Inheritance of spheroid body and plastid in the raphid diatom *Epithemia* (Bacillariophyta) during sexual reproduction

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ABSTRACT

Diatoms belonging to the family Epithemiaceae have endosymbiont “spheroid bodies’’, which have received attention as a model to provide new insights into the early stages of organelle evolution. Uniparental organelle inheritance, known in a wide range of sexually reproducing eukaryotes, is considered to be one of the key characteristics acquired during the evolution of an endosymbiont into an organelle. However, there has been no information about the inheritance of spheroid bodies. The aim of the present study was, therefore, to investigate the inheritance modes of the spheroid bodies and plastids in the isogamous diatom *Epithemia gibba* var. *ventricosa*, which we established to be heterothallic. We induced sexual reproduction of *E. gibba* var. *ventricosa* in culture, using sexually compatible mating strains that differed with respect to nucleotide polymorphisms. The F1 strains were genotyped to reveal the parental origin of the spheroid bodies and plastids using parent-specific polymorphisms. The results suggested that inheritance of the spheroid bodies was uniparental (*i.e.* progeny have the spheroid body genome from either parent but not both) and random (*i.e.* with an unbiased ratio of parental origins), while that of the plastids was more complex, being predominantly uniparental, but with a few biparental cases. This study is the first to report the inheritance pattern of the spheroid body which will contribute to better understand the evolutionary state of this organelle.

Key words: evolution; uniparental inheritance; organelle; endosymbiont; sexual reproduction

INTRODUCTION

Diatoms are unicellular algae distributed widely in aquatic environments, playing a predominant role in oceanic primary production and the biogeochemical cycling of carbon and silica (Nelson *et al.* 1995; Mann 1999). Their need for silicon arises because they use it to construct their cell walls, which in turn, because of their limited flexibility, leads in most species to an inexorable decrease in cell size during the vegetative phase (Round *et al.* 1990). When cells become smaller than a certain size threshold, many diatoms become sexually potent, *i.e.* they can perform sexual reproduction when external conditions permit. Sexual reproduction ends up with the formation of an enlarged zygote, called the auxospore, in which a new vegetative cell is formed, called an initial cell, which represents the largest cell size in their life cycle.

Diatom species belonging to the family Epithemiaceae have unique intracellular structures, the "spheroid bodies" (Drum & Pankratz 1965; Geitler 1977), which are evolutionarily derived from nitrogen-fixing cyanobacterial symbionts (Prechtl *et al.* 2004; Nakayama *et al.* 2011). While mitochondria and plastids are believed to have been acquired from endosymbiotic prokaryotes over a billion years ago (Dyall *et al.* 2004; Yoon *et al.* 2004; Gould *et al.* 2008; Archibald 2009; Parfrey *et al.* 2011), the spheroid body is much younger, with an estimated origin at ~12 Ma, according to the fossil record and molecular phylogenetic analysis (Nakayama *et al.* 2011).

Although the spheroid body has received attention as a good model to provide new insights into the early stages of organelle evolution (Kneip *et al.* 2007; Trapp *et al.* 2012; Nowack & Weber 2018), there has been no supporting information to infer what phase of organellar evolution the spheroid body represents – this could be elucidated with further information, such as the presence or absence of a targeting system for transporting and importing protein products between the symbiont and the host (Dyall *et al.* 2004; Theissen & Martin 2006; Keeling 2011), or the development of uniparental inheritance, which is commonly seen in both mitochondria and plastids (Birky 1995, 2001; Kuroiwa 2010). In this paper we focus on the latter.

Sexual reproduction in eukaryotes involves not only the fusion of gametes and cell nuclei,

but also the transmission of parental organelles to the next generation. The phenomenon of uniparental organellar inheritance might have already been acquired in the eukaryotic common ancestor, which showed sexual reproduction (Goodenough & Heitman 2014). The mode of inheritance of organelle DNA has been investigated in a wide range of organisms, *e.g.* chlorophyte and streptophyte (Miyamura 2010), brown algae (Peters *et al.* 2004; Kato *et al.* 2006), mosses (Jankowiak-Siuda *et al.* 2008), ferns (Gastony & Yatskievych 1992), fungi (Kawano *et al.* 1987; Yang & Griffiths 1993), higher plants (Mogensen 1996) and animals (Ankel-Simons & Cummins 1996; Sutovsky & Schatten 2000). Such studies have shown that non-Mendelian, uniparental inheritance of organelle DNA is the general rule, but that there are many exceptions (Xu 2005). In diatoms the mode of inheritance has been determined in only three cases so far – two dealt with mitochondria and the other one with plastids. Gastineau *et al.* (2013) examined the mode of mitochondrial inheritance in *Haslea ostrearia* (Gaillon) Simonsen, using *cox1* as a genetic marker, and revealed that the mitochondria are strictly transmitted uniparentally to F1 progeny. Bagmeta *et al.* (2020) also found uniparental inheritance in *Nitzschia palea* (Kützing) W. Smith, again using *cox1*. Plastid inheritance has been studied using *rbcL* as a marker. Ghiron *et al.* (2008) studied inheritance in *Pseudo-nitzschia delicatissima* (Cleve) Heiden and found that the plastids are transmitted randomly to the F1 progeny.

In this study, we aimed at revealing the mating system of *Epithemia gibba* var. *ventricosa* (Kützing) Grunow (formerly *Rhopalodia gibba* var. *ventricosa*) and the mode of the inheritance of its spheroid body, to elucidate whether a selective digestion or segregation mechanism has evolved so that the body from one parent is eliminated during sexual reproduction. This could be a criterion to assess the degree of "organellogenesis" in *Epithemia*. Using the same pair of parental strains, we also tried to reveal the inheritance of plastids and mitochondria, in order to know whether these organelles and the spheroid body from one parent are inherited or eliminate together, or whether their fates are determined independently. To do this we successfully genotyped parental and F1 strains with respect to polymorphisms detected within the spheroid body and plastid genomes using

high throughput sequencing technology. In the case of uniparental inheritance of the spheroid body, *i.e.* its organellogenesis is equivalent to that of established organelles, we can expect that genetic markers in the F1 generation will be derived from a single parent; in contrast, biparental inheritance will be supported if markers of both parental strains are present in the F1.

MATERIAL AND METHODS

Culture

Samples were collected from Nakaikemi Wetland, Tsuruga, Fukui Prefecture, Japan (35°39'27.7"N 136°05'23.5"E) on April 12, 2016. *Epithemia gibba* var. *ventricosa* vegetative cells were attached to bogbean *Menyanthes trifoliata* Linnaeus and isolated by capillary pipette to establish strains. All the strains were grown at 18 °C under the photoperiod L:D=12:12 with cool white light ca. 50 μmol photons m^{-2} s^{-1}, with CSi medium (Nakayama *et al.* 2011) with an extra 1.0 mM $NaNO_3$ added to the base water collected from the field (which contained 1–5 μM of nitrogen, determined with autoanalyzer TRAACS 2000, Bran & Luebbe, Norderstedt, Germany), pre-filtered and adjusted to pH 6.2 with HCl, and sterilized through a 0.2-μm pore membrane filter (Advantec Toyo, Tokyo, Japan).

Microscopy

For light microscopy, living cells were observed with an Axio Imager A2 (Zeiss, Oberkochen, Germany) with Axiocam 506 color digital camera (Zeiss) and differential interference contrast (DIC) optics. For confocal laser scanning microscopy, cells were placed in Lab-Tek Chambered Coverglasses (1.0 borosilicate coverglass, Thermo Fisher Scientific, Massachusetts, USA) and observed with LSM780 (Zeiss) to determine the number of plastids（due to the complex 3-dimensional shape of the plastid, it was difficult to determine the number under light microscopy or epifluorescence microscopy). SYBR Green I Nucleic Acid Gel Stain (Takara Bio, Shiga, Japan)

was added to observe spheroid bodies and left 20 min at room temperature at the final concentration 0.1 µl l^{-1}.

Genome sequencing and SNP detection

Cells of the sexually compatible strains K03 and K06 were harvested from culture plates using cell scrapers (Sumitomo Bakelite, Tokyo, Japan), transferred into microtubes, and centrifuged to remove supernatant. Cell pellets were homogenized using BioMasher II (Nippi, Tokyo, Japan). DNA was extracted by DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). Each of the DNA solutions obtained was measured for concentration using Qubit 4 fluorometer and Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). Approximately 2.0 µg and 1.6 µg of DNA were obtained from K03 and K06, respectively, and used for high throughput sequencing on an Illumina HiSeq platform (paired-end, insert size 350 bp, read length 150 bp). Sequencing yielded 95.5 Gb in K03 and 45.3 Gb in K06. These read pools were preprocessed through fastp 0.14.1 (Chen *et al.* 2018) with default parameters to remove the adapters and error-prone reads. Then, we used Platanus 1.2.4 (Kajitani *et al.* 2014) with default parameters for *de novo* assembly for each strain. Similarity search was done with local blastn 2.9.0 (Zhang *et al.* 2000) to find organellar contigs, against known organellar genome sequences of *Epithemia* or those of related raphid diatoms as queries, i.e. spheroid body (AP012549, *E. turgida*), plastid (NC_015403; *Fistulifera solaris* S.Mayama, M.Matsumoto, K.Nemoto & T.Tanaka) and mitochondria (MF997423; *Surirella* sp.). Because contigs showing high similarities (Evalue =0) to each reference genome were longer in the K06 assembly, we mapped the trimmed reads of both K03 and K06 strains onto the contigs using bwa 0.6.1 (Li & Durbin 2009) to visually search for a single nucleotide polymorphism (SNP) between two parental strains with Integrative Genomics Viewer 2.4.3 (Thorvaldsdóttir *et al.* 2013), and to evaluate its credibility, i.e. whether the site is covered by unambiguously mapped reads and the coverages were high. As a result, we detected SNP only for the plastid contig, and thus, we further extended the spheroid body and mitochondrial contigs with NOVOPlasty 2.2.2 (assembly type = ‘’mito’’, Dierckxsens *et al.*

2016), using the longest contig for each spheroid body and mitochondrial genome as seeds. This procedure successfully extended the contigs, i.e. from 2,995 bp to 1,073,272 bp for the spheroid body genome, and from 10,305 bp to 21,052 bp for the mitochondrial one, and SNPs were detected only from the spheroid body genome. All the SNPs were further confirmed by Sanger sequencing with the primers listed in Table S1, and the product sequences are available as supplemental data. Raw reads of K03 and K06 are deposited to Sequence read archive (accession will be added afterwards).

Induction of sexual reproduction and establishment of F1 strains

Sexual reproduction was induced by mixing K03 and K06 in a plastic petri dish (90 mm diameter, STAR SDish9015 ver.2, Rikaken, Nagoya, Japan). The isolation of progenies to establish the F1 strains was less straightforward, as the two initial cells were enclosed in a robust capsule of mucilage, which was visualized with Indian ink (Fig. S1A), and thus essentially inseparable by pipette. Initially, therefore, we isolated pairs of initial cells to make a number of ”progeny strains” (i.e. derived from two initial cells). These were left for about a week until two initial cells were liberated from the perizonia, and divided mitotically for ca 2 times to make the cells free from the mucilage capsule which physically hampered the isolation of a cell. Then, from the progeny strains, we further isolated a single cell to make the F1 strain, which was genuinely clonal.

Molecular analysis for F1 strain genotyping

The F1 strains were grown in a petri dish for ca 1 month to form a visible pellet when cells were collected by centrifugation. The cells were homogenized with a bioMasher II (Nippi, Tokyo, Japan) and briefly spun down (*ca.* 10 sec with a desk-top centrifuge). Supernatant was used as PCR template for amplification of genetic marker regions. Primers were designed using Primer3Plus ver. 2.4.2 (https://primer3plus.com/ cgi-bin/dev/primer3plus.cgi, Untergasser *et al.* 2012) and are listed in Table S1. Each PCR was performed in a 25-µl reaction volume that contained a final

concentration of 0.625 U MightyAmp DNA Polymerase (Takara Bio), 1 × MightyAmp Buffer Ver.3 (Mg^{2+}, dNTP plus) (Takara Bio), 0.5 μM of forward primer, 0.5 μM of reverse primer, and 2.0 μl of template. T100 Thermal Cycler (Bio Rad, California, USA) was used, with the reaction conditions as follows; an initial denaturation at 98°C for 2 min followed by 35 cycles of denaturation at 98℃ for 10 s, annealing at 60℃ for 15 s and extension at 68℃ for 1 min. PCR products were purified using ExoSAP-IT Express (Thermo Fisher Scientific) and sequenced with the PCR primers through Eurofins Genomics (Tokyo, Japan). Electropherograms were inspected using ATGC ver. 7 (Genetyx Corporation, Tokyo, Japan).

RESULTS

Heterothallic sexual reproduction and behavior of the spheroid body

Sexual reproduction was studied in culture using seven clones isolated from single cells. Reproduction never occurred in monoclonal cultures, only in particular mixtures of actively growing clones, and the results of preliminary mating experiments (Table S2) could be accounted for on the basis that *E. gibba* var. *ventricosa* is heterothallic, with two mating types. The longest cells capable of sexual reproduction in our experiments were 70.6 μm long, the smallest 15.7 μm. Of the seven clones we selected two for further study and for experiments on spheroid body and plastid inheritance, based on their vigorous growth and reproduction.

Despite the differentiation into two mating types, no differences were observed between mating cells with respect to gamete morphology and behavior: *E. gibba* var. *ventricosa* is isogamous. The vegetative cells of each strain showed motility. Details of the sexual process were examined in mating experiments using a mixture of clones K03 (valve length, average ± SD = 35.3 μm ± 0.3, n=10) and K06 (45.2 μm ± 0.7, n=10). Within a few days after making a cross, compatible cells made pairs: mating was obviously not intraclonal, judging from the size

differences between paired cells (Fig. 1). Within a couple of days after pairing, two zygotes were formed by each pair of gametangia (Fig. 2). The zygotes then expanded bipolarly to form long ±linear auxospores orientated perpendicular to the long axes of the parental cells (Figs 3, 4). After several days of expansion of the auxospore, an initial cell was liberated from each auxospore and returned to the vegetative phase to repeat mitotic cell divisions (Fig. 5). The valve lengths of the initial cells were 105.0–185.0 μm (n = 100). During the entire process of auxosporulation, from pairing to auxospore maturation, the cells were enclosed in a mucilage capsule (Fig. 3, arrows indicate the limit of the capsule). The presence of two spheroid bodies and one plastid per cell was confirmed in both the parental and F1 cells with light and confocal microscopy (Figs 5–7). No quantitative data were collected on spheroid body numbers but we never observed a cell with no spheroid bodies; most of them had two, and only occasionally one or more than two during the study period. We could only stain DNA in the spheroid body in dead cells in which the nucleus remained unstained or had been lost (Figs 6, S1B).

SNP detection

Sequence comparison of organellar genomes between the parental strains K03 and K06 revealed that the genomic fragments derived from the spheroid bodies and plastids contained single nucleotide polymorphisms (SNPs) that could be used to determine the parental origin of each genome in the F1 strains; unfortunately, no SNP was detected in the mitochondrial genomic fragments, so that we were unable to examine its inheritance pattern. The SNPs used to discriminate the parental strains K03 and K06 were TT and AA for the spheroid bodies, and A and C for the plastids, respectively (Fig. 8).

Because our strains were not axenic, the read pools certainly contained bacterial reads. Therefore, it was possible that mis-assemblies took place likely due to high similarities between the contaminate bacterial and spheroid body/plastid genomes. However, for the spheroid body marker, we found the presence of the conserved synteny at the SNPs region along with other spheroid body

genomes publicly available, *E. turgida* (Nakayama *et al*. 2014) and *E. gibberula* (Nakayama & Inagaki 2017), in which at least 2 genes were shared and arranged in the same orientations to flank both 5' and 3' directions of the SNPs site (Fig. S2). The synteny is only found in the two spheroid body genomes sequenced so far, and not in any other bacterial genomes. The blastn search of the intergenic region (between ORF2 and 3 in Fig. S2), which contained our spheroid body SNPs, resulted in no hit to any GenBank entries. For the plastid marker, NCBI nucleotide blastn search (megablast, works best for the target percent identity of 95%, according to the instruction given under blastn suite) resulted in many hits with diatom plastid genomes. Thus, it is reasonable to assume that the target sequences were not present in other bacterial genomes *i.e.* our genotyping was not affected by contaminant bacteria.

Mode of inheritance of the spheroid body and plastid

Using the 39 F1 strains that were successfully established, the flanking regions of SNPs for each organelle genome were PCR amplified and Sanger sequenced to determine the strain genotype. Almost all the electropherograms exhibited single peaks at the SNP alleles, indicating the fact that each F1 strain possessed only one genotype for each organelle (Fig. 8; the two exceptions are described below). The ratio of parental origins for the spheroid body in the F1 strains was K03:K06=14:15 (Table 1), and that of plastid was 12:25 (Table 1). In 10 out of 39 F1 strains, we failed to determine the genotype of the spheroid bodies because of PCR/sequencing failures.

Among the 29 F1 strains successfully genotyped for both spheroid body and plastid, joint inheritance of K03 organelles, *i.e.* both the spheroid bodies and the plastid derived from clone K03, was found in three F1 strains, whereas that of K06 organelles occurred in eight F1 strains. On the other hand, mixed inheritance, *i.e.* spheroid bodies from K03 and the plastid from K06, or vice versa, was found in 11 and 6 strains respectively (Table 1).

A puzzling pattern was found in two F1 strains (F1#19 and #39), which showed ambiguity at the plastid SNP site with biparental signals derived from both K03 and K06 strains (*i.e.* the site

had double peaks in the electropherogram, of both A and C) (Fig. 8), although vegetative cells only have a single plastid.

DISCUSSION

The discovery of heterothallism in *Epithemia gibba* var. *ventricosa* brings yet another genus in line with the view, developed during and since the seminal work of Roshchin (Roshchin 1994, Chepurnov *et al.* 2004), that pennate diatoms are fundamentally heterothallic organisms, though with many homothallic and automictic exceptions. The heterothally and isogamous sexual reproduction of *E. gibba* var. *ventricosa*, including pairing via the ventral sides and expansion of the auxospores perpendicular to the gametangia, agree closely with *Amphora* (Mann & Poulíčková 2010), which is consistent with their phylogenetic proximity according to molecular data (Sato *et al.* 2013; Stepanek & Kociolek 2014).

The morphology and cytology of sexual reproduction in *Epithemia* (including in species formerly classified in *Rhopalodia*) were studied in some depth by Klebahn (1896) and Geitler (1932, 1977), following much earlier but very brief reports of the process by Smith (1853–6) and others. Both Klebahn and Geitler recorded the continuity of the spheroid bodies through the sexual phase (though Klebahn misinterpreted them as pyrenoids) and from their observations and illustrations it would appear that none of the bodies are lost by degradation (autodigestion): the only structures they recorded (either in the text or in their illustrations) as aborting were the two superfluous nuclei from meiosis II (Klebahn 1896, figs 9, 11; Geitler 1977, figs 1D, E) and the superfluous nucleus from each of the acytokinetic mitoses associated with the formation of the initial valves (Geitler 1977, fig. 1n). In *E. gibba*, Klebahn's observations indicate that, two spheroid bodies were present in each parent cell and that these were segregated one into each gamete (see also Geitler 1977, fig. 3e). Although we did not observe the formation, rearrangement and fusion of gametes in our material *E. gibba* var. *ventricosa*, it is reasonable to reject the possibility that the two zygotes were formed via self-fertilization of each parental cell – if this had been the case, each

auxospore should have been encased within an individual mucilage capsule; however, we observed two sibling auxospores in a single capsule (Fig. S1A). Each zygote consequently had two bodies, one inherited from each compatible gamete: no division nor fusion of spheroid body was mentioned throughout the entire process of sexual reproduction he observed. However, in our F1 strains of *E. gibba* var. *ventricosa*, all the electropherograms of the spheroid body marker regions showed unambiguous single peaks at the two polymorphic sites, as seen in the parental strains, and that each F1 strain had the genotype of one parental strain, either K03 or K06. This result shows that both mating types can potentially transmit the spheroid body to the F1, but that inheritance is nevertheless strictly uniparental in this taxon. How this is achieved is unclear. Given that each zygote receives at least one spheroid body from each parent, it should contain one K03 and one K06 body and it might be expected that at least some of the F1 strains would retain this heteroplasmy. Hence, the absence of any heteroplasmic strains among the 29 F1 strains we genotyped and the 1:1 ratio of K03 and K06 in the strains suggests either (1) the two gametic bodies segregate at the first division of the initial cell, (2) one spheroid body is digested at random in the zygote sometime during the later development of the auxospore, before the first division of the initial cell (although neither Klebahn nor Geitler nor ourselves observed any degenerating spheroid bodies) or (3) sorting (either random or directed) occurs during the first few vegetative divisions after auxosporulation, *i.e.* heteroplasmy may be eliminated stochastically (*cf.* the plastid gene inheritance model of VanWinkel-Swift 1980; also Greiner *et al.* 2014). We cannot rule out the last-mentioned since we were unable to genotype the initial cells themselves (see Material and Methods: we explain that isolating individual initial cells was impractical because of the mechanical strength of the mucilage capsule holding the auxospores, see Fig. S1A). Whatever the means by which heteroplasmy is avoided, the inheritance of the spheroid body is clearly not from only one of the mating types of *E. gibba* var. *ventricosa*, unlike the inheritance of plastids or mitochondria in a number of other heterothallic eukaryotes (reviewed by Xu 2005), and indeed, there is no bias as to which parental spheroid body is selected.

DeYoe *et al.* (1992) reported that the number of spheroid bodies per vegetative cell varied in *Epithemia*, depending on the availability of nitrogen; however, in our parental and F1 strains, cells predominantly contained two spheroid bodies per cell in the presence of dissolved nitrogen in our modified CSi medium, indicating tight control of spheroid body division and segregation. Other *Epithemia* species have different numbers of spheroid bodies per cell and in some at least, the number varies during the life cycle. For example, *E. porcellus* cells generally have 4–8 bodies per cell, depending on the cell size and stage of the cell cycle, but up to 16 in initial cells and as few as 2 in the smallest cells (Geitler 1977, as *E. zebra* var. *porcellus* (Kützing) Grunow); *E. turgida* also contains up to 16 per cell. In such taxa, it could be easier than in our species to check the mechanisms by which heteroplasmy is avoided, since stochastic loss of one parental genotype would be slower with a larger number of spheroid bodies, allowing its detection if present.

In this study, as in previous studies of Epithemiaceae (*e.g.* Klebahn 1896; Geitler 1932, 1977), we observed only one plastid in vegetative cells of *E. gibba* var. *ventricosa*, both in the parental and F1 strains, under light and confocal microscopy. During gametogenesis, the plastids divided and segregated, so that when the cell divided at the end of meiosis I, each gamete received one plastid, as in the related genus *Amphora* (Mann 1994; Mann & Poulíčková 2010). Not surprisingly, therefore, there were two plastids in expanding auxospores and initial cells, representing the plastids inherited from the gametes. Later, as in *Amphora* and other diatoms with one plastid per cell in normal vegetative cells, the initial cell appeared to partition the two parental plastids between the daughter cells when it divided. Given all this, it was unexpected to discover two F1 strains possessing a double peak (A/C) for the plastid marker, indicating inheritance from both parental strains. We do not know how this happened. Possibly F1 strains #19 and #39 anomalously harbored an extra plastid from one parent, so that when the initial cell divided, one daughter established a lineage containing plastids from both parents. Unfortunately we were unaware of the heteroplasmy in #19 and #39 until afterwards, when checks with confocal microscopy were no longer possible. However, close examination of the upper auxospore in Fig. 4

suggests that this may have contained three plastids, since at the left there is a plastid fragment that appears to be separate from two larger plastids to the right. Alternatively, heteroplasmy may arise by fusion of plastids to create a single organelle with genomes from both parents to create chimaeric plastids, as in *Chlamydomonas* (e.g. VanWinkel-Swift 1980). Occasional breakdown of uniparental transmission has been suggested to be advantageous for avoiding the build-up of deleterious mutations in a lineage (through Müller's ratchet: Greiner *et al*. 2014) and even in higher plants, biparental plastid inheritance has occasionally been detected in genera such as *Passiflora* (Hansen *et al.* 2007), *Zantedeschia* (Snijder *et al.* 2007), and *Medicago* (Matsushima *et al.* 2008).

Because of relatively recent establishment of the spheroid body endosymbiosis within Epithemiaceae (Nakayama *et al.* 2011), better understanding of the nature of the spheroid body could be a key to understanding the early development of endosymbionts into organelles. Fully established organelles, such as plastids and mitochondria, divide at the same pace as the host cell and are segregated accurately into new cells, so that the organelle is a permanent resident and further acquisition of new endosymbionts is unnecessary (Rodríguez-Ezpeleta & Philippe 2006). The diatom-diazotroph associations *Hemiaulus–Richelia* and *Rhizosolenia–Richelia* are apparently functionally equivalent to the endosymbiotic relationship of the spheroid body and *Epithemia*, in the sense that atmospheric N is fixed and supplied to the host (Foster *et al.* 2011). The existence of these associations suggests that the nitrogen-mediated interaction between bacteria and diatoms confers ecological advantages, particularly under nitrogen depleted conditions. Nevertheless, the striking difference between these endosymbionts and the spheroid body is that the diatom–diazotroph associations are facultative – the endosymbionts are transmitted to daughter cells through vegetative division but only for several generations, and they are occasionally absent from the hosts (Rai *et al.* 2000).

Organelles typically show smaller genome sizes than free living relatives, as a result of losing genes via transfer to the host nucleus or by being lost entirely from the organism (Timmis *et al.* 2004; Archibald 2009; Rodríguez-Ezpeleta & Philippe 2006). The reduced gene complement

means that the organelle has limited functionality and is dependent on the host nucleus, and thus, unable to survive outside the host cell anymore. Furthermore, what is also striking for characterizing organelles is uniparental inheritance, which is a mechanism interpreted to be a system controlled by the host nuclear genome for maintaining homoplasmy of cytoplasmic DNA and inhibiting the evolution of the organelles via recombination, (*e.g.* Birky 2008; Kuroiwa 2010). Synchronized division and genome size reduction have already been reported in the spheroid body (Geitler 1977; Nakayama & Inagaki 2017; Nakayama *et al*. 2014), and in the "cyanelle" of *Paulinella chromatophora* Lauterborn, which is a primary plastid analogous to the plastids of Archaeplastida (Marin *et al.* 2005; Rodríguez-Ezpeleta & Philippe 2006). Genome size reduction is also known in the candidatus *Atelocyanobacterium thalassa* (UCYN-A), a nitrogen-fixing cyanobacterium suggested to have established a symbiotic relationship relatively recently with haptophytes (Thompson *et al.* 2012), although no information on the mode of its division (*i.e.* synchronized with host cell division or not) is available. To our knowledge, however, no attempt has been made to reveal the mode of inheritance of these "young" organelles during the sexual phase. Our data indicate that the spheroid body in *Epithemia gibba* var. *ventricosa* is uniparentally inherited, even though there is no link with mating type. Along with the other characteristics of the spheroid body as mentioned above, we would suggest it is at a rather late stage in organellogenesis, despite its young evolutional age compared to plastid and mitochondria.

We detected F1 strains bearing two organelles with different parental origins, that is, spheroid bodies from strain K03 and plastids from K06, or vice versa. Although this type of heterogenic origin of plastids and mitochondria has been reported from higher plants, *e.g.* in bananas (Fauré *et al.* 1994) and kiwi plants (Testolin & Cipriani 1997), this is the first report to show that diatoms can also show such patterns of inheritance of organelles/endosymbionts. We do not know how common these are, since there are still very limited numbers of studies of organelle inheritance in diatoms, *i.e.* of mitochondria by Gastineau *et al.* (2013) and Bagmet *et al.* (2020); and plastids by Ghiron *et al.* (2008), all of which dealt with a single organellar inheritance. Further

investigation is needed with *Epithemia* to know how the mitochondria are inherited. Unfortunately, no information on the pattern of the mitochondrial inheritance was obtained in our study, due to the lack of SNPs in our genomic fragments in the parental strains.

It should be added that the results presented here were based on a single pair of the parental strains, K03 and K06, since the markers regions showed no SNPs in other parental strains. One could, of course, argue that the inheritance pattern of the organelles observed in this study is just one of several diverse modes of inheritance, as in the slime mould *Physarum polycephalum* Schwein (Moriyama & Kawano 2003). Our current results need to be interpreted carefully because of the sample size. However, it is still worth noting that no single strain exhibited biparental inheritance of the spheroid body among the F1 strains established in this study. This implies that the host diatom has already developed a system that ensures uniparental inheritance of this newly established organelle.

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Table 1. Parental origin of spheroid body and plastid in total 39 F1 strains. "-" denotes sequencing failure.

F1 strain #	Spheroid body	Plastid
1	K06	K06
2	K06	K03
3	K06	K06
4	K06	K03
5	K03	K06
6	K03	K06
7	K03	K06
8	-	K03
9	K06	K06
10	K03	K06
11	K03	K03
12	K03	K06
13	K06	K06
14	K03	K03
15	K06	K03
16	K03	K06
17	K06	K03
18	K03	K06
19	K06	K03 and K06
20	K06	K06
21	-	K06
22	-	K03

23	K03	K03
24	K03	K06
25	K06	K03
26	-	K03
27	K03	K06
28	K06	K06
29	K06	K03
30	K06	K06
31	-	K06
32	-	K06
33	-	K06
34	-	K06
35	-	K06
36	K06	K06
37	K03	K06
38	K03	K06
39	-	K03 and K06
Numbers of F1 strains with spheroid bodies and plastid(s) inherited from		
K03	14	12
K03 and K06	0	2
K06	15	25
Total	29	39

Figure legends

Figs 1–5. Sexual reproduction and auxosporulation of *Epithemia gibba* var. *ventricosa*. Scale bars = 10 μm. Figs 1–4 share the same bar.

Fig. 1. Sexually compatible cells come next to each other by ventral side to form a pair. Note that cell sizes are markedly different, ruled out clonal pairing.

Fig. 2. Rearranging zygotes after gametes fusion. Two zygotes formed per pair.

Fig. 3. Zygotes expand perpendicularly along long axis of parental cells. Arrows indicate the limit of mucilage capsule.

Fig. 4. Two auxospores formed per pair.

Fig. 5. Cell in F1 strain. Arrowheads indicate spheroid bodies.

Figs 6, 7. Observation of spheroid bodies and plastid with confocal laser scanning microscopy. Scale bar = 10 μm.

Fig. 6. Two spheroid bodies stained with SYBR Green. A fluorescent image is merged with a bright field image.

Fig. 7. Autofluorescence of a single plastid. The plastid has two lobes, connected by a narrow bridge indicated by arrowhead. Note that the spheroid bodies are stained with dead cell, whereas complex plastid shape is illustrated with healthy cell.

Fig. 8. Examples of electropherograms from amplified DNA marker regions in spheroid body and plastid of F1 strains generated by K03×K06.

Supplemental tables

Table S1. Primers used to amplify and sequence marker regions.

Primer name	Sequence (5' – 3')	Target	Product length
SB_04_F	TCGTCAACAATTAGACCAGATCA	Spheroid body SNPs region	513 bp
SB_04_R	CTGTGGTCAACAGGGGTAGC		
CP_03_F	TTGCTAAATCAGCACCAGAGAA	Plastid SNP region	443 bp
CP_03_R	GTTGCAGGTTCCGAATTTGT		

Table S2. Results of mating experiments using 7 parental strains. Each plus and minus indicate the pairs that sexual reproduction occurred or not, respectively.

Parental strains	K01	K03	K04	K05	K06	K07	K08
K01	-						
K03	-	-					
K04	+	+	-				
K05	+	+	-	-			
K06	+	+	-	-	-		
K07	-	-	+	+	+	-	
K08	-	-	+	+	+	-	-

Legends for supplemental figures

Fig. S1. Observation of *Epithemia gibba* var. *ventricosa* (A) Thick mucilage capsule encasing two sibling auxospores. Mucilage is visualized by Indian ink. Scale bar = 50 μm. (B) Confocal laser scanning microscopy; bright field (upper) and fluorescence (lower) showing spheroid bodies stained with SYBR Green. The merged image is shown in Fig. 6. Scale bar = 10 μm.

Fig. S2. Schematic diagram of the genome architecture around SNPs found in spheroid body genome.

The SNPs found between the strains K03 and K06 in *E. gibba* var. *ventricosa* are located between the ORF2 and 3 (arrow). The gene orders flanking the SNPs is same among all spheroid body genomes, whereas the order is not conserved in *Rippkaea orientalis*, a closest relative of the spheroid body in free living cyanobacteria (Prechtl *et al.* 2004). Homologous ORFs (Evalue < $2E^{-170}$ with NCBI blastn search) are shown in same color.

Fig.1

Click here to access/download;Figure;fig1.tiff

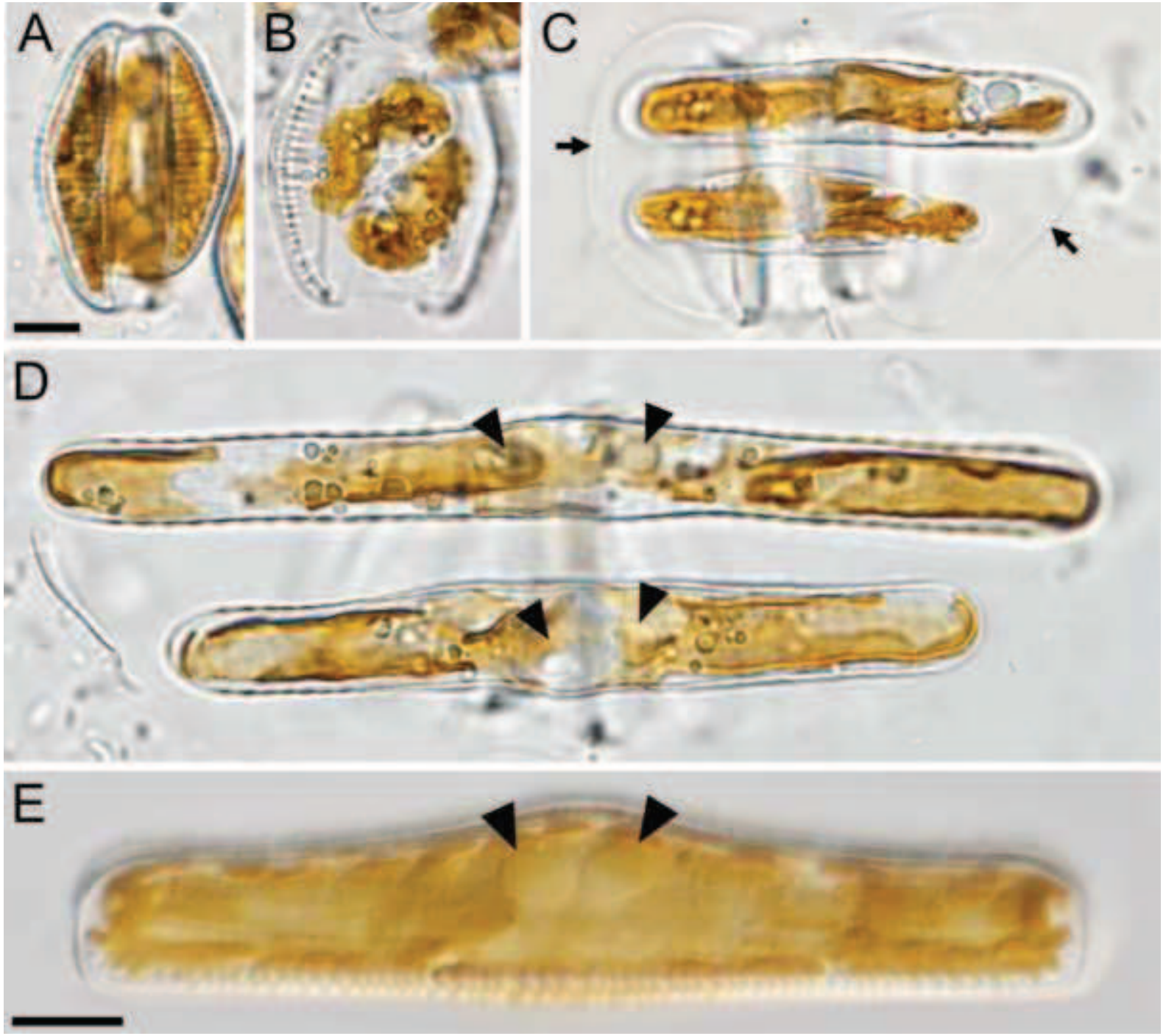
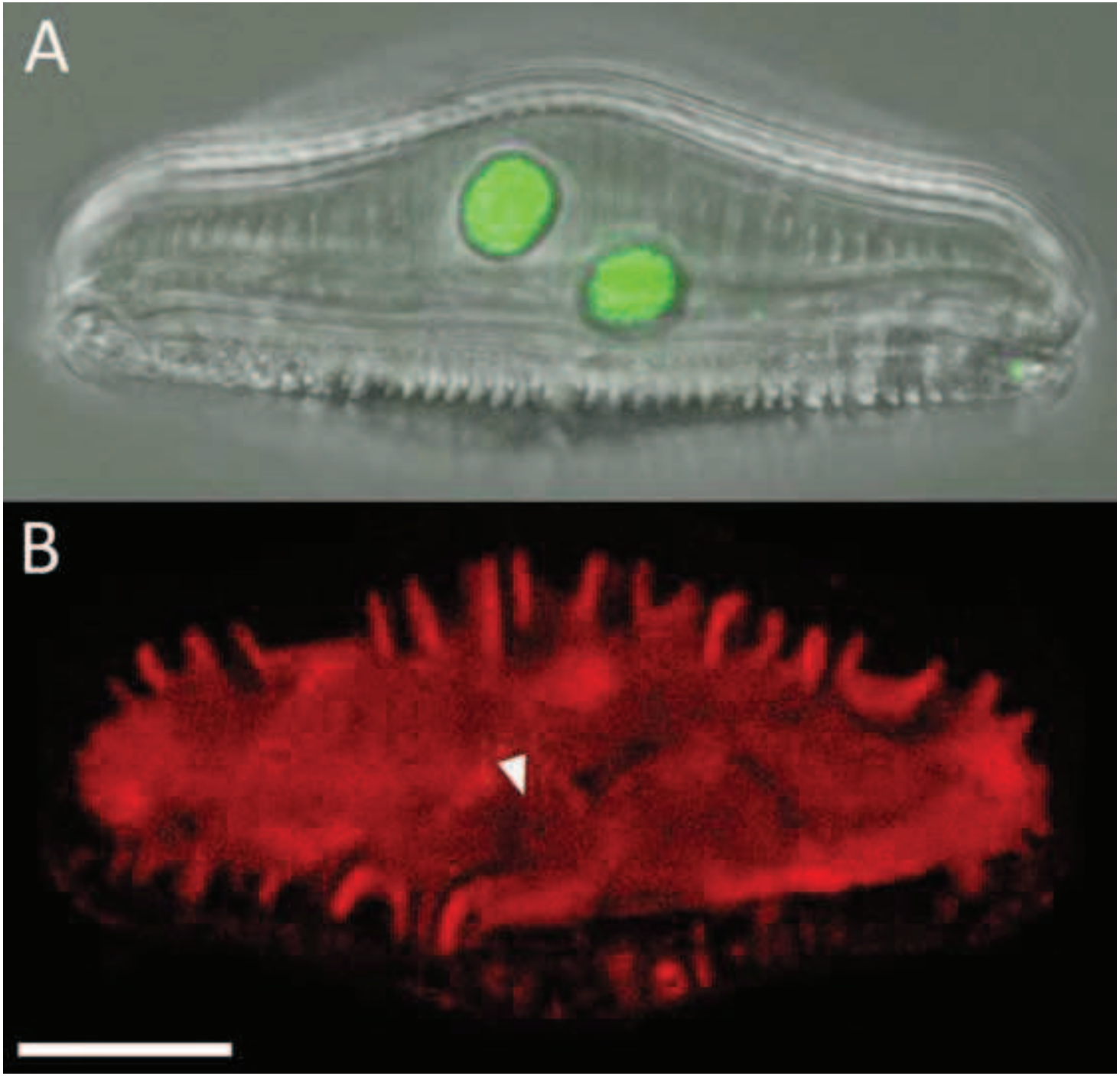


Fig.2

Click here to access/download;Figure;fig2.tiff



2

Fig.3

Click here to access/download;Figure;fig3.tiff

3