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ORIGINAL ARTICLE

The sexual phase of the diatom *Pseudo-nitzschia multistriata*: cytological and time-lapse cinematography characterization

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Abstract *Pseudo-nitzschia* is a thoroughly studied pennate diatom genus for ecological and biological reasons. Many species in this genus, including *Pseudo-nitzschia multistriata*, can produce domoic acid, a toxin responsible for amnesic shellfish poisoning. Physiological, phylogenetic and biological features of *P. multistriata* were studied extensively in the past. Life cycle stages, including the sexual phase, fundamental in diatoms to restore the maximum cell size and avoid miniaturization to death, have been well described for this species. *P. multistriata* is heterothallic; sexual reproduction is induced when strains of opposite mating type are mixed, and proceeds with cells producing two functionally anisogamous gametes each; however, detailed cytological information for this process is missing. By means of confocal laser scanning microscopy and nuclear staining, we followed the nuclear fate during meiosis, and using time-lapse cinematography, we timed every step of the sexual reproduction process from mate pairing to initial cell hatching. The present paper depicts cytological aspects during gametogenesis in *P. multistriata*, shedding light on the chloroplast behaviour during sexual reproduction, finely describing the timing of the sexual phases and providing reference data for further studies on the molecular control of this fundamental process.

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Keywords Chloroplasts · Diatoms · Life cycle · *Pseudo-nitzschia multistriata* · Sexual reproduction · Time-lapse cinematography

Introduction

The genus *Pseudo-nitzschia* includes 45 species of marine planktonic diatoms that are important members of the phytoplankton communities in both coastal and open oceanic waters (Trainer et al. 2012; Teng et al. 2014). A considerable amount of information has been gathered in the last decades on the distribution of the different species, their physiology, toxicology and genetic diversity, making them one of the best known genera of marine phytoplankton (see reviews by Lelong et al. 2012; Trainer et al. 2012). This interest stems from the fact that some *Pseudo-nitzschia* species produce domoic acid (Mos 2001), a neurotoxin responsible for the amnesic shellfish poisoning syndrome (Pulido 2008). Since 1989, when the first paper describing the life cycle of a *Pseudo-nitzschia* species was published (Davidovich and Bates 1998), information has been gained on the life cycle features of 14 different species and 1 variety (reviewed in Lelong et al. 2012). Almost all the investigated species have a heterothallic life cycle; i.e. sexual reproduction was obtained only when strains with opposite mating type get in contact (Fig. S1). Up to now, the only documented exception is *Pseudo-nitzschia brasiliana* Lundholm, Hasle and Fryxell, where sexual stages were observed in clonal cultures (Quijano-Scheggia et al. 2009). The basic mode of the sexual phase of the life cycle is conserved among *Pseudo-nitzschia* species (Lelong et al. 2012). Upon mixing two strains of compatible mating type and in the cell size window for sexualization, some cells align side to side and differentiate into gametangia. Two gametes are produced within each gametangium; one gametangium produces *active*

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(+) gametes that migrate towards the *passive* (−) partners and conjugate. The zygote transforms into an auxospore, which is not surrounded by the rigid frustule, and within the auxospore, a large-sized initial cell is produced (Fig. S1).

To date, various aspects of the life cycle of these pennate diatoms have been investigated, spanning from the description of the sexual phase in different species (e.g. Amato et al. 2005; Amato and Montresor 2008; D'Alelio et al. 2009) to investigations on mating compatibility to test the biological species concept (Amato et al. 2007; Casteleyn et al. 2008; Quijano-Scheggia et al. 2008; Amato and Orsini 2015) and to studies on the variability of toxin production among F1 generation strains (Amato et al. 2010) and chloroplast inheritance mode (Levialdi Ghiron et al. 2008). Experimental studies have addressed the effect of different light/dark cycles on the success of sexual reproduction (Hiltz et al. 2000) and the dynamics of the sexual phase in relation to different concentrations of the parental strains (Scalco et al. 2014). Evidence for sexual events involving *Pseudo-nitzschia* species in the natural environment has been provided (Holtermann et al. 2010; Sarno et al. 2010), and the timing of sexual events has also been estimated by following cell size patterns of natural populations over time (D'Alelio et al. 2010).

We investigated the progression of the sexual phase of the marine pennate diatom *Pseudo-nitzschia multistriata* (Takano) Takano by means of light microscopy (LM), confocal laser scanning microscopy (CLSM) and time-lapse microscopy (TLM). TLM and CLSM technologies represent powerful tools to decipher cytological features of sexual reproduction and cell cycle in diatoms (Sato et al. 2011; Laney et al. 2012; Edgar et al. 2014). This technique was used to follow sexual events in other organisms, e.g. in the red alga *Bostrychia* Montagne to track nuclear fate and plasmogamy during sexual reproduction (Pickett-Heaps and West 1998), or to study the formation and release of gametes in *Ulva* Linnaeus (Wichard and Oertel 2010). Here, we focus on gametogenesis and conjugation and describe the nuclear behaviour during meiosis. Time-lapse cinematography allowed to estimate the time required for the formation of gametes, to describe their conjugation and to illustrate chloroplast division and segregation in the two gametes produced within the gametangial cell. *P. multistriata* has been successfully genetically transformed using a biolistic method (Sabatino et al. 2015), enabling a more detailed follow-up of the present study using e.g. fluorescent protein fusions with markers of different subcellular compartments to follow each organelle during meiosis, gametogenesis and plasmogamy/karyogamy. A de novo genome sequencing project is in progress for this species, and a number of transcriptomes are already available (M.I. Ferrante, unpublished data). These resources and techniques applied on a species whose sexual cycle is well established and easily manipulated will enable functional studies and further investigations on life cycles which are not currently possible in other diatom model species.

Material and methods

Six strains of *P. multistriata* were used for the experiments and the observations in time-lapse microscopy (Table S1). Strains were isolated and cultured as illustrated in Scalco et al. (2014).

Time course of sexual reproduction and confocal laser scanning microscopy

Two independent mating experiments were carried out with one couple of strains of opposite mating type. The average cell size of Pm^- and Pm^+ strains was different but always below the threshold size for sexualization in order to monitor parental cells of the two mating types (Table S1). For each experiment, a culture flask containing 100 mL of F/2 culture medium (Guillard 1975) was inoculated with both parental strains at a final concentration of 5×10^3 cells mL^{-1} each. Aliquots of 4 mL were dispensed in three 6-well culture plates, for a total of 18 wells. Plates were incubated in a growth chamber at the same conditions at which strains were grown (18 °C, 60 μmol photons m^{-2} s^{-1}, 12:12 h dark/light photocycle). Every 12 h and for a total of six sampling points, the content of 3 wells was fixed with neutralized formaldehyde at a final concentration of 1.6 % *v/v*. In order to visualize the nuclear behaviour, the samples were stained with SYBR Green I (S7567, liquid; Molecular Probes, Leiden, The Netherlands) to a final dilution of 1:10,000 for 15 min in the dark at room temperature. The concentration of the different life cycle stages (i.e. large and small parental cells), meiotic cells with segregated cytoplasm, round gametes with one nucleus, round zygotes with two nuclei, auxospores and initial cells was estimated using the Utermöhl method (Edler and Elbrächter 2010) using the Zeiss Axiovert 200 epifluorescence microscope equipped with the FS09 filter (exCitation, 450 to 490 nm; emission, 515 nm) at ×400 magnification.

A parallel experiment, at the same conditions and with the same strains, was run in glass bottom Petri dishes (WillCo Wells B.V., Amsterdam, The Netherlands) for CLSM. Petri dishes were inspected at a laser excitation of 488 nm, with 1–2 % of laser intensity, and a BP 500–550 acquisition filter was used. Images of the different life cycle stages were captured at different magnifications with by Zeiss LSM 510 META CLSM (Carl Zeiss, Oberkochen, Germany).

Time-lapse microscopy

Microcinematography was carried out in bright field (BF) with a Leica DMI 6000B time-lapse microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica

DFC360 FX photocamera. Two pairs of strains, differing in their average apical axis length, were used (Table S1), and co-cultures of the two parental strains in glass bottom Petri dishes were started at the same cell concentration as above. Time-lapse sessions were carried out at different time points, i.e. starting from 24 and 48 h after the inoculum of the parental strains and lasting 16 h. A frame frequency of 1.33 frames min^{-1} (one frame every 45 s) and the *best focus* option were selected.

Two controls were set up: (i) one Petri dish was kept in the growth chamber to confirm that strains underwent sexual reproduction with the expected timing and (ii) another Petri dish was placed in the microscope room at the moment of time-lapse experiment but was wrapped in aluminium foil to avoid exposition to the light flashes of the microscope; this control was meant to confirm that the conditions in the microscope room did not impair the success and timing of sexual reproduction. Control Petri dishes were inspected using a Zeiss Axiovert 200 light microscope (Carl Zeiss, Oberkochen, Germany) at ×400 magnification to check for the presence of sexual stages.

Results

Sexual reproduction: time course

The results of two parallel experiments in which parental strains of opposite mating type were co-cultured and monitored for three consecutive days are shown in Fig. 1. Within each pair, strains of opposite mating type differed in cell size, in order to track them in the culture vessels. The concentration of parental strains remained almost constant during the whole experiment. Paired gametangia were observed 12 h after the inoculum. At the same time, paired gametangia showing cleavage of the cytoplasm (meiotic cells) were observed, when vegetative cell concentration was $\geq 5 \times 10^3$ cells mL^{-1} (Fig. 1a). Meiotic cells reached a maximum concentration of 287.1±234.5 cells mL^{-1} after 60 h from the inoculum with an average maximum percentage of 3.24±3.3 % (Fig. 1a). Observation in CLSM of samples stained with the nuclear stain SYBR Green I after 24 and 36 h provided evidence that these are stages where cells undergo meiotic division (Fig. 2a–f). Meiosis occurred asynchronously in the two mating types, with the Pm^+ mating type (in this case, the smaller cell) starting first (Fig. 2a–f). In Fig. 2a, b, two paired gametangia are shown: the nucleus of the larger Pm^- cell on the left had lost its rounded shape and had begun to expand, approaching the early stage of meiotic prophase. The smaller Pm^+ gametangium on the right contained two protoplasts, each of them with a nucleus. Meiosis I was completed in Pm^+ and cytokinesis that is visible as a cleavage separating the two portions of the gametangial cell took place. During the second meiotic division, only karyokinesis occurred in the Pm^+ gametangium, with the formation of two haploid nuclei within each protoplast (Fig. 2c, d). The small Pm^+ gametangium shown in Fig. 2e, f contained two protoplasts with one nucleus each, suggesting that pyknosis, i.e. the degeneration of one of the two nuclei originating from meiosis II, had already taken place, while the large Pm^- gametangium had completed meiosis II and had two haploid nuclei in each protoplast. Cells with cleaved cytoplasm, i.e. gametangia undergoing meiosis, were detected till the end of the experiment (Fig. 1a). Round gametes with one nucleus encased in the gametangial frustule (Fig. 2g) were first detected 24 h after the inoculum (Fig. 1b), while zygotes and auxospores were observed after 36 h (Fig. 1b, c). At times, gametangia detached from each other and/or gametes detached from their gametangium were then observed free in the medium. Round stages with two nuclei and generally attached to the gametangial frustule were identified as zygotes. Zygotes had a well-defined rounded shape due to the presence of the primary zygote wall. Gametes and zygotes reached the maximum average percentage of 15.2±9.6 and 3.06±1.9 %, respectively, after 48 h from the inoculum (Fig. 1b, c). The conjugation of gametes of opposite mating type (see the following section for details) produced a zygote, called auxospore (Fig. 2h), with two haploid nuclei that fused only after completion of auxospore elongation. The maximum auxospore concentration (>1×10^3 cells mL^{-1}, 7.13 %) was observed after 60 h from the inoculum (Fig. 1c), at the same time when initial cells were detected (Fig. 2i, j).

A triploid zygote and a tetraploid auxospore were also observed, the former bearing six chloroplasts and three nuclei (Fig. S2a-b), the latter with eight chloroplasts and four nuclei (Fig. S2c-d).

Time-lapse microscopy

In order to follow the behaviour of Pm^+ and Pm^- during mate search and the first steps of gametogenesis, mating experiments with strains considerably different in apical axis length were carried out (Table S1). The longer strain, regardless of the mating type, seemed more active and moved more rapidly than the shorter one. Thus, in experiments involving longer Pm^-, these scanned the environment in search for the Pm^+. In experiments involving longer Pm^+, it was the opposite (Movie S1).

In the paired gametangia, cytoplasmic movement was evident during the process of gametogenesis, which included the two meiotic divisions illustrated above as well as the rearrangement of cytoplasmic organelles (Movie S2). The observations in time-lapse cinematography (Movies S3 and S4) allowed following the movement of chloroplasts, which is also illustrated in Fig. 3, prepared with frames extracted from Movie S3 and with schematic drawings (Fig. 4) that help to follow the process. At the beginning of meiosis I, plastokinesis occurred and four daughter chloroplasts were clearly visible in each gametangium (Figs. 3c, d and 4b). After completion of meiosis I and before

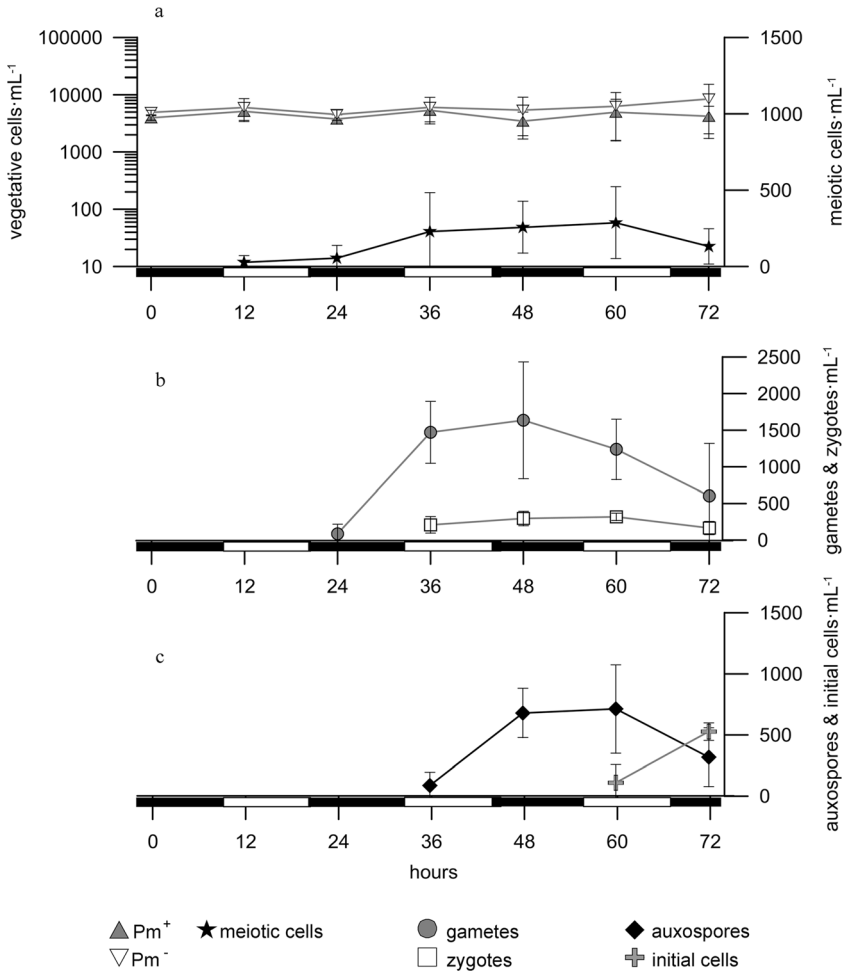

Fig. 1 Time course of sexual reproduction in *P. multistriata* and cell concentration during the different stages: *a* parental cells (*left axis*, log scale; Pm^+, *grey up-triangles*; Pm^-, *white down-triangles*) and meiotic cells (*right axis*; *black stars*); *b* gametes, *grey circles*, and zygotes, *white squares*; and **c** auxospores, *black diamonds*, and initial cells, *grey crosses. Each point* represents the average of six replicated counts± standard deviation. *The black and white bars on the x-axis* indicate the light and dark periods

plasmokinesis, each chloroplast slid along the frustule in concert with the other chloroplasts: the two daughter chloroplasts on the left side slid upwards and the other two on the right, downwards (Figs. 3e–j and 4c). Plasmokinesis occurred that is visible as a cleavage in the middle portion of the gametangial cell (Fig. 3k–m). The two portions of cytoplasm, i.e. the two gametes, thus contained two daughter chloroplasts, each of them deriving from one mother chloroplast of the gametangial cell (Figs. 3n–p and 4d, Movies S3 and S4) and one nucleus that will subsequently undergo meiosis II (Fig. 2c, d). The duration of gametogenesis at the selected experimental conditions was estimated as the time elapsing from the first evidence of cytoplasmic movement in the paired gametangia and the complete formation of two round gametes in each gametangium and lasted, on average, 1 h and 59 min and 24 s±26 min and 51 s (data gained from 5 time-lapse movies). Observations in TLM showed that gametogenesis always started in the Pm^+ gametangium.

Once gamete formation was completed, the gametangial frustules opened up and set the active gametes (Pm^+) free to join the passive ones (Pm^-) (Fig. 4e, Movie S5). Active gametes always conjugated with the passive gametes located on the opposite gametangium, type IA2 functional anisogamy sensu Geitler (1973), in a cross fashion (Fig. 4e, f). The conjugation process yielded one zygote bearing four chloroplasts. Within each zygote, all the four plastidial genomes of the parental cells were thus represented (Figs. 3 and 4f). The duration of the conjugation process at the selected experimental conditions was estimated as the time elapsing from the completion of gamete formation and the completion of the conjugation process of the two pairs of gametes and lasted, on average, 1 min and 35±47 s (data gained from 12 time-lapse movies).

The zygote expanded in a bipolar way to produce the auxospore (Fig. 4g, Movie S2). The two haploid nuclei, which remained separated until the completion of expansion, were close to each other and located in the central portion of the auxospore (Fig. 2h). In the expanded auxospore, the four chloroplasts became very elongated (Movie S2). The entire auxospore elongation was never followed because the individual movies only lasted 16 h. However, an approximate calculation of auxospore elongation was possible using different clips. At the beginning, the elongation was faster with 4.78 μm h^{-1} while, at the end the elongation speed, dropped

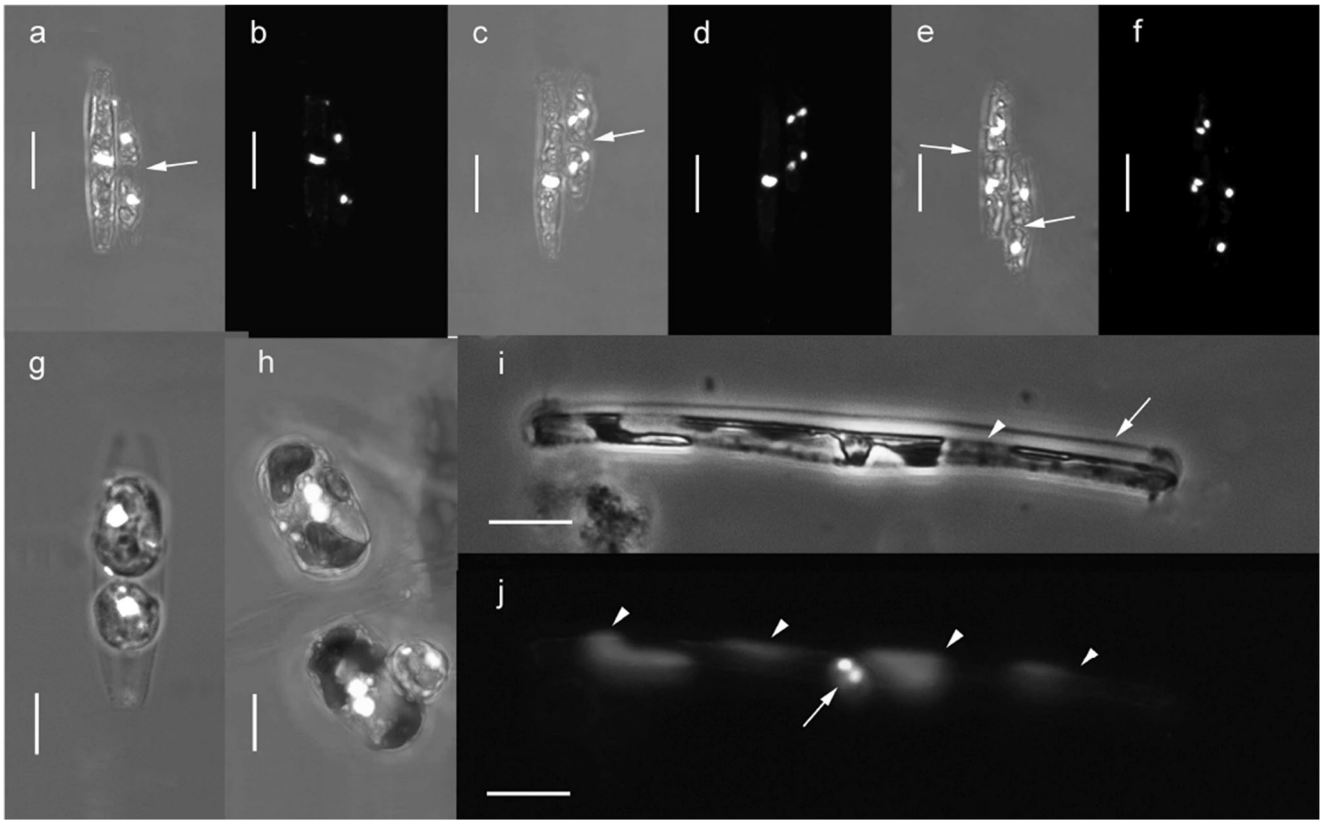

Fig. 2 Confocal Z-stack projections (**a**–**h**) and LM micrographs (**i**, **j**) of different sexual stages of *P. multistriata*. Nuclei are stained with SYBR Green I. Gametogenesis (**a**–**f**), each pair of pictures includes the merge of a bright-field image with the corresponding 488 nm excitation image (**a**, **c**, **f**) and the 488 nm argon/2 laser excitation image only (**b**, **d**, **e**). **a**, **b** The small gametangium on the right has gone through meiosis I and has one nucleus inside each protoplast; the cytoplasm segregation is *arrowed* in **a. c**, **d** The small gametangium on the right has completed meiosis II and has two nuclei inside each protoplasm; the cytoplasm segregation is *arrowed* in **c. e**, **f** The small gametangium on the right has completed meiosis II, and one of the two nuclei in each protoplasm has degenerated; the large gametangium on the *left* has completed meiosis II, and two nuclei are still present in each protoplasm. The cytoplasm segregation in both gametangia is *arrowed* in **e. g** Two round gametes with one nucleus each. **h** Two early auxospores, each one with two nuclei. **i**, **j** Formation of the initial cell inside the auxospore. **i** The new epi-valve (*arrowhead*) has been synthetized inside the perizonium (*arrow*), phase contrast micrograph. **j** The same cell in epifluorescence illumination, with the four chloroplasts (*arrowheads*) and the two nuclei (*arrow*). *Scale bars*=10 μm (**a**–**f**, **i**, **j**) and 5 μm (**g**, **h**)

to 1.5 μm h^{-1}, with an average of 3.9±2.4 μm h^{-1} (data gained from 15 time-lapse movies). It took about 20.5 h for a complete auxospore expansion. When the auxospore expansion was completed, the two nuclei fused to produce the diploid nucleus. The frustule of the initial cell, which contained four chloroplasts, was subsequently deposited within the auxospore (Fig. 2i, j), and the initial cell escaped the perizonium (Fig. 4h). At the first mitotic division that is not accompanied by plastokinesis, the four chloroplasts segregated into the two post-initial cells. The first mitotic division took place when the initial cell was still surrounded by the perizonium or after the initial cell hatched.

Reorganization into gametes of cell content is a crucial process for a correct gametogenesis to occur. A failure in this precisely orchestrated process impairs gametogenesis and, eventually, conjugation. Evidence for these failures was provided by the observation of chloroplast displacement (about 30 % of the total observation), where the two daughter chloroplasts from one mother chloroplast took the wrong direction in the gametangium. In the example illustrated with schematic drawings in Fig. 5 and shown in Movies S6 and S7, one of the two daughter chloroplasts (indicated in white, Fig. 5) went upwards while the other one went downwards (Fig. 5c). This produced the formation of three gametes instead of two (Fig. 5d, Movies S6 and S7). The gamete in the central part of the gametangium contained two daughter chloroplasts (one black and one white in Fig. 5), while on opposite poles of the gametangium, two smaller protoplasts were produced containing one daughter chloroplast each (Fig. 5d). Nuclear behaviour was not followed in this experiment, but it can be hypothesized that the central gamete contained one nucleus and one of the two apical gametes contained the other one. One alternative possibility is that the central gamete contained both nuclei and the two apical protoplasts were akaryotic. The central gamete conjugated with one gamete of the opposite gametangium and produced an apparently

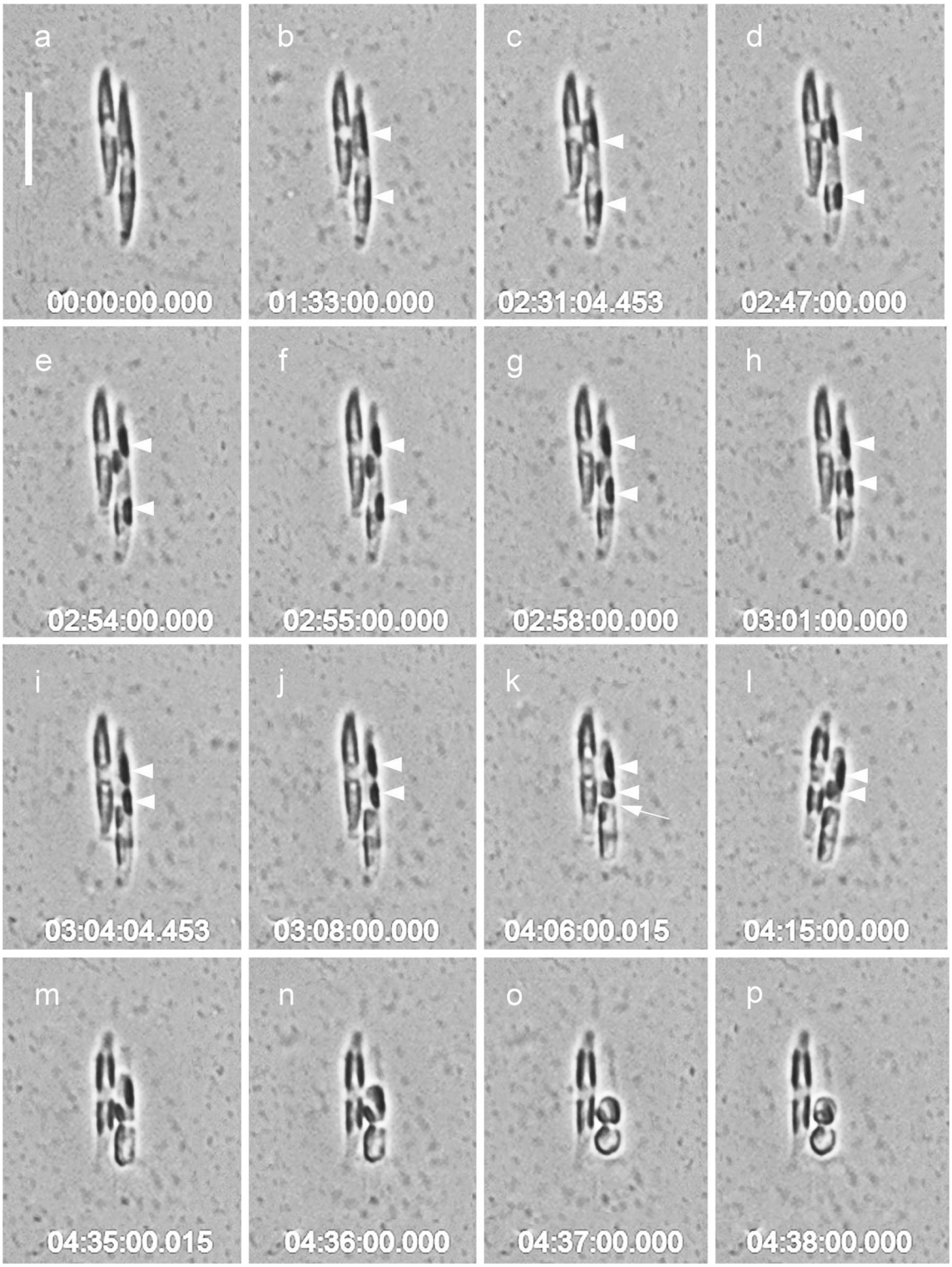

Fig. 3 Chloroplast translation and gametogenesis in *P. multistriata*. Pictures are selected frames of Movie S3. Gametogenesis is not synchronous in *P. multistriata* and occurs first in the gametangial cell on the *right*. *Arrowheads* indicate the two chloroplasts on the *right side* of this gametangium. On the *bottom* of each picture, the time elapsed from the beginning of the time-lapse experiment is reported. The schematic drawings in Fig. 4 help the reader to follow the main steps of the processes. **a** Two parental cells of opposite mating type aligned side to side (Fig. 4a). Note that the chloroplasts already started dividing. **b** Chloroplast division was completed, and each cell contains four daughter chloroplasts. **c**, **d** In the cell on the *right*, chloroplasts contract to prepare translation (Fig. 4b). **e** Chloroplast translation starts; the chloroplasts on the *right-hand side* will slide upwards (*arrowheads*), while the other couple will slide downwards. **f**–**j** The process of chloroplast translation (Fig. 4c) is accomplished in 14 s [from 2 min and 54 s (**e**) to 3 min and 8 s (**j**)]. **k**–**m** Plasmokinesis occurs, marked with an *arrow* on **k. n**–**p** Gametes round up (Fig. 4d); each gamete contains two chloroplasts, deriving from the division of the parental chloroplasts. *Scale bar*=20 μm

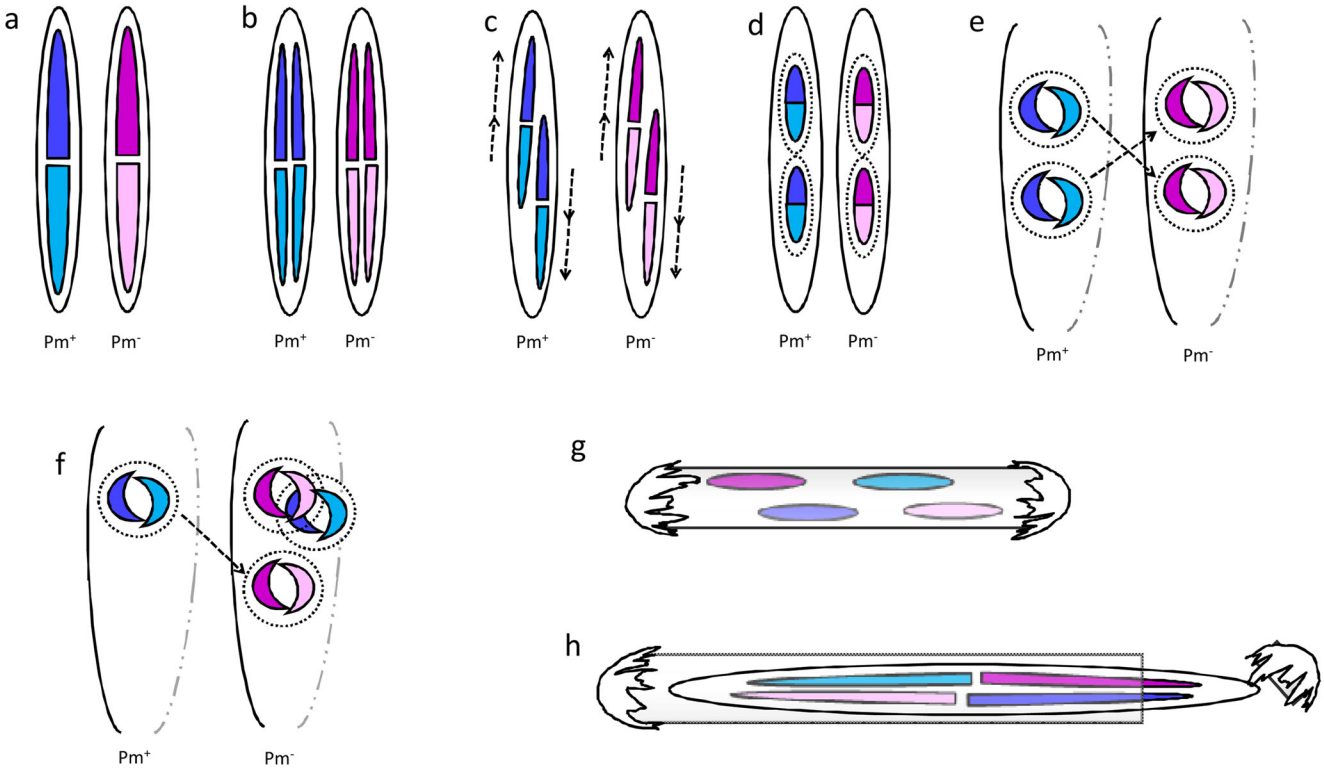

Fig. 4 Schematic drawings of the sexual stages of *P. multistriata* with focus on chloroplast behaviour. Pm^+ and Pm^- indicating the different mating types. **a** Paired gametangia with two chloroplasts each. **b** Chloroplast division in each gametangium. **c** Chloroplasts migration in opposite directions indicated with *arrows*. **d** Cytoplasm cleavage and formation of two round gametes, each of them with two chloroplasts. **e** The frustule opens, with the *arrows* indicating the movement of the gametes and chloroplast. **f** Conjugation of the first two gametes. **g** The elongated auxospore with four chloroplasts. **h** The initial cell escapes from the perizonium

normal zygote with four chloroplasts, while one apical gamete degenerated immediately after formation. The conjugation of the other apical gamete led to a zygote with only three chloroplasts.

One case of automixis was observed, where two gametes from the same gametangium conjugated and produced a zygote that, soon after, degenerated (Movie S8).

Discussion

Sexual reproduction: time course experiment and time-lapse cinematography

The results of the two short-term time course experiments showed that parental cells did not significantly increase in number when sexual stages were produced, thus confirming previous observations (Scalco et al. 2014): vegetative division is inhibited when the two strains of opposite mating type get in contact. The arrest of the cell cycle in conjunction with the onset of the sexual phase has been reported also in fungi, where it has been interpreted as a response to pheromone signalling (Cote and Whiteway 2008; Garcia-Muse et al. 2003).

The examination of samples stained with the nuclear stain SYBR Green I allowed to distinguish gametangia in the process of producing gametes and early-stage zygotes. Experiments started with a cell concentration of 5×10^3 mL^{-1} for each parental strain, and the first appearance of gametes was recorded after 24 h from co-culturing, corroborating previous findings (see Fig. 2 relative to experiment 2 in Scalco et al. 2014). Gametangia undergoing meiosis were detected earlier, 12 h after co-culturing started, and were still present after 2 days, albeit in lower concentrations. The various sexual stages appeared in sequence: the gametes after 24 h, reaching their maximum number after 48 h and subsequently decreasing; the first auxospores after 36 h and constantly increasing till 60 h; and the first initial cells after 60 h. A standardized experimental set-up is of fundamental importance to address questions related to the regulation of the life cycle, e.g. the mechanisms that trigger gamete attraction or the presence of molecular checkpoints during the formation of sexual stages.

The observations in time-lapse microscopy of co-cultures of strains of opposite mating type showed an active behaviour of the large-sized strains that seem to actively search for cells of the opposite mating type. This behaviour is apparently not

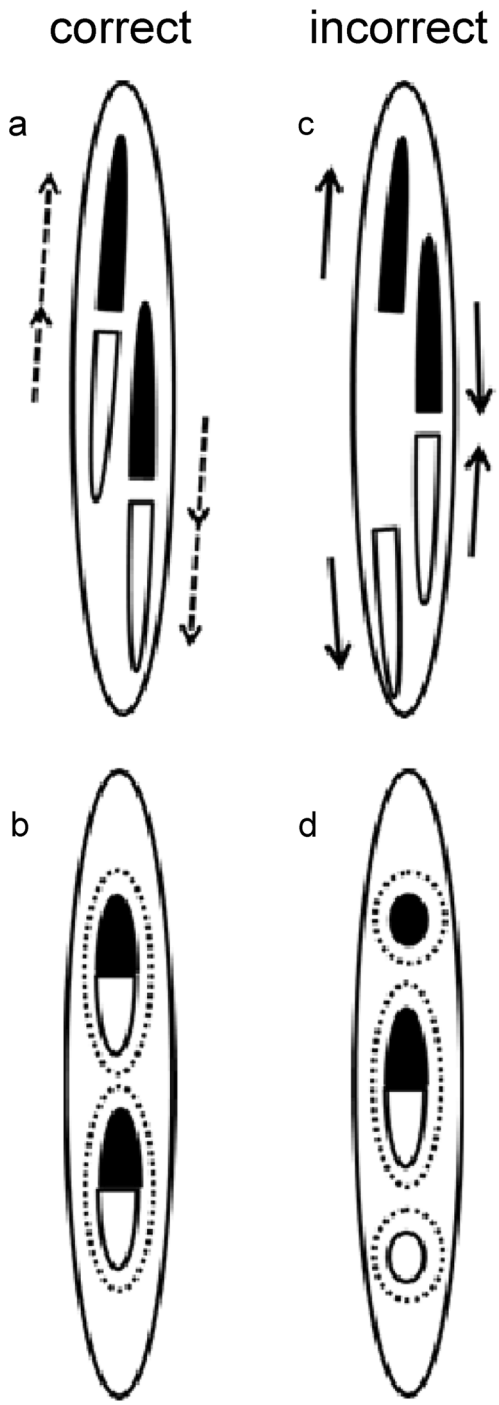

Fig. 5 Schematic drawings illustrating the correct (*a*, *b*) and the incorrect (*c*, *d*) chloroplast movement during gametogenesis in *P. multistriata*: *a* correct chloroplast behaviour and *b* formation of two gametes with two chloroplasts each; *c* anomalous chloroplast behaviour and *d* formation of one gamete with two chloroplasts and two round bodies with one chloroplast each

related to the mating type but to cell size and markedly differs from what is reported for the benthic pennate raphid diatom *Seminavis robusta* Danielidis and Mann, where MT^+ cells actively moved around an attracting MT^- cell (Gillard et al. 2013).

We have estimated by time-lapse microscopy the time required for the formation of gametes (between 1 h and 19 min and 2 h and 23 min), and the very fast conjugation process only lasted a couple of minutes. This raises the question of how planktonic cells can perceive each other and pair (i.e. gametangial pairing) in the water column. It has been hypothesized that sex might occur in thin layers of physical discontinuity (Rines et al. 2002), where density gradients can facilitate encounters (Amato et al. 2005; Scalco et al. 2014) or that hydrodynamic interactions at low Reynolds number between sinking cells or chains might favour contacts between them (Botte et al. 2013).

Chloroplast arrangement during gametogenesis

In *P. multistriata*, like in other diplastidic biraphid pennate diatoms (Mann 1996; Round et al. 1990), chloroplasts divide along the apical axis of the cell during mitotic division and segregate in the two daughter cells. In several pennate diatoms, chloroplasts can rotate before mitosis in order to reach the proper position at the moment of cytokinesis (chloroplast translation).

A series of observations of co-cultures of opposite mating type in time-lapse microscopy allowed tracking the behaviour of chloroplasts during gametogenesis. In the gametangial cell, the two chloroplasts divide and two sibling plastids of each parental chloroplast migrate at the opposite poles of the cell. When cytokinesis occurs after the first meiotic division, each gamete thus inherits sibling plastids from both chloroplasts present in the gametangial cell (biparental inheritance). We observed abnormal chloroplast translation that yielded one central gamete with two daughter chloroplasts and two residual bodies at the poles of the cell with one chloroplast each. We could follow the conjugation of the normal gamete and one of the two apical bodies with the two gametes of the opposite gametangium but could not assess if the zygotes were viable. These irregular movements of chloroplasts (and, most probably, other organelles, including nuclei) and consequent production of malfunctioning gametes were observed in culture; if this also occurs in natural populations, it might represent an additional cost of sex for diatoms (Lewis 1983).

The pattern of chloroplast segregation into gametes has implications for the inheritance of plastidial genomes. In higher plants, a considerable diversity of plastid inheritance patterns exists and both uniparental and biparental models are known (Greiner et al. 2015). Few are the studies on chloroplast inheritance patterns for micro- and macro-algae. In the green micro-algae *Chlamydomonas reinhardtii* Dangeard and *Volvox* Linnaeus (Chlorophyta), plastids are inherited from the *maternal* mating type (Miyamura 2010). In brown algae that, together with diatoms, belong to Stramenopiles, inheritance of chloroplasts is biparental in isogamous species while it is

maternal in oogamous ones (Motomura et al. 2010). The male flagellate gametes of centric diatoms do not have chloroplasts or have very reduced ones (Jensen et al. 2003), whose fate in the zygote remains, however, unknown. The chloroplast inheritance in diatoms depends on two processes: their segregation pattern during gamete formation and the segregation pattern that occurs during the first mitotic division of the initial cell. Establishing how the two steps occur is critical, especially for diplastidic diatoms, where each gamete can inherit one of the two chloroplasts of the maternal cell or copies of both of them. Time-lapse microscopy observations provided, for the first time, information on chloroplast transmission mode in the gametes of the pennate diatom *P. multistriata*. Levialdi Ghiron et al. (2008) took advantage of sexually compatible strains of *Pseudo-nitzschia arenysensis* Quijano-Scheggia, Garcés and Lundholm (*Pseudo-nitzschia delicatissima* (Cleve) Heiden in the paper) with a distinct rbcL ribotype to examine the inheritance pattern in several cultures established from the isolation of large F1 cells. It was shown that chloroplasts segregate stochastically during the first mitotic division of the initial cell. We add to this study the information that each of the two auxospores bears an identical chloroplast assortment; i.e. it has chloroplasts originating from both parental cells. This inheritance mechanism guarantees the highest diversity. Evolution is driven by mutations and fixation: therefore, inheriting two different chloroplast genomes can boost evolution but the presence of a backup chloroplast reduces the risks of fixing a deleterious mutation.

During gametogenesis, we observed daughter chloroplast translation that places the four newly divided plastids in the proper position for gametogenesis. Conversely, during vegetative division, no chloroplast displacement was observed. In other pennate diatoms, chloroplasts move within the cell at mitosis (Mann 1996) while *P. multistriata* does not show such movements at mitosis but at gametogenesis, which occurs very rarely (D'Alelio et al. 2010). Minimizing chloroplast displacement could play a role in optimizing the cellular energetic budget during cell cycle.

Polyploidization and automixis

When observing samples with stained nuclei in CLSM, we could detect a triploid zygote and a tetraploid auxospore in *P. multistriata* co-cultures. They should be the product of the conjugation of three and four gametes, respectively, or of the conjugation between gametes in which pyknotic degeneration of nuclei after the second meiotic division did not occur. The production of polyploid auxospores and initial cells was reported for *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle (Chepurnov et al. 2005), *Dickieia ulvacea* Berkeley ex Kützing (Mann 1994), *S. robusta* (Chepurnov et al. 2002) and other diatoms (reviewed in Chepurnov et al. 2004). Only for *D. ulvacea* the viability of these polyploid stages was assessed, but their capability to undergo sexual reproduction has not been determined (Mann 1994). Evidences of polyploidization in diatoms are scarce (Kociolek and Stoermer 1989; von Dassow et al. 2008; Koester et al. 2010), but the production of anomalous sexual stages might represent a mechanism through which genome content duplicates and polyploid lineages arise.

Time-lapse microscopy provided also evidence for automixis, i.e. conjugation of gametes produced within the same gametangium, in *P. multistriata*. Zygotes, though, rapidly degenerated after their formation. This fits with the fact that sexual stages were never observed in monoclonal cultures of *P. multistriata* (D'Alelio et al. 2009; Scalco et al. 2014), and suggests that automixis is a very sporadic event in this species.

A very intriguing question to be addressed would be the definition of the intricate cytological and molecular machinery regulating chloroplast displacement.

Microscopy studies focusing on diatom life cycles date back to more than 150 years ago (e.g. Thwaites 1847), and since then, a large amount of information has been produced describing different cell processes in many species. Advanced tools in microscopy, and most importantly emerging molecular tools, are now allowing to leap forward in the description and understanding of key phases in diatom life cycles. *P. multistriata*, with the extensive information available in the literature and because of the recent development of molecular tools (Sabatino et al. 2015) and genomic resources, represents a promising model species to address different questions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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