**In vivo** Conformation of Mitochondrial DNA Revealed by Pulsed-Field Gel Electrophoresis in the True Slime Mold, *Physarum polycephalum*

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**Abstract**

Pulsed-field gel electrophoresis (PFGE) was used to examine the in vivo and in vitro conformations of *Physarum polycephalum* mitochondrial DNA (mtDNA). We used plugs containing isolated mitochondria, isolated mitochondrial nucleoids (mt-nuclei), and isolated mtDNA, in addition to whole cells. The mtDNA contained in the myxamoebae, plasmodia, isolated mitochondria, and isolated mt-nuclei was circular, but most of the isolated mtDNA had been site-specifically fragmented and linearized during DNA preparation and storage under low ionic strength conditions. Restriction mapping of *Physarum* mtDNA by the direct digestion of the isolated mt-nuclei from two different strains, DP89xAI16 and KM88xAI16, resulted in the circular form. A linear mitochondrial plasmid, mF, is known to promote mitochondrial fusion and integration of itself into the mtDNA in *Physarum*. Linearization of mtDNA by the integration of the mF plasmid was demonstrated when we used PFGE to analyze isolated mitochondria from the plasmodial strain DP89xNG7 carrying the mF plasmid (mF+). The PFGE system can be used not only to determine whether the form of mtDNA is linear or circular but also to analyze the dynamic conformational changes of mtDNA.

**Key words:** Mitochondrial DNA (mtDNA); Pulsed-field gel electrophoresis (PFGE); Mitochondrial plasmid; *Physarum polycephalum*

1. **Introduction**

There are many instances in which the results of restriction mapping and electron microscopy (EM) are apparently contradictory for fungal and plant mtDNA. Evans and Suskind (1971)1 characterized mtDNA from *Physarum polycephalum* and found it to be in the form of linear fragments up to about 46 kb in size with a base composition of about 26% G+C. Bohnert (1977)2 found 10% of the mtDNA from the M3 strain of *Physarum* to be circular molecules with a circumference of approximately 19 μm, corresponding to a size of about 63 kb. In contrast, Kawano et al. (1982)3 characterized the mtDNA recovered from the mitochondrial nucleoid of a strain PPO-1 by EM and found linear mtDNA molecules about 22 μm long. They also determined the order of *Xho* I and *EcoR* I fragments and the location of a number of additional restriction enzyme sites; the resulting restriction map was linear with a length of about 69 kb. Kawano et al. (1987)4 digested the mtDNA from 19 different *Physarum* strains with six different restriction enzymes and, although no restriction map was produced, several restriction fragment length polymorphisms were evident and the size of the mtDNA ranged from 60.1 to 68.2 kb. In 1990, Jones et al.5 constructed circular maps of the mtDNA of strains M3, CL, ΔM3, and 2SL, reporting lengths of 60, 61, 56, and 62 kb, respectively. On the other hand, Takano et al. (1990)6 constructed an 86-kb linear mtDNA map with a long tandem duplication of 19.6 kb on its end using a strain CH934×CH938, which is largely isogenic with the CL strain used by Jones et al. (1990).5

The use of other methods to investigate the in vivo conformation of mtDNA is indispensable, since restriction mapping and EM alone cannot be used to confirm the conformation of mtDNA. Pulsed-field gel electrophoresis (PFGE) is an effective method for this analysis, as PFGE permits the resolution of relatively intact mtDNA, and...
2. Materials and Methods

2.1. Yeast strains and growth conditions

Yeast strain YPH499 was provided by Y. Ohya (University of Tokyo), strains MH41-7B, CBS1707, CBS1600, and CBS2887 were provided by I. Miyakawa (Yamaguchi University). Cells were grown in YPD medium at 30°C with shaking until early stationary phase.

2.2. Physarum polycephalum strains and growth conditions

Myxamoebal strains used in this study were DP89, obtained from D. Pallota (Laval University); KM88, which is the derivative of CL (Colonia isolate); AI16 from J. Dee; and NG7 that was isolated from plasmodial strain Ng. Myxamoebae were cultured on PGY plates in association with live bacteria KM88xAI16, and DP89xNG7 were used. They were cultured in semi-defined medium as described by Daniel and Baldwin. Cultures were grown, with shaking, at 23°C, in 500-ml flasks that contained 200 ml of medium, and cells were used at the late exponential phase.

2.3. Isolation of mitochondria, mt-nuclei, and mtDNA

Mitochondria and mt-nuclei were isolated from microplasmodia by the method of Sasaki et al. (1998) with minor modification. NE1-S buffer without phenylmethylsulfonyl fluoride was used as an isolation buffer in this study. Isolated mitochondria and mt-nuclei were suspended in the isolation buffer and prepared for sample plugs and restriction analysis. Isolation of mtDNA was performed using two methods. Mitochondria recovered from Percoll by the modified method of Sasaki et al. were diluted in 50 ml of isolation buffer and pelleted by centrifugation at 18,500 g for 3 min. The pellet was washed again and re-suspended in 10x Tris/Saline (100 mM Tris, 100 mM EDTA, pH 8.0, 150 mM NaCl) containing 1% SDS, 1 mg/ml proteinase K (Merck, Germany). After incubation of the suspension at 37°C for 2 hr, 1 mg/ml CsCl and 300 μg/ml ethidium bromide were added. The CsCl-dye mixture was loaded in ultracentrifuge tubes and spun at 260,000 g for 12 hr. mtDNA was visualized as a band and recovered, extracted with isopropanol to remove the ethidium bromide, diluted three fold with distilled water (DW) and precipitated by the addition of two volumes of ethanol. Isolated mtDNA was dissolved and stored in either TE (10 mM Tris, pH 8.0, 1 mM EDTA), STE (TE containing 150 mM NaCl), or 0.1 mM EDTA. Other mtDNA isolations were performed according to Takano et al. (1990).

2.4. Preparation of sample plugs

Yeast plugs were prepared according to the manufacturer's instructions (Bio-Rad, USA) with minor modification. Modified proteinase K reaction buffer (100 mM EDTA, 10 mM Tris, pH 8.0, 1% SDS, 1 mg/ml proteinase K) was used in this study. Yeast cells in YPD medium were pelleted by centrifugation and washed twice in cell suspension buffer (20 mM NaCl, 10 mM Tris-HCl, pH 7.2, 50 mM EDTA). A 20- to 40-ng pellet of yeast cells was embedded for each 1 ml plug to be made. Myxamoebae on PGY plates were suspended in DW using a spreader. The cells were pelleted by centrifugation and washed twice with DW. Microplasmodia in semi-defined medium were harvested by centrifugation. A 25-ng pellet of myxamoebae and microplasmodia was embedded for each 1 ml plug. Next, 2.5 mg of mitochondria and the mt-nuclei recovered from 6 mg of mitochondria were embedded for each 1 ml plug to be made. The amount of the mitochondria was estimated by a Bio-Rad protein assay (Bio-Rad, USA) using γ-globulin as a standard. All plugs were treated with five volumes of modified proteinase K reaction buffer at 50°C for 1-2 days and subjected to four 1-hr washes in 50 volumes of wash buffer (20 mM Tris-HCl, pH 8.0, 50 mM EDTA) at room tem-
perature with gentle agitation. They were stored in wash buffer at 4°C until use.

2.5. PFGE

PFGE was performed using a contour-clamped homogeneous electric field apparatus (CHEF Mapper; Bio-Rad USA). The migration conditions were optimized for the detection of mtDNA: 1% agarose gel, 0.5xTBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), pulse time 1 to 12 sec for 15 hr (linear ramp) at 6 V/cm and 12°C. Low range PFGE marker (New England BioLabs, USA) was used as a size standard.

2.6. Hybridization procedure

DNA separated by PFGE was transferred to Hybonc N+ nylon membrane (Amersham Pharmacia Biotech) using a vacuum blotting system (Amersham Pharmacia Biotech). Labeling, hybridization, and detection were performed with a Gene Imaging kit (Amersham Pharmacia Biotech) according to the supplier's instructions. Probes were made using a 3.5-kb EcoKVI-XbaI fragment from *Physarum* mtDNA, a 4.0-kb EcoRV mF plasmid fragment, a 2.6-kb fragment of *Physarum* rDNA encoding most of the 26s gene amplified by PCR using primers 5’GATAAGGATTGGCTCTATAGGCTGG3’ and 5’CGACATCGAAGGATCAAAAAGCCAC3’, and the yeast *coxl* gene provided by H. Fukuhara (Centre Universitaire Paris XI).

2.7. Restriction enzyme analysis

Isolated mt-nuclei were directly cut with restriction enzymes before DNA extraction. After digestion, 20 mM EDTA, 1% SDS, 0.2 mg/ml proteinase K, and 0.1 mg/ml RNase A were added to the solution and incubated at 37°C for 2 hr. Phenol/ chloroform/ isoamylalcohol (25:24:1, v/v/v) extraction and chloroform/ isoamylalcohol (24:1, v/v) extraction were performed with gentle inverting to mix; the recovered DNA solution was used directly for electrophoresis. Isolated mtDNA was digested with restriction enzymes according to the supplier’s instructions (Takara Shuzo Co., Ltd., Japan). Digested mt-nuclei and mtDNA were then electrophoresed in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at approximately 2 V/cm for 12 hr using Sty I-digested bacteriophage lambda DNA as a size standard.

2.8. Densitometric assay

Gels stained with ethidium bromide were scanned with Image Saver (AE-6905C; ATTO Co., Japan), and Lane Analyzer Ver. 2.2 (ATTO Co., Japan) was used to measure the relative intensity of each band.

Figure 1. PFGE of yeast strains with linear and circular mtDNA. A, ethidium bromide-stained gel. B, profile of Southern hybridization with a *coxl* gene probe. Lanes: 1, *Saccharomyces cerevisiae* YPH499; 2, *S. cerevisiae* MM41-7B; 3, *Willeopsis mrakii* CBS1707; 4, *Pichi jadinii* CBS1600; 5, *P. pijperi* CBS2887. The compression zone (cz) and linear mtDNA size markers in kb are indicated on the left. Arrowheads in B indicate the bands of monomer units of linear mtDNA estimated by restriction mapping of mtDNA.8

3. Results and Discussion

3.1. PFGE of yeast strains with linear- and circular-mapping mtDNA

To analyze the mobility of mtDNA in PFGE, we subjected whole-cell DNA preparations of five yeast strains with linear- and circular-mapping mtDNA to PFGE and Southern hybridization of the gel with a *coxl* gene probe was performed (Fig. 1). The strains, mapping forms, and sizes of the mtDNA are listed in Table 1. In the *Saccharomyces cerevisiae* strains, which have circular mtDNA, most of the hybridization signals were seen in the well with faint hybridization signals in the compression zone (lanes 1 and 2). On the other hand, for the three strains with linear mtDNA, several bands appeared in the separation region and they were found to be mtDNA by hybridization. Strong and weak signals were also seen in the well and the compression zone (lanes 3, 4, and 5). The size of each band detected in the separation region is shown in Table 1. In *P. jadinii* and *P. pijperi*, dimers were also detected in addition to monomers. For *S. cerevisiae* strains, a distinct 5-kb band appeared. Since the 5-kb bands never appeared after RNase treatment of the plugs, they may be the RNA molecules of the L mycoviral genome (data not shown).26
mobility differences to examine the in vivo conformation of Physarum mtDNA.

3.2. PFGE of Physarum mtDNA

To examine the in vivo and in vitro conformation of Physarum mtDNA, we electrophoresed several sample preparations, including whole cells of myxamoebae and plasmodia, isolated mitochondria, isolated mt-nuclei, and isolated mtDNA from the plasmodia using the conditions described above (Fig. 2A). Since our group has shown that isolated mtDNA from Physarum is unstable and fragmented under low ionic strength conditions, we electrophoresed the samples after washing the plugs in either TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) or 0.1 mM EDTA as well as the normal wash buffer (20 mM Tris-HCl, pH 8.0, 50 mM EDTA).

For the myxamoebae (Fig. 2A; a), linear DNA molecules of about 70 kb appeared as a band in the separation region in addition to the bands in the compression zone and the well. No difference in the migration pattern was observed between the plugs stored in wash buffer (lane 1), washed in TE (lane 2), or 0.1 mM EDTA (lane 3). On the other hand, for the plasmodia (Fig. 2A; b) the same patterns as the myxamoebae were seen with the wash buffer (lane 1) and TE (lane 2), but some of the DNA retained in the well seemed to migrate into the gel as a smear for the sample washed with 0.1 mM EDTA (lane 3). For the isolated mitochondria (Fig. 2A; c) and mt-nuclei (Fig. 2A; d), almost all the mtDNA remained in the well and no discrete bands were observed in the other regions with the wash buffer (lane 1) and TE (lane 2), but some mtDNA from the 0.1 mM EDTA sample (lane 3) migrated to the separation region. Smears and some discrete bands were visible in the 70–80 kb range. For the isolated mtDNA (Fig. 2A; e), most of the DNA formed smears in the 7–40 kb range of the separation region, with some discrete bands visible.

Table 1. Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Restriction map of mtDNA</th>
<th>Detected bands (kb) in the separation region</th>
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<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>YPH499</td>
<td>Circular</td>
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</tr>
<tr>
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<td>MH41-7B</td>
<td>Circular</td>
<td>75</td>
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<td>CBS1707</td>
<td>Linear</td>
<td>58</td>
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<tr>
<td>Pichia jadinii</td>
<td>CBS1600</td>
<td>Linear</td>
<td>40</td>
</tr>
<tr>
<td>Pichia pijperi</td>
<td>CBS2887</td>
<td>Linear</td>
<td>24</td>
</tr>
</tbody>
</table>
| *Data for restriction maps of mtDNA from Wesolowski and Fukuhara (1979)²⁵ and Fukuhara et al. (1993).*²
| *The assignment of the sizes of detected bands was made from Fig. 1.*²

Southern hybridization of gels with the whole-cell DNA of the myxamoebae (Fig. 2C; a) and the plasmodia (Fig. 2C; b) was also performed using a 26s rDNA gene probe. The 70-kb linear bands in the separation region hybridized with the probe and were therefore extrachromosomal rDNA. The migration patterns of the myxamoebal rDNA did not change under any of the three conditions used (Fig. 2C; a-lanes 1–3). The migration patterns of the plasmodial rDNA also did not change under any of the conditions (Fig. 2C; b-lanes 1–3).

Under normal PFGE conditions (regular wash buffer) the linear rDNA of the myxamoebae and plasmodia appeared as a band in the separation region while the mtDNA remained in the well, suggesting that the mtDNA of Physarum is circular in vivo. When the plugs were electrophoresed after being washed in 0.1 mM EDTA, the plasmodial mtDNA entered the gel and it is possible that circular mtDNA fragmented under low ionic strength conditions resulting in the appearance of linear fragments. However, this fragmenting could not be due to digestion with nuclease, because the migration pattern of the plasmodial rDNA was unaltered under these conditions. In contrast, this breakage was not observed when myxamoebal mtDNA plugs were washed in 0.1 mM EDTA and electrophoresed, probably because the mtDNA molecules of myxamoebae have little single-strand breakage in vivo, unlike those of plasmodia.

When the plugs of plasmodia, isolated mitochondria,
Figure 2. PFGE of a *Physarum* myxamoebal strain DP89 (a), plasmodial strain DP89×A116 (b), isolated mitochondria (c), mt-nuclei (d), and mtDNA (e) from DP89×A116. A, ethidium bromide-stained gel. B, profiles of Southern hybridization with a 3.5-kb mtDNA fragment as a probe. C, profiles of Southern hybridization with rDNA-26s gene probe. Plugs stored in wash buffer (lane 1), washed in TE (lane 2) and washed with 0.1 mM EDTA (lane 3) were electrophoresed. The compression zone (cz) and linear DNA size markers in kb are indicated on the left.

and mt-nuclei were electrophoresed after being washed in 0.1 mM EDTA, some of the mtDNA entered the gel. It is possible that site-specific breakage of the mtDNA occurred and produced linear fragments of defined sizes, since some bands could be distinguished separate from the smears of mtDNA in the gel. The changes of the migration patterns of mtDNA from isolated mitochondria and mt-nuclei under the three conditions were similar to that of plasmodia. Therefore, mtDNA contained in them could reflect the condition of the plasmodial mtDNA, and the molecules retain that circular conformation when stored in high ionic strength conditions as wash buffer. On the other hand, isolated plasmodial mtDNA fragmented even when kept in high ionic strength conditions during DNA extraction and it does not reflect the *in vivo* conformation. These results suggest that proteins present in mt-nuclei play an important role in keeping the structure of the mtDNA intact. The naked mtDNA is easily fragmented during isolation because the proteins have been removed. This may be the reason that genome-sized circular molecules of mtDNA are rarely seen with EM, yet the restriction maps of most fungal and plant mtDNA are circular. 28

3.3. Differences in the restriction pattern between isolated mt-nuclei and mtDNA

We also sought to examine the effect on restriction digest patterns of breakage of isolated mtDNA during preparation and storage. The plasmodial strains DP89×A116 and KM88×A116 were used in these experiments. mt-nuclei stored in isolation buffer and mtDNA stored in STE (TE containing 150 mM NaCl), TE, and 0.1 mM EDTA were digested and electrophoresed (Fig. 3A). The mtDNA was isolated using two methods (see Materials and Methods). mt-nuclei in isolation buffer can be directly cut with restriction enzymes. 24 Twelve stoichiometric and distinct bands were observed in the 0.3–14.5 kb range when the isolated mt-nuclei from DP89×A116 were digested with *Xba*I (Fig. 3A; a-lane 1). In contrast, a faint band of 4.5 kb (asterisk) was observed in the restriction patterns of the isolated mtDNA from DP89×A116, in addition to the 12 distinct bands (Fig. 3A; a-lanes 2–5). When mt-nuclei from KM88×A116 were digested with *Xba*I, 12 stoichiometric and distinct bands were observed in the 0.3–18.4 kb range (Fig. 3A; b-lane 1). The faint 4.5-kb band (asterisk) was also observed in the restriction patterns of the isolated mtDNA from KM88×A116 in addition to the 12 distinct bands (Fig. 3A; b-lanes 2–5). In either case, the intensity of each band seemed to be different between the mt-nuclei and the mtDNA, and these patterns seem to be influenced by the conditions in which the mtDNA was dissolved (STE, TE, 0.1 mM EDTA) and by the method of mtDNA isolation.

To quantitatively analyze the restriction pattern differ-
3.4. Construction of circular mtDNA restriction maps

We thought that the breakage of mtDNA during preparation and storage, resulting in the appearance of the artificial 4.5-kb band and the diminution of the 10.2-kb band intensity, should make it possible to construct a linear restriction map of Physarum mtDNA. The faint band of 4.5-kb was thought to be derived from the terminal region of linear mtDNA molecules. We used restriction patterns of the mt-nuclei to make an accurate restriction map reflecting the in vivo conformation. Figure 4A shows the restriction maps of DP89xA116 and KM88xA116 with XbaI, SphI, and XhoI. These maps show that Physarum mtDNA is circular. Restriction fragment length polymorphisms were seen in the two strains. In addition to the loss or addition of restriction enzyme sites, two regions of approximately 1.8 kb and 2.0 kb were only found in KM88xA116. We sequenced the regions of the deletions/insertions in both DP89xA116 and CH934xCH938 (which is isogenic to strain KM88xA116) and compared them (Fig. 4B). The sequence data shows that the deletions or insertions of 1842 and 2020 bp occurred between 11 bp and 3 bp repetitive sequences, respectively.

3.5. PFGE detection of a linearized recombinant resulting from mtDNA and mF plasmid recombination

Our PFGE system using plugs containing isolated mitochondria revealed the in vivo conformation of the mitochondrial genome. The mF plasmid that promotes mitochondrial fusion and integration of itself into mtDNA in Physarum provided an opportunity to test the usefulness of the system. When this linear plasmid recombines with circular mtDNA, linear recombinants must arise (Fig. 5A). To obtain mF− and mF+ plasmodia, mF−
myxamoebal strain DP89 was crossed with mF⁻ myxamoebal strain AI16 and mF⁺ myxamoebal strain NG7. We subjected total mtDNA contained in the isolated mitochondria from the mF⁻ plasmodia (DP89×AI16) and the mF⁺ plasmodia (DP89×NG7) to PFGE (Fig. 5B; a). In DP89×AI16 (mF⁻), no discrete band was observed outside the well (lane 1). On the other hand, in DP89×NG7 (mF⁺), two distinct bands appeared at about 16 and 70 kb, in addition to the band at the well (lane 2).

Southern hybridization using a 4.0 kb EcoRV fragment from the mF plasmid as a probe was performed (Fig. 5B; b). In the mF⁻ plasmid (DP89×AI16), no hybridization signal was detected (lane 1). In the mF⁺ plasmid (DP89×NG7), the bands of 16 and 70 kb were detected by hybridization in addition to the band in the well. Southern hybridization using a 3.5-kb mtDNA EcoRV-XhoI fragment as a probe was then performed (Fig. 5B; c). In DP89×AI16, the hybridization signal was detected only at the well (lane 1). On the other hand, in DP89×NG7 (mF⁺), the hybridization signal was detected at 70 kb and at the well (lane 2). This shows that the 16 kb and 70 kb bands could represent the free mF plasmid and the recombinant, respectively, as shown in Fig. 5A. These results demonstrate not only the usefulness of the system, but also the in vivo linearization of mtDNA with mF plasmid.

Linear mitochondrial plasmids have been identified in numerous fungi, protozoa, and plants. These plasmids replicate autonomously in the mitochondria, independently of the mtDNA, and may be responsible for the diversity among mitochondrial genomes. It has been demonstrated that some fungal and plant linear mitochondrial plasmids are integrated into mtDNA and cause structural changes in the mtDNA. The mitochondrial genomes of the S-type cytoplasm of male-sterile maize contain two short linear plasmids, S1 and S2, in addition to the usual circular mtDNA, and they could recombine with homologous sequences present on the circular mtDNA to convert a high proportion of the circular mtDNA into linear molecules with S1 and S2 covalently linked to one end. There are also reports that mtDNA of maize and Physarum linearized by the integration of these plasmids seem to be recircularized through further recombination. However, a particular linear mitochondrial plasmid (pAL2-1), which integrates to linearize the mtDNA, is known to be in Podospora anserina. The almost full-length copy of pAL2-1 integrates at one site in the mtDNA and that the mtDNA is not linearized by their integration. However, a particular linear mitochondrial plasmid (pAL2-1), which integrates to linearize the mtDNA, is known to be in Podospora anserina. The almost full-length copy of pAL2-1 integrates at one site in the mtDNA and produces linear fusion elements (terminating linear plasmids) and circular recombinants containing both sequences of mtDNA and pAL2-1. The mode of integration of this plasmid is thought to be similar to...
In vivo Conformation of mtDNA in Physarum polycephalum

Figure 5. An in vivo conformational change of mtDNA demonstrated by PFGE. A, a model of recombination between circular mtDNA and mF plasmid. The mtDNA of DP89xNG7 and the mF plasmid recombine through their homologous sequences, indicated by open rectangles, and a linear recombinant is the result. The open arrows indicate the terminal inverted repeats present on the ends of the mF plasmid. B, PFGE of isolated mitochondria from plasmodial strains DP89xAI16 (mF−; lane 1) and DP89xNG7 (mF+; lane 2). a, ethidium bromide-stained gel; b, profiles of Southern hybridization with a 4.0-kb fragment of mF plasmid as a probe; c, profiles of Southern hybridization with a 3.5-kb mtDNA fragment as a probe. The linear DNA size markers in kb are indicated on the left. Open and closed arrowheads indicate the mF and the linearized recombinant, respectively.

that suggested for integration of linear plasmids into the mtDNA of Neurospora strains. Giant linear plasmids of about 50 kb and 70 kb have been demonstrated by PFGE analysis.

The linearization and recircularization of the mtDNA integrated with plasmids are important sources of the conformational and gene-organizational diversity of mitochondrial genomes. The PFGE system can be used not only to determine whether the mtDNA conformation is linear or circular, but also to analyze the dynamic conformational changes of mtDNA in vivo. Our future work will concentrate on elucidating the molecular mechanism of the linearization of Physarum mtDNA and on analyzing the relationship of linearization and recircularization of mtDNA.

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