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ORIGINAL ARTICLE

Cytoplasmic inheritance of mitochondria and chloroplasts in the anisogamous brown alga *Mutimo cylindricus* (Phaeophyceae)

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Abstract

Based on the morphology of gametes, sexual reproduction in brown algae is usually classified into three types: isogamy, anisogamy, and oogamy. In isogamy, chloroplasts and chloroplast DNA (chlDNA) in the sporophyte cells are inherited biparentally, while mitochondria (or mitochondrial DNA, mtDNA) is inherited maternally. In oogamy, chloroplasts and mitochondria are inherited maternally. However, the patterns of mitochondrial and chloroplast inheritance in anisogamy have not been clarified. Here, we examined derivation of mtDNA and chlDNA in the zygotes through strain-specific PCR analysis using primers based on single nucleotide polymorphism in the anisogamous brown alga *Mutimo cylindricus*. In 20-day-old sporophytes after fertilization, mtDNA and chlDNA derived from female gametes were detected, thus confirming the maternal inheritance of both organelles. Additionally, the behavior of mitochondria and chloroplasts in the zygotes was analyzed by examining the consecutive serial sections using transmission electron microscopy. Male mitochondria were isolated or compartmentalized by a double-membrane and then completely digested into a multivesicular structure 2 h after fertilization. Meanwhile, male chloroplasts with eyespots were observed even in 4-day-old, seven-celled sporophytes. The final fate of male chloroplasts could not be traced. Organelle DNA copy number was also examined in female and male gametes. The DNA copy number per chloroplast and mitochondria in male gametes was lower compared with female organelles. The degree of difference is bigger in mtDNA. Thus, changes in different morphology and DNA amount indicate that maternal inheritance of mitochondria and chloroplasts in this species may be based on different processes and timing after fertilization.

Keywords Anisogamy · Brown algae · Cytoplasmic inheritance · Organelle DNA copy number · Strain specific PCR · Ultrastructure

Introduction

In 1909, the non-Mendelian inheritance was firstly discovered in the higher plants *Mirabilis jalapa* and *Pelargonium zonale* (Baur 1909; Correns 1909). Interestingly, these discoveries were two entirely different patterns in inheritance of leaf phenotypes. The offspring leaf color in *P. zonale* shows biparental inheritance, while the pattern of the next generation is always defined by the maternal parent in *M. jalapa*. Since then, over the past 100 years, the pattern and the mechanism of cytoplasmic inheritance have been studied in many organisms.

In algae, there is a diversity of sexual reproduction types based on the different morphology (shape and size) of male and female gametes. Green and brown algae have three patterns in sexual reproduction: isogamy, anisogamy, and oogamy (Wynne and Loiseaux 1976; Graham and Wilcox 2000). In contrast, the type of sexual reproduction in red algae is oogamy (Hommersand and Fredericq 1990). In the red algae, mitochondria DNA (mtDNA) and chloroplast DNA (chlDNA) maternally inherited in hybrids between different haplotypes of organellar DNA in *Pyropia yezoensis* and *Bostrychia moritziana* (Zuccarello et al. 1999a, 1999b; Choi et al. 2008). In most cases of oogamy in brown algae and

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green algae, both mitochondria and chloroplasts are maternally inherited (Sun et al. 1988; Adams et al. 1990; Motomura 1990; Kraan and Guiry 2000; Coyer et al. 2002; Kimura et al. 2010b).

In isogamy and anisogamy, organelle inheritance patterns are different in organisms. In the isogamous green alga *Chlamydomonas reinhardtii*, zygotes inherited chlDNA from plus mating type, while mtDNA is from minus mating type exclusively (Sager and Lane 1972; Boynton et al. 1987). In anisogamous green algae *Bryopsis maxima* and *Derbesia tenuissima*, chlDNA and mtDNA in male gametangium show preferential digestion during gametogenesis before the release of gametes, since only organelle DNAs of female gametes remain in zygotes (Kuroiwa and Hori 1986; Kuroiwa et al. 1991; Lee et al. 2002). The isogamous brown algae *Scytosiphon lomentaria* and *Ectocarpus siliculosus* were confirmed to inherit maternal mitochondria, because preferential degradation of mtDNA derived from male gamete after fertilization was shown (Peters et al. 2004; Kato et al. 2006; Kimura et al. 2010a). However, the fate of paternal mitochondria without DNA after fertilization has not yet been clarified. In isogamous species, chloroplasts are inherited biparentally, and two chloroplasts in the zygote from the male and female gametes are randomly distributed into the daughter cells during cytokinesis (Nagasato et al. 2000; Peters et al. 2004; Kato et al. 2006; Kimura et al. 2010a). There are few cytological reports regarding fertilization of anisogamous brown algae (Nagasato et al. 1998). Nagasato et al. (1998) observed the fertilization of *Mutimo cylindricus* focusing on the paternal inheritance of centrioles, but the cases of chloroplasts and mitochondria were not mentioned.

In eukaryotic cells, autophagy and the lysosome-mediated degradation process of intercellular proteins and structures are the major pathways to remove the damaged or unwanted mitochondria (mitophagy) for maintaining the fundamental processes in eukaryotic development (Green and Levine 2014; Redmann et al. 2014). Moreover, in autophagy, mitochondrial shape changes by elongation and branching (Gomes and Scorrano 2013). In *Caenorhabditis elegans*, the paternal mitochondrial is eliminated by autophagy after fertilization (Sato and Sato 2011). Meanwhile, in the green alga *C. reinhardtii*, mitochondria are fused with the opposite mating types in the zygote, and the plus mating type (maternal) mtDNA is selectively eliminated during maturation (Aoyama et al. 2006). In the brown algal oogamous species, *Saccharina angustata* and *Undaria pinnatifida*, paternal mitochondria and chloroplasts are digested within lysosomes in one-celled stage (Motomura 1990; Kimura et al. 2010b). Autophagy is involved in the degradation of chloroplast materials (Zhuang and Jiang 2019); however, there has been no report of the elimination of uniparental chloroplasts by autophagy.

In this study, we examined the cytoplasmic inheritance of mitochondria and chloroplasts including the organelle DNA and structure in the anisogamous brown alga, *M. cylindricus*, to compare with the cases of isogamous and oogamous species of the brown algae. To achieve this purpose, we performed observations of the behavior of mitochondria and chloroplasts using transmission electron microscopy, genotyping, and PCR analysis using the strain-specific primers of the juvenile sporophyte derived from a single zygote to estimate organelle DNA copy number using real-time PCR. Our results suggest that mitochondria and chloroplasts (or chlDNA) were inherited maternally in the anisogamous brown alga *M. cylindricus*, and the exclusion manner of paternal mitochondria organelle is similar to the oogamous cases.

Material and methods

Culture material

Unialgal cultures of *M. cylindricus* (Okamura) H. kawai and T. Kitayama (Kawai et al. 2012) were established from gametes or trichothallic hairs of male and female gametophytes collected at Katsuma Coast, Fukuoka, Fukuoka, Japan (33°41′ N 130°17′ E) and Odanohama, Toba, Mie, Japan (34°45′ N 136°87′ E), respectively, in March 2015 and incubated in the sterilized seawater containing half strength Provasoli's enriched seawater (PES) medium (Provasoli 1968). Female and male strains from Fukuoka or Mie were named Fuk-F, -M or Mie-F, -M, respectively. Gametogenesis and maturation were induced under the following conditions: 15 °C, using 20–40 μmol photons m^{-2} s^{-1}, long-day conditions (14 h light:10 h dark), using white light-emitting diodes (LED). The release of gametes was stimulated by exchange of fresh culture medium the day before and by light on the next day. Male and female gametes were separately collected into microtubes on ice. Fertilization was induced by added male gametes to settled female gametes on the cover glasses.

Design for the strain-specific primers in nuclear, mitochondrial, and chloroplastidal DNA

To design the molecular markers in nuclear, mitochondrial, and chloroplastidal DNA for each strain, DNA was amplified using the common primers among strains in each region as follows: Pha18EF (5′- AGGAAGGTGAAGTCGTAACAAGGTTT -3′) and Pha5.8ER (5′- AACAGACACTCCGACAAGCATGCTCCC -3′) for the internal transcribed spacer 1 (ITS1) region of nuclear ribosomal DNA (nrDNA) (Uwai et al. 2001); Mc_nad2 (5′- TAGGNYTADGBYTTHTVDTTT -3′) and Mc_nad9 (5′- ADDTCCCAAATTTCACGTTCC -3′) which were based on *nad2* and *nad9* nucleotide sequences of brown algae *Fucus vesiculosus* (NC_007683), *Dictyota dichotoma* (NC_007685), *Desmarestia viridis* (NC_007684),

Saccharina japonica (NC_013476), *Ectocarpus siliculosus* (NC_030223), and *Pylaiella littoralis* (NC_003055) for cytochrome c oxidase subunit 1 (*cox1*) gene; and psaA130F (5′-AACWACWACTTGGATTTGGAA -3′) and psaA970R (5′-GCYTCTARAATYTCTTTCA -3′) for the PSI P700 chlorophyll a apoprotein A1 (*psaA*) gene of chloroplast DNA (Yoon et al. 2002).

Total genomic DNA was extracted from gametophytes of Fuk-F, Fuk-M, Mie-F, and Mie-M using the DNeasy plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The templates were amplificated by TaKaRa Ex Taq (TaKaRa, Shiga, Japan) in a TaKaRa PCR Thermal Cycler Dice (TaKaRa) using 30 cycles of 96 °C for 30 s, annealing at appropriable temperature for primers for 30 s, and an extension at 68 °C for 1 or 3 min depending on the predicted product sizes. The details of primers and the concentration used in PCR reaction mixture is in Supplementary Table S1. Amplified products were purified and eluted to carry out sequencing directly using an ABI 3730xl DNA analyzer or 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Based on the sites of single nucleotide polymorphisms, we designed the six primer pairs for strain-specific PCR. The Mc_optFuknrF (5′-CGAGAGAGGAAGCGAGAACC -3′) and Mc_optMienrF (5′- CGAGAGAGGAAGCGAGAACA- 3′) as forward primers were used for detection of nrDNA for Fukuoka- (LC573962) or Mie-strains (LC573963). The corresponding reverse primer used the common primer Pha5.8ER. Mc_optFukmtF (5′- TTTTATAACCACCATTTTTCAC -3′) and Mc_optFukmtR (5′- AAATCCTGGTAAAATTATTC TA -3′) were used for detection of mtDNA of Fukuoka-strain (LC573964), and Mc_optMiemtF (5′- TTTTATAA CCACCATTTTTCAT -3′) and Mc_optMiemtR (5′-AAATCCTGGTAAAATTATTCTG -3′) for mtDNA detection of Mie-strain (LC573965), Mc_optFukcpF (5′-GGACAAGAAATATTAAATGGAGATTTA -3′) and Mc_optFukcpR (5′- CCAAGCAAGACAACCTCAC -3′) for chlDNA detection of Fukuoka-strain, Mc_optMiecpF (5′- GGACAAGAAATATTAAATGGAGATTTG -3′) and Mc_optMiecpR (5′- CCAAGCAAGACAACCTCAA -3′) for chlDNA detection of Mie-strains for the strain specific PCR (the list is in Table 1). For optimizing the specificity, we created a mismatch artificially at the third base from the 3′end by substituting a G for a T residue (or an A for a C) (Hayashi et al. 2004).

We also designed the three primer pairs for quantitative real-time PCR (qPCR) to quantify the DNA copy numbers included in gametes of Fukuoka- and Mie-strains. Mc_ITS1QF (5′- TGTCTTTGTGGGTGTTCGTG -3′) and Mc_ITS1QR (5′- CGCATAACAAGGGACAAGAAA -3′) primers were used for nrDNA, Mc_cox1QF (5′-CGCCCCAGGAATGAGCA -3′) and Mc_cox1QR (5′-CAGGAAGTGAAAGCAAAAGCA -3′) for mtDNA, and Mc_psaAQF (5′- GGTTCCAAAATGCTGAATCG -3′) and Mc_psaAQR (5′- TGATGACCTGACCAAGCAAG -3′) for chlDNA for the qPCR assay (the list is in Table 1).

PCR with the strain-specific primers

Inheritance of mitochondrial and chloroplastidal DNA in 20-day-old hybrid sporophytes with Fuk-F and Mie-M, or Mie-F and Fuk-M, was assayed by PCR with the strain-specific primers. Sporophyte DNA was extracted according to the method proposed by Xin et al. (2003). Before DNA extraction, the sporophytes were fixated with 70% ethanol overnight at 4 °C; then, each sporophyte was transferred into a different microtube. Each sporophyte was incubated in 50-μl lysis buffer A consisting of 100-mM NaOH and 2% Tween 20 at 95 °C for 20 min and neutralized with 50-μl buffer B (pH is about 2.0) consisting of 100-mM Tris-HCl and 2-mM ethylenediaminetetraacetic acid (EDTA). Extracted DNA was purified by ethanol precipitation and resuspended in 10 μl of MilliQ water.

Cytoplasmic inheritance of the sporophyte was assayed by PCR using the strain-specific primer pairs for mtDNA and chlDNA with TaKaRa Ex Taq (TaKaRa). Detection of male and female nuclear DNA to confirm the success of fertilization was done using the primer pairs, Mc_optFuknrF or Mc_optMienrF, and Pha5.8ER with AmpliTaq Gold Master Mix (Applied Biosystems). The cycling conditions were as follows: 40 cycles of 95 °C for 5 s and 60 °C for 60 s. For the detection of nrDNA, unspecific amplification was seen by PCR with TaKaRa Ex Taq; thereby, different polymerases were used to minimize this. The concentration of primers and templates used in PCR reaction mixture are shown in Supplementary Table S1. Amplification PCR products were detected by staining with GelRed (Biotium, Hayward, USA) on a 2% agarose gel.

Examination of *cox1* and *psaA* copies in gametes

Copy numbers of *cox1* and *psaA* genes included in female and male gametes were examined using Fukuoka- and Mie-strains. The PCR fragments amplified with TaKaRa Ex Taq include the regions of *cox1*, by using Mc_cox1F (5′-TCATCTATCTGGTGCAGCTTCT -3′) and Mc_cox1R (5′-TACCTGTAGGAACAGCTAT -3′) and *psaA*, by using Mc_psaAF (5′-GCAATCATATTTTTATGGATAAGTGG -3′) and Mc_psaAR (5′- TTGATAAGAAACTCATATGG TAAAGG -3′). The PCR products (494 bp in *cox1* region and 500 bp in *psaA* region) were ligated into the pT7blue vector (Novagen, Madison, USA), and transformation was performed in *E. coli* JM109 competent cells (TaKaRa). The copy number of the plasmids was calculated as the following formula: 6.02×10^{23} (copies·mol^{-1}) × amount of recombinant plasmid DNA (g)/DNA length (bp) × 660 (g·mol^{-1}·bp^{-1})

Table 1 Primers used in this study

Location/target	No.	Primer name	Sequence (5′ − 3′)	AT[a] (°C)
Primers for next-generation sequencing				
nrDNA/ITS1	1	Pha18EF	AGGAAGGTGAAGTCGTAACAAGGTTT	58
	2	Pha5.8ER	AACAGACACTCCGACAAGCATGCTCCC	58
mtDNA/cox1	3	Mc_nad2	TAGGNYTADGBYTTHTVDTTT	55
	4	Mc_nad9	ADDTCCCAAATTTCACGTTCC	55
chlDNA/*psaA*	5	psaA130F	AACWACWACTTGGATTTGGAA	50
	6	psaA970R	GCYTCTARAATYTCTTTCA	50
Primers[b] for strain-specific PCR				
Fuk, nrDNA/ITS1	7	Mc_optFuknrF	CGAGAGAGGAAGCGAGAACC	58
Mie, nrDNA/ITS1	8	Mc_optMienrF	CGAGAGAGGAAGCGAGAACA	58
Fuk, mtDNA/cox1	9	Mc_optFukmtF	TTTTATAACCACCATTTTTCAC	45
	10	Mc_optFukmtR	AAATCCTGGTAAAATTATTCTA	45
Mie, mtDNA/cox1	11	Mc_optMiemtF	TTTTATAACCACCATTTTTCAT	45
	12	Mc_optMiemtR	AAATCCTGGTAAAATTATTCTG	45
Fuk, chlDNA/*psaA*	13	Mc_optFukcpF	GGACAAGAAATATTAAATGGAGATTTA	50
	14	Mc_optFukcpR	CCAAGCAAGACAACCTCAC	50
Mie, chlDNA/*psaA*	15	Mc_optMiecpF	GGACAAGAAATATTAAATGGAGATTTG	50
	16	Mc_optMiecpR	CCAAGCAAGACAACCTCAA	50
Primers for absolute quantitative real-time PCR				
nrDNA/ITS1	17	Mc_ITS1QF	TGTCTTTGTGGGTGTTCGTG	60
	18	Mc_ITS1QR	CGCATAACAAGGGACAAGAAA	60
mtDNA/cox1	19	Mc_cox1QF	CGCCCCAGGAATGAGCA	60
	20	Mc_cox1QR	CAGGAAGTGAAAGCAAAAGCA	60
chlDNA/*psaA*	21	Mc_psaAQF	GGTTCCAAAATGCTGAATCG	60
	22	Mc_psaAQR	TGATGACCTGACCAAGCAAG	60

[a] *AT* annealing temperature

[b] An artificial mismatch (underline) was created at the 3rd base of the 3′ end by substituting G for T (or A for C)

(Whelan et al. 2003). Serial dilutions of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 of the copies of recombinant plasmid DNA were subjected to real-time PCR, using a 10-μl volume containing 5 μl of SYBR Premix Ex Taq II (TaKaRa) and 0.25 μM of each primer. Real-time PCR was performed on the Eco Real-Time PCR System (Illumina, San Diego, USA) using the following conditions: initial polymerase activation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 1 min. A standard curve was established from the recombinant plasmid with known copy numbers by plotting the quantification cycle (Cq) values against the copy number of recombinant plasmid DNA.

Gametes (20 μl) were fixated with 1.5% glutaraldehyde in seawater for density determination using a hemocytometer. A volume of 2 μl of gametes were fixated with 70% ethanol for DNA extraction using the DNeasy plant mini kit (QIAGEN). To evaluate the efficiency of gamete DNA extraction of female and male gametes, we performed the qPCR targeting the nrDNA marker, ITS1, using a DNA amount equivalent to the one of forty gametes. The primer pair Mc_ITS1QF and Mc_ITS1QR was used for verification. The results showed that the Cq values were almost the same between the DNA equivalent of 100 gametes in female and male gametes of each strain (Fuk-F, 21.17; Fuk-M, 21.13; Mie-F, 21.14; Mie-M, 21.12). An amount of DNA equivalent to 50, 100, and 200 gametes were used as a template for real-time PCR as described above and the obtained Cq value was substituted into the formula to obtain the copy number per gamete.

Preparation of samples for TEM

To obtain a lot of female and male gametes from Mie, mature gametophytes were collected at Odanohama, Toba, Mie, Japan (April, 2019) and the release of gametes was induced in the laboratory. Protocol for rapid freezing and freeze substitution in TEM samples was done according to a previous

report (Nagasato and Motomura 2002). Gametes were centrifugated and the pellet was placed on a formvar-coated gold ring. Fertilization and development of zygotes were induced on a gel support film (ATTO, Tokyo, Japan). The 2- and 6-hour and 1-, 2- and 4-day-old zygotes were fixated with the film. All samples were rapidly frozen by introducing the samples into liquid propane which was previously cooled down to − 180 °C in liquid nitrogen, and immediately transferred into liquid nitrogen. Then they were transferred into cooled acetone (− 85 °C) containing 2% of osmium tetroxide and stored at − 85 °C for 2 days. After that, the fixated samples were gradually allowed to rise in temperature by being placed at − 20 °C for 2 h, at 4 °C for 2 h, and at room temperature for 30 min, sequentially. The samples were then washed with acetone several times at room temperature and embedded in the low-viscosity Spurr's epoxy resin (Polysciences, Warrington, USA) on the aluminum foil dishes. Serial sections were cut with a diamond knife on an ULTRACUT ultramicrotome (Reichert-Jung, Vienna, Austria) and mounted on formvar-coated slot grids. The sections were stained with EM stainer (Nisshin EM, Tokyo, Japan) and lead citrate. Observation was done by using a JEM-1011 electron microscope (JEOL, Tokyo, Japan). In this experiment, we examined the consecutive serial sections of each sample.

Results

Feature of gametes and development of zygotes

The life cycle of *M. cylindricus* shows an alternation of generations between a macroscopic haploid male and female gametophyte and a microscopic diploid sporophyte (Kitayama et al. 1992). Sexual reproduction is carried out by fusion of a large female gamete and a small male gamete. Female gametes are within 12–15 μm in length and 7–8 μm in width, which are approximately twice the size of the male gametes (Supplementary Fig. S1a, b). There was no difference in size between the female and male gametes in Fukuoka- and Mie-strains used in this study. Both gametes swim with two flagella, an anterior long flagellum and a posterior short one. Female gametes swim and attach on the substratum and secrete sexual pheromones to attract male gametes. The fertilization is judged by counting the number of eyespots, which is equal to the number of gametes involved in fertilization. Two eyespots derived from male and female gametes were observed in a zygote, 6 h after fertilization (Supplementary Fig. S1c). A 2-day-old zygote completed cytokinesis and two eyespots were found in the daughter cells (Supplementary Fig. S1d). At 4 days after fertilization, the zygote symmetrically divided and entered the four-celled stage. The eyespots became smaller in this stage when compared with the one- and two-celled stages (Supplementary Fig. S1e). In an 8-celled stage sporophyte, only one eyespot remained (Supplementary Fig. S1f). For 20 days after fertilization, each zygote grew into a crustose thallus (Fig. S1g). Supplementary Fig. S1c–g shows the development of the same zygote.

Mitochondria and chloroplasts in male and female gametes

The number of mitochondria and chloroplasts in female and male gametes was examined in complete serial sections using TEM (male in Fig.1a–d and female in Fig. 1e–l). Each five female and male gametes were observed. In the female gametes, 20–30 mitochondria were observed, while the male gamete usually had 4–5 mitochondria (Table 2). Mitochondria in male and female gametes presented a globular shape with 650–1000 nm of diameter (Fig. 1; Supplementary Fig. S2a, b). Through the observation of mitochondrial cross-sections using TEM, all of the mitochondria contained linear materials inside the tubular cristae both in male and female gametes (Supplementary Fig. S2c, d). There is only a single chloroplast in male gametes, while female gametes contain 7–12 chloroplasts (Table 2). In female gametes, one of the chloroplasts has an eyespot. A chloroplast having an eyespot is tightly associated with the nucleus both in male and female gametes (Supplementary Fig. S2e, f). There were 3–4 thylakoid lamellae and girdle lamella enclosing chloroplast stroma in the chloroplasts of male gametes and 6–10 in the chloroplasts of female gametes (Supplementary Fig. S2e, f).

Behavior of mitochondria after fertilization

We observed the five zygotes 2 h after fertilization. Three zygotes finished karyogamy. In 2-hour-old zygotes, two chloroplasts with eyespots derived from female and male gametes could be observed (Fig. 2a, b; Supplementary Fig. S3). Mitochondria enclosed in double-membranous structures were frequently observed and were always found near the male chloroplasts and a pair of centrioles derived from male gametes (Fig. 2b; Supplementary Fig. S3h). Those mitochondria were found around condensed male nucleus before karyogamy and chloroplast with eyespot was tightly associated with male nucleus (Supplementary Fig. S4). The structures indicated as m1 in Fig. 2c and d seemed to indicate the most considerably degraded mitochondrion, and m2 in Fig. 2e and f would follow the same outcome. In those mitochondria, many vesicles arose inside, and cristae became obscure. Other mitochondria surrounded with a double-membrane (m3 in Fig. 2e–h, m4 in Fig. 2h, i, and m5 in Fig. 2j) appeared to show the early stage of degradation, as seen by m1 and m2 in Fig. 2c–f.

At 2 h after fertilization, each zygote contained 29 mitochondria and 10 chloroplasts on average. The number of mitochondria included intact and digesting mitochondria. It was

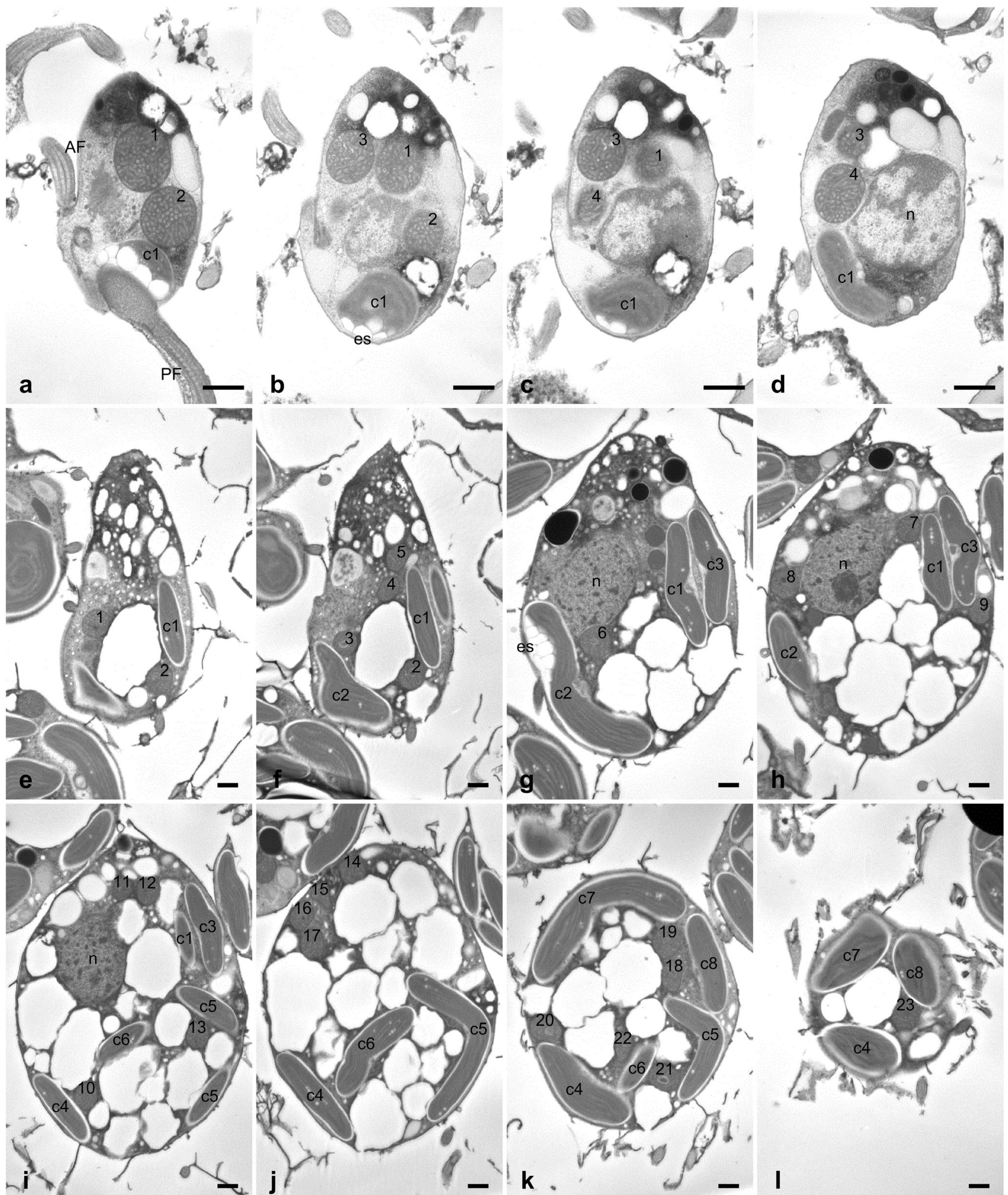


Fig. 1 Serial section of male and female gametes of *Mutimo cylindricus*. **a–d** A male gamete. A total of four mitochondria are numbered, and a chloroplast is indicated as c1. **e–l** A female gamete. A total of 23 mitochondria are numbered, and a total of eight chloroplasts are indicated by their serial number with C. AF, anterior flagellum; es, eyespot; n, nucleus; PF, posterior flagellum. Scale bars: 500 nm

Table 2 Number of mitochondria and chloroplasts in gamete and zygote in *Mutimo cylindricus*

	Male gamete		Female gamete		2 h zygote		6 h zygote	
	mt[a]	chl[b]	mt	chl	mt	chl	mt	chl
Sample 1	4	1	20	7	25	8	20	8
Sample 2	4	1	20	8	27*	9	21	9
Sample 3	5	1	23	8	28**	9	22	9
Sample 4	5	1	28	10	29	9	29	10
Sample 5	5	1	30	12	36*	13	30	12
Average[c]	5	1	24	9	29	10	24	10

[a] Mitochondria

[b] Chloroplasts

[c] Rounding off in average

* One digesting mitochondrion was found

** Two digesting mitochondria was found

Each value of the number of mitochondria or chloroplasts is counted by complete serial sections

coincident with the sum of mitochondria and chloroplasts in both gametes (Table 2).

At 6 h after fertilization, mitochondria enclosed with a double membrane were no longer observed in our complete serial sections (Supplementary Fig. S5). The mean number of mitochondria was about 5 lower than that of the 2-hour-old zygote, which may be the result of digestion of male gamete mitochondria (Table 2).

Chloroplasts in four- and eight-celled sporophytes

Three days after fertilization, most zygotes had developed into symmetrical 4-celled crustose sporophytes (Fig. 3a). Two small eyespots were usually found in different cells (Fig. 3b). Both of them contained 6–8 thylakoid lamellae within the girdle lamella (Fig. 3c–h). It was difficult to distinguish male and female chloroplasts from the number of thylakoid lamellae as in the early zygotes. Four days after fertilization, most sporophytes became about 8-celled (Supplementary Fig. S6a, b). Two eyespots at this stage could not be detected in all sporophytes (Supplementary Fig. S6c, d).

PCR using strain-specific primers

To examine the inheritance pattern of mtDNA and chlDNA in sporophytes, we performed PCR using strain-specific primers against 20-day-old artificial hybrids of Fuk-F and Mie-M or Mie-F and Fuk-M. The sporophyte phase was prepared for four individuals.

Strain-specific primers within the ITS region were amplified with Pha18EF and Pha5.8ER. The polymorphism region within it was only one base. As the strain-specific primer, a forward primer containing SNP in the 3′ site was designed, and the common primer was used as the reverse primer. The PCR product targeted by these primers was 238 bp in length (Supplementary Fig. S7a). The electrophoresis showed that these primers had high specificity for the strains, and four sporophytes at the age of 20 days were confirmed as the fertilized thalli (Fig. 4a).

In the strain-specific primers targeting the *cox1* region, the forward and reverse primers could include a SNP in the 3′ site. The PCR product size was 265 bp (Supplementary Fig. S7b). It was confirmed that the primers did not show affinity against the different strains from the result of PCR using gamete DNA, and only maternal mtDNA was detected in the sporophytes (Fig. 4b). Male mtDNA marker was not amplified in either hybrid combination.

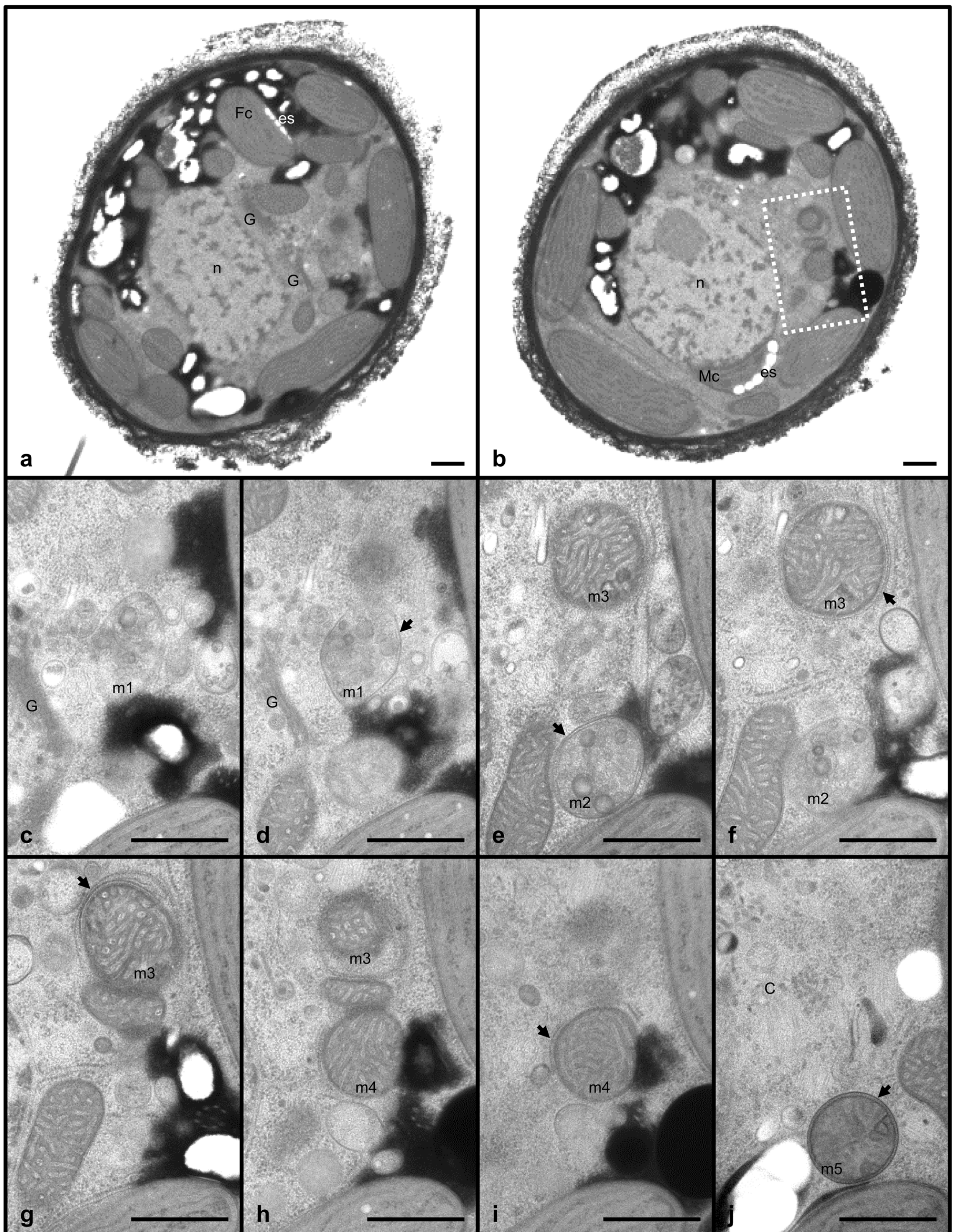
Polymorphism within *psaA* was found only in the female gamete of the Fukuoka-strain (related DNA sequences are not shown in this study). Therefore, the chlDNA markers we designed were valid in only one hybrid combination, Fuk-F and Mie-M. The PCR product, a 237-bp band, appeared only for Fuk-F from this hybrid combination (Fig. 4c; Supplementary Fig. S7c).

Copy number of mtDNA and chlDNA in male and female gametes

Cox1 and *psaA* are single-copy genes in mtDNA and chlDNA. We estimated the copy number of these organelles in a single mitochondrion and in a chloroplast of female and male gametes using quantitative PCR (qPCR). To validate the efficiency of the primer sets for qPCR, the plasmid-inserted target region was diluted (10^8–10^2 copies) and qPCR was performed. Linear regressions of the standard curves and corresponding efficiencies for each primer set against the number of plasmid copies of the target genes are shown in Supplementary Fig. S8. A calibration curve was used for the examination of genes' copy number using the Cq values from qPCR of gamete DNA equivalent to 50, 100, and 200 gametes. The mean value and standard deviation of the samples are shown in Table 3. In *coxI* gene, 1110 copies in female gametes of both strains, 13 copies in Fuk-M and 24 copies in Mie-M were estimated, while copies of *pasA* gene were calculated as 1145 or 1138 copies in Fuk-F or Mie-F and 85 or 78 in Fuk-M or Mie-M.

Discussion

This study displayed maternal inheritance of mitochondria and chloroplasts in the anisogamous brown alga, *M. cylindricus*. This is the first report showing the organelle inheritance of the anisogamous species of brown algae, and as a result, the missing part in brown algae relating to organelle


Fc
es
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m1
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f
m3
g
m3
m4
h
m4
i
C
m5
j

◀ **Fig. 2** Digestion of male mitochondria in a two-hour-old zygote. **a** Whole cell image including female chloroplast with eyespots (Fc). **b** Whole cell image including a male chloroplast with eyespots (Mc). **c–j** Partial serial sections of a magnified view from the white rectangular region in **b**. Mitochondria labeled with m1–m5 are isolated by double-membranes (arrows) and inside different destruction degrees of mitochondria can be observed. The most degraded mitochondrion is m1, because cristae are hardly seen. Cristae in m2 are stained faintly. C, centriole; es, eyespot; G, Golgi body; n, nucleus. Scale bars: 500 nm

inheritance was finally filled. In the cases of isogamy and oogamy, mitochondria are maternal, so it was easily expected that mitochondria are derived from female gametes in the anisogamous species of brown algae. Finally, the results have confirmed it. We noted the way in which exclusion of mitochondria was similar to the case of oogamy. Meanwhile, the chloroplast inheritance pattern in the anisogamous species was maternal, but the fate of chloroplast structure was not determined like in the fate of male mitochondria in the case of isogamy in brown algae (Kimura et al. 2010a).

Degradation of mitochondria derived from male gamete

Degraded paternal mitochondria and chloroplasts within lysosomes were observed during one-cell stage of the zygote in the oogamous species of brown algae, *Saccharina angustata* and *Undaria pinnatifida* (Motomura 1990; Kimura et al. 2010b). In those species, sperm mitochondria are distinguished from female mitochondria. Cristae of motile sperm mitochondria contain a tubular structure like other brown algal swarmers (Henry and Cole 1982). Sperm mitochondria and chloroplasts always stay close to the sperm nucleus, even after fertilization. In the case of anisogamous species, *M. cylindricus*, both the female and male gametes are motile with two heterogeneous

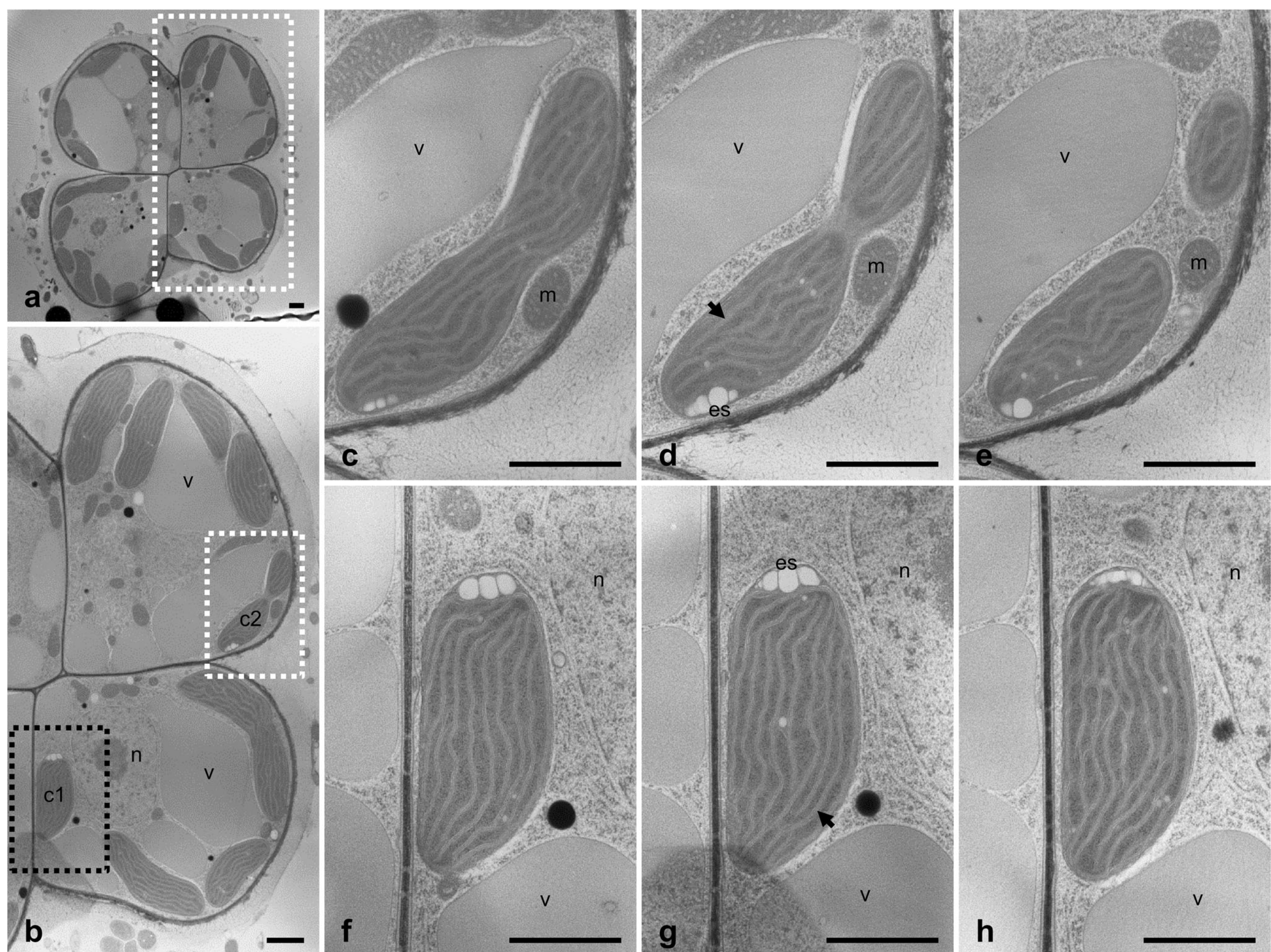

Fig. 3 Ultrastructure of a four-celled crustose sporophyte. **a** Whole image of a 4-celled crustose sporophyte. **b** The enlarged view from the white rectangular region in **a**, two chloroplasts with eyespots (c1 and c2) exist in the different cells of the sporophyte. **c–e** Partial serial sections of magnified views of chloroplast from the white rectangular region in **b**. About 6 lamellae (arrow) surrounded by girdle lamella in the chloroplast. **f–h** Partial serial sections of magnified views of chloroplast from the black rectangular region in **b**. About eight lamellae (arrow) surrounded by girdle lamella in the chloroplast. es, eyespot; m, mitochondrion; n, nucleus; v, vacuole. Scale bars: 500 nm (**a**, **b**); 1 µm (**c**–**h**)

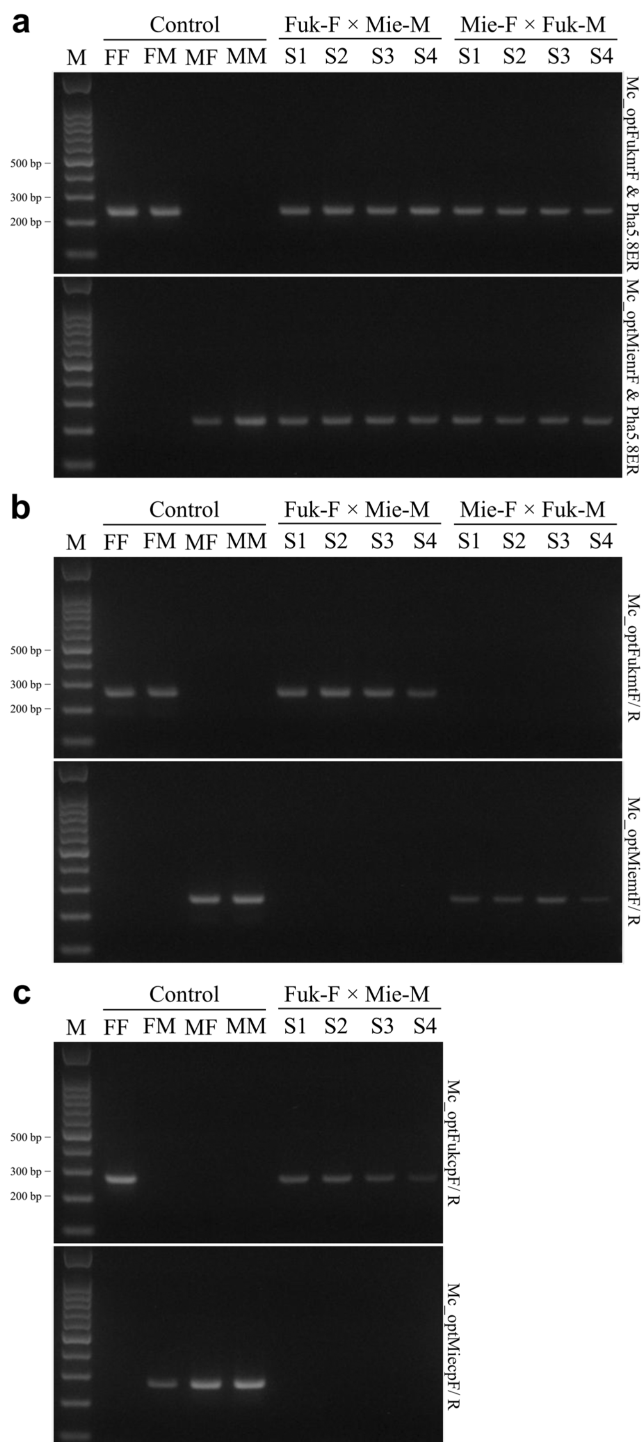

Fig. 4 PCR using strain-specific primers for nuclear, mitochondrial and chloroplast DNA
a PCR using the ITS1 nuclear marker. **b** PCR using a mitochondrial marker (*cox1* gene). **c** PCR using chloroplastal marker (*psaA* gene). Upper electrophoresis image shows PCR amplified by the Fuk-strain specific primers and the lower images show PCR amplified by the Mie-strain specific primers. M; 100 bp ladder marker, FF, Fuk-F; FM, Fuk-M; MF, Mie-F; MM, Mie-M; Mie-F × Mie-M S1, S2, S3, S4, hybridized sporophyte with Fuk-F and Mie-M; Mie-F × Fuk-M S1, S2, S3, S4; hybridized sporophyte with Mie-F and Fuk-M

flagella (Supplementary Fig. S1a, b); therefore, a tubular structure within the mitochondrial cristae was observed in both gametes. In addition, the size of mitochondria between the female and male gametes was almost identical. In this study, mitochondria enclosed with double membranes showing different degrees of destruction were observed in 2-hour-old zygotes (Fig. 2; Supplementary Fig. 3 h, m–p). We concluded that digested mitochondria were derived from male gametes due to the following reasons: Firstly, male mitochondria and chloroplasts stayed close to the male nucleus before and after karyogamy like in the case of oogamous species (Fig. 2, Supplementary Fig. 3, 4); next, a pair of centrioles derived from a male gamete (Nagasato et al. 1998) was always found in the vicinity of degraded mitochondria; finally, the total number of mitochondria declined about five in 6-hour old zygotes compared with the total number of mitochondria in female and male gametes, namely, by the exclusion of male mitochondria in anisogamous species shortly after fertilization.

There are different mechanisms of uniparental mitochondrial inheritance. It has been reported that uniparental mitochondria are disassembled by autophagy in metazoan *Caenorhabditis elegans*. Sperm-derived paternal mitochondria enter the oocyte cytoplasm and gradually degrade with autophagy in the early embryos (Al-Rawi et al. 2011; Sato and Sato 2011). While autophagy is not contributed to the elimination of sperm mitochondria in mice (Luo et al. 2013; Luo and Sun 2013), degradation of sperm mtDNA takes place before fertilization, and sperm mitochondria are packed into one blastomere before 4-cell embryo. This process is a mechanism for maternal inheritance of mitochondria in mice (Luo et al. 2013; Luo and Sun 2013). In fungal species *Ustilago maydis* and the unicellular green alga *Chlamydomonas*, it has been confirmed that mutants of autophagy-related genes are not affected by the uniparental inheritance of mitochondria (Wagner-Vogel et al. 2015; Kajikawa et al. 2019). In the basidiomycete *Cryptococcus neoformans*, autophagy-deficient (ATG8) strains showed that autophagy is not responsible for uniparental inheritance of mitochondria; however, destruction of mitochondria structure lacking mt DNA was delayed (Nishimura et al. 2020). In our study, paternal mitochondria were engulfed with a double membrane structure, and degraded mitochondria were observed within it. In *Mutimo cylindricus*, autophagy was thought to be an important mechanism for maternal inheritance of mitochondria together with degradation of male mtDNA before fertilization.

Preferential degradation of DNA in paternal mitochondria

Preferential degradation of paternal mtDNA before fertilization has been clarified in green algae. In the anisogamous

Table 3 Absolute quantitative real-time PCR for mtDNA and chlDNA

	Assay name	DNA equivalent (gametes)	Cq	Copies[a]/ gamete	Mean[b]/ gamete	Mean[b]/ organelle[c]
mtDNA (*cox1* gene)	Fuk-F	200	21.11	1162	1110 ± 26	–
		100	22.18	1089		
		50	23.17	1080		
	Fuk-M	200	27.24	15	17 ± 1	–
		100	28.05	17		
		50	28.91	19		
	Mie-F	200	21.16	1121	1110 ± 276	44 ± 1
		100	22.22	1058		
		50	23.08	1151		
	Mie-M	200	26.21	31	30 ± 1	6 ± 0
		100	27.19	31		
		50	28.28	29		
chlDNA (*psaA* gene)	Fuk-F	200	21.17	1122	1145 ± 19	–
		100	22.18	1130		
		50	23.13	1184		
	Fuk-M	200	26.45	31	40 ± 5	–
		100	27.14	39		
		50	27.77	51		
	Mie-F	200	21.20	1099	1138 ± 29	113 ± 3
		100	22.19	1122		
		50	23.12	1194		
	Mie-M	200	26.15	38	37 ± 1	37 ± 1
		100	27.15	39		
		50	28.33	35		

[a] Rounding off is used in average calculation

[b] Mean ± s.d., $n = 3$

[c] Based on counting data by TEM

green alga, *Bryopsis maxima*, maternal inheritance of organelle DNA was shown using the DNA specific dye 4-6-diamidino-2-phenylindole (DAPI) (Kuroiwa and Hori 1986; Kuroiwa et al. 1991). In that case, the nucleoid of the mitochondria in male gametangia disappeared completely during gametogenesis. Also, in another anisogamous green alga, *Derbesia tenuissima*, degeneration of mtDNA in mature male gametangia was observed by DAPI staining and immunoelectron microscopy using anti-DNA antibody (Lee et al. 2002).

In our study, the copy number of mtDNA and chlDNA in female and male gametes of *M. cylindricus* was estimated by real-time PCR. According to the data, 1110 copies of mtDNA were contained in Mie-F, and 44 copies were contained in a single mitochondrion. In contrast, about 24 copies of mtDNA were contained in Mie-M, and each mitochondrion carried only 5 copies into the zygote (Table 3). Thus, the difference between the copy number of mtDNA in female and male gametes occurred before fertilization. This is the first report to count the copy number of organelles DNA and to display the difference of copy number in a single mitochondrion between female and male gametes in anisogamous brown alga.

A decrease of paternal mtDNA was reported in a freshwater fish, *Oryzias latipes* (Nishimura et al. 2006). The number of mtDNA nucleoids gradually decreases during spermatogenesis. After fertilization, the complete digestion of paternal mtDNA is followed by the destruction of the paternal mitochondrial structure. In our study, it is obscure whether the destruction of the mitochondrial sheath occurs before or after mtDNA disappearance in *M. cylindricus*. However, it is confirmed that the male mitochondria have a different condition from the female mitochondria when incorporated into the zygote. It would be related to the fact that male mitochondria were targeted by the autophagy system and destroyed in the early zygotes. In the oogamous species of brown algae, destruction of the paternal mitochondrial structure was observed in the early developmental stage, compared with the case of isogamy (Motomura 1990; Kimura et al. 2010a, 2010b). In *Undaria pinnatifida*, DNA was detected in sperm mitochondria and chloroplasts (Kimura et al. 2010b); however, the

differences of DNA amounts between the two organelles have not been examined. It is strongly suggested that sperm mtDNA will be also decreased during spermatogenesis, and male mtDNA in isogamy is not affected by the degradation process. Contrarily to isogamous species, a different elimination mechanism of paternal mtDNA and its structure may exist in oogamous and isogamous species.

Maternal inheritance of chloroplasts in anisogamous species

Little is known about the relationship between the sexual reproduction and the cytoplasmic inheritance. However, there is a novel hypothesis that the uniparental mode should have arisen at the late stage of endosymbiosis and replaced the biparental mode being predominant during eukaryotic evolution (Zhang and Sodmergen 2010). In the green algae, oogamy and anisogamy are the advanced types of sexual reproduction, compared with isogamy that always exhibits striking maternal inheritance of chloroplasts (Kuroiwa 2010; Miyamura 2010). A similar phenomenon is also seen in the brown algae (this study; Motomura et al. 2010). Concerning the maternal inheritance of chloroplasts, there are two major mechanisms that exist in the green and brown algae that occur along with the degeneration of uniparental mitochondria: (1) digestion of chlDNA during male gametogenesis or after fertilization and (2) disintegration of the chloroplast structure after fertilization (Miyamura 2010; Motomura et al. 2010). However, in our study, the chlDNA copy number per chloroplast in female gamete was 1.4 times as it is in a male gamete (Table 3). Ultrastructural observation showed the differences in size and the number of lamellae between male and female chloroplasts (Supplementary Fig. S2). Therefore, the difference in the copy number of chlDNA between male and female gametes might be caused by the body size, not result of digestion. Male and female chloroplasts with eyespots could be chased until they reached the four-celled stage, infrequently in the eight-celled stage when the appearance of chloroplasts was almost identical, and it was difficult to distinguish a male chloroplast from a female one. Therefore, if, while growing in sporophyte, male chloroplasts lost their eyespots, they would not be detected as male chloroplasts. In this study, the fate of male chloroplasts was not shown. In *Chlamydomonas*, chlDNA is inherited maternally, and mtDNA is inherited paternally. It is known that uniparental organelles that have lost their DNA fuse with opposite maturation type organelles (Cavalier-Smith 1970; Nishimura et al. 2002). In the case of the brown algae, two more extra membranes (the secondary endosymbiotic origin) exist in chloroplasts of brown algae in addition to the chloroplast envelope. Further observation is needed to be clear whether male chloroplasts can fuse to female chloroplasts beyond the barrier of this additional membrane or if they are digested within lysosome-like structures in male mitochondria.

Conclusion

In this study, the maternal inheritance of mitochondria and chloroplast in the anisogamous brown alga, *M. cylindricus*, was revealed for the first time. Autophagy was related to the destruction of male mitochondria, which was similar to the case of oogamous species of the brown algae. Moreover, it was highly speculated that the amount of male mtDNA had decreased before fertilization. On the other hand, the timing of the paternal male chlDNA loss and the fate of male chloroplasts remained unclear. The mechanism for uniparental organelle DNA degradation and the targeting system of uniparental organelles have to be revealed to better understand organelle inheritance in brown algae.

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Author contributions Yuan Shen and Chikako Nagasato designed the experiment and maintained the strains of *M. cylindricus*. Toyoki Iwao collected the fresh samples in the field. Taizo Motomura analyzed the data and critically reviewed this manuscript. All authors wrote and edited this manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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