The Origin of a Linear Mitochondrial Plasmid (mF) that Promotes Mitochondrial Fusion in Laboratory Strains of *Physarum polycephalum*

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Abstract: The mitochondrial-fusion promoting (mF) plasmid first found in the slime mold *Physarum polycephalum* is a parasitic, selfish mitochondrial plasmid, and is capable of manipulating mitochondria behavior as indicated by its name. To investigate the origin of the mF plasmid, we collected many *Physarum* laboratory strains as are available at present throughout the world, and classified their origin on the basis of the results of mating-type analysis and restriction endonuclease analysis of their mtDNA. They include 8 plasmidial strains obtained from mating crosses with 22 different myxamoebal strains and two sclerotia. One of the two sclerotia is originated at W. Seifriz's laboratory in the University of Pennsylvania, and is the oldest laboratory strain in the world. The mtDNA of each strain formed a single 80–90 kbp band but the mF⁺ strains yielded a 14–16 kbp mF plasmid band in addition to this main band. Southern hybridization using labeled plasmid DNA as the probe allowed the mF[−] strains to be classified into two categories: simple mF[−] having only an mID sequence and Δ mF having some part of the mF plasmid integrated into the mtDNA. The mID sequence is almost identical to a 475-bp sequence (pID) of the mF plasmid. To confirm this finding, we designed PCR primers for amplifying certain parts of the mF plasmid. This PCR system enabled us to determine whether apparent mF[−] strains and sclerotia possessed the free or integrated mF plasmids. The sclerotium derived from Seifriz's laboratory is simple mF[−], but the other sclerotium, which was originally derived from the North Carolina Biological supply house is complete mF⁺. Moreover, except this derivative of North Carolina Biological, DP12 $\times$ DP13, which derived from a stock at the University of Iowa is Δ mF. Such scattered distribution of mF⁺ and Δ mF suggests that the mitochondria occasionally acquired the mF plasmid after the establishment of *P. polycephalum* as a species.

Key words: Plasmid; origin; mitochondria; fusion; *Physarum polycephalum*.

Introduction. Linear and circular mitochondrial plasmids are common in natural isolates of fungi and plants.¹⁾ Their definitive function remains obscure although they have been extensively studied and a number of them have been sequenced. Such plasmids are interesting in themselves as examples of selfish or parasitic DNA. They may also provide insights into the biology and evolution of their hosts, mitochondria.

Separate strains of the true slime mold, *Physarum polycephalum*, whose mitochondria fuse and do not fuse were isolated, and these mitochondria were the first to show evidence of a relationship between fusion and recombination. All strains of *P. polycephalum* that display mitochondrial fusion possess a specific mitochondrial-plasmid, in other words, this plasmid promotes mitochondrial fusion.²⁾ This mitochondrial-fusion-promoting (mF)

plasmid has a 475-bp sequence (pID) that is identical to a 479-bp sequence (mID) of mtDNA, with the exception of deletions of a total of only four base pairs at three sites.³⁾ With these identical sequences, the mF plasmid is constitutively crossed over with mtDNA to integrate itself into the mtDNA. This is very similar to that proposed as the initial stage in the evolution of sex, as pointed out by Hurst (1991).⁴⁾ The existence of the mF plasmid may serve as direct support for the idea that parasitic, selfish genes are capable of manipulating their hosts (mitochondria) in the manner proposed by Hickey and Rose (1988).⁵⁾

To investigate the origin of mF plasmid, in the present study, we collected many *Physarum* laboratory strains as are available at present throughout the world, and classified their origin on the basis of the results of mating-type analysis and restriction endonuclease analysis of their mtDNA. The trace harboring the mF plasmid was found in only two original strains.

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Table I. List of plasmodial strains of *P. polycephalum* classified by mtDNA RFLPs and PCR of the mF plasmid

Plasmodial strain	mtDNA		mF Plasmid			
	Type	mID ¹⁾	Free	Sou ²⁾	Integrated mF pID ³⁾	ter ⁴⁾
1 NG7×RA669	N7+P	yes	yes	com	yes	yes
2 OZ12×RA669	RM+P	yes	yes	com	yes	yes
3 OZ35×RA669	RM+P	yes	yes	com	yes	yes
4 NG11×RA669	N11+P	yes	yes	com	yes	yes
5 NG7×OX110	RM+P ⁵⁾	yes	yes	com	yes	yes
6 NG7×OX115	N7	yes	no	del	no	yes
7 NG27×RA669	N27	yes	no	del	no	yes
8 NG19×RA669	N19	yes	no	del	no	yes
9 NG15×RA669	N15	yes	no	del	no	yes
10 HI10×RA669	H10	yes	no	del	no	yes
11 HI14×RA669	H14	yes	no	del	no	yes
12 NC8×RA669	B8	yes	no	del	no	yes
13 DP12×DP13	I	yes	no	del	no	yes
14 DP14×DP15	WA	yes	no	no	no	no
15 DP74×DP75	B7	yes	no	no	no	no
16 DP89×DP90	W	yes	no	no	no	no
17 OS60×RA669	O	yes	no	no	no	no
18 OX110×OX115	M	yes	no	no	no	no
19 Sclero 1	— ⁶⁾	yes	—	—	no	no
20 Sclero 2	—	yes	—	—	yes	yes

¹⁾–⁴⁾ “mID”, “Sou”, “pID”, and “ter” indicate the results of PCR with mID primers, Southern hybridization, and PCR with pID and ter primers, respectively. ⁵⁾ The free mF plasmid was not detected on the agarose gel stained with ethidium bromide but was detected by PCR.

⁶⁾ The mtDNA from sclerotia could not be analyzed by agarose gel electrophoresis and Southern hybridization.

Materials and methods. *Strains.* The plasmodial and myxamoebal strains of *Physarum polycephalum* are listed in Tables I and II, respectively.^{6),7)} Two pieces of sclerotia were obtained from N. Kamiya (Osaka University, Sclero 1) and F. Matsumura (Nagoya University, Sclero 2). Plasmodia were cultured on MEA plates, on wet filter paper with compressed oats, and in semi defined liquid medium (SDM) as microplasmodia.^{8),9)} Sporulation and germination of spores were induced as described previously.^{8),9)} Myxamoebae were cultured with *Escherichia coli* on SM-1 plates.⁸⁾

Genotype analysis. The myxamoebal progeny of plasmodia were tested for their ability to form plasmodia in mating-type analysis with tester strains representative of certain known *matA* and *matB* alleles.^{8),10)} Two distinct mitochondrial phenotypes, mitochondrial fusion (*mif*⁺) and mitochondrial fusion-deficient (*mif*[−]) were classified to observe the frequency of mitochondrial fusion in the spores.^{2),11)}

Restriction endonuclease analysis. mtDNA was isolated from myxamoebal and plasmodial strains by density centrifugation on Hoechst 33258/CsCl.¹²⁾ The isolated

mtDNA was analyzed with certain restriction endonucleases also as described previously.²⁾ Southern hybridization analysis with the ECL labeling kit (Amersham) was carried out as described previously.¹¹⁾

PCR analysis. Three pairs of primers that amplify the DNA regions of identical sequences of mtDNA (mID) and the mF plasmid (pID), and the terminal region of the mF plasmid (ter) were used here. The primers of mID 1 and 2 are 5'GTTACTCTACCCGAATATCC3' and 5'CTATCC-TGCTCATTTATGTC3'; the primers of pID 1 and 2 are 5'GGATCCTTTTCATCGTAGCAC3' and 5'CTCGGCA-TTCCAAGATATA3'; the primers of ter 1 and 2 are 5'T-CCTTCTTCCGAAAATTTGG3' and 5'CGTAATAGACA-GTTAACACG3'. Polymerase chain reaction (PCR) with Taq polymerase (Takara Ex Taq, Takara Shuzo Co., Ltd., Japan) was performed in an automated Thermal Cycler according to the condition recommended by the manufacture (Takara). Total DNA of sclerotia for PCR was extracted with phenol and chloroform from small pieces of Sclero 1 and 2 which were soaked in Tris Saline EDTA (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 100 mM EDTA) including 2% SDS.

Table II. List of myxamoebal strains of *P. polycephalum* classified by mating type, mF plasmid, and origin of laboratory strains

Amoebal strain	Genotype	mtDNA type	Plasmid	Origin
Group I				
Sclero 1		—	mF ⁻	Pennsylvania
Progeny of Os-1				
OS60	<i>matA18matB14 mif⁻</i>	O	mF ⁻	Osaka
Group II				
Sclero 2		—	mF ⁺	North Carolina
Progeny of Ng				
NG7	<i>matA12matB6 mif⁺</i>	N7+P	mF ⁺	Nagoya
NG11	<i>matA11matB5 mif⁺</i>	N11+P	mF ⁺	Nagoya
NG15	<i>matA12matB5 mif⁻</i>	N15	ΔmF	Nagoya
NG19	<i>matA12matB6 mif⁻</i>	N19	ΔmF	Nagoya
NG27	<i>matA11matB5 mif⁻</i>	N27	ΔmF	Nagoya
Progeny of Hi				
HI10	<i>matA12matB6 mif⁻</i>	H10	ΔmF	Hitotsubashi
HI14	<i>matA11matB5 mif⁻</i>	H14	ΔmF	Hitotsubashi
Progeny of NG×RA				
OZ12	<i>matA12matB6 mif⁺</i>	RM+P	mF ⁺	Nagoya/McArdle
OZ35	<i>matA12matB6 mif⁺</i>	RM+P	mF ⁺	Nagoya/McArdle
Progeny of Nc				
NC8	<i>matA12matB5 mif⁻</i>	B8	ΔmF	North Carolina
Group III; Colonia isogenic				
XO110	<i>matA2matB1 mif⁻</i>	M	mF ⁻	McArdle/Indiana
XO115	<i>matA3matB1 mif⁻</i>	M	mF ⁻	McArdle/Indiana
RA669	<i>matA1matB4 mif⁻</i>	M	mF ⁻	McArdle/Indiana
Group IV; DP-series				
DP12	<i>matA5matB10 mif⁻</i>	I	ΔmF	Iowa
DP13	<i>matA6matB11 mif⁻</i>	I	ΔmF	Iowa
DP14	<i>matA7matB7 mif⁻</i>	WA	mF ⁻	Ward
DP15	<i>matA8matB8 mif⁻</i>	WA	mF ⁻	Ward
DP74	<i>matA12matB5 mif⁻</i>	B7	mF ⁻	Boreal
DP75	<i>matA11matB6 mif⁻</i>	B7	mF ⁻	Boreal
DP89	<i>matA15matB12 mif⁻</i>	W	mF ⁻	Wisconsin
DP90	<i>matA16matB13 mif⁻</i>	W	mF ⁻	Wisconsin

Results. We obtained 22 amoebal strains, which are available as laboratory strains (Table II) and two sclerotia which may be original strains of the OS series (Sclero 1) and NG series (Sclero 2).⁶⁾ Each natural isolate tends to carry two different alleles for *matB* locus as well as for the *matA* locus.⁷⁾ To verify the origin of these laboratory strains, we identified *matA* and *matB* alleles of all strains used except Sclero 1 and 2 (Table II), neither of which could be revived by any available means because they had been stocked for at least 56 and 16 years, respectively. Every amoebal strain investigated here was confirmed to have specific *matA* and *matB* alleles that identify the source of the isolate (Table II).

The extent of restriction fragment length polymorphisms (RFLPs) in mtDNA among these strains of *P. polycephalum* provides a useful source of cytoplasmic

genetic markers that indicate the origin of the isolate.⁸⁾ To confirm the origin of the strains used here on the basis of mtDNA RFLPs, we obtained 18 plasmodial strains by inbreeding and outbreeding with RA669, XO110, and XO115, and isolated their mtDNA. In *matA* hierarchy in transmission pattern of mtDNA,¹³⁾ the myxamoebal strain RA669 (*matA1matB4*) locates in the lower status and plays as male with respect to the transmission of mtDNA.¹³⁾ This allows us to directly determine the RFLPs of other myxamoebal strains from the mtDNA of the plasmodia yielded by the mating cross with RA669. Fig. 1b shows the patterns of *Hind*III restriction fragments of mtDNA from 18 representative plasmodial strains. Sixteen distinct types of mtDNA were detected (Table I). The fragments specific for the strains having the free mF plasmid (mF⁺) cannot be detected at first glance (Fig. 1b). However, agarose gel

Fig. 1. Profiles of undigested (a) and *Hind*III-digested (b) mtDNAs from 18 plasmodial strains after electrophoresis on agarose gels. Southern hybridization of the same gels was carried out using labeled plasmid DNA as the probe (c and d). Closed and open arrowheads indicate bands of mtDNA and the mF plasmid, respectively. Lanes 1-4 are mF⁺, lanes 5-13 are Δ mF, and lanes 14-18 are mF⁻. The plasmodial strains used here are listed in Table I. The plasmodial strain of lane 5 (NG7×OX110) was later confirmed to be mF⁺ by PCR.

electrophoresis of undigested mtDNA (Fig. 1a) allowed classification of the mtDNAs into two types according to whether they are mF⁺ or mF⁻. The mtDNA of each mF⁺ strain also formed a single band of 80-90 kbp, but, in addition to this main band, mF⁺ strains yielded a second band of approximately 14-16 kbp.^{2),14)}

Southern hybridization using labeled plasmid DNA as

Fig. 2. Profiles after electrophoresis on agarose gels of PCR products obtained with mID 1 and 2 primers (mID), pID 1 and 2 primers (pID), and ter 1 and 2 primers (ter), which amplify 317 bp, 1130 bp, and 769 bp of both identical sequences of mtDNA (mID) and the mF plasmid (pID), and a near terminal region of the mF plasmid, respectively. Lanes 1-5 and 20 are mF⁺, lanes 6-13 are Δ mF, and lanes 14-19 are mF⁻. The plasmodial strains used here are listed in Table I.

the probe was carried out to determine whether any of the sequences of the mF plasmid were represented in the mitochondrial genomes of the mF⁻ strains. The labeled plasmid probe hybridized to some bands of mtDNA not only in all of the mF⁺ strains but in some of the mF⁻ strains as well (Fig. 1d). This suggests that a portion of the mF plasmid integrated into mtDNA is deleted in some mF⁻ strains. Moreover, in mF⁻ strains not having strong hybridized bands, the probe faintly hybridized to the whole mitochondrial genome (Fig. 1c) and the largest *Hind*III fragment of the mitochondrial genome (Fig. 1d). This suggests that the mtDNA of not only mF⁺ but mF⁻ possesses an mID sequence. Accordingly, the mF⁻ strains were classified into two categories: simple mF⁻ having only the mID sequence and Δ mF having some part of the mF plasmid integrated into mtDNA.

To confirm the results of Southern hybridization, we designed PCR primers to amplify a part (317 bp) of the mID sequence of M-type mtDNA. Fig. 2 shows that the mID primers could amplify mID sequences in the mtDNA of all strains, including Sclero 1 and 2 (lanes 19 and 20 in mID). The mtDNAs for Sclero 1 and 2 used here were small amounts of degraded total DNA extracted from pieces of Sclero 1 and 2. This finding not only confirms that the mtDNA of all strains possesses the mID sequence but shows that a specific sequence of mtDNA, such as mID, could be amplified by our PCR system using the degraded total DNA of scleroticia. Fig. 2 also shows the results of PCR using pID and ter primers. Since the pID primers

amplify the sequence (1,130 bp) including border sequences of pID, only free mF plasmids not integrated mF plasmids can be detected by using them (Table I). On the other hand, since the *ter* primers amplify the sequence (769 bp) that locates in the near terminal region of the mF plasmid, the mF plasmid can be detected regardless of whether integrated or free (Table I). Therefore, the PCR system using these two pairs of primers allows determination of whether apparent mF⁻ strains and sclerotia contain the free and integrated mF plasmid. For example, this PCR system showed the free mF plasmid in NG7×OX110 (lane 5 in Fig. 2), instead of the presence of a complete sequence of the integrated mF plasmid, although its presence was not detected by either agarose gel electrophoresis of mtDNA or Southern hybridization (lane 5 in Fig. 1). Furthermore, the results shown in lanes 19 and 20 of Fig. 2 confirmed that Sclero 1 is simple mF⁻ but Sclero 2 is complete mF⁺.

Discussion. *P. polycephalum* appears to be characterized by three unique alleles at each of the unlinked multiallelic, mating-type loci *matA*, *matB*, and *matC*,^{15–17} making mating-type alleles good markers of nuclear genes for determining the particular isolate from which any given strain is derived. To cross efficiently, myxamoebae must carry different alleles, at least at *matA* and *matB*. For each of these loci, full compatibility results when any two alleles are combined among a set of at least fifteen. Each natural isolate tends to carry two different alleles at *matB* as well as at the *matA* locus.⁷ In this study, 22 amoebal strains that had been mated to form 18 plasmodia and two sclerotia were classified into four groups according to mating types (Table II). Although the two sclerotia could not be revived, Sclero 1 and 2 were included in Group I and II, respectively. Based on its mating type and mtDNA type, we have practically confirmed that a plasmodial strain, Os-1 originated at Seifriz's laboratory in the University of Pennsylvania.⁶ Since Os-1 is thought to be one of the plasmodial strains descended from Sclero 1 at Kamiya's laboratory in Osaka University, Sclero 1 was classified in the same group as OS60 (Table II). This group is the oldest laboratory strain in the world.⁶

The extent of mtDNA RFLPs among the strains of *P. polycephalum* is higher than in other organisms and provides a useful source of cytoplasmic genetic markers.¹⁸ The mtDNA types are usually consistent with the mating types and the isolate origins of myxamoebal strains, as shown in Group IV (Table II). On the other hand, although all myxamoebal strains included in Group II have the same combination of mating types (*matA*11, 12, *matB*5, 6), their mtDNA types varied considerably (lanes 1–12 in Fig. 1,

Tables I and II). This is because they have harbored or currently harbor the mF plasmid. Some Δ mF strains harbor certain sequences of the integrated mF plasmid in spite of being determined to be *mif*⁻ and mF⁻ according to the phenotype of mitochondrial fusion and the results of restriction endonuclease analysis (Table II). Takano *et al.* (1996)⁹ have shown that recombination occurs between only 12-nucleotide or six repeats of AAT of homologous sequences in both mtDNA and the integrated mF plasmid, and the regions lying between these homologous sequences are deleted to yield various mtDNA RFLPs. Δ mF seems to be caused by such deletions combined with a marked decrease in free mF plasmids, as shown in NG7×OX110. The presence of some Δ mF strains in Group II suggests that this deletion combined with loss of the free mF plasmid occurs frequently.

The free mF plasmid was first found in NG7, a progeny of the plasmodial strain Ng.² However, it is impossible to determine whether Ng contains the free mF plasmid, since the original strain cultured at Nagoya University was ruined for itself for aging. Japanese Physarumists mainly used three plasmodial strains that had descended from strains in Seifriz's laboratory, and the Ward Biological and North Carolina Biological supply houses.⁶ Neither Sclero 1 derived from Seifriz's laboratory nor the amoebal strains (DP14 and DP15) derived from Ward Biological have the mF plasmid. Only Sclero 2, a progenitor of Ng derived from North Carolina Biological, according to the mating types has the free mF plasmid (Table II). These findings suggest that the origin of the mF plasmid is North Carolina Biological. However, the amoebal strains (HI10, HI14, and NC8) isolated from the plasmodia from North Carolina Biological are Δ mF but contain no free mF plasmids. It is possible that only one plasmodium purchased from North Carolina Biological during a very limited period had the free mF plasmid.

When Collins (1975)²⁰ first analyzed the *matA* alleles of plasmodia, he found that the plasmodium from North Carolina Biological carried the *matA*9 and *matA*10 alleles and that the Turtox plasmodium carried the *matA*11 and *matA*12 alleles. However, Kirouac-Brunet *et al.* (1981)⁷ obtained plasmodia from the North Carolina, Turtox, and Boreal Biological supply houses in 1978 and found that they all contain the same four alleles: *matA*11, *matA*12, *matB*5, and *matB*6. Kawano *et al.* (1987)¹⁸ also confirmed that the plasmodium from North Carolina Biological had been replaced by the plasmodium that was originally known as "Turtox isolate" (*matA*11, 12; *matB*5, 6). The myxamoebal strains (DP74 and DP75) derived from the Boreal plasmodium replaced by the Turtox isolate at almost same

time as North Carolina Biological were confirmed to be simple mF⁻ in this study (Table II). This shows that the Turtox isolate did not have the free mF plasmid at least until it was transplanted to North Carolina Biological.

DP12 and DP13 are Δ mF and contain some regions of the integrated mF plasmid (Table II). This shows that the free mF plasmid was present in their progenitor. Their original strains are Ppll.1.17–100 and Ppll.1.17–80, which were isolated by Collins (1975)²⁰ from a stock at the University of Iowa. This stock may be derived from materials collected by Howard (1931)²¹ while at the University of Iowa. There is no evidence showing that these strains were related to the North Carolina plasmodium. There may be two lines having the free mF plasmid. Such scattered distribution of the free mF plasmid suggests that its origin is not evolutionary but occasional.

At first glance this seems to be inconsistent with the fact that the mF plasmid has a sequence (pID) that is almost completely identical to that of mtDNA (mID). This mID is common to the mtDNA of all strains (Table I). The homology between the sequences of pID and mID suggests a common origin for these sequences. It is reasonable to conclude that the mF plasmid was integrated into the mtDNA and that almost all of the sequences of the integrated mF plasmid were subsequently deleted leaving a very short sequence such as pID in the mtDNA, i.e., mID originated in pID. Alternatively, the mF plasmid acquired mID from the mtDNA after its entry into a mitochondrion i.e. pID originated in mID.²² Since all strains of *P. polycephalum* possess mID, if the former hypothesis is true, the origin of the mF plasmid should be older than the establishment of *P. polycephalum* as a species. However, the scattered distribution of the free mF plasmid shown in this work suggests that the mitochondria occasionally acquired the mF plasmid after the establishment of *P. polycephalum*. To trace the true origin of the mF plasmid, in the future, we will need sequential information on mID and pID based on such a pedigree analysis of *Physarum* laboratory strains.

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