors and the asexual development in vertebrate host. Coccidian parasites have, nevertheless, complex developmental cycles: first, oocysts excreted from the hosts undergo differentiation (sporulation) in the environment and become infective. When ingested by a host animal, oocysts undergo rounds of discrete, expansive asexual reproduction (merogony and schizogony) in the intestine, followed by sexual differentiation, fertilization and shedding of unsporulated oocysts (Jeurissen et al., 1996). *Eimeria tenella* is one of the most important *Eimeria* species, as it causes intestinal coccidiosis in domestic fowls (*Gallus gallus*), imposing enormous economic losses (Shirley et al., 2004). In *E. tenella*, two extrachromosomal DNAs have been demonstrated by pulsed-field gel electrophoresis (Dunn et al., 1998): one is the 35 kb apicoplast genome (Cai et al., 2003) and the other is a smaller size mt genome. The primary structure and the gene organization of *E. tenella* mt genome, however, remain undetermined. In this study, we report the mt genome sequence of *E. tenella*, and show that the *E. tenella* mt genome has the form of a tandemly repeated linear element or concatemer structure, and contains 19 rRNA fragments as well as three protein-coding genes. This finding indicates that the mt genomes of both *Eimeria* and *Plasmodium* retain common structural features. We discuss evolution of structural divergence in the apicomplexan mt genomes. Additionally, we identified nuclear genome DNA fragments that are shared by the mt genome of *E. tenella*.

2. Materials and methods

2.1. Blast search for mt genome sequence

A contig of *Eimeria tenella* (Houghton strain), containing mtDNA, was retrieved from *Eimeria tenella* GeneDB (http://www.genedb.org/Homepage/Etenella) using the following gene names: cytochrome *c* oxidase subunit 1, cytochrome *c* oxidase subunit 3 and cytochrome *b*. Two unfinished genomic sequences (EIMER_contig_00018071 and EIMER_contig_00018452), encoding putative COX1 and putative COB, respectively, were obtained from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/E_tenella/).

2.2. DNA sequencing

E. tenella NIAH strain was maintained at Tohoku University by routine passage through chickens. Oocyst stage parasites were collected from feces of infected chickens and purified by the centrifugation method (Nakai et al., 1993). Purified oocysts were subjected to 5 times repeated freeze–thawing. Parasite genomic DNA was isolated using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Genomic DNA of *Plasmodium gallinaceum* (8A strain) was kindly provided by the late M. Shahabuddin (NIAID/NIH, USA). Nucleotide sequences of the *E. tenella* mt genome; small subunit (SSU) and large subunit (LSU) rRNA genes of the *E. tenella* apicoplast genome; *cox1* and *cob* of the *P. gallinaceum* mt genome were determined by direct sequencing of polymerase chain reaction (PCR) products using specific primers (Supplementary Table 1A) designed from retrieved sequences. Amplification conditions, PCR product purification (QIAquick PCR purification kit, QIAGEN) and DNA sequencing of two independent PCR products were carried out as previously described (Hikosaka et al., 2010). Sequencing primers were designed to cover target regions in both directions. The sequences obtained in this study have been deposited in DDBJ/EMBL/GenBank with the following accession numbers: AB564272 to AB564276.

2.3. Gene annotation

Nucleotide sequences of *E. tenella* were aligned with reported sequences from *P. falciparum* (GenBank accession # M76611), *P. mexicanum* (EF079653), *B. bovis* (AB499088), *T. parva* (AB499089) and *T. annulata* (NW_001091933) by CLUSTAL W (Thompson et al., 1994) with manual corrections. Protein-coding genes were predicted using previously annotated sequences from the five parasite species. Putative rRNA genes were identified essentially as described (Hikosaka et al., 2010). MtDNA sequence or annotated rRNA gene fragments from *P. falciparum* (M76611) were used as query under suggested algorithm parameters (Freyhult et al., 2007) in NCBI BLAST 2.2 (Altschul et al., 1990). The termini of the candidate genes were assigned using aligned sequences and putative base-pairings between fragments proposed for *P. falciparum* mt rRNA fragments, and secondary structure predicted by CentroidHomfold (Hamada et al., 2009).

2.4. Southern blot hybridization

Genomic DNA of *E. tenella*, either undigested or digested with *Hind*III or *Pvu*II, were electrophoresed on 1.0% agarose gels in TAE (40 mM Tris–acetate, 1 mM EDTA) and transferred to a positively charged nylon membrane (Amersham Hybond-N+, GE Healthcare, Little Chalfont, England). PCR products specifically amplified for target regions of the *E. tenella* mt genome were labeled with digoxigenin-dUTP using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Rotkreuz, Switzerland). After overnight hybridization with the DIG-labeled DNA probes, blots were washed twice with 2× SSC, 0.1% SDS and twice with 0.5× SSC, 0.1% SDS, at 65 °C for 15 min. Hybridization signals were detected using Detection Starter Kit II. Southern blot hybridization was also done in parallel with *P. falciparum*, whose mt genome structure is known to be circular or linear concatenated with numerous branching off (Preiser et al., 1996).

2.5. Phylogenetic analysis

The concatenated amino acid sequences of COX1 and COB (755 sites) from 15 apicomplexan parasites (Supplementary Table 2) were used for phylogenetic analysis. A free-living ciliate, *Tetrahymena thermophila* (Brunk et al., 2003), was included as an outgroup. COX3 was not used, since *cox3* of *T. thermophila* is present in the nuclear (but not mt) genome. We constructed the maximum likelihood (ML) phylogenetic trees by the PROML program in PHYLIP version 3.68 (Felsenstein and Churchill, 1996). CODEML program in PAML version 4.2 (Yang, 2007) was used to estimate the Γ shape parameter value α. Bootstrap analysis was done by applying PROML to 100 re-sampled datasets produced by SEQBOOT program in PHYLIP.

A phylogenetic tree of LSU sequences of the mt genomes (LSUE, LSUF, LSUG and RNA10, 379 sites in total) was constructed using the ML method implemented in PAUP* 4.0 b10 (Swofford, 2002). SSU/LSU sequences of the apicoplast genomes (3045 sites) (Supplementary Table 2) were also used for phylogenetic analysis. The non-photosynthetic flagellate, *Astasia longa*, was included as an outgroup instead of *T. thermophila* which lacks a plastid genome. Bootstrap probability was estimated from 1000 heuristic replicates. For statistical comparisons among the best-tree and its alternatives, p-values of the KH test (Kishino and Hasegawa, 1989), the SH test (Shimodaira and Hasegawa, 1999) and the AU test (Shimodaira, 2002) were obtained.

2.6. Search for nuclear mitochondrial DNA in E. tenella

In some eukaryotes, nuclear genomes contain DNA segments which have a high sequence similarity to mtDNA (Caro et al., 2010; Hazkani-Covo et al., 2010). These sequences are considered to have been derived from mtDNA and thus designated as nuclear mtDNAs

(NUMTs) (Richly and Leister, 2004). The contigs (File version; assembly 2007_05_08.gz) of *E. tenella* were retrieved from the Wellcome Trust Sanger Institute, and the whole mt sequence obtained in this study was used as query under cut-off conditions of >50 nucleotides and >95% identity. We identified 21 NUMTs and confirmed these by direct sequencing (Supplementary Table 3). Two NUMTs, Emt3 and Emt4 occurring in contigs 00028951 and 00029260, respectively (Supplementary Table 1B), were used for Southern blot hybridization analysis against *E. tenella* DNA. Copy number of *E. tenella* mt genome was estimated using dot blot hybridization. Briefly, DNA fragments of the mt genome and the contig (contig_00029260) were amplified by PCR using specific primers (Supplementary Table 1C), and DNA amount was measured. Serial known dilutions of control PCR products were dot-blotted onto a nylon membrane, following heat denaturation (99 °C, 10 min). Genomic DNA were electrophoresed on agarose gels and then transferred to a nylon membrane. A PCR product specifically amplified from target regions of the *E. tenella* mt genome (Supplementary Table 1B) was labeled as described. Chemiluminescence signals were quantitated using LAS-4000mini.

3. Results and discussion

3.1. Mitochondrial genome organization

We obtained a mt genome sequence (6213 bp) from *E. tenella*, in which three protein-coding genes, *cob*, *cox1* and *cox3*, and 12 fragments of the large subunit (LSU) rRNA gene and 7 fragments of the small subunit (SSU) rRNA gene were identified (Fig. 1A). These genes and rRNA gene fragments are also present in the *P. falciparum* (Fig. 1B) (Feagin et al., 1997), although gene arrangements greatly differ between the two mt genomes.

Southern blot hybridization with a *cox3* probe (Emt1) against undigested DNA produced a smeared signal from around 4 kb to 20 kb. Hybridization against DNA digested with *Hind*III gave a major band at 6.2 kb which tailed-off to lower contiguous fragments. Hybridization against DNA digested with *Pvu*II yielded a clear signal at 2.6 kb. These signal sizes matched to those predicted from the *E. tenella* mt sequence (Fig. 2A and C). A *cox1* probe (Emt2) gave similar results (not shown). Southern blot hybridization using a *P. falciparum* probe (Pmt1) revealed a smeared signal from 6 kb to 23 kb against *P. falciparum* undigested DNA, a distinct band at 1.3 kb against *Hind*III-digested DNA, and a predominant 6.0 kb single band which tailed-off to a smear against *Pvu*II-digested DNA (Fig. 2B and D); yielding a similar hybridization pattern to *E. tenella*. This suggests that the *E. tenella* mt genome structure is similar to that of *P. falciparum*. The long tailing-off smears observed in both *E. tenella* and *P. falciparum* were not found in the mt genomes of *Babesia* and *Theileria* (Hikosaka et al., 2010), which have monomeric linear structures. The tailing-off smears probably reflect DNA fragments of various sizes branching off from polydispersed linear DNA molecules with various length termini (Preiser et al., 1996), which seems to be characteristics of a polydispersed concatenated mtDNA. In *E. tenella*, this tailing was somewhat longer than in *P. falciparum*, probably due to fragmentation caused by repeated freeze–thawing to disrupt the oocyst wall, which is highly rigid and not permeable to common solvents used for disruption. The absence of specific restriction fragments smaller than 6.2 kb after digestion with single-site enzymes suggests that the ends of the linear concatemers are not defined by telomere-like unique sequences, as seen in *Babesia* and *Theileria* (Kairo et al., 1994; Hikosaka et al., 2010). These results strongly suggest that the bulk of *E. tenella* mtDNA consist of polydisperse head-to-tail tandem arrays of the 6.2 kb element as in *P. falciparum*.

3.2. Phylogeny

The ML tree of concatenated COX1 and COB amino acid sequences revealed monophyly of the genera *Babesia* and *Theileria*, and of the genus *Plasmodium* with high BP values of 98 and 89%, respectively (Fig. 3A). *E. tenella* was positioned close to *Plasmodium* with a moderate BP value (75%). The ML tree of LSU sequences showed the same topology to that of the *cox1*+*cob* tree (Supplementary Fig. 1): *E. tenella* positioned close to *Plasmodium* with a low BP value (55%). ML tree using SSU and LSU sequences of the apicoplast genome, however, yielded a topology with *E. tenella* and *T. gondii* branching off from a common ancestor of *Plasmodium* and *Babesia/Theileria* with 100% BP (Fig. 3B). Thus, topologies of the mt trees and the apicoplast tree are not consistent. The inconsistency was not due to differences in the number of taxa used for tree construction because BP value changed little (76%) even when the number of taxa in the *cox1*+*cob* tree was reduced to the same as the apicoplast tree (data not shown).

The two mt tree topologies are also not consistent with phylogenetic trees constructed using 18S rRNA gene or hundreds of protein-coding nuclear genes (Morrison and Ellis, 1997; Philippe et al., 2004; Kuo et al., 2008), whereas the apicoplast tree is consistent with trees of nuclear genes. Since the positions of *E. tenella* in the two mt trees were not well supported with high BP values, we tested other possibilities of *E. tenella* position. The KH, the SH or the AU tests did not reject these alternative positions of *E. tenella* placed at a common ancestor of *Plasmodium* and *Babesia/Theileria* (arrow a in Fig. 3A) or at a common ancestor of *Babesia/Theileria* (arrow b in Fig. 3A) (Supplementary Table 4). Phylogenetic position of *E. tenella*, thus, remains unresolved with the mt dataset. *Eimeria* and other intestinal coccidians belong to the class Coccidea, and *Plasmodium* and *Babesia/Theileria* belong to the class Haematozoea. The two classes show remarkably different life cycles (Hausmann and Hülsmann, 1996). This taxonomical classification is consistent with phylogenetic trees

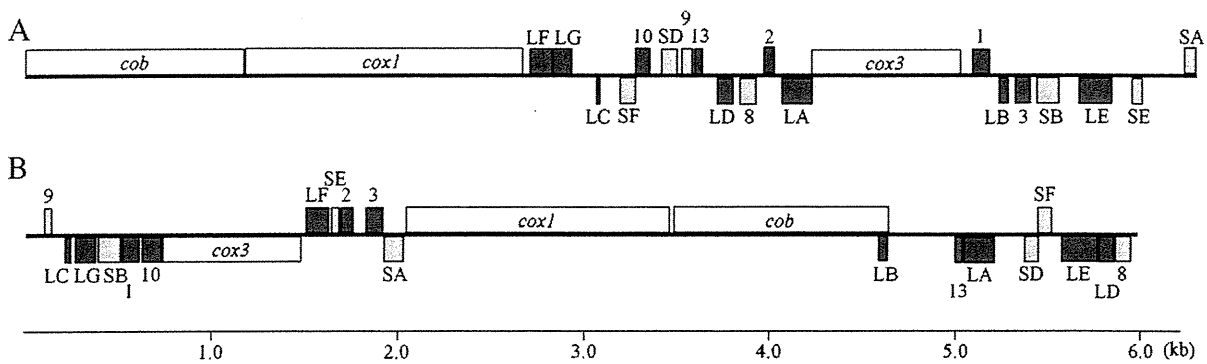


Fig. 1. Mitochondrial genome structure of *Eimeria tenella* (A) and *Plasmodium falciparum* (B). Genes shown above the bold line in each genome have predicted transcriptional directions from left to right; and those below, from right to left. Because the 6.2 kb element of *E. tenella* mt genome is tandemly repeated, both termini are arbitrary. For details refer to GenBank accession numbers AB564272 and M76611. White boxes indicate protein-coding genes (*cox1*, *cox3* and *cob*); fragments of LSU (LA–LG, 1, 2, 3, 10 and 13) and SSU (SA, SB, SD–SF, 8 and 9) rRNA genes are shown by dark and light gray boxes, respectively.

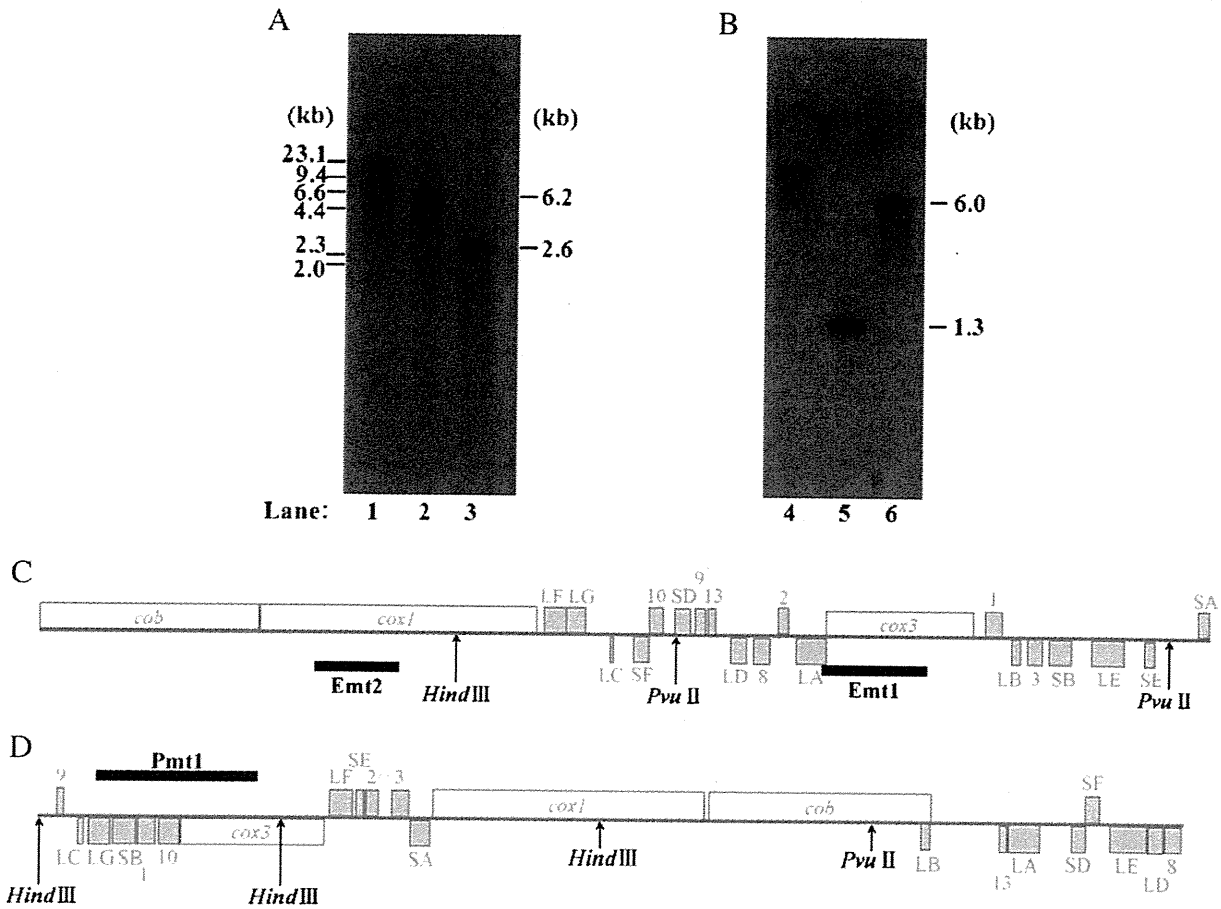


Fig. 2. Southern blot hybridization showing the mitochondrial (mt) genomes of *Eimeria tenella* (A) and *Plasmodium falciparum* (B). *E. tenella* probes (Emt1 and Emt2) and a *P. falciparum* probe (Pmt1), whose positions are shown in (C) and (D), were hybridized against undigested DNA of *E. tenella* and *P. falciparum*, respectively (lanes 1 and 4) and DNA digested with *Hind*III (lanes 2 and 5) or *Pvu*II (lanes 3 and 6).

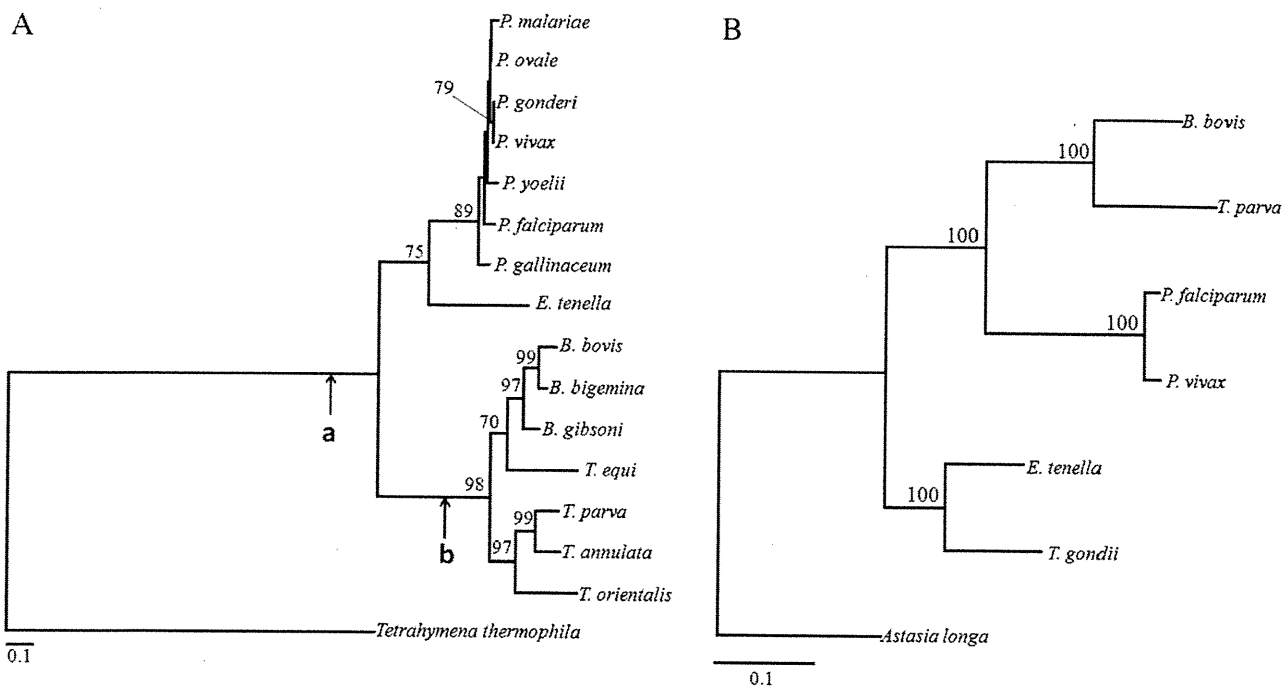


Fig. 3. Maximum likelihood phylogenetic trees of mitochondrial genes, *cox1* and *cob*, from *Plasmodium*, *Eimeria tenella*, *Babesia* and *Theileria* with *Tetrahymena thermophila* as an outgroup (A); and of apicoplast small and large subunits (SSU and LSU) of rRNA genes from six apicomplexan species with *Astasia longa* as an outgroup (B). For *cox1*+*cob* tree, concatenated amino acid sequences (755 sites) were used with 1000 heuristic replicates under a Jones, Taylor, and Thornton model (Jones et al., 1992) ($\alpha = 0.86$). For apicoplast SSU+LSU tree, concatenated nucleotide sequences (3045 sites in total: 1037 bp for SSU; 2008 bp for LSU) were used with 1000 heuristic replicates under a GTR+Γ model ($\alpha = 1.22$). Numbers shown along nodes represent bootstrap values. Arrows a and b indicate alternative positions of *E. tenella*. Both possibilities were statistically compared by the SH, KH and AU tests.

constructed using nuclear genes (Morrison and Ellis, 1997; Philippe et al., 2004; Kuo et al., 2008) and the apicoplast genome tree (Fig. 3B). We therefore consider it likely that *E. tenella* branched off from a common ancestor of *Plasmodium* clade and *Babesia/Theileria* clade.

Adopting this phylogenetic relationship allows us to infer a scenario for evolutionary trajectory of the mt genome structure of apicomplexans. Since both *Eimeria* and *Plasmodium* possess concatenated mtDNA, a common ancestor of these two parasites might have had a concatenated form of the mt genome, and the monomeric linear mt genomes of *Babesia/Theileria* were generated in the lineage. The present finding that *Eimeria* has the same 19 rRNA gene fragments as seen in the *Plasmodium* mt genome supports this scenario. Although we favor this scenario, we cannot completely rule out the possibility that a monomeric linear structure was an ancestral form and concatenated genome structures of *Eimeria* and *Plasmodium* evolved independently in each lineage. The likelihood is supported by *Tetrahymena* which has linear mt genomes, similar to those found in mt genomes of *Babesia/Theileria*. However, evolutionary distance between *Tetrahymena* and Apicomplexa is too far to gain insights into an ancestral form of the apicomplexan mt genome and, likewise, changes in molecular architecture of mt genomes are very frequent (Nosek and Tomaska, 2003). Nevertheless, it should be noted that mt genome architecture is conserved and does not change frequently within genus of the phylum: thus, in apicomplexan mt genome sequences available to date, all eight *Babesia/Theileria* species have the form of linear structure (Hikosaka et al., 2010), and all 23 *Plasmodium* species have the form of concatemer structure (Hikosaka et al., unpublished data). This within-genus stability of mt genome structure should allow us to infer an ancestral form of the apicomplexan mt genomes. In order to clarify evolutionary trajectory of the mt genome of Apicomplexa, further analysis of mt genomes of algae, closely related to apicomplexans such as *Chromera velia* and CCMP3155 (an undescribed species) would be required (Janouskovec et al., 2010).

3.3. Nuclear mitochondrial DNAs (NUMTs) in E. tenella

Blast search identified 21 sequence segments similar to the *E. tenella* mtDNA with lengths from 51 to 146 nucleotides in the *E. tenella* contigs (Supplementary Table 5). In contigs containing multiple NUMTs, several NUMTs were found arrayed in direct junction or in close proximity. Southern blot hybridization using probes Emt3 and Emt4, which contain NUMTs, gave signals derived from the *E. tenella* mt genome. We were, however, unable to detect signals derived from the nuclear genome by a similar procedure (Supplementary Fig. 2). In contrast, a probe specific to the nuclear genome (Enu1) hybridized at predicted sizes against either undigested DNA or DNA digested with *HindIII* or *Eco*RI, when a large amount of gDNA was used (data not shown). Copy number estimation analysis using Southern hybridization showed that *E. tenella* cells contained around 50 copies of the 6.2 kb element per haploid nuclear genome. The failure of detecting NUMTs in the nuclear genome with Emt3 and Emt4 was thus likely due to this copy number difference, with potential signals from the nuclear genome being masked in a smear of mtDNA.

4. Conclusion

This study suggests that the mt genome of ancestral apicomplexan parasites had a concatenated structure containing 19 rRNA gene fragments as well as three protein-coding genes and that the monomeric linear mt genome of *Babesia/Theileria* was generated in the lineage of the genera. Elucidation of a molecular mechanism, by which a linear mt genome with terminal inverted repeats on both ends was established, should help to further understand the evolution and divergence of mt genomes.

Supplementary data to this article can be found online at doi:10.1016/j.mito.2010.10.003.

Acknowledgements

This work was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18073013) and from Japan Society for Promotion of Sciences (18GS03140013 and 20390120). We would like to thank the *Eimeria tenella* genome project at the Sanger Institute, UK (http://www.sanger.ac.uk/Projects/E_tenella) for partial sequences in the design of some PCR primers.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.
Brunk, C.F., Lee, L.C., Tran, A.B., Li, J., 2003. Complete sequence of the mitochondrial genome of *Tetrahymena thermophila* and comparative methods for identifying highly divergent genes. Nucleic Acids Res. 31 (6), 1673–1682.
Cai, X., Fuller, A.L., McDougald, L.R., Zhu, G., 2003. Apicoplast genome of the coccidian *Eimeria tenella*. Gene 321, 39–46.
Caro, P., Gomez, J., Arduini, A., Gonzalez-Sanchez, M., Gonzalez-Garcia, M., Borras, C., Vina, J., Puertas, M.J., Sastre, J., Barja, G., 2010. Mitochondrial DNA sequences are present inside nuclear DNA in rat liver and increase with age. Mitochondrion 10 (5), 479–486.
Dunn, P.P., Stephens, P.J., Shirley, M.W., 1998. *Eimeria tenella*: two species of extrachromosomal DNA revealed by pulsed-field gel electrophoresis. Parasitol. Res. 84 (4), 272–275.
Feagin, J.E., 1992. The 6-kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes. Mol. Biochem. Parasitol. 52 (1), 145–148.
Feagin, J.E., Mericle, B.L., Werner, E., Morris, M., 1997. Identification of additional rRNA fragments encoded by the *Plasmodium falciparum* 6 kb element. Nucleic Acids Res. 25 (2), 438–446.
Felsenstein, J., Churchill, G.A., 1996. A Hidden Markov Model approach to variation among sites in rate of evolution. Mol. Biol. Evol. 13 (1), 93–104.
Freyhult, E.K., Bollback, J.P., Gardner, P.P., 2007. Exploring genomic dark matter: a critical assessment of the performance of homology search methods on noncoding RNA. Genome Res. 17 (1), 117–125.
Gray, M.W., Lang, B.F., Burger, G., 2004. Mitochondria of protists. Annu. Rev. Genet. 38, 477–524.
Hamada, M., Kiryu, H., Sato, K., Mituyama, T., Asai, K., 2009. Prediction of RNA secondary structure using generalized centroid estimators. Bioinformatics 25 (4), 465–473.
Hausmann, K., Hülsmann, N., 1996. Protozoology, 2nd Edition. Georg Thieme Pub.
Hazkani-Covo, E., Zeller, R.M., Martin, W., 2010. Molecular poltergeists: mitochondrial DNA copies (numts) in sequenced nuclear genomes. PLoS Genet. 6 (2), e1000834.
Hikosaka, K., Watanabe, Y., Tsuji, N., Kita, K., Kishine, H., Arisue, N., Palacpac, N.M., Kawazu, S., Sawai, H., Horii, T., Igarashi, I., Tanabe, K., 2010. Divergence of the mitochondrial genome structure in the apicomplexan parasites, Babesia and Theileria. Mol. Biol. Evol. 27 (5), 1107–1116.
Janouskovec, J., Horak, A., Obornik, M., Lukes, J., Keeling, P.J., 2010. A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. Proc. Natl Acad. Sci. USA 107 (24), 10949–10954.
Jeurissen, S.H., Janse, E.M., Vermeulen, A.N., Vervelde, L., 1996. *Eimeria tenella* infections in chickens: aspects of host–parasite: interaction. Vet. Immunol. Immunopathol. 54 (1–4), 231–238.
Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8 (3), 275–282.
Kairo, A., Fairlamb, A.H., Gobright, E., Nene, V., 1994. A 7.1 kb linear DNA molecule of *Theileria parva* has scrambled rDNA sequences and open reading frames for mitochondrially encoded proteins. EMBO J. 13 (4), 898–905.
Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. J. Mol. Evol. 29 (2), 170–179.
Kuo, C.H., Wares, J.P., Kissinger, J.C., 2008. The Apicomplexan whole-genome phylogeny: an analysis of incongruence among gene trees. Mol. Biol. Evol. 25 (12), 2689–2698.
Lau, A.O., 2009. An overview of the *Babesia*, *Plasmodium* and *Theileria* genomes: a comparative perspective. Mol. Biochem. Parasitol. 164 (1), 1–8.
Levine, N.D., 1988. Progress in taxonomy of the Apicomplexan protozoa. J. Protozool. 35 (4), 518–520.
Mogi, T., Kita, K., 2010. Diversity in mitochondrial metabolic pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. Parasitol. Int. 59 (3), 305–312.
Morrison, D.A., Ellis, J.T., 1997. Effects of nucleotide sequence alignment on phylogeny estimation: a case study of 18S rDNAs of apicomplexa. Mol. Biol. Evol. 14 (4), 428–441.
Nakai, Y., Edamura, K., Kanazawa, K., Shimizu, S., Hirota, Y., Ogimoto, K., 1993. Susceptibility to *Eimeria tenella* of chickens and chicken embryos of partly inbred lines possessing homozygous major histocompatibility complex haplotypes. Avian Dis. 37 (4), 1113–1116.
Nosek, J., Tomaska, L., 2003. Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. Curr. Genet. 44 (2), 73–84.
Ossorio, P.N., Sibley, L.D., Boothroyd, J.C., 1991. Mitochondrial-like DNA sequences flanked by direct and inverted repeats in the nuclear genome of *Toxoplasma gondii*. J. Mol. Biol. 222 (3), 525–536.
Palmer, J.D., Soltis, D., Soltis, P., 1992. Large size and complex structure of mitochondrial DNA in two nonflowering land plants. Curr. Genet. 21 (2), 125–129.

Perkins, S.L., 2008. Molecular systematics of the three mitochondrial protein-coding genes of malaria parasites: corroborative and new evidence for the origins of human malaria. Mitochondrial DNA 19 (6), 471–478.
Philippe, H., Snell, E.A., Bapteste, E., Lopez, P., Holland, P.W., Casane, D., 2004. Phylogenomics of eukaryotes: impact of missing data on large alignments. Mol. Biol. Evol. 21 (9), 1740–1752.
Preiser, P.R., Wilson, R.J., Moore, P.W., McCready, S., Hajibagheri, M.A., Blight, K.J., Strath, M., Williamson, D.H., 1996. Recombination associated with replication of malarial mitochondrial DNA. EMBO J. 15 (3), 684–693.
Richly, E., Leister, D., 2004. NUMTs in sequenced eukaryotic genomes. Mol. Biol. Evol. 21 (6), 1081–1084.
Shimodaira, H., 2002. An approximately unbiased test of phylogenetic tree selection. Syst. Biol. 51 (3), 492–508.
Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16 (8), 1114–1116.
Shirley, M.W., Ivens, A., Gruber, A., Madeira, A.M., Wan, K.L., Dear, P.H., Tomley, F.M., 2004. The *Eimeria* genome projects: a sequence of events. Trends Parasitol. 20 (5), 199–201.
Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, MA.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 (22), 4673–4680.
Ward, B.L., Anderson, R.S., Bendich, A.J., 1981. The mitochondrial genome is large and variable in a family of plants (cucurbitaceae). Cell 25 (3), 793–803.
Yang, Z., 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24 (8), 1586–1591.

Parasitology International 60 (2011) 175–180

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Highly conserved gene arrangement of the mitochondrial genomes of 23 *Plasmodium* species

Kenji Hikosaka [a], Yoh-ichi Watanabe [b], Fumie Kobayashi [c], Seiji Waki [d], Kiyoshi Kita [b], Kazuyuki Tanabe [a,*]

[a] Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan
[b] Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
[c] Department of Infectious Diseases, Faculty of Medicine, Kyorin University, Mitaka, Tokyo, Japan
[d] Gunma Prefectural College of Health Sciences, Maebashi, Gunma, Japan

ARTICLE INFO

Article history:
Received 22 November 2010
Received in revised form 25 January 2011
Accepted 7 February 2011
Available online 15 February 2011

Keywords:
Mitochondrion
Plasmodium
Apicomplexa
Genome structure
Inverted repeat sequence
Evolution

ABSTRACT

Mitochondrial (mt) genomes from diverse phylogenetic groups vary considerably in size, structure and organization. The genus *Plasmodium*, the causative agent of malaria, has the smallest mt genome in the form of a tandemly repeated, linear element of 6 kb. The *Plasmodium* mt genome encodes only three protein genes (*cox1*, *cox3* and *cob*) and large- and small-subunit ribosomal RNA (rRNA) genes, which are highly fragmented with 19 identified rRNA pieces. The complete mt genome sequences of 21 *Plasmodium* species have been published but a thorough investigation of the arrangement of rRNA gene fragments has been undertaken for only *Plasmodium falciparum*, the human malaria parasite. In this study, we determined the arrangement of mt rRNA gene fragments in 23 *Plasmodium* species, including two newly determined mt genome sequences from *P. gallinaceum* and *P. vinckei vinckei*, as well as *Leucocytozoon caulleryi*, an outgroup of *Plasmodium*. Comparative analysis reveals complete conservation of the arrangement of rRNA gene fragments in the mt genomes of all the 23 *Plasmodium* species and *L. caulleryi*. Surveys for a new rRNA gene fragment using hidden Markov models enriched with recent mt genome sequences led us to suggest the mtR-26 sequence as a novel candidate LSU rRNA fragment in the mt genomes of the 24 species. Additionally, we found 22–25 bp-inverted repeat sequences, which may be involved in the generation of lineage-specific mt genome arrangements after divergence from a common ancestor of the genera *Eimeria* and *Plasmodium/Leucocytozoon*.

1. Introduction

Mitochondria are essential organelles required for energy transduction and cellular functions, and are ubiquitous in almost all eukaryotic cells. Like nuclear genomes, mitochondrial (mt) genomes exhibit remarkable variation in structure and size [1]. The largest mt genome is found in land plants, in which the size ranges from 180 to 2400 kb [2,3]. The smallest mt genome is 6 kb and is found in the genus *Plasmodium*, the causal agent of malaria, which belongs to the phylum Apicomplexa. Almost all members of the Apicomplexa are clinically and/or economically important pathogenic parasites [4]. The mt genomes of apicomplexan parasites are highly diverse in structure and organization. In *Plasmodium*, the mt genome at the asexual erythrocytic stages is a tandemly repeated linear 6-kb element [5]. The 6-kb element contains only three protein-coding genes (cytochrome *c* oxidase subunit I [*cox1*] and III [*cox3*] and cytochrome *b* [*cob*]) and large subunit (LSU) and small subunit (SSU) ribosomal RNA (rRNA) genes. The two rRNA genes are highly fragmented with 19 identified rRNA sequences [6]. In *Babesia* and *Theileria*, genera that are closely related to *Plasmodium* [7], the mt genomes are monomeric linear, with sizes ranging from 6.6 kb to 8.2 kb, and contain terminal inverted repeats on both ends [8,9]. Although the *Babesia/Theileria* mt genomes encode the same three protein-coding genes as *Plasmodium*, gene arrangements and transcriptional direction are different from *Plasmodium*. Furthermore, only six fragmented LSU rRNA sequences have been identified in the *Babesia/Theileria* mt genomes, and the pattern of fragmentation differs drastically from *Plasmodium* [9]. Thus, the mt genomes of *Plasmodium* and *Babesia/Theileria* are structurally highly divergent.

We have recently shown that the mt genome of *Eimeria tenella*, which is distantly related to *Plasmodium* and *Babesia/Theileria*, has a concatemeric form and contains the same three protein-coding genes and 19 rRNA gene fragments as *Plasmodium*. This suggests that a concatemeric structure is an ancestral form of *Plasmdoium* and *Eimeria* mt genomes [10]. In spite of this similarity, the gene arrangements and transcriptional direction greatly differ between the *Plasmodium* and *Eimeria* mt genomes. It remains unknown how these differences were generated. Furthermore, even though the complete mt genome sequences are available for 21 *Plasmodium* species, little is known

* Corresponding author at: Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 4260; fax: +81 6 6879 4262.
E-mail address: kztanabe@biken.osaka-u.ac.jp (K. Tanabe).

1383-5769/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2011.02.001

about the variation in arrangement of the mt rRNA gene fragments. In this study, we report the arrangement of mt rRNA gene fragments in 23 *Plasmodium* species, including two newly determined mt genome sequences from *P. gallinaceum* and *P. vinckei vinckei*, as well as *Leucocytozoon caulleryi*, an outgroup of *Plasmodium*. There is complete conservation of the arrangement of rRNA gene fragments in the mt genomes of all the *Plasmodium* species and *L. caulleryi*. A novel candidate LSU rRNA fragment was found in the mt genomes of the parasite species. Additionally, 22- to 25-bp inverted repeat sequences were detected, which may be involved in the generation of lineage-specific mt genome arrangements.

2. Materials and methods

2.1. DNA sequencing

Genomic DNA of *P. gallinaceum* (A8 strain) was kindly provided by the late M. Shahabuddin (NIAID/NIH, USA). Genomic DNA of *P. vinckei vinckei* was extracted using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) as previously described [11]. Nucleotide sequences of *P. gallinaceum* and *P. vinckei vinckei* mt genomes were determined by direct sequencing of polymerase chain reaction (PCR) products using conserved primers (Supplementary Table 1), which were designed by aligning the mt genome sequences of *P. falciparum* (DDBJ/EMBL/GenBank accession number M76611), *P. chabaudi* (AB379663), *P. relictum* (AY733089) and *P. mexicanum* (AB375765). The PCR amplification conditions, product purification and DNA sequencing protocol were the same as previously described [11]. Sequencing primers were designed to cover target regions in both directions. DNA sequencing was conducted on a 3130 Genetyx Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA) and was with at least two independent amplification products and sequencing runs. The sequences obtained in this study have been deposited in DDBJ/EMBL/GenBank with the following accession numbers: AB599930 (*P. gallinaceum* mt genome) and AB599931 (*P. vinckei vinckei* mt genome).

2.2. Gene annotation

Nucleotide sequences of the mt genomes from *P. gallinaceum* and *P. vinckei vinckei* and their deduced amino acid sequences were aligned with the mt genome sequences of *P. falciparum* (M76611) and other 20 *Plasmodium* species plus *L. caulleryi* retrieved from GenBank (Table 1) using ClustalW [12] with manual corrections. Protein-coding genes were predicted using previously annotated sequences from *P. falciparum*.

Putative rRNA gene fragments were identified using the annotated rRNA gene fragments from *P. falciparum* (M76611) as a query sequence [6] under suggested algorithm parameters [13] in NCBI BLAST 2.2 [14]. The termini of candidate gene fragments were determined using aligned sequences. The accession numbers of mt genomes used for identifying rRNA gene fragments are shown in Table 1.

Hidden Markov model of bacterial rRNA has recently been suggested to be useful to search for fragmented rRNA genes in metagenomic data [15]. In addition, the alpha-proteobacterial origin of mitochondria is widely accepted [16], and mt and bacterial rRNAs are closely related. We thus applied this model to identify novel rRNA gene fragments in *P. falciparum*. We also performed BLAST searches using *Tetrahymena pyriformis* mt rRNA fragments (AF160864). *T. pyriformis*, a member of ciliates, is closely related to the phylum Apicomplexa [17], and its mt rRNAs are fragmented [18,19]. Putative regions, missing from the *P. falciparum* mt rRNA, were surveyed with possible consensus sequence/structure from mt rRNAs [20] using scan_for_matches [21]. In addition, hidden Markov models constructed using mt sequences from *Babesia gibsoni* (AB499087), *Theileria orientalis* (AB499090) and *Theileria equi* (AB499091) [9] with HMMER 1.8.3 [22] were used to survey for mt genomes from representative *Plasmodium*/*Leucocytozoon* species (*P. falciparum*, *P. fragile*, and *L. caulleryi*).

2.3. Search for direct and inverted repeat sequences

Repeat sequences, such as an inverted repeat sequence, can potentially form secondary structures and may be involved in rearrangement events of genomes. Therefore, searches for repeat sequences were performed on the mt genomes for the 24 species used in this study (Table 1) and *E. tenella* (AB564272), which contains the same three protein-coding genes and 19 rRNA gene fragments as *Plasmodium* [10]. These searches were performed using the program REPFIND (http://zlab.bu.edu/repfind/) [23] under cut-off conditions of >10 nucleotides and a *P*-value<0.0001. Inverted repeat sequences were searched for using a 'self against self' BLASTN search [24] under cut-off conditions of >10 nucleotides. Furthermore, additional searches for repeats and

Table 1
Twenty three *Plasmodium* species and one *Leucocytozoon* species used in this study.

Species	Strain	Host	Accession number
Plasmodium gallinaceum	A8 strain	Bird	AB599930 (this study)
Plasmoidum vinckei vinckei	–	Rodent	AB599931 (this study)
Plasmodium falciparum	C10 line	Human	M76611
Plasmodium vivax	Salvador I	Human	NC_007243
Plasmodium malariae	Uganda I	Human	AB354570
Plasmodium ovale	Nigeria II	Human	AB354571
Plasmodium reichenowi	CDC1	Chimpanzee	NC_002235
Plasmodium hylobati	WAK (ATCC30194)	Gibbon	AB354573
Plasmodium cynomolgi	Langur	Monkey	AB434919
Plasmodium simiovale	(ATCC 30140)	Monkey	AB434920
Plasmodium fieldi	N-3 strain (ATCC30163)	Monkey	AB354574
Plasmodium inui	IM-Perak (ATCC30156)	Monkey	AB354572
Plasmodium fragile	Hackeri	Monkey	AY722799
Plasmodium coatneyi	CDC strain	Monkey	AB354575
Plasmodium knowlesi	Malayan strain (ATCC30192)	Monkey/Human	NC_007232
Plasmodium gonderi	(ATCC 30045)	Monkey	AB434918
Plasmodium yoelii	17XNL	Rodent	MALPY00209
Plasmodium chabaudi	AS strain	Rodent	AB379663
Plasmodium berghei	ANKA strain	Rodent	ANKA contig 5406
Plasmodium relictum	(ATCC 30141)	Bird	AY733089
Plasmodium juxtanucleare	–	Bird	AB250415
Plasmodium mexicanum	–	Lizard	AB375765
Plasmodium floridense	–	Lizard	NC_009961
Leucocytozoon caulleryi	–	Bird	AB302215

inverted repeats were performed using GENETYX software (Version 8; SDC, Tokyo, Japan).

3. Results

3.1. Mitochondrial genome organization of *P. gallinaceum* and *P. vinckei vinckei*

We sequenced the mt genomes from *P. gallinaceum* (6.0 kb) and *P. vinckei vinckei* (5.9 kb), and identified three protein-coding genes (Fig. 1A and B). These genes are syntenic to the *P. falciparum* mt genome (Fig. 1C).

Using the *P. falciparum* mt genome sequence as a query, we also identified 12 fragments of LSU rRNA gene and seven fragments of SSU rRNA gene in the mt genomes of *P. gallinaceum*, *P. vinckei vinckei*, and 20 other *Plasmodium* species and *L. caulleryi* (Supplementary Table 2). Several approaches were undertaken to explore putative missing sequences of *Plasmodium* mt rRNA gene fragments. Searches with bacterial rRNA hidden Markov models and BLAST searches with *T. pyriformis* mt rRNA sequences both failed to detect any additional fragmented rRNA candidates. Additional searches using hidden Markov models constructed with *Babesia/Theileria* sequences revealed that six nucleotides in mtR-26, a recently identified *P. falciparum* transcript [25], are complementary to a region in RNA1, one of the mt LSU rRNA fragments of *P. falciparum*, if G–U pairs are allowed (Fig. 2A). G–U "wobble" base pairing is one of the most frequently found "mismatches" in various RNAs including rRNAs [26]. Thus, the interaction between RNA1 and mtR-26 including G–U pair(s) seems to be very likely. The mtR-26 corresponds to *E. coli* LSU rRNA positions between 579–584 and flanking regions, and is perfectly conserved in the mt genomes of the 23 *Plasmodium* species and *L. caulleryi* (Fig. 2B) (Supplementary Table 2). Thus, we suggest that *P. falciparum* mtR-26 and its putative homologs in 22 *Plasmodium* species (as well as *L. caulleryi*) are a fragmented LSU rRNA. The organization and predicted transcriptional direction of rRNA gene fragments including mtR-26 are completely conserved among the 24 parasite species examined here.

3.2. Comparison of mt genome sequences

Pairwise sequence identity of *cox3*, *cox1* and *cob* is comparable among the 23 *Plasmodium* species and *L. caulleryi* (Table 2): 79.9–98.9% for *cox3*, 85.0–99.2% for *cox1* and 82.5–99% for *cob* at the nucleotide sequence level, and 79.6–99.6% for COX3, 90.6–100% for COX1 and 82.2–100% for COB at the amino acid sequence level. In contrast to these protein-coding genes, pairwise sequence identity of 20 rRNA gene fragments is very high, 94.4–99.9% (94.7–100% for 13 LSUs and 93.8–100% for seven SSUs) among the 24 species. Pairwise sequence identity of intergenic regions is also high, 89.5–99.4% among the 24 species. Pairwise sequence identity of 10 representative parasites is given in Supplementary Tables 3–5.

Small differences in size of the 24 mt genomes (5948 bp of *P. vinckei vinckei* to 6014 bp of *P. juxtanucleare*) are due to variations in the number of A or T homopolymers, and insertion/deletion of A or T in rRNA gene fragments and intergenic regions.

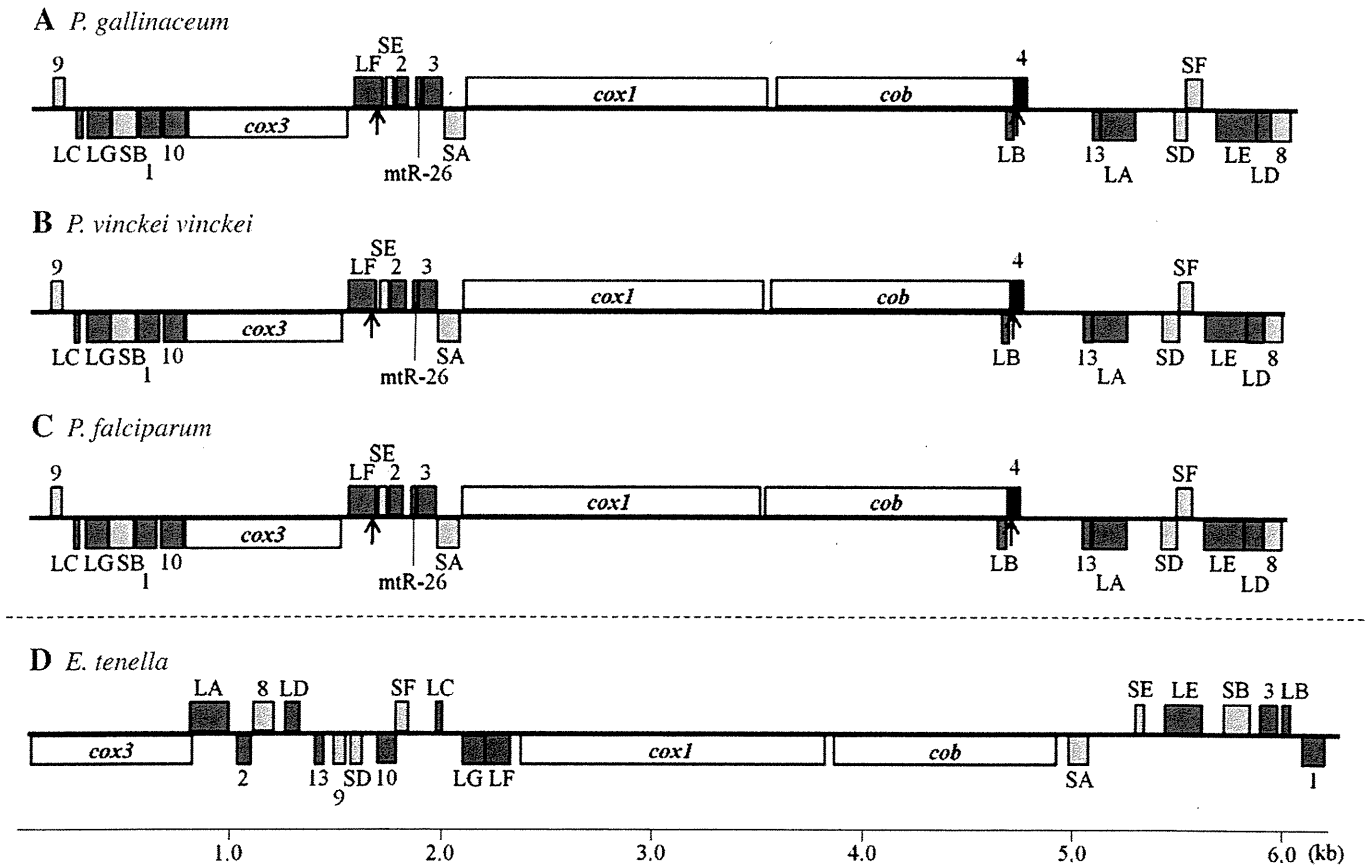


Fig. 1. Structure of the mitochondrial (mt) genomes of *P. gallinaceum* (A) and *P. vinckei vinckei* (B). Shown for comparison are the mt genomes of *P. falciparum* (C) (M76611) and *Eimeria tenella* (D) (AB564272). Elements within these genomes are tandemly repeated, so the designation of both termini is arbitrary. Genes shown above the bold line in each genome are transcribed left to right and those below are transcribed from right to left. Dark and light grey boxes indicate fragments of LSU and SSU rRNA genes, respectively. Black boxes indicate transcript, RNA4, which has not been annotated (M76611). Arrows indicate the position of inverted repeat sequences. *cox1*, cytochrome *c* oxidase subunit I; *cox3*, cytochrome *c* oxidase subunit III; *cob*, cytochrome *b*.

A

```
RNA1     5' G C U G A C 3'
             .  *  .  .  *  *
mtR-26   3' U G G U U U G 5'
```

B

```
P. falciparum mtR-26  T G T T A T G T T G T T G G T T T A A G C C C
P. gallinaceum        . . . . . . C . . . . . . . . . . . . . . . .
P. vinckei vinckei    . . . . . . . . . . . . . . . . . . . . . . .
P. vivax              . . . . . . . . . . . . . . . . . . . . . . .
P. malariae           . . . . . . . . . . . . . . . . . . . . . . .
P. ovale              . . . . . . . . . . . . . . . . . . . . . . .
P. reichenowi         . . . . . . . . . . . . . . . . . . . . . . .
P. hylobati           . . . . . . . . . . . . . . . . . . . . . . .
P. cynomolgi          . . . . . . . . . . . . . . . . . . . . . . .
P. simiovale          . . . . . . . . . . . . . . . . . . . . . . .
P. fieldi             . . . . . . . . . . . . . . . . . . . . . . .
P. inui               . . . . . . . . . . . . . . . . . . . . . . .
P. fragile            . . . . . . . . . . . . . . . . . . . . . . .
P. coatneyi           . . . . . . . . . . . . . . . . . . . . . . .
P. knowlesi           . A . . . . . . . . . . . . . . . . . . . . .
P. gonderi            . . . . . . . . . . . . . . . . . . . . . . .
P. yoelii             . . . . . . . . . . . . . . . . . . . . . . .
P. chabaudi           . . . . . . . . . . . . . . . . . . . . . . .
P. berghei            . . . . . . . . . . . . . . . . . . . . . . .
P. relictum           . . . . . . . C . . . . . . . . . . . . . . .
P. juxtanucleare      . . . . . . . C . . . . . . . . . . . . . . .
P. mexicanum          . . . . . . . C . . . . . . . . . . . . . . .
P. floridense         . . . . . . . C . . . . . . . . . . . . . . .
L. caulleryi          . . . . . . . C . . . . . . . . . . . . . . .
```

Fig. 2. Complementarity between *P. falciparum* mtR-26 and RNA1 (A), and nucleotide sequence alignments of *P. falciparum* mtR-26 and its homologs from 22 *Plasmodium* species and *Leucocytozoon caulleryi* (B). In panel A, the six nucleotides of mtR-26 correspond to *E. coli* LSU rRNA positions between 579 and 584. Dots and asterisks indicate G–U base pairing motifs and A–U/G–C pairs, respectively. In panel B, a predicted sequence region complementary to RNA1 is boxed. Dots indicate identical nucleotides to *P. falciparum* mtR-26.

3.3. Repeat sequences of the Plasmodium mt genomes

Searches for direct repeat sequences identified one to six repeats with lengths of 10 to 15 bp in the mt genomes of all species examined here, except *P. gallinaceum* and *P. floridense*. These repeat sequences are not conserved among the 23 species but one pair of inverted repeat sequences of 22 to 25 bp is conserved in all *Plasmodium* species and *L. caulleryi* (Fig. 3). These inverted repeat sequences were located at the 3′ region of LSUF, which has been identified as a LSU rRNA gene fragment and at the 5′ region of RNA4, the function of which in the *P. falciparum* mt genome (M76611) is unknown (Feagin, personal communication). The nucleotide positions of RNA4 in each species examined here are shown in Supplementary Table 2. In contrast, no inverted repeat sequences were found in the *E. tenella* mt genome.

4. Discussion

This study shows that the organization and predicted transcriptional direction of mt protein-coding genes and mt rRNA gene fragments are highly conserved among the 23 *Plasmodium* species and *L. caulleryi* examined. In addition, arrangements and nucleotide sequences of intergenic regions ranging from 953 bp to 1011 bp, which represent approximately one-sixth of the 6-kb genome, are also highly conserved. Recently, transcription of almost all intergenic regions of *P. falciparum* mt genome has been demonstrated [25]. These highly conserved sequence regions may thus code for functional RNAs. This study suggests that one of the transcripts, mtR-26, is a fragment of the LSU rRNA gene.

The high degree of conservation of the *Plasmodium* mt genome structure may be due to structural constraints on the genome. In general, mt genomes display a tendency of size reduction or deletional bias [27]. Since the *Plasmodium* mt genome is the smallest so far known, a further reduction and rearrangement of sequences may deleteriously affect the function of their genomes. Consistent with this proposition, the size and gene arrangement of vertebrate mt genomes remain virtually unchanged over long evolutionary time; e.g. the gene arrangements of human and trout mt genomes are nearly identical [28]. Although vertebrate mt genomes are much larger (16 kb) than the *Plasmodium* mt genome, the high conservation of vertebrate mt genome structures supports our proposition that structural constraints limit mt genome change.

In the present study, an inverted repeat sequence was identified in all *Plasmodium* species and *L. caulleryi*. The presence of this inverted repeat sequence allows us to infer a scenario for the evolutionary trajectory of the mt genome structures of *Plasmodium* and *E. tenella*. A comparison of the arrangements of three protein coding genes between *Plasmodium* and *Eimeria* suggests that at least one inversion event of a region containing *cox1/cob* or *cox3* must have occurred in an ancestral lineage leading to either *Plasmodium* or *Eimeria*. The inverted repeat sequences are located 5′ to *cox1* and 3′ to *cob*. Therefore, these inverted repeats may have been involved in an inversion event of either *cox1/cob* or *cox3* in an ancestral lineage. The present data set does not predict whether the inverted repeats were inserted into an ancestral lineage of *Plasmodium* or deleted from an ancestral lineage of *Eimeria*.

Table 2
Sequence identity of protein-coding genes, rRNA gene fragments and the intergenic region of the mitochondrial genomes of 23 *Plasmodium* species and *Leucocytozoon caulleryi*.

Region		No. of sites	Sequence identity	
			Mean ± SE[a] (%)	Range (%)
Protein-coding gene	*cox3* + *cox1* + *cob*	3318 nt	89.4 ± 0.35	83.0–98.9
	(Amino acid sequence)	(1106 aa)	(92.0 ± 0.47)	(85.6–99.5)
	cox3	753 nt	88.0 ± 0.61	79.9–98.9
	(Amino acid sequence)	(251 aa)	(88.4 ± 1.14)	(79.6–99.6)
	cox1	1434 nt	89.6 ± 0.42	85.0–99.2
	(Amino acid sequence)	(478 aa)	(94.7 ± 0.61)	(90.6–100)
	Cob	1131 nt	89.9 ± 0.45	82.5–99.0
	(Amino acid sequence)	(377 aa)	(91.0 ± 0.85)	(82.2–100)
rRNA gene fragment	LSU + SSU	1624 nt	97.8 ± 0.20	94.4–99.9
	LSU	1091 nt	97.7 ± 0.26	94.7–100
	SSU	533 nt	97.9 ± 0.30	93.8–100
Intergenic regions		884 nt	94.3 ± 0.39	89.5–99.4

[a] Standard error (SE) estimates were obtained by a bootstrap procedure (500 replicates) using MEGA4 [30].

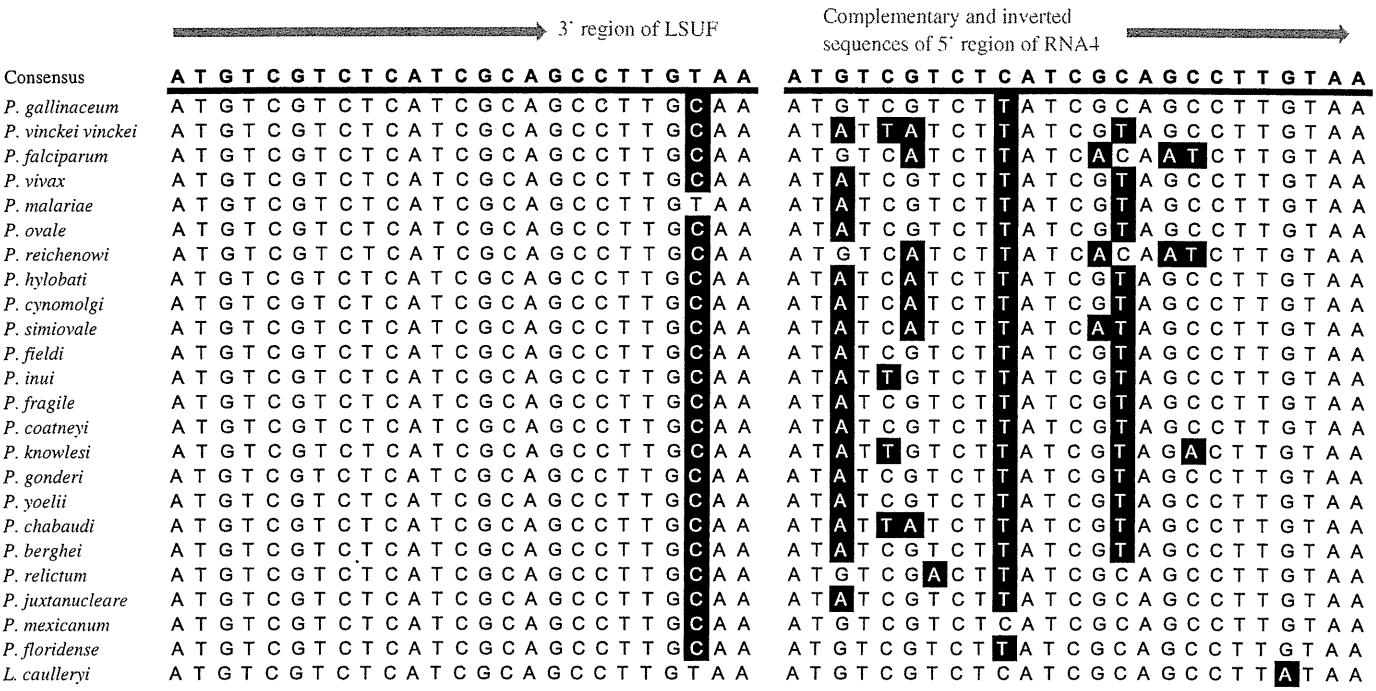


Fig. 3. Nucleotide sequence alignments of inverted repeats in the mt genomes of 23 *Plasmodium* species and *Luecocytozoon caulleryi*. Alignments are located at the 3′ region of LSUF (left) and 5′ region of RNA4 (right). RNA4 sequences shown are complementary and inverted. White characters highlighted in black indicate nucleotides differing from the consensus sequence.

A closer examination of the gene order of *cox1* and *cob*, as well as many of the 19 rRNA gene fragments, reveals that it is considerably different between *Plasmodium* and *Eimeria*. This difference cannot be explained by an inversion event. It is generally assumed that the duplication and random loss (TDRL) model [28] accounts for changes in gene order. According to the TDRL model, genes or gene fragments are duplicated and some of these are subsequently lost thereby generating a new gene order. For example, a duplication of a segment containing *cox1/cob* in the *Plasmodium* mt genome, followed by the loss of the 5′-end *cox1* and the 3′-end *cob*, can generate the gene order of *cob/cox1*, which is found in the *Eimeria* mt genome (shown in a complementary direction, in Fig. 1). However, rearrangements of rRNA gene fragments are very extensive between the mt genomes of *Plasmodium* and *Eimeria* (Fig. 1). Therefore, TDRL model may be too simple to adequately account for the observed rearrangements of rRNA gene fragments.

Complete mt genome sequences are now available for 23 *Plasmodium* species but the biological function of their very compact mt genome remains largely unknown [29]. It is extremely difficult to purify and therefore characterize mitochondria from *Plasmodium*. The high conservation of mt genomes among *Plasmodium* species may shed some light on the importance of mt function. Further molecular and biochemical studies are required to determine the significance of this sequence conservation.

Acknowledgements

This work was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18073013) and from Japan Society for Promotion of Sciences (18GS03140013 and 20390120).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.parint.2011.02.001.

References

[1] Gray MW, Lang BF, Burger G. Mitochondria of protists. Annu Rev Genet 2004;38:477–524.
[2] Ward BL, Anderson RS, Bendich AJ. The mitochondrial genome is large and variable in a family of plants (cucurbitaceae). Cell 1981;25:793–803.
[3] Palmer JD, Soltis D, Soltis P. Large size and complex structure of mitochondrial DNA in two nonflowering land plants. Curr Genet 1992;21:125–9.
[4] Levine ND. Progress in taxonomy of the apicomplexan protozoa. J Protozool 1988;35:518–20.
[5] Wilson RJ, Williamson DH. Extrachromosomal DNA in the apicomplexa. Microbiol Mol Biol Rev 1997;61:1–16.
[6] Feagin JE, Mericle BL, Werner E, Morris M. Identification of additional rRNA fragments encoded by the *Plasmodium falciparum* 6 kb element. Nucleic Acids Res 1997;25:438–46.
[7] Lau AO. An overview of the *Babesia*, *Plasmodium* and *Theileria* genomes: a comparative perspective. Mol Biochem Parasitol 2009;164:1–8.
[8] Kairo A, Fairlamb AH, Gobright E, Nene V. A 7.1 kb linear DNA molecule of *Theileria parva* has scrambled rDNA sequences and open reading frames for mitochondrially encoded proteins. EMBO J 1994;13:898–905.
[9] Hikosaka K, Watanabe Y, Tsuji N, Kita K, Kishine H, Arisue N, et al. Divergence of the mitochondrial genome structure in the apicomplexan parasites, Babesia and Theileria. Mol Biol Evol 2010;27:1107–16.
[10] Hikosaka K, Nakai Y, Watanabe Y, Tachibana SI, Arisue N, Palacpac NM, et al. Concatenated mitochondrial DNA of the coccidian parasite *Eimeria tenella*. Mitochondrion 2011;11:273–8.
[11] Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. Exp Parasitol 2001;97:50–4.
[12] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
[13] Freyhult EK, Bollback JP, Gardner PP. Exploring genomic dark matter: a critical assessment of the performance of homology search methods on noncoding RNA. Genome Res 2007;17:117–25.
[14] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[15] Huang Y, Gilna P, Li W. Identification of ribosomal RNA genes in metagenomic fragments. Bioinformatics 2009;25:1338–40.
[16] Gray M, Spencer DF. Organellar Evolution. Evolution of microbial life, 54. Cambridge University Press; 1997. p. 109–26.
[17] Cavalier-Smith T. Kingdom protozoa and its 18 phyla. Microbiol Rev 1993;57:953–94.
[18] Schnare MN, Heinonen TY, Young PG, Gray MW. A discontinuous small subunit ribosomal RNA in *Tetrahymena pyriformis* mitochondria. J Biol Chem 1986;261:5187–93.

[19] Heinonen TY, Schnare MN, Young PG, Gray MW. Rearranged coding segments, separated by a transfer RNA gene, specify the two parts of a discontinuous large subunit ribosomal RNA in *Tetrahymena pyriformis* mitochondria. J Biol Chem 1987;262:2879–87.
[20] Cannone JJ, Subramanian S, Schnare MN, Collett JR, D'Souza LM, Du Y, et al. The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinform 2002;3:2.
[21] Dsouza M, Larsen N, Overbeek R. Searching for patterns in genomic data. Trends Genet 1997;13:497–8.
[22] Eddy SR. Profile hidden Markov models. Bioinformatics 1998;14:755–63.
[23] Betley JN, Frith MC, Graber JH, Choo S, Deshler JO. A ubiquitous and conserved signal for RNA localization in chordates. Curr Biol 2002;12:1756–61.
[24] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[25] Raabe CA, Sanchez CP, Randau G, Robeck T, Skryabin BV, Chinni SV, et al. A global view of the nonprotein-coding transcriptome in *Plasmodium falciparum*. Nucleic Acids Res 2010;38:608–17.
[26] Gutell RR, Larsen N, Woese CR. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microb Rev 1994;58:10–26.
[27] Andersson SG, Kurland CG. Reductive evolution of resident genomes. Trends Microbiol 1998;6:263–8.
[28] Boore JL. Animal mitochondrial genomes. Nucleic Acids Res 1999;27:1767–80.
[29] Mogi T, Kita K. Diversity in mitochondrial pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. Parasitol Int 2010;59:305–12.
[30] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.

Tetrahedron 67 (2011) 6582–6586

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Ukulactones A and B, new NADH-fumarate reductase inhibitors produced by *Penicillium* sp. FKI-3389

Mihoko Mori [a,b], Hiromi Morimoto [c], Yong-Pil Kim [a], Hideaki Ui [a,b], Kenichi Nonaka [a], Rokuro Masuma [a,b], Kimitoshi Sakamoto [d], Kiyoshi Kita [d], Hiroshi Tomoda [c], Kazuro Shiomi [a,b,*], Satoshi Ōmura [a,*]

[a] *Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*
[b] *Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*
[c] *School of Pharmacy, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*
[d] *Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

ARTICLE INFO

Article history:
Received 29 April 2011
Received in revised form 20 May 2011
Accepted 20 May 2011
Available online 27 May 2011

Keywords:
Electron transport enzyme inhibitor
NADH-fumarate reductase
Penicillium
Ukulactone

ABSTRACT

Screening for NADH-fumarate reductase inhibitors led to the isolation of the new polyketide compounds, ukulactones A and B (**1** and **2**, Fig. 1) from a culture broth of *Penicillium* sp. FKI-3389. The structure of ukulactone A was elucidated as a methylated derivative of prugosene A1, which was produced by *Penicillium rugulosum* and NOESY experiment revealed ukulactone B was a stereoisomer of ukulactone A. Ukulactone A showed potent inhibitory activity against NADH-fumarate reductase of the roundworm *Ascaris suum* in vitro.

1. Introduction

NADH-fumarate reductase (NFRD), consisting of mitochondrial complexes I and II, is an electron transport system involved in a unique energy metabolic pathway found in many anaerobic organisms, such as helminths.[1] This system is used to generate ATP in the absence of oxygen and allows helminths to live in anaerobic circumstances inside the host. Therefore, a selective inhibitor of NFRD is expected to be a good anthelmintic. We have screened for NFRD inhibitors in culture broths of fungi using helminth (*Ascaris suum*) mitochondria, and found some potent inhibitors, such as nafuredin,[2] atpenins,[3] verticipyrone,[4] and paecilaminol.[5] During this screening we obtained new NFRD inhibitors, ukulactones A and B (**1** and **2**, Fig. 1), from the culture broth of the terrestrial fungus *Penicillium* sp. FKI-3389. In this report, we describe the isolation, structural elucidation, and biological activities of these two ukulactones.

2. Results and discussion

2.1. Isolation and structure elucidation of ukulactones A (1) and B (2)

A solid culture of *Penicillium* sp. FKI-3389 was extracted with EtOAc and the extract was purified by silica gel column chromatography and HPLC. From 3 kg of the solid broth, 515 mg of **1** and 3.5 mg of **2** were isolated.

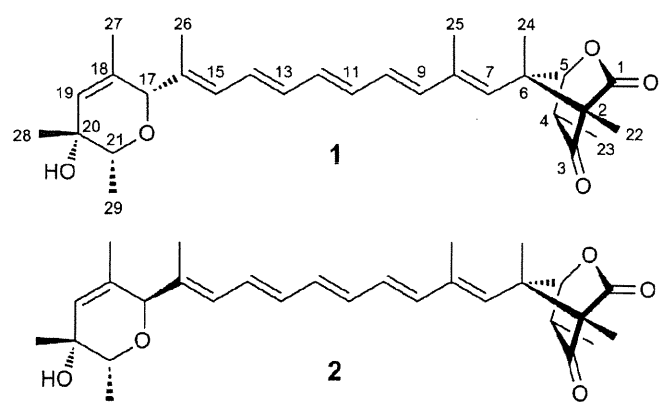


Fig. 1. Structures of ukulactones A (**1**) and B (**2**).

Ukulactone A (**1**) was obtained as a yellow syrup. The molecular formula was elucidated to be $C_{29}H_{38}O_5$ by HRFABMS (observed $[M]^+$ at 466.2722, calcd $[M]^+$ 466.2719). The characteristic absorption maxima at 325, 340, and 358 nm in the UV spectrum, strongly suggested that **1** has a pentaene in the structure. The IR absorptions indicated the presence of hydroxyl (3730 cm^{-1}) and carbonyl (1795 and 1745 cm^{-1}) groups.

* Corresponding authors. E-mail addresses: shiomi@lisci.kitasato-u.ac.jp (K. Shiomi), omuras@insti.kitasato-u.ac.jp (S. Ōmura).

0040-4020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2011.05.090

Table 1
^{1}H and ^{13}C NMR data of **1** and **2** (in $CDCl_3$)

Position	**1**[a]		**2**[b]	
	δ_H (Int., mult., J in Hz)	δ_C mult.	δ_H (Int., mult., J in Hz)	δ_C mult.
1		171.4 s		171.4 s
2		70.1 s		70.1 s
3		207.6 s		207.6 s
4	2.65 (1H, qd, 7.4, 2.2)	44.3 d	2.66 (1H, qd, 7.4, 2.2)	44.3 d
5	5.15 (1H, d, 2.2)	85.2 d	5.16 (1H, d, 2.2)	85.2 d
6		57.9 s		57.9 s
7	5.21 (1H, s)	127.7 d	5.22 (1H, s)	127.8 d
8		138.8 s		138.8 s
9	6.12 (1H, d, 14.4)	136.1 d	6.13 (1H, d, 14.4)	136.2 d
10	6.32 (1H, dd, 14.4, 10.9)	133.5 d	6.38 (1H, dd, 14.4, 10.9)	134.2 d
11	6.27 (1H, dd, 14.4, 10.9)	132.7 d	6.24 (1H, dd, 14.4, 10.9)	132.8 d
12	6.37 (1H, dd, 14.4, 10.9)	134.3 d	6.31 (1H, dd, 14.4, 10.9)	133.6 d
13	6.30 (1H, dd, 14.4, 10.9)	129.5 d	6.28 (1H, dd, 14.4, 10.9)	129.5 d
14	6.47 (1H, dd, 14.4, 10.9)	129.1 d	6.49 (1H, dd, 14.4, 10.9)	129.2 d
15	6.16 (1H, d, 10.9)	130.5 d	5.82 (1H, d, 10.9)	128.9 d
16		136.3 s		134.9 s
17	4.36 (1H, s)	85.2 d	4.30 (1H, s)	80.7 d
18		136.3 s		134.8 s
19	5.68 (1H, s)	130.8 d	5.66 (1H, s)	130.1 d
20		67.0 s		67.3 s
21	3.47 (1H, q, 6.3)	77.2 d	3.51 (1H, q, 6.3)	71.0 d
22	1.21 (3H, s)	4.8 q	1.21 (3H, s)	4.8 q
23	1.18 (3H, d, 7.4)	11.6 q	1.19 (3H, d, 7.4)	11.6 q
24	1.40 (3H, s)	16.8 q	1.40 (3H, s)	16.8 q
25	1.89 (3H, s)	14.3 q	1.90 (3H, s)	14.4 q
26	1.70 (3H, s)	12.0 q	1.90 (3H, s)	16.2 q
27	1.47 (3H, s)	18.5 q	1.62 (3H, s)	20.1 q
28	1.14 (3H, s)	23.5 q	1.13 (3H, s)	23.7 q
29	1.22 (3H, d, 6.3)	14.2 q	1.15 (3H, d, 6.3)	14.0 q

[a] Recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C).
[b] Recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C).

The ^{1}H and ^{13}C NMR data of **1** are shown in Table 1. Analysis of the ^{1}H and ^{13}C NMR, DEPT, and HSQC spectra revealed the presence of eight methyl groups, one sp^3 methine carbon, three quaternary carbons, three oxymethine carbons, nine sp^2 methine carbons, three sp^2 quaternary carbons, one carboxyl group, and one carbonyl group. The COSY correlations showed the presence of a spin system of seven olefin protons (from H-9 to H-15) in a pentaene moiety suggested by UV spectrum. The configurations of the olefins were assigned to be *trans* by their coupling constants (J=14.4 and 10.9 Hz). In the pentaene moiety, NOE correlations between H-7 and H-9 and between H-14 and H-26 were observed (Fig. 2). In addition, ^{13}C chemical shifts of the olefin methyl carbons C-25 (δ 14.3) and C-26 (δ 12.0) suggested the configuration of the pentaene moiety was all-*trans*.[6]

HMBC correlations from methyl proton H-25 to C-7, C-8, and C-9 and from H-26 to C-15, C-16, and C-17 were observed (Figs. 3 and 5), thus two methyl moieties were attached to C-8 and C-16 of the pentaene substructure of **1**, respectively. By the HMBC correlations in Fig. 3, another partial structure was deduced to be 4,6,7-trimethyl-2-oxabicyclo[2.2.1]heptane-3,5-dione contained in shimalactones A and B,[7] prugosenes A1–A3,[8] coccidiostatin A[9] and wartmannilactones E, F, and H.[10] The ^{1}H and ^{13}C chemical shifts of this substructure were in good accordance with those of shimalactones A and B in $CDCl_3$.[7] The HMBC correlations from the methyl proton H-24 revealed that this bicyclo unit connected to C-7 of the pentaene (Fig. 3). We conducted a NOESY experiment to analyze relative configuration of the bicyclo unit. The observed NOE correlations are shown in Fig. 4. The data indicated the relative configuration of this unit was (2*S*,4*R*,5*S*,6*S*) the same as prugosenes A1–A3.[8]

HMBC correlations (Fig. 5) clarified **1** had the 3,6-dihydro-3-hydroxy-2,3,5-trimethyl-2*H*-pyran ring also contained in prugosene A1[8] and wortmannilactones E and F.[10] The relative conformation of the dihydropyran ring was (17*S*,20*R*,21*R*), indicated by NOE correlations from H-17 to H-21, H-26 and H-27, and those from H-28 to H-19 and H-21 (Fig. 6). Therefore, ukulactone A was deduced to be an 8-methyl derivative of prugosene A1.

Ukulactone B (**2**) was also obtained as a yellow syrup. The molecular formula was elucidated to be $C_{29}H_{38}O_5$, the same value as ukulactone A (**1**) by HRFABMS. UV and IR spectra were almost identical, but the sign of $[\alpha]_D$, which was opposite to that of **1**, suggested **2** is a stereoisomer of **1**. As compared with ^{1}H and ^{13}C

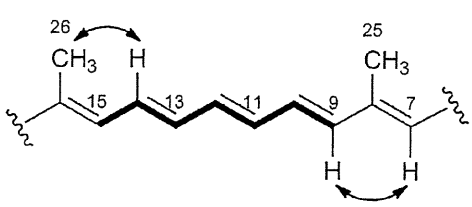


Fig. 2. COSY (bold line) and key NOESY (arrow) correlations of pentaene unit of **1**.

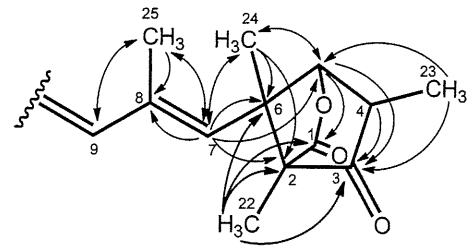


Fig. 3. HMBC correlations of the trimethyloxabicyclo[2.2.1]heptane unit of **1**.