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Sexual reproduction, mating system, chloroplast dynamics and abrupt cell size reduction in
Pseudo-nitzschia pungens from the North Sea (Bacillariophyta)

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Sexual reproduction, mating system, chloroplast dynamics and abrupt cell size reduction in *Pseudo-nitzschia pungens* from the North Sea (Bacillariophyta)

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Clonal cultures of *Pseudo-nitzschia pungens* were isolated at various times from seven sites in the North Sea. During the mitotic cell cycle, the two plate-shaped chloroplasts were girdle-appressed during interphase and mitosis. After cytokinesis, the chloroplasts moved onto the parental valve and remained there during the formation of the new hypovalve and until separation and re-arrangement of the sibling cells within the cell chain had been completed. Clones were almost always heterothallic and cultures of opposite mating type isolated from different localities were compatible. Meiosis I was cytokinetic and accompanied by chloroplast division. Meiosis II involved karyokinesis but not cytokinesis and preceded the rearrangement and contraction of the two gametes. Sexual reproduction involved physiological anisogamy. With one exception, gamete behaviour was clone-specific, gametes being active in clones of one mating type but passive in clones of the other mating type. Auxospore development was accompanied by deposition of a transverse and then a longitudinal perizonium. Infrequently, triploid auxospores and presumably haploid auxospores were produced. The four chloroplasts of diploid auxospores did not divide, and behaved synchronously during the two acytokinetic mitotic cycles accompanying the deposition of the initial thecae. Just before the first division of the initial cell, the chloroplasts shifted onto the valves (two per valve). The division of the initial cell was not accompanied by chloroplast division and so the two daughter cells received two chloroplasts each. Two modes of abrupt cell size reduction were detected. One occurred during initial cell formation when part of the expanded auxospore aborted. The other pattern was more gradual and was observed in growing cultures; during successive cell divisions a frustule constriction appeared and intensified, one chloroplast split into two, and part of the protoplast aborted. A simple naming system is proposed for mating types in pennate diatoms.

Key words: abrupt size reduction, auxosporulation, Bacillariophyta, chloroplast, diatoms, heterothally, mating system, *Pseudo-nitzschia*, sexual reproduction

Introduction

Species of the fibulate pennate diatom *Pseudo-nitzschia* H. Peragallo in H. Peragallo et Peragallo are common components of phytoplankton in marine coastal and open-ocean waters world-wide (e.g. Hasle, 2002). During the last 20 years, *Pseudo-nitzschia* has attracted much attention because some species produce the neurotoxin domoic acid and blooms can have severe adverse consequences for marine biota and humans (e.g. Bates, 2000). So far, nine species of *Pseudo-nitzschia* have been shown to be actually or potentially toxic (Fryxell & Hasle, 2003).

The economic significance of *Pseudo-nitzschia* species has accelerated study of various aspects of their biology and ecology (e.g. Parsons et al., 2002; Maldonado et al., 2002; Fehling et al., 2004; Lundholm et al., 2004; Orellana et al., 2004), to help establish a basis for monitoring, understanding and countering *Pseudo-nitzschia* blooms. Consequently, *Pseudo-nitzschia* species have rapidly become some of the best-studied of all diatoms. However, as von Stosch commented, “You only know a species if you know its complete life cycle” (Elbracht, 2003, p. 629) and long-term maintenance of diatoms and completion of the
life-cycle in culture require that the characteristics of the mating system are understood (Mann & Chepurnov, 2004; Chepurnov et al., 2004). In these respects there is still much to be learned about *Pseudo-nitzschia* (Mann, 2002; Mann & Bates, 2002). Fortunately, *Pseudo-nitzschia* species are easily isolated into culture.

Some information on sexual reproduction and mating systems is available for five *Pseudo-nitzschia* species, all of them potentially toxic (Fryxell & Hasle, 2003). The best studied is *Pseudo-nitzschia multiseries* (Hasle) Hasle (Davidovich & Bates, 1998; Hiltz et al., 2000), which proved to be heterothallic. Davidovich and Bates (1998) also reported heterothallic sexual reproduction in *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle from two localities, namely the Black Sea and the American coast of the Atlantic Ocean. Recent re-examination of their cultures by scanning electron microscopy (Lundholm et al., 2003) revealed, however, that the Black Sea strains belong to the newly described species *Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle; the Atlantic clones are "either *P. pseudodelicatissima* or *P. cuspidata*; the identity could not be finally established because the shape of the valve is uncertain" (Lundholm et al., 2003). Sexual reproduction and heterothallic mating behaviour have also been reported in *Pseudo-nitzschia delicatissima* (Cleve) Heiden in Heiden et Kolbe from the Mediterranean Sea (Amato et al., 2005) and *Pseudo-nitzschia fraudulenta* (Cleve) Hasle from the North Sea (Chepurnov et al., 2004). No information, however, is currently available on life-cycle traits in *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle, which is a very widely distributed, perhaps cosmopolitan species (Hasle, 2002) and which is also potentially toxic (Fryxell & Hasle, 2003). We therefore isolated a series of monoclonal cultures from various localities in the North Sea where *P. pungens* is abundant (e.g. Vrieling et al., 1996). Sexual reproduction was successfully initiated experimentally and we describe its characteristics, together with some other aspects of the cell and life-cycle that have not been reported previously for any *Pseudo-nitzschia* species.

**Materials and methods**

**Sampling, mating experiments and microscopy**

Planktonic samples containing living cells of *P. pungens* were collected from seven locations in the North Sea (Fig. 1) and 24 clonal cultures were established (Table 1) by isolating single colonies by micropipette. Each colony was placed in a separate well of a Repli dish (24-well plates, Greiner bio-one, Frickenhausen, Germany, No. 662160) with 2.5 ml of f2 culture medium (Guillard, 1975), based on filtered and sterilized seawater (c. 32 %) collected from the North Sea. The clones were subsequently grown in 24-well Repli dishes or 50-mm Petri dishes in an incubator at 18 °C with 12:12 h light-dark period and 25–50 μmol photons m⁻² s⁻¹ from cool-white fluorescent lights. Cells were re-inoculated into fresh medium every 7–10 days.

For mating experiments, mixed cultures were prepared by inoculating clones (in exponential growth phase) together into Repli wells in all possible pair-wise combinations. The cultures were examined daily for 6–8 days, using a Zeiss Axiosvert 135 inverted microscope (Zeiss, Jena, Germany), until they reached the stationary phase of growth.

For DAPI (4'-6-diamidino-2-phenylindole) staining of nuclei during gametogenesis, mixed cultures of clones of opposite mating type were fixed during gametogenesis with Lugol’s iodine, formalin and sodium thiosulphate solutions, according to Rassoulzadeh’s method (Sherr & Sherr, 1993). After a few hours’ fixation, cultures were stained with DAPI (0.5 μg ml⁻¹) for 20 min and filtered gently onto black polycarbonate filters (pore size, 0.2 μm; Isopore GTBP membranes; Millipore, Massachusetts, USA) at low vacuum (< 10kPa). The filters were then mounted in a drop of low fluorescence (halogen-free) immersion oil (Zeiss, Jena, Germany).

Frustules were cleaned by oxidation with hydrogen peroxide and glacial acetic acid and washed repeatedly with distilled water before being mounted in Naphrax (PhycoTech, St Joseph, MI, USA). Light microscopical (LM) observations of live cells, cleaned frustules and preparations stained with DAPI were carried out using

![Fig 1. Location of the sampling stations (st 1–st 7) in the North Sea.](image-url)
Table 1. List of clones used. Each clone was isolated within one or two days of sampling

<table>
<thead>
<tr>
<th>Clone</th>
<th>Voucher</th>
<th>Sampling station</th>
<th>Date of sampling</th>
<th>Date</th>
<th>Size* (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trnz-1</td>
<td>st 4</td>
<td>24.05.2002</td>
<td>06.06.2002</td>
<td>127–130 (128.6±0.96)</td>
</tr>
<tr>
<td>2</td>
<td>Trnz-2</td>
<td>st 4</td>
<td>24.05.2002</td>
<td>06.06.2002</td>
<td>131–134 (132.3±1.04)</td>
</tr>
<tr>
<td>6</td>
<td>LP-6</td>
<td>st 1</td>
<td>01.05.2000</td>
<td>08.12.2003</td>
<td>78–85 (81.0±2.22)</td>
</tr>
<tr>
<td>14</td>
<td>LP-14</td>
<td>st 2</td>
<td>25.04.2002</td>
<td>08.12.2003</td>
<td>117–120 (119.0±1.16)</td>
</tr>
<tr>
<td>16</td>
<td>LP-16</td>
<td>st 3</td>
<td>22.05.2002</td>
<td>08.12.2003</td>
<td>111–120 (115.7±2.72)</td>
</tr>
<tr>
<td>17</td>
<td>LP-17</td>
<td>st 3</td>
<td>22.05.2002</td>
<td>08.12.2003</td>
<td>115–122 (118.0±1.77)</td>
</tr>
<tr>
<td>18</td>
<td>LP-18</td>
<td>st 3</td>
<td>22.05.2002</td>
<td>08.12.2003</td>
<td>108–114 (111.0±1.95)</td>
</tr>
<tr>
<td>19</td>
<td>LP-19</td>
<td>st 3</td>
<td>22.05.2002</td>
<td>08.12.2003</td>
<td>118–122 (119.5±1.51)</td>
</tr>
<tr>
<td>20</td>
<td>ZL130-20</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>93–97 (94.2±1.32)</td>
</tr>
<tr>
<td>21</td>
<td>ZL130-21</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>84–88 (86.2±1.48)</td>
</tr>
<tr>
<td>22</td>
<td>ZL130-22</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>94–99 (95.4±1.17)</td>
</tr>
<tr>
<td>23</td>
<td>ZL130-23</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>80–83 (81.4±1.18)</td>
</tr>
<tr>
<td>24</td>
<td>ZL130-24</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>84–87 (85.1±0.99)</td>
</tr>
<tr>
<td>26</td>
<td>ZL130-26</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>85–89 (86.8±1.23)</td>
</tr>
<tr>
<td>27</td>
<td>ZL130-27</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>92–95 (93.3±0.95)</td>
</tr>
<tr>
<td>28</td>
<td>ZL130-28</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>86–89 (87.7±1.16)</td>
</tr>
<tr>
<td>29</td>
<td>ZL130-29</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>92–96 (94.2±1.32)</td>
</tr>
<tr>
<td>30</td>
<td>ZL130-30</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>85–91 (88.4±2.46)</td>
</tr>
<tr>
<td>33</td>
<td>ZL130-33</td>
<td>st 5</td>
<td>30.06.2003</td>
<td>23.09.2003</td>
<td>95–97 (95.8±1.14)</td>
</tr>
<tr>
<td>40</td>
<td>GFC-40</td>
<td>st 6</td>
<td>13.08.2003</td>
<td>24.09.2003</td>
<td>73–77 (75.4±1.84)</td>
</tr>
<tr>
<td>44</td>
<td>GFC-44</td>
<td>st 6</td>
<td>13.08.2003</td>
<td>24.09.2003</td>
<td>82–86 (84.2±1.55)</td>
</tr>
<tr>
<td>52</td>
<td>Ambt-52</td>
<td>st 7</td>
<td>13.09.2003</td>
<td>26.09.2003</td>
<td>90–92 (91.6±0.84)</td>
</tr>
<tr>
<td>57</td>
<td>Ambt-57</td>
<td>st 7</td>
<td>13.09.2003</td>
<td>26.09.2003</td>
<td>90–92 (90.6±0.84)</td>
</tr>
</tbody>
</table>

*Values are range (means ± SD) of 10 measurements.

Table 2. Pseudo-nitzschia pangens: dimensions and stria and fibula densities* from integrated literature data (Fryxell & Hasle, 2003), one of our field samples (from station 5) and four clonal cultures

<table>
<thead>
<tr>
<th>Material analysed</th>
<th>Apical length (µm)</th>
<th>Width (µm)</th>
<th>Striae (in 10 µm)</th>
<th>Fibulae (in 10 µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrated data</td>
<td>74–174</td>
<td>2.4–5.3</td>
<td>9–16</td>
<td>9–16</td>
</tr>
<tr>
<td>Sample (N = 50)</td>
<td>86.3–160.8 (104.6±10.42)</td>
<td>3.7–5.3 (4.5±0.35)</td>
<td>10–13 (11.1±0.68)</td>
<td>10–16 (12.8±1.40)</td>
</tr>
<tr>
<td>Clone 1 (N = 10)</td>
<td>127.0–129.6 (128.6±0.96)</td>
<td>3.8–4.4 (4.2±0.18)</td>
<td>11–12 (11.3±0.35)</td>
<td>11–13 (11.9±0.57)</td>
</tr>
<tr>
<td>Clone 2 (N = 10)</td>
<td>130.9–133.7 (132.3±1.04)</td>
<td>3.8–4.7 (4.3±0.34)</td>
<td>11–13 (11.9±0.74)</td>
<td>11–14 (12.1±1.17)</td>
</tr>
<tr>
<td>Clone 24 (N = 20)</td>
<td>86.2–94.9 (90.3±2.00)</td>
<td>3.1–4.4 (3.6±0.36)</td>
<td>11–13 (12.1±0.67)</td>
<td>10–15 (13.2±1.24)</td>
</tr>
<tr>
<td>Clone 29 (N = 20)</td>
<td>97.1–101.4 (99.0±1.25)</td>
<td>3.1–4.3 (3.6±0.29)</td>
<td>11–14 (12.2±0.71)</td>
<td>12–15 (13.3±0.93)</td>
</tr>
</tbody>
</table>

*Values are range (means ± SD).

a Zeiss Axioplan 2 Universal microscope (Zeiss, Jena, Germany) equipped with a digital camera (VIP III, Hamamatsu Photonics Deutschland, Herrsching, Germany). The morphometric measurements presented in Table 2 were made with the aid of ImageJ software version 1.29x (http://rsb.info.nih.gov/ij/). Scanning electron microscopy (SEM) was performed using a JEOL JSM5600LV (JEOL, Tokyo, Japan).

Voucher specimens of cleaned material of the original natural samples and clonal cultures have been deposited in the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium.

**Terminology of mating types**

The discovery of heterothally in many pennate diatoms during the last decade (Chepurnov et al., 2004) has been a considerable surprise, and terminology has not kept pace with advances in knowledge. For example, there is currently no consistency in the naming of mating types. Mating types have sometimes been referred to as ‘male’ and ‘female’, where there is a constant difference between them in the behaviour of the gametes (e.g. in araphid pennate diatoms: Chepurnov & Mann, 2004; Chepurnov et al., 2004). However, in some heterothallic diatoms (e.g. Eunotia Ehrenberg: Mann et al., 2003 and Seminavis D.G. Mann in Round, Crawford et D.G. Mann; Chepurnov et al., 2002) the gametes produced by opposite mating types behave alike and look identical, so that designation as ‘male’ or ‘female’ is impossible. Furthermore, there is no evidence as to whether, for example, the ‘male’ mating types are equivalent in different taxa, even though this seems likely among closely related species. Therefore, we suggest a simple convention for naming mating types in diatoms, via an acronym consisting of a few letters representing the genus and species, followed by (1) + or −, if the mating system is simple and...
gametangia produce either active or passive gametes, or (2) a number, if the gametangia are not obviously differentiated, or (3) some other symbol that has meaning in relation to the characteristics of mating. In *P. pungens*, the two mating types differ in gamete behaviour and so we propose to designate them as PNP⁺ and PNP⁻, ‘PNP’ being a simple contraction of the species binomial.

Results

Morphology and identification

In a freshly collected marine planktonic sample, cells of *Pseudo-nitzschia* can easily be recognized by their characteristic motile ‘stepped’ colonies (e.g. Hasle & Syvertsen, 1996; Vrieling et al., 1996; Orsini et al., 2004). In our samples, *P. pungens* was sometimes accompanied by other *Pseudo-nitzschia* species already known to occur in the North Sea (e.g. Vrieling et al., 1996; Hasle, 2002), including *P. fraudulenta*, *Pseudo-nitzschia turgidula* (Hustedt) Hasle and *P. delicatissima*. However, even in live samples, it was quite easy to identify *P. pungens* colonies. Cells of *P. turgidula* and *P. delicatissima* were generally smaller and when the samples were disturbed, *P. turgidula* and *P. delicatissima* colonies disintegrated almost completely into single cells. In contrast, the colonies of *P. pungens* and *P. fraudulenta* were robust. Even at low magnification, *P. fraudulenta* could be separated from *P. pungens* through the extent to which the cells overlapped in the stepped colonies (c. 1/5 to 1/3 of the cell length in *P. pungens* but by c. 1/6 to 1/8 of the cell length in *P. fraudulenta*) (Figs 9–12 and see Throndsen et al., 2003). In addition, the chains of *P. fraudulenta* were straight whereas those of *P. pungens* were always slightly bent when seen in valve view (Fig. 2). Finally, frustule morphology and morphometric measurements made with LM.

Figs 2–6. *Pseudo-nitzschia pungens* vegetative cells: LM (Figs 2, 3) or SEM (Figs 4–6). Fig. 2. Stepped chain of cells (live) in girdle view, clone 18. Scale bar: 50 μm. Fig. 3. Valve, clone 29. Note the coarse striae and the absence of a central interspace (the raphe slit is not interrupted by the central nodule). Scale bar: 20 μm. Figs 4–6. Valve ultrastructure. Note the presence of two rows of poroids per stria (Figs 4, 5), the single row of paired poroids on the proximal mantle (thick arrow on Figs 4–6), and single row of poroids (spaced like those of the valve face) on the distal mantle. The girdle of a complete theca consists of three bands (Fig. 6, thin arrows), each with a row of single poroids. Figs 4, 5. Clone 2: valve face in internal (Fig. 4) and external (Fig. 5) views. Fig. 6. Clone 23: frustule in girdle view (the epitheca is marked with an arrowhead). Scale bar: 2 μm.
(Table 2, Fig. 3) and SEM (Figs 4–6) showed that all our isolates could be confidently identified as *P. pungens* (e.g. Hasle, 1995; Hasle & Syvertsen, 1996; Fryxell & Hasle, 2003; Throndsen *et al*., 2003).

### Chloroplasts of vegetative cells

The nucleus lay between the chloroplasts in the centre of the cell and did not migrate at any stage of the mitotic cell cycle. Vegetative cells possessed two simple, plate-like chloroplasts, one in each polar half of the cell (Figs 7–12). During interphase, the chloroplasts lay adjacent to the girdle, with their margins extending slightly beneath the valves (Figs 7–9). Usually, both were appressed to the same side of the girdle (Fig. 7), but occasionally they were located on opposite sides (Fig. 8). During cytokinesis, each chloroplast was split into two by the cleavage furrow (Fig. 10), so that each daughter cell obtained half of each parental plastid. Following cytokinesis, the chloroplasts shifted onto the valves of the parental frustule (the epivalves of the daughter cells) and remained valve-appressed (Fig. 11) until after the new valve had been completed and the sibling cells had separated and slid apart to establish the stepped configuration of the mature colony (Fig. 12). Soon after the translocation of the sibling cells had been completed, however, the chloroplasts returned to the girdle-appressed position illustrated in Fig. 9.

### Abrupt cell size reduction

In culture, *P. pungens* clones generally reduced in size by c. 5 µm (cell length) per month. In addition, however, cell size sometimes reduced abruptly, even by about half the cell length within a division. We observed the process in detail in clone 23. Abrupt size reduction was preceded by progressive changes during several mitotic divisions. The first sign was the appearance of a slight irregularity in frustule morphology near the cell centre, normally in the form of a small bulge (Fig. 8). In subsequent cell divisions, cells became constricted in valve and girdle view, through the formation and progressive deepening of an indentation (Figs 13, 14), which finally caused the neighbouring chloroplast to split in two (Fig. 15). Cells containing three plastids remained viable and continued to divide (Fig. 16). Next, the constriction became so deep as to divide the entire protoplast into two parts (Fig. 17), causing abortion of the part that contained one product of the divided chloroplast but no nucleus (Fig. 18). During the next cell division, therefore, hypovalves were formed that were shorter than the epivalves by the length of the aborted part (Fig. 19). Such cells with unequal thecae could not move apart from each other. At subsequent divisions, however, cells were formed with equal, shortened valves and although shortened cells often remained together during a few cell cycles, forming very characteristic clusters.
Fig 13–22. Abrupt cell size reduction in living vegetative cells, clone 23. Fig. 13. Cell of abnormal shape, valve view. Fig. 14. Two sibling cells of abnormal shape soon after division, girdle view. Note the constriction of the cells at the centre (arrow). Fig. 15. The lower chloroplast has been split into two, valve view. Fig. 16. Two sibling cells just after cell division, each containing three chloroplasts, girdle view. Fig. 17. Separation of the proplast into two parts (arrow), girdle view. Fig. 18. The separated protoplast fragment, which contained one product of a divided chloroplast but lacked a nucleus (arrow), has aborted; valve view. Fig. 19. Division of a cell with a partly aborted protoplast has resulted in the formation of short hypothecae, girdle view. Fig. 20. Typical cluster of abruptly reduced cells formed by three mitoses subsequent to abrupt size reduction, girdle view. Figs 21, 22. Stepped colonies of abruptly reduced cells in valve (Fig. 21) and girdle views (Fig. 22). Scale bars: 20 μm.
regardless of mating type, but those produced behaviour, which is described below.

one clone (clone 40) exhibited particularly unusual cultures and auxosporulation was initiated only in reduced clones and grew it separately (clone 23s), when we isolated a short colony of abruptly the cells corrected the asymmetry (Fig. 22) and where the smaller plastid lay (Figs 19, 20). Later, nucleus shifted from the cell centre towards the end (Fig. 20), they soon began to form typical motile, stepped colonies (Figs 21, 22).

During and immediately after abrupt size reduction, the chloroplasts were unequal in size and the division, the chloroplasts were unequal in size and the tions (only clone 23 was not included in all tests) revealed that 12 clones belonged to one mating type (PNp+) and 11 to the opposite mating type (PNp−) (Table 3). However, not all combinations of PNp+ and PNp− led to auxosporulation and one clone (clone 40) exhibited particularly unusual behaviour, which is described below.

The gametes were morphologically identical, regardless of mating type, but those produced by PNp+ gametangia were active (‘male’); they migrated out of the gametangial frustule to fuse with the gametes of PNp− gametangia, which were passive (‘female’), remaining within the compartment formed by the gametangial frustule (Figs 30, 31).

The mating behaviour of clone 40 was anomalous. Clone 40, like other clones, was isolated as a single chain and, as with other clones, never reproduced sexually in monoclonal cultures, so that there was no indication of heterogeneity within it. However, cells of clone 40 became sexualized if grown together with any other clone, whether of PNp+ or PNp−. When clone 40 was mixed with larger-celled clones (see Table 1), e.g., PNp+ clones 6 (Figs 23–26, 28) or 20, or PNp− clones 17 (Figs 27, 29–32) or 52, it was possible to investigate how cells of each clone behaved sexually in mixed cultures. These experiments showed that cells of clone 40 were able to pair amongst themselves and reproduce successfully, as well as with the PNp− and PNp+ clones. Clone 40 cells behaved as ‘male’ when crossed with ‘female’ PNp− clones and as ‘female’ in crosses with ‘male’ PNp+ clones. In intraclonal pairings, sexual reproduction was physiologically anisogamous, as in crosses between normal PNp− and PNp+ clones, so that some of the clone 40 cells behaved as male and others as female.

### Table 3. Results of mating experiments

<table>
<thead>
<tr>
<th>Mating type</th>
<th>Clone 1 16 17 19 24 29 30 52 28 14 23* 2 6 18 20 21 22 26 27 33 44 50 57 40s 40**</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNp−</td>
<td>1 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>16 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>17 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>19 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>24 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>29 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>30 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>52 0 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>28 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>14 0 0 0 0 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>23* nt nt nt nt nt nt nt nt nt nt 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>2 + + + + + + + + + + + + + + + + + + + + 0 0 nt [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>6 + + + + + + + + + + + + + + + + + + + + nt 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>18 + + + + + + + + + + + + + + + + + + + + + + nt 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>20 + + + + + + + + + + + + + + + + + + + + 0 nt 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>21 + + + + + + + + + + + + + + + + + + + + 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>22 + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>26 + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>27 + + + + + + + + + + + + + + + + + + + + 0 nt 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>33 + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>44 + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>50 + + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>57 + + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>PNp−</td>
<td>40s + + + + + + + + + + + + + + + + + + + + + + + nt 0 0 0 0 0 0 0 0 0 0 [0]</td>
</tr>
<tr>
<td>?</td>
<td>40** + + + + + + + + + + + + + + + + + + + + + + + nt + + + + + + + + + + + + + + + + + nt 0 [0]</td>
</tr>
</tbody>
</table>

+, sexual reproduction occurred in mixed culture; ++, crosses where we could determine (because of size differences between the clones) that all matings were interclonal; 0, sexual reproduction absent in mixed culture; [0], sexual reproduction absent in monoclonal culture; nt, cross not made; *, subculture of clone 23, which was obtained after abrupt size reduction (see text); **, the complex mating behaviour is described in the text.
Remarkably, clone 40 was also capable of intraclonal reproduction in mixed cultures with monoclonal cultures of either mating type of *P. fraudulenta* (which is also heterothallic: Chepurnov et al., 2004) and when grown with a strain of *P. multiseries*. In these cases, however, unlike in *P. pungens* crosses, there was no interclonal pairing; cells belonging to different species could not interbreed.

After the anomalous behaviour of clone 40 was confirmed by the results of repeated set of crosses (all possible pair-wise combinations with the other *P. pungens* clones available (Table 3) and with *P. fraudulenta* and *P. multiseries*), we decided to re-isolate a subclone from a single short (four-cell) colony. Surprisingly, the new culture (subclone 40s) exhibited strictly heterothallic behaviour and its cells always behaved as 'males', like those of a normal PNp$^+$ clone, producing gametangia with active gametes while interacting with PNp$^+$ clone 6 (Figs 23–26, 28) and PNp$^-$ clone 17 (Figs 27, 29–32).

Figs 23–32. Pairing, gametogenesis, plasmogamy and early auxospore development in crosses between anomalous clone 40 (smaller) and PNp$^+$ clone 6 (Figs 23–26, 28), and clone 40 (smaller) and PNp$^-$ clone 17 (Figs 27, 29–32). Fig. 23. Valve-to-valve pairing. Fig. 24. Pairing between cells still connected in chains. In the left pair, plastokinesis and cytokinesis have been completed, following meiosis I. Figs 25, 26. Pairs of gametangia stained with DAPI, after meiosis I (Fig. 25) and after meiosis II (Fig. 26, upper gametangium). Fig. 27. Triplet of gametangia. Two cells of clone 40 (smaller cells) have paired with one cell of clone 17. The topmost clone 40 gametangium and the clone 17 gametangium below it are undergoing gamete re-arrangement; the gametes in the lower clone 40 gametangium have completed re-arrangement and contracted. Fig. 28. Late gametogenesis, DAPI staining: the gametes of the upper gametangium are in early gamete re-arrangement, while those of the lower gametangium have re-arranged and begun to contract. Both sibling haploid nuclei are still visible and equal (those of the left gamete of the lower gametangium are not in focus). Fig. 29. Gametangia after re-arrangement and partial contraction of the gametes. Fig. 30. Fully contracted gametes. Note attachment of the right gamete of the upper gametangium to one theca of the parental cell. Fig. 31. Zygotes after allogamous fusion of gametes. The zygotes lie within the compartment formed by the frustule of the upper gametangium. Fig. 32. Early auxospore expansion. Note the cap-like structures at the tips of auxospores (arrows), which represent the ruptured primary auxospore cell wall. Scale bars: 20 μm.
clone 40, no signs of sexualization were noted, as within clone 40 itself. Clones 14 and 28 were also anomalous. Both were unisexual PNp− clones (Table 3) but would not interbreed with all of the available PNp+ clones. Clone 14 bred only with PNp+ clones 6, 18, 22 and 50 and never interacted sexually with other PNp+ clones. Cells of clone 28 were less selective but still recognized only PNp+ clones 6, 18, 20, 22, 26 and 40 s as compatible. Unsuccessful crosses of female clones 14 and 28 with PNp+ clones were repeated at least four times for each pair-wise combination and were made alongside other PNp+ × PNp− crosses, using the same subcultures and conditions (usually different wells of a single Repli dish) as for crosses in which the same clones exhibited sexual activity.

The effect of abrupt size reduction on sexual competence was examined using clone 23s (see above). Clone 23s cells were 42–44 μm in length, in contrast to the original clone 23 cells from which they were derived, which were 76–79 μm. Despite this, clone 23s, like clone 23, exhibited strictly female behaviour while crossed with PNp+ clone 26 (Table 3), and produced viable offspring.

Pattern of sexual reproduction

The first signs of transition to sexual reproduction usually appeared 2–4 days after compatible clones were mixed and involved mass disintegration of the stepped colonies into single cells or (more rarely) short 2–3-cell chains, and sinking of most cells to the bottom of the container. Hence, pairing was mostly between single cells (Fig. 23), but the few cells that remained connected in chains were also capable of sexualization (Fig. 24). Formation of triplets, where a single cell interacted with two cells of the opposite mating type, also occurred (Fig. 27).

The predominant configuration of sexual partners was valve-to-valve (Figs 23, 24); infrequently, valve-to-girdle and girdle-to-girdle configurations were also observed (not illustrated). Meiosis I in gametangia was accompanied by equal plasto- and cytokinesis (Figs 24–26), which visually resembled the equivalent mitotic events (Fig. 10). Meiosis II followed shortly after the first meiotic division and involved karyokinesis alone (Fig. 26, upper gametangium). Next, the gametes became rearranged within the parental frustule (Figs 27, 28) and gradually contracted (Figs 27, 29). Finally, they became spherical (Fig. 30) and at this stage DAPI staining revealed that most gametes contained only one ‘functional’ nucleus; the other haploid product had already begun to abort, becoming smaller and condensed, or had already disappeared completely (not illustrated).

Following the completion of gametogenesis, the gametes from one gametangium migrated towards the other gametangium and allogamous fusion of gametes occurred within the compartment formed by the gametangium (Fig. 31). Sexual reproduction was therefore physiologically anisogamous and the gametangia could be regarded as ‘male’ (usually only from PNp+ clones) and female (usually only from PNp− clones). Sometimes, female gametes retained contact with the thecae of the parental frustule (Fig. 30) but complete separation of female gametes from the walls of gametangial frustule, before plasmogamy, was also observed regularly. After a few hours the zygotes started to expand, becoming auxospores. Expansion was bipolar and the auxospores remained with their tips fixed in the space between the thecae of the female (PNp−) gametangia (Fig. 32), probably indicating the presence of mucous material, which, however, could not be detected by LM in our unstained material. The sibling auxospores tended to expand parallel to each other and perpendicular to the apical axes of the parental gametangia. The auxospores possessed cap-like structures on their tips, representing the ruptured organic wall of the zygote (Fig. 32), and expansion was accompanied by the formation of a perizonium of delicate transverse bands (Fig. 42).

The two functional haploid nuclei inherited from the gametes became and remained closely associated with each other at the centre of auxospores and were easily visible throughout auxospore expansion (Fig. 33). Fusion seemed to take place after expansion was complete. In the early stages of expansion, the four chloroplasts were arranged apparently at random. Later, they became aligned in a single longitudinal row, with two on either side of the (still unfused) nuclei (Fig. 33).

Initial cell formation

After expansion, the auxospore contracted away from the transverse perizonium and the initial epivalve was laid down on this side while the line of chloroplasts lay opposite (Fig. 34). Following this, the chloroplasts shifted onto the girdle region (as defined by the position of the initial epitheca), stayed there for a while (Fig. 35) and then, still in a single line, moved synchronously onto the epivalve (Fig. 36). Next, the hypovalve was laid down (Fig. 37) after a second, lesser contraction of the protoplast. The chloroplasts then moved back to the girdle (as in the configuration shown for the earlier stage in Fig. 35) but subsequently became re-arranged within the initial cell, the two apical chloroplasts shifting onto one valve while the two central ones moved onto the other (Figs 38, 39).

The first division of the initial cell followed.
This division, unlike other mitotic cytokineses, was not accompanied by plastokinesis (Fig. 40), so that the two daughter cells contained two chloroplasts, just like ordinary vegetative cells. The first division of the initial cell could occur either before (Fig. 40) or after escape from the perizonium, which ruptured at one pole allowing the initial cell to slide out (Fig. 41). Once the initial cell had escaped, it became obvious that a longitudinal perizonium is present in *P. pungens,*
Life history and mating system of Pseudo-nitzschia

**Table 4.** Parental and initial cell lengths* for two crosses, clone 6 × clone 40 (21.12.2003) and clone 16 × clone 18 (21.01.2004)

<table>
<thead>
<tr>
<th>Parental clone</th>
<th>Cell length** (µm)</th>
<th>Initial cell length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>72.3–77.9 (74.4 ± 2.38)</td>
<td>157.1–176.3</td>
</tr>
<tr>
<td>40</td>
<td>61.4–63.5 (62.5 ± 0.65)</td>
<td>165.6 ± 4.24 N = 50</td>
</tr>
<tr>
<td>16</td>
<td>107.1–112.0 (109.5 ± 1.63)</td>
<td>159.4–175.2</td>
</tr>
<tr>
<td>18</td>
<td>101.9–107.8 (104.6 ± 1.86)</td>
<td>167.7 ± 4.77 N = 26</td>
</tr>
</tbody>
</table>

*Values are range (means ± SD); **20 cell measurements per clone.

appearing in LM as a single, elongate, smooth strip (Fig. 42).

During initial cell formation, the nucleus was central. Deposition of each initial valve was preceded by an acytokinetic mitosis, with quick abortion of one of the products (not illustrated).

‘Cardinal’ points of the life-cycle

The cardinal points represent critical points in the life-cycle, where changes in physiological status occur in relation to cell size (Geitler, 1932; von Stosch, 1965; Chepurnov et al., 2004). The first cardinal point represents the size of the initial cells. 76 normally-formed initial cells (for abnormalities in initial cell formation, see below) were measured, giving a range of 157.1–176.3 µm (Table 4). To check for any relationship between gametangium size and initial cell size, we measured initial cells in two mixed cultures of *P. pungens*, in which the gametangia were large (clone 16 × clone 18: gametangia 101–112 µm long) or relatively small (clone 6 × clone 40: gametangia 61–78 µm long). The lengths of the initial cells were almost identical (Table 4).

The second cardinal point is the upper size threshold for sexual reproduction, below which cells are sexually inducible given appropriate growth conditions. The largest cells that transformed into gametangia were in PNp− clone 19. This clone, isolated when its cells were c. 120 µm long (Table 1), exhibited no signs of sexualization in repeated crossing experiments until the cells had reduced to 109.4–115.3 µm (112.9 ± 1.59) long. In a cross between clone 19 and clone 40s, the largest gametangium of clone 19 was c. 115 µm.

The third cardinal point is the critical minimal size for sexualization, or the critical minimal size below which cells are not viable, whichever comes first. In *P. pungens*, cells died when they were c. 25–30 µm long.

Atypical behaviour during sexual reproduction

During the development of the initial cell, part of the protoplast adjacent to one tip of the cell sometimes aborted (Fig. 43). This process, which did not involve loss of any of the chloroplasts, did not harm the further development of the initial cell, which was therefore much shorter than the auxospore containing it (Fig. 44).

Once, an expanding auxospore was found that contained only two plastids and a single nucleus (Fig. 45). This auxospore had presumably developed from a single unfertilized gamete. We rarely but regularly (in almost every vigorously reproducing mixed culture that we examined in detail) observed the formation of triploid auxospores, by fusion of three gametes. The triploid cells were capable of auxospore expansion, even though they contained one more nucleus and two more plastids than normal auxospores (Fig. 46, compare Fig. 33). We did not follow the complete development of any individual triploid auxospores. However, we saw several initial cells with complete frustules containing six chloroplasts (Fig. 47), instead of the four usually present (Figs 38, 39). These initial cells had presumably developed from the triploid auxospores.

Discussion

Chloroplast division and arrangement

Chloroplast division is brought about in *P. pungens* by inward growth of the cleavage furrow during cytokinesis. This imposes chloroplast division so there are two lineages of chloroplasts within each clone of *P. pungens* cells, ‘dual’ inheritance *sensu* Mann (1996). Dual inheritance occurs in many other Bacillariaceae (Mann, 1996 and unpublished data), and in some *Nitzschia* Hassall species (e.g. *Nitzschia sigmoidea* (Nitzsch) W. Smith and *Nitzschia linearis* W. Smith: Pickett-Heaps & Tippit, 1980; Round et al., 1990) chloroplast division is imposed by the cleavage furrow, as in *Pseudo-nitzschia*. Elsewhere, however, chloroplast division is at least partly autonomous, constriction of the chloroplast into two being achieved just before the cell is divided by the cleavage furrow in *Nitzschia palea* (Kützing) W. Smith and two other *Nitzschia* species (Geitler, 1975).

A linear arrangement of the chloroplasts, resembling that in *P. pungens* auxospores (Figs 33–37), has been shown in the young auxospores of *N. palea* (Geitler, 1928, fig. 11, as *Nitzschia subtilis* (Kützing) Grunow: for notes on the identification, see Geitler, 1973), *Nitzschia amphibia* Grunow (Geitler, 1969, fig. 2), *Nitzschia frustulum* var. *perpusilla* (Rabenhorst) Grunow in Van Heurck (Geitler, 1970) and *Nitzschia recta* Hantzsch ex Rabenhorst (Mann, 1986, fig. 14). The presence of a similar arrangement in such a wide spread of taxa may indicate that the arrangement is typical for
Bacillariaceae (or at least the biplastidic species). However, further information is desirable. The orchestrated movements of the chloroplasts from side to side within the expanded auxospore as the initial cells form have not been reported previously from Bacillariaceae, as far as we are aware. The pattern of translocation of pairs of chloroplasts prior to division of the initial cell indicates tight control on chloroplast inheritance, though we cannot say whether this leads to mixing of the two chloroplast lineages from each gametangium or ensures their segregation.

Size changes during the life-cycle and abrupt size reduction

The maximal length of *P. pungens* initial cells obtained experimentally (176.3 μm) agrees well with the maximum length found in nature, i.e. 174 μm (Fryxell & Hasle, 2003; see also Table 2). The variation in size of the initial cells (of c. 30 μm) is not surprising (e.g. Mann *et al.*, 1999, 2003; Edlund & Bixby, 2001). In some diatoms, a correlation has been reported between the sizes of the initial cells and those of the gametangia producing them: small gametangia tend to produce small initial cells (e.g. Roshchin, 1994; Davidovich, 1994, 2001; Nagai *et al.*, 1995; Edlund & Bixby, 2001). However, this is not a universal rule, as demonstrated here in *P. pungens* and also in some other diatoms, such as *Tabularia tabulata* (C. Agardh) Snoeijts (Roshchin, 1994), where initial cell length remains ± constant, despite considerable variation in gametangium size. In *Neidium ampliatum* (Ehrenberg) Krammer, in which each gametangium produces one active and one passive gamete (*trans* physiological anisogamy), the size of the initial cell is related to the size of the gametangium in which it forms (i.e. the gametangium contributing the passive gamete), but not to the size of the gametangium contributing the active gamete (Mann & Chepurnov, 2005).

As in most diatom studies so far, vegetative multiplication of *P. pungens* was generally

Figs 43–47. Anomalous development during auxosporulation, clone 40 × clone 6. Figs 43, 44 Abrupt size reduction at initial cell formation. Fig. 43. Partial abortion of auxospore cell contents at one pole (arrow). Fig. 44. The initial cell is shorter than the developed auxospore and longitudinal perizonium (arrow). Fig. 45. Auxospore containing two chloroplasts and a single nucleus. Fig. 46. Auxospore containing six chloroplasts and three nuclei (at centre). Fig. 47. Initial cell containing six chloroplasts. Scale bars: 20 μm.
accompanying by gradual reduction in cell size (according to the Macdonald–Pfitzer rule: Crawford, 1981; Chepurnov et al., 2004). However, abrupt size reduction also occurred. Abrupt cell size reduction has previously been reported in several lineages of both centric and pennate diatoms (e.g. von Stosch, 1965; Roshchin, 1994; Mann et al., 2003; Chepurnov et al., 2004). In diatom cultures, abrupt size reduction can occur spontaneously or can be initiated experimentally by regulation of the culture density, nutrient limitation, or delicate ‘surgical intervention’ (e.g. von Stosch, 1965; Chepurnov et al., 2004). The ecological significance of this phenomenon is not understood. However, two consequences of this phenomenon are obvious – reduction of the duration of the life-cycle and, if abrupt size reduction concerns large cells from the sexually ‘insensitive’ part of life-cycle, the possibility of rapid or immediate sexualization (e.g. Mann et al., 2003).

In *P. pungens*, two methods of abrupt cell size reduction were detected. The first occurred during initial cell formation (see Figs 43, 44) and will shorten the purely vegetative phase of the life-cycle. The second occurred in clones that were already within the sexual size range (Figs 13–20), although we cannot exclude the possibility that similar reduction may occur in larger cells as well. The appearance of a constriction that intensified and finally led to abrupt cell size reduction could be regarded as an artefact of culturing. However, the cells of the original colony from which clone 23 was initiated already contained a small but visible irregularity in shape at the centre and similar abnormalities, as depressions or slight bulges at the cell centre, were also noted (although very infrequently) in cells from natural samples. Similar abnormalities in cell shape have also been illustrated in cultures of other *Pseudo-nitzschia* species, e.g., *P. multiseries* (Subba Rao et al., 1991, figs 1B and 1C). Thus, abrupt size reduction is likely to occur here as well.

**Pattern of sexual reproduction**

Sexual reproduction has now been reported for six species of *Pseudo-nitzschia* (Davidovich & Bates, 1998; Chepurnov et al., 2004; Amato et al., 2005). In all of them, the pattern of auxosporulation corresponds to Geitler’s (1973) type IA2, in which the gametangia produce two gametes apiece and are differentiated into ‘male’ (producing active gametes) and ‘female’ (producing passive gametes). *Pseudo-nitzschia* species are also similar in pairing configuration, gametogenesis and the association of the developing auxospores with the frustule of the female gametangium. The species studied include representatives of all three of the major clades within the genus that have been detected by analysis of rDNA sequence data (from the ITS1–5.8S–ITS2 region: Lundholm et al., 2003). Hence we can predict that type IA2 auxosporulation will be found to be characteristic of the whole genus. The uniformity of sexual reproduction within a single, morphologically well-defined, monophyletic genus is not surprising and has been found, for example, in *Liemophora* C. Agardh (Chepurnov & Mann, 2004), *Eunottia* (Mann et al., 2003), *Sellaphora* Mereschkowsky (Mann, 1989), *Achnanthes* C. Agardh (Sabbe et al., 2004), *Cocconeis* Ehrenberg, *Cymbella* C. Agardh and *Gomphonema* C. Agardh (Geitler, 1973). However, the uniformity within *Pseudo-nitzschia* contrasts strongly with the variation already evident within the closely related genus *Nitzschia*, despite the fact that few *Nitzschia* species (c. 12) have been examined in detail (Geitler 1932, 1973; Mann, 1986; Roshchin, 1994). Some *Nitzschia* species pair side-to-side, as in *Pseudo-nitzschia* (examples are *N. recta* and *N. longissima* (Brébisson ex Kützing) Grunow: Karsten, 1897; Mann, 1986; Chepurnov in Roshchin, 1994), but *N. amphibia* pairs end-to-end. Plasmogamy occurs within a diffuse mucilage envelope in *N. fonticola* Grunow in Van Heurck (Geitler, 1932) and *N. linearis* (Mann, unpublished data), as in *Pseudo-nitzschia*, but in the type species of *Nitzschia*, *N. sigmoidea*, and in several other species, plasmogamy occurs via narrow copulation tubes formed at the centre (*N. sigmoidea*) or at one pole (*N. amphibia*) (Geitler, 1969; Mann, 1986).

Some species produce one gamete per gametangium, others two (Geitler, 1932, 1973). However, the Bacillariaceae, to which *Nitzschia* and *Pseudo-nitzschia* belong, is an ancient group that was already diverse in the upper Eocene (Schrader, 1969; Desikachary & Sreelatha, 1989) and, in the partial LSU rDNA gene tree presented by Lundholm et al. (2002), *Nitzschia* is paraphyletic with respect to *Pseudo-nitzschia*. Hence heterogeneity in sexual reproduction within *Nitzschia* may simply reflect the fact that the revision of *Nitzschia* by Round et al. (1990), in which *Tryblionella* W. Smith and *Psammodictyon* D.G. Mann in Round, Crawford et D.G. Mann as well as *Pseudo-nitzschia* and *Fragilariopsis* Hustedt in A. Schmidt et al. were separated from *Nitzschia*, did not go far enough.

Kaczmarska et al. (2000) made a detailed SEM investigation of the gametes, auxospores and initial cells of *P. multiseries*, which is apparently the closest relative of *P. pungens* (Lundholm et al., 2003), but found no longitudinal perizonium, only a transverse perizonium. A longitudinal perizonium is present in *P. pungens*, however
(Figs 42, 44), suggesting that re-examination of *P. multiseries* may be worthwhile.

**Mating behaviour**

Understanding mating systems, including whether mating occurs randomly or assortatively and how sexual partners are related to each other (outbreeding v. inbreeding), is key to the interpretation of data on the genetic structure of species and their evolutionary potential. Remarkably, all six *Pseudo-nitzschia* species studied so far are heterothallic (Davidovich & Bates, 1998; Chepurnov et al., 2004; Amato et al., 2005). The only exceptions are the anomalous bisexual behaviour of our *P. pungens* clone 40 and a report of sexual reproduction in a mixture of clones of a single mating type in the otherwise heterothallic *P. calliantha* (Davidovich & Bates, 1998, as 'P. pseudodelicatissima' from the Black Sea). In contrast, the few other complexes of closely related pennate species that have been studied intensively with respect to breeding behaviour, in *Sellaphora, Achnanthes* and *Eunotia*, are more variable in their mating systems, exhibiting both intra- and interspecific variation in mating behaviour (Mann, 1999; Chepurnov *et al.*, 2004; Mann et al., 2004; Sabbe et al., 2004).

The behaviour of clone 40 raises interesting questions about mating type and sex determination. The fact that clone 40 never reproduced in monoclonal culture (either as pure clone 40 or with subclone 40s) indicates that it was not expressing all features of both mating types. Yet it was able to mate with both PNp+ and PNp− clones in biclonal cultures. Its behaviour as a ‘normal’ male PNp+ clone after re-isolation (as subclone 40s) from a single chain is even more curious. Though there is little secure evidence, most observations made so far suggest that sex or mating type determination in pennate diatoms is basically genetic (Chepurnov et al., 2004). The behaviour of clone 40 suggests perhaps that some mating types are heteroallelic and that some mating type alleles in these heteroallelic mating types are usually epigenetically silenced. Occasionally, however, suppression (or expression) is incomplete or temporary, leading to ‘abnormal’ or inconsistent behaviour. The behaviour of clones 14 and 28 show further complexities. Clone 14 was among the longest-celled clones isolated and previous observations (e.g. Mann et al., 1999; Chepurnov et al., 2002) have suggested that sexualization becomes progressively easier as cells become significantly smaller than the sexual size threshold. Hence, some failures might be expected among tests involving clones such as clone 14, at least initially. However, clone 14 was initially similar in size to clone 19, which was ‘well behaved’, and clone 28 was relatively small-celled when isolated. Furthermore, clones 14 and 28 both failed to reproduce when mixed with the initially small-celled clone 44.

The message from our data, therefore, is that interesting aspects of the mating system may be missed if only a few clones are isolated and tested. Although the overall picture is clear — that *P. pungens* is heterothallic — there are subtleties in mating type determination, which may be significant.

Further descriptive studies of mating behaviour are needed in *Pseudo-nitzschia* species but significant progress in understanding the microevolution and geographical distribution of the genus will depend more on population genetic studies and developing molecular markers for sex-related events in the natural environment (Armbrust & Galindo, 2001; see also Swanson & Vacquier, 2002; Barrett, 2002). However, confirmation of heterothally (which will enforce outbreeding) in six *Pseudo-nitzschia* species is consistent with the demonstration of high genotypic diversity in *P. calliantha* (Skov *et al.*, 1997, as *P. pseudodelicatissima*), *P. pungens* (Lundholm *et al.*, 2003, p. 811; Evans *et al.*, 2004a) and *P. multiseries* (Evans *et al.*, 2004b).

**What is *P. pungens*?**

A recent review of species-level taxonomy in diatoms concluded that species boundaries have often been drawn too widely (Mann, 1999). The extent to which this is true has been examined using various systems, including the freshwater genus *Sellaphora* and the marine diatoms *Skeletonema* Greville, *Achnanthes* and *Diploneis* Ehrenberg ex Cleve (see Mann, 1999). Knowledge gained from such systems can then be used to help interpret variation elsewhere, where time and money do not permit detailed examination using less intensive (and admittedly less sure) approaches. *Pseudo-nitzschia* has now become established as another system, because of the growing corpus of work stemming from taxo-
logical concerns.

Recently, two distinctive, widely distributed ‘morphotypes’ of *Pseudo-nitzschia* have been studied for correspondence between genetic and mating data. Based on purely morphological data, definition of these two diatoms – *P. pseudodelicatissima* and *P. delicatissima* — remained ambiguous (Hasle, 2002). Large-scale studies of *P. pseudodelicatissima* were therefore undertaken, using morphology and molecular data (ITS sequences) and taking into account the results of some crossing experiments (Davidovich & Bates, 1998). The outcome was the description of two new species, *P. calliantha* and *P. caciantha*, and...
emendation of *P. pseudodelicatissima* and the morphologically similar *Pseudo-nitzschia cuspidata* (Hasle) Hasle (Lundholm et al., 2003). Subsequently, intensive studies were made of *P. delicatissima* from the Gulf of Naples (Mediterranean Sea), again using a mixture of morphological, mating and molecular approaches (Orsini et al., 2004). The data showed the presence of five distinct lineages within what had appeared to be a single morphospecies; this cryptic diversity within *P. delicatissima* requires further study and may lead to the description of several new species.

*P. pungens* is distinctive morphologically, has been reported worldwide (e.g. Hasle, 2002), and appears to be the sister group to *P. multiseries* (Lundholm et al., 2003). We undertook the present work as a preliminary to a molecular genetic study of *P. pungens*, particularly along the Belgian coastline but also from other localities. Evans et al. (2004c) have developed microsatellite markers for *P. pungens* revealing high levels of genetic diversity but apparently no variation that would indicate cryptic speciation. Among our 24 clonal cultures, isolated at different times and from different localities off the north-western coast of Europe, there was no sign of heterogeneous morphological variation, and the sexual compatibility of clones and the viability of the F1 progeny (our unpublished observations) also suggest that all the clones investigated belong to a single species. We have obtained ITS1-5.8S-ITS2 sequences from at least one clone from each of the seven locations of Fig. 1, including clones 14, 28 and 40, and all proved to be identical (Casteleyn et al., 2004).

Regular sampling and isolation of multiple (>6) clones during blooms of *P. pungens* along the Belgian coast were undertaken during spring 2004 and preliminary analyses of morphology, molecular sequences and results of crossing experiments give no indication of any taxonomically significant variation. We have also investigated clones from the Atlantic coast of Spain, New Zealand and the Pacific coast of the USA and found all to be sexually compatible, with no obvious loss of viability in the F1 generation.

**Polyploidy and haploid parthenogenesis**

Polyploidy and haploid parthenogenesis have not been reported previously in *Pseudo-nitzschia* but seem to be not uncommon among diatoms (e.g., Mann, 1994; Chepurnov et al., 2004). The significance and evolutionary consequences of such changes in ploidy are not clear, although during a review of the few chromosome counts available for diatoms, Kociolek and Stoermer (1989) suggested that polyploidy might play an important role in diatom evolution. Polyploidy is a regular though infrequent event in sexually reproducing cultures of *P. pungens*. It occurred more frequently during mating experiments in *P. fraudulenta* (unpublished). The ease with which sexuality can be initiated experimentally in *Pseudo-nitzschia* and the regularity with which ploidy changes occur during auxosporulation in *P. pungens* and *P. fraudulenta* make *Pseudo-nitzschia* an attractive system in which to study polyploidy in greater detail.

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**References**


Life history and mating system of Pseudo-nitzschia


