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論文内容の要旨

Amitochondoriate protists are unicellular eukaryotes that lack mitochondria. Diplomonadida (including *Giardia*), Palabasala (including *Trichomonas*), Entamoebidae (including *Entamoeba*), Pelobionta (including *Mastigamoeba*), and Microsporidia (including *Encephalitozoon*) are well known as major lineages. Morphological evidence and findings on the ribosomes showing 'primitive eukaryotic' features for these lineages led a proposal of the 'Archezoa' hypothesis that several amitochondriate protist lineages (Archezoa) diverged preceding the endosymbiotic origin of proto-mitochondria, and thus have been living relics of the early phase of eukaryotic evolution.

Early studies on the eukaryotic phylogenies based on the small subunit (SSU) ribosomal RNA (rRNA) and on the translation elongation factors (EF) supported this hypothesis, placing three amitochondriate protists lineages, Microsporidia, Parabasala and Diplomonadida, at the basal position of the eukaryotic tree. However, phylogenies based on other genes, such as tubulins, mitochondorial-type heat shock protein 70 (HSP70mit) and the largest subunit of RNA polymerase II (RpoII), suggested that Microsporidia are not deep branching eukaryotes but are closely related to Fungi. Furthermore, phylogenies of various genes based on the recent accumulation of many sequence data from various protist lineages sometimes gave conflicting results with each other, indicating that the phylogenetic relationships among major eukaryotic lineages have still been an open problem.

On the other hand, mitochondrion-related genes that are coded in nuclear DNA and target for mitochondria were isolated from Entamoebidae, Microsporidia, Parabasala and Diplomonadida. The findings suggested that ancestors of these amitochondriate lineages once harbored mitochondria and lost them secondarily during their evolution.

On these backgrounds of the studies on early eukaryotic evolution, this work was intended to elucidate an evolutionary status of the amitochondriate protists. First, in order to establish a robust placement of the amitochondriate protist lineages in the eukaryotic tree, phylogenetic relationships among major eukaryotic lineages including amitochondriate protists were statistically analyzed in detail by applying a combined maximum likelihood (ML) method to the sequence data of multiple genes. Next, in order to establish whether the ribosomal features of potentially early-branching lineages are 'primitive eukaryotic', the ribosomes of the amitochondriate protists, *Giardia intestinalis* (Diplomonadida) and *Trichomonas vaginalis* (Parabasala), were analyzed, and the components were compared to those of other eukaryotic organisms including amitochondriate protists.

Chapter 1 of this article described the analyses of the phylogenetic relationships among major eukaryotic lineages including amitochondriate protists, with the reports on 27 original sequence data of various genes mostly derived from amitochondriate protists.

At first, phylogenetic positions of Microsporidia and of stramenopiles were analyzed. In order to clearly settle a phylogenetic position of Microsporidia among major eukaryotic lineages, a combined ML analysis was performed using 6,391 positions from 10 genes for which data from Microsporidia were available. These genes were EF-1áEF-2, valyl- and isoleucyl- tRNA synthetases (ValRS, IleRS), RpoII, Actin, átubulin, âtubulin, SSUrRNA,

and large subunit (LSU) rRNA. Although several phylogenies based on individual genes, such as EF-1áIleRS, and SSUrRNA, did not support a close relationship between Microsporidia and Fungi, the combined analysis clearly demonstrated a relationship, (Metazoa, (Fungi, Microsporidia)) with a very high statistical support. In addition, another combined ML analysis was performed to examine a relationship between stramenopiles and Alveolata, using 5,423 positions from eight genes for which data from stramenopiles were available (EF-1á EF-2, cytosolic-type HSP70 (HSP70c), non-catalytic 'B' subunit of vacuolar ATPase, Actin, âtubulin, SSUrRNA, and LSUrRNA). The analysis demonstrated also with a very high statistical support that stramenopiles and Alveolata were the closest relatives with each other.

In the next, the phylogenetic position of the Pelobiont *Mastigamoeba balamuthi* was analyzed in relation to the position of *E. histolytica*. A combined ML analysis using 3,935 positions from four genes, SSUrRNA, LSUrRNA, EF-1áand EF-2, suggested that *M. balamuthi* was the closest relative of *E. histolytica* and that Mycetozoa were placed at the sistergroup to the common ancester of *M. balamuthi* and *E. histolytica*. These findings supported the notion, which had previously been proposed primarily on cytological evidence, that both *M. balamuthi* and *E. histolytica* are closely related to the Mycetozoa and that these three together represent a major eukaryotic lineage (Conosa).

Finally, on the basis of the findings as mentioned above and as currently reported in the literatures, 13 major eukaryotic lineages were divided into seven groups: 1. (Metazoa, (Fungi, Microsporidia)), 2. (Mycetozoa, (Pelobionta, Entamoebidae)) [Conosa],
3. (Viridiplantae, Rhodophyta), 4. (stramenopiles, Alveolata), 5. Euglenozoa,
6. Diplomonadida, and 7. Parabasala. Phylogenetic relationships among these groups with an outgroup were examined by a combined ML analysis of the genes, EF-1áEF-2, ribosomal protein (Rp) S14, RpS15a, RpL5, RpL8, RpL10a, IleRS, ValRS, RpoII, chaperonin 60, HSP70mit, endoplasmic reticulum-type HSP70, HSP70c and cytosolic-type HSP90, chaperonin-containing testis complex polypeptide-1 subunit (CCT) áCCTãCCTäCCTæ Actin, átubulin, âtubulin, SSUrRNA, and LSUrRNA. The combined ML analysis clearly supported with statistical confidence that Diplomonadida and Parabasala diverged earlier than other five groups in the eukaryotic tree, although the branching order between these two lineages were still open for further analysis. In addition, especially when among-site rate heterogeneity was taken into consideration, it was clearly supported that (Metazoa, (Fungi, Microsporidia)), Conosa, and (Viridiplantae, Rhodophyta) were the first, the second and the third earliest offshoots among the five groups excluding Diplomonadida and Parabasala. The analysis at the first time demonstrated robustly that Diplomonadida and Parabasala are the early branching eukaryotes, although presence of a potential artefact derived from a long branch attraction could not be ruled out entirely.

Chapter 2 of this article described the analyses of the ribosomes of potentially early branching amitochondriate protists, *G. intestinalis* and *T. vaginalis*.

Sedimentation analyses demonstrated that the sedimentation coefficients of these ribosomes were larger than that of *Escherichia coli* and smaller than that of *Saccharomyces cerevisiae* or *Artemia salina*. Based on the radical free and highly reduced two dimensional polyacrylamide gel electrophoresis analysis, N-terminal sequencing analysis, and/or similarity

search on the public database, the number of ribosomal proteins were estimated to be at least 74 for *G. intestinalis* and approximately 80 for *T. vaginalis*. These numbers were comparable with that of a 'typical' eukaryote (about 80) and larger than that of *E. coli* (about 55). The N-terminal sequences of the protein spots and alignment analyses of all the ribosomal proteins currently available revealed that the sequences of *G. intestinalis* and *T. vaginalis* are clearly of 'typical' eukayotic type with no exception.

On the other hand, sequence comparison analyses of rRNAs revealed that the SSU and LSUrRNAs of *G. intestinalis* and *T. vaginalis* were remarkably shorter in length than those of 'typical' eukaryotes. All the helices that belong to the universal core, however, were strictly conserved also in *G. intestinalis* and *T. vaginalis*. In contrast, variable regions of both rRNAs were reduced to be short in *G. intestinalis* and *T. vaginalis*.

As far as these results are concerned, the protein components and the essential parts of the rRNAs of the *G. intestinalis* and *T. vaginalis* ribosomes are clearly of 'typical' eukaryotic type. No 'primitive eukaryotic' features are found in the ribosomes of these amitochondriate protists. The smaller sedimentation coefficients of the ribosomes of *G. intestinalis* and *T. vaginalis* than those of 'typical' eukaryotes are due to the smaller size of rRNAs with shortened variable regions. These findings give additional evidence for fully developed eukaryotic nature of *G. intestinalis* and *T. vaginalis*. Probably Diplomonadida and Parabasala already had obtained major eukaryotic properties commonly found in the 'typical' eukaryotes.

論文の審査結果の要旨

本論文の主要な貢献は以下の点である。

(1)　ミトコンドリアをもたないものを含む原生生物6種から、翻訳、転写、シャペロン等に関連する15種類の分子について合計27個の遺伝子を単離しそれらの配列を決定した。オリジナルデータを含め25種類の分子それぞれについて、アライメント、最尤法による分子系統樹推定を行なうとともに、個々の分子の解析結果を総合評価し、ミトコンドリアをもたない原生生物の5つの分類群を含む真核生物の大きな分類群相互の系統的位置関係を詳細に検討した。その結果、1) Microsporidiaは真菌類に近縁、2) EntamoebidaeとPelobiontaは近縁でこれらの共通祖先にはMycetozoaが近縁、3) DiplomonadidaとParabasalaは他の真核生物の分類群よりも分岐が早い、4) Alveolata/Stramenopiles, Viridiplantae/Rhodophyta, Euglenozoaの3群は単系統群を形成、などの点を明らかにし、ミトコンドリアをもたない分類群の系統樹上での位置づけに関し明確な結論を得た。

(2)　ミトコンドリアをもたない原生生物 *G.intestinalis* (Diplomonadida) と *T. vaginalis* (Parabasala)からリボソームを精製し、沈降係数の分析、2次元電気泳動法による蛋白成分の分離、蛋白スポットに対するN末端アミノ酸配列の決定などを行った。さらに、リボソーム蛋白質の網羅的アライメント、リボソームRNAの2次構造の推定などの結果を踏まえ、これら生物種を含めミトコンドリアをもたない原生生物のリボソーム構成成分を他生物種のそれと比較解析した。その結果、*G. intestinalis*や*T. vaginalis*のリボソームは明らかに通常の真核生物型であり、沈降係数が通常の真核生物型とされる80Sより小さいのはRNAの可変領域が縮小しているためであるとの結論を得た。(3)(1)と(2)解析結果をもとに最近の研究動向を踏まえて、ミトコンドリアをもたない原生生物の進化的位置づけに関し現時点での研究の到達点をまとめた。

本研究において、複数の分子系統樹の解析結果を総合評価するというアプローチにより、これまで単独の分子による解析では明らかに出来なかった多くの新たな系統学的知見を得たことは評価される。真核生物の系統進化の解析上重要な多くのオリジナル配列データを明らかにした点も評価される。また、「原核生物型」あるいは「原核生物から真核生物への移行型」と見なされてきた*G. intestinalis*や*T. vaginalis*のリボソームが通常の真核生物型であることを明確に示し、これまでの議論の混乱を収束させる結論を示した点も重要である。

以上の評価より、本論文の内容は博士(理学)に十分に値するものであると判定した。なお、本学位申請論文の内容に関する2編の原著論文(申請者は筆頭著者)が、国際学術誌である*J. Eukaryot. Microbiol.*に掲載予定となっている。また1編の原著論文(申請者は9人中第4著者)が、国際学術誌である*Mol. Biochem. Parasitol.*に掲載済みである。

先導科学研究科における課程博士の授与に係わる論文審査等に関する規定に基づき、公開の論文発表会を開催した。研究内容は分かり易くまとめられ、質疑応答も適切なものであった。その後審査委員による口頭試問を行った。その結果、申請者は、遺伝子解析、蛋白解析等の実験的研究を進める十分な技術力を有していると同時に、配列データを解析する能力にも秀でていると認められた。さらに、申請者が生命体科学及び関連する分野に関して十分な学識を有することも認められた。また、論文の英語が明快であることから判断して、申請者が十分な英語力を有することも認められた。以上の結果から試験に合格したと判定した。