Species structure and biogeography of *Pseudo-nitzschia pungens*

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Soortstructuur en biogeografie van *Pseudo-nitzschia pungens*

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Contents

1

General introduction and thesis outline

A brief biology of diatoms

Diatoms are the most species-rich and productive group of eukaryotic algae. They are extremely abundant in all aquatic ecosystems, occurring in the plankton and benthos of marine and fresh waters (as freeliving organisms or as endosymbionts in e.g. dinoflagellates and foraminifers), and in terrestrial environments, such as damp soils and moist surfaces of rocks and plants, from the tropics to the polar regions (Round et al. 1990). Diatoms have global ecological significance in the carbon and silicon cycles and probably provide 20% of global photosynthetic fixation of carbon (Field et al. 1998, Mann 1999).

Diatoms are unicellular, although they can also exist as colonies in the shape of filaments (e.g. *Pseudo-nitzschia*), fans (*Meridion*), zigzags (*Tabellaria*), or stars (*Asterionella*). The hallmark of the diatom cell is its intricately shaped and ornamented silica cell wall, called the frustule, which consist of two overlapping thecae, each in turn consisting of a valve and a number of hoop-like or segmental girdle bands. The frustules show a wide diversity in shape, form and ornamentation, which has been the basis of the traditional diatom taxonomy (Fig. 1).

Diatoms have a diplontic life cycle with a prolonged vegetative phase during which the cells divide mitotically. Their unique cell wall structure (two overlapping halves) and division pattern in which new cell wall components are formed within the parental cell, cause diatoms to gradually reduce their cell size in the course of the mitotic part of the life cycle (MacDonald-Pfitzer rule) (Round et al. 1990). Cell size is restored through the development of a special expanding cell called the auxospore, which normally results from sexual reproduction (Chepurnov et al. 2004) (Fig. 2). As a result, sexual

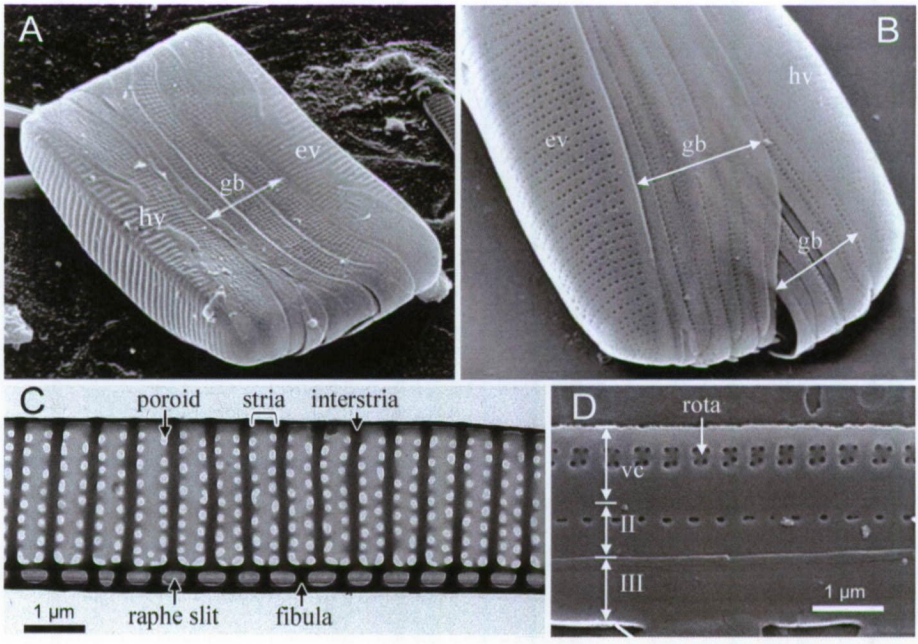

Fig. 1. Architecture of a diatom cell (A) *Eunotia*, whole frustule showing the two valves (hv and ev = hypovalve and epivalve) separated by sets of girdle bands (gb) (arrow). (B) *Parlibellus*, slightly disrupted frustule (A, B from Mann & Evans 2007). (C) *P. pungens* var. *aveirensis* (from Churro et al. in press) (D) *P. pungens* var. *cingulata* showing girdle bands (vc = valvocopula, II and III, second and third girdle band, respectively)

reproduction is firmly integrated into the life cycle of diatoms because periodical sexual events are vital to re-establish the initial cell size and to avoid becoming too small for survival (Chepurnov et al. 2004). As genetic recombination is also achieved through sexual reproduction, the obligatory nature of sex in their life cycle may be linked with the evolutionary and ecological success of diatoms.

Species boundaries in diatoms

Fossil records and molecular data indicate that diatoms have a relatively young evolutionary history. No fossil data have been found before the Mesozoic, 250 Ma (Kooistra and Medlin 1996, reviewed in Sims et al. 2006, Sorhannus 2007). They have since diversified into hundreds of genera and thousands of species. Traditionally, species

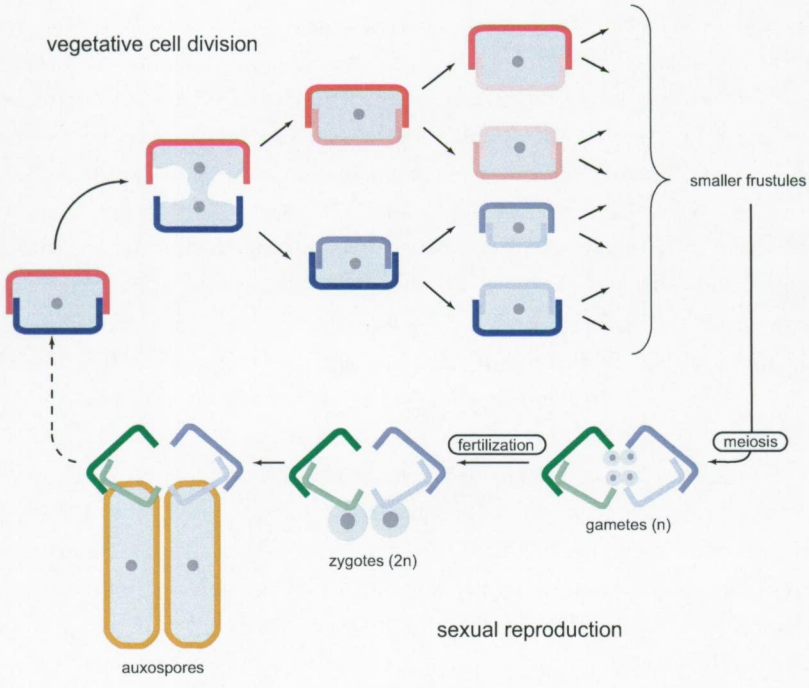

Fig. 2. Life cycle of a pennate diatom.

boundaries have largely been based on discontinuities in morphological and ultrastructural characters of the frustule without a full understanding of the underlying causes of morphological variation. During the last decades however, it has become clear that subtle discontinuous morphological variation patterns, which were previously assumed to be taxonomically insignificant, can be correlated with variation in reproductive, molecular, physiological, and ecological characters (see review by Mann 1999; Behnke et al. 2004, Beszteri et al. 2005a, b, Créach et al. 2006, Lundholm et al. 2006, Amato et al. 2007, Vanormelingen et al. 2007, 2008). These studies suggest that many traditionally circumscribed diatom species contain several distinct entities that are worth taxonomic recognition at the species level, and that, as a consequence, diatom species diversity has been severely underestimated (Mann 1999). The total diversity of diatoms may therefore be an order of magnitude higher than the current number of described species, with possibly up to 200,000 species (Mann & Droop 1996).

A better insight into diatom species taxonomy and evolution can be obtained by studying and comparing variation patterns in various sets of e.g. morphological, reproductive and molecular features (the so-called multi-method approach) (Mann 1999), as has been shown in various other groups of organisms (Dettman et al. 2003,

Agapow et al. 2004). Different properties, such as reproductive isolation, morphological distinctness and monophyly, can result in different species delimitations because they arise at different times during the process of speciation (de Queiroz 2007). For example, Coleman's (2005) study on the ciliate *Paramecium aurelia* showed that reproductive isolation of sexual forms is not always accompanied by divergence in spacer regions of the genome (i.c. ITS) and therefore that lack of significant ITS variation does not imply that speciation has not occurred. It is suggested that this may be linked by whole genome duplication events (Aury et al. 2006). On the other hand, taxa delimited on the basis of monophyletic groups could be part of the same reproductive unit, as has been shown in fungi (Dettman et al. 2003). One of the most significant outcomes from studies that incorporated both molecular and breeding data was the discovery of morphologically indiscernible groups (cryptic species) that are genetically and/or reproductively separate. Cryptic diversity has been observed in various diatoms (Amato et al. 2007, Kooistra et al. 2008) and many other groups of eukaryotes (Fernandez et al. 2006, Hebert et al. 2004, Slapeta et al. 2006, Darling & Wade 2008). Therefore, different lines of evidence (i.e. phenotypic discontinuities, sexual incompatibility or genetic distinctiveness) can help to detect and infer separately evolving lineages, the primary defining property of the species category (de Queiroz 2007). Although the presence of any one of these properties can be regarded as evidence for lineage separation and thus for the existence of a species, a highly corroborated hypothesis of lineage separation requires multiple lines of evidence.

Only a few diatom species have been subjected to an integrative approach by analysis of morphological, molecular and/or mating data. The freshwater epipelic (i.e. in or attached to sediments) species complex *Sellaphora pupula* has been the most thoroughly investigated. It was shown that the complex comprises different previously undetected species, which are morphologically similar but reproductively isolated. They also possess different mating systems, exhibit different degrees of genetic relationships to each other and differ in sensitivity to parasites (Behnke et al. 2004, Evans et al. 2007, 2008, Mann 1999, Mann & Droop 1996, Mann et al. 1999, 2004, 2008). Another freshwater diatom that has been investigated using a combination of morphological, molecular and breeding data is the freshwater epiphyte (i.e. growing attached to other plants or algae) *Eunotia bilunaris*. It was found that *Eunotia bilunaris* clones isolated from New Zealand and Tasmania, 450–2000 km apart, were reproductively compatible despite some differentiation in the ITS rDNA region (Vanormelingen et al. 2007). Phylogenetic relationships and pre- and postzygotic reproductive barriers were also studied in morphologically heterogeneous, sympatric clones of *Eunotia bilunaris* sensu lato. These data suggest the presence of several species (Vanormelingen et al. 2008). In the freshwater planktonic diatom *Cyclotella meneghiniana*, Beszteri et al. (2005a, b,

2007) revealed several distinct genetic entities (based on rDNA sequence and AFLP analyses), only some of which could be corroborated by subtle morphometric differences. Detailed studies on the cosmopolitan marine planktonic diatom *Skeletonema costatum* have also revealed extensive cryptic diversity, with some of the newly uncovered species having a confined geographical distribution (Medlin et al. 1991, Sarno et al. 2005, Godhe et al. 2006, Kooistra et al. 2005, 2008). These examples show that diatom species diversity is much higher than envisaged before. One of the most intensively studied diatom genera is the marine planktonic genus *Pseudo-nitzschia*, which will be described in detail below.

A good understanding of species boundaries is essential to understand patterns in diatom diversity and biogeography, their evolutionary history, and their relationship to environmental conditions. Good knowledge of species limits is also a prerequisite for further studies at the species level and below, including investigation of within-species genetic variation and hybridization events. Hybridization events between genetic distinguishable taxa may have an important role in creating new diversity either by introgression of single alleles, or by the establishment of recombinant genotypes as a new species (Barton 2001). Hybridization events may not be phenotypically visible (Byrne & Anderson 1994; Harper & Hart 2007; Lamb & Avise 1987), and as a result the identification of species on the sole basis of morphology may lead to hybrids being undetected. Joint comparisons of morphology and genotypic constitution may therefore be necessary when studying natural hybridization (Lamb & Avise 1987).

The genus *Pseudo-nitzschia*

The genus *Pseudo-nitzschia* (H. Peragallo, *in* H. & M. Peragallo 1900) is characterized by long, needle-shaped, weakly silicified cells, which form typical step-shaped colonies with overlapping cell ends (Hasle 1994) (Fig. 3). Molecular phylogenetic studies based on LSU rDNA revealed that the genus is paraphyletic and forms, together with the smaller genus *Fragilariopsis*, a clade within the *Bacillariaceae* (Lundholm et al. 2002a, b). More recent phylogenies based on *rbc*L and *psb*C genes showed that *Pseudo-nitzschia* and *Fragilariopsis* constitute two monophyletic sister groups (Kooistra, unpublished results).

Pseudo-nitzschia is one of the most common marine phytoplankton diatom genera. It occurs in phytoplankton communities in both neritic and oceanic environments over a wide latitudinal range from polar to temperate and equatorial waters (Hasle 2002).

Scientific interest in the genus arose since 1987, when *P. multiseries* was related to a domoic acid poisoning incident in Canada that caused the death of three elderly people and over a hundred illnesses after consumption of intoxicated cultivated mussels (Bates et al. 1989). Several *Pseudo-nitzschia* species (up to twelve of the now about 30 described species) have been shown to produce the neurotoxin domoic acid. This water soluble amino acid ($C_{15}H_{21}NO_6$) causes severe gastro-intestinal responses and neurological disorders in vertebrates, including disorientation and memory loss (Todd 1993). Because of the memory loss that could persist indefinitely, the syndrome was named Amnesic Shellfish Poisoning (ASP). Blooms of *Pseudo-nitzschia* may result in accumulation of the toxin in a wide array of organisms in the marine food web, thereby affecting humans, sea birds and marine mammals consuming them (e.g. Bates et al. 1989, Bates et al. 1998, Scholin et al. 2000, Bates & Trainer 2006). These harmful algal blooms are also a severe threat for economically important sectors like fisheries and aquaculture (danger for human health by consumption of shellfish) (LIFEHAB 2001).

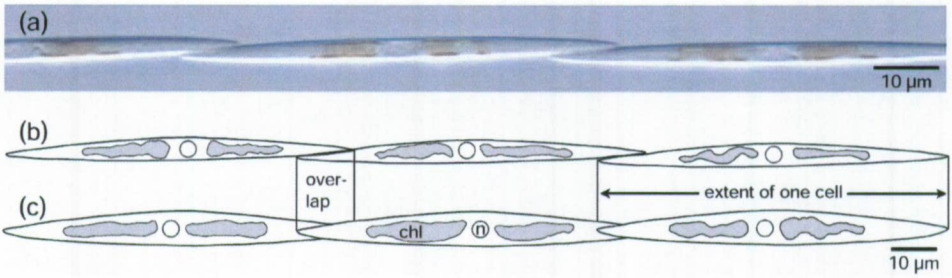

Fig. 3. Chain of *Pseudo-nitzschia multiseries* cells. (a) light microscope image showing girdle (side) view. (b) Drawing of girdle view. (c) Drawing of valve (top) view. chl = chloroplast; n = nucleus (from Trainer et al. 2008).

The economic significance of *Pseudo-nitzschia* species has accelerated the study of various aspects of their biology and ecology, in order to help establish a sound basis for monitoring, understanding and countering *Pseudo-nitzschia* blooms (e.g. Miralto et al. 1999, Parsons et al. 2002, Maldonado et al. 2002, Fehling et al. 2004a, Lundholm et al. 2004, Orellana et al. 2004, Mengelt & Prézelin 2005, Bejarano et al. 2008). Worldwide monitoring programs are being undertaken. Since accurate identification of the different *Pseudo-nitzschia* species is only possible by electron microscopy, which is time-consuming and labor-intensive, molecular probes are used for fast identification of toxic species (Scholin et al. 2003). Unfortunately the use of these probes has not always been successful (e.g. Parsons et al. 1999, Orsini et al. 2002). In addition, differences in toxicity between strains of the same species were observed and contrasting data on the

toxicity of certain species were reported (Bates et al. 1998). Possible explanations are the occurrence of intra-specific genetic variation, whether or not related to geographical or temporal factors (cf. Parsons et al. 1999), or the presence of (semi)-cryptic species within established species. As a consequence, systematic studies in *Pseudo-nitzschia* have received considerable attention motivated by the need to determine the capacity of different species to produce domoic acid and to develop fast and reliable molecular methods to identify *Pseudo-nitzschia* species.

In the last decade, detailed studies on genetic and morphological diversity in *Pseudo-nitzschia* have been carried out, most notably in the toxic *P. pseudodelicatissima/P. cuspidata* species complex (Lundholm et al. 2003, Amato et al. 2007), *P. delicatissima* (Orsini et al. 2004, Lundholm et al. 2006, Amato et al. 2007) and *P. galaxiae* (Cerino et al. 2005), which have resulted in the description of several new *Pseudo-nitzschia* species. In addition, the life cycle of various *Pseudo-nitzschia* species has been thoroughly investigated, revealing a heterothallic sexual cycle (i.e. sexual reproduction is possible only between two sexually compatible strains belonging to opposite mating types), which permits crossing experiments to test mating compatibility (Davidovich & Bates 1998, Kaczmarska et al. 2000, Chepurnov et al. 2004, Amato et al. 2005, Amato & Montresor 2008, D'Alelio et al. 2009a). Amato et al. (2007) combined mating tests and various molecular markers to elucidate the species structure of *P. delicatissima* and *P. pseudodelicatissima* in the Gulf of Naples. They showed general concordance between ITS2 sequence clusters, reproductive compatibility, and slight differences in morphological features. A similar study was carried out for *P. multistriata* in the Gulf of Naples (D'Alelio et al. 2009b). Different ITS types were found but, in contrast to the study of Amato et al. (2007), mating tests showed that they belonged to the same reproductive unit. Likewise, no morphological differences were observed. It was suggested that the ITS polymorphism may be related to the contemporary occurrence of different but still interbreeding populations which either diverged recently or originated in different geographic areas and became sympatric in the studied area. The above-mentioned studies have provided increasing evidence for cryptic (morphologically identical but genetically distinct entities) or pseudo-cryptic (minor ultrastructural differences only detectable by detailed morphological analyses besides genetic divergence, Mann & Evans 2007) species, having important implications for harmful algal bloom monitoring of *Pseudo-nitzschia*, since (pseudo)cryptic species may vary in toxicity and ecological preferences. The original economic motivation for studying *Pseudo-nitzschia* has rendered it one of the best studied diatom genera.

To date, *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Hasle (Fig. 4) has received relative little attention, despite being one of the most commonly reported, potentially toxic representatives of the genus, occuring worldwide in cold to tropical coastal waters

(Hasle 2002). Clones of *P. pungens* isolated from various geographic areas exhibit different abilities to produce domoic acid. Toxic clones have been reported from New Zealand (Rhodes et al. 1996), Washington State (Trainer et al. 1998) and Monterey Bay, California (Bates et al. 1998), whereas no toxic clones have so far been reported from the Atlantic Ocean (e.g. Vrieling et al. 1996).

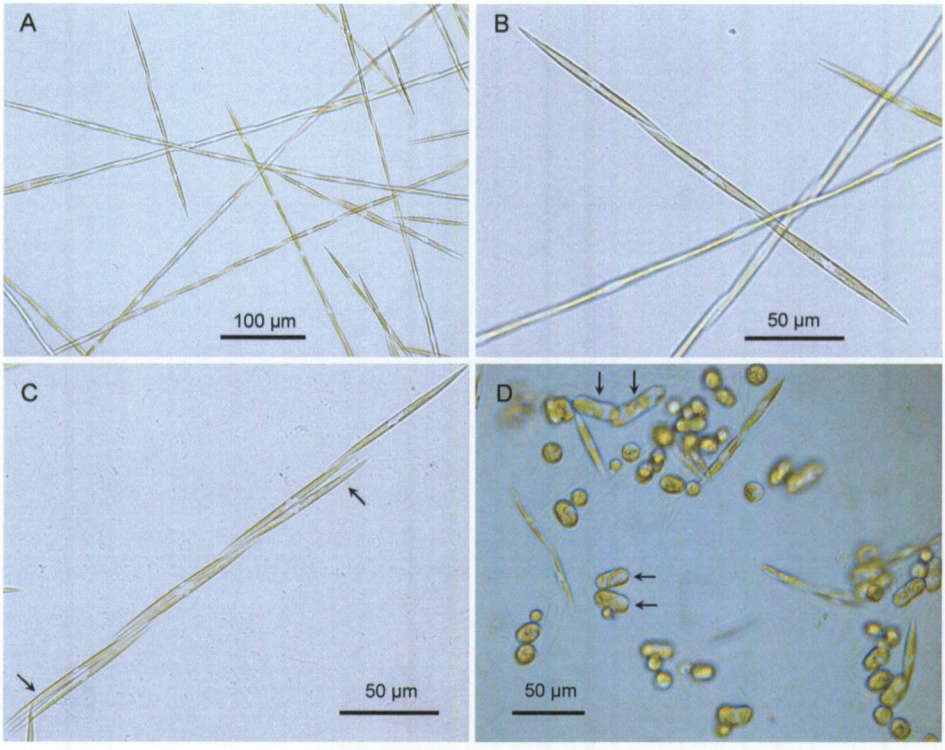

Fig. 4. Cultures of *Pseudo-nitzschia pungens*. (A) Stepped chain-like colonies. (B) Detail of cell chain showing cell overlap. (C) Vegetative cell division in cell chain (arrows). (D) Sexual reproduction showing expanding auxospores (arrows).

Biogeography of marine microbial plankton

In contrast to terrestrial or freshwater environments, the marine planktonic realm was long believed to be a vast homogeneous habitat because of the apparent lack of barriers and many marine species were viewed as having great dispersal abilities, and therefore large geographical ranges, large population sizes, high gene flow and slow rates of evolution (Palumbi 1994, Carr et al. 2003). This was especially emphasized for free-living micro-organisms, which were believed to be comprised of a limited number of ubiquitously distributed species (Baas-Becking 1934, Fenchel & Finlay 2004). Advances in our understanding of oceanography, however, indicate considerable oceanographic structure with ecological and evolutionary consequences (Longhurst 1998, Follows et al. 2007). In addition, the commonplace application of molecular tools has transformed our understanding of marine biodiversity, particularly regarding micro-organisms. In many marine planktonic groups, including copepods (Bucklin et al. 2000, 2002, Goetze 2005), foraminifers (de Vargas et al. 1999, 2002, Darling et al. 2004, Darling & Wade 2008), coccolithophores (Saez et al. 2003, Iglesias-Rodriguez et al. 2006), dinoflagellates (John et al. 2003, Montresor et al. 2003, Lilly et al. 2007, Nagai 2007) and diatoms (Lundholm et al. 2003, 2006, Sarno et al. 2005, Godhe et al. 2006, Amato et al. 2007, Kooistra et al. 2008) several cryptic species and high degrees of geographical structuring were revealed within morphologically circumscribed species that were previously regarded as cosmopolitans. These studies indicate that several barriers to dispersal are indeed present in the marine environment (e.g. ocean currents and vast geographic distances) and in addition distribution ranges may not be limited by the ability to disperse, but rather by the inability to maintain viable populations in areas where environmental conditions are not optimal (De Vargas et al. 1999, 2002, Goetze 2005, Sáez et al. 2003, Darling et al. 2007, Darling & Wade 2008).

As already mentioned earlier, it is clear that accurate species delimitation based on different lines of evidence is important to determine variation patterns in biogeography, because the use of species complexes without recognizing hidden taxonomic complexity can overestimate the geographical distribution of species. For example, it was shown that the marine planktonic diatom *Skeletonema costatum,* previously regarded as a single cosmopolitan species, constitutes several morphologically and genetically distinct species, some of which are geographically confined (Kooistra et al. 2008). Recently, several new diatom species have been described (primarily based on molecular and morphological data and sometimes breeding data), but at present their distributional ranges remain unknown due to insufficient sampling. In conclusion, accumulating evidence is refuting the notion that the marine environment contains a limited number of ubiquitously distributed micro-organisms.

Population genetic structure of marine micro-organisms

In order to understand the causes of marine microbial diversity, the evolutionary processes occurring at the population level should be investigated. The actual evolving units of a species are local populations, i.e. groups of organisms of the same species living within a sufficiently restricted geographical area so that any member can potentially mate with any other member of the opposite sex. It is within such units that local adaptive evolution takes place through systematic changes in allele frequencies (Hartl & Clark 2007).

The field of population genetics studies the genetic variation within and among populations, focusing on the processes that affect genotypic and allele frequencies. These processes include mutation, migration, selection, and drift which structure populations genetically, i.e. produce a non-random distribution of genetic variation (Hartl & Clark 2007). While mutation, random genetic drift and selection at the local scale may cause the divergence of populations, migration and gene flow between locations homogenize them. The level of population differentiation then depends on the balance between selection and gene flow for adaptive traits (Slatkin 1987, Lenormand 2002) and between genetic drift and gene flow for neutral loci (Slatkin 1985). Population genetics for the most part does not focus on phenotypes, since the genes and alleles underlying most phenotypic traits are unknown (Conner & Hartl 2004).

The distribution of allelic and genotypic frequencies is mainly revealed through molecular markers. To be potentially useful, a genetic marker must vary intraspecifically and have a known means of inheritance. Finding that two distant populations share a single fixed allele provides no information about movement between populations (Hellberg et al. 2002). Microsatellites have become increasingly popular for detecting genetic variation within populations. Microsatellites are polymorphic and presumably neutral markers which are scattered throughout the genomes of all eukaryotes, including diatoms (Rynearson & Armbrust 2000). They consist of tandem repeats of 2 – 10 base pair nucleotide motifs and are useful to population geneticists because the length of the repeat region (i.e., the number of repeats) can vary between individuals of a species and so can act as part of a fingerprint to distinguish one individual from another. They are more variable than allozymes and more reliable than RAPDs, and they exhibit co-dominant inheritance (i.e. in diploid individuals, both alleles at a locus can be detected and so it can be determined whether an individual is homozygous or heterozygous at each locus), which increases the information yielded (compared to AFLPs, for example) (Mann & Evans 2007).

Population genetic data for diatoms are scarce and the only detailed investigations using microsatellite markers in marine diatoms include studies of the species *Ditylum*

brightwelii, *Pseudo-nitzschia multiseries* and *P. pungens* (Evans et al. 2004, Evans et al. 2005, Rynearson et al. 2006). All three studies were restricted to local scales. So far, only a single study has investigated population genetic structure of a diatom species on a wide spatial scale using highly discriminatory markers (Evans et al. in press). In this study, highly differentiated populations of the benthic freshwater diatom *Sellaphora capitata* within Europe and between Europe and Australia were observed using ten microsatellite loci. To date, worldwide population genetic studies are lacking for marine planktonic diatoms, which are expected to show less differentiation than benthic or freshwater representatives.

Aims and outline

The main objective of this thesis was to obtain a better understanding of the species structure and biogeography of the marine planktonic diatom species *Pseudo-nitzschia pungens*. More specifically, we wanted to find out (1) whether *P. pungens* is a single species or comprises multiple (semi)cryptic species, by analyzing variation patterns in selected molecular markers, sexual compatibility and morphological characteristics on local, regional and global scales; (2) whether the distribution of *P. pungens* is truly cosmopolitan or whether potential (semi)cryptic entities show different, possible restricted, geographic distributions; and (3) whether intraspecific, geographical genetic structuring could be detected by investigating population genetic diversity and differentiation on both regional and global scales.

In the first part of this thesis we elucidated the taxonomic status and biogeography of *P. pungens*.

In **chapter 2** the life cycle and sexual reproduction of *P. pungens* was studied. Therefore, a series of monoclonal cultures from various localities in the North Sea was isolated. Sexual reproduction was successfully initiated experimentally and its characteristics, together with some other aspects of the cell and life cycle were described.

In **chapter 3** genetic, morphological and reproductive variation in global populations of *P. pungens* was investigated to assess potential intraspecific variation and biogeographic distribution patterns. We extended the sampling from the North Sea and adjoining areas by including *P. pungens* isolates from five geographically distant areas in the Atlantic and Pacific Oceans (193 isolates in total). We identified genetic variation by using sequences of the ITS1 – 5.8S – ITS2 rDNA region, documented morphological

differences, and involved all available clones in crossing experiments to reveal mating barriers.

In **chapter 4** we demonstrate natural hybridization between two sympatric and genetically distinct *P. pungens* clades revealed in chapter 3, corresponding to two varieties (var. *pungens* and var. *cingulata*) by analysing molecular markers in two different genomic compartments (nucleus and chloroplast) and examining morphological features for strains. We expected (1) that populations found with microsatellite markers by Adams (2006) would correspond to the two *P. pungens* varieties (2) that hybrids would be present in natural samples, which could be revealed by molecular markers: hybrids should have both ITS types and be homo- or heteroplastidial, and (3) that the hybrids would be morphologically intermediate to the parents.

In the second part of this thesis we studied the genetic structure at the population level. We concentrated on *P. pungens* var. *pungens*, which was found to have a widespread distribution in temperate areas in the Atlantic and the Pacific Ocean.

In **chapter 5** the spatial and temporal genetic composition of *P. pungens* var. *pungens* in the heterogeneous Southern Bight of the North Sea was investigated with six nuclear microsatellite markers developed by Evans & Hayes (2004). We isolated 310 cells in spring 2007 from water bodies with different environmental conditions (marine, estuary, saline lake) and with different degrees of connectivity (from complete isolation to supposedly free exchange between environments) over a scale of ca. 100 km. Our hypothesis was that different environmental conditions or partial isolation could result in genetic differentiation between populations. In addition, temporal differentiation was assessed by comparing a marine population from 2004 with one from 2007. It was expected that even more genetic differentiation would appear on a larger geographical scale. To that end, isolates from a wider area were included (ca. 650 km).

In **chapter 6** the geographical scale was expanded to different localities in the Atlantic and the Pacific. The same microsatellite loci were used on 242 isolates from all over the world. Based on the results of chapter 5 (no population structure) and in combination with the uniformity in ITS and *rbc*L sequences and the sexual compatibility between global var. *pungens* populations, we wanted to test the hypothesis that *P. pungens* var. pungens consists of a single homogenous global population.

Finally, the main conclusions are summarized and discussed in **chapter 7** and perspectives for future research are provided.

The **appendix** provides a detailed account of all *Pseudo-nitzschia* species occurring in a large lagoon on the west coast of Portugal, Ria de Aveiro. Members of *P. pungens* clade 3 revealed in chapter 3, were studied in detail.

2

Life history and mating system of *Pseudo-nitzschia pungens* [1]

Clonal cultures of *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle 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, as in ordinary vegetative cells. 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.

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[1] Adapted from: Chepurnov VA, Mann D, Sabbe K, Vannerum K, Casteleyn G, Verleyen E, Peperzak L & Vyverman W (2005) Sexual reproduction, mating system, chloroplast dynamics and abrupt cell size reduction in *Pseudo-nitzschia pungens* from the North Sea (Bacillariophyceae). European Journal of Phycology 40, 379-395.

Contribution to this chapter by GC: help with data collection and revision of the article.

Introduction

Species of the fibulate pennate diatom *Pseudo-nitzschia* H. Peragallo in H. Peragallo et Peragallo are common components of phytoplankton in marine coastal 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, Bates & Trainer 2006). So far, up to twelve of the now about 30 described species of *Pseudo-nitzschia* have been shown to be actually or potentially toxic (Trainer et al. 2008).

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. 2004a, Lundholm et al. 2004, Orellana et al. 2004, Mengelt & Prézelin 2005, Bejarano et al. 2008), in order 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 H. A. von Stosch commented, "You only know a species if you know its complete life cycle" (see Elbrächter 2003, p. 629) and long-term maintenance of diatoms and completion of the life cycle in culture requires 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[2] *Pseudo-nitzschia* species, all of them potentially toxic (Fryxell & Hasle 2003). The best studied is *P. multiseries* (Hasle) Hasle (Davidovich & Bates 1998, Hiltz et al. 2000, Kaczmarska et al. 2000), which proved to be heterothallic. Davidovich and Bates (1998) also reported heterothallic sexual reproduction in *P. 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 *P. 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". Sexual reproduction and heterothallic mating behaviour have also been reported in *P. delicatissima* (Cleve) Heiden in Heiden et Kolbe from the Mediterranean Sea (Amato et al. 2005) and *P. fraudulenta* (Cleve) Hasle from

[2] Information on sexual reproduction and mating system is now extended to eight *Pseudo-nitzschia* species, including *P. pungens* (this chapter), *P. mannii* (Amato & Montresor 2008) and *P. multistriata* (D'Alelio et al. 2009a)

the North Sea (Chepurnov et al. 2004 and unpublished). No information, however, is currently available on life cycle traits in *P. 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.

Fig. 1. Location of the sampling stations (st 1 – st 7) in the North Sea.

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 Repli

dish well with 2.5 mL of f2 culture medium (Guillard, 1975), based on filtered and sterilized sea water (*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 reinoculated into fresh medium every 7-10 days.

Table 1. List of clones used. Each clone was isolated within one or two days of sampling in nature.

Clone	Voucher	Sampling station	Date of sampling	First measurement of cell length	
				Date	Size* (µm)
1	Trnz-1	st 4	24.05.2002	06.06.2002	127–130 (128.6 ± 0.96)
2	Trnz-2	st 4	24.05.2002	06.06.2002	131–134 (132.3 ± 1.04)
6	LP-6	st 1	01.05.2000	08.12.2003	78–85 (81.0 ± 2.22)
14	LP-14	st 2	25.04.2002	08.12.2003	117–120 (119.0 ± 1.16)
16	LP-16	st 3	22.05.2002	08.12.2003	111–120 (115.7 ± 2.72)
17	LP-17	st 3	22.05.2002	08.12.2003	115–122 (118.0 ± 1.77)
18	LP-18	st 3	22.05.2002	08.12.2003	108–114 (111.0 ± 1.95)
19	LP-19	st 3	22.05.2002	08.12.2003	118–122 (119.5 ± 1.51)
20	ZL130-20	st 5	30.06.2003	23.09.2003	93–97 (94.2 ± 1.32)
21	ZL130-21	st 5	30.06.2003	23.09.2003	84–88 (86.2 ± 1.48)
22	ZL130-22	st 5	30.06.2003	23.09.2003	94–94 (95.4 ± 1.17)
23	ZL130-23	st 5	30.06.2003	23.09.2003	80–83 (81.4 ± 1.18)
24	ZL130-24	st 5	30.06.2003	23.09.2003	84–87 (85.1 ± 0.99)
26	ZL130-26	st 5	30.06.2003	23.09.2003	85–89 (86.8 ± 1.23)
27	ZL130-27	st 5	30.06.2003	23.09.2003	92–95 (93.3 ± 0.95)
28	ZL130-28	st 5	30.06.2003	23.09.2003	86–89 (87.7 ± 1.16)
29	ZL130-29	st 5	30.06.2003	23.09.2003	92–96 (94.2 ± 1.32)
30	ZL130-30	st 5	30.06.2003	23.09.2003	85–91 (88.4 ± 2.46)
33	ZL130-33	st 5	30.06.2003	23.09.2003	95–97 (95.8 ± 1.14)
40	GFC-40	st 6	13.08.2003	24.09.2003	73–77 (75.4 ± 1.84)
44	GFC-44	st 6	13.08.2003	24.09.2003	82–86 (84.2 ± 1.55)
50	Ambl-50	st 7	13.09.2003	26.09.2003	98–102 (101.0 ± 1.33)
52	Ambl-52	st 7	13.09.2003	26.09.2003	90–92 (91.6 ± 0.84)
57	Ambl-57	st 7	13.09.2003	26.09.2003	90–92 (90.6 ± 0.84)

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

For mating experiments, mixed cultures were prepared by inoculating clones (in exponential growth phase) together into Repli wells in all possible pairwise combinations. The cultures were examined daily for 6–8 days, using a Zeiss Axiovert

135 inverted microscope (Zeiss, Jena, Germany), until they reached the stationary phase of growth.

For DAPI staining of nuclei during gametogenesis, mixed cultures of clones of opposite mating type were fixed during gametogenesis with Lugol's iodine, formalin and sodium thiosulfate solutions, according to Rassoulzagedan's method (Sherr & Sherr 1993). After a few hours' fixation, cultures were stained with DAPI (0.5 $\mu g\ ml^{-1}$) for 20 min and filtered gently onto black polycarbonate filters (pore size, 0.2 μm; Isopore GTBP membranes; Millipore, Massachusetts, USA) at low vacuum (< 10 kPa). 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 observations of live cells, cleaned frustules and preparations stained with DAPI were carried out using a Zeiss Axioplan 2 Universal microscope (Zeiss, Jena, Germany) equipped with a Hamamatsu (VIP III) digital camera (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 was performed using a JEOL JSM5600LV (JEOL, Tokyo, Japan).

Voucher specimens of cleaned material of the original natural samples and clonal cultures are kept 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, Vanormelingen et al. 2007 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.

Table 2. *Pseudo-nitzschia pungens*: linear dimensions (µm) and stria and fibula densities* (in 10 µm) from integrated literature data (Fryxell & Hasle 2003), one of our field samples (from station 5) and four selected clonal cultures.

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

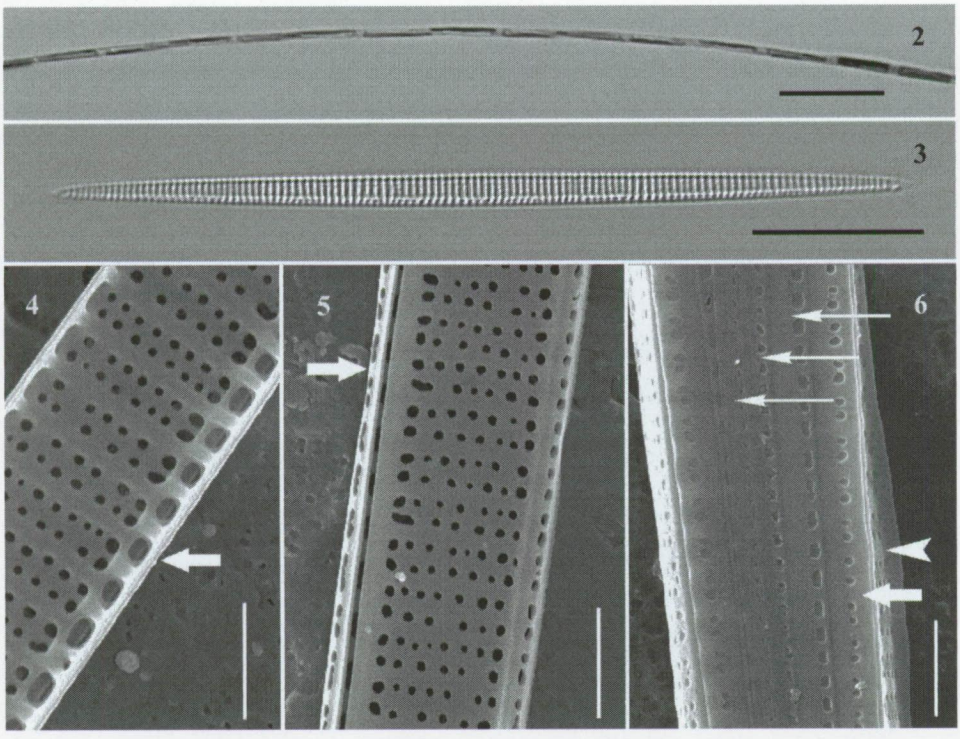
*Values are range (means ± SD)

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*, *P. 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.*

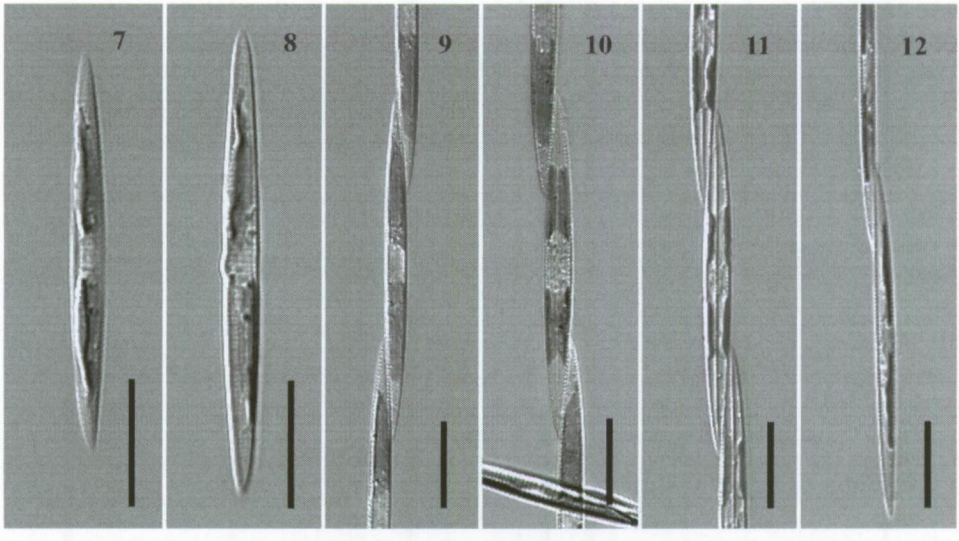
delicatissima colonies disintegrated almost completely into single cells. In contrast, the colonies of *P. pungens* and *P. fraudulenta* were robust. *P. fraudulenta* could be separated from *P. pungens*, even at low magnification, through the extent to which the cells overlapped in the stepped colonies (ca. 1/5 to 1/3 of the cell length in *P. pungens* but by ca. 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 by LM (Table 2, Fig. 3) and SEM studies (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).



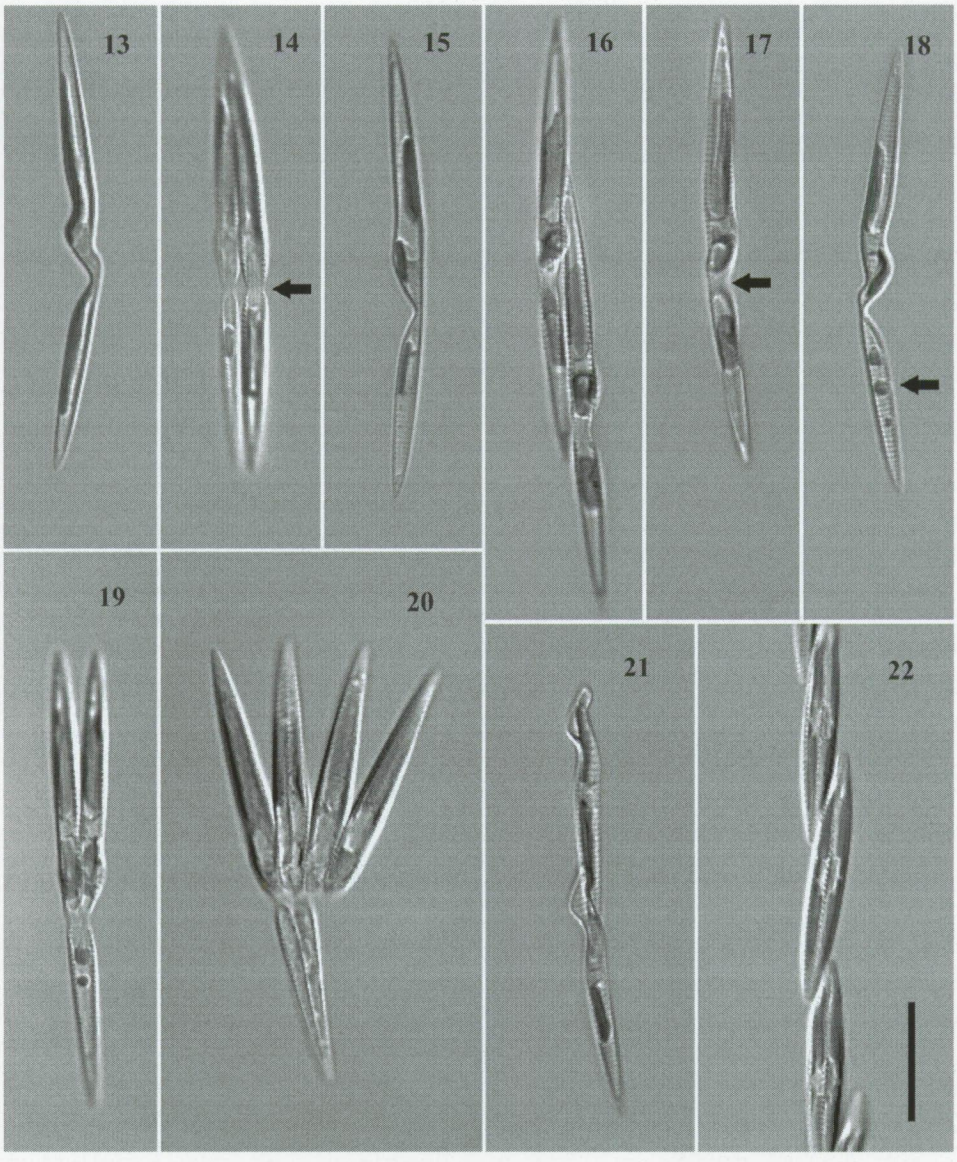
Figs 2–6. *Pseudo-nitzschia pungens* vegetative cells: LM (Figs 2, 3) or SEM (Figs 4–6). Fig. 2. Stepped chain of cells (live) in girdle view, clone 18. Fig. 3. Valve, clone 29. Note the coarse striae and the absence of a central interspace. Figs 4–6. Valve ultrastructure. Note the presence of two rows of poroids per striae (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 bars represent: Fig. 2, 50 µm; Fig. 3, 20 µm; Figs 4-6, 2 µm.

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.



Figs 7–12. Live vegetative cells. Figs 7–9. Interphase chloroplasts. Figs 7, 8. Cells in valve view: the chloroplasts are appressed to the same (Fig. 7) or opposite sides (Fig. 8) of the girdle. Fig. 9. Cell chain in girdle view. Fig. 10. Chloroplast division at mitotic cytokinesis, girdle view. Fig. 11. Cell division is complete and the chloroplasts have moved onto the epithecae of the newly formed cells, girdle view. Fig. 12. Two sibling cells, following rearrangement into the characteristic stepped configuration, girdle view. Note that the chloroplasts are still appressed to the epivalves. Scale bars represent 20 µm.



Figs 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. Split of the protoplast into two parts (arrow), girdle view. Fig. 18. The separated fragment of protoplast, which contained a half-chloroplast and was without a nucleus (arrow), has aborted; valve view. Fig. 19. Division of a cell with a partly aborted protoplast has resulted in formation of short hypothecae, girdle view. Fig. 20. Typical cluster of abruptly reduced cells formed by a few 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 bar (for all) represents 20 µm.

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 of 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 neighboring 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 half a 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. 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 nucleus shifted from the cell centre towards the end where the small plastid lay (Figs 19, 20). Later, the cells corrected the asymmetry (Fig. 22) and when we isolated a short colony of abruptly reduced cells and grew it separately (clone 23s), it appeared healthy and grew well, though a little more slowly than unreduced clones. Long chains of the short cells often spiraled.

Mating behaviour

Sexual reproduction never occurred in monoclonal cultures and auxosporulation was initiated only in particular combinations of clones: *P. pungens* is heterothallic. Results of repeated crosses of the clones in most of the possible pairwise combinations (only clone 23 was not included fully in 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.

The mating behaviour of clone 40 was anomalous. Clone 40, like other clones, was isolated as a single chain and, as with other clones, clone 40 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.

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, in all possible pairwise combination with the other *P. pungens* clones available (Table 3) including *P. fraudulenta* and *P. multiseries,* we then 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^- (Table 3, Figs 30–32). However, in a cross between subclone 40s and the parental 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 did interbreed only with some 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 as compatible only PNp^+ clones 6, 18, 20, 22, 26 and 40s. Unsuccessful crosses of female clones 14 and 28 with PNp^+ clones were repeated at least four times for each pairwise combination and were made

alongside other $PNp^+ \times PNp^-$ 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), with production of viable offspring.

Table 3. Results of mating experiments.

Mating type	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**
PNp^-	1	[0]																								
PNp^-	16	0	[0]																							
PNp^-	17	0	0	[0]																						
PNp^-	19	0	0	0	[0]																					
PNp^-	24	0	0	0	0	[0]																				
PNp^-	29	0	0	0	0	0	[0]																			
PNp^-	30	0	0	0	0	0	0	[0]																		
PNp^-	52	0	0	0	0	0	0	0	[0]																	
PNp^-	28	0	0	0	0	0	0	0	0	[0]																
PNp^-	14	0	0	0	0	0	0	0	0	0	[0]															
PNp^-	23*	nt	nt	nt	nt	0	nt	nt	nt	nt	nt	[0]														
PNp^+	2	++	++	++	++	++	++	++	++	0	0	nt	[0]													
PNp^+	6	+	++	++	++	+	+	+	+	+	++	nt	0	[0]												
PNp^+	18	++	+	+	+	++	++	++	++	++	+	nt	0	0	[0]											
PNp^+	20	+	++	++	++	+	+	++	+	++	0	nt	0	0	0	[0]										
PNp^+	21	++	++	++	++	+	++	+	++	0	0	nt	0	0	0	0	[0]									
PNp^+	22	+	++	++	++	++	+	++	+	++	++	nt	0	0	0	0	0	[0]								
PNp^+	26	++	++	++	++	+	+	+	+	+	0	++	0	0	0	0	0	0	[0]							
PNp^+	27	+	++	++	++	+	+	+	+	0	0	nt	0	0	0	0	0	0	0	[0]						
PNp^+	33	+	++	++	++	+	+	++	+	0	0	nt	0	0	0	0	0	0	0	0	[0]					
PNp^+	44	++	++	++	++	+	++	+	++	0	0	nt	0	0	0	0	0	0	0	0	0	[0]				
PNp^+	50	++	++	++	++	++	++	++	++	0	++	nt	0	0	0	0	0	0	0	0	0	0	[0]			
PNp^+	57	+	++	++	++	+	+	+	+	0	0	nt	0	0	0	0	0	0	0	0	0	0	0	[0]		
PNp^+	40s	++	++	++	++	++	++	++	++	++	0	nt	0	0	0	0	0	0	0	0	0	0	0	0	[0]	
?	40**	+	+	+	+	+	+	+	+	+	+	nt	+	+	+	+	+	+	+	+	+	+	+	+	0	[0]

+, sexual reproduction occurred in mixed culture; ++, crosses where sexual reproduction occurred in mixed cultures and where we could determine confidently (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, was used in crosses (see text); **, the complex mating behaviour of clone 40 is described in the text.

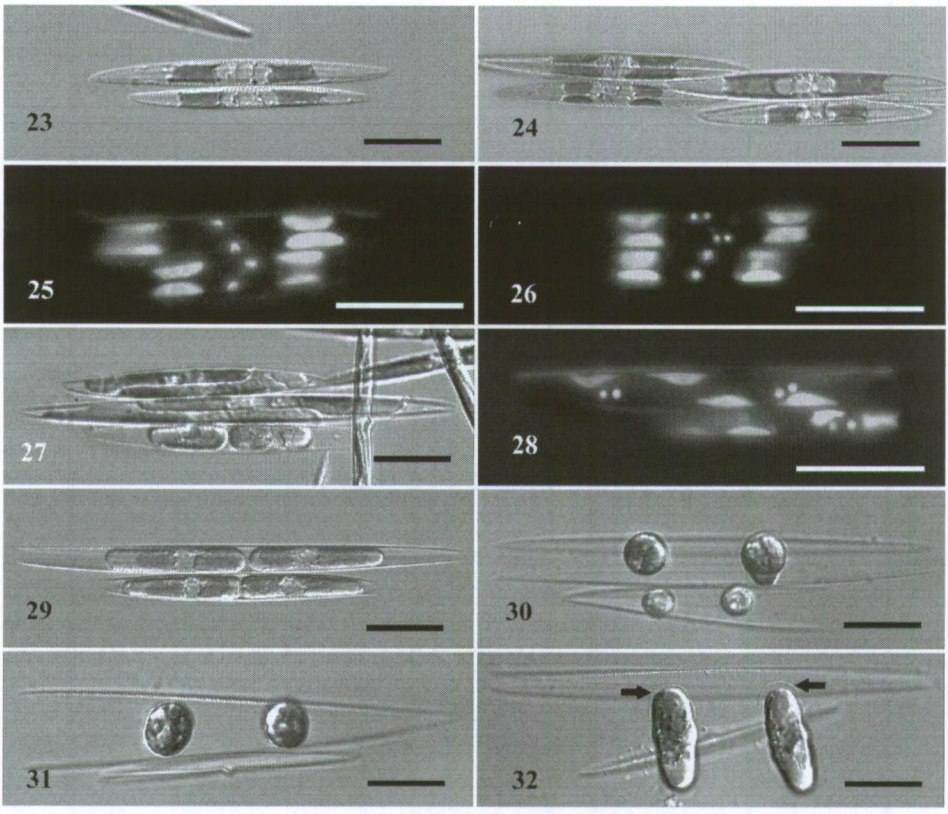
Pattern of sexual reproduction

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 contract (Figs 27, 29). Finally, they became spherical (Fig. 30) and at this stage DAPI staining reveals 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).



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) or clone 40 (smaller) and PNp^{-} clone 17 (Figs 27, 29–32). Fig. 23 Valve-to-valve pairing between single cells. Fig. 24. Pairing between cells still connected in chains. In the left pair, plastokinesis and cytokinesis have been completed, following meiosis I. Fig. 25, 26. Pairs of gametangia stained with DAPI, after the first meiotic division (Fig. 25) and after the second meiotic division (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 at the stage of gamete re-arrangement; the gametes in the lower clone 40 gametangium have already completed re-arrangement and have contracted. Fig. 28. Late gametogenesis, DAPI staining: The gametes of the upper gametangium are at early stage of gamete re-arrangement, while those of the lower gametangium have re-arranged and have begun to contract. Note that both members of each pair of 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, which have developed synchronously. Fig. 30. Fully contracted ± spherical gametes. Note the attachment of the right gamete of the upper gametangium to one theca of the parental cell. Fig. 31. Almost spherical 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 represent 20 μm.

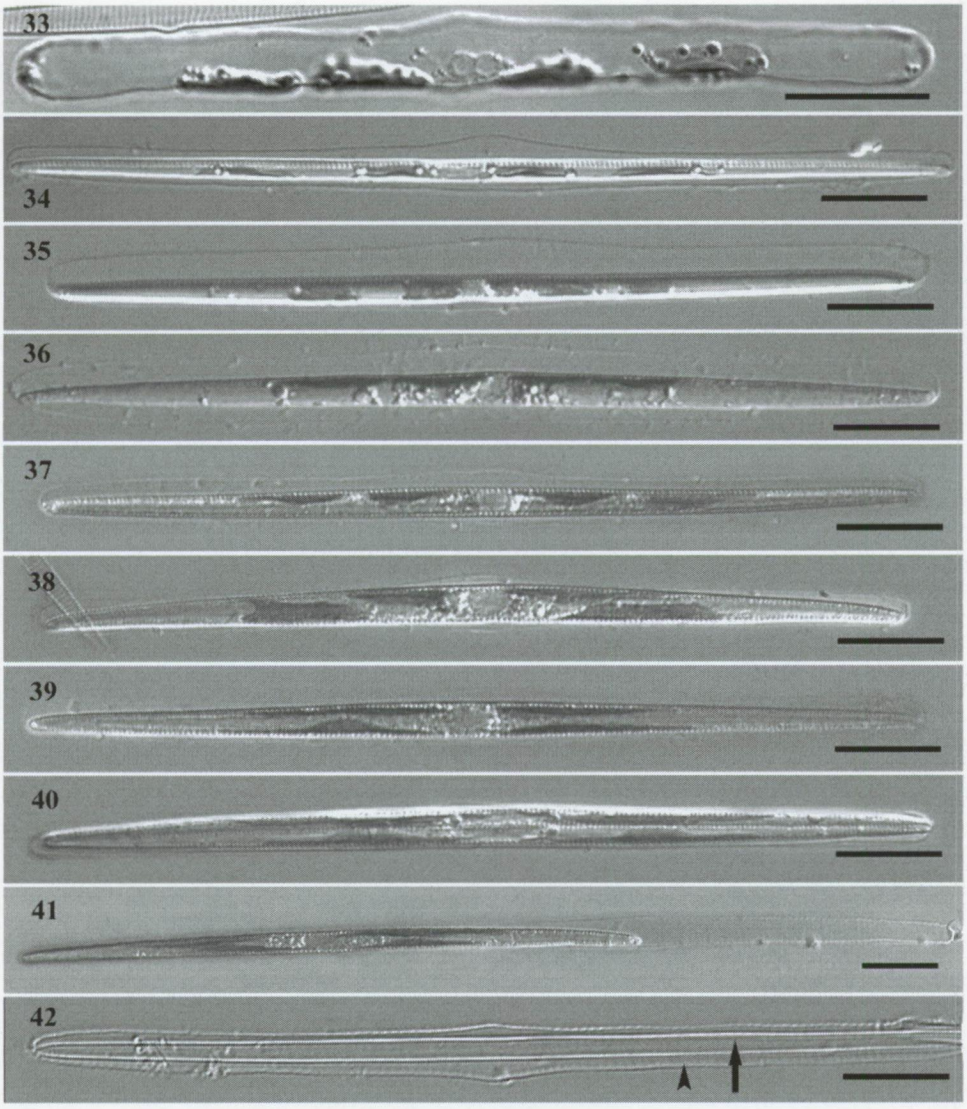


Fig. 33–42. Auxospore and initial cell formation, in a cross between clones 40 and 6. Fig. 33. Auxospore at a late stage of expansion. Note the two unfused haploid nuclei at the center. Figs 34–39. Initial cell formation, girdle view. Fig. 34. The contents of auxospore have contracted and the epivalve has been laid down. The chloroplasts lie on the cell side opposite to the epitheca. Fig. 35. Shift of the chloroplasts onto the girdle of the future initial cell. Fig. 36. The chloroplasts have moved onto the epivalve. Fig. 37. Completion of initial hypovalve. Fig. 38. Re-arrangement of the chloroplasts: the two marginal plastids move onto the hypovalve and the two central plastids onto the epivalve. Fig. 39. Re-alignment of chloroplasts complete. Fig. 40. Division of initial cell within the transverse perizonium. Fig. 41. Initial cell escaping from the perizonium before the first cell division. Fig. 42. Transverse perizonium (arrowhead) and longitudinal perizonium (arrow), after the escape of the initial cell. Scale bars represent 20 µm.

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 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*, 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; 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).

Table 4. Initial cell size* (µm) in two crosses, clone 6 × clone 40 (21.12.2003) and clone 16 × clone 18 (21.01.2004).

Parental clone	Cell size**	Initial cells
6	72.3–77.9 (74.4 ± 2.38)	157.1–176.3 (165.6 ± 4.24) N=50
40	61.4–63.5 (62.5 ± 0.65)	
16	107.1–112.0 (109.5 ± 1.63)	159.4–175.2 (167.7 ± 4.77)
18	101.9–107.8 (104.6 ± 1.86)	

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

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 which 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.

Fig. 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 length of initial cell is shorter than that of the developed auxospore and longitudinal perizonium (arrow). Fig. 45. Auxospore containing two chloroplasts and a single nucleus. Figs 46. Auxospore containing six chloroplasts and three nuclei (at center). Fig. 47. Initial cell containing six chloroplasts. Scale bars represent 20 µm.

Discussion

Chloroplast division and arrangement

Chloroplast division is brought about in *P. pungens* by inward growth of the cleavage furrow during cytokinesis. Chloroplast division is thus imposed and there are two lineages of chloroplasts within each clone of *P. pungens* cells: there is 'dual' inheritance of chloroplasts (sensu Mann 1996). Dual inheritance occurs in many other Bacillariaceae (Mann 1996 and unpublished) and in some *Nitzschia* Hassall species (e.g. *N. sigmoidea* (Nitzsch) W. Smith and *N. 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 is at least partly autonomous, constriction of the chloroplast into two being achieved just before the cell is divided by the cleavage furrow in *N. palea* (Kütz.) 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 *N. subtilis*: for notes on the identification, see Geitler ,1973), *N. amphibia* Grun. (Geitler, 1969, fig. 2), *N. frustulum* var. *perpusilla* (Rabenh.) Grun. in Van Heurck (Geitler, 1970) and *N. recta* Hantzsch ex Rabenh. (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[3]. 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[4].

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

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[3] But see Amato et al. (2005). Auxospores of *Pseudo-nitzschia delicatissima* also contained four chloroplasts which migrated along the auxospore during the maturation process. The initial cell produced inside the auxospore on the other hand seemed to contain only two chloroplasts.

[4] Levialdi Ghiron et al. (2008) showed for *P. delicatissima* that chloroplasts are inherited randomly from the two parents. See also chapter 4.

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. Ag.) Snoeijs (Roshchin 1994), where initial cell length remains ± constant, despite considerable variation in gametangium size. In *Neidium ampliatum* (Ehrenb.) 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 diatoms studies so far, vegetative multiplication of *P. pungens* was generally accompanied 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 artifact 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 center, 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[5].

Pattern of sexual reproduction

Sexual reproduction has now been reported for six[6] species of *Pseudo-nitzschia* (see Introduction). 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 genus is not surprising and has been found, for example, in *Licmophora* C. Agardh (Chepurnov & Mann 2004), *Eunotia* (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* (Bréb. ex Kütz.) Grun.: 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* Grun. 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. (2002a), *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*

[5] An abrupt decrease in cell size was also sporadically detected in *P. multistriata* cultures but the mechanism was not studied (D'Alelio et al. 2009a)

[6] See footnote 2

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 vs. inbreeding), is key to the interpretation of data on the genetic structure of species and their evolutionary potential. Remarkably, all eight *Pseudo-nitzschia* species studied so far are heterothallic (Davidovich & Bates 1998, Chepurnov et al. 2004, Amato et al. 2005, Amato & Montresor 2008, D'Alelio et al. 2009a). 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, Vanormelingen et al. 2008).

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 eight *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. 2005) and *P. multiseries* (Evans et al. 2004).

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* (our unpublished observations). 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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3

Pseudo-nitzschia pungens: a cosmopolitan diatom species? [1]

Genetic, reproductive and morphological variation were studied in 193 global strains of the marine diatom species *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle to assess potential intraspecific variation and biogeographic distribution patterns. Genetic differentiation between allo- and sympatric strains was investigated using the ITS1 – 5.8S – ITS2 rDNA region. Three ITS clades were found. Clones of opposite mating type were sexually compatible within clades I or II, and viable F1 hybrid offspring were produced in crosses between them. The molecular differences between these clades were correlated with slight but consistent morphological differences. At present, nothing can be said about morphology and mating behavior for clade III clones because only ITS data were available. The three ITS clades showed different geographic distributions. Clade II was restricted to the NE Pacific, whereas clones belonging to clade III originated from geographically widely separated areas (Vietnam, China and Mexico). ITS clade I was recovered in all locations studied: the North Sea (Belgium, The Netherlands, France), the eastern and western N Atlantic (Spain, Canada), the NW and S Pacific (Japan, New Zealand) and the NE Pacific (Washington State). Clade I thus appears to be globally distributed in temperate coastal areas and provides the first strong evidence to date for the global distribution of a biologically, genetically and morphologically defined diatom species.

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[1] Published as: Casteleyn G, Chepurnov V, Leliaert F, Mann DG, Bates SS, Lundholm N, Rhodes L, Sabbe K & Vyverman W (2008) *Pseudo-nitzschia pungens* (Bacillariophyceae): a cosmopolitan diatom species? Harmful Algae 7: 241-257.

Introduction

During the last two decades, the pennate diatom genus *Pseudo-nitzschia* H. Peragallo has been the focus of much attention. Representatives of this genus are a typical component of marine phytoplankton worldwide and, based on morphology, most appear to have a cosmopolitan distribution (Hasle 2002). The genus contains at least 12 species that are able to produce domoic acid, a neurotoxin responsible for amnesic shellfish poisoning (Bates 2000, Bates & Trainer 2006), and 'harmful algal blooms' (HABs) involving these organisms have caused a series of environmental and public health problems (e.g. Bates & Trainer 2006). As a consequence, the genetic and morphological diversity of *Pseudo-nitzschia* has received much attention because, without confident delineation of species boundaries, efforts to understand, monitor and predict events occurring in natural populations (especially during HABs) are severely compromised.

Recent taxonomic research on diatoms suggests that traditional species boundaries, based largely on variation in the morphology of the cell's siliceous exoskeleton (the frustule), have been drawn too widely and that real species diversity has probably been greatly underestimated (Mann 1999). As for other groups of organisms (Dettman et al. 2003, Agapow et al. 2004), it is expected that better insight into diatom species taxonomy and evolution can be obtained through comparison of biological, phylogenetic and morphological species circumscriptions (using breeding, molecular and morphological data). *Pseudo-nitzschia* is one of the few diatom genera that has been subject to this approach. This has led to the revision of existing species and the description of several new species (Lundholm et al. 2003, Orsini et al. 2004, Hasle & Lundholm 2005, Lundholm et al. 2006, Amato et al. 2007). To date, *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Hasle has received little attention, despite being one of the most commonly reported, potentially toxic representatives of the genus worldwide (Hasle 2002). Clones of *P. pungens* isolated from various geographic areas exhibited different abilities to produce domoic acid. Toxic clones have only been reported from New Zealand (Rhodes et al. 1996), Washington State (Trainer et al. 1998) and Monterey Bay, California (Bates et al. 1998). No toxic clones have so far been reported from the North Sea (Vrieling et al. 1996), where *P. pungens* is the most common representative of the genus.

In a recent study, we obtained 24 clones of *P. pungens* from the North Sea and the Westerschelde estuary and experimentally examined the principal cell and life cycle features, including auxosporulation and the mating system (Chepurnov et al. 2005, chapter 2 of this thesis). North Sea *P. pungens* is heterothallic and must therefore outbreed, which is consistent with the high genetic diversity observed in North Sea populations by Evans et al. (2005). In the present study, we aimed at assessing genetic,

morphological and reproductive variation in global populations of *P. pungens*. We extended the sampling from the North Sea and adjoining areas and also included *P. pungens* clones from five geographically more distant areas in the Atlantic and Pacific Oceans. We identified genetic variation by using sequences of the ITS1 – 5.8S – ITS2 rDNA region, documented morphological differences, and involved all available clones in crossing experiments to reveal mating barriers.

Materials and methods

Sampling and culturing

A first set of *P. pungens* clones, established in 2002–2003, contained 24 isolates from different sites in the southern North Sea (the Netherlands, Belgium and northern France). These strains are listed by Chepurnov et al. (2005) and were used for intensive study of the life cycle and mating system. Fourteen of these strains were also used in the present study.

In 2004, planktonic samples were collected monthly on the Belgian North Sea coast (five stations, 91 strains isolated), an adjoining coastal lagoon at Oostende (Spuikom, 12 strains isolated) and in the Westerschelde estuary at Terneuzen and Vlissingen (2 stations, 36 strains isolated) (Fig. 1A) using a plankton net with mesh size 20 μm. Within a few hours, an aliquot of the material was transferred to polystyrene 50-mm Petri dishes and examined under an Axiovert 135 inverted microscope (Zeiss, Jena, Germany). Monoclonal cultures were established by isolating single chains by micropipette and were grown in f/2 medium (Guillard, 1975) based on filtered and sterilized seawater (~30-32 psu) from the North Sea (18 °C, 12:12-h light-dark period and 25-50 μmol photons m^{-2} s^{-1} from cool-white fluorescent lights).

Other *P. pungens* clones (Fig. 1B and Table 1) originated from the North-East Atlantic (Spain, 4 strains), North-West Atlantic (Canada, 16 strains), North-East Pacific (Washington State, USA, 12 strains), North-West Pacific (Japan, 5 strains) and the South Pacific (New Zealand, 3 strains). Detailed information about the precise sampling locations and dates, suppliers, initial strain designations and EMBL accession numbers, is available in the Supplementary Table S1.

Table 1. List of *P. pungens* monoclonal cultures involved in the present study. Strains that were sequenced are shown in bold. Strains of which PCR products were cloned are shown with an [a]. Strains for which no live cells but only sequences were available are shown with a [b].

Clone	General region	Specific location	Sampling date
1, **2**	Westerscheldt, The Netherlands	Terneuzen	24/05/02
6	North Sea, The Netherlands	Terschelling	1/05/00
14	North Sea, The Netherlands	Marsdiep	25/04/02
16, **18**, **19**	North Sea, The Netherlands	Noordwijk	22/05/02
20, **24**, **28**	North Sea, Belgium	VLIZ station 130	30/06/03
40s, **44**	North Sea, Belgium	Ijzermonding Nieuwpoort	13/08/03
52, **57**	North Sea, northern France	Ambleteuse	13/09/03
V120(3)1, 2, 3, 4, **5**, 6, 7, 8	North Sea, Belgium	VLIZ station 120	24/03/04
V120(8)1, 2, 3, 4, 5, 6, 7, 8	North Sea, Belgium	VLIZ station 120	16/08/04
V215(3)1, **2**, 3, 4, **5**, 6, 7	North Sea, Belgium	VLIZ station 215	24/03/04
V215(7)1, 2, **3**, 4, 5, 6, **7**[a], 8, 9	North Sea, Belgium	VLIZ station 215	19/07/04
V215(10)1	North Sea, Belgium	VLIZ station 215	18/10/04
V215(12)1, 2, 3, 4, 5, 6, 7, 8, 9, 10	North Sea, Belgium	VLIZ station 215	08/12/04
V330(3)1, 2, 3, 4	North Sea, Belgium	VLIZ station 330	24/03/04
V700(4)1, 2, 3, 4, 5, 6, 7, 8	North Sea, Belgium	VLIZ station 700	19/04/04
V700(7)1, 2, 3, 4, **5**, 6, 7, 8, 9, 10, **11**, 12	North Sea, Belgium	VLIZ station 700	19/07/04
V421(3)1, 2, 3, **4**, 5, 6	North Sea, Belgium	VLIZ station 421	29/03/04
V421(8)1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	North Sea, Belgium	VLIZ station 421	23/08/04
V421(10)1, 2, 3, 4, 5, 6, 7	North Sea, Belgium	VLIZ station 421	19/10/04
S(4)1, 2, 3, 4, 5, **6**[a], 7, 8, 9	Oostend Lagoon, Belgium	Spuikom Oostende	7/04/04
S(5)1, 2	Oostend Lagoon, Belgium	Spuikom Oostende	18/05/04
S(7)1	Oostend Lagoon, Belgium	Spuikom Oostende	13/07/04
W1(4)1, 2, 3, 4, 5, 6, 7, 8	Westerscheldt, The Netherlands	Breskens	21/04/04
W1(7)1, 2, 3, 4, 5, **6**[a], 7, 8, 9, 10	Westerscheldt, The Netherlands	Breskens	14/07/04
W4(4)1, 2, 3, 4, 5, 6, 7, 8	Westerscheldt, The Netherlands	Terneuzen	21/04/04
W4(7)1, **2**, 3, 4, 5, 6, 7, 8, 9, 10	Westerscheldt, The Netherlands	Terneuzen	14/07/04
Vigo-1, **Vigo-2**, **Vigo-3**[a], **Vigo-4**	NE Atlantic, Spain	Bay of Vigo	1/04/04
Cn-172	NW Atlantic, Canada	Cardigan River, Prince Edward Island	5/09/02
Cn-181, Cn-184	NW Atlantic, Canada	Oak Point, Miramichi Bay, New Brunswick	23/09/02
Cn-193	NW Atlantic, Canada	Deadmans Hbr., Bay of Fundy, New Brunswick	9/10/02
Cn-196, Cn-200	NW Atlantic, Canada	Egg Island, Miramichi Bay, New Brunswick	28/10/02
Cn-201, Cn-202	NW Atlantic, Canada	Egg Island, Miramichi Bay, New Brunswick	15/09/03
Cn-204, **Cn-205**	NW Atlantic, Canada	Malpeque Bay, Prince Edward Island	27/10/03
Cn-213, Cn-214	NW Atlantic, Canada	Brudenell River, Prince Edward Island	7/09/04
Cn-215, **Cn-216**	NW Atlantic, Canada	Cardigan River, Prince Edward Island	7/09/04
Cn-217, **Cn-218**	NW Atlantic, Canada	Boughton River, Prince Edward Island	7/09/04
US-77	NE Pacific, USA	La Push, WA	18/09/01
US-93	NE Pacific, USA	Eld Inlet, Mud Bay, WA	17/06/02
US-94[a], **US-96**	NE Pacific, USA	Sequim Bay State Park, WA	8/08/02
US-115, **123**[a], 125, 126,**132**, 134, **135**, 136	NE Pacific, USA	ECOHAB I (coastal Washington)	/06/03
Jp-1	NW Pacific, Japan	Ofunato Bay, Iwate Prefecture	7/08/00
Jp-11	NW Pacific, Japan	Ofunato Bay, Iwate Prefecture	25/06/01
Jp-14	NW Pacific, Japan	Ofunato Bay, Iwate Prefecture	3/12/01
Jp-01	NW Pacific, Japan	Okkirai Bay, Iwate Prefecture	25/07/00
Jp-02	NW Pacific, Japan	Okkirai Bay, Iwate Prefecture	28/07/00
NZ-49	S Pacific, New Zealand	Steels Reef, North Island	/10/00
NZ-67	S Pacific, New Zealand	Big Glory Bay, Stewart Island	/02/03
NZ-74	S Pacific, New Zealand	Taylor's Mistake, South Island	2004
DQ166533[b]	NW Pacific, Vietnam	Hai Phong	
AY544769[b]	NW Pacific, China		
DQ0626665 (KBH2)[b]	NW Pacific, Vietnam	Khan Hoa Bay	
AY257846 (Mex 18)[b]	Gulf of Mexico, Mexico	Near Tuxpam	

Fig.1. Locations of sampling stations. (A) The North Sea and Westerschelde estuary; (B) World-wide. ▲: sampling locations. The numbers indicate the sampling station according to the VLIZ monitoring campaign (www.vliz.be). Terschelling (135), Marsdiep and Noordwijk (10) are monitoring stations named according to the RIKZ (Rijksinstituut voor Kust en Zee) (www.waterbase.nl), numbers between brackets indicate number of km offshore.

DNA extraction, amplification and sequencing

DNA of *P. pungens* cells was extracted using a bead-beating method with phenol extraction and ethanol precipitation (Zwart et al. 1998). The internal transcribed spacer region (ITS1 – 5.8S – ITS2) of rDNA was amplified by PCR, using the primers 1800F (Friedl 1996) and ITS4 (White et al. 1990). PCR mixtures contained 1–5 µl of template DNA, primers at a concentration of 0.5 µM, deoxynucleoside triphosphates at 200 µM each, bovine serum albumin (BSA) at 0.4 µg μl^{-1}, 5 µl of 10 X PCR buffer [Tris-HCl, $(NH_4)_2SO_4$, KCl, 15 mM $MgCl_2$, pH 8.7 at 20 °C; "Buffer I", Applied Biosystems, Foster City, USA] and 2.5 units of Taq polymerase (AmpliTaq, Perkin-Elmer, Wellesley, USA); mixtures were adjusted to a final volume of 50 µl with sterile water (Sigma, St-Louis, USA). The cycling parameters were: initial denaturation at 94 °C for 7 min; 30 cycles of 1 min at 94 °C, 1 min at 48 °C and 2 min at 72 °C; and finally 72 °C

for 10 min. Amplified products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced with the aid of a Big Dye™ Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Foster City, USA), using the same forward and reverse primers as applied for PCR amplifications. Sequencing was done with a capillary sequencer (ABI3100, Applied Biosystems, Foster City, USA). Sequences run in both directions were overlapping. The sequence data were checked and edited using BioNumerics (Applied Maths, Belgium) software. Strains that gave no or a very weak PCR product required a new PCR, with nested primer combinations. First a PCR was run with primers and cycling conditions as described by Lundholm et al. (2003). A nested PCR was performed on these PCR products with the same primers and cycling conditions mentioned above. To study sequence variation within a single strain, purified PCR products were cloned using the pGEM-T Kit (Promega, Madison, USA), as recommended by the manufacturer. Vector primers T7 and SP6 were used as sequencing primers. For 6 strains (see Table 1, Table S1), 5 to 7 cloned sequences were analyzed.

Sequence alignment and phylogenetic analysis

Sequences were aligned using BioNumerics (Applied Maths, Belgium) and corrected manually. The boundaries of the ITS regions and 5.8S gene were identified by comparison with published sequences (Manhart et al. 1995, Cangelosi et al. 1997, Lundholm et al. 2003). The alignment comprised ITS sequences of *P. pungens* that were obtained in the present study (direct and cloned sequences) and those available in GenBank [accession numbers AY257845 (Portugal), AY257846 (Mexico), AY544769 (China), DQ062665 (Vietnam), DQ166533 (Vietnam)]. Both to test the accuracy of our results and to check those sequences already available for *P. pungens* in GenBank, we obtained three *P. pungens* strains for which ITS sequences had been deposited in GenBank (AY257845, from Portugal; AY257846, from Mexico; and DQ062665, from Vietnam). The DNA was re-extracted and the ITS region sequenced. Our sequences and those from GenBank were identical for the Mexican and the Vietnamese strains but different for the Portuguese isolate (our Portuguese sequence was identical to the North Sea sequences, while the sequence found in GenBank was identical with the Mexican sequence). We therefore omitted the Portuguese sequence from the phylogenetic analysis.

Phylogenetic signal among parsimony-informative sites was assessed by comparing the measure of skewedness (g1-value; PAUP*) with the empirical threshold values in Hillis and Huelsenbeck (1992). All phylogenetic analyses were performed on the total ITS1 – 5.8S – ITS2 rDNA region with the aid of PAUP 4.0*beta test version 10

(Swofford 2002). Maximum parsimony (MP) analyses were carried out using heuristic search options with random stepwise addition of taxa (1000 repetitions) and the tree bisection-reconnection branch-swapping algorithm. Gaps were treated as missing data. Maximum Likelihood (ML) and Neighbour Joining (NJ) analyses were performed using a Hasegawa-Kishino-Yano nucleotide substitution model (Hasegawa et al. 1985), as determined by the Akaike Information Criterion (AIC) using Modeltest Version 3.06 (Posada & Crandall 1998). ML analyses were performed using the same heuristic search settings as in the MP analyses. Bootstrap analyses (1000 replicates) were performed to test the robustness of tree topologies (Felsenstein 1985).

P. multiseries (Hasle) Hasle was used to root all trees since it has always been recovered with high bootstrap support as the sister taxon of *P. pungens* (Lundholm et al. 2003, Hasle & Lundholm 2005).

Secondary structures of rDNA ITS1 and ITS2

Common secondary structural motifs of the ITS1 and ITS2 sequences of *P. pungens* were initially recognized by folding each sequence using the Mfold software (http://www.bioinfo.rpi.edu/; Zuker 2003). Foldings were conducted at 25 °C using a search within 10% of thermodynamic suboptimality. Paired regions were then recognized by comparing the ITS secondary structures of different *Pseudo-nitzschia* taxa (including *P. australis* Frenguelli, *P. decipiens* Lundholm et Moestrup, *P. galaxiae* Lundholm et Moestrup, *P. multiseries*, *P. obtusa* (Hasle) Hasle & Lundholm and *P. seriata* (Cleve) Peragallo) and by identifying compensatory base changes (CBCs: change of the nucleotides at both sides of a double-stranded helix, preserving the pairing) and hemi-CBCs (change of a nucleotide at one side of a stem, but still preserving the pairing) as proposed by Mai and Coleman (1997). The common structural patterns were then annotated in a manual alignment with DCSE v. 2.60 (De Rijk & De Wachter 1993). This DCSE file was used to create the secondary structure diagrams with RnaViz (De Rijk et al. 2003). The helices of ITS2 were numbered according to Mai and Coleman (1997).

Mating experiments

All crosses were carried out in the Laboratory of Protistology and Aquatic Ecology (Ghent University, Belgium). Crosses were performed by inoculating pairs of clones (in exponential growth phase; see Davidovich & Bates 1998) into wells of 24-well Repli plates (Greiner Bio-One, Kremsmuenster, Austria). The mixed cultures were examined daily for 6–8 days, using a Zeiss Axiovert 135 inverted microscope, until they reached the stationary phase of growth and started to die. Previous investigations (Chepurnov et al. 2005) had shown that North Sea *P. pungens* is heterothallic and that clones of

opposite mating type differed in the behavior of gametes they produced. Clones can be designated as PNp^+ ('PNp' is an acronym representing the genus and species; '+' indicates that the gametangia produce two active gametes) or PNp^- (the gametangia produce two passive gametes). As in many other diatoms (e.g. Geitler, 1932, Drebes, 1977, Chepurnov et al. 2004), induction of sexuality in North Sea *P. pungens* clones was size-dependent, the upper threshold for sexual induction being about 115 µm (Chepurnov et al. 2005).

Out of the 24 strains from 2002–2003, six reference clones of known sexuality (i.e. clones 1, 14, 19 of mating type PNp^- and clones 2, 6 and 18 of opposite mating type PNp^+) were selected to cross with other strains available (Supplementary Table S1). Crosses between clones derived from various geographic areas were also performed (Tables 3 and 4).

Morphological analysis

The combination of a few morphological characters, including cell size, robustness and curvature of cell chains and the extent of overlap of cells in the 'stepped' colonies (Chepurnov et al. 2005), is sufficient to distinguish *P. pungens* from other co-occurring *Pseudo-nitzschia* species [e.g. *P. turgidula* (Hustedt) Hasle, *P. delicatissima* (Cleve) Heiden and *P. fraudulenta* (Cleve) Hasle] under an inverted microscope. After isolation, every clone was assessed for valve ornamentation and the stria and fibula densities characteristic of *P. pungens* via light microscopy; this was possible even in live cells in water mounts, without cleaning the frustule by oxidation (see also Hasle & Syvertsen 1996). In addition, however, 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, USA). Light microscopical (LM) observations were carried out using a Zeiss Axioplan 2 Universal microscope (Zeiss, Jena, Germany). Scanning electron microscopy (SEM) was performed using a JEOL JSM5600LV (Tokyo, Japan) on cleaned material dried onto aluminium stubs and coated with gold-palladium.

Detailed morphological analyses had already been performed for the 24 clones isolated from the North Sea, including the reference clones (Chepurnov et al. 2005). In addition, morphometric data for 11 clones (7 from clade I and 4 from clade II) are presented in Table 5. Statistical analyses (*t*-tests) between measurements of these 11 clones were performed using STATISTICA version 5.0 for Windows (StatSoft, Tulsa, USA).

Results

ITS diversity

ITS1 – 5.8S – ITS2 rDNA sequences were obtained by direct sequencing of PCR products for 54 *P. pungens* strains representing different localities and seasons (Table 1). For 6 strains the PCR products were cloned; 37 sequences were obtained (Table 1 and S1). All newly obtained sequences of *P. pungens* were identical in length, with ITS1: 260 bp, 5.8S: 171 bp and ITS2: 264 bp. The complete alignment, including *P. pungens* sequences from GenBank and *P. multiseries* as the outgroup, comprised 718 characters, of which 144 positions were variable and 36 parsimony-informative. However, many of these sites reflect the separation between *P. pungens* and *P. multiseries* and the total number of variable sites in the ingroup was 61, among which 32 were parsimony-informative. Comparison of the measure of skewedness with the empirical threshold values in Hillis and Huelsenbeck (1992) revealed that the data contained significant phylogenetic signal [g1 = -1.50; threshold value g1 = - 0.19 ($p = 0.01$) for 15 taxa and 50 variable characters].

Intra-isolate sequence variation, studied in six strains by cloning of PCR products, was extremely low (0–0.7%). Changes involved autapomorphic point mutations that were not phylogenetically informative, except at two sites in US-123 (US-123/a and b) and one site in Vigo-3 (Vigo-3/a and b) and US-94 (US-94/e and f), where partially homogenized mutations were found.

Maximum parsimony (MP) analysis resulted in three most parsimonious trees (tree length = 165, consistency index [CI] = 0.94). Neighbor joining and maximum likelihood analysis (for ML, –log likelihood = 1835.13) yielded similar tree topologies. One of the MP trees is illustrated in Fig. 2. Two sister clades (I and II) were always recovered with high bootstrap support, whereas the basal branches either clustered in a clade (NJ, one MP tree, Fig. 2 A) or formed a grade (ML and two of the MP trees, Fig. 2 B and C). Analyses with ingroup sequences only (data not shown) always recovered the three clades (NJ; ML; MP: 1 tree found, CI = 1) with very high bootstrap support. Clade I included the largest number of clones, representing all of the major geographic areas investigated, i.e. the North Sea (Belgium, the Netherlands, northern France) and adjoining areas (Westerschelde and Spuikom), NE Atlantic (Spain), NW Atlantic (Canada), NW Pacific (Japan), S Pacific (New Zealand) and two clones (US-94 and US-96) from the NE Pacific (USA). Clade II exclusively comprised strains from the NE Pacific (USA). Clade III contained sequences found in GenBank from Vietnam, China and Mexico.

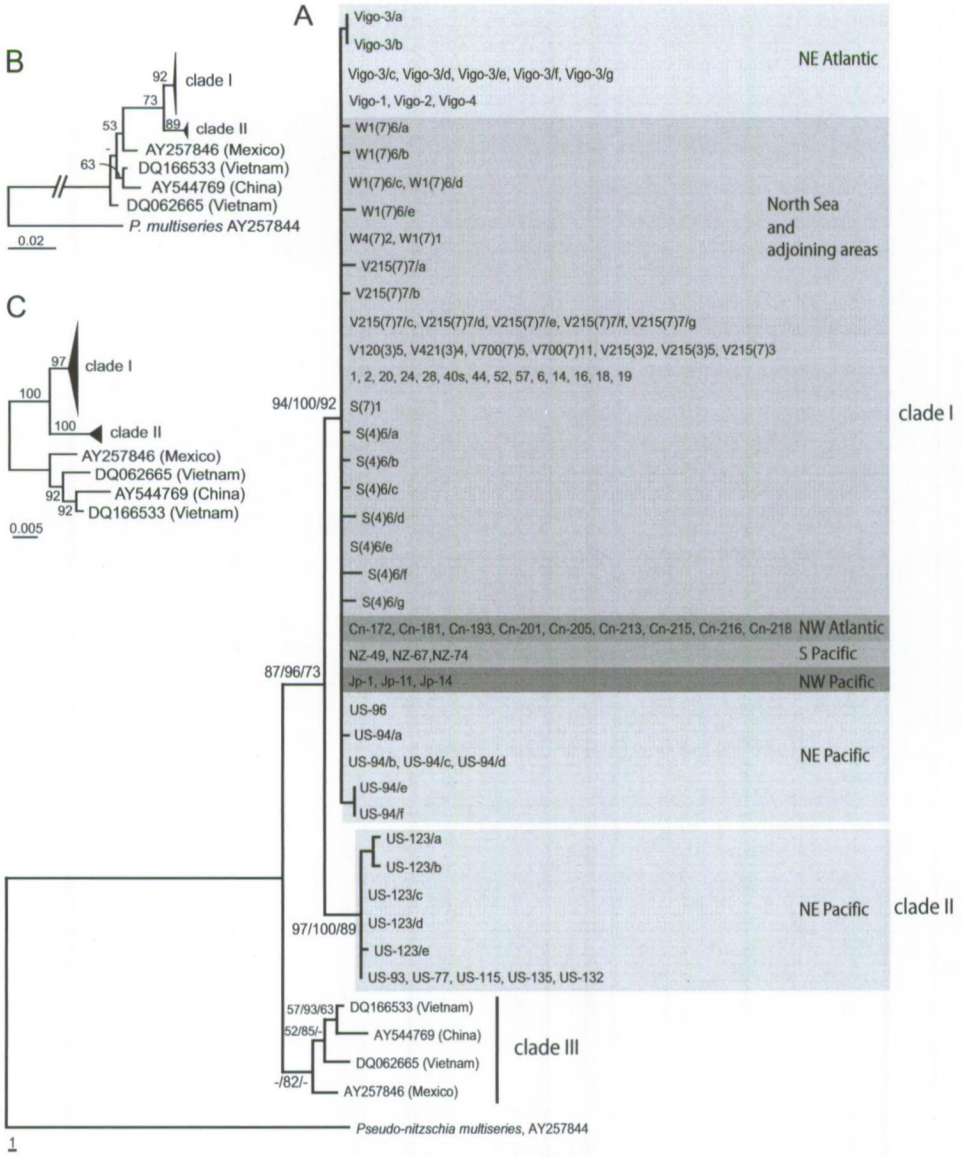

Fig. 2. *Pseudo-nitzschia pungens*. (A) Maximum parsimony tree of *P. pungens* isolates inferred from ITS-5.8S-ITS2 sequence data, and rooted with *P. multiseries*. Bootstrap values (MP/NJ/ML) are indicated above branches. (B) Maximum likelihood tree, rooted with *P. multiseries*, with indication of boostrap values. (C) Phylogenetic tree resulting from ML analysis of ingroup sequences only, rooted at the midpoint, with indication of bootstrap values.

Sequence divergences (uncorrected p-values), both within and between the three clades, are shown in Table 2. Between clades I and II, whole ITS1 – 5.8S – ITS2 region differed by 1.3% (only direct sequences included), corresponding to 3 substitutions (1

transversion and 2 transitions) in ITS 1 and 6 substitutions (all transitions) in ITS 2. Within each of clades I and II, the direct sequences were all identical and the differences seen were entirely due to intraclonal variation (< 0.7%). The divergence between clade III and the rest of the ingroup was 2.7–4.4%.

Table 2. Sequence divergence (uncorrected p-values) within and amongst the three clades shown in Fig. 2. Shown as: minimum - maximum (average). Note the three clades are characterized by a different number of sequences, preventing precise comparisons of genetic variability among them.

	Clade I			**Clade II**			**Clade III**		
	ITS1 – 5.8S – ITS2	ITS1	ITS2	ITS1 – 5.8S – ITS2	ITS1	ITS2	ITS1 – 5.8S – ITS2	ITS1	ITS2
Clade I (n = 76)	0 - 0.007 (0.001)	0 - 0.019 (0.001)	0 - 0.008 (0.001)						
Clade II (n = 10)	0.013 - 0.022 (0.014)	0.012 - 0.031 (0.014)	0.023 - 0.034 (0.024)	0 - 0.006 (0.002)	0 - 0.012 (0.003)	0 - 0.008 (0.002)			
Clade III (n = 4)	0.027 - 0.039 (0.031)	0.023 - 0.050 (0.030)	0.045 - 0.053 (0.048)	0.032 - 0.044 (0.035)	0.027 - 0.050 (0.034)	0.053 - 0.064 (0.056)	0 - 0.019 (0.013)	0 - 0.023 (0.015)	0 - 0.019 (0.014)

ITS1 and ITS2 secondary structures

In the seven *Pseudo-nitzschia* species compared (*P. australis*, *P. decipiens*, *P. galaxiae*, *P. multiseries*, *P. obtusa, P. pungens*, and *P. seriata*), a common overall organization of the ITS1 secondary structure could be identified, despite large sequence divergence and considerable variation in secondary structural motifs; this common structure comprised a large loop and five paired regions (helices I to V) (Fig. 3). Within *P. pungens*, the ITS1 secondary structures of all genotypes had an identical organization. Eight base changes among the three *P. pungens* clades were situated in unpaired regions, thus having no effect on the secondary structure of the ITS1 RNA molecule. Six HCBCs could be identified. Three were in helix II and occurred between clade I and some strains of clade III, and three others were in helix V, occurring between clade I and clades II and III.

The ITS2 secondary structures were also organized similarly in all *Pseudo-nitzschia* species, with a loop and five helices (I, II, IIa, III and IV). Within *P. pungens*, the organization of all ITS2 secondary structures was identical. Most of the base changes were situated in unpaired regions. No CBCs or HCBCs were found between clades I and II, but one CBC and one HCBC, both in helix III, were recognized between clade III and the other two.

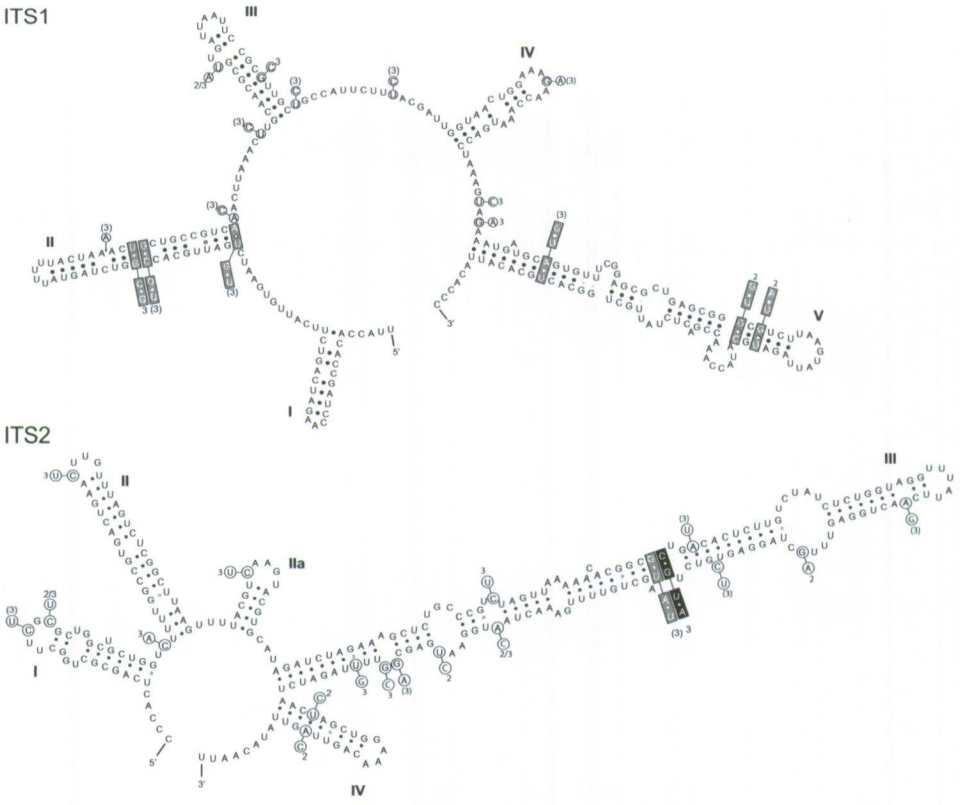

Fig. 3. Diagrams of secondary structures of the ITS1 and ITS2 transcripts of *Pseudo-nitzschia pungens* (clone 1, EMBL accession number AM778733) derived by comparisons among seven *Pseudo-nitzschia* species. Base changes between the different genotypes of *P. pungens* are indicated: the base pair marked in a black box indicates a CBC; base pairs marked in grey boxes indicate HCBCs; single base changes are marked in circles. The numbers next to the boxes and circles specify the *P. pungens* clade in which the base changes occurred; numbers in brackets indicate that the base change occurred in only some sequences of the clade.

Crossing experiments

All 193 isolated *P. pungens* clones – from both clade I and clade II – were involved in breeding tests with at least some other clones. None of the clones exhibited intraclonal sexual reproduction. The six selected reference clones from the North Sea were used to determine the mating types of other clones, revealing no contradictory results (i.e. each clone behaved consistently, either as PNp^{+} or PNp^{-}). Where sex was induced, it occurred exclusively in mixed cultures of clones that belonged to opposite mating types (Fig. 4, Tables 3 and 4, Supplementary Table S1).

Most of the new clones isolated from the North Sea and adjoining waters in 2004 (the V, W and S series of clones) that had cells smaller than 115 µm were able to reproduce sexually with the reference clones. However, a few clones that were well below 115 µm long (the threshold size for sexual reproduction estimated by Chepurnov et al. 2005) did not exhibit any sign of sexualization (see Supplementary Table S1). Cells that were longer than 115 µm did not reveal any signs of sexuality when crossed with the reference strains (Table S1).

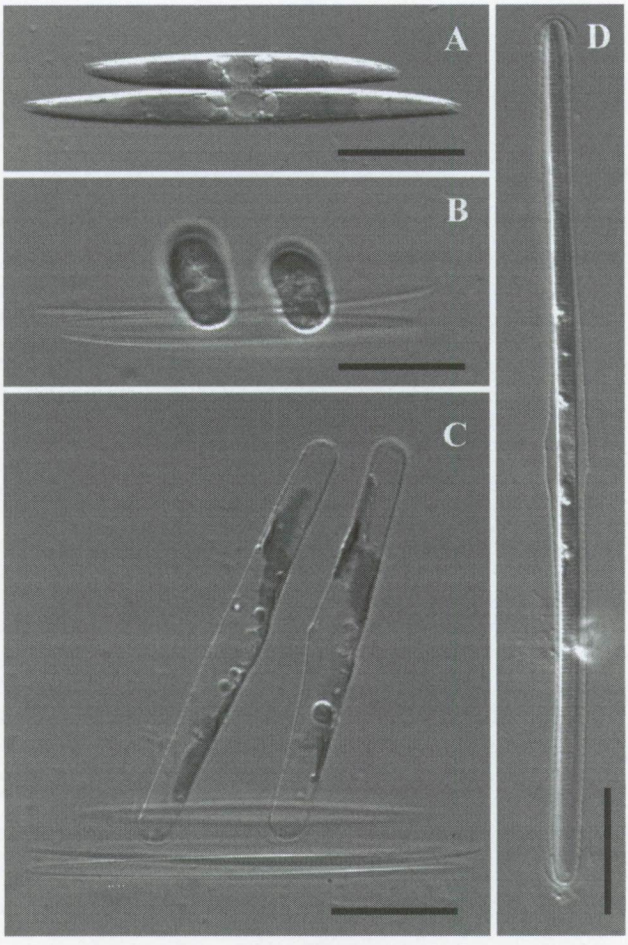

Fig. 4. Heterothallic sexual reproduction in *Pseudo-nitzschia pungens*: pairing (A), auxospores (B and C) and initial cell (D) in crosses between the clones derived from widely spaced geographical areas. (A) PNp^+ clone 6 (smaller in size, The North Sea) and PNp^- clone Vigo-1 (Spain). (B) PNp^+ clone Vigo-2 (Spain) and PNp^- clone NZ-67 (New Zealand). (C and D) PNp^+ clone 18 (North Sea) and PNp^- clone NZ-67 (New Zealand). Scale bar, 25 µm.

Table 3. *Pseudo-nitzschia pungens*: Canadian clones were crossed between themselves and selected clones from distant geographic areas. +, sexual reproduction occurred in mixed culture; 0, sexual reproduction absent in mixed culture; [0], sexual reproduction absent in monoclonal culture; ns, no sequence available. Expected positive crosses between Pnp^- and PNp^+ clones were shown in black boxes. Where no sexual behaviour was detected, no matingtype could be determined. Shaded cells show successful crosses between clade I and clade II clones.

			Cn-172	Cn-195	Cn-204	Cn-213	Cn-214	Cn-184	Cn-193	Cn-200	Cn-201	Cn-202	Cn-205	Cn-215	Cn-217	Cn-218	Cn-181	Cn-216
Clade I	PNp^+	Cn-172	[0]															
ns	PNp^+	Cn-195	0	[0]														
ns	PNp^+	Cn-204	0	0	[0]													
Clade I	PNp^+	Cn-213	0	0	0	[0]												
ns	PNp^+	Cn-214	0	0	0	0	[0]											
ns	PNp^-	Cn-184	+	+	+	+	+	[0]										
Clade I	PNp^-	Cn-193	+	+	+	+	+	0	[0]									
ns	PNp^-	Cn-200	+	+	+	+	+	0	0	[0]								
Clade I	PNp^-	Cn-201	+	+	+	+	+	0	0	0	[0]							
ns	PNp^-	Cn-202	+	+	+	+	+	0	0	0	0	[0]						
Clade I	PNp^-	Cn-205	+	+	+	+	+	0	0	0	0	0	[0]					
Clade I	PNp^-	Cn-215	+	+	+	+	+	0	0	0	0	0	0	[0]				
ns	PNp^-	Cn-217	+	+	+	+	+	0	0	0	0	0	0	0	[0]			
Clade I	PNp^-	Cn-218	+	+	+	+	+	0	0	0	0	0	0	0	0	[0]		
Clade I	?	Cn-181	0	0	0	0	0	0	0	0	0	0	0	0	0	0	[0]	
Clade I	?	Cn-216	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	[0]
Clade I	PNp^-	Jp-11	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp^-	NZ-67	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp^-	US-94	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp^-	Vigo-1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp^-	1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp^+	Jp-14	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade I	PNp^+	NZ-74	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade II	PNp^+	US-93	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade I	PNp^+	Vigo-2	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade I	PNp^+	2	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade II	?	US-123	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clade II	?	US-132	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ns	?	US-134	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Sex was induced in all clones from Spain (4), Japan (5) and New Zealand (3). Sexual reproduction was successfully initiated in mixed cultures of opposite mating-type clones, both within and between each of these three geographical groups (Tables 3 and 4). These clones also proved to be sexually compatible with the reference clones from the North Sea (Tables 3 and 4, Table S1 and Fig. S1). The characteristics of sexual reproduction (which involved physiological anisogamy) were identical to those that were reported previously (Chepurnov et al. 2005).

Among 16 clones collected in the NW Atlantic (Canada), 14 were capable of sexual reproduction. Each exhibited compatibility with the clones of corresponding mating type from this region and geographically distant locations (Tables 3 and S1). Sex could not be

Table 4. *Pseudo-nitzschia pungens*: results of crosses between the clones originating from Washington State, Japan, New Zealand, Spain and the Netherlands. +, sexual reproduction occurred in mixed culture; 0, sexual reproduction absent in mixed culture; [0], sexual reproduction absent in monoclonal culture; nt, cross not made; ns, no sequence available. Shaded cells show expected positive crosses between Pnp^- and PNp^+ clones. Dark shaded cells show successful crosses between clade I and clade II clones.

			US-123	US-132	US-134	US-135	US-115	US-94	US-96	US-125	US-126	US-136	US-77	US-93	Jp-11	Jp-21	Jp-22	Jp-01	Jp-14	NZ-49	NZ-67	NZ-74	Vigo-1	Vigo-4	Vigo-2	Vigo-3
Clade II	?	US-123	[0]																							
Clade II	?	US-132	0	[0]																						
ns	?	US-134	0	0	[0]																					
Clade II	?	US-135	0	0	0	[0]																				
Clade II	PNp^-	US-115	0	0	0	0	[0]																			
Clade I	PNp^-	US-94	0	0	0	0	0	[0]																		
Clade I	PNp^-	US-96	0	0	0	0	0	0	[0]																	
ns	PNp^-	US-125	0	0	0	0	0	0	0	[0]																
ns	PNp^-	US-126	0	0	0	0	0	0	0	0	[0]															
ns	PNp^-	US-136	0	0	0	0	0	0	0	0	0	[0]														
Clade II	PNp^+	US-77	0	0	0	0	0	+	+	+	+	+	[0]													
Clade II	PNp^+	US-93	0	0	0	0	+	+	+	+	+	+	0	[0]												
Clade I	PNp^-	Jp-11	0	0	0	0	0	0	0	0	0	0	+	+	[0]											
Clade I	PNp^-	Jp-21	0	0	0	0	0	0	nt	nt	nt	nt	nt	+	0	[0]										
Clade I	PNp^-	Jp-22	0	0	0	0	0	0	nt	nt	nt	nt	nt	+	0	0	[0]									
Clade I	PNp^+	Jp-01	0	0	0	0	0	+	nt	+	+	+	0	0	+	nt	nt	[0]								
Clade I	PNp^+	Jp-14	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	[0]							
Clade I	PNp^-	NZ-49	0	0	nt	0	0	nt	nt	nt	nt	0	nt	nt	nt	nt	nt	nt	nt	[0]						
Clade I	PNp^-	NZ-67	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	+	0	[0]					
Clade I	PNp^+	NZ-74	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	0	nt	+	[0]				
Clade I	PNp^-	Vigo-1	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	+	0	0	+	[0]			
Clade I	PNp^-	Vigo-4	0	0	0	0	0	0	nt	nt	nt	nt	+	+	nt	nt	nt	nt	nt	0	0	nt	0	[0]		
Clade I	PNp^+	Vigo-2	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	0	+	+	0	+	+	[0]	
Clade I	PNp^+	Vigo-3	0	0	0	0	0	+	nt	nt	nt	nt	0	0	nt	nt	nt	nt	nt	+	+	nt	+	+	0	[0]
Clade I	PNp^-	1	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	+	0	0	+	0	0	+	+
Clade I	PNp^-	19	0	0	0	0	0	0	0	0	0	0	+	nt	0	0	0	+	+	nt	0	+	0	0	+	+
Clade I	PNp^+	2	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	0	+	+	0	+	+	0	0
Clade I	PNp^+	6	0	0	0	0	0	+	+	+	+	+	0	nt	+	+	+	0	0	nt	+	0	+	+	0	0

initiated in two Canadian strains, Cn-181 and Cn-216, which were at opposite ends of the size spectrum. When used in mating experiments, cells of Cn-216 were 122–129 µm long and hence above the upper sexual threshold (~115 µm) for *P. pungens* clones from the North Sea. Intriguingly, however, two other large-celled Canadian clones, Cn-202 and Cn-215, were readily capable of sexual reproduction when crossed with various clones of opposite mating type, even though they were 118-124 and 126-131 µm long, respectively. The other sexually recalcitrant clone, Cn-181, had abnormally curved, small cells (only 36–56 µm long) and multiplied very slowly (at least twice as slowly as clones with larger cells). Furthermore, Cn-181 cells were typically solitary, only rarely forming the stepped colonies characteristic of *Pseudo-nitzschia* species and then of only a few cells.

All of the above clones, from the seas bordering Atlantic Canada, Europe, Japan and New Zealand, belonged to molecular clade I. Clade II clones were present only among the NE Pacific clones from Washington State, USA, and breeding tests involving 12 clones from this area gave more 'irregular' and ambiguous results, compared to those among isolates from the other five regions. Three clones belonging to clade II (US-123, US-132 and US-135), plus US-134, for which we have no molecular data, did not show any signs of sexualization in any of the crosses tried (Table 4 and S1), although all four were well below the 115-µm sexual threshold determined for *P. pungens* from the North Sea. Another clade II clone that we had expected to become sexual – US-115 (89–90 µm) – became sexual only once, when mated with clone US-93. The remaining clones examined, comprising US-77 and US-93 (clade II), US-94 and US-96 (clade I), and, US-125, US-126, and US-136 (all three of unknown ribotype), were crossed successfully with each other, regardless of the clade attribution. They could also mate with reference cultures from the North Sea and clones from other distant geographic areas (Tables 4 and S1).

In all mixed cultures where sexual reproduction occurred, auxospores successfully developed into viable initial cells, regardless of whether the clones originated from the same or different regions. The F1 cells were capable of further vegetative divisions, without any visible signs of growth impairment.

Table 5. Morphometric measurements (dimensions and stria, fibula and poroid densities) of selected clones. Range (average ± SD). n = number of measured valves per clone.

Clone	Clade	Apical length (µm)	Width (µm)	Striae (in 10 µm)	Fibulae (in 10 µm)	Poroids* (in 1 µm)
US-132 (n = 10)	Clade II	108.2 - 110.8 (109.4 ± 0.74)	3.4 - 4.2 (3.8 ± 0.27)	10 - 13 (11.6 ± 0.88)	11 - 15 (12.8 ± 1.40)	4.0 – 5.0 (4.34 ± 0.47)
US-115 (n = 10)	Clade II	87.9 - 91.1 (89.9 ± 1.02)	3.8 - 4.4 (4.1 ± 0.22)	11 - 13 (11.75 ± 0.68)	11.5 - 14 (12.5 ± 1.03)	3.0 – 5.0 (3.96± 0.46)
US-123 (n = 11)	Clade II	98.6 - 102.4 (100.2 ± 1.21)	3.5 - 4.7 (3.9 ± 0.28)	10 - 12 (11.2 ± 0.68)	11 - 15 (12.5 ± 1.29)	
US-135 (n = 10)	Clade II	103.5 - 108.7 (106.7 ± 1.35)	3.6 - 4.2 (3.9 ± 0.19)	11 - 13 (12.0 ± 0.69)	11.5 - 14 (12.9 ± 0.82)	
US-94 (n = 10)	Clade I	83.9 - 93.1 (88.3 ± 3.76)	3.2 - 3.8 (3.5 ± 0.22)	10.5 - 12 (11.3 ± 0.49)	11 - 14 (11.7 ± 0.97)	2.0 – 4.0 (2.89 ± 0.54)
NZ-67 (n = 10)	Clade I	68.2 - 75.3 (72.7 ± 2.97)	2.4 - 3.2 (2.7 ± 0.28)	10.5 - 12 (11.2 ± 0.63)	11 - 13 (11.9 ± 0.52)	2.0 – 4.0 (3.0 ± 0.52)
NZ-49 (n = 10)	Clade I	24.4 - 28.8 (27.4 ± 1.27)	2.8 - 3.7 (3.2 ± 0.24)	9 - 12 (10.7 ± 1.16)	10 - 14 (12.0 ± 1.25)	
Vigo-2 (n = 10)	Clade I	77.6 - 78.8 (78.2 ± 0.35)	2.6 - 2.9 (2.7 ± 0.14)	10 - 11 (10.8 ± 0.42)	10.5 - 12 (11.4 ± 0.57)	3.0 – 4.0 (3.3 ± 0.38)
Vigo-3 (n = 10)	Clade I	84.7 - 86.7 (85.8 ± 0.69)	2.8 - 3.5 (3.3 ± 0.19)	10 - 11.5 (10.7 ± 0.54)	10 - 14 (12.1 ± 1.21)	
Vigo-4 (n = 10)	Clade I	81.5 - 83.3 (82.7 ± 0.56)	2.6 - 3.2 (3.0 ± 0.17)	11 - 12 (11.7 ± 0.47)	11 - 13 (11.9 ± 0.70)	
Cn-216 (n = 10)	Clade I	117.3 - 121.0 (118.8 ± 1.12)	3.4 - 4.2 (3.8 ± 0.25)	11 - 13 (11.8 ± 0.68)	11 - 14 (12.5 ± 0.85)	2.0 – 4.0 (2.98 ± 0.45)
Clade I (average ± SD)		79.1 ± 8.7	3.2 ± 0.4	11.1 ± 0.5	11.9 ± 0.3	3.0 ± 0.5
Clade II (average ± SD)		101.5 ± 27.2	3.9 ± 0.1	11.6 ± 0.3	12.7 ± 0.2	4.2 ± 0.5

*: 50 measurements per clone.

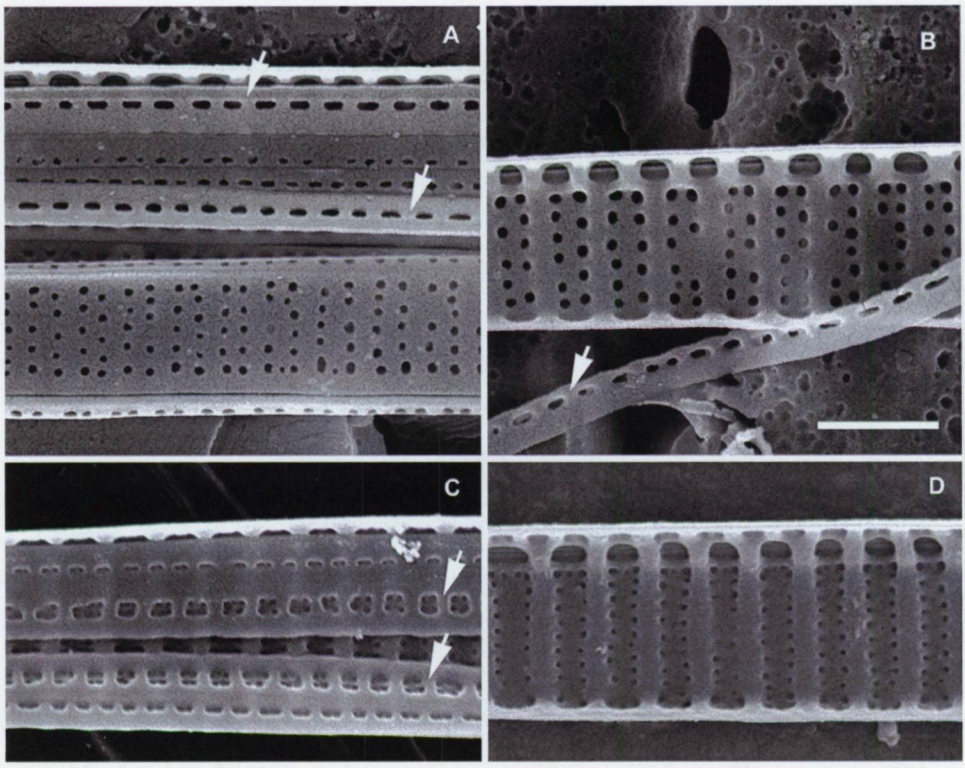

Fig. 5. *Pseudo-nitzschia pungens* frustules, SEM (valvocopulae are marked with an arrow). (A) Clade I (clone Vigo-2): valve face in external view (lower cell) and girdle bands (copulae) on internal valve face (upper cell). (B) Clade I (clone NZ-67): valve face in internal view and valvocopula. (C) Clade II (clone US-115): copulae lying on internal face of valve. (D) Clade II (clone US-132): valve face in internal view. Scale bar, 2 µm

Morphology

To assess possible morphological differentiation between the clades, the frustule morphology of 11 global strains from clades I (7) and II (4) was investigated in detail using LM and SEM. For the third clade, we had no access to material for morphological analysis. Morphometric measurements made with LM (Table 5) showed that, although length did not differ between clades I and II (*t*-test, $p = 0.15$), there were some subtle but significant differences in width ($p = 0.005$) and fibula density ($p = 0.004$). There may also be a trend towards a higher stria density in clade II ($p = 0.09$). Under SEM, the differences between the clades were more pronounced (Fig. 5). All three girdle bands (copulae) of clade I cells (Fig. 5, A and B) had one row of simple large oval poroids (Fig. 5, A and B, see also Fig. 6 in Chepurnov et al. 2005). In clade II clones, the poroids of the valvocopulae were typically square or rectangular and were always occluded by

rotae (usually consisting of four radial bars per poroid) (Fig. 5C). The valvar striae of strains of both clades were composed of two rows of poroids and sometimes included a third incipient row. However, the poroid density in 1 μm (measured for 2 strains of clade II and 4 strains of clade I) was significantly higher ($p << 0.01$) in clones of clade II (Table 5, Fig. 5D) than in clade I (Fig. 5B).

Discussion

Mating behavior

The extent of our crossing experiments probably exceeds that in any other diatom study to date. The data obtained were unambiguous in relation to heterothally and, in the vast majority of crosses, the dependence of sexual inducibility on cell size. However, a few cases were aberrant and require comment and further study. First, some clones did not reveal sexual activity when crossed with the reference clones of both mating types, even though the clones were below the 115-μm upper threshold for the sexually inducible size range of North Sea *P. pungens* (Chepurnov et al. 2005). These clones included several of clade II, but also some belonging to clade I. Some other clones, although clearly unisexual (we found no evidence in the present study for bisexual clones like those present in *Achnanthes*: Chepurnov & Mann 1997), mated with only some of the clones of opposite mating type with which they were mixed: these included clones 14 and 28 (clade I) from the North Sea studied by Chepurnov et al. (2005) and clone US-115 in clade II (Table 3). Neither molecular nor morphological data suggested the existence of any other specific differences between these 'atypical' clones and others belonging to the same ITS clade and isolated from the same geographic locations.

Lack of sexualization where it was expected suggests that something essential is still missing in our knowledge of the genetic control or experimental initiation of sex in *P. pungens*. Indeed, there is remarkably little information on how the environment affects the induction of sexual reproduction in diatoms, once the cells are of the 'right' size and the partner of opposite sex is present (Chepurnov et al. 2004). Nor are there any data on the molecular genetic mechanisms underpinning sex in heterothallic pennate diatoms in general, and *Pseudo-nitzschia* in particular.

The behavior of clones Cn-202 and Cn-215 shows that the sexual size threshold, specified as the apical cell length (though it may be cell volume or the cell volume: nuclear volume ratio or some other parameter that is actually perceived by the cell), can vary among closely related diatoms. In these clones, sexualization occurred at lengths of

118–124 and 126–131 μm, respectively, whereas none of the clones from the North Sea has ever auxosporulated above the 115-μm threshold, even though numerous large-celled cultures have been checked in mating experiments (Table S1). It is possible, therefore, that Canadian *P. pungens* are genetically differentiated from other clone I populations with respect to size range, despite sharing the same ribotype.

Variation in molecular, breeding and morphological data

Although ITS rDNA sequences have proven to be a valuable phylogenetic marker at the species level and below, their use has also been criticized (Alvarez & Wendel 2003). The prevalent complication for phylogenies is the existence of extensive sequence variation, arising from ancient or recent array duplication events, genomic harboring of pseudogenes in various states of decay, incomplete intra- or inter-array homogenization and/or hybridization events. These phenomena can create a network of paralogous sequence relationships that can potentially confound phylogenetic reconstruction. We tested six of our strains for intraclonal ITS variation. The degree of variation found was very low (maximum 0.7%), and most changes involved autapomorphic point mutations, except at a few sites where partially homogenized mutations were found. These point mutations may represent recently arisen mutations that the homogenization process has not yet excluded or promoted; the partially homogenized mutations are apparently in the process of homogenization (Dover et al. 1993). When the different paralogues of a strain were included in the phylogenetic analysis (Fig. 2), the sequence variation was too minor to affect the division in three separate clades. We therefore regard the ITS1 – 5.8 S – ITS2 region as a useful tool to investigate relationships between *P. pungens* strains. In addition, our sequencing results never revealed any ambiguities through direct sequencing (see above). Homogenization of ribosomal cistrons seems sufficiently complete within *P. pungens* as to offer no hindrance to utilizing direct sequencing results.

Our data show that at least three distinct ITS entities exist within *P. pungens*. Mating tests demonstrated sexual compatibility among the strains of clade I, regardless of their geographic origin (North Sea region, Spain, Canada, Japan, New Zealand or the Pacific coast of North America) (Fig. 2). Some clones of clade II (US-93 and US-77) provided unambiguous evidence that clones from clade I and II are sexually compatible in the laboratory and can produce viable hybrid offspring. The molecular differences between these two ITS clades are correlated with slight but consistent morphological differences. At present, nothing can be said about morphology and mating behavior for clade III clones because only ITS data were available.

In *Pseudo-nitzschia*, differences between well-supported ITS clades, when accompanied by morphological differences, have been suggested to be of taxonomic significance at the species level and have led to the description of new species (Manhart et al. 1995, Lundholm et al. 2003, 2006). Mostly, sequence divergence between these clades was high (> 10%). However, some studies of *Pseudo-nitzschia* have discriminated species on the basis of a much lower ITS sequence divergence. For example, Hasle & Lundholm (2005) raised *P. seriata* f. *obtusa* to species level (*P. obtusa*) with ITS divergence of only 6%, and the morphologically distinguishable *P. seriata* and *P. australis* have an ITS sequence divergence as low as 2.6% (Fehling et al. 2004b, Hasle & Lundholm 2005). In comparison, the divergence between *P. pungens* clades I and II (1.3%) is lower than the variation found between any currently accepted congeneric species, while the divergence between clade III and the rest of the ingroup (2.7 – 4.4%) is comparable with the ITS separation between *P. seriata* and *P. australis*.

Although ITS sequence comparisons have been used several times to help delimit diatom species in cases where morphological differences are subtle or difficult to interpret (e.g. Zechman et al. 1994, Behnke et al. 2004, Beszteri et al. 2005b, Godhe et al. 2006), including examples in *Pseudo-nitzschia* (see above), diatom studies that combine breeding tests with molecular analyses are still scarce (Mann 1999, Behnke et al. 2004, Vanormelingen et al. 2007). In the freshwater diatoms *Sellaphora pupula* (Kützing) Mereschkowsky (Behnke et al. 2004) and *Eunotia bilunaris* (Ehrenberg) Mills (Vannormelingen et al. 2007), a correlation has been found between sexual compatibility and ITS sequence divergence. Organisms capable of interbreeding in the laboratory show low variation in ITS sequences (at most 7.3% in *Sellaphora pupula* and 4.3% in *Eunotia bilunaris*), whereas in those that cannot interbreed, the ITS sequence divergence is much higher. To date, mating tests have been used alongside both morphological and molecular data in only one taxonomic study of *Pseudo-nitzschia*, of the *P. delicatissima* and *P. pseudodelicatissima* (Hasle) Hasle species complexes in the Gulf of Naples (Amato et al. 2007). In that study, only ITS2 divergence was a consistently good discriminator for reproductively isolated groups. The present study is the first for the genus that combines molecular approaches and multiple crossing experiments on a global scale.

A correlation between the evolution of ITS2 secondary structure and sexual compatibility has been shown for green algae of the order Volvocales and for the ciliate *Paramecium aurelia* Ehrenberg (e.g. Fabry et al. 1999, Coleman 2000, 2005). These studies demonstrated that if two organisms mate successfully, they are found to have no CBCs or HCBCs, whereas the converse is not always true: some pairs of *Paramecium* syngens are reproductively isolated, despite having identical sequences (Coleman 2005). The *P. delicatissima*-like and *P. pseudodelicatissima*-like strains from the Gulf of

Naples fit this hypothesis (Amato et al. 2007): reproductively isolated strains belonged to different CBC-clades (group of organisms where there are no CBCs). This appears to be true also in *Sellaphora* where ITS2 sequences of the 'rectangular' and 'pseudocapitate' demes of *S. pupula* differ only in HCBCs and can mate in the laboratory (Behnke et al. 2004). In the present study, sexual compatibility was illustrated between clades I and II, and ITS2 secondary structure comparisons revealed no CBCs and HCBCs between these clades. On the other hand a CBC and HCBC were detected between clades {I + II} and III. Thus, if Coleman's idea is correct, i.e. that CBCs act as a marker for the cessation of gene flow, we can predict that members of clade III will be reproductively isolated from clades I and II.

In the present study, the ITS difference between clades I and II was accompanied by morphological dissimilarities. The morphological features of strains belonging to clade II correspond to *P. pungens* var. *cingulata* Villac as described by Villac and Fryxell (1998) from Monterey Bay (California), in the NE Pacific, along the same coast (dominated by the California Current System) where the isolates of clade II were found. By contrast, clade I clones correspond to the morphology of the nominate form of *P. pungens*, as observed in previous studies (Hasle 1995, Hasle & Syvertsen 1996, Chepurnov et al. 2005). In addition, there may be differences between clades I and II in the upper sexual size threshold, or in the external factors that permit sexualization, judging by the surprising lack of sexual response in clones US-123, US-132, US-134, US-135 and US-115.

Taken together, our evidence suggests that clades I and II could represent slightly differentiated populations of the same species (consistent perhaps with Villac and Fryxell's use of the variety category). However, members of both clades occur sympatrically in Washington State waters. We found no signs of mixtures of ITS genotypes (after cloning to detect intra-isolate ITS variation), indicating no hybridization between clades (cf. Coleman 2002), but the number of isolates studied is still too small to allow definite conclusions concerning gene flow in nature. There is apparently little or no intrinsic pre-zygotic isolation between the two ITS clades, but the existence of post-zygotic reproductive isolation (inability to produce an F2 or backcross to parental strains) cannot yet be excluded. Various forms of post-zygotic isolation have been demonstrated in other organisms, ranging from hybrid sterility to a decrease in fitness of hybrids to even non-viability of hybrids (Coyne & Orr 2004).

Speciation in this group might be ongoing and very recent, so that pre-zygotic isolation is not yet complete. Populations can diverge genetically despite their potential for gene exchange. Studies in fungi have shown that genetic isolation always precedes morphological or reproductive isolation (Taylor et al. 2006). Even if there is no intrinsic

barrier to hybridization, it is still possible that interbreeding between clades I and II is rare or absent in nature, even where they co-occur. Clade I and II strains are still able to recognize each other but assortative mating could account for the absence of hybrids in nature (Coyne & Orr 2004). Another explanation could be that populations of the clades are temporally separated, blooming in different seasons while maintaining a low concentration throughout the remainder of the year, as shown for sympatric *P. delicatissima* ITS types in parts of the Mediterranean (Orsini et al. 2004). There could also be some ecological selection against hybrids in nature (Schluter 2001). Amato et al. (2007) have suggested that small morphological differences may be ecologically relevant in *Pseudo-nitzschia* species and this could apply also in *P. pungens*. The significance of pseudocryptic variation is better understood in foraminifera, where it has been shown that morphologically very similar species can have quite different ecological requirements and geographical distributions, with significant implications for the use of these species in paleooceanographical reconstruction (e.g. Kucera & Darling 2002).

Geographic distribution

Recently, the debate has been resurrected over whether most microorganisms are cosmopolitan, because their vegetative cells or propagules may have virtually unlimited dispersal potential (Finlay 2002, Fenchel & Finlay 2004). Traditionally, tentative judgements about whether a species is cosmopolitan or has a restricted geographic distribution have been based purely on morphological data (e.g. Hasle 2002, Finlay 2002). In *Pseudo-nitzschia*, most species have been reported worldwide, while others seem to be restricted to certain latitudinal zones, e.g. *P. seriata* only occurs in the north Atlantic and *P. obtusa* is restricted to Artic regions (Hasle 2002, Hasle & Lundholm 2005). With the introduction of molecular approaches, many morphologically-defined 'cosmopolitan' protist species, including members of the genus *Pseudo-nitzschia*, are now known to be composed of multiple (semi- or pseudo-) cryptic species, whose distribution is unknown and may be restricted (Lundholm et al. 2003, 2006, Amato et al. 2007).

Like most *Pseudo-nitzschia* species, the morphologically distinct entity currently known as '*Pseudo-nitzschia pungens*' has been reported worldwide (Hasle 2002). However, the three ITS clades within *P. pungens* have different geographic distributions. Clade III strains have hitherto been isolated only from warm waters (annual mean sea surface temperatures ~25–30 °C), whereas the other two *P. pungens* clades were all derived from more temperate waters (annual mean sea surface temperatures ~10–20 °C). The second ITS clade has been found so far only in the NE Pacific, whereas clade I was found in the North Sea, the eastern and western N Atlantic, and the NW, NE and S

Pacific. While we have insufficient data to make any conclusive statements about the biogeography of clades II and III, the data for clade I are highly significant. Clade I is a lineage with a very broad oceanic distribution, perhaps with no barriers to global dispersal. In addition, crossing experiments demonstrated sexual compatibility among the strains of clade I, regardless of their geographic origin: this is important because uniformity in ITS regions does not always correspond to reproductive compatibility (e.g. *Paramecium*; Coleman 2005). Clade I is therefore widespread within temperate zones in both hemispheres and supplies the most conclusive evidence so far for the global distribution of a biologically, genetically and morphologically defined diatom species.

It is yet unclear if this distribution is natural or anthropogenic. A few other HAB taxa found globally have also been reported with very low intraspecific genetic diversity in the ITS region, e.g. the raphidophytes *Heterosigma akashiwo* and *Fibrocapsa japonica* (Connell 2000, Kooistra et al. 2001). In these, the apparent lack of genetic differentiation has been suggested to reflect geologically recent spreading between oceanic regions, possibly mediated by humans. Viable cells of *Pseudo-nitzschia* species, and of *P. pungens* in particular, have been found in ballast water samples (Gollasch et al. 2000, Rhodes et al. 1998) and so it is possible that *P. pungens* could be spread around the world through shipping. The co-occurrence of clade I and II in the NE Pacific could therefore reflect recent breakdown of a previously effective geographical separation by land (Pacific–Atlantic) and sea (NW–NE Pacific) barriers. To test this further, genetic differentiation between widely separated populations needs to be explored with other genetic markers that evolve faster than ITS. Six microsatellite markers developed for *P. pungens* by Evans et al. (2005) revealed weak genetic differentiation over the temporal and spatial scales sampled (18 months and 100 km) and suggested that *P. pungens* in the German part of the North Sea exists as a single, largely unstructured population. We are now using such markers to explore the genetic differentiation among widely separated *P. pungens* populations and to discover possible dispersion routes.

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Supplementary table

Table S1. Clonal cultures of *P. pungens* used in the present study, with voucher information (supplier and initial strain designation) and EMBL accession numbers. In bold: strains that were sequenced. [a]: strains of which PCR products were cloned. Clones could be designated as PNp^+ or PNp^-; 0, sexual reproduction absent in mixed culture. Vouchers beginning with VLIZ are sampled by the Flemish Marine Institute, vouchers beginning with Spuikom are sampled by the Flemish Environment Agency and vouchers beginning with WS are sampled in association with the Netherlands Institute of Ecology (NIOO).

Clone	Sampling		Voucher	Size	Mating type	EMBL accession number
	Location	Date				
1	Westerscheldt, The Netherlands, Terneuzen	24/05/02	K. Sabbe, Trz1	83-86	PNp^-	AM778733
2	Westerscheldt, The Netherlands, Terneuzen	24/05/02	K. Sabbe, Trz2	92-95	PNp^+	AM778734
6	North Sea, The Netherlands, Terschelling	01/05/00	L. Peperzak, T0105Psnpun2	78-85	PNp^+	AM778735
14	North Sea, The Netherlands, Marsdiep	25/04/02	L. Peperzak, NZ-P5	110-115	PNp^-	AM778736
16	North Sea, The Netherlands, Noordwijk	22/05/02	L. Peperzak, NB-P1	111-120	PNp^-	AM778737
18	North Sea, The Netherlands, Noordwijk	22/05/02	L. Peperzak, NB-P4	108-114	PNp^+	AM778738
19	North Sea, The Netherlands, Noordwijk	22/05/02	L. Peperzak, NB-P5	109-115	PNp^-	AM778739
20	North Sea, Belgium, VLIZ station 130	30/06/03	K. Muylaert, ZL130 - 20	93-97	PNp^+	AM778740
24	North Sea, Belgium, VLIZ station 130	30/06/03	K. Muylaert, ZL130 - 24	84-84	PNp^-	AM778741
28	North Sea, Belgium, VLIZ station 130	30/06/03	K. Muylaert, ZL130 - 28	86-89	PNp^-	AM778742
40s	North Sea, Belgium, Ijzermonding Nieuwpoort	13/08/03	K. Sabbe, GFC - 40s	73-77	PNp^+	AM778743
44	North Sea, Belgium, Ijzermonding Nieuwpoort	13/08/03	K. Sabbe, GFC - 44	82-86	PNp^+	AM778744
52	North Sea, northern France, Ambleteuse	13/09/03	F. Leliaert, Ambl - 52	90-92	PNp^-	AM778745
57	North Sea, northern France, Ambleteuse	13/09/03	F. Leliaert, Ambl - 57	90-92	PNp^+	AM778746
V120(3)1	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 1	158-160	0	
V120(3)2	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 2	86-87	PNp^+	
V120(3)3	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 3	78-79	PNp^-	
V120(3)4	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 4	91-93	PNp^-	
V120(3)5	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 5	89-92	PNp^-	AM778747
V120(3)6	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 6	104-106	PNp^+	
V120(3)7	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 7	87-89	PNp^+	
V120(3)8	North Sea, Belgium, VLIZ station 120	24/03/04	VLIZ120 (24/03/04) - 8	154-156	0	
V120(8)1	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 1	106-109	PNp^-	
V120(8)2	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 2	138-141	0	
V120(8)3	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 3	125-128	0	
V120(8)4	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 4	135-138	0	
V120(8)5	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 5	134-138	0	
V120(8)6	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 6	130-134	0	
V120(8)7	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 7	140-144	0	
V120(8)8	North Sea, Belgium, VLIZ station 120	16/08/04	VLIZ120 (16/08/04) - 8	145-149	0	
V215(3)1	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 1	149-151	0	
V215(3)2	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 2	78-80	PNp^-	AM778748
V215(3)3	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 3	145-147	0	
V215(3)4	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 4	75-78	PNp^+	
V215(3)5	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 5	80-82	PNp^+	AM778749
V215(3)6	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 6	81-83	PNp^-	
V215(3)7	North Sea, Belgium, VLIZ station 215	24/03/04	VLIZ215(24/03/04) - 7	88-91	PNp^-	
V215(7)1	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) -1	92-93	0	
V215(7)2	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 2	147-148	0	
V215(7)3	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 3	84-85	0	AM778750
V215(7)4	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 4	149-150	0	

Table S1. Continued.

V215(7)5	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 5	155-156	0	
V215(7)6	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 6	149-150	0	
V215(7)7[a]	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 7	152-153	0	AM778751 - AM778757
V215(7)8	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 8	89-90	0	
V215(7)9	North Sea, Belgium, VLIZ station 215	19/07/04	VLIZ215(19/07/04) - 9	88-90	PNp$^+$	
V215(10)1	North Sea, Belgium, VLIZ station 215	18/10/04	VLIZ215(18/10/04) - 1	130-132	0	
V215(12)1	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 1	80-82	PNp$^-$	
V215(12)2	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 2	145-147	0	
V215(12)3	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 3	140-142	0	
V215(12)4	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 4	135-137	0	
V215(12)5	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 5	151-153	0	
V215(12)6	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 6	91-94	PNp$^-$	
V215(12)7	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 7	130-133	0	
V215(12)8	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 8	88-90	PNp$^-$	
V215(12)9	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 9	84-87	PNp$^-$	
V215(12)10	North Sea, Belgium, VLIZ station 215	08/12/04	VLIZ215(08/12/04) - 10	141-142	0	
V330(3)1	North Sea, Belgium, VLIZ station 330	24/03/04	VLIZ330(24/03/04) - 1	89-91	PNp$^-$	
V330(3)2	North Sea, Belgium, VLIZ station 330	24/03/04	VLIZ330(24/03/04) - 2	84-86	PNp$^-$	
V330(3)3	North Sea, Belgium, VLIZ station 330	24/03/04	VLIZ330(24/03/04) - 3	77-78	PNp$^-$	
V330(3)4	North Sea, Belgium, VLIZ station 330	24/03/04	VLIZ330(24/03/04) - 4	132-134	0	
V700(4)1	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 1	84-85	PNp$^+$	
V700(4)2	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 2	86-87	PNp$^+$	
V700(4)3	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 3	95-96	PNp$^-$	
V700(4)4	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 4	99-100	PNp$^+$	
V700(4)5	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 5	84-85	PNp$^-$	
V700(4)6	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 6	86-87	PNp$^-$	
V700(4)7	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 7	81-82	PNp$^-$	
V700(4)8	North Sea, Belgium, VLIZ station 700	19/04/04	VLIZ700(19/04/04) - 8	78-79	PNp$^-$	
V700(7)1	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 1	78-80	PNp$^+$	
V700(7)2	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 2	89-91	PNp$^-$	
V700(7)3	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 3	142-144	0	
V700(7)4	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 4	155-156	0	
V700(7)5	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 5	144-146	0	AM778758
V700(7)6	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 6	136-137	0	
V700(7)7	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 7	142-143	0	
V700(7)8	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 8	134-135	0	
V700(7)9	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 9	89-91	PNp$^-$	
V700(7)10	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 10	84-85	PNp$^-$	
V700(7)11	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 11	86-88	0	AM778759
V700(7)12	North Sea, Belgium, VLIZ station 700	19/07/04	VLIZ700(19/07/04) - 12	89-90	PNp$^-$	
V421(3)1	North Sea, Belgium, VLIZ station 421	29/03/04	VLIZ421(29/03/04) - 1	91-94	PNp$^+$	
V421(3)2	North Sea, Belgium, VLIZ station 421	29/03/04	VLIZ421(29/03/04) - 2	93-96	PNp$^+$	
V421(3)3	North Sea, Belgium, VLIZ station 421	29/03/04	VLIZ421(29/03/04) - 3	151-154	0	
V421(3)4	North Sea, Belgium, VLIZ station 421	29/03/04	VLIZ421(29/03/04) - 4	93-96	PNp$^+$	AM778760
V421(3)5	North Sea, Belgium, VLIZ station 421	29/03/04	VLIZ421(29/03/04) - 5	89-92	PNp$^+$	
V421(3)6	North Sea, Belgium, VLIZ station 421	29/03/04	VLIZ421(29/03/04) - 6	87-89	0	
V421(8)1	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 1	142-145	0	
V421(8)2	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 2	124-127	0	
V421(8)3	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 3	135-137	0	
V421(8)4	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 4	109-113	PNp$^-$	
V421(8)5	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 5	130-133	0	
V421(8)6	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 6	134-137	0	
V421(8)7	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 7	127-130	0	
V421(8)8	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 8	134-138	0	
V421(8)9	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 9	135-137	0	

Table S1. Continued.

V421(8)10	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 10	140-143	0	
V421(8)11	North Sea, Belgium, VLIZ station 421	23/08/04	VLIZ421(23/08/04) - 11	143-147	0	
V421(10)1	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 1	115-118	0	
V421(10)2	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 2	99-102	PNp^-	
V421(10)3	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 3	125-128	0	
V421(10)4	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 4	110-113	0	
V421(10)5	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 5	130-132	0	
V421(10)6	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 6	122-124	0	
V421(10)7	North Sea, Belgium, VLIZ station 421	19/10/04	VLIZ421(19/10/04) - 7	104-106	PNp^+	
S(4)1	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 1	83-86	PNp^+	
S(4)2	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 2	85-88	PNp^+	
S(4)3	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 3	88-91	PNp^+	
S(4)4	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 4	88-91	PNp^-	
S(4)5	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 5	85-88	PNp^+	
S(4)6 [a]	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 6	87-90	PNp^+	AM778761 - AM778767
S(4)7	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 7	85-88	PNp^+	
S(4)8	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 8	88-91	PNp^-	
S(4)9	Oostend lagoon, Belgium, Spuikom Oostende	07/04/04	Spuikom(07/04/04) - 9	75-78	PNp^+	
S(5)1	Oostend lagoon, Belgium, Spuikom Oostende	18/05/04	Spuikom(18/05/04) - 1	75-78	PNp^-	
S(5)2	Oostend lagoon, Belgium, Spuikom Oostende	18/05/04	Spuikom(18/05/04) - 2	88-91	PNp^+	
S(7)1	Oostend lagoon, Belgium, Spuikom Oostende	13/07/04	Spuikom(13/07/04) - 1	131-132	0	AM778768
W1(4)1	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 1	88-90	PNp^-	
W1(4)2	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 2	87-89	PNp^+	
W1(4)3	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 3	89-91	PNp^+	
W1(4)4	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 4	80-82	PNp^-	
W1(4)5	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 5	80-82	PNp^+	
W1(4)6	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 6	92-94	PNp^-	
W1(4)7	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 7	89-92	PNp^-	
W1(4)8	Westerscheldt, The Netherlands, Breskens	21/04/04	WS1(21/04/04) - 8	85-86	PNp^+	
W1(7)1	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 1	146-148	0	AM778769
W1(7)2	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 2	75-77	PNp^+	
W1(7)3	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 3	98-100	PNp^-	
W1(7)4	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 4	86-88	0	
W1(7)5	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 5	83-86	PNp^-	
W1(7)6 [a]	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 6	81-83	0	AM778770 - AM778774
W1(7)7	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 7	106-108	PNp^+	
W1(7)8	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 8	115-117	0	
W1(7)9	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 9	91-94	PNp^+	
W1(7)10	Westerscheldt, The Netherlands, Breskens	14/07/04	WS1(14/07/04) - 10	83-85	PNp^-	
W4(4)1	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 1	84-85	PNp^-	
W4(4)2	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 2	89-90	PNp^+	
W4(4)3	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 3	81-82	PNp^+	
W4(4)4	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 4	92-93	PNp^+	
W4(4)5	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 5	92-93	PNp^+	
W4(4)6	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 6	85-86	PNp^+	
W4(4)7	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 7	89-90	PNp^-	
W4(4)8	Westerscheldt, The Netherlands, Terneuzen	21/04/04	WS4(21/04/04) - 8	92-93	PNp^+	
W4(7)1	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 1	91-94	0	
W4(7)2	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 2	143-145	0	AM778775
W4(7)3	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 3	146-148	0	
W4(7)4	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 4	146-148	0	
W4(7)5	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 5	96-98	PNp^-	
W4(7)6	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 6	130-132	0	
W4(7)7	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 7	141-144	0	

Table S1. Continued.

W4(7)8	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 8	143-146	0	
W4(7)9	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 9	127-129	0	
W4(7)10	Westerscheldt, The Netherlands, Terneuzen	14/07/04	WS1(14/07/04) - 10	138-141	0	
Vigo-1	E Atlantic, Spain, Bay of Vigo	01/04/04	M. Lastra	98-102	PNp$^-$	AM778776
Vigo-2	E Atlantic, Spain, Bay of Vigo	01/04/04	M. Lastra	82-85	PNp$^+$	AM778777
Vigo-3 [a]	E Atlantic, Spain, Bay of Vigo	01/04/04	M. Lastra	95-98	PNp$^+$	AM778778 - AM778784
Vigo-4	E Atlantic, Spain, Bay of Vigo	01/04/04	M. Lastra	90-93	PNp$^-$	AM778785
Cn-172	NW Atlantic, Canada, Cardigan River, Prince Edward Island	05/09/02	S. Bates, CL-172	71-72	PNp$^+$	AM778786
Cn-181	NW Atlantic, Canada, Oak Point, Miramichi Bay, New Brunswick	23/09/02	S. Bates, CL-181	36-56	0	AM778787
Cn-184	NW Atlantic, Canada, Oak Point	23/09/02	S. Bates, CL-184	39-43	PNp$^-$	
Cn-193	NW Atlantic, Canada, Deadmans Hbr., Bay of Fundy, New Brunswick	09/10/02	S. Bates, CL-193	42-53	PNp$^-$	AM778788
Cn-196	NW Atlantic, Canada, Egg Island, Miramichi Bay, New Brunswick	28/10/02	S. Bates, CL-196	65-74	PNp$^+$	
Cn-200	NW Atlantic, Canada, Egg Island	28/10/02	S. Bates, CL-200	47-53	PNp$^-$	
Cn-201	NW Atlantic, Canada, Egg Island	15/09/03	S. Bates, CL-201	47-53	PNp$^-$	AM778789
Cn-202	NW Atlantic, Canada, Egg Island	15/09/03	S. Bates, CL-202	118-124	PNp$^-$	
Cn-204	NW Atlantic, Canada, Lennox Channel, Prince Edward Island	27/10/03	S. Bates, CL-204	73-82	PNp$^+$	
Cn-205	NW Atlantic, Canada, Lennox Channel	27/10/03	S. Bates, CL-205	73-79	PNp$^-$	AM778790
Cn-213	NW Atlantic, Canada, Brudenell River, Prince Edward Island	07/09/04	S. Bates, CL-213	73-82	PNp$^+$	AM778791
Cn-214	NW Atlantic, Canada, Brudenell River	07/09/04	S. Bates, CL-214	76-85	PNp$^+$	
Cn-215	NW Atlantic, Canada, Cardigan River, Prince Edward Island	07/09/04	S. Bates, CL-215	126-131	PNp$^-$	AM778792
Cn-216	NW Atlantic, Canada, Cardigan River	07/09/04	S. Bates, CL-216	122-129	0	AM778793
Cn-217	NW Atlantic, Canada, Boughton River, Prince Edward Island	07/09/04	S. Bates, CL-217	78-85	PNp$^-$	
Cn-218	NW Atlantic, Canada, Boughton River	07/09/04	S. Bates, CL-218	89-95	PNp$^-$	AM778794
US-77	NE Pacific, North America, La Push, WA	18/09/01	B. Bill, NWFSC-077	88-91	PNp$^+$	AM778795
US-93	NE Pacific, North America, Eld Inlet, Mud Bay, WA	17/06/02	B. Bill, NWFSC-093	72-75	PNp$^+$	AM778796
US-94 [a]	NE Pacific, North America, Sequim Bay State Park, WA	08/08/02	B. Bill, NWFSC-094	91-93	PNp$^-$	AM778797 - AM778802
US-96	NE Pacific, North America, Sequim Bay State Park, WA	08/08/02	B. Bill, NWFSC-096	90-94	PNp$^-$	AM778803
US-115	NE Pacific, North America, ECOHAB I (coastal Washington)	/06/03	B. Bill, NWFSC-115	89-90	0	AM778804
US-123 [a]	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-123	99-100	0	AM778805 - AM778809
US-125	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-125	96-99	PNp$^-$	
US-126	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-126	84-88	PNp$^-$	
US-132	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-132	109-111	0	AM778810
US-134	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-134	103-107	0	
US-135	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-135	104-107	0	AM778811
US-136	NE Pacific, North America, ECOHAB I	/06/03	B. Bill, NWFSC-136	100-104	PNp$^-$	
Jp-01	NW Pacific, Japan, Ofunato Bay	07/08/00	Y. Kotaki, OFPp 001	51-55	PNp$^+$	AM778812
Jp-11	NW Pacific, Japan, Ofunato Bay	25/06/01	Y. Kotaki, OFPp 011	64-68	PNp$^-$	AM778813
Jp-14	NW Pacific, Japan, Ofunato Bay	3/12/01	Y. Kotaki, OFPp 014	74-79	PNp$^+$	AM778814
Jp-21	NW Pacific, Japan, Okkirai Bay	25/07/00	Y. Kotaki, OKPp 001	49-55	PNp$^-$	
Jp-22	NW Pacific, Japan, Okkirai Bay	28/07/00	Y. Kotaki, OKPp 002	41-48	PNp$^-$	
NZ-49	S Pacific, New Zealand, Steels Reef, North Island	/10/00	L. Rhodes, CAWB49	37-40	PNp$^-$	AM778815
NZ-67	S Pacific, New Zealand, Big Glory Bay, Stewart Island	/02/03	L. Rhodes, CAWB67	72-75	PNp$^-$	AM778816
NZ-74	S Pacific, New Zealand, Taylor's Mistake, South Island	/04	L. Rhodes, CAWB74	83-86	PNp$^+$	AM778817

4

Natural hybrids in *Pseudo-nitzschia pungens*: genetic and morphological evidence[1]

Hybridization between genetically distinguishable taxa provides opportunities for investigating speciation. While hybridization is a common phenomenon in various macro-organisms, natural hybridization among micro-eukaryotes is barely studied. Here we used a nuclear and a chloroplast molecular marker and morphology to demonstrate the presence of natural hybrids between two genetically and morphologically distinct varieties of the marine planktonic diatom *Pseudo-nitzschia pungens* (vars. *pungens* and *cingulata*) in a contact zone in the northeast Pacific. Cloning and sequencing of the rDNA internal transcribed spacer region revealed strains containing ribotypes from both varieties, indicating hybridization. Both varieties were found to also have different chloroplast encoded *rbc*L sequences. Hybrid strains were either hetero- or homoplastidial, as demonstrated by Denaturing Gradient Gel Electrophoresis, which is in accordance with expectations based on the mode of chloroplast inheritance in *Pseudo-nitzschia*. While most hybrids are probably first generation, there are also indications for further hybridization. Morphologically, the hybrids resembled var. *pungens* for most characters rather than having an intermediate morphology. Further research should focus on the hybridization frequency, by assessing the spatial and temporal extent of the contact zone, and hybrid fitness, to determine the amount of gene flow between the two varieties and its evolutionary consequences.

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Introduction

Hybridization is generally defined as the crossing of genetically distinguishable taxa leading to viable individuals of mixed ancestry (Mallet 2005). Hybridization plays an important role in creating new diversity either by introgression of single alleles, or by the establishment of recombinant genotypes as a new species (Barton 2001). Regions where divergent populations meet and interbreed are referred to as hybrid zones. They are useful in studying the process of speciation as they provide natural examples of, through their recognizable and limited geneflow, the continuum between a single species with multiple populations and multiple reproductively isolated species. Hybridization events have traditionally been studied through morphological comparisons, under the assumption that various hybrid offspring are predictably intermediate to parents (Arnold 1997). However heterospecific crosses sometimes produce offspring that more closely resemble the phenotype of one of the parental taxa (Byrne & Anderson 1994, Harper & Hart 2007, Lamb & Avise 1987). In these cases genetic markers independent of morphological traits can often reveal allele or haplotype sharing that is consistent with hybridization and introgression. Therefore, when studying natural hybridization, joint comparisons of morphology and genotypic constitution are recommended (Lamb and Avise 1987).

The importance of hybridization and introgression has long been recognized in plants (reviewed in Arnold 1997) but only relatively recently in animals (Mallet 2005). While the majority of hybridization examples are from terrestrial species, Gardner (1997) suggested that hybridization might be a common phenomenon in marine environments as well. Well-studied examples of hybridization among marine organisms are those of the blue mussel *Mytilus edulis* species complex (Bierne et al. 2003), the brown seaweed *Fucus* (Coyer et al. 2007) and various corals (Willis et al. 2006, Slattery et al. 2008). In contrast, there are hardly any studies investigating natural hybridization in aquatic micro-eukaryotes. One reason is the problematic delineation of species, often based solely on morphology, which leads to hybrids being undetected. Studies on natural hybridization are also hampered by difficulties in the experimental manipulation of microscopic organisms together with the need to establish clonal cultures if one wants to study different aspects of a clone (e.g. different molecular markers and morphological features). There are indications for natural inter-specific hybridizations in dinoflagellates based on rDNA markers but the results are not decisive (Edvardsen et al. 2003, Hart et al. 2007). In diatoms, one of the most speciose taxa of aquatic protists, there are a number of studies on experimental hybridization (overview given by Vanormelingen et al. 2008), but only a single recent study addressed intraspecific hybridization in the field (D'Alelio et al. 2009b).

The pennate diatom *Pseudo-nitzschia pungens* is a common component of marine phytoplankton assemblages worldwide (Hasle 2002). In a recent study, at least three distinct but closely related genetic entities were found within *P. pungens* based on nrDNA internal transcribed spacers (ITS) sequences (Casteleyn et al. 2008, chapter 3 of this thesis). These three ITS clades showed slight but consistent morphological differences. Strains of clade I (referred to hereafter as ribotype I) were found to correspond morphologically to *P. pungens* var. *pungens* while strains of clade II (ribotype II) fit *P. pungens* var. *cingulata* (Casteleyn et al. 2008). Recently, members of the third clade were described as a new variety, var. *aveirensis* (Churro et al. in press, appendix of this thesis). *P. pungens* var. *pungens* is distributed in temperate waters worldwide while var. *cingulata* seems to be restricted to the NE Pacific Ocean where it co-exists with the former variety. Both varieties are sexually compatible in the laboratory and produce viable offspring. Despite the fact that apparently few or no intrinsic prezygotic barriers between both varieties exist, no signs of mixtures of ribotypes or strains with intermediate morphologies in nature have previously been recovered from the NE Pacific (Casteleyn et al. 2008), possibly because the number of strains studied was too low. Recently, a microsatellite study using a more extensive set of *P. pungens* strains from the Washington coast revealed two populations of *P. pungens* (Adams 2006). Interestingly, some strains with an intermediate probability of belonging to one or the other population were recovered.

In the present study, we demonstrate natural hybridization between *Pseudo-nitzschia pungens* var. *pungens* and var. *cingulata* by analysing molecular markers in two different genomic compartments (nucleus and chloroplast) and examining morphological features for a subset of strains used by Adams (2006). We first investigated whether the two populations found by Adams (2006) correspond to the two *P. pungens* varieties and if natural hybridization between the two varieties does indeed occur in nature. We used the ITS region as a diagnostic marker to identify the two varieties. It was already shown by Casteleyn et al. (2008) that intragenomic ITS variation is negligible so that each variety contains a single characteristic sequence type. Since 18S–26S rDNA arrays reside in the nuclear genome, ITS sequences are biparentally inherited. A cross between the two varieties will therefore produce an F_1 hybrid with the two sequence types. In addition, we investigated whether the varieties were also differentiated with respect to a chloroplast marker (*rbc*L) and characterized hybrid strains using this chloroplast marker. Plastid DNA is known to have a different pattern of inheritance than nuclear markers. Although plastid inheritance in *Pseudo-nitzschia* is also biparental, F_1 cells contain two maternal or two paternal plastids, or one from both parents, due to random plastid inheritance in the F_1 progeny (Levialdi Ghiron et al. 2007). We also examined whether putative hybrids had intermediate morphologies or if they more closely resembled one or the other of the parental varieties.

Material and methods

Cultures

A subset of 57 strains from a study of *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle in the NE Pacific (Adams 2006) was used for this investigation. We included a large random sample of strains that were clearly assignable (with probability > 0.9) to one of the two populations revealed in a cluster analysis (STRUCTURE) and all 12 still available strains with an intermediate probability that did not allow them to be confidently assigned to either population and thus potential hybrids (NA116, NA169, NA177, NA178, NA179, NA192, NA200, NA204, NA231, NA233, NA235, NA242). Living material for these cultures is no longer available because of size reduction in diatoms, but DNA and ITS clones for these strains are available upon request. For 37 additional strains from other geographic areas and the Pacific from a previous study (Casteleyn et al. 2008), the *rbc*L type was determined (Table S1). Cultures were maintained in f/2 medium (Guillard, 1975) based on natural seawater (~30-32 psu) at 18 °C, 60-80 µmol photons m^{-2} s^{-1} irradiance and a 12:12 hour light:dark cycle.

DNA extraction, ITS cloning and sequencing

Genomic DNA of the 57 strains was extracted from pelleted cells using a bead-beating method with phenol extraction and ethanol precipitation (Zwart et al. 1998). The internal transcribed spacer region (ITS1 – 5.8S – ITS2) of the rDNA was amplified using the primers 1800F (Friedl 1996) and a newly designed reverse primer, ITSRpsn (5'- TCC TCT TGC TTG ATC TGA GAT CCG -3'). PCR mixtures of 25 µL were as in Casteleyn et al. (2008). Thirty cycles (1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C) were performed, with an initial denaturation step of 7 min at 94 °C and a final elongation step of 10 min at 72 °C. Amplified products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced in both directions using the PCR primers. PCR products that gave ambiguities on certain nucleotide positions through direct sequencing, were cloned into plasmids using the pGEM-T Kit (Promega, Madison, WI, USA). Also PCR products of five strains that gave no ambiguities were cloned as a control (Table 2). Three to ten plasmids per strain were sequenced using the universal vector primers T7 and SP6. Sequencing was conducted in ABI 3130xl DNA (Applied Biosystems, Foster City, CA, USA) automated sequencer with the aid of a Big Dye™ Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Foster City, USA). The sequences were assembled, edited and aligned using BioNumerics (Applied Maths, Belgium) software. The boundaries of the ITS sequences were determined by comparison with Casteleyn et al. (2008).

*rbc*L sequencing and DGGE analysis

Sequences of the plastid gene that encodes for the large subunit of RuBisCo (*rbc*L) were obtained for six strains, two of each ITS ribotype and two hybrids. The *rbc*L gene was amplified using the primers DP*rbc*L1 and DP*rbc*L7 (Daugbjerg and Andersen 1997). PCR mixtures were the same as for the ITS region. Twenty-five cycles (1 min at 94 °C, 1 min at 52 °C, 1.5 min at 72 °C) were performed, with an initial denaturation step of 3 min at 94 °C and a final elongation step of 5 min at 72 °C. PCR products were sequenced using amplification primers and two internal primers 15R and 17R (Jones et al. 2005). Sequences were assembled and aligned with Bionumerics. Inspection of the first, second and third codon positions was conducted using MEGA 3.0 (Kumar et al. 2004) and compared with published sequences (Amato et al. 2007, Kowallik et al. 1995).

Two *rbc*L haplotypes were revealed, differing in four point mutations. Primers *rbc*L-PNp-F (5'- TTA TTA CAC TTA CAC CGT GCT GG -3') and *rbc*L-PNp-R (5'- TTT ACC TAC AAC TGT TCC AGC G -3') were designed to amplify a short part of the *rbc*L (135 bp) comprising two of these mutations. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified *rbc*L fragments was developed to separate the different haplotypes. This allowed a rapid screening of all Pacific strains (Casteleyn et al. 2008 and this study) along with strains of other geographic areas (North Sea and adjoining waters, Denmark, Ireland, Spain, Canada, Japan and New Zealand, Table S1, online supplementary material), without the need to clone and sequence PCR products. The *rbc*L fragments were amplified using a nested PCR protocol: the first PCR was as for sequencing of the *rbc*L gene and the second PCR used the designed primers with the forward primer having a GC-clamp. The PCR program started with a denaturation step of 5 min at 94 °C, followed by 30 cycles. Cycle step times were 30s each for denaturation (94 °C), annealing (53 °C) and extension (72 °C). A final extension step was performed for 10 min at 72 °C. PCR samples were loaded onto 8% polyacrylamide gels, 1 mm thick, in 1×TAE (20 mM Tris–acetate (pH 7.4), 10 mM acetate, 0.5 mM disodium EDTA). The denaturing gradient contained 35-45% denaturant (100% denaturant corresponded to 7 M urea and 40% formamide). Equal amounts of PCR products were applied to the DGGE gel. Electrophoresis was performed for 16 h at 75 V and a temperature of 60 °C. DGGE gels were stained with ethidium bromide and photographed on a UV trans-illumination table with a CCD camera.

The production of PCR artefacts (e.g. chimeras and heteroduplexes) is a potential risk when mixed templates of related sequences are amplified by PCR (Wang and Wang 1996, Thompson et al. 2002). This leads to an overestimation of the genetic variation through the amplification of non-existent sequences (Hugenholtz & Huber 2003, von Wintzingerode et al. 1997). To detect PCR artefacts, DNA of two strains with one

haplotype each (confirmed by sequencing of complete *rbc*L gene) were mixed before PCR amplification (to mimic strains with both *rbc*L haplotypes) and loaded on the DGGE-gel. As a negative control the separate PCR products of these 2 strains were mixed after PCR.

Morphology and morphometrics

In order to evaluate morphological variation and differentiation between *P. pungens* var. *pungens* and *P. pungens* var. *cingulata* Villac and their putative hybrids, frustules of 16 strains (Table 2) were studied. Six strains of ribotype/haplotype I and six strains of ribotype/haplotype II and four hybrids (all with both ribotypes and three with both haplotypes and one with a single haplotype) were studied in detail. The strains were randomly chosen within each group and were all grown in the same culture conditions for the same period before morphological measurements were carried out. Therefore morphological differentiation by phenotypic responses to different environmental conditions can be ruled out. Frustules were cleaned by oxidation with hydrogen peroxide and glacial acetic acid, filtered onto 2 µm pore-size, 25 mm diameter polycarbonate filters (Whatman Inc., Florharm Park, NJ, USA) and repeatedly washed with distilled water. Filters were dried and fixed onto coverslips, which were attached to aluminium stubs using conductive tape, coated with gold, and examined with a JEOL JSM5600LV scanning electron microscope (JEOL, Ltd., Tokyo, Japan) (slides available upon request). Photographs were taken by the SEM videograbber and measurements were done on the digital images using ImageJ software version 1.34s (http://rsb.info.nih.gov/ij/). For morphometric analysis, valve length, width, stria density, fibula density, poroid density and band stria density of the valvocopulae were measured for at least 10 valves per strain. For poroid densities a minimum of 150 measurements for 10 valves were made to reduce measurement errors. In addition, the structure of the valvocopula, which differs between the two varieties (Villac and Fryxell 1998), was studied.

Principal component analysis (PCA) on average valves was carried out to investigate patterns of morphological variation. PCA does not employ any pre-existing classification of objects, but can be used to discover discontinuities between groups of objects. For PCA of morphological measurements, we used CANOCO vs. 4.5 for Windows (Biometrics — Plant Research International, Wageningen, The Netherlands). Prior to analysis, the morphological variables were standardized using the center and standardize option for these variables in CANOCO and the linearity of the response curves was checked by looking at the lengths of gradient of a detrended correspondence analysis which were well below a threshold value of 3 (Leps & Smilauer 2003). Analysis

of Variance (ANOVA) of PCA sample scores on the first and second axes was carried out with the ribotypes used as a priori groups (type 1, type 2, hybrid). Post hoc Tukey's honest significant difference (HSD) tests were used to identify significant differences in PCA scores between the three groups. Since valve length varies considerably through the life cycle in diatoms, correlations between morphological variables and length can confound differences between groups. To include the effects of length on the different morphological variables, we used a "homogeneity of slopes" design to test whether length and ribotype interacted in influencing the putative differences in morphology. Post hoc Tukey's HSD tests were used to identify characters showing significant differences between means of the three groups. In all analyses, $P < 0.05$ was considered significant. All statistical analyses were performed using STATISTICA version 6.0 for Windows (StatSoft, Tulsa, USA).

Results

Molecular analyses

Examination of the electropherograms obtained from direct sequencing of the ITS region of 57 NE Pacific strains of *Pseudo-nitzschia pungens* did not reveal any ambiguous nucleotides for 47 of these strains. The sequences could be readily attributed to either ribotype I (30 strains) or ribotype II (17 strains), differing in three point mutations in ITS1 and six point mutations in ITS2 (Table 1). Uniformity of ITS within each variety was already shown by Casteleyn et al. (2008). For the remaining ten strains ambiguities were encountered on these positions, indicating the presence of intragenomic variation in ITS in these strains. Cloning of these strains revealed three types of sequences within clones: ITS ribotype I and II, and several recombinant ribotypes of the two

Table 1. Nucleotide variable sites between the different rDNA ITS ribotypes found in *P. pungens* var. *pungens* (ribotype I) and var. *cingulata* (ribotype II).

	Nucleotide variable sites											EMBL accession numbers
	ITS1				ITS2							
position	49	100	202	219	452	554	611	643	649	669	684	
ribotype I	G	T	G	C	C	A	G	A	T	T	A	FM207591
ribotype II	G	A	A	T	T	A	A	C	C	C	C	FM207590
recombinant types	G	A/T	A/G	C/T	C/T	A	G/A	A/C	C/T	C/T	A/C	FM207592 - FM207611
novel type II	A	A	A	T	T	T	A	C	C	C	C	FM207612

Table 2. ITS ribotype and *rbc*L haplotype composition of 57 *P. pungens* strains. ITS ribotypes were determined by direct sequencing or after cloning (when ambiguities were revealed through direct sequencing + 5 control strains). For cloned strains, number of sequences type I / number of sequences type II and-or novel type II / number of recombinant sequences, are provided. Complete sequences of *rbc*L were obtained for six strains (two of each ITS ribotype and two hybrids). For the remaining strains the *rbc*L haplotype was determined by DGGE. In bold: strains used for morphometric analysis. Light grey shaded cells show hybrid strains based on ITS and *rbc*L. Dark grey shaded cell shows the strain that was not recognized as intermediate by Adams (2006) but that showed some recombinant ribotypes indicating a past hybridization event.

	ITS		***rbc*L**	
Strains	Sequencing	ITS ribotype	Method of screening	rbcL haplotype
NA2, NA19, NA21, NA98, NA99, NA100, NA102, **NA104**, NA110, NA175, NA180, NA183, NA185, NA203, NA207, NA208, **NA212**, NA236, NA237, NA240, NA243, NA245, NA246, NA247	directly	I	DGGE	I
NA22	after cloning[b]	3/0/0	DGGE	I
NA205	after cloning[b]	7/0/0	DGGE	I
NA169[a]	after cloning[b]	10/0/0	DGGE	I
NA235[a]	after cloning[b]	10/0/0	DGGE	I
NA95, NA213	directly	I	sequencing	I
NA241	after cloning	4/0/2	DGGE	I
NA1, **NA9**, **NA11**, NA14, NA15, **NA16**, **NA172**, NA174, NA199, NA206, **NA211**, NA216, NA234, NA250	directly	II	DGGE	II
NA108, **NA244**	directly	II	sequencing	II
NA177[a]	after cloning[b]	0/7/3	DGGE	both
NA116[a]	after cloning	4/3/3	DGGE	both
NA178[a]	after cloning	2/5/3	DGGE	both
NA179[a]	after cloning	3/6/1	sequencing	both
NA192[a]	after cloning	1/7/2	DGGE	both
NA200[a]	after cloning	4/2/4	DGGE	both
NA204[a]	after cloning	3/7/0	DGGE	I
NA231[a]	after cloning	8/2/0	DGGE	both
NA233[a]	after cloning	3/5/2	DGGE	I
NA242[a]	after cloning	3/7/0	sequencing	I

[a] strains that could not be confidently assigned to one of the two populations found in a STRUCTURE analysis (i.e. with probability of < 0.9 of belonging to one of the two populations) (Adams 2006)

[b] no ambiguities through direct sequencing (control strains)

parental types (Tables 1 and 2). The existence of both ribotypes in these strains indicates a hybrid origin, and we will refer to them as "hybrids" from here on. Apart from some autapomorphies in sequences of both ribotypes (which are typically revealed through cloning, Thornhill et al. 2007), some novel synapomorphic base pair changes were found in ribotype II sequences: two new point mutations (on position 49 in ITS1 and on position 554 in ITS2, Table 1) were found in six strains (17 sequences in total). The

cloned sequences of five control strains (no ambiguities revealed through direct sequencing) were identical to those of the direct sequencing results except for one strain (NA177) for which three sequences of recombinant origin were recovered after cloning of the PCR product (Table 2).

Direct sequencing of the *rbc*L chloroplast gene of six strains revealed the presence of two haplotypes and one sequence (NA179) with ambiguities. The two haplotypes differed in four bases (Table 3), all situated on third codon positions and resulting in synonymous substitions. With DGGE, the five strains having one haplotype gave a single clear band while strain NA179 showed both bands plus some additional bands (Fig. 1, arrowheads). To verify that these additional bands resulted from PCR artefacts, two strains with different haplotypes were mixed together and then amplified by PCR. These PCR products gave the same banding pattern on DGGE as the strain with both haplotypes. When the PCR products of separate haplotypes were mixed and analysed with DGGE no additional bands appeared, confirming that the additional bands were PCR amplification artefacts and could be ignored. The remaining strains were screened with DGGE to determine which haplotype they possess (Fig. 1). Ribotype groups observed in the nuclear ITS sequences corroborated the *rbc*L haplotype groupings (Table 2), except for the hybrid strains. Hybrid genotypes, containing two ribotypes, had either both haplotypes (six strains) or only haplotype I (three strains). Strain NA177, which harboured three mixed ribotype sequences also contained two haplotypes. The 30 var. *pungens* strains from other geographic areas (North Sea and adjoining waters, Denmark, Ireland, Spain, Canada, Japan and New Zealand) were found to consistently contain only haplotype I plastids (Table S1). The seven Pacific strains from a previous study (Casteleyn et al. 2008) showed the haplotype corresponding to their ribotype (Table S1).

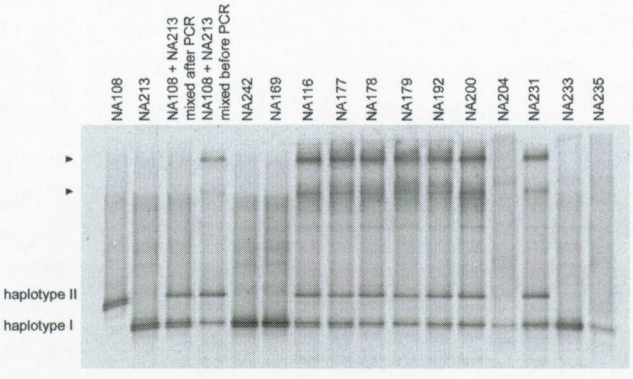

Fig. 1. DGGE gel of *rbc*L fragments of some *P. pungens* strains. Bands represent *rbc*L haplotypes I and II. Arrowheads indicate PCR chimeras.

Table 3. Nucleotide variable sites in the two *rbc*L haplotypes found in *P. pungens* var. *pungens* (haplotype I) and var. *cingulata* (haplotype II). The position numbering corresponds to the complete *rbc*L sequence of *Odontella sinensis* (GenBank accession number NC001713).

	Nucleotide variable sites				EMBL accession numbers
Position	312	660	912	984	
Haplotype I	C	G	T	C	FM207548
Haplotype II	T	A	C	T	FM207547

Morphological analyses

Results of principal components analysis of morphometric data showed that the first and second axes account for 66% and 17% of the total variation respectively. Analysis of variance showed a significant separation ($P = 0.009$) of two groups along the first axis: var. *pungens* and hybrid strains on the left side and var. *cingulata* strains on the right side (Fig. 2). Cell width, stria, fibula, poroid and band striae density contributed most to the separation along the first axis. Length was associated with the second axis but did not result in further differentiation of groups. A homogeneity of slopes model was used to account for size-dependent variations among the three groups (var. *pungens*, var. *cingulata* and hybrids). Univariate tests of significance showed that the effect of length on stria, fibula and band stria densities was different for the three groups (Table 4). In var. *cingulata* length was significantly correlated with stria, fibula and band stria densities, whereas for var. *pungens* strains and hybrid strains no significant correlations were found (P-values > 0.05). Post hoc Tukey HSD tests pointed out that while there was no significant difference in length among the three groups, cell width did significantly differ among them. For the other morphological characters, var. *pungens* and var. *cingulata* are significantly different but only var. *cingulata* could be discriminated from the hybrids (Table 4).

Detailed examination of the band structure of the valvocopula, showed that strains of var. *pungens* had one row of simple large oval poroids (Fig. 3C, see also fig. 4A and B in Casteleyn et al. 2008), while var. *cingulata* strains had square or rectangular poroids which were always occluded by rotae (Fig. 3F, see also fig. 4C in Casteleyn et al. 2008). The bands of the hybrid strains showed poroids with an irregular form, in between the oval and square to rectangular poroids characteristic of each variety. Rotae were usually present except in a few cases but they were less pronounced than in var. *cingulata* strains (Figs. 3I-L).

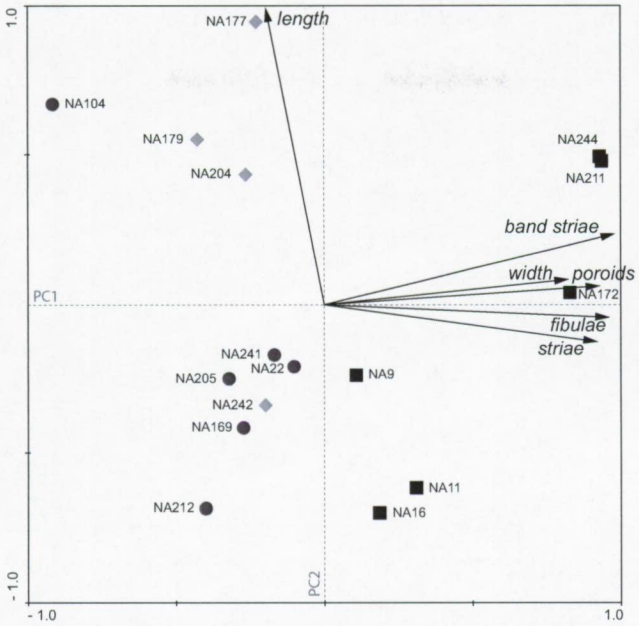

Fig. 2. PCA of morphological measurements on *Pseudo-nitzschia pungens* var. *pungens* (circles), *P. pungens* var. *cingulata* (squares) and hybrids strains (diamonds). The direction of each arrow indicates increasing values of the corresponding morphological parameter. The first two axes are shown, explaining 66% and 17% of total variation respectively.

Table 4. Homogeneity of slopes model (HSM) and Tukey HSD tests for morphometric trait variation between *P. pungens* var. *pungens* (type I), *P. pungens* var. *cingulata* (type II) and hybrids. In bold: statistically significant at $P = 0.05$

	Type I	Type II	Hybrids	HSM *P*-values			Probabilities for Tukey HSD-tests		
	Average ± SD	Average ± SD	Average ± SD	type	length	type * length	between type I and II	between type I and hybrids	between type II and hybrids
Length (µm)	109.5 ± 15.3	105.0 ± 16.3	127.9 ± 18.3				0.86	0.18	0.081
Width (µm)	2.9 ± 0.1	3.8 ± 0.2	3.3 ± 0.3	0.71	0.99	0.91	**0.0004**	**0.043**	**0.033**
Striae density (in 10µm)	11.0 ± 0.6	11.8 ± 0.4	11.0 ± 0.1	0.073	0.83	**0.033**	**0.017**	1.00	**0.028**
Fibulae density (in 10µm)	11.4 ± 0.6	12.2 ± 0.6	11.4 ± 0.2	**0.023**	0.91	**0.0089**	**0.012**	0.95	**0.014**
Poroid density (in 1µm)	3.2 ± 0.1	4.3 ± 0.6	3.2 ± 0.1	0.91	0.42	0.52	**0.0012**	1.00	**0.0026**
Band striae density (in 10µm)	14.4 ± 1.1	19.8 ± 3.8	15.0 ± 0.5	0.10	0.092	**0.026**	**0.0011**	0.85	**0.0052**

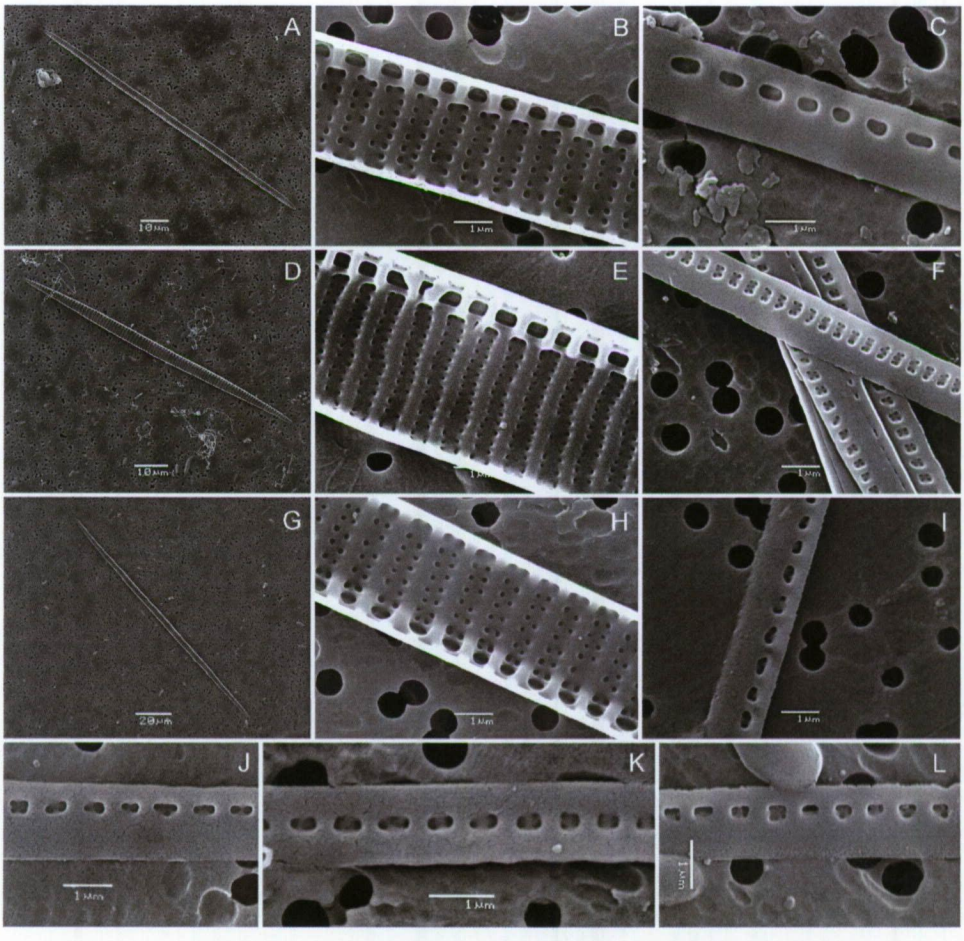

Fig. 3. *Pseudo-nitzschia pungens* frustules and valvocopulae, SEM. **(A–C)** *P. pungens* var. *pungens*. Complete frustule (strain NA205) (A), valve face in internal view (strain NA212) (B) and valvocopula with one row of simple large oval poroids (strain NA104) (C). **(D–F)** *P. pungens* var. *cingulata*. Complete frustule (strain NA11) (D), valve face in internal view (strain NA211) (E) and valvocopula with square or rectangular poroids occluded by rotae (strain NA244) (F). **(G–L)** Hybrid strains. Complete frustule (strain NA177) (G), valve face in internal view (NA179) (H) and valvocopula with more irregular poroids (strains NA177, NA179, NA204 and NA242) (I–L).

Discussion

Genetics

The notion that the two *P. pungens* varieties represent genetically well-differentiated entities (Casteleyn et al. 2008) is reinforced by the observation that, next to having divergent ITS rDNA sequences, they can also be distinguished by diagnostic base pair

differences in the plastid encoding *rbc*L gene. Moreover, the two *P. pungens* populations outlined in a microsatellite study by Adams (2006) using STRUCTURE (Pritchard et al. 2000) also correspond to the two varieties, as was shown in the present study by determining the ITS ribotypes of the same strains, thereby demonstrating that the varieties are also differentiated for these microsatellite loci. In *Pseudo-nitzschia,* the *rbc*L and ITS loci are not always genealogically concordant within species. Amato et al. (2007) demonstrated that in *Pseudo-nitzschia delicatissima* strains two distinct *rbc*L haplotypes could be found whereas all strains have a single ITS genotype. In contrast, *Pseudo-nitzschia pseudodelicatissima*-like strains with different ITS sequences can share the same *rbc*L haplotype (Amato et al. 2007).

The fact that we found strains with both ribotypes together with the uniformity of ITS within each variety (Casteleyn et al. 2008), clearly demonstrates the occurrence of natural hybrids between both *P. pungens* varieties. In a recent study on *Pseudo-nitzschia multistriata*, strains with different ITS types were also shown to hybridize in the field (D'Alelio et al. 2009b). However, in *P. multistriata* there were no indications that the strains with a different ITS type belonged to separate lineages. We can expect that with a better understanding of species boundaries based on sequence data, natural hybrids are likely to be found increasingly in the future.

The hybrid strains in our study also contain novel ITS ribotypes, either resulting from recombination of both ribotypes or characterized by two new point mutations in ribotypes II. The recombinant ribotypes found in our hybrids could either be real recombinants resulting from crossing-over during gamete formation in F_1 or mere PCR artefacts (e.g. chimeras or heteroduplexes) or a combination of both. On the basis of the available data we were not able to distinghuish between these possibilities. However we suspect most recombinants to be PCR artefacts. It was shown that up to 30% of sequences could be chimeras when mixed templates of closely related sequences are amplified by PCR (Wang and Wang 1996) and 9 out of 20 of our recombinants showed mosaic patterns typical for heteroduplexes (Thompson et al. 2002). Yet it is not known whether parental ribotypes after formation of F_1 hybrids are maintained without recombination or whether they are maintained and undergo various degrees of recombination, both possibilities having been demonstrated in plants (Alvarez & Wendel 2003). It is also possible that one ribotype rises to dominance within a genome as a consequence of concerted evolution (Dover 1982, Liao 1999); this may occur as fast as one generation after the combination of two parental ITS types in plants (Aguilar et al. 1999). A way to study the fate of the ITS repeats in hybrids would be by creating artificial hybrids to explore if and how fast concerted evolution acts on the ITS region of early generation hybrids and introgressants. Despite the fact that Casteleyn et al. (2008) demonstrated sexual compatibility within and between the two *P. pungens* varieties, we

have been unable to induce sexual reproduction in our cultures despite multiple trials to cross ribotype I, ribotype II and hybrid strains in all possible combinations under various culture conditions (Tables S2 and S3).

Chloroplast loci are inherited in a different way than nuclear DNA. Based on cytological observations, plastid inheritance has been shown to be biparental in several centric and in all studied pennate diatoms (Jensen et al. 2003, Round et al. 1990). In *P. pungens*, the vegetative cells possess two plate-like chloroplasts. During mitosis they divide clonally and segregate over the daughter cells. In the meiotic cycle, each gamete contains two plastids and the two parental plastids are retained in the zygote, so all four plastids arrive in the initial cell and then segregate two by two during the first mitotic division of the initial cell into the two daughter cells (Chepurnov et al. 2005, chapter 2 of this thesis). The origin of plastids in the F_1 generation was studied in *P. delicatissima* using *rbc*L haplotypes by Levialdi Ghiron et al. (2007). They showed that the F_1 cells inherit either two maternal plastids or two paternal ones, or one paternal and one maternal one in a ratio of 1/6, 1/6 and 2/3 respectively. Our results are in agreement with their findings: hybrid strains (containing two ribotypes) with either 2 haplotypes or only one haplotype were found although no strains with only haplotype II (var. *cingulata*) chloroplasts were recovered. This might be attributed to the low number of hybrid strains examined. Although Levialdi Ghiron et al. (2007) showed that cells with different *rbc*L haplotypes interbreed freely in the laboratory and F_1 hybrids are viable and fertile, no strains with both haplotypes were found in their field samples. This could point to the existence of prezygotic mating barriers in the field. In the present study, heteroplastidial strains were recovered in our field samples from the NE Pacific, indicating that prezygotic barriers are weak in *P. pungens*. Since chloroplasts are biparently inherited we could not use the *rbc*L marker to distinguish the paternal and maternal species.

Analysis of a combination of ITS and *rbc*L did not allow us to discriminate between hybrid classes (F_1, F_2, backcrosses, …), because strains containing both ribo- and one or two haplotypes can obviously be found in each hybrid class and the uncertain origin of the recombinant ribotypes complicates proving the presence of further hybridization and recombination. Nevertheless, comparison with a population genetic study of *P. pungens* strains from the NE pacific using four microsatellite markers (Adams 2006) yields some further insights. That study revealed sixteen strains that could not be confidently assigned to one of the two populations found in a STRUCTURE analysis (i.e. with probability of < 0.9 of belonging to one of the two populations) (Adams 2006). For ten out of twelve of these strains used in the present study, we have now shown that they are the result of hybridization between the two *P. pungens* varieties. Eight of these strains had a probability between 0.4-0.6 of belonging to one or the other population in the STRUCTURE analysis, suggesting that they are first generation hybrids (e.g. Coyer et al.

2007). However, one strain had only ribotype I (through direct sequencing) and both *rbc*L haplotypes, indicative of further hybridization since F_1 hybrids should have both ribotypes. The four other strains had a probability of 0.8-0.9 of belonging to one or the other cluster which points to backcrosses to either one or the other population (e.g. Coyer et al. 2007). Two of them (NA231 and NA242) were recognised as hybrids in our study and according to the STRUCTURE analysis they could be backcrosses to var. *cingulata*. This suggests that not all hybrids are F_1's and that further hybridization is taking place. The presumable backcrosses to var. *pungens* (NA169 and NA235) were not recognised as hybrids in our analysis. We also found one strain that was not recognised as intermediate in the microsatellite analysis but that harboured some recombinant ribotypes in the nucleus which may indicate a past hybridization. These results corroborate the notion that a relatively large number of microsatellite loci (12 at least) are needed to clearly distinguish between backcrosses, F_1 hybrids and purebred parental individuals (Vaha and Primmer 2006).

Morphology

Various morphological features of the frustules were compared between the hybrid strains and the two *P. pungens* varieties. Of the six morphometric characters studied, only valve width was found to be intermediate between the parents in the hybrids. In addition, a detailed examination of the structure of the valvocopula also revealed intermediate characteristics. For the other morphometric characters, the hybrids could not be discriminated from var. *pungens* although both varieties could be readily distinguished. Artificial F_1 progeny between genetically and morphologically closely related species of the diatom *Eunotia bilunaris* were intermediate with respect to all measured morphological characters (Vanormelingen et al. 2008). Despite the fact that most of our hybrids are presumably F_1's, we did not observe an intermediate morphology. It thus appears that factors involved in valve morphology do not cooperate in an additive way in the hybrids. Instead, genetic factors with dominant and/or epistatic effects appear to be involved to a varying degree in the genetic architecture of valve morphology when parental genomes are combined in hybrids. To date, however, it remains an open question how particular genes or interactions between genes effect the development of a morphological/phenotypic character in diatoms.

Conclusions and perspectives

The present study clearly demonstrates the occurrence of natural hybridization between two genetically well-differentiated varieties of *P. pungens* in the NE Pacific. The evolutionary outcome of such hybridization events depends on the hybridization

frequency and the fitness of the resulting progeny, as this will determine the amount of gene flow between lineages (Barton and Hewitt 1985). Further analysis of the spatial and temporal range of the contact zone by extensive sampling along the NE Pacific coast, where both varieties co-occur, and the use of multiple biparentally inherited nuclear markers is necessary to investigate the hybridization frequency and the extent of the hybrid zone. In addition, physiological experiments and detailed studies of population dynamics should be conducted to determine hybrid fitness. Together, these data will allow defining the amount of gene flow between the *P. pungens* varieties and its evolutionary consequences.

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Supplementary tables

Table S1. Strains of *P. pungens* from other geographic areas for which the *rbc*L haplotype is determined by DGGE. ITS sequences and EMBL accession numbers are from a previous study (Casteleyn et al. 2008).

strain	sampling location	sampling date	sample supplier and initial strain designation	*P. pungens* variety	EMBL accession number for ITS	*rbc*L haplotype determined by DGGE
18	North Sea, The Netherlands, Noordwijk	22 May 2002	L. Peperzak, NB-P4 (2002)	var. pungens	AM778738	I
57	North Sea, northern France, Ambleteuse	13 Sep. 2003	F. Leliaert, Ambl - 57	var. pungens	AM778746	I
28	North Sea, Belgium, VLIZ station 130	30 Jun. 2003	K. Muylaert, ZL130 - 28	var. pungens	AM778742	I
V215(3)2	North Sea, Belgium, VLIZ station 215	24 Mar. 2004	Flemish Marine Institute, VLIZ215(24/03/04) - 2	var. pungens	AM778748	I
V700(7)5	North Sea, Belgium, VLIZ station 700	19 Jul. 2004	Flemish Marine Institute, VLIZ700(19/07/04) - 5	var. pungens	AM778758	I
V7(5)10	North Sea, Belgium, VLIZ station 700	4 May 2007	Flemish Marine Institute, VLIZ700(04/05/07)-10	var. pungens		I
V330(3)4	North Sea, Belgium, VLIZ station 330	24 Mar. 2004	Flemish Marine Institute, VLIZ330(24/03/04) - 4	var. pungens		I
V421(10)4	North Sea, Belgium, VLIZ station 421	19 Oct. 2004	Flemish Marine Institute, VLIZ421(19/10/04) - 4	var. pungens		I
S(7)1	Oostend lagoon, Belgium, Spuikom Oostende	13 Jul. 2004	Flemish Environment Agency, Spuikom(13/07/04) - 1	var. pungens	AM778768	I
1	Westerscheldt, The Netherlands, Terneuzen	24 May 2002	K. Sabbe, Trz1	var. pungens	AM778733	I
W4(7)2	Westerscheldt, The Netherlands, Terneuzen	14 Jul. 2004	Netherlands Institute of Ecology, WS1(14/07/04) - 2	var. pungens	AM778775	I
W1(7)1	Westerscheldt, The Netherlands, Breskens	14 Jul. 2004	Netherlands Institute of Ecology, WS1(14/07/04) - 1	var. pungens	AM778769	I
Vlissingen 1	Westerscheldt, The Netherlands, Vlissingen	15 May 2007	G. Casteleyn, vlissingen 1	var. pungens		I
Terneuzen 1	Westerscheldt, The Netherlands, Terneuzen	15 May 2007	G. Casteleyn, terneuzen 1	var. pungens		I
Hfdplaat 1	Westerscheldt, The Netherlands, Hoofplaat	15 May 2007	G. Casteleyn, hfdplaat 1	var. pungens		I
schore 23	Westerscheldt, The Netherlands, Schore	15 May 2007	G. Casteleyn, schore 23	var. pungens		I

Table S1. Continued.

zijpe 1	Oosterscheldt, The Netherlands, Zijpe	15 May 2007	G. Casteleyn, zijpe 1	var. pungens		I
Neeltje Jans 1	Oosterscheldt, The Netherlands, Neeltje Jans	4 July 2007	G. Casteleyn, neeltje jans 1	var. pungens		I
Zeedijk 1	Lake Grevelingen, The Netherlands, Zeedijk	15 May 2007	G. Casteleyn, zeedijk 1	var. pungens		I
Osse 1	Lake Grevelingen, The Netherlands, Osse	15 May 2007	G. Casteleyn, osse 1	var. pungens		I
Denmark 1	Limfjord, Denmark, Løgstær	27 Aug. 1997	N. Lundholm, L7	var. pungens		I
Ierse Zee H8-1	Irish Sea, Ireland, Belfast Harbour	Jul. 2007	V. Creach, HAB8-1	var. pungens		I
Vigo-1	E Atlantic, Spain, Bay of Vigo	1 Apr. 2004	M. Lastra	var. pungens	AM778776	I
Cn-218	NW Atlantic, Canada, Boughton River, Prince Edward Island	7 Sep. 2004	S. Bates, CL-218	var. pungens	AM778794	I
Cn-2	NW Atlantic, Canada, March Water, New London Bay, Prince Edward Island	21 Nov. 2005	S. Bates, IS-2	var. pungens		I
Cn-254	NW Atlantic, Canada, Miramichi Bay, New Brunswick	18 Sep. 2007	S. Bates, CL-254	var. pungens		I
Jp-01	NW Pacific, Japan, Ofunato Bay, Iwate Prefecture	7 Aug. 2000	Y. Kotaki, OFPp 001	var. pungens	AM778812	I
OFPp06-54	NW Pacific, Japan, Ofunato Bay, Iwate Prefecture	13 Nov. 2006	Y. Kotaki, OFPp06-54	var. pungens		I
NZ-74	SW Pacific, New Zealand, Taylor's Mistake, South Island	2004	L. Rhodes, CAWB74	var. pungens	AM778817	I
NZ25	SW Pacific, New Zealand, Gisborne Harbour, North Island	16 Apr. 2007	L. Rhodes, NZ25	var. pungens		I
US-94	NE Pacific, North America, Sequim Bay State Park, WA	8 Aug. 2002	B. Bill, NWFSC-094	var. pungens	AM778798	I
US-96	NE Pacific, North America, Sequim Bay State Park, WA	8 Aug. 2002	B. Bill, NWFSC-096	var. pungens	AM778803	I
US-135	NE Pacific, North America, ECOHAB I (coastal Washington)	Jun. 2003	B. Bill, NWFSC-135	var. cingulata	AM778811	II
US-132	NE Pacific, North America, ECOHAB I (coastal Washington)	Jun. 2003	B. Bill, NWFSC-132	var. cingulata	AM778810	II
US-115	NE Pacific, North America, ECOHAB I (coastal Washington)	Jun. 2003	B. Bill, NWFSC-115	var. cingulata	AM778804	II
US-93	NE Pacific, North America, Eld Inlet, Mud Bay, WA	17 Jun. 2002	B. Bill, NWFSC-093	var. cingulata	AM778796	II
US-77	NE Pacific, North America, La Push, WA	18 Sep. 2001	B. Bill, NWFSC-077	var. cingulata	AM778795	II

Table S2. Culture conditions used to induce sexual reproduction in all pairwise combinations within and between ribotype I and ribotype II strains (for a list of strains used see Table S3).

conditions	
light	10-25-50-100 µmol photons m^{-2} s^{-1} Natural daylight – window on the north 24h or 48h dark arrest continous light
Nutrient concentrations	100%, 50%, 25%, 10% f/2 filtered seawater without addition of nutrients L1 medium (contains additional trace metals in comparison with f/2)
temperature	8-22°C
salinity	20 – 30 – 35 - 40 psu
pH	7,5 – 7,8 – 8 – 8,3
Culture density	Low versus high
Culture volume	96-, 24-, 6- well plates (0.025 – 3 -15 ml), 50 ml culture flasks

Table S3. Cell size of *P. pungens* strains at first crosses. Trials to cross strains were done until the cultures were below the viable size, also for the not measured cultures (nm). Length measurements were done at 14.02.2006. Monthly attempts were conducted to cross ribotype I, ribotype II and hybrid strains in all possible combinations in the same culture conditions successfully applied previously (Chepurnov et al. 2005, Casteleyn et al. 2008). Upper sexual size threshold for North Sea strains = 115 µm (Chepurnov et al. 2005).

ribotype	strain	Mean cell length in µm (n=5)
I	NA2	92.5
	NA19	90
	NA21	67.5
	NA22	102.5
	NA95	137.5
	NA98	130
	NA99	137.5
	NA100	140
	NA102	142.5
	NA104	135
	NA110	100
	NA169	96
	NA175	122
	NA180	100
	NA183	135
	NA185	128.5
	NA203	130
	NA205	105
	NA207	117.5
	NA208	137.5
	NA212	90
	NA213	127.5
	NA235	nm
	NA236	107.5
	NA237	135

Table S3. Continued.

I	NA240	97.5
	NA243	135
	NA245	140
	NA246	nm
	NA247	nm
hybrid?	NA241	102.5
II	NA1	67.5
	NA9	110
	NA11	82.5
	NA14	77.5
	NA15	82.5
	NA16	85
	NA108	120
	NA172	100
	NA174	117.5
	NA199	nm
	NA206	nm
	NA211	117.5
	NA216	nm
	NA234	nm
	NA244	121
	NA250	nm
hybrids	NA116	nm
	NA177	142.5
	NA178	nm
	NA179	118.5
	NA192	nm
	NA200	nm
	NA204	127.5
	NA231	nm
	NA233	nm
	NA242	96

5

Lack of population genetic structuring in *Pseudo-nitzschia pungens* in a heterogeneous area in the Southern Bight of the North Sea[1]

Several marine holoplanktonic organisms show a high degree of geographically structured diversity for which it often remains unclear to what extent this differentiation is due to the presence of cryptic taxa. For the genetically distinct diatom *Pseudo-nitzschia pungens* var. *pungens*, we used six microsatellite markers to investigate the spatial and temporal genetic composition in the heterogeneous Southern Bight of the North Sea. Although our sampling area (ca. 100 km) comprised water bodies with different environmental conditions (marine, estuary, saline lake) and different degrees of connectivity (from complete isolation to supposedly free exchange between environments), no evidence of genetic differentiation was found. Expanding our sampling area (ca. 650 km), suggested a homogenous population structure over even larger areas in the North Sea. Our results suggest that the population structure of this diatom is mainly shaped by strong homogenizing effects of gene flow preventing genetic drift, even in water bodies with limited connectivity.

[1] Accepted manuscript: Casteleyn G, Evans KM, Backeljau T, D'hondt S, Chepurnov VA, Sabbe K, Vyverman W. Lack of population genetic structuring in the marine planktonic diatom *Pseudo-nitzschia pungens* (Bacillariophyceae) in a heterogeneous area in the Southern Bight of the North Sea. Marine Biology.

Introduction

Marine organisms with high dispersal potential are expected to show little spatial variation in genetic structure given the perceived lack of geographical barriers in the sea. However, recent studies on genetic variation within marine taxa with free-living larval stages have shown that many are more structured than would be expected (Palumbi 1994; Barber et al. 2000; Hellberg et al. 2002). Even in marine holoplanktonic taxa, which have very large population sizes and drift in water currents throughout their life cycle, a high degree of geographically structured species and population diversity has been shown (e.g. de Vargas et al. 1999; Bucklin et al. 2000; Darling et al. 2004; Goetze 2005; Peijenburg et al. 2006; Rynearson and Armbrust 2004; Rynearson et al. 2006; Nagai et al. 2007; Darling and Wade 2008). To understand how this diversity in marine species, and in planktonic organisms in particular, could have arisen, a better understanding of the spatial and temporal scales at which populations are genetically structured is necessary.

Population genetic surveys using sufficiently discriminatory molecular markers can provide important information on the genetic diversity within and the connectivity between populations. However, in order to interpret such information, it is essential to distinguish between intraspecific population structuring and cryptic taxonomic diversity, as these two issues may mimic each other. It is therefore important to elucidate species boundaries before assessing variation at the population level. Detailed analyses of species limits have been conducted for the marine, planktonic diatom *Pseudo-nitzschia* through a combination of morphological, molecular and breeding data (Amato et al. 2007; Casteleyn et al. 2008). For *Pseudo-nitzschia pungens*, analysis of rDNA internal transcribed spacer (ITS) sequences from samples worldwide revealed three distinct genetic entities, corresponding to the morphological entities var. *pungens*, var. *cingulata* and var. *aveirensis* with different geographical distributions (Casteleyn et al. 2008; Churro et al., in press).

P. pungens var. *pungens* appears to be distributed globally in temperate areas, and ITS and *rbc*L sequence data strongly suggest genetic homogeneity within this variety (Casteleyn et al. 2008; Casteleyn et al. 2009). As for most diatoms (Chepurnov et al. 2004), *P. pungens* has a diplontic life cycle, with a prolonged vegetative phase (in which the cells divide mitotically) and a short obligate stage of sexual reproduction (Chepurnov et al. 2005). Sexual compatibility between *P. pungens* var. *pungens* isolates from widely separated populations has been shown, demonstrating the potential for global gene-flow between populations (Casteleyn et al. 2008). At a much smaller spatial scale (ca. 100 km) over a period of 18 months, a population genetic study of 464 isolates of *P. pungens* var. *pungens* using six nuclear microsatellite loci, revealed a single, largely unstructured

population in the homogeneous open-water environment of the German part of the North Sea (Evans et al. 2005).

In this study we investigate the spatial and temporal genetic composition of *P. pungens* var. *pungens* in the environmentally more heterogeneous Southern Bight of the North Sea with the same six nuclear microsatellite markers used by Evans et al. (2005). This area is known to only harbor var. *pungens* (Casteleyn et al. 2008; Casteleyn et al. 2009). We isolated 310 strains in spring 2007 from water bodies with different environmental conditions (marine, estuary, saline lake) and with different degrees of connectivity between them (from complete isolation to supposedly free exchange between environments) over a scale of ca. 100 km. We were particularly interested to know if different environments or partial isolation could restrict gene flow between populations. Within this area genetically well-differentiated populations of marine fish with planktonic larval stages (Pampoulie et al. 2004) and of free-living nematodes with expected passive dispersal capacity through rafting on decomposing algae (Derycke et al. 2005, 2007a) were already documented. To assess temporal differentiation, we compared a marine population from the Belgian North Sea in 2004 with one from 2007. In addition, we estimated differentiation on a larger geographic scale (ca. 650 km) to assess if and on what scale *P. pungens* var. *pungens* populations could be differentiated in the North Sea.

Materials and Methods

Sampling locations and isolation of strains

Pseudo-nitzschia pungens cells were isolated in spring 2007 from planktonic samples taken at ten locations in four different water entities: the Belgian North Sea (station 120 and 700), the Westerschelde Estuary (Vlissingen, Hoofdplaat, Terneuzen and Schore), the Oosterschelde Estuary (Neeltje Jans and Zijpe) and Lake Grevelingen (Den Osse and Zeedijk) (Fig. 1, Table 1). Different degrees of connectivity are present in our sampling design: The North Sea and Westerschelde are in open connection with each other, while the Oosterschelde is occasionally separated from the sea by the storm surge barrier and Lake Grevelingen is isolated from the North Sea by geographical and man-made barriers (The Grevelingendam).

The four areas also exhibit different environmental conditions. (1) The coastal region of the Belgian North Sea is characterised by shallow waters (<20m depth) with a vertically homogeneous water column (Lee, 1980) with relatively high concentrations of suspended particulate matter (Van Raaphorst et al., 1998) and overall high nutrient

concentrations (De Galan et al., 2004). (2) The Westerschelde is a turbid, eutrophic estuary that is heavily polluted by domestic and industrial waste (De Wolf et al. 2000). Maximum tidal currents are 1–1.5 m s^{-1} and the average residence time of the water is about 75 days (Heip 1989), causing a gradual salinity gradient. The gradient of decreasing pollution towards the sea is paralleled with an increasing downstream salinity gradient. (3) The Oosterschelde is since the construction of the storm surge barrier in 1987 a relatively clean tidal basin with a more or less constant salinity (Gerringa et al. 1996). Since then, the inflow of North Sea water has decreased and the tidal height differential reduced leading to decreasing current velocities and an increasing water transparency (Smaal and Nienhuis 1992). The water residence time varies between 10 days in the western part and 100 to 200 days in the eastern parts. (4) Lake Grevelingen is cut off from the North Sea since 1971 and has become a nontidal saltwater lake with fairly constant salinity and with a water residence time of circa 10 years (Nienhuis 1978).

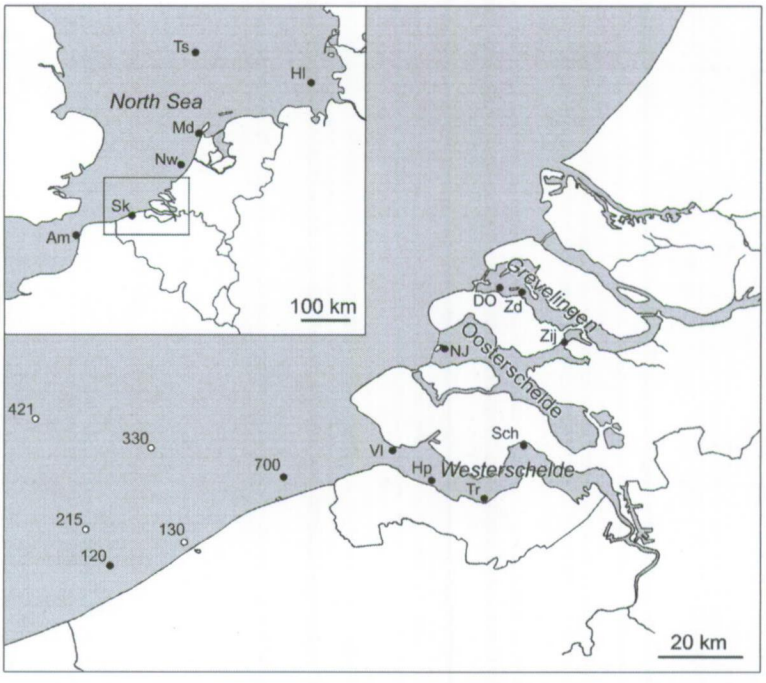

Fig. 1. Sampling locations. The numbers indicate the sampling station according to the Flanders Marine Institute's (VLIZ) monitoring campaign (http://www.vliz.be/). Vl: Vlissingen, Hp: Hoofdplaat, Tr: Terneuzen, Sch: Schore, NJ: Neeltje Jans, Zij: Zijpe, DO: Den Osse, Zd: Zeedijk, Hl: Helgoland, Ts: Terschelling, Md: Marsdiep, Nw: Noordwijk, Sk: Spuikom, Am: Ambleteuse.

Table 1. Details of the *P. pungens* var. *pungens* isolates used in this study: sampling location (Fig. 1) and date, number of isolates sampled and number of unique multilocus genotypes (MLGs) per sampling location (for more details see Supplementary Table S1).

waterbody	sampling location	sampling date	number of isolates	number genotyped	Number of unique MLGs
Isolates from 2007					
Belgian North Sea	120	04.05.07	52	52	50
	700	04.05.07	52	52	50
Westerschelde	Vlissingen	15.05.07	22	22	22
	Hoofdplaat	15.05.07	22	22	22
	Terneuzen	15.05.07	22	22	22
	Schore	15.05.07	22	22	22
Oosterschelde	Neeltje Jans	04.07.07	53	53	47
	Zijpe	15.05.07	18	18	16
Lake Grevelingen	Den Osse	15.05.07	11	11	11
	Zeedijk	15.05.07	36	36	35
Isolates from 2000-2006					
Belgian North Sea	120	24.03.04 – 16.08.04	16 [a]	15	15
	700	19.04.04 – 19.07.04	17 [a]	17	16
	215	24.03.04 – 18.10.04	15 [a]	12	12
	421	29.03.04 – 19.11.04	23 [a]	19	19
	330	24.03.04	4 [a]	4	4
	130	30.06.03	3 [a]	3 [b]	3
	120	27.03.06	3 [a]	3 [b]	3
	215	27.03.06	1 [a]	1 [b]	1
Coastal lagoon	Oostende, Spuikom	07.04.04	10 [a]	10 [b]	9
German North Sea	Helgoland	*.*.03	10	10 [b, c]	10
Dutch North Sea	Terschelling	01.05.00	1 [a]	1 [b]	1
	Marsdiep	25.04.02	1 [a]	1 [b]	1
	Noordwijk	22.05.02	3 [a]	3 [b]	3
French North Sea	Ambleteuse	13.09.03	3 [a]	3 [b]	3
total			420	412	397

[a] strains used for mating tests and ITS rDNA sequencing in Casteleyn et al. (2008)
[b] only used in STRUCTURE analysis
[c] strains used in the study of Evans et al. 2005

P. pungens isolates from a monthly sampling in the Belgian North Sea in 2004, together with isolates from the Belgian North Sea in 2003 and 2006, a coastal lagoon (Oostende, Spuikom), the French North Sea (Ambleteuse) and the Dutch North Sea (Terschelling, Marsdiep, Noordwijk) from a previous study (Casteleyn et al. 2008) were

also included (Fig. 1). In addition, ten randomly chosen isolates from Evans et al. (2005) from the German North Sea (Helgoland) were used (Table 1). Detailed information about the sampling locations is available in Supplementary Table S1.

Isolation of *P. pungens* cells and culture conditions were as described in Casteleyn et al (2008). All isolations were done by the same person (Victor Chepurnov) using consistent isolation procedures within 24 hours after sampling (to reduce post-sampling divisions).

Determination of genotypes

Cells from 50 mL cultures in exponential growth were harvested by centrifugation and stored at -80°C. Genomic DNA was extracted from pelleted cells using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol.

Six nuclear microsatellite loci (PP1, PP2, PP3, PP4, PP5 and PP6) were amplified with primers developed by Evans and Hayes (2004). These loci were amplified in separate PCR reactions with fluorescently labeled primers (6FAM, VIC, NED; Applied Biosystems, Foster City, USA) following PCR conditions as described in Evans and Hayes (2004) with some modifications. PCR mixtures contained 1–2 µL of template DNA, primers at a concentration of 0.5 µM, deoxynucleoside triphosphates at 200 µM each, bovine serum albumin (BSA) at 0.4 µg μL^{-1}, 2 µL of 10× PCR buffer (GeneAmp® 10× PCR Buffer II, Applied Biosystems), 2.5 or 3 mM $MgCl_2$ for PP1, PP3, PP4, PP5, PP6 or PP2, respectively, and 2.5 units of Taq polymerase (AmpliTaq, Perkin-Elmer, Wellesley, USA); mixtures were adjusted to a final volume of 20 µL with sterile water (Sigma, St-Louis, USA). The cycling conditions were: initial denaturation at 94°C for 3 min; 30 cycles of 1 min at 94°C, 1 min at 56°C (PP1, PP3, PP4) / 60°C (PP2) / 55°C (PP5, PP6) and 30s at 72°C; and a final elongation at 72°C for 30 min.

Three different fluorescently labeled PCR products were combined prior to fragment detection. One µl of the PCR mixture was added to 8.5 µl of formamide and 0.5 µl of internal size standard GS 600LIZ (Applied Biosystems). The resulting mixture was denaturated for 2 min at 95°C, placed on ice and electrophoresed for 50 min on an ABI 3130xl (Applied Biosystems) genetic analyzer. If the signal was weak or ambiguous, the PCRs were replicated to ensure accurate designation of alleles. To reduce genotyping errors, positive and negative controls were included and scoring of alleles/genotypes was done by the same person, using GENEMAPPER 4.0 software (Applied Biosystems).

Analysis of microsatellite data

Characterization of microsatellite loci and genetic diversity

For characterization of the microsatellite loci, we focused on 310 isolates from 2007. Potential scoring errors due to stuttering (a PCR artifact caused by slippage of the polymerase during amplification), or large allele dropout (the preferential amplification of small alleles) were assessed using the program MICRO-CHECKER (Van Oosterhout et al. 2004). To identify identical multilocus genotypes (MLGs), the Excel add-in MS TOOLS (Park 2001) was utilized. Clonal diversity was assessed by comparing the number of unique MLGs with the total number of MLGs. GIMLET 1.3.3 (Valiere 2002) was used to assess the resolving power of the microsatellite markers and to calculate matching probabilities of isolates with identical MLGs. The probability of identity, $P_{(ID)}$ (the probability of sampling two unrelated individuals from the same population with identical genotypes) and the probability of identity among siblings, $P_{(ID)sib}$ (this takes into account the genetic similarity among related individuals and is used as a conservative upper bound for the probability of observing identical genotypes between two individuals sampled from a population, Waits et al. 2001) were calculated for each locus and all loci combined. Waits et al. (2001) suggest that $P_{(ID)}$ or $P_{(ID)sib}$ values between 0.01 and 0.0001 indicate that the markers possess sufficient resolving power.

Numbers of alleles, allele frequencies, observed heterozygosity (H_o), and expected heterozygosity (H_e), were calculated for each locus using GENEPOP 4.0 (Rousset 2008). Since estimates of the number of alleles in a population are highly dependent on sample size, ARES (Van Loon et al. 2007) was used to assess what fraction of alleles was captured with our sample size. ARES is based on a model that extrapolates an incidence-based accumulation. Using this model, allelic richness estimates can be obtained with confidence bounds for any sample size.

GENEPOP 4.0 (Rousset 2008) was used to detect deviations from Hardy-Weinberg equilibrium (HWE) at each locus and across all loci. Exact tests (Guo and Thompson 1992) with a markov chain method were conducted to estimate the exact *P*-values at each locus (dememorisation number: 10000, 300 batches with 5000 iterations per batch; to keep the standard errors < 0.01). Fisher's exact tests were performed to test HWE across loci. F_{IS} was used as an indicator of heterozygote excess or deficit (Weir and Cockerham 1984). Exact tests for linkage disequilibrium (LD) between loci were also performed using GENEPOP 4.0 as outlined above. Isolates with identical MLGs were excluded from tests of LD, since clonal reproduction mimics LD between loci (Halkett et al. 2005). In all cases where multiple tests were run, corrected *P*-values were calculated using the sequential Bonferroni technique at the 5% significance level (Rice 1989).

Population structure

Population structure was assessed by two different approaches: (1) using predefined populations that may be genetically distinct and (2) using no a priories with respect to population subdivision. Only isolates with unique MLGs were included in population structure analysis.

To assess spatial differentiation, the 310 isolates of 2007 were grouped (1) into four populations corresponding with the four water bodies on a geographic scale of ca. 100 km and (2) into 10 populations corresponding to the sampling locations that were separated from each other by 5-100 km. Temporal differentiation was assessed by comparing cells isolated from the Belgian North Sea in 2007 to isolates from the Belgian North Sea in 2004 (Table 1). Exact tests of allelic and genotypic differentiation were applied to each locus between all pairs of predefined populations and overall *P*-values across loci were calculated according to Fisher's method. These analyses were done with GENEPOP, using default settings. Weir and Cockerham's (1984) estimates of Wright's (1951) *F*-statistics were calculated in FSTAT 2.9.3 (Goudet 2001). Permutation tests were used to determine whether F_{ST} values were significantly different from zero. *P*-values were corrected using the sequential Bonferroni technique.

To infer population structure without a predefined population subdivision, a Bayesian clustering analysis was conducted using STRUCTURE 2.2 (Pritchard et al. 2000), which probabilistically assigns individuals to populations based on their MLGs. In this analysis all isolates were used, including isolates from 2007 (297 isolates with unique MLGs), from the Belgian North Sea in 2003-2006 (73 isolates), a Belgian coastal lagoon (9 isolates), from the Dutch North Sea (5 isolates), the French North Sea (3 isolates) and the German North Sea (10 isolates) covering a geographic scale of ca. 650 km (Table 1).

The most likely number of populations (*K*) was estimated by performing 10 independent runs for each value of *K* from 1 to 24 (corresponding with the number of sampling locations) with a burn-in and run length of 100,000 repetitions and using the model with correlated allele frequencies, non-informative priors and assuming admixture. Examination using the parameter for independent allele frequencies did not qualitatively change the results. The posterior probability was then calculated for each mean value of *K* over 10 runs using the mean estimated log-likelihood of *K*. The estimated "Ln Probability of Data (Ln P(D))" for each *K* was used as a criterion to infer the number of clusters represented by our sample (Pritchard et al. 2007). In addition, a principal coordinate analysis (PCO) was used to identify clusters based on multilocus similarity among isolates. Pairwise linear genetic distances (Peakall et al. 1995) between all MLGs were calculated in GENALEX v. 6 (Peakall and Smouse 2006) and then subjected to PCO in GENALEX.

Results

Characterization of microsatellite loci and genetic diversity

All 310 isolates collected in 2007 were successfully genotyped at all six loci (Table 2). For the remaining 110 isolates (Table 1), eight could not be confidently genotyped and nine isolates had missing data at one locus (seven isolates for PP4, one isolate for PP1 and one isolate for PP6) and one isolate had missing data at two loci (PP1 and PP3). Analysis with MICRO-CHECKER indicated no evidence for scoring errors.

Of the 310 MLGs from 2007, 296 were different, corresponding to a clonal diversity of 95% (Table 1). Isolates sharing a MLG were isolated from the same sampling location at the same time except in one case where isolates originated from different water bodies sampled 11 days apart, i.e. from the North Sea (station 120) and Lake Grevelingen (Zeedijk). The 14 pairs of isolates with identical MLGs had $P_{(ID)}$ and the $P_{(ID)sib}$ values ranging from 8.05×10^{-12} to 4.57×10^{-6} and from 2.54×10^{-3} to 1.05×10^{-2}, respectively (Table S2). For 12 pairs, the matching probabilities were well below the upper limit of 0.01 as suggested by Waits et al. (2001) ($\leq 9.47 \times 10^{-3}$, Table S2), indicating that these pairs of isolates were likely the result of asexual reproduction. For the pairs from station 700 and Neeltje Jans, the values slightly exceeded this upper limit (0.0105 and 0.0101, respectively) so it is uncertain whether these strains were truly clonal. Nevertheless, the six microsatellite loci possessed enough resolving power to distinguish individuals, because the $P_{(ID)}$ and the $P_{(ID)sib}$ values across all loci (1.80×10^{-6} and 6.52×10^{-3}, respectively) were clearly within the limits suggested by Waits et al. (2001) (Table 1).

Table 2. Number of *Pseudo-nitzschia pungens* var. *pungens* isolates genotyped (*n*), size ranges of the alleles (bp), number of alleles (N_A), observed (H_o) and expected (H_e) heterozygosity, F_{IS} according to Weir and Cockerham (1984) and probabilities of identity ($P_{(ID)}$ and $P_{(ID)sib}$) for each locus and across loci.

locus	n	bp	N_A	*Ho*	*He*	F_{IS}	$P_{(ID)}$	$P_{(ID)sib}$
PP1	310	217-246	12	0.71	0.74	0.04	9.58E-02	4.04E-01
PP2	310	166-248	10	0.63	0.60	-0.05	2.42E-01	5.12E-01
PP3	310	191-254	17	0.86	0.86	0.00	3.36E-02	3.30E-01
PP4	310	133-209	15	0.80	0.84	0.04	4.09E-02	3.42E-01
PP5	310	186-198	5	0.54	0.54	0.01	2.44E-01	5.41E-01
PP6	310	195-236	5	0.58	0.59	0.00	2.31E-01	5.16E-01
across loci			64	0.69	0.69	0.01	1.80E-06	6.52E-03

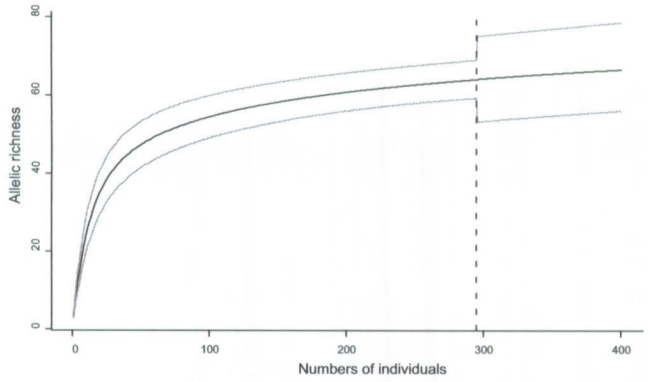

Fig. 2. Mean allelic richness curve with 95% confidence bounds, computed with ARES which extrapolates beyond the sample size (based on 297 unique multilocus genotypes).

Allele size ranges, numbers of alleles, observed and expected heterozygosities are shown in Table 2. In total 64 alleles were recorded for 310 isolates across six loci. The ARES analysis indicated that a vast amount of alleles were recovered with our sample size (Fig. 2). Neither significant deviations from HWE nor significant linkage disequilibria were observed at any locus (results not shown).

Population differentiation

F_{ST} values between the four water bodies were very small (max. 0.0038) and not significantly different from zero (Table 3). Likewise, no significant differentiation was found between the ten sampling locations, with highest $F_{ST} = 0.0077$ between Zijpe and Schore (Table 4). Results of allelic and genotypic differentiation tests were consistent with F statistics. Comparison between Belgian North Sea isolates of 2004 (66 isolates) and 2007 (100 isolates) revealed a non-significant F_{ST} value of 0.0046 ($P = 0.05$), whereas allelic and genotypic tests over all loci were significant ($P = 0.007$ and $P = 0.019$, respectively). This was due to allelic heterogeneity at loci PP4 and PP6 ($P = 0.030$ and $P = 0.027$, respectively) and genotypic heterogeneity at locus PP6 ($P = 0.047$).

The STRUCTURE analysis of the complete data set (Table 1) revealed no population structuring. The most likely number of subpopulations identified was $K = 1$ and higher values of K returned lower likelihoods (results not shown). Also the proportion of a strain assigned to each population was roughly symmetric, corresponding to $1/K$ in each population, indicating that no real population structure is present (Pritchard et al. 2007). The PCO of this data set showed much overlap among sampling locations, indicating no clear substructuring, consistent with the results of the STRUCTURE analysis (Fig. 3).

Table 3. F_{ST} values between the four waterbodies in 2007. No value was significantly different from zero after sequential Bonferroni correction. Negative F_{ST} values can be caused by corrections for unequal sample size in the method of Weir and Cockerham (Feder et al. 1990). NS: North Sea, WS: Westerschelde, OS: Oosterschelde, LG: Lake Grevelingen. In parentheses, the number of unique multilocus genotypes.

	WS (88)	OS (63)	LG (46)
NS (100)	-0.0007	0.0033	-0.0022
WS (88)		0.0027	-0.0025
OS (63)			0.0038

Table 4. F_{ST} values between the ten locations sampled in 2007. No value was significantly different from zero after sequential Bonferroni correction. In parentheses, the number of unique multilocus genotypes. Sample location abbreviations as in Fig. 1.

	700 (50)	Vl (22)	Hp (22)	Tr (22)	Sch (22)	NJ (47)	Zij (16)	DO (11)	Zd (35)
120 (50)	0.0015	-0.0067	-0.0029	0.0026	-0.0089	0.0037	0.0038	-0.0043	-0.006
700 (50)		0.0043	0.0034	0.0025	0.0005	0.0017	0.0031	0.0033	-0.0002
Vl (22)			-0.0029	-0.0002	-0.0016	-0.0032	0.0031	-0.0022	-0.0043
Hp (22)				-0.001	-0.0023	0.003	-0.0042	-0.0025	-0.0027
Tr (22)					-0.0004	0.0019	0.0005	-0.0034	-0.0015
Sch (22)						0.0045	0.0077	-0.0128	-0.006
NJ (47)							-0.0042	0.0063	0.0014
Zij (16)								0.0036	0.0008
DO (11)									-0.0046

Discussion

In total 64 alleles were recorded in 310 *P. pungens* var. *pungens* isolates across six loci (average of 10.7 alleles per locus), whereas Evans et al. (2005) found 77 alleles in 464 isolates from the German part of the North Sea (average of 12.5 alleles per locus). This small difference could be attributed to the larger sample size in the latter study as indicated by the ARES analysis. Levels of overall heterozygosity (mean H_e = 0.69) were similar to Evans et al. (2005) (mean H_e = 0.73). As in our study, no deviations of HWE were detected by Evans et al. (2005).

These high levels of genetic variation are consistent with the fact that sexual reproduction is firmly integrated into the life cycle of diatoms. Because diatoms gradually reduce their cell size in the course of their mitotic (clonal) part of the life

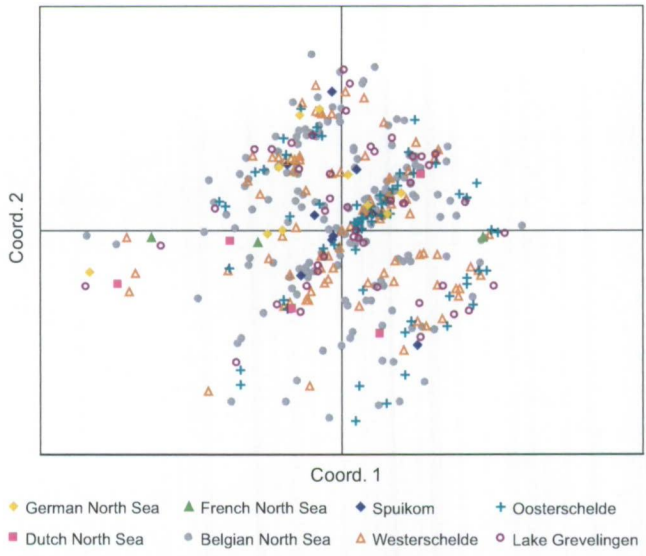

Fig. 3. Principal coordinate analysis based on multilocus distance among isolates. Each point on the figure corresponds to a multilocus genotype.

cycle, they are forced to undergo periodical sexual events to re-establish their initial cell size (Chepurnov et al. 2004). The high genetic variation can furthermore be explained by the fact that *P. pungens* is heterothallic and therefore outbreeding (Chepurnov et al. 2005). We detected 296 isolates with unique MLGs out of 310 isolates (95%). This is comparable with the clonal diversity found for this species in the German part of the North Sea (98%; Evans et al. 2005) and with another microsatellite analysis based on three loci of the marine diatom *Ditylum brightwelli* in two connected estuaries in the NE Pacific (87%; Rynearson and Armbrust 2005). Most isolates with identical MLGs in this study were likely members of a clonal lineage, as indicated by the low P_{ID} values for the matching pairs.

We found no evidence for spatial differentiation in our sampling area, while allelic and genotypic tests suggested slight temporal variation between the Belgian North Sea isolates from 2004 and 2007. Yet, this differentiation is not very pronounced as the degree of temporal genetic differentiation (F_{ST} = 0.0046, P = 0.05) was similar to the spatial differentiation between the ten sampling locations in 2007 (highest F_{ST} = 0.0077 between Zijpe (Oosterschelde) and Schore (Westerschelde)). Similarly, Evans et al. (2005) only found marginally significant differentiation between the two sampled locations in the German part of the North Sea, Helgoland and Sylt, separated by ca. 100 km (F_{ST} = 0.0016, P = 0.022). They analyzed samples from three periods (spring 2002, spring 2003 and autumn 2003) and only found slight temporal heterogeneity between

spring 2003 and autumn 2003 (F_{ST} = 0.0018, P = 0.013). Overall the authors concluded that there was neither a marked spatial nor temporal pattern of genetic structure (Evans et al. 2005). This is in agreement with our findings, despite the fact that our sampling area comprised water bodies with different environmental conditions and different degrees of connectivity. The migration rate (gene flow) is apparently high enough to make the sampling area of ca. 100 km act as a single unstructured population. The water residence times are presumably not long enough to result in population differentiation in this highly dynamic region. Residence times of about 10-20 days were calculated for the stations from Vlissingen to Schore (Westerschelde) (Soetaert and Herman 1995) and comparable residence times were documented for the western part of the Oosterschelde, viz. Neeltje Jans, but residence times for Zijpe (Oosterschelde) are higher (up to 100 days, Gerringa et al. 1998). In Lake Grevelingen, which is disconnected from the sea, residence times are much higher (circa 10 years, Nienhuis 1978) but also no differentiation was detected. The fact that clonal MLGs were found in the disconnected water bodies of the North Sea and Lake Grevelingen, indicates that there is either enough inflow of North Sea water (for example during occasional storms), or that migration of *P. pungens* is not restricted to marine currents, but may also be mediated by human activity or via other vectors, such as birds.

Alternatively, *P. pungens* populations may not yet have had the time to differentiate as the Oosterschelde and Lake Grevelingen were only separated from the North Sea since 1970s-1980s and drift processes may be very slow in plankton populations with presumed high population sizes. However, genetically well-differentiated populations of fish and nematodes have recently been documented in the same area (Pampoulie et al. 2004; Derycke et al. 2005, 2007a). For example, the marine goby, *Pomatoschistus minutus*, was expected to be genetically homogenous because of the high dispersal ability of its larvae and its large population sizes (Pampoulie et al. 2004). Nevertheless, based on seven microsatellites, this species showed an overall F_{ST} value of 0.026 resulting from a spatial population structuring in four differentiated subunits, viz. Belgian North Sea (coastal and marine), Westerschelde and Oosterschelde (Pampoulie et al. 2004). In the same area similar patterns were observed in free-living nematodes with supposed high passive dispersal capacity through rafting on decomposing algae (Derycke et al. 2005, 2007a). Within the nematode *Rhabditis (Pellioditis) marina*, four distinct mitochondrial lineages (cryptic species) were revealed, but within each lineage, there was significant population structure between different water bodies. For example, populations of the most abundant lineage were differentiated between the North Sea, the Oosterschelde and the Westerschelde (φ_{ST} = 0.19, $P < 0.0001$ in analysis of molecular variance) and even between sampling sites within the Westerschelde (φ_{ST} = 0.09, $P <$ 0.0001). A lineage that was present in the North Sea and in Lake Grevelingen, also

showed significant differentiation between the two areas (φ_{ST} = 0.47, P < 0.0001) (Derycke et al. 2005). The organisms mentioned above, viz. the marine fish and nematodes, spend only a fraction of their life in the plankton and for the nematodes it was suggested that genetic structure could be attributed to founder effects and genetic bottlenecks in these unstable and patchily distributed populations, rather than to restricted gene flow (Derycke et al. 2007b). Hence, differences in life-habit characteristics could explain the contrasting results with our study on the holoplanktonic *P. pungens*.

However, population genetic studies of planktonic organisms in other areas over comparable geographic scales revealed differentiated populations. Rynearson et al. (2006) showed the maintenance of genetically distinct populations of the diatom *Ditylum brightwellii* in connected and daily intermixing estuaries along the NW coast of America. However, it can not be excluded that their results might have been confounded by the presence of two cryptic species as is suggested by ITS sequence divergence values, differences in valve diameter, and deviations of HWE in most samples. Nevertheless, Rynearson et al. (2006) assumed that populations were interbreeding, but unfortunately this was not confirmed by mating tests.

A number of studies on planktonic marine organisms have shown that population differentiation starts to appear on wider geographical scales. For example, the dinoflagellate *Alexandrium tamarense* showed population genetic structure over ca. 1600 km along the Japanese and Korean coast (based on nine microsatellites and 520 isolates) (Nagai et al. 2007). Genetic differentiation between most of the sampling locations was found, with a correlation between genetic distance and geographic distance. Similarly, the holoplanktonic chaetognath *Sagitta setosa* shows strong genetic differentiation between the NE Atlantic, the Mediterranean and the Black Sea, while in the NE Atlantic no structuring is apparent (based on mitochondrial and four microsatellite markers; Peijnenburg et al. 2006). Expanding our sampling area by the inclusion of *P. pungens* var. *pungens* isolates from more distant locations from German, French and Dutch parts of the North Sea in our STRUCTURE analysis, revealed no spatial differentiation over ca. 650 km. These results suggest no differentiation of *P. pungens* var. *pungens* over wider scales in the North Sea, although it should be noted that this analysis was based on relatively few isolates and should be confirmed with more extensive sampling of populations. Interestingly, Evans et al. (2005) included three Canadian strains in their *P. pungens* study in the German part of the North Sea. These Canadian isolates contained two private alleles at the least polymorphic loci, which suggest that differentiation, although possibly limited, might occur at a much wider scale.

In conclusion, we found no evidence for genetic structuring in the holoplanktonic diatom *P. pungens* var. *pungens*. Even between water bodies with very limited connectivity, populations appeared homogenous, possibly because there is sufficient gene flow to counteract genetic drift, although additional and similar effects imposed by recent separation of populations (water bodies) can not be ruled out entirely. The absence of differentiation at neutral loci, however, does not preclude the existence of more pronounced differences at loci affected by selection (Cousyn et al. 2002; Koskinen et al. 2002). Since selected and non-selected genes can have different effective migration rates, adaptive differences could persist in spite of significant neutral gene flow. It would be very interesting to compare our results with ecologically relevant markers to find out if adaptation is present in the different environments of our sampling area.

Acknowledgments

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Supplementary tables

Table S1. Clonal cultures of *Pseudo-nitzschia pungens* used in the present study, with information about sampling location and date and the total number of strains used per population. Salinity values for the stations sampled in the North Sea in 2003-2006 are from the MIDAS database (www.vliz.be). Other salinity values are own measurements.

waterbody	sampling location	latitude	longitude	sampling date	environment	Salinity (psu)	number of isolates	Total per population
North Sea (isolates form 2007)	station 120	51.185°	2.70117°	04-05-2007	coast	29.6	52	
	station 700	51.3767°	3.22°	04-05-2007	coast	29.7	52	104
Westerschelde	Vlissingen	51.4389°	3.57637°	15-05-2007	estuary	28.4	22	
	Hoofdplaat	51.3755°	3.66475°	15-05-2007	estuary	26	22	
	Terneuzen	51.3358°	3.80942°	15-05-2007	estuary	19.6	22	
	Schore	51.4616°	3.97627°	15-05-2007	estuary	19.6	22	88
Oosterschelde	Neeltje Jans	51.6364°	3.71372°	04-07-2007		29.5	53	
	Zijpe	51.654°	4.09947°	15-05-2007		27.8	18	71
Lake Grevelingen	Den Osse	51.7438°	3.8903°	15-05-2007		28.3	11	
	Zeedijk	51.7335°	3.94425°	15-05-2007		28.3	36	47
North Sea (isolates from 2003-2006)	station 120	51.185°	2.70117°	24-03-2004	coast	34.1	7	
				16-08-2004		33.4	9	
				27-03-2006		31.2	3[a]	
	station 700	51.3767°	3.22°	19-04-2004	coast	31.2	7	
				19-07-2004		33.3	10	
	station 130	51.2708°	2.905°	30-06-2003	coast		3[a]	
	station 215	51.2767°	2.61337°	24-03-2004	coast	34.3	6	
				19-07-2004		34.5	8	
				18-10-2004			1	
				27-03-2006		32.6	1[a]	
	station 330	51.4333°	2.80833°	24-03-2004	coast-marine	34.2	4	
	station 421	51.4805°	2.45°	29-03-2004	marine	33.1	4	
				23-08-2004		32.7	11	
				19-10-2004		34.9	7	
				19-11-2004			1	82[b]
North Sea	Helgoland, Germany	54.1508°	7.81093°	2003	marine		10[a]	10[b]
North Sea	Terschelling, the Netherlands	54.1822°	3.42253°	01-05-2000	marine		1[a]	
	Marsdiep, the Netherlands	52.9596°	4.65059°	25-04-2002	coast		1[a]	
	Noordwijk, the Netherlands	52.2653°	4.3695°	22-05-2002	coast		3[a]	5[b]
Lagoon	Spuikom, Belgium	51.2251°	2.94601°	07-04-2004	lagoon		9[a]	
				13-04-2004			1[a]	10[b]
North Sea	Ambleteuse, France	50.8055°	1.60081°	13-09-2003	coast		3[a]	3[b]

[a] strains only used in STRUCTURE analysis
[b] strains used for mating tests and ITS rDNA sequencing in Casteleyn et al. 2008

Table S2. Matching MLGs isolates with their matching probabilities based on allelic frequencies in the population from which the MLGs originated.

			Matching probability	
population	isolate 1	Isolate 2	$P_{(ID)}$	$P_{(ID)sibs}$
North Sea	V120(5)38	V120(5)42	5.68E-07	7.70E-03
	V120(5)5	V120(5)8	8.05E-12	2.54E-03
	V700(5)3	V700(5)57	7.55E-07	9.74E-03
	V700(5)44	V700(5)45	8.73E-07	1.05E-02
Oosterschelde	NeeltjeJans11	NeeltjeJans32	4.57E-06	1.01E-02
	NeeltjeJans12	NeeltjeJans18	1.96E-07	4.63E-03
	NeeltjeJans13	NeeltjeJans8	1.25E-06	7.22E-03
	NeeltjeJans29	NeeltjeJans43	7.92E-10	2.61E-03
	NeeltjeJans31	NeeltjeJans9	1.35E-07	6.48E-03
	NeeltjeJans35	NeeltjeJans39	7.04E-08	4.27E-03
	Zijpe10	Zijpe11	2.15E-07	8.28E-03
	Zijpe19	Zijpe6	1.23E-06	6.96E-03
Lake Grevelingen	Zeedijk16	Zeedijk17	2.85E-08	6.50E-03
	V120(5)57	Zeedijk2	3.30E-06	7.07E-03

6

Macrogeographic population genetic differentiation in the cosmopolitan marine planktonic diatom *Pseudo-nitzschia pungens* var. *pungens* [1]

Recent studies have demonstrated that the marine diatom *Pseudo-nitzschia pungens* var. *pungens* has a cosmopolitan distribution. Therefore we analyzed six polymorphic microsatellite loci in 242 strains from various localities in the Atlantic and the Pacific Ocean to assess whether or not this taxon consists of a single homogenous global population. Despite the assumed high dispersal ability of *P. pungens* var. *pungens*, our population genetic analyses reveal significant population differentiation, suggesting restricted gene-flow on a large geographical scale. Our results suggest that, in contrast to the current paradigm, allopatric processes may have an important role in the diversification of marine plankton, such as diatoms. This study is a first step in understanding intraspecific genetic structuring and in determining the biogeographic extent of planktonic micro-organisms.

[1] Unpublished manuscript: Casteleyn G, Leliaert F, Backeljau T, Debeer AE, Kotaki Y, Rhodes L, Lundholm N, Sabbe K & Vyverman W. Macrogeographic population genetic differentiation in the cosmopolitan marine planktonic diatom *Pseudo-nitzschia pungens* var. *pungens*

Introduction

Marine planktonic organisms have long been thought to have limited potential for allopatric speciation because of their huge population sizes, their high dispersal potential and the putative lack of apparent dispersal barriers in marine environments (Palumbi 1992, 1994, Finlay 2002, Carr et al. 2003). As a result, marine environments were believed to sustain only a limited number of ubiquitously distributed planktonic species that lack spatial population genetic structuring. However, the use of molecular tools indicate that many planktonic species formerly regarded as cosmopolitans turn out to be complexes of several genetically distinct but morphologically highly similar species (John et al. 2003, Montresor et al. 2003, Saez et al. 2003, Lilly et al. 2007, Lundholm et al. 2003, 2006, Sarno et al. 2005, Amato et al. 2007, Kooistra et al. 2008), whose distribution may be restricted by both physical and ecological barriers (de Vargas et al. 1999, 2002, Darling et al. 2004, Goetze 2005, Darling & Wade 2008).

To understand how geographical structuring in marine planktonic taxa could have arisen, genetic diversity within and the connectivity between populations should be investigated. Population genetic surveys, that allow differentiating among individuals and/or populations within species, can improve our understanding of the underlying mechanisms that may have caused this previously unrecognized structuring since local populations are the evolving units of a species. However, the use of species complexes without considering hidden taxonomic complexity can confound the interpretation of such population genetic surveys. Therefore, the boundary between intraspecific population structuring and cryptic taxonomic diversity should first be elucidated. The cosmopolitan marine planktonic diatom *Pseudo-nitzschia pungens*, for example, comprises three genetically distinct entities based on rDNA ITS sequence data. These three taxa correspond to the morphological varieties *pungens*, *cingulata* and *aveirensis* (Casteleyn et al. 2008, chapter 3 of this thesis, Churro et al. in press, appendix of this thesis). The distribution of var. *aveirensis* appears non-overlapping with the other two varieties, occurring in tropical to warm-temperate waters of the Pacific and Atlantic Oceans. The var. *pungens* is widely distributed in temperate waters and is found on both sides of the northern Atlantic and Pacific Oceans and the south west Pacific Ocean. The var. *cingulata* seems restricted to the NE Pacific where it co-occurs with var. *pungens*. Although the latter two varieties are genetically well-differentiated (ITS, *rbc*L and four microsatellite markers), occasional hybrids between both varieties were recorded in the NE Pacific (Adams 2006, Casteleyn et al. 2009, chapter 4 of this thesis).

Within var. *pungens*, ITS and *rbc*L sequence data show no global differentiation (i.e. all strains have identical sequences) and strains from widely separated populations reproduce sexually under laboratory conditions (Casteleyn et al. 2008, 2009). This

suggests historic and/or current transoceanic gene-flow between distant populations of var. *pungens*. On a local scale in the North Sea (650 km), high levels of genetic diversity and gene flow were shown with nuclear microsatellite markers (Evans et al. 2005, Casteleyn et al. accepted, chapter 5 of this thesis) but the population genetics on a global scale remain unexplored. The current study was designed to assess population genetic structure of *P. pungens* var. *pungens* populations on a worldwide scale by means of six nuclear polymorphic microsatellite loci. We analyzed 242 strains from localities in the Atlantic and the Pacific Ocean to test the ITS- and *rbc*L-based hypothesis that *P. pungens* var. *pungens* is a single, globally homogenous and unstructured population.

Material and methods

Isolation of strains

Pseudo-nitzschia pungens var. *pungens* strains were isolated from seven geographic areas in the Atlantic (Belgium, Denmark, Ireland, Canada) and the Pacific Ocean (USA, Japan, New Zealand) forming a nested set of sampling area's (Fig. 1, Table 1). In total 242 strains were surveyed, ranging from 20-52 strains per area. Detailed sample information is given in Table 1. Isolation of strains and culture conditions were as described in Casteleyn et al. (2008).

Molecular analysis

Cells from a 50 mL growing batch culture were harvested by centrifugation and stored at -80°C. Genomic DNA was extracted from pelleted cells using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Six nuclear microsatellite loci (PP1, PP2, PP3, PP4, PP5 and PP6) were amplified with primers developed by Evans and Hayes (2004). These loci were amplified in separate PCR reactions with fluorescently labelled primers (6FAM, VIC, NED; Applied Biosystems, Foster City, USA) following PCR conditions as described in Evans and Hayes (2004) and modified as outlined in Casteleyn et al. (accepted). PCR products were analyzed on an ABI 3130xl genetic analyzer (Applied Biosystems) according to Casteleyn et al. (accepted). To reduce genotyping errors, positive and negative controls were used and scoring of alleles was done by the same person. Genotypes were scored using Genemapper 4.0 software (Applied Biosystems).

Table1. Strains used in this study

water basin	Pre-defined population	sampling location	latitude	longitude	sampling date	isolation by	Accession no.		n	n_{tot}
							ITS	rbcL		
Atlantic Ocean										
North Sea	Belgium	VLIZ 120	51.185°	2.701°	4 May 2007	V. Chepurnov	= AM778747		50	50
Limfjord	Denmark		56.8629°	8.94292°	Aug-Sep 1997	N. Lundholm	***	= FM207548	20	20
Irish Sea	Ireland	Belfast Harbour, HAB6	54.738°	-5.653°	7 July 2007	G. Casteleyn			29	
		Belfast Harbour, HAB7	54.728°	-5.68°	7 July 2007	G. Casteleyn			20	
		Belfast Harbour, HAB8	54.713°	-5.723°	7 July 2007	G. Casteleyn		= FM207548	3	52
NW Atlantic	Canada	Cardigan River, PEI	46.2231°	-62.5708°	Sep 2002	C. Léger	AM778786		1	
		Miramichi Bay, NB	47.1153°	-65.1659°	Sep-Oct 2002	C. Léger	AM778787		3	
		Bay of Fundy, NB	44.9944°	-65.7945°	Oct 2002	C. Léger	AM778788		1	
		Miramichi Bay, NB	47.1153°	-65.1659°	Sep 2003	C. Léger	AM778789		2	
		Malpeque Bay, PEI	46.533°	-63.8°	Oct 2003	C. Léger	AM778790		2	
		Brudenell River, PEI	46.2232°	-63.4842°	Sep 2004	C. Léger	AM778791		2	
		Cardigan River, PEI	46.2231°	-62.5708°	Sep 2004	C. Léger	AM778792		2	
		Boughton Bay, PEI	46.1656°	-62.4769°	Sep 2004	C. Léger	AM778794	= FM207548	2	
		Boughton Bay, PEI	46.1656°	-62.4769°	Sep 2005	C. Léger			3	
		Malpeque Bay, PEI	46.533°	-63.8°	Nov 2005	C. Léger			2	
		New London Bay, PEI	46.4906°	-63.4601°	Nov 2005	I. Sahraoui		= FM207548	1	
		Miramichi Bay, NB	47.1153°	-65.1659°	Sep 2007	C. Léger		= FM207548	5	26
Pacific Ocean										
NE Pacific	USA	BS	48.148°	-125.248°	Oct 2004	N. Adams	FM207591	FM207548	3	
		E3	48.2°	-125.883°	Sep 2005	N. Adams	FM207591	FM207548	5	
		E1	48.039°	-125.616°	Sep 2005	N. Adams	FM207591	FM207548	5	
		E2	48.131°	-125.72°	Sep 2005	N. Adams	FM207591	FM207548	6	
		E4	48.074°	-126.141°	Sep 2005	N. Adams	FM207591	FM207548	8	27
NW Pacific	Japan	Ofunato Bay	39.0617°	141.732°	7 Aug 2000	Y. Kotaki	AM778812	= FM207548	1	
					25 Jun 2001	Y. Kotaki	AM778813		1	
					3 Dec 2001	Y. Kotaki	AM778814		1	
					13 Nov 2006	Y. Kotaki		= FM207548	21	24
SW Pacific	New Zealand	Steels Reef, North Island	-37.9442°	177.058°	Oct 2000	L. Rhodes	AM778815		1	
		Big Glory Bay, Stewart Island	-46.9824°	168.1°	Feb 2003	L. Rhodes	AM778816		1	
		Taylor's Mistake, South Island	-43.5788°	172.79°	Aug 2004	L. Rhodes	AM778817	= FM207548	1	
		Collingwood, South Island	-40.686°	172.69°	Oct 2004	L. Rhodes			1	
		Nydia Bay, South Island	-41.1653°	173.786°	Aug 2005	L. Rhodes			1	
		Cannon Bay, North Island	-41.0987°	174.895°	Sep 2005	L. Rhodes			1	
		Gisborne Harbour, North Island	-38.7°	178°	18 Apr 2007	L. Rhodes		= FM207548	37	43
										253

NB: New Brunswick; PEI: Prince Edward Island; n: number of strains; n_{tot}: total number of strains per population.

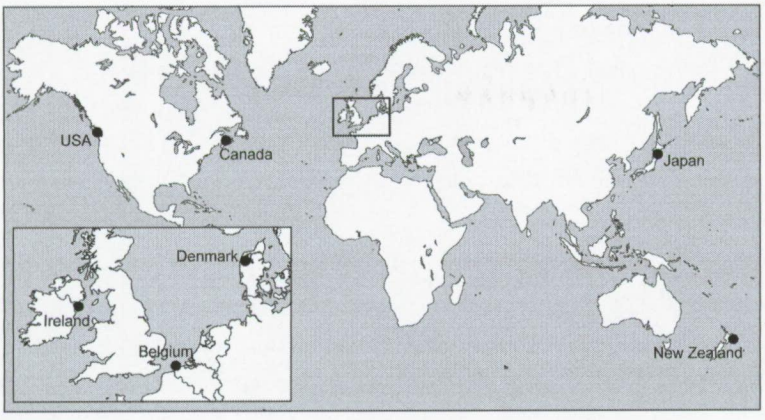

Fig. 1. Sampling locations. Inset indicates sampling sites in the North Sea and Irish Sea.

Analysis of microsatellite data

Genetic diversity

For analysis of the microsatellite data, seven pre-defined populations were considered, based on the geographical area sampled: Belgium, Denmark, Ireland, Atlantic Canada, Pacific USA, Japan and New Zealand. Previous studies (Evans et al. 2005, Casteleyn et al. accepted) showed that temporal variation does not exceed spatial variation for *P. pungens* var. *pungens*. Therefore populations sampled in different seasons or years from the same geographic area were pooled.

Possible scoring errors due to stuttering or large allele dropout were assessed using the program MICRO-CHECKER (Van Oosterhout et al. 2004). CONVERT 1.31 (Glaubitz 2004) was used to format the multi-locus genotype (MLG) data and to identify private alleles for each population. To identify matching MLGs, the Excel add-in MS TOOLS (Park 2001) was utilized. Identical MLGs from the same sampling location and sampling date were removed from the dataset because isolations were not always done within 24 hours after sampling. This is a potential problem because asexual (i.e. clonal) reproduction may take place between the time of field sampling and the moment of isolating the strains, leading to a potential underestimation of diversity. Therefore, we did not estimate clonal diversities (number of unique genotypes relative to sample size) per population. GIMLET 1.3.3 (Valiere 2002) was used to calculate the probability of identity, $P_{(ID)}$, and the probability of identity for siblings, $P_{(ID)sib}$, for each locus and over all loci. Waits et al. (2001) suggested that $P_{(ID)}$ or $P_{(ID)sib}$ values between 0.01 and 0.0001 indicate that the markers possess sufficient resolving power to distinguish unrelated individuals.

Numbers of alleles, allele frequencies, number of genotypes, observed heterozygosity (H_o), and expected heterozygosity (H_e), were calculated per population for each locus using GENEPOP 4.0 (Rousset 2008). To compare the number of alleles between populations with different sample sizes, the software ARES (Van Loon et al. 2007) was used. Unlike existing tools based on rarefaction, ARES extrapolates beyond the sample size.

Departures from Hardy-Weinberg Equilibrium (HWE) at each locus in every population were tested using an exact test (Guo & Thompson 1992) with a markov chain method to estimate exact *P*-values (dememorisation number: 10000, 300 batches with 5000 iterations per batch; to keep the standard errors < 0.01). F_{IS} was used as an indicator of heterozygote excess or deficit (Weir & Cockerham 1984). Fisher's exact tests were performed to test across loci within each population. HWE tests were done in GENEPOP 4.0 which was also used for testing linkage disequilibrium (LD) between all pairs of loci per population using exact tests as outlined above. Multiple test problems were dealt with by calculating corrected *P*-values using the sequential Bonferroni technique at the 5% significance level (Rice 1989).

Population structure

Population differentiation was evaluated with allelic and genotypic exact tests in GENEPOP using default settings. Tests were run for each locus between all pairs of pre-defined populations and combinations of *P*-values across loci were obtained according to Fisher's method. The degree of genetic differentiation between pairs of populations was quantified using Weir and Cockerham's (1984) estimate of F_{ST} in FSTAT 2.9.3 (Goudet 2001). Permutation tests were used to determine whether F_{ST} values were significantly different from zero. F_{ST} values depend on allelic diversities, therefore standardized F_{ST} values were calculated using RECODEDATA 0.1 (Meirmans 2006). In all cases, *P*-values were corrected using a sequential Bonferroni technique. A principal component analysis (PCA) was performed to visualize differentiation among populations (F_{ST}) using GENALEX v. 6 (Peakall & Smouse 2006).

To infer population structure without a pre-defined population subdivision, the Bayesian clustering program STRUCTURE 2.2 (Pritchard et al. 2000) was used. STRUCTURE divides sampled individuals into a number of clusters (K) independent of locality information (i.e. based only on MLGs), so as to minimize deviations from Hardy–Weinberg and linkage equilibrium. Individuals are probabilistically assigned to one cluster or more than one cluster if they are genetically admixed. The most likely number of populations (*K*) was estimated by performing ten independent runs for each

value of K from 2 to 10 with a burn-in and run length of 100,000 repetitions and using the model with correlated allele frequencies, noninformative priors and assuming admixture. The estimated "Ln Probability of Data (Ln P(D))" given K was used as a criterion to select the most likely number of populations (K) represented by our data by looking for either a maximum value or a more or less plateau for increasing K (Pritchard et al. 2007). The lower value of K showing such behaviour was considered as representative of the most apropriate clustering model (Pritchard et al. 2007). For the selected K value, we evaluated the individual membership coefficient (q_{ind}) to the inferred clusters. Individuals with a proportion of membership to each cluster $q_{ind} < 0.90$ (admixed individual) were assigned to more than one cluster whereas individuals with $q_{ind} \geq 0.90$ were assigned to only one cluster. CLUMPP 1.1.1 (Jakobsson and Rosenberg 2007) was utilized to line up the cluster labels across runs and to estimate the degree of congruence between independent runs, by calculating the Symmetric Similarity Coefficient (SSC) for pairs of runs at each K value, resulting in an average pairwise similarity measure named H (the nearer H is to one, the higher the degree of congruence between independent runs). The *Fullsearch* algorithm was used for K=2 and K=3 and the *Greedy* algorithm for greater values of K (testing a pre-defined number of random sequences: 100,000 for K=4; 10,000 for K=5 and 1,000 for K>6). DISTRUCT 1.1 (Rosenberg 2004) was used to visualize the results from the Bayesian analyses.

Isolation by distance

To test whether genetic distance was correlated to geographical distance (isolation by distance) the program IBDWS (Jensen et al. 2005) was used. Nonparametric Mantel tests were performed to test for nonrandom associations between matrices of genetic distances between all population pairs and matrices of pairwise geographical distances. Geographical distance between sampling areas was measured as the shortest continuous water surface distance using Google Earth (earth.google.com). Genetic distances were computed using Slatkin's (1993) similarity measure ($M = ((1/F_{ST}) - 1)/4$). The significance of the major axis regression was assessed by 10000 permutations of the data.

Results

Genetic diversity

Of the 242 *P. pungens* strains from seven areas, 241 were successfully genotyped at all six loci. Only one strain from Canada had a missing genotype at locus PP4. The $P_{(ID)}$ and the $P_{(ID)sib}$ across all loci for the pooled populations (3.75×10^{-9} and 1.95×10^{-3} respectively, Table 2) were within the guidelines suggested by Waits et al. (2001), indicating that our six microsatellite markers contained enough resolving power to distinguish between strains.

The number of alleles per locus across the seven populations ranged from nine (locus PP5) to 39 (locus PP3). In total 118 alleles were recovered in 242 strains. Results of the allelic richness analysis showed that Japan had the highest and the USA the lowest numbers of alleles (Fig. 2). Private alleles in populations were nearly always due to single strains. However, private alleles at PP3 and PP6 in Japan were found in two to four strains. The same was true for private alleles at PP2, PP3, PP5 and PP6 in Canada. In New Zealand private alleles at PP2, PP3, PP4 and PP6 were shared by multiple strains with up to 17 strains for PP4 and 39 strains for PP6 (Fig. 3).

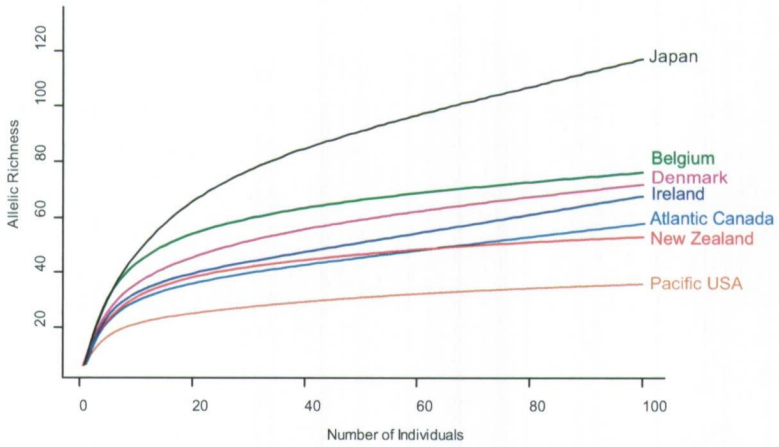

Fig. 2. Comparison of allelic richness between *P. pungens* var. *pungens* populations from different geographical areas. Allelic richness was inferred from MLGs (six microsatellite loci) and extrapolated beyond the sample size using ARES.

Table 2. Number of *Pseudo-nitzschia pungens* var. *pungens* strains genotyped (n), size ranges of the alleles (bp), number of alleles (N_A), number of private alleles (N_{priv}), number of unique genotypes observed (G_o), observed (H_o) and expected (H_e) heterozygosity, F_{IS} according to Weir and Cockerham (1984) and probabilities of identity ($P_{(ID)}$ and $P_{(ID)sib}$) across loci for every population and overall populations. In bold: significant deviation from HWE (after sequential Bonferroni correction at the 5% significance). Totals per locus, where appropriate are shown in the last column.

locus	populations Belgium	Denmark	Ireland	Canada	USA	Japan	New Zealand	Total
PP1								
n	50	20	52	26	27	24	43	242
bp	217-246	217-246	217-246	217-246	225-246	217-250	227-248	217-250
N_A	9	9	10	7	6	12	9	15
N_{priv}	0	0	0	0	0	2	1	
G_o	17	12	25	12	9	15	15	
H_o	0.76	1.00	0.81	0.77	0.78	0.75	0.63	
H_e	0.78	0.84	0.81	0.81	0.70	0.84	0.70	
F_{IS}	0.03	-0.18	0.01	0.05	-0.12	0.11	**0.11**	
PP2								
n	50	20	52	26	27	24	43	242
bp	166-240	168-240	168-240	175-233	166-190	168-190	168-233	166-240
N_A	9	5	6	4	4	5	7	13
N_{priv}	2	0	0	1	0	0	1	n/a
G_o	13	6	9	6	5	8	11	28
H_o	0.70	0.65	0.52	0.50	0.74	0.67	0.81	
H_e	0.68	0.62	0.56	0.49	0.58	0.68	0.71	
F_{IS}	-0.03	-0.04	0.07	-0.02	-0.28	0.01	-0.14	
PP3								
n	50	20	52	26	27	24	43	242
bp	191-254	191-246	191-262	212-254	196-236	197-257	205-244	191-262
N_A	12	9	15	9	7	26	8	39
N_{priv}	1	0	2	1	2	11	2	n/a
G_o	30	15	36	12	13	21	9	97
H_o	0.86	0.75	0.92	0.96	0.63	0.83	0.81	
H_e	0.87	0.87	0.89	0.81	0.79	0.97	0.69	
F_{IS}	0.02	0.14	-0.04	-0.19	0.20	**0.14**	-0.19	
$P_{(ID)}$								
$P_{(ID)sib}$								
PP4								
n	50	20	52	25	27	24	43	241
bp	133-203	133-203	133-209	133-205	133-205	133-218	155-216	133-218
N_A	10	9	16	10	3	18	13	29
N_{priv}	0	0	0	2	0	5	3	n/a
G_o	26	15	28	17	4	17	26	93
H_o	0.74	0.80	0.92	0.80	0.63	0.71	0.86	
H_e	0.84	0.86	0.88	0.87	0.51	0.93	0.87	
F_{IS}	0.12	0.07	-0.05	0.08	-0.23	**0.23**	0.01	
PP5								
n	50	20	52	26	27	24	43	242
bp	186-198	186-198	186-202	186-200	192-198	188-202	192-198	186-202
N_A	5	6	7	6	3	7	4	9
N_{priv}	0	0	0	1	0	0	0	n/a
G_o	9	10	10	8	5	10	7	21
H_o	0.60	0.55	0.42	0.62	0.48	0.83	0.40	
H_e	0.60	0.62	0.48	0.61	0.54	0.74	0.56	
F_{IS}	0.01	0.11	0.11	-0.01	0.11	-0.13	0.30	
PP6								
n	50	20	52	26	27	24	43	242
bp	195-236	195-236	195-236	191-218	191-258	191-262	191-216	191-262
N_A	3	3	7	5	2	7	5	13
N_{priv}	0	0	0	1	1	1	1	n/a
G_o	6	6	10	7	2	14	8	35
H_o	0.54	0.70	0.58	0.65	0.04	0.71	0.74	
H_e	0.57	0.65	0.69	0.60	0.04	0.83	0.63	
F_{IS}	0.06	-0.08	0.16	-0.09	0.00	0.14	-0.18	
Across loci								
n	50	20	52	26	27	24	43	242
N_A [a]	8.0	6.8	10.2	6.8	4.2	12.5	7.7	19.7
H_o	0.70	0.74	0.70	0.72	0.55	0.75	0.71	
H_e	0.72	0.75	0.72	0.70	0.53	0.83	0.69	
F_{IS}	0.03	0.00	0.03	-0.03	-0.04	**0.10**	**-0.02**	
$P_{(ID)}$	4.70E-07	8.48E-08	2.01E-07	6.04E-07	1.67E-04	5.57E-12	1.79E-06	3.75E-09
$P_{(ID)sib}$	5.26E-03	4.87E-03	5.24E-03	6.95E-03	2.87E-02	2.07E-03	7.21E-03	1.95E-03

[a] average number of alleles accross the 6 loci

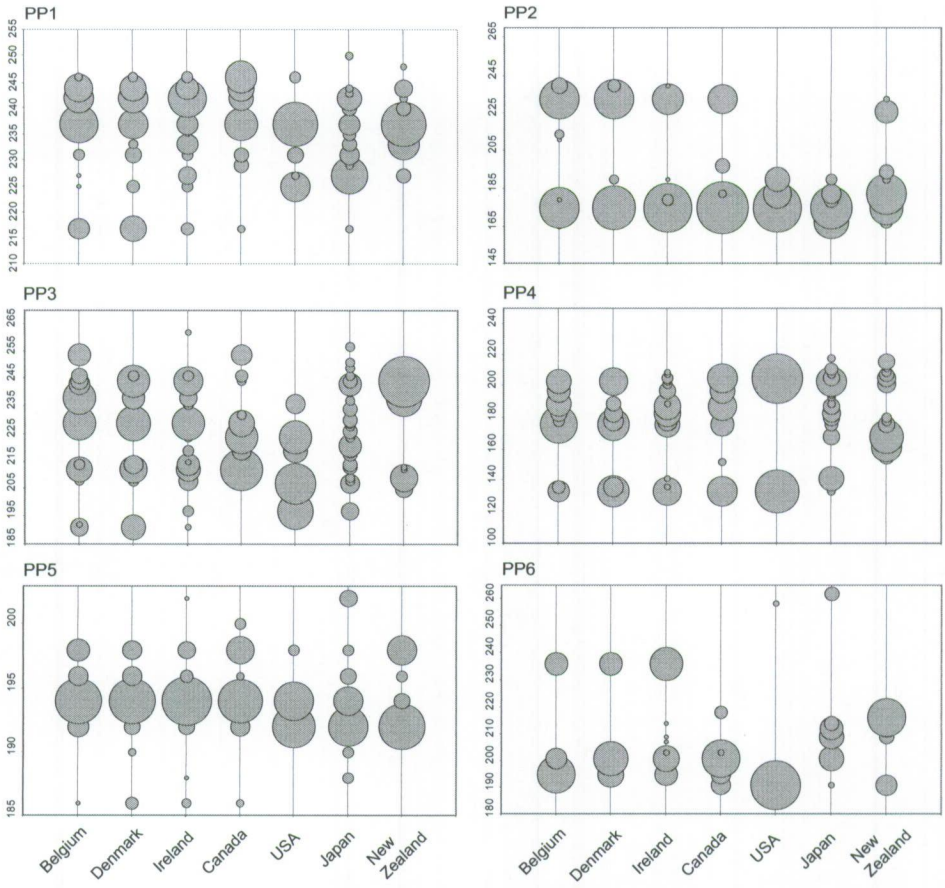

Fig. 3. Allele frequencies for the seven predefined populations: Belgium, Denmark, Ireland, Atlantic Canada, Pacific USA, Japan and New Zealand. Individual circles are centred at a specific allele size. The area of a particular circle is proportional to the frequency of that allele.

H_o averaged over all loci for each population ranged from 0.55 (USA) to 0.75 (Japan) and was similar to H_e except in Japan, where lower H_o values were found (Table 2). Hence HWE tests revealed significant deviations at PP3 and PP4 in the Japanese population. In the New Zealand population there was also a significant HWE deviation at PP1 (Table 2). There was no evidence that scoring errors due to large allele drop-out or stutter contributed to the observed HWE deviations. Exact tests for linkage disequilibrium yielded eight significant values out of 105 pairwise tests (7.6 %), six of them were found in the Japanese population (five of them involving PP3 and PP4). This suggests that LD is not a particularly important characteristic of this dataset.

Population differentiation

There was a large overlap in allele size classes for all six loci but the allele frequency distributions were not identical across populations (Fig. 3). Allelic and genotypic differentiation tests and F_{ST} statistics were used to detect genetic differentiation among populations. The results of the allelic and genotypic tests for each locus and over all loci yielded the same results (Table 3). Pairwise F_{ST} values between populations are shown in Table 3. The values were relatively high (ranging from 0.06 to 0.76) and all, except Belgium versus Denmark, were significant after sequential Bonferroni adjustment. PCA of pairwise F_{ST} values showed that the first and second principal components accounted

Table 3. Estimates of standardized multilocus Fst values among pairs of populations (above diagonal). The loci that were significantly different among populations in the single locus tests (allelic and genotypic tests) are given below the diagonal. In bold: significant differences after sequential Bonferroni correction between pairwise comparisons.

	Belgium	Denmark	Ireland	Canada	USA	Japan	New Zealand
Belgium		0.06	**0.18**	**0.31**	**0.74**	**0.59**	**0.73**
Denmark	6		**0.06**	**0.18**	**0.73**	**0.49**	**0.74**
Ireland	1, 2, 3, 4, 6	1, 4		**0.23**	**0.70**	**0.47**	**0.76**
Canada	1, 2, 3, 4, 6	1, 2, 3, 4, 6	1, 2, 3, 4, 6		**0.57**	**0.50**	**0.74**
USA	all 6	all 6	all 6	all 6		**0.66**	**0.65**
Japan	all 6	all 6	all 6	all 6	all 6		**0.57**
New Zealand	all 6	all 6	all 6	all 6	all 6	all 6	

Fig. 4. Principal component analysis of pairwise F_{ST} values among the seven populations of *P. pungens* var. *pungens*. The first and second principal components accounted for 59.47 % and 24.58 %, respectively of the total variation.

for 59.47 % and 24.58 %, respectively of the total variation. Pacific and Atlantic populations were separated along the first axis. The PCA also suggested that populations from the Atlantic were more genetically similar than those from the Pacific (Fig. 4). This is in agreement with Mantel test for matrix correlation between log(genetic similarity (M)) and log(geographic distance) ($Z = 16.8975$, $r = -0.8707$, one-sided $p = 0.0044$) and regression analyses ($r^2 = 0.758$, 95% CI: 0.450-0.889) that showed significant isolation by distance (Fig. 5).

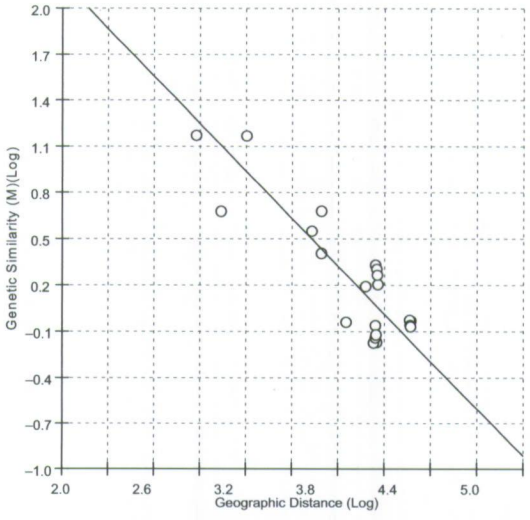

Fig. 5. Isolation by distance relationship. Scatter plot of genetic similarity versus geographic distances (in km) of seven *P. pungens* var. *pungens* populations showing significant correlation between geographic and genetic distance.

Cluster analyses using STRUCTURE revealed the highest log-likelihood value for $K = 6$ (Fig. 6). For this model, clustering results were highly consistent among runs (H=0.99). Using the six cluster model, we found three clusters that showed little admixing and three clusters with higher levels of admixing (Fig. 7). Strains from the Pacific USA, Japan and New Zealand could be readily assigned to the clusters corresponding to their geographic origin except in a few rare cases. The North Atlantic clusters showed more admixing. For Canadian strains, 18 out of 26 strains had a $q_{ind} > 0.9$ with respect to a "Canadian" cluster. The NE Atlantic strains showed higher levels of admixture. Of the Belgian strains, 23 out of 50, could be assigned to the "purple" cluster with a probability > 0.9. The majority of Belgian strains had the highest probability of belonging to the "purple" cluster ($q_{ind} > 0.6$). Only five Danish strains had a $q_{ind} > 0.9$ of belonging to one cluster (purple) and almost an equal amount of strains had highest q_{ind}

(> 0.6) in the purple or the green cluster. Of the Irish strains, almost half of the strains could be readily assigned to the green cluster ($q_{ind} > 0.9$) and 42 strains had the highest probability ($q_{ind} > 0.6$) in that cluster.

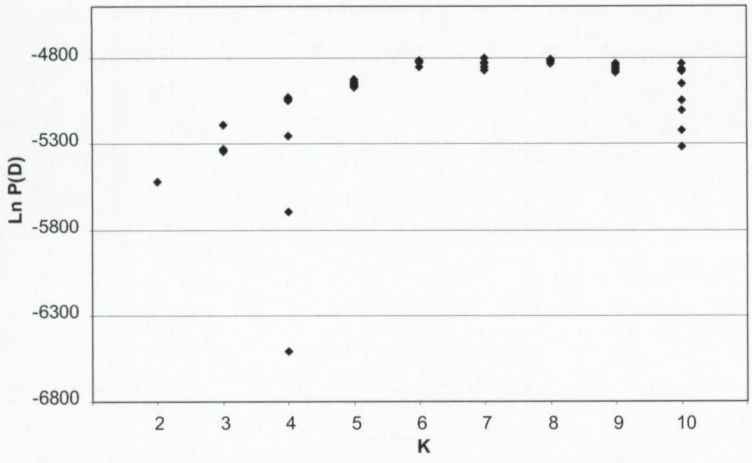

Fig. 6. Results of STRUCTURE analysis for 1-10 populations in the complete dataset. Estimated Ln Probability of Data (Ln P(D)) over ten runs as a function of K is presented.

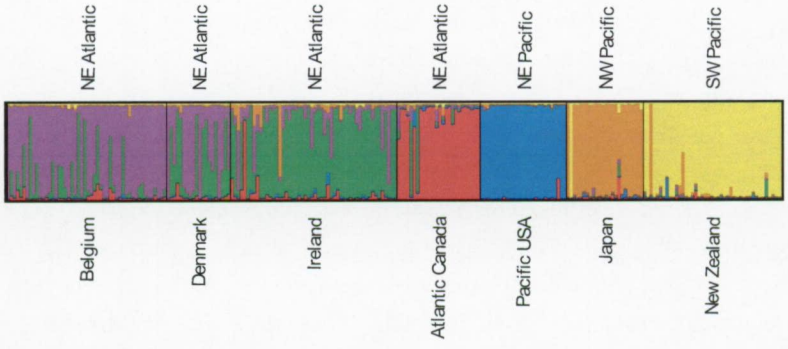

Fig. 7. Structure plot for K=6. Each individual is depicted by a vertical line that is partitioned into K coloured sections, with the length of each section proportional to the estimated membership coefficient (q_{ind}) of the strain to each cluster.

Discussion

This study refutes the ITS and *rbc*L based hypothesis that *Pseudo-nitzschia pungens* var. *pungens* constitutes a single, globally homogenous population. This is initially indicated by the higher allelic diversity found within var. *pungens* on a global scale compared with local studies in the North Sea (Evans et al. 2005, Casteleyn et al. accepted). We recorded 118 alleles in 242 strains (19.7 alleles per locus on average), while in the North Sea a maximum of 77 alleles were found in almost twice as many var. *pungens* strains (12.5 alleles per locus on average, Evans et al. 2005). This shows that the higher number of alleles recorded in the present study is not a matter of sample size, but is due to new variants in other geographic areas. Interestingly, the highest and lowest allelic richness and H_e (0.83 vs. 0.53) were found on opposite sides of the N Pacific, respectively in Japan and Pacific USA. Information on historical distribution patterns will be needed to elucidate whether this pattern is due to a spread of var. *pungens* from a NW Pacific centre of origin to other locations and whether the NE pacific population is the result of a recent range expansion of var. *pungens* (where it occurs sympatrically with var. *cingulata*) across a historically effective dispersal barrier. The other populations showed intermediate H_e values comparable with values found in the North Sea (mean 0.71, Evans et al. 2005, Casteleyn et al. accepted) and H_e estimates in another marine planktonic diatom, *Ditylum brightwellii* (0.71, Rynearson and Armbrust 2005). The HWE tests showed only significant departures from HWE in the New Zealand population at locus PP1 and in the Japanese population at locus PP3 and locus PP4 which could probably be attributed to the higher allelic richness at loci PP3 and PP4 in Japan compared to other populations.

Significant geographic genetic structuring was revealed on a global scale (Table 3). The PCA analysis of pairwise F_{ST} values showed that New Zealand and Pacific USA are most differentiated from the other populations and that populations within the Atlantic are less divergent. This is in agreement with the IBD analysis, which showed a significant negative correlation between genotypic similarity and geographical distance. It should be noted that we used the shortest sea connections in our IBD analysis which may not necessarily reflect the actual dispersal distance in complex oceanic circulations (see below). The results of the STRUCTURE analyses were in agreement with the results based on pre-defined populations. Six genetic clusters were revealed, indicating that the amount of genetic structure was strong enough to assign strains to different groups and that migration rates/gene flow between the sampling locations are not high enough to make the sampling locations act as a single unstructured population (Pritchard et al. 2007). Strains from the Atlantic showed a more admixed background, while Pacific strains clustered mainly according to their geographic origin. Yet, even on the largest

geographical scale in our sampling design, some admixing was suggested by the STRUCTURE analysis, indicating that long-distance dispersal may occur, but not frequently enough to counteract population differentiation. Whether these occasional long-distance dispersal events are natural or human-mediated (e.g. by means of ballast water or translocation of aquaculture stocks) can as yet not be deduced from our data. Additional data on divergence times between the var. *pungens* populations, historical distribution records, and testing the observed geographical patterns with oceanic models (Dawson et al. 2005, Bolch & de Salas 2007) may help to elucidate this question.

In contrast to the high levels of gene flow found in *P. pungens* var. *pungens* populations in the North Sea (Evans et al. 2005, Casteleyn et al. accepted), our results suggest that on a larger geographical scale gene flow and thus population mixing is more restricted. This implies that there may be limits to the actual dispersal of *P. pungens* var. *pungens* and that genetic differentiation in allopatry plays an important role in the observed population structure. Isolation by (large) distance (i.e. thousands of kilometres) could allow for genetic divergence even in high-dispersal organisms, such as planktonic diatoms. A number of studies on marine fishes and invertebrates have indeed demonstrated IBD over large to small geographic scales (e.g. Nishida & Lucas 1988, Palumbi & Metz 1991, Pogson et al. 2001). Apart from distance, various other isolating mechanisms, including present-day physical or ecological barriers, are potentially at play in the apparently homogeneous marine environment. *Pseudo-nitzschia pungens* var. *pungens* has until now only been found with certainty in temperate coastal regions (Casteleyn et al. 2008). This apparently anti-tropical distribution indicates that geographic variation in seawater temperature might act as an ecological barrier for trans-tropical dispersal, as has been shown in some fishes and foraminifers (Bowen & Grant 1997, Darling et al. 2004, 2007). This hypothesis remains to be tested through physiological experiments and extensive sampling in tropical and subtropical seas. Also oceanic dispersal might be more problematic than previously assumed, given that coastal environments and the open ocean have very different physical and biological characteristics (Nuwer et al. 2008). For example, differentiation of East and West Pacific populations of coastal sardines was attributed to vast expanses of open ocean (Bowen & Grant 1997). Physical separation as a consequence of prevailing water currents provides another possible explanation for the observed genetic isolation among global populations. Tidal currents and geographical isolation were invoked as explanations for fine scale population genetic structure of the planktonic dinoflagellate *Alexandrium tamarense* along the Japanese and Korean coasts (Nagai et al. 2007). Similarly, in a population study of the chaetognath *Sagitta setosa*, partially enclosed gyral currents within the Mediterranean Sea were shown to maintain population differentiation, while no structure was found in the well-mixed NE Atlantic populations (Peijnenburg et al.

2006). In our study, no significant F_{ST} value was found between var. *pungens* populations from the Belgian North Sea and a Danish inland fjord connected with this Sea. On the other hand, strains from the Irish Sea did show differentiation with the adjacent North Sea strains. Waters from the Irish Sea and North Sea are known to be separated by sea fronts which limit exchange between both seas (Pingree & Griffiths 1978). Similarly, a population genetic survey of the coccolithophore *Emiliania huxleyi* revealed two well-differentiated populations in two water masses (a Norwegian fjord in connection with the North Sea and an oceanic population on the west of Ireland) (Iglesias-Rodriguez et al. 2006). On a macrogeographical scale, large-scale features of the ocean circulation and continental land masses may act as effective barriers to gene flow among ocean basins and between ocean circulation centres (Palumbi 1994). Such barriers may explain the reduced gene flow observed in the var. *pungens* populations between the Atlantic and Pacific, and between the northern and southern Pacific Ocean. Also in the globally distributed oceanic copepod sister species *Eucalanus hyalinus* and *E. spinifer*, oceanic gyres and continental land masses were the two features observed to act as effective barriers to gene flow between populations (Goetze 2005). Yet, the impact of specific barriers on population genetic structure was found to vary between both sister species (Goetze 2005). Similarly, in well-studied pelagic marine foraminifer species complexes, ecological and geographical barriers to gene flow producing biogeographic structure were shown to be species-specific (Darling & Wade 2008). This is related to the fact that individual species have unique evolutionary histories and ecological requirements resulting in diverse biogeographies (Planes & Fauvelot 2002, Goetze 2005, Darling & Wade 2008).

The results presented here show that within var. *pungens*, significant population differentiation exists at the largest macrogeographic scales, indicating that allopatric processes have an important role in diversification. Denser geographical sampling, wider scanning of the genome and knowledge on the species ecology will be needed to reveal on what scale differentiation takes place and to better understand the underlying causes of the observed differentiation in *Pseudo-nitzschia pungens*. Population genetic studies in other *Pseudo-nitzschia* species and in diatoms in general, may shed light on the degree to which patterns observed here are species-specific. This will yield important insights in the role of allopatric events in speciation of planktonic protists, which has long been underrated because of the large population sizes and high dispersal ability of micro-organisms (Finlay et al. 2004).

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7

General discussion

This thesis focuses on the species structure and biogeography of the marine planktonic diatom *Pseudo-nitzschia pungens*. This species has been reported worldwide in coastal regions over a wide temperature range (Hasle 2002) and toxic clones have been reported from New Zealand (Rhodes et al. 1996), Washington State (Trainer et al. 1998) and Monterey Bay, California (Bates et al. 1998). However, given the high (pseudo)cryptic species diversity in diatoms in general (Mann 1999) and *Pseudo-Nitzschia* in particular (Lundholm et al. 2003, 2006; Amato et al. 2007, Amato & Montresor 2008), *P. pungens* might actually comprise several species with unknown geographic distributions and environmental preferences. A prerequisite for studying biogeography in diatoms is therefore to obtain a good understanding of taxon boundaries, preferably based on different lines of evidence. To this end, local, regional and global variation in selected molecular markers, sexual compatibility and morphological characteristics were studied in *Pseudo-nitzschia pungens*, after which the geographic distribution of the resulting lineages was assessed. To understand intraspecific genetic structure in *P. pungens*, genetic diversity and differentiation at the population level were studied using microsatellites on both regional and global scales.

Pseudo-nitzschia pungens: one or multiple species?

Despite the wide acceptance of the idea that species represent a fundamental unit of biological organization (Mayr 1982), there has been a great deal of disagreement with regard to the criteria used to delimit species. This disagreement has led to a proliferation of different species concepts, followed by endless discussions on their respective value and applicability (Mayden 1997). More recently, however, important conceptual progress has been made in thinking about species concepts (de Queiroz 1998, 2007). De Queiroz argues that all species concepts share the common view that species are "separately evolving lineages". Other species concepts (including the morphological,

biological, and phylogenetic species concept) then become criteria for species recognition. In other words, different lines of evidence (e.g. phenotypic discontinuities, sexual incompatibility or genetic distinctiveness) can help the inference of these lineages, which are the primary defining properties of the species category. De Queiroz (2007) postulated that the presence of any one of these properties can be regarded as evidence for lineage divergence and thus for the existence of a species, because different characters may arise at different times during the process of speciation. However, strong support for a hypothesis of lineage separation requires multiple lines of evidence.

To elucidate whether *P. pungens* comprises a single or multiple lineages, we have studied genetic and morphological variation and reproductive compatibility in a large collection of isolates. An initial investigation of the life-cycle and sexual reproduction in *P. pungens* pinpointed the cardinal points of the life-cycle and showed that the species has a heterothallic mating system (chapter 2), concordant with results from other *Pseudo-nitzschia* species (Davidovich & Bates 1998, Kaczmarska et al. 2000, Chepurnov et al. 2004, Amato et al. 2005, D'Alelio et al. 2009a). This allowed us to test reproductive compatibility by crossing experiments.

Genetics

Phylogenetic analysis of rDNA internal transcribed spacer (ITS) sequences from strains of the NE and NW Atlantic, and the NE, NW and SW Pacific revealed the presence of at least three closely related but distinct clades in *P. pungens* (chapter 3). Additional genetic study showed that clades I and II are also differentiated for the *rbc*L chloroplast gene (chapter 4). Moreover, this agrees well with another study finding two distinct populations in *P. pungens* using four microsatellite loci (PP2, PP3, PP5 and PP6) (Adams 2006), which were shown to correspond to clades I and II (Chapter 4). Standardized F_{ST} value between the two clades was 0.97 (recalculated from Adams 2006), which is in agreement with very little overlap in allele size classes. This F_{ST} value was much higher than pairwise F_{ST} values between global clade I populations found in our population genetic study (even when we used only the same four loci used by Adams 2006 to calculate F_{ST} values) (chapter 6).

Morphology

These clades can also be distinguished by subtle but consistent morphological differences, visible under the scanning electron microscope, and correspond to the nominal variety of *P. pungens* (clade I), the previously described variety *cingulata* by Villac and Fryxell (1998) (clade II) and the newly described variety *aveirensis* by Churro et al. (in press, see appendix) (clade III).

Reproductive compatibility

Mating tests revealed that sympatric strains of opposite mating type were sexually compatible within these clades (chapter 3, appendix). In clade I, sexual compatibility was also demonstrated among allopatric strains regardless of their geographic origin (North Sea region, Spain, Canada, Japan, New Zealand or the Pacific coast of North America). This is relevant because studies in other eukaryotic groups have shown that uniformity in ITS sequences does not always correspond to reproductive compatibility (e.g. *Paramecium*; Coleman, 2005, see chapter 1). Our results thus indicate that members of clade I can also be regarded as belonging to one single lineage based on their sexual compatibility. Strains belonging to clade I and clade III were sexually incompatible (appendix). It should be noted, however, that negative test results for reproductive isolation in culture should be interpreted with care. We experienced that sometimes strains failed to mate even though they were known to be reproductively compatible, based on previous positive crossings. In a number of cases sexual activity could not be induced at all in crosses with reference clones of both mating types, although these strains belonged to the same clade, originated from the same locality and were below the known sexual size threshold. Different factors could explain this absence of mating; for example the presence of an internal clock, the presence or absence of certain bacteria, or other environmental factors that could influence the induction of sexual reproduction once cells have the sexual inducible size (Lewis 1984, Hiltz et al. 2000, Chepurnov et al. 2004, D'Alelio et al. 2008).

In contrast to the apparent reproductive isolation between clades I and III, strains of clades I and II were sexually compatible in laboratory conditions and produce viable hybrid offspring (chapter 3). In addition, natural hybrids (based on ITS and *rbc*L sequence data) were discovered in samples from the NE Pacific where members of both clades co-occur (chapter 4). However, the occurrence of hybrids and hence gene flow between taxa, does not inevitably imply complete fusion of the taxa involved (Barton and Hewitt 1989). The evolutionary outcome of such hybridization events depends on the hybridization frequency and the fitness of the resulting progeny, as this will determine the amount of gene flow between lineages (Barton and Hewitt 1985). The sympatric persistence of two distinct *P. pungens* clades capable of forming hybrids, suggests that the hybrids may suffer a disadvantage or that there is limited spatial or temporal overlap between the two clades. Low hybrid fitness can be intrinsic (i.e. the F1 progeny is not viable or sterile) or the parental taxa that hybridize could remain distinct despite the absence of intrinsic postzygotic isolation in F1 hybrids. Low hybrid fitness might then be extrinsic (dependent on the environment) or may only appear in generations beyond the F1 (Coyne and Orr 2004, Price and Bouvier 2002). In the case of the *P. pungens* hybrids, the F1 progeny being not viable or sterile seems unlikely

because the F1 isolates from the field samples grew well in culture and there were also indications for further hybridisation beyond F1 in the field. Unfortunately, we could not test the fertility of the F1 generation because we were not able to induce sexual reproduction in our NE Pacific cultures (cf. above). Extrinsic factors or possible postzygotic isolation (sterility or inviability) of later generation hybrids (Coyne and Orr 2004) should be further investigated. Despite the occurrence of occasional hybridisation between clades I and II, both clades appear to persist, even in sympatry, and can therefore be regarded as two independently evolving lineages.

In conclusion, *P. pungens* comprises three closely related, independently evolving lineages, currently described as varieties (var. *pungens*, var. *cingulata*, var. *aveirensis*). Different lines of evidence support the recognition of the three lineages as different species. These results are in concordance with other studies in diatoms that revealed genetically and biologically distinct, but often closely related species within morphologically defined taxa (see chapter 1 for references).

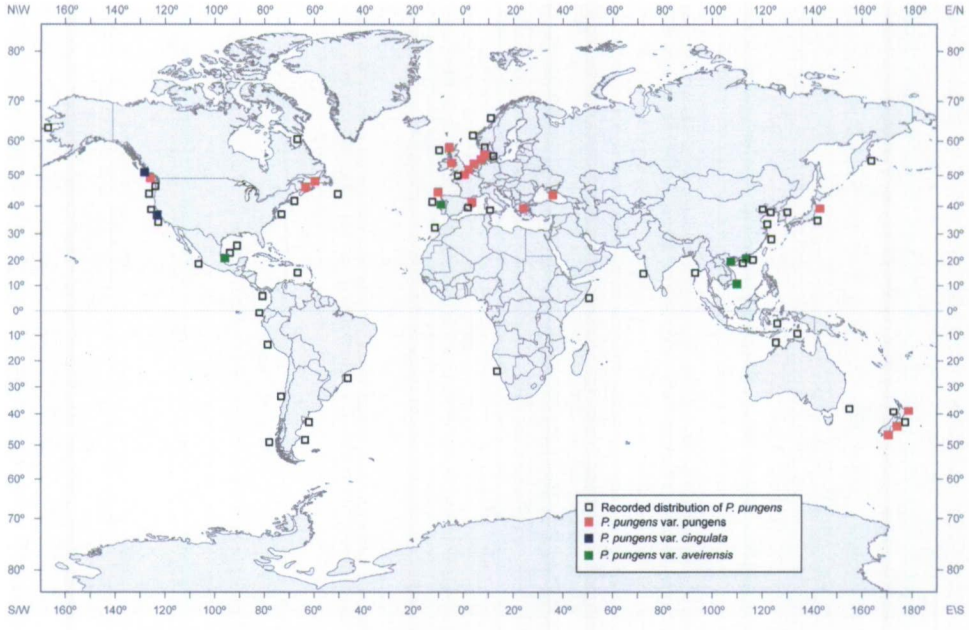

Fig. 1. Geographical distribution of *Pseudo-nitzschia pungens*: morphology-based records (open squares) and verified isolates based on DNA sequencing (filled squares). Redrawn and updated from Trainer et al. (2008).

Biogeography

The marine planktonic environment appears to lack strong isolating barriers, and was therefore believed to sustain only a limited number of ubiquitously distributed species (Palumbi 1992, 1994, Finlay 2002, Carr et al. 2003). This belief, however, has been mainly based on morphological taxon delineations (Finlay, 2002). Recently, the use of molecular tools suggests that many planktonic protist species which were formerly regarded as cosmopolitans are in fact complexes of several genetically distinct but morphologically identical (cryptic) or strongly similar (semi-cryptic) species (de Vargas et al. 1999, 2002, John et al. 2003, Montresor et al. 2003, Saez et al. 2003, Darling et al. 2004, Goetze 2005, Sarno et al. 2005, Lundholm et al. 2003, 2006, Amato et al. 2007, Lilly et al. 2007, Darling & Wade 2008, Kooistra et al. 2008). In several cases these (semi)cryptic species were found to be geographically restricted by both physical and ecological barriers (de Vargas et al. 1999, 2002, Darling et al. 2004, Goetze 2005, Darling & Wade 2008). Yet, cryptic diversity does not necessarily preclude wide distribution of species, as is exemplified by the existence of multiple, globally distributed ancient cryptic lineages in picoplanktonic green algae (Šlapeta et al. 2006).

Species of the pennate diatom genus *Pseudo-nitzschia* are common components of phytoplankton in coastal and open-ocean waters worldwide and, based on morphology, most appear to have a cosmopolitan distribution (Hasle, 2002). Phylogenetic studies have revealed cryptic variation in a number of *Pseudo-nitzschia* species, but most of these studies were restricted to local-regional scales and consequently the distribution of the newly described species remains unknown (e.g. Amato et al. 2007, Amato & Montresor 2008). A few studies on a wider spatial scale only included a limited number of strains, resulting in incomplete geographic coverage (Lundholm et al., 2003, 2006). One of the most comprehensive species-level phylogenies in diatoms includes a study on the globally distributed planktonic taxon *Skeletonema costatum* (Kooistra et al. 2008). This study revealed multiple cryptic species, some of which are found in both the northern and southern temperate latitudes whereas others appear to have only subtropical to tropical ranges and still others seem to have more restricted geographical ranges (e.g. only in USA waters) (Kooistra et al. 2008).

Likewise, *P. pungens* has been reported worldwide from subpolar to tropical coastal waters (Hasle 2002). The three distinct lineages/varieties however appear to have different geographic distributions (Fig. 1). A large geographical sampling, carried out in the framework of this study, confirmed the presence of *P. pungens* var. *pungens* in coastal temperate Atlantic and Pacific waters in both hemispheres (NE and NW Atlantic, NE and NW Pacific, SW Pacific) (Fig. 1), and thus appears to have an antitropical distribution. *P. pungens* var. *cingulata* has so far only been recorded from the NE Pacific

Ocean where it co-occurs with var. *pungens*. *P. pungens* var. *aveirensis* appears to be present in warmer seas as it has to date been recorded from Mexico, Vietnam, China and a Portuguese lagoon. Additional sampling will likely reveal a wider geographical distribution. Interestingly, a well-studied and extensively sampled coastal lagoon in Aveiro, Portugal, only harboured var. *aveirensis* and no other *P. pungens* variety (see appendix). On the other hand, var. *aveirensis* was never encountered in other well-sampled coastal temperate areas (chapter 3-6, Quijano-Scheggia et al. 2008) suggesting that the distribution of var. *aveirensis* might be non-overlapping with the other two varieties. This may be related to different ecological preferences or tolerances for this variety (e.g. temperature). Current knowledge about the ecology of *P. pungens* is insufficient to assess what environmental factors may underlie the different distributions of the varieties. It has recently been shown that the niche range of different cryptic marine microplankton species is remarkably smaller than previously thought, e.g. in marine foraminiferans (Darling & Wade 2008) and the picoplanktonic green alga *Micromonas pusilla* (Foulon et al. 2008). As a result, many species, even if they are widely distributed, may be limited to areas with a certain temperature, light or surface ocean productivity (de Vargas et al. 1999, Darling & Wade 2008).

How may the observed distribution patterns of the three *P. pungens* varieties have come about? Present-day geographical distributions of organisms are affected by both historical (e.g. past climatic or geological) and contemporary (e.g. environmental) events. The establishment of a feasible hypothesis of historical biogeography not only requires a realistic knowledge of present-day geographical distributions but also a robust, well-covered species-level phylogeny with dated divergence times. The latter is problematic since the age of the *Pseudo-nitzschia* species (and *P. pungens* varieties) is unknown due to the lack of a reliable fossil record. We tried to make a rough estimate of the divergence times between the three varieties based on an extrapolation of a diatom molecular clock-study (Sorhannus 2007), which estimated the split of *P. pungens* with its closest relative *P. multiseries* at 5 Mya (Fig. 2, see the figure legend for analytical details) (the resulting chronogram should be interpreted with care, as different molecular clock studies have shown variation in divergence times between major diatom lineages; Kooistra & Medlin 1996, Sorhannus 2007). From this analysis, we infer that var. *aveirensis* first diverged from the other two varieties around 1.3 - 0.5 Mya and that var. *pungens* and var. *cingulata* diverged from each other somewhere between 500.000 - 100.000 years ago (Fig. 2). The divergence of *P. pungens* into three distinct taxa may thus have taken place during the Pleistocene, a period characterized by a series of advances and retreats of polar ice sheets as the climate fluctuated between cold (glacial) and warm (interglacial) periods. The sea level rose during the melting of the glaciers, then dropped again during the next long cold period (ice formation). The common

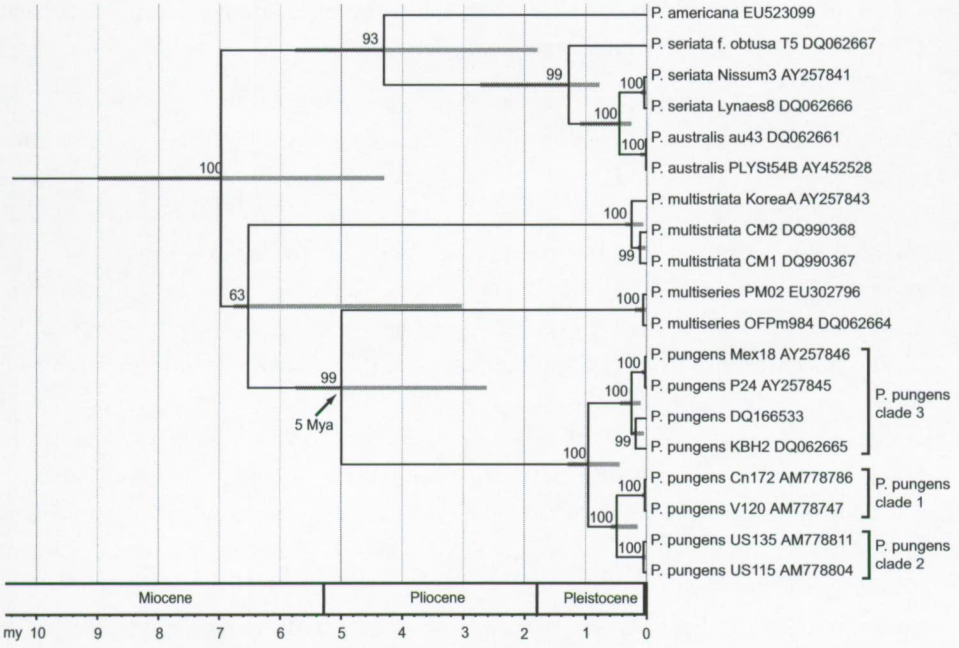

Fig. 2. Dated phylogeny of *Pseudo-nitzschia* clade I (as circumscribed by Lundholm et al. 2006) based on a Bayesian analysis of *rbc*L, LSU rDNA and ITS sequence data. For each species a single *rbc*L and LSU sequence was selected from GenBank (except in *P. pungens*, where three *rbc*L sequences were used for each variety). Accession numbers refer to ITS sequences only. Divergence times were estimated under a relaxed molecular clock using an uncorrelated lognormal (ULLN) model in BEAST v1.4.6 (Drummond & Rambaut 2007). The Markov chain Monte Carlo (MCMC) analysis was run for 5 million generations, sampling every 1000, and summary statistics and trees were generated using the last 3 million generations. Uncertainty in the tree topology and divergence times are indicated by grey bars on internal nodes, corresponding to the 95% highest posterior density (HPD) of node ages. The single calibration point (estimated split of *P. pungens* with its closest relative *P. multiseries* at 5 Mya, based on Sorhannus, 2007) is indicated by an arrow. Time is given in millions of years before present.

ancestor of *P. pungens* may have expanded its range during colder periods, while during a successive warmer period, ecological diversification by oceanographic changes in water temperature could have arisen, leading to diversification in geographically non-overlapping genetic entities, i.e. var. *aveirensis* and the ancestor of var. *pungens* and var. *cingulata*. The antitropical distribution of var. *pungens–cingulata* clade could thus have arisen during the Ice Ages. The increased cooling and upwelling along the equatorial divergences in both the Pacific and the Atlantic allowed temperate forms to disperse from one hemisphere to the other. A following warmer interglacial period would then result in an antitropical separation (Crame 1993). This hypothesis should be formally

tested through controlled physiological experiments to establish the temperature tolerance of the different varieties and extensive sampling in tropical and subtropical seas to confirm the exclusive presence of var. *aveirensis*.

According to our molecular clock estimates, var. *pungens* and var. *cingulata* are recent taxa and in an early stage of divergence as indicated by their low sequence divergences (ITS: 1.3% and rbcL: 0.3%). The absence of prezygotic isolation mechanisms between both taxa appears to corroborate this observation. To our knowledge, var. *cingulata* is endemic to the NE Pacific. Alternatively, it may be extinct elsewhere. Several (albeit speculative) hypotheses, related to the specific climate histories and sizes of the Atlantic and Pacific oceans, can be put forward to explain the observed biogeographies of both varieties. As stated in Jacobs et al. (2004), climate induced perturbations during the Pliocene and Pleistocene were more extreme in the N Atlantic and along the west coast of South America than in the NE Pacific, which led to regional extinctions in the former areas. In addition, the Atlantic is a smaller ocean, providing less surface area within which evolutionary novelties may appear and disperse (Cox and Moore 2005). This may explain why for marine fauna the N Atlantic is much less diverse and has fewer endemic forms than the N Pacific. Because the Atlantic is narrower than the Pacific, a greater proportion of species is shared between both sides of the Atlantic in comparison with the Pacific (Cox and Moore 2005). More detailed studies on fishes and marine cladocerans have shown that the vast expanse of the Pacific and subsequent glacial/interglacial separations and connections between NE and NW Pacific or between Pacific and Atlantic via the Arctic Ocean, have generated significantly differentiated populations on both sites of the Pacific (Bowen & Grant 1997, Durbin et al. 2008). On a more regional scale, Nuwer et al. (2008) showed that coastal subspecies populations of the planktonic copeopod *Calanus pacificus* are differentiated along the NE Pacific coast, which indicates historical or recent barriers (physical or ecological) along the NE Pacific coast. This might also explain the divergence of *P. pungens* into two varieties along this coast.

The geographic distribution patterns of the two *P. pungens* varieties and their regional co-occurrence in the NE Pacific can tentatively be explained by two different scenarios: (1) the two *P. pungens* varieties evolved sympatrically along the NE Pacific coast; or (2) a secondary contact arose after para- or allopatrical differentiation of the two varieties (either along the same coast or in distant geographical regions). Both scenarios are notoriously difficult to separate (Barluenga et al. 2006, Savolainen et al. 2006). Evidence from microsatellite data (chapter 6) appears to favour the hypothesis that *P. pungens* var. *cingulata* originated in the NE Pacific and later var. *pungens* came in secondary contact with limited genetic admixture. Based on the maximum microsatellite allelic richness and H_e observed in Japan compared with the other

locations in the Atlantic and the Pacific, we could hypothesize that var. *pungens* has spread from the NW Pacific to other regions. Centers of origin are generally characterized by a higher genetic diversity than newly invaded areas (Dlugosch & Parker 2008). The lowest allelic richness and H_e revealed in the Washington State (NE Pacific) could then be seen as a recent range expansion of var. *pungens* to the NE Pacific across a previously effective dispersal barrier by land/ice (Pacific–Atlantic) or sea/ice (NW–NE Pacific). In addition, var. *cingulata* revealed a much higher allelic richness and H_e in comparison to var. *pungens* in the NE Pacific, based on four microsatellite loci (Adams 2006). H_e and allelic richness for var. *cingulata* were comparable with values for var. *pungens* from Japan, when calculated with the same four microsatellite loci as in the study of Adams (2006) (Fig. 3), which may suggest that var. *cingulata* originated in the NE Pacific. It is obvious that these hypotheses should be thoroughly tested. Future phylogeographical studies using rapidly evolving and rapidly sorting DNA markers (e.g. fast evolving spacer regions), could yield important insights in the historical distribution events. In addition, comparative phylogeographical analysis of multiple, co-distributed taxa are needed to differentiate general events (e.g., vicariance or dispersal) from taxon-specific events (e.g. long-distance dispersal and speciation of peripheral isolates) (Riddle & Hafner 2007).

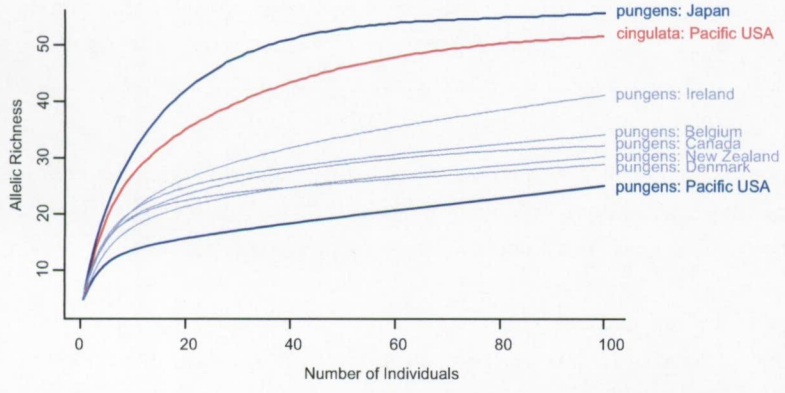

Fig. 3. Comparison of allelic richness between *P. pungens* var. *cingulata* from the NE Pacific (red line), and *P. pungens* var. *pungens* from both sides of the northern Pacific (blue lines) and other regions (light blue lines). Allelic richness was inferred from multilocus genotypes (microsatellite loci PP2 PP3 PP5 PP6), and extrapolated beyond the sample sizes using ARES (Van Loon et al. 2007).

Population genetics

The first population genetic study on marine diatoms was performed by Gallagher (1980) using allozymes on 457 isolates of *Skeletonema costatum*. This study gave a first indication of genetic diversity among diatom isolates, although several cryptic species might have been involved. The physiological variability observed between individuals with the same allozyme genotype showed that even greater genetic variation existed within populations that went undetected with the relatively insensitive protein techniques (Gallagher 1982). Skov et al. (1997) found that 10 isolates of *Pseudo-nitzschia pseudodelicatissima* (now *P. callianta*) each had a different protein banding pattern suggesting that blooms were composed of a large number of genetically different clones. The use of microsatellite markers has recently resulted in a better understanding of genetic diversity within diatom populations. In a first study using two microsatellite loci, Rynearson & Armbrust (2000) showed that 22 out of 23 strains of *Ditylum brightwellii* isolated from one water sample had a unique genotype. High clonal and genetic diversity were also found in subsequent studies based on a larger number of isolates (Rynearson & Armbrust 2004, 2005, Rynearson et al. 2006) and in microsatellite surveys of *Pseudo-nitzschia multiseries* and *P. pungens* (Evans et al. 2004, Evans et al. 2005). These findings are consistent with the fact that sexual reproduction is firmly integrated into the life cycles of these diatoms generating high levels of genetic diversity. Because diatoms gradually reduce their cell size in the course of the mitotic (clonal) part of their life cycle, periodical sexual events are essential for re-establishing the initial cell size (Chepurnov et al. 2004). Moreover, theoretical work suggests that a small number of sexual individuals per generation is sufficient to make an apparently asexual population genotypically highly variable (Bengtsson 2003).

Population genetic surveys using sufficiently discriminatory molecular markers on a sufficiently large sample size also provide information on gene flow (and hence connectivity) between populations. Two local-scale microsatellite studies using numerous isolates revealed contrasting results. For *Ditylum brightwellii*, the maintenance of differentiated populations was shown in connected and daily intermixing estuaries along the NW coast of America (Rynearson et al. 2006). This differentiation between populations was attributed to a combination of hydrology and differential selection (Rynearson & Armbrust 2004). In contrast, for *Pseudo-nitzschia pungens* strains from an open water environment in the German Bight of the North Sea over a similar spatial scale, apparent panmixis was found (Evans et al. 2005). We extended the study of Evans et al. (2005) by investigating the spatial and temporal genetic composition in the environmentally and hydrographically heterogeneous Southern Bight of the North Sea to assess whether different environments or partial isolation could restrict gene flow

between populations of *P. pungens* as was seen for *Ditylum* (chapter 5). Despite the fact that our sampling ranging over ca. 100 km comprised water bodies with different environmental and hydrographic conditions (marine, estuary, saline lake, tidal vs non-tidal) and with different degrees of connectivity between them (from near-complete isolation to supposedly free water exchange between environments), no evidence for genetic differentiation was found. This indicated that separation between water masses was not complete and gene flow too high (e.g. via water birds) or that isolation was too recent to allow *P. pungens* populations to diverge. Our findings appear to be in stark contrast with the results of Rynearson et al. (2006). However, it can not be excluded that their results might have been confounded by the presence of two cryptic species as is suggested by relative high levels of ITS sequence divergence (1.1 %), twofold differences in valve diameter, and deviations of HWE in most samples. It is imperative to elucidate species boundaries before assessing variation at the population level because intraspecific population structuring and cryptic taxonomic diversity may mimic each other. Cryptic diversity was not an issue in our study because we showed the existence of only one single lineage (var. *pungens*) in our sampling area. Extending our sampling area by the inclusion of isolates from the German, French and the Dutch parts of the North Sea (ca. 650 km apart), also did not reveal significant structuring, suggesting the presence of a homogenous population structure for *P. pungens* var. *pungens* in large parts of the North Sea.

It has been suggested that *P. pungens* s.l. may be far more homogeneous on a wide geographic scale than it close relative *P. multiseries* (Evans et al. 2005, Lundholm & Moestrup 2006). In a microsatellite study of *P. multiseries* involving 25 isolates, it was shown that a single Russian isolate (Sea of Japan) introduced 11 new alleles at six loci, relative to NW Atlantic Canadian material (Evans et al., 2004). In contrast, a *P. pungens* study showed that three Canadian isolates possessed only two alleles not found among 464 German isolates (Evans et al. 2005). This appeared to be surprising given the considerable geographical separation and the conflicting results for its close relative *P. multiseries* and was interpreted as a first indication that *P. pungens* s.l. may be truly cosmopolitan, being able to tolerate a wide range of environmental conditions with few barriers impeding its dispersal (Evans et al. 2005). However, our population genetic survey of var. *pungens* on a global scale (chapter 6) showed that the results in *P. multiseries* and *P. pungens* var. *pungens* might be very similar. We also found that Japanese isolates introduced more new alleles when compared to Canada and that Canadian isolates have few private alleles in comparison with the North Sea. This is probably related with fewer obvious dispersal barriers present between the NE and NW Atlantic than between the NW Atlantic and the Pacific (see above "Biogeography"). Indeed our global analysis of var. *pungens* populations revealed a pattern of isolation by

distance with much more genetic admixing between NE and NW Atlantic populations than between Atlantic and Pacific populations and than within Pacific populations. These results show that within var. *pungens*, significant population differentiation exists at the largest macrogeographic scales and that there may be limits to the actual dispersal of *P. pungens* var. *pungens*, driven by oceanographic or ecological factors (see chapter 6). This provides support for the role of geographic isolation in generating microbial diversity, which was believed to be of minor importance in the marine microbial environment (Finlay et al. 2004). However, a wider scanning of the genome and knowledge on the species ecology will be needed to reveal the underlying causes of the observed differentiation. After all, the impact of specific barriers on population genetic structure is found to be species-specific, as shown for example for sister species of oceanic copepods (Goetze 2005). Similarly, in well-studied pelagic marine foraminifer species complexes, species-specific ecological and geographical barriers to gene flow producing biogeographic structure were shown (Darling & Wade 2008). Indeed individual species have unique evolutionary histories and ecological requirements resulting in diverse biogeographies (Planes & Fauvelot 2002, Goetze 2005, Darling & Wade 2008). Population genetic studies in other *Pseudo-nitzschia* species and in diatoms in general, will yield further insight into the degree to which patterns observed in *P. pungens* var. *pungens* are species-specific.

Recently, it is widely accepted that the geographic distributions of protists may be modified by human introductions. The potential of ballast water as a major vector for marine introductions has since long recognized (Ostenfeld 1908). However, finding evidence that links suspected introduced species with a source population is more challenging. There is one study on toxic dinoflagellates, based on multiple lines of evidence (historical distribution records, sediment dating studies, comparison of observed patterns of genetic and biochemical variation with paleobiogeographical scenarios) that indicates the introduction of certain dinoflagellate species into Australian and New Zealand waters during the past 100 years. This was most probably by means of ballast water from bulk-cargo shipping from Japan and/or south-east Asia (Bolch & de Salas 2007). On a much smaller scale, a microsatellite survey of the dinoflagellate *Alexandrium tamarense* along the Japanese and Korean coasts (ca. 1600 km), attributed the lack of differentiation between wide separated locations to human assisted dispersal, based on disturbance of a generally observed correlation between genetic distance and geographic distance, along with circumstantial evidence from long term monitoring (Nagai et al. 2007). In our global *P. pungens* var. *pungens* microsatellite study, we found a clear correlation between genetic similarity and geographic distance (chapter 6), although it should be noted that only a few disjunct populations were analyzed, potentially biasing our results. Interestingly, we also found some indications for long-

distance dispersal in our global dataset, but these events are not frequent enough to counteract population differentiation. Given the present data, it remains unclear whether long-distance dispersal has resulted from natural dispersal or human-mediated transport (e.g. by means of ballast water or translocation of aquaculture stocks). Simultaneous estimates of migration and population divergence times might give better insight in this matter (cfr. Cunningham 2008). Ideally, information on divergence times between the var. *pungens* populations could be used to test for human introductions by comparing the appearance and distribution of populations with human activity. Unfortunately, information on mutation rates of our microsatellites loci are lacking and therefore it is impossible to estimate the divergence times of our populations. The use of oceanic models may help to elucidate if the observed geographical patterns are possible via natural dispersal in oceanic currents (cfr. Dawson et al. 2005). By integrating dated molecular phylogenetic information of the marine meroplanktonic moon-jellyfish *Aurelia* and global biophysical ocean models, Dawson et al. (2005) were able to show that disjunct distribution patterns of this organism were attributed to human aided introductions worldwide, rather than natural long-distance dispersal.

Suggestions for future research

A good understanding of biogeography and global patterns of population structure requires broad and dense geographical sampling. In this thesis, sampling has perhaps not been sufficiently dense geographically to test some of our hypotheses adequately (e.g. antitropical distribution, global population differentiation, IBD). There is a big hiatus in our sampling in the southern Atlantic, southern East Pacific and Indian Ocean, especially tropical and subtropical regions. A better understanding of historical distribution events of the three varieties and their different populations could be obtained by phylogeographic analysis based on rapidly evolving and sorting DNA markers (more variable than ITS, e.g. fast evolving spacer regions). The available complete genome sequences of the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, and the running genome projects for *Fragilariopsis cylindrus* and *Pseudo-nitzschia multiseries* (sister species of *P. pungens* s.l.) will increase the amount of information available and will aid the development of markers (both neutral and those potentially under selection) in these species and their closest relatives (Mann & Evans 2008).

Knowledge about the ecology of the three varieties and their different populations may enhance our understanding of their current distribution. Controlled ecophysiological experiments may assess differential adaptation of geographically separated populations

to environmental conditions and may find relations with toxicity of different populations. Together, this information could be used in oceanic models to help elucidate if the observed geographical patterns are possible via natural dispersal in oceanic currents or if they are human-mediated (cfr. Dawson et al. 2005).

Population genetic studies in other *Pseudo-nitzschia* species and in diatoms in general, will yield insight on the degree to which patterns observed in *P. pungens* var. *pungens* are species-specific. Population genetics studies in protists will be easier in the future by the development of single-cell PCR (Frommlet & Iglesias-Rodriguez 2008), circumventing the need to establish clonal cultures.

Our study revealed the NE Pacific coast to be a very interesting study area because of the co-occurrence of two *P. pungens* varieties. Extensive sampling along the NE Pacific coast, together with the use of multiple biparentally inherited nuclear markers, will give better insight in the spatial and temporal range of the contact zone and hybridization frequency. Controlled culture experiments should be conducted to assess whether the F1 progeny is fertile and whether further hybrids (F2 and backcrosses) are also viable and fertile or whether certain environmental factors reduce their fitness. Together, these data will allow defining the amount of gene flow between the *P. pungens* varieties and its evolutionary consequences.

In order to better understand mechanisms for induction of sexual reproduction, reproductive compatibility and mating barriers, detailed genome-based studies will be required. Future studies using information from EST libraries may aid in finding genes which are correlated with sexual induction and compatibility.

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Appendix

Pseudo-nitzschia in Aveiro lagoon, Portugal

Species diversity and description of *P. pungens* var. *aveirensis* var. nov.[1]

The syndrome Amnesic Shellfish Poisoning (ASP) is the result of ingesting sea food contaminated by a neurotoxin, domoic acid. The responsible organisms for the production of this toxin are diatoms of the genus *Pseudo-nitzschia* and *Nitzschia*. A total of eleven species of *Pseudo-nitzschia* were identified during two surveys of the plankton at the entrance and in the southern channels of Ria de Aveiro, the largest lagoon on the west coast of Portugal. During the first survey, conducted from February to October 2000, the following species were identified: *P. australis, P. cuspidata, P. delicatissima, P. dolorosa, P. fraudulenta, P. pseudodelicatissima, P. subpacifica* and a new variety of *P. pungens*. The second survey took place from September 2003 to April 2004, except for a break in December 2003-January 2004, with one complementary sample in October 2004; it revealed three additional species, *P. americana, P. calliantha* and *P. multistriata*. In contrast, *P. cuspidata, P. dolorosa* and *P. subpacifica* were not found during the second survey. Five species: *P. subpacifica, P. americana, P. calliantha, P. multistriata* and *P. cuspidata* were reported for the first time in Portuguese waters. Overall, *P. australis*, *P. pseudodelicatissima* and a new variety of *P. pungens* were the most common species, the latter being present in all samples except October 2000. The highest concentrations of *Pseudo-nitzschia* cells registered were 90,000 cells L^{-1} in August 2000 and 106,000 cells L^{-1} in March 2004, with *P. australis* accounting for 65-75% of the cells. Cell concentration was highest near the entrance of the lagoon and decreased along the channels with decreasing salinity. Cultures of *P. australis*, *P. americana*, *P. fraudulenta*, *P. multistriata* and a new variety of *P. pungens* were established and tested for the production of domoic acid, but the results were negative. Morphological differences were found between our strains of *P. pungens* and two other previously described varieties of *P. pungens* regarding valve width, densities of striae, fibulae, poroids and band striae as well as differences in the morphology of the band striae. These differences supported by phylogenetic analyses of ITS rDNA sequences, existence of a compensatory base change and mating studies indicate the presence of a separate taxon, which is presently described as a new variety, *P. pungens* var. *aveirensis* var. nov.

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[1] Paper in press: Churro CI, Carreira CC, Rodrigues FJ, Craveiro SC, Calado AJ, Casteleyn G, Lundholm N. Diversity and abundance of potentially toxic *Pseudo-nitzschia* Peragallo in Aveiro coastal lagoon, Portugal and description of a new variety, *P. pungens* var. *aveirensis* var. nov. Diatom Research.

Contribution to this chapter by GC: involved in the *P. pungens* part: text, morphometric and phylogenetic analyses.

Introduction

Pseudo-nitzschia H. Peragallo is a widely distributed genus of marine planktonic diatoms (Hasle et al. 1996, Hasle 2002). The genus has received much attention recently because of the capacity of some species to produce the toxin domoic acid (DA), which accumulates through the food web causing intoxication in humans and wildlife (Bates et al. 1989, Bates *et al.* 1998, Fryxell & Hasle 2003). While most species in the genus form characteristic stepped chain colonies that make them readily identifiable at the generic level in the light microscope, identification at species level often requires the observation of minute characters of the cleaned valves, only visible with electron microscopy (Skov et al. 1999). Measures to prevent human poisoning with *Pseudo-nitzschia*-derived toxins include detection of DA in sea food and are currently part of the monitoring programme for marine toxins in many countries; DA analysis of shellfish tissue from the Portuguese coast has been routinely conducted since 1996. The monitoring has shown that the presence of DA in shellfish is a recurring phenomenon that affects shellfish several times of the year, especially in spring and autumn and not seldom at levels above the acceptance limit of 20 μg DA g^{-1} (Vale & Sampayo 2001).

Ria de Aveiro is the largest tidal coastal lagoon of Portugal, extending 45 km N-S and about 10 km E-W, between the city of Aveiro and the Atlantic Ocean. It receives marine water from a single connection with the sea and freshwater from two main rivers and several smaller streams (Fig. 1), whereby a gradient of salinity is established throughout its channels (Dias et al. 1999). This aquatic system is an important resource for aquaculture, including production of shellfish. Contamination by phytoplankton toxins including DA has been reported (Vale et al. 1998, Vale & Sampayo 2001).

Although several species of *Pseudo-nitzschia* have long been known to occur in Portuguese coastal waters (e.g. Carrisso 1911, Candeias 1939, Silva & Pinto 1948), an account of the genus using modern identification methods has never been published for the area. In a phytoplankton list for the Portuguese coast, Moita & Vilarinho (1999) mention *P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. pseudodelicatissima* and *P. pungens*. The species most often reported in Portuguese coastal waters is *P. seriata*, as *Nitzschia seriata* (Silva & Pinto 1948, Silva 1952, Pinto & Silva 1956, Silva 1990). However, *P. seriata* is a cold water species appearing north of 45°N (Hasle 1972). The Portuguese records of *P. seriata* therefore probably represent the morphologically similar *P. fraudulenta* or *P. australis*. Additionally, Hasle et al. (1996) reported *P. subfraudulenta* off the Portuguese coast.

Hence, presently, there are no detailed records of *Pseudo-nitzschia* species in Aveiro coastal waters or even along the Portuguese coast. In the present paper *Pseudo-*

nitzschia species found in Ria de Aveiro are described in detail using light and electron microscopy. Furthermore, quantification of the species throughout the sampling period and occurrence along the southern channels of the lagoon are reported. Strains presumably belonging to *P. pungens* will be discussed because they deviate from the nominal *P. pungens* variety and will here be described as a new variety.

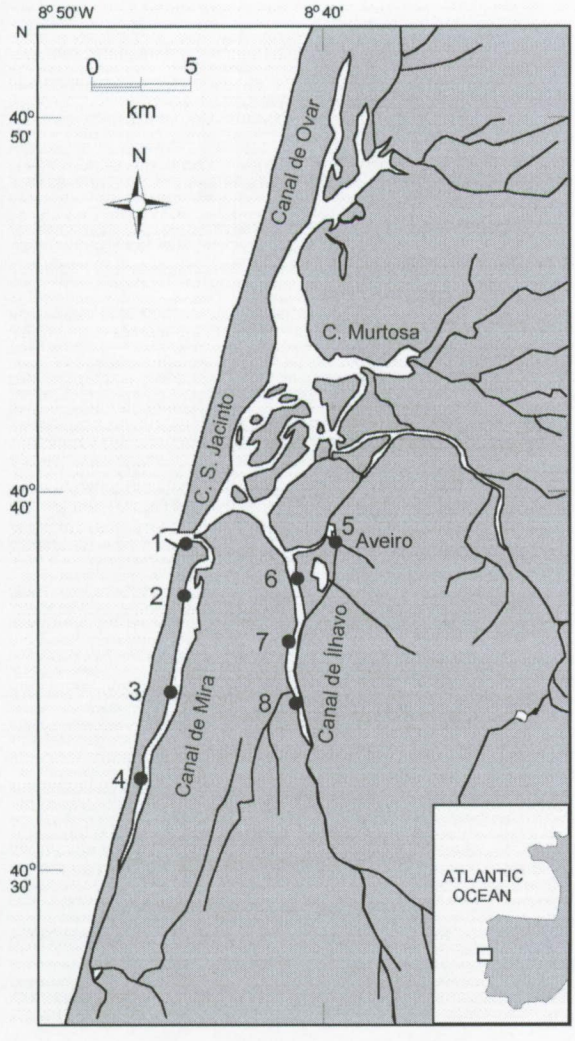


Fig. 1. Map showing sampling localities in the Aveiro coastal lagoon "Ria de Aveiro", Atlantic coast of Portugal. 1, Barra (entrance of the lagoon); 2, Costa Nova; 3, Vagueira; 4, Areão; 5, Rossio; 6, Ílhavo; 7, Vista Alegre; 8, Vagos.

Table 1. Strains of *Pseudo-nitzschia* isolated from Aveiro lagoon and used for molecular and toxin analyses as well as other strains (below the line) used for molecular and statistical analyses.

Species	Strain designation	Isolation date / place	Accession number
P. americana	Phi5	Oct 7, 2004	Not sequenced
P. australis	Delta2	March 4, 2004	EU684233
P. fraudulenta	Pi2	April 2, 2004	EU684232
P. multistriata	Alfa 5	Oct 10, 2003	EU684237
P. pungens var. *aveirensis*	Alfa 1	Oct 10, 2003	EU684234
P. pungens var. *aveirensis*	Alfa 3	Oct 10, 2003	EU684235
P. pungens var. *aveirensis*	Alfa 7	Oct 13, 2003	As Alfa 1
P. pungens var. *aveirensis*	Alfa 10	Oct 13, 2003	As Alfa 1
P. pungens var. *aveirensis*	Alfa 11	Oct 13, 2003	As Alfa 1
P. pungens var. *aveirensis*	Alfa 12	Oct 13, 2003	As Alfa 1
P. pungens var. *aveirensis*	Alfa 13	Oct 13, 2003	As Alfa 1
P. pungens var. *aveirensis*	Alfa 14	Oct 13, 2003	As Alfa 1
P. pungens var. *aveirensis*	Theta 4	Oct 13, 2003	EU684236
P. pungens var. *aveirensis*	Alfa10Theta4	Alfa 10 x Theta 4	As Theta 4
P. pungens var. *aveirensis*	Mex18	Gulf of Mexico	AY257846
P. pungens var. *aveirensis*	P-24	Portugal	AY257845
P. pungens var. *aveirensis*		Vietnam	DQ166533
P. pungens var. *aveirensis*	KBH2	Vietnam	DQ062665
P. pungens var. *cingulata*	US-123/a	NE Pacific, USA	AM778805
P. pungens var. *cingulata*	US-115	NE Pacific, USA	AM778804
P. pungens var. *cingulata*	US 132	NE Pacific, USA	
P. pungens var. *pungens*	V120(3)5	North Sea, Belgium	AM778747
P. pungens var. *pungens*	Cn172	NW Atlantic, Canada	AM778786
P. pungens var. *pungens*	W1(7)6/a	Westerscheldt, The Netherlands	AM778770
P. pungens var. *pungens*	US-94/a	NE Pacific, USA	AM778797
P. pungens var. *pungens*	Vigo 2	NE Atlantic, Spain	
P. pungens var. *pungens*	NZ67	S Pacific, New Zealand	
P. pungens var. *pungens*	Cn216	NW Atlantic, Canada	

Materials and Methods

Sampling and cultures

The samples originated mainly from two sampling periods: (1) from February to October 2000, with samples taken every fortnight from the mouth of Ria de Aveiro (designated Barra) and from several locations along the southern channels of the lagoon (summarized in Fig. 1); (2) from September to November 2003, and from February to April and in October 2004, with regular monthly samples complemented by collections

taken irregularly during the period, all from Barra. Sampling was performed approximately one hour before the end of flood tide to ensure the predominance of coastal water entering the lagoon; additional samples were taken near the end of ebb tide during sampling period (1) to get a view of the plankton exiting the lagoon. Qualitative samples were obtained with a 25-µm plankton net towed just below the surface, one duplicate being immediately fixed in 5% neutral formalin and the other refrigerated and transported live to the laboratory. One-litre samples of unconcentrated surface water, immediately fixed with neutral Lugol's solution, were used for cell counts. Water temperature was measured with a hand thermometer and salinity estimated with an Atago S/Mill Refractometer.

Non-axenic cultures of *Pseudo-nitzschia* species (Table 1) were established by single cell pipetting into f/2 medium (Guillard 1975) based on filtered sea water (salinities between 28 and 35 psu). The single cells were washed in medium and grown at 15-20°C, under a 12:12h light:dark cycle and a photon flux density of 15-50 µmol m^{-2} s^{-1}.

Morphological species identification and quantification

Clean frustules were obtained from field samples and cultures by removing the organic material with 40% nitric acid and potassium dichromate, followed by repeated washing with distilled water; or by addition of sulphuric acid and saturated potassium permanganate in water as described by Lundholm & Moestrup (2000).

Species identification was based on the electron microscopical observation of cleaned frustules, which was routinely done with a Jeol-JSM 5400 scanning electron microscope (Department of Biology, University of Aveiro, Portugal) operated at 10-15 kV for sputter-coated material, or at 2 kV for uncoated frustules directly deposited onto graphite foil. Supplementary SEM observations were made on a Jeol-JSM 6310F field-emission scanning electron microscope (CEMUP, Porto). TEM observations were made by deposition of cleaned material onto Formvar-coated grids and examination with a Jeol JEM 1010 transmission electron microscope (Department of Biology, University of Copenhagen, Denmark). The cleaned material was also used to make permanent slides mounted in Naphrax, and observed in the light microscope. Cell counts of about 400 cells during periods of abundance, or less than 100 cells at low cell concentrations, were either done on 10 times concentrated Lugol-fixed samples using 10 mL Kolkwitz chambers in the inverted microscope or in Sedgewick-Rafter chambers in an upright microscope equipped with a long-working-distance 40x objective. Counting was always preceded by the observation of the material in the electron microscope. This enabled

determination of the probable identity of most specimens counted based on general morphometric features. Length measurements were performed on printed LM micrographs whereas the remaining characters were measured on printed EM micrographs.

Type material of *P. pungens* var. *pungens*

Pseudo-nitzschia pungens first "appeared as a nomen nudum in the text to slide No. 307 of a Challenger sample of surface diatoms from Yeddo (=Tokyo) Bay, Japan (Cleve & Möller 1882)." (Hasle et al. 1996). Cleve (1897, p. 24, pl. 2) described and illustrated *P. pungens* referring to the Cleve and Möller slide. *Pseudo-nitzschia pungens* was later lectotypified by Hasle et al. (1996) on a slide made by Hustedt on a subsample of the Challenger material, which he received from the P.T. Cleve Collection. A small subsample of this material was kindly provided by Friedel Hinz, the Hustedt collection, Bremerhaven and examined using TEM.

Toxin analysis

Non-axenic strains of *P. americana*, *P. australis*, *P. fraudulenta*, *P. multistriata* and a new variety of *P. pungens* were grown in 500 mL sterilised Erlenmeyer flasks containing 400 mL of Si-depleted f/2 medium (32 psu) at 15ºC, with photon irradiance of 100 µmol photons m^{-2} s^{-1} and a 16:8 light:dark cycle. Ten-mL subsamples were taken at different growth stages of each batch culture, immediately frozen at -20ºC and analysed for DA by the FMOC-HPLC method (see Kotaki et al. 2000) at the Institut für Ernährungswissenschaften, Friedrich-Schiller-Universität Jena, Germany.

Molecular methods

Cells were harvested by centrifugation and frozen at -20°C. Afterwards the DNA was extracted and the ITS region of the nuclear ribosomal DNA was amplified and sequenced following Lundholm et al. (2003). The sequences were aligned into the alignment of Lundholm et al. (2006). A large data set including all the Portuguese strains was initially analysed using neighbour joining (NJ). It confirmed the morphological identification of *P. australis, P. fraudulenta* and *P. multistriata.* Several clades of *P. pungens* have been identified by Casteleyn et al. (2007), hence further focus was put on this species and a smaller alignment including only *P. pungens* and *P. multiseries* was analysed. All the *P. pungens* strains sequenced for the present study were identical and therefore all except two were deleted from the analyses. The alignment included 863 characters and all were included in the final analyses. 30 characters were parsimony

informative. The analyses were rooted with *P. multiseries* as it is firmly established as a sister taxon of *P. pungens* (e.g. Lundholm et al. 2003). All analyses were performed using PAUP* version 4.0b.8 (Swofford 2001). Distance analyses were performed using NJ (1000 replicates) with the same parameters as in maximum likelihood (ML). The optimal model for ML and NJ analyses was found with a 99% level of significance using Modeltest version 3.7 (Posada & Crandall 1998) to be the HKY85 (Hasegawa Kishino Yano) model with application of among site rate heterogeneity (gamma distribution). ML analyses were performed doing heuristic searches with 100 random addition replicates and the tree bisection reconnection (TBR) branch-swapping algorithm, MP analyses with 1000 replicates. One thousand bootstrap replicates were performed in MP and NJ and 100 in ML. The secondary structure of nuclear ITS2 sequences was predicted using the mfold server at http://frontend.bioinfo.rpi.edu/ (Zuker 2003). Helices were recognised by comparing the ITS regions of *P. pungens*. The helices were named according to Mai & Coleman (1997) and Amato et al. (2007).

Mating experiments

Mating experiments were performed among 10 strains of *P. pungens*: four Portuguese strains (alfa7, alfa10, alfa14, theta4), five strains from Karadag, south east coast of Crimea, Ukraine (DO212-B, DO212-F, DO242-C3, DO214-7, DO218-5) and one strain from Sequim Bay, WA, USA (NWFSC094). 0.5 mL of exponentially growing strains were mixed in 5 cm petri dishes containing 1 mL f/2 medium of 32 psu. The mixed cultures were placed at 15°C and 70 µmol photons m^{-2} s^{-1} at a 16:8 light:dark cycle and examined daily for 5 days.

Morphometrics

To assess possible morphological differentiation between *P. pungens* strains, detailed morphological data of the type material of var. *pungens* and our strains were combined with previously published data (Casteleyn et al. 2008). Three characters were analysed for all 17 strains: valve width, striae density and fibulae density. The poroid density and the band striae density were available for a smaller dataset of 12 strains. Principal Component Analysis (PCA) and analyses of variance (ANOVA) were performed using STATISTICA version 6.0 for Windows (StatSoft, Tulsa, USA). For analyses of variance, ITS rDNA clades found in the molecular phylogeny were used as a priori groups (ITS clades). One-way ANOVAs followed by post hoc Tukey's honest significant difference (HSD) tests were used to identify characters showing significant differences between means of the three ITS-clades. In all analysis, $P < 0.05$ was considered significant.

Results

Salinity and temperature

During the sampling periods, salinity during flood tide was always about 35 psu at the mouth of the lagoon (Barra; see Fig. 1) and decreased along Mira channel to 19-35 psu at Costa Nova, 15-31 psu at Vagueira and 2-15 psu at Areão; in the Ílhavo channel salinity was 23-35 psu near Ílhavo and 12-34 psu near Vagos. Water near Rossio, Aveiro (locality 5 in Fig. 1), which is connected to the sea through a large navigation channel, showed flood tide salinities between 26 and 35 psu. The decrease in salinity during ebb tide was only very slight at Barra (about 1 psu), except during April-May 2000, when heavy rainfall and a marked increase in the flow of the freshwater tributaries brought salinity down to a minimum of 17 psu. Other sites showed salinity reductions between flood and ebb tide ranging in general from 4 to about 10 psu, except during the above-mentioned period of heavy rainfall when freshwater influence was paramount. Water temperature at Barra was mostly between 14°C and 17°C, with the lowermost winter temperatures ~12°C and late summer temperatures up to 19°C. Flood tide–ebb tide temperature variation was mostly 1-2°C, with water temperature increasing during residence in the lagoon, except in the colder winter months.

Pseudo-nitzschia occurrence

Over the course of the two surveys, a total of eleven *Pseudo-nitzschia* species was identified (Fig. 2). The following five species were found during both sampling periods: *P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. pseudodelicatissima* and a new variety of *P. pungens*. The species *P. dolorosa, P. cuspidata* and *P. subpacifica* were only found in 2000, whereas *P. americana*, *P. calliantha* and *P. multistriata* were only detected in 2003-04. Five species: *P. subpacifica, P.americana, P. calliantha, P. multistriata* and *P. cuspidata* were reported for the first time in Portuguese waters.

The concentration of *Pseudo-nitzschia* cells found during the first survey was always largest in samples collected near the mouth of the lagoon. The innermost sites of the two southern channels (Areão and Vagos) contained at most few unhealthy-looking cells that always failed to grow when isolated into culture medium. The reduction in concentration of *Pseudo-nitzschia* cells between Barra and samples taken near Rossio, Aveiro, was slighter than the decrease observed along the two southern channels.

The highest concentration of *Pseudo-nitzschia* cells was found at Barra, in March 2004, with about 106,000 cells L^{-1}, of which ~75% were *P. australis*. In 2000, the highest concentration found was 90,000 cells L^{-1} in August, with *P. australis* making up

about 2/3 of the cells. Autumn-winter maxima were much lower: about 900 cells L^{-1} in February 2000 and 10,000 cells L^{-1} in November 2003. The variation in abundance of *Pseudo-nitzschia* cells (all species) and the periods of occurrence of each species are summarized in Fig. 2.

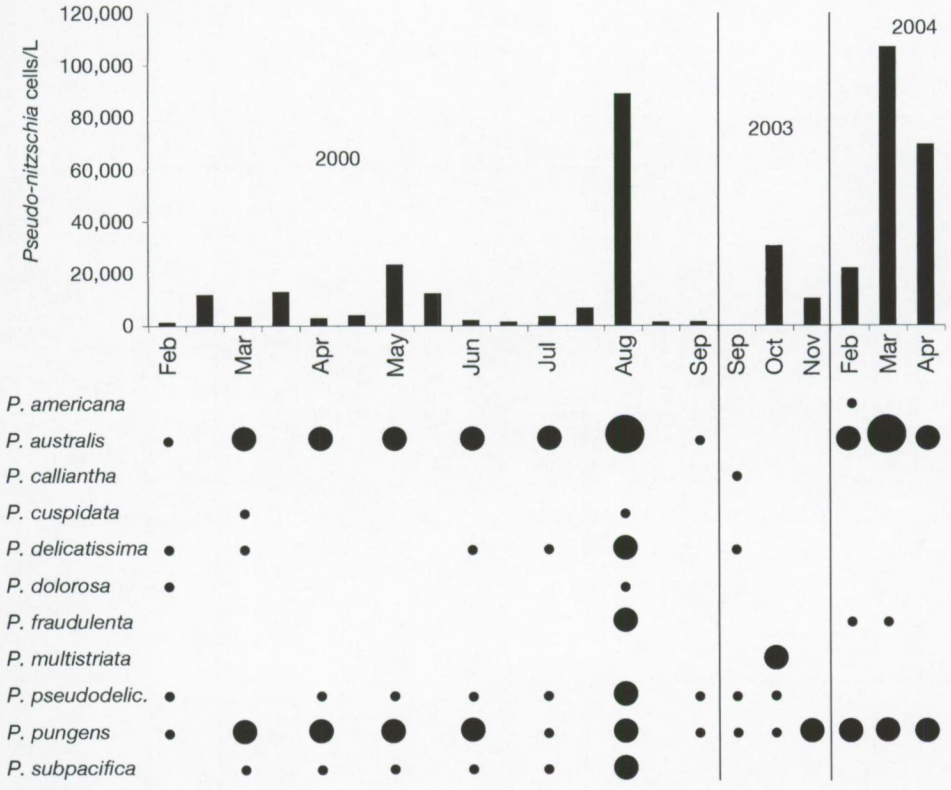
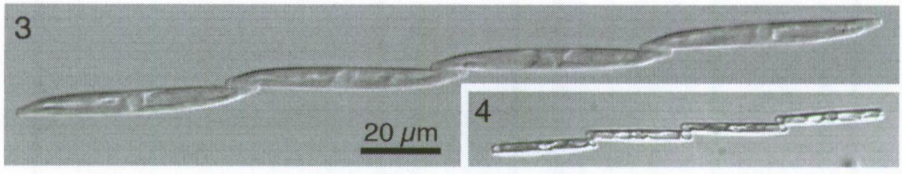


Fig. 2. Diagram showing cell densities of *Pseudo-nitzschia* spp. during the two sampling periods and below a semi-quantitative indication of the frequency of the different species during the same periods. October 2000 is not included because no *Pseudo-nitzschia* cells were found on that date. Although *P. americana*, *P. australis*, *P. multistriata* and *P. pungens* were detected in October 2004, it is not included in the diagram because the sample was not quantified. Small dots represent densities of ca. 1000 cells L^{-1} or lower; medium dots, ca. 1000-50,000 cells L^{-1}; large dots, higher than 50,000 cells L^{-1}.

Table 2. Morphometric parameters of *Pseudo-nitzschia* cells from field samples and cultures from Aveiro lagoon during the present study. * indicates only one specimen measured from field samples.

Species name	Length (μm)	Width (μm)	Fibulae in 10 μm	Striae in 10 μm	Rows of poroids	Poroids in 1 μm	Central nodule	Overlap
P. americana	31-36	2.5-2.8	19-22	27-33	2	8-9	Absent	1/10
P. australis	69-126	4.5-7	14-18	14-18	2	4-5	Absent	1/3-1/4
P. calliantha *	---	2	22	38-39	1	6	Present	---
P. cuspidata *	54	2.5	17	37	1	6	Present	---
P. delicatissima	30-63	1.8-2.3	18-25	32-39	2	7-11	Present	1/8
P. dolorosa	30-39	2.5-3.0	18-22	35-36	1-2	5-8	Present	1/6
P. fraudulenta	54-86	4-6	20-22	22-24	2-3	6	Present	1/5
P. multistriata	51-70	2.7-3	24-26	36-39	2	10-11	Absent	1/8
P. pseudodelicatissima	75-82	1.2-1.6	22-26	38-41	1	5	Present	1/5-1/6
P. pungens var. *aveirensis*	47-100	2.7-3.7	13-16	13-16	2-3	3-5	Absent	1/5-1/6
P. subpacifica	37-58	4.1-5.5	16-20	28-32	2	7-10	Present	1/5-1/6



Figs 3, 4. Light micrographs of *Pseudo-nitzschia* colonies in girdle view. The scale bar applies to both figures. Fig. 3. *P. multistriata*, field material. Fig. 4. *P. americana*, cultured material.

Morphology of individual species

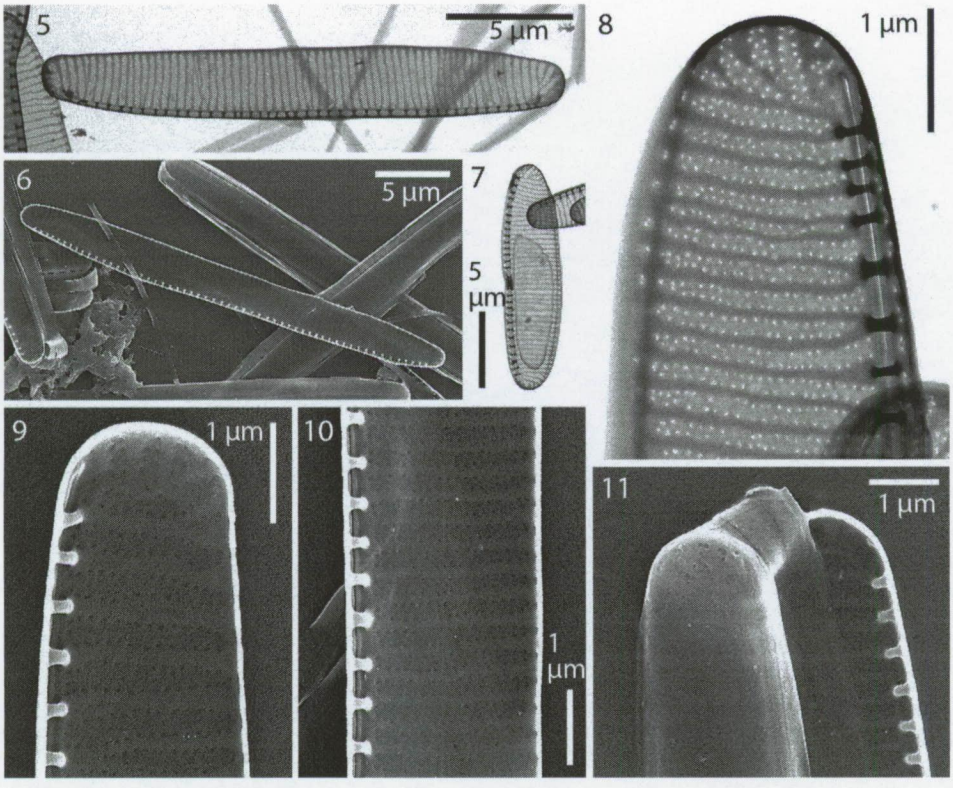
All *Pseudo-nitzschia* species detected formed stepped colonies (Figs 3-4). The morphometric parameters determined from the cells found during the 2000 and 2003-2004 surveys are presented in Table 2 and the descriptions of the species are found below.

Pseudo-nitzschia americana (Hasle) G.A. Fryxell (Figs 4-11)

This species forms short colonies of three to four cells with a small overlap of 1/10 of the cell length (Fig. 4). The valves are linear and symmetrical in relation to the apical plane in longer individuals and more swollen in shorter cells. The frustules are 31-33 μm

long and 2.5-2.8 µm wide in valve view (Figs 5-7). The fibulae are regularly spaced, 16-22 in 10 µm, whereas the striae are denser, 27-28 in 10 µm. The striae are perpendicular to the valve edges except at the rounded apices where they curve up and radially surround the apical raphe endings (Figs 8, 9, 11). There is no central nodule (Fig. 10). The interstriae sometimes fuse close to the base of the fibulae, apparently more commonly in shorter specimens. The striae are perforated by two rows of small circular poroids (8-9 in 1 µm) close to the interstriae.

Pseudo-nitzschia americana survived very well in culture at 35 psu and the reduction in length over 10 months was 43% (15.5 µm). It was found in February and October 2004 in concentration lower than 1000 cells L^{-1} .

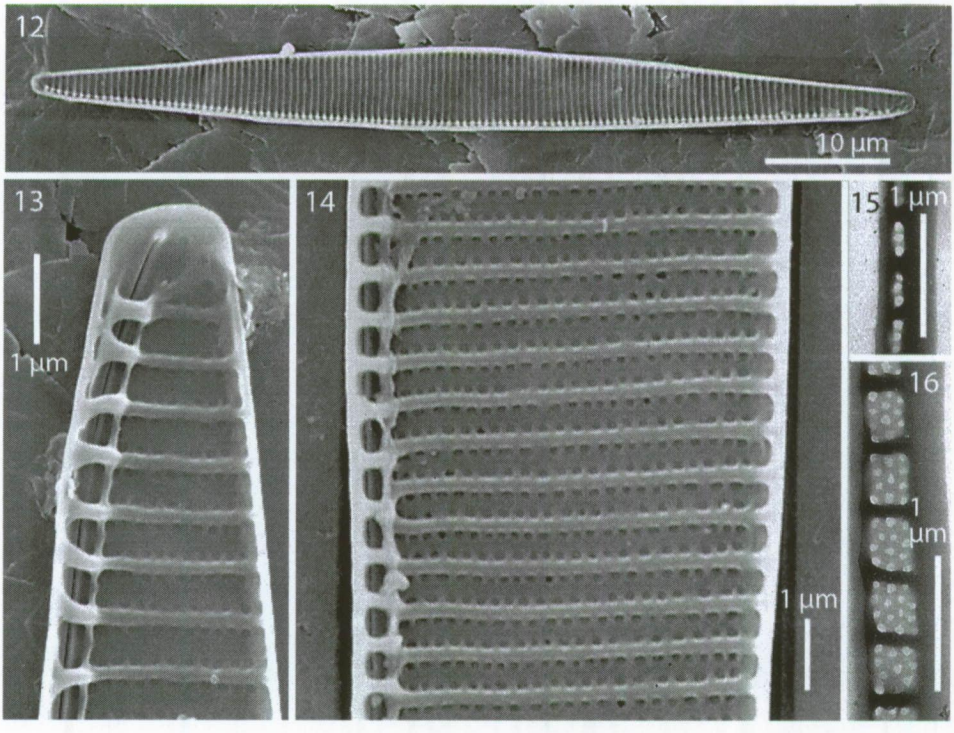


Figs 5-11. *Pseudo-nitzschia americana*, cultured material. Fig. 5. Whole valve from recent culture. TEM. Fig. 6. Internal and external views of whole valves. SEM. Fig. 7. Whole valve showing the size reduction of cultured material. TEM. Fig. 8. TEM showing oblique striations at the valve tip. Fig. 9. Valve extremity, internal view. SEM. Fig. 10. Middle part of valve, internal view. SEM. Fig. 11. External view of valve end. SEM.

Pseudo-nitzschia australis Frenguelli (Figs 12-16)

The cells are linear to lanceolate, symmetric to slightly asymmetric in valve view, with rounded ends. In the colonies, the overlap is about 1/3 to ¼ of the cell length. The length is 69-126 µm and the valve width 4.5-7.0 µm. The valve lacks a central nodule (Figs 12, 14). The interstriae are in continuity with the fibulae and fibulae and striae appear in approximately equal numbers, 14-18 in 10 µm. The striae contain two rows of circular poroids (4-5 in 1 µm) (Figs 13-14). Two main types of cingular bands were observed. One type with the striae perforated by one or two rows of three poroids corresponding to the one or two bands closest to the valve (Fig. 15) and a second type with striae (21 in 10 µm) perforated by three or four rows of four poroids each (Fig. 16).

This species was present through all the sampling period in 2000 (except in October) and from February to April and October 2004 (Fig. 2).



Figs 12-16. *Pseudo-nitzschia australis*. Figs 12-14. Internal view of whole valve, valve end and middle part of valve, respectively. SEM of field material. Figs 15, 16. Two different types of girdle bands. TEM of cultured material.

Pseudo-nitzschia calliantha Lundholm, Moestrup et Hasle (Figs 17-18)

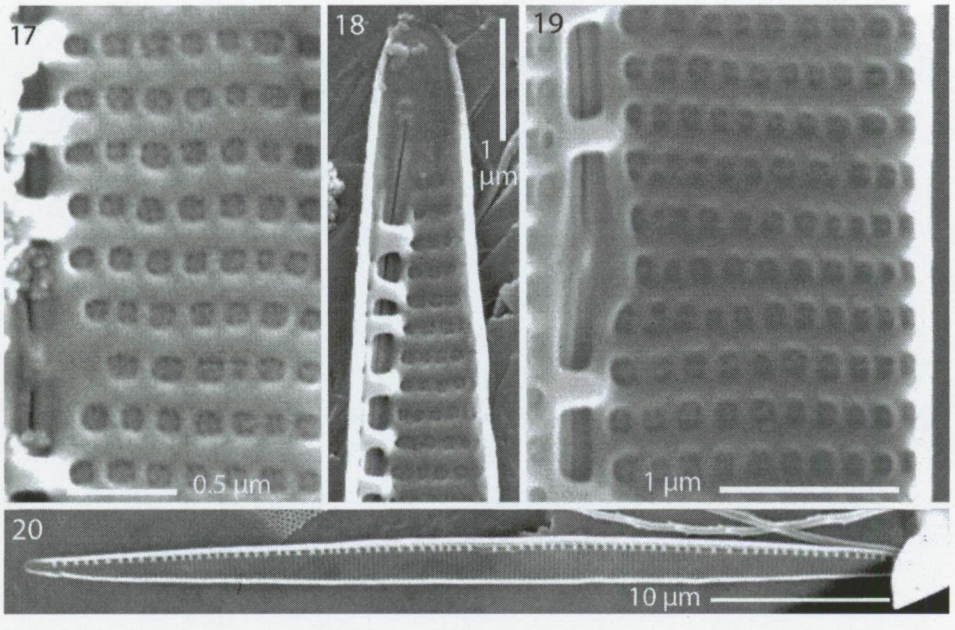
The appearance of the cells is linear. The width of the valve is 2 µm. The valve has 22 fibulae in 10 µm and 38-39 striae in 10 µm and the raphe is divided in the middle by a central nodule (Figs 17-18). Each stria contains one row of round-squared poroids, 6 in 1 µm. The poroid pattern resembles a flower, the hymen being perforated by 7-10 sectors arranged in a circle and often with a sector in the middle (Fig. 17).

This species was found only once, in September 2003, and only a few cells (not quantifiable).

Pseudo-nitzschia cuspidata (Hasle) Hasle *emend.* Lundholm, Moestrup et Hasle (Figs 19-20)

The valves are lanceolate, 54 µm long and 2.5 µm wide. The density of the striae is much higher (37 in 10 µm) than the density of the fibulae (17 in 10 µm). The raphe has a central nodule (Fig. 20). Each stria contains one row of poroids (6 in 1 µm), which are divided into two sectors (Fig. 19).

This species was found in low numbers in March and August 2000 (not quantifiable).



Figs 17-20. *Pseudo-nitzschia calliantha* and *P. cuspidata*, SEM. Field material. Figs 17, 18. Internal view of central part and valve end of *P. calliantha*. Figs 19, 20. Internal view of central part and whole valve of *P. cuspidata*.

Pseudo-nitzschia delicatissima (Cleve) Heiden (Figs 21-22)

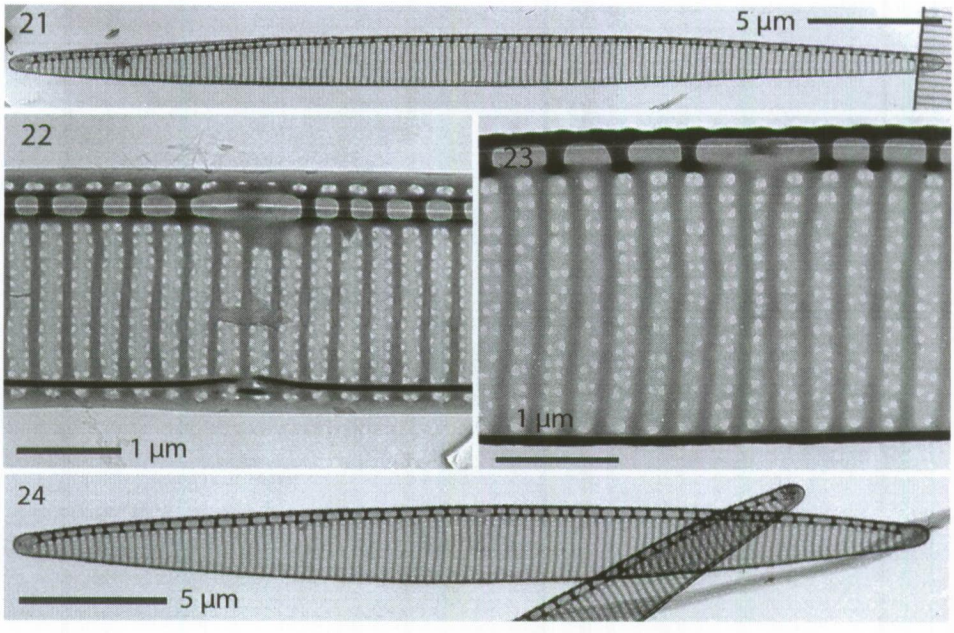
In valve view, the cells are narrow and linear to lanceolate (Fig. 21). There is an extremely short overlap between cells in colonies, 1/7 to 1/10 of the cell length. The valve is 34-63 µm long and 1.8-2.3 µm wide. The valve has 32-39 striae in 10 µm and 18-25 fibulae in 10 µm. A central nodule is present (Fig. 22). The striae are perforated by two rows of small round poroids (7-11 in 1 µm) positioned close to the interstriae.

Pseudo-nitzschia delicatissima, or maybe a mixture of *P. delicatissima* and *P. cuspidata*, was found irregularly through the 2000 sampling period (February, March and June to August) and in September 2003 (Fig. 2).

Pseudo-nitzschia dolorosa Lundholm et Moestrup (Figs 23-24)

In valve view, the cells are lanceolate with the valve width varying between 2.5 and 3 µm and the length between 30 and 39 µm. The number of striae (35-36 in 10 µm) is higher than the number of fibulae (18-22 in 10 µm). The valve possess a central nodule (Fig. 24). Each stria contains one or two rows of small poroids (5-8 in 1 µm) (Fig. 23).

This species was found only twice, in February and August 2000 (Fig. 2), in qualitative samples (not quantifiable).

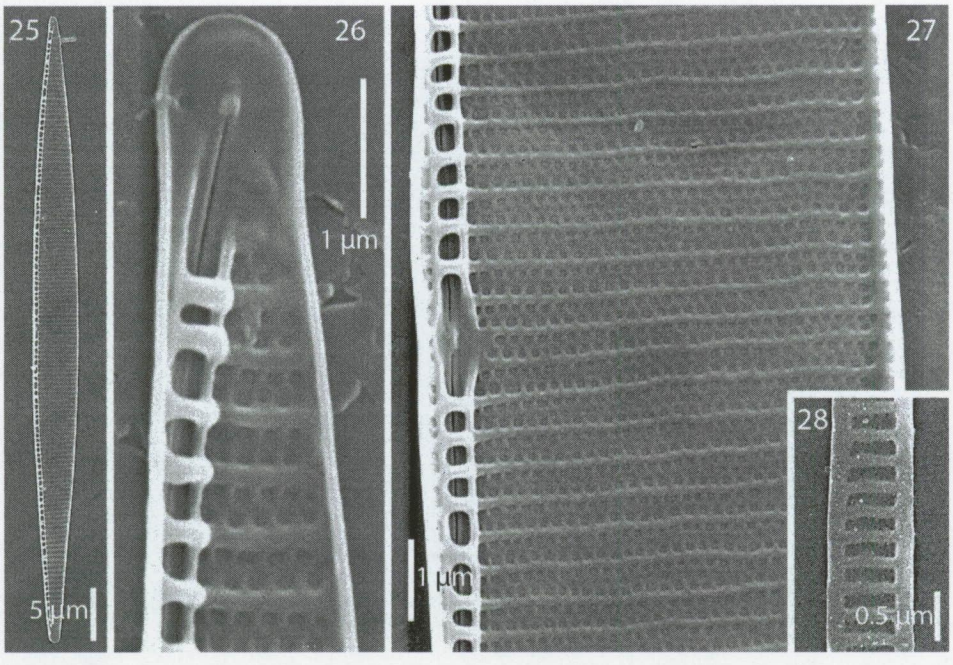


Figs 21-24. *Pseudo-nitzschia delicatissima* and *P. dolorosa*, TEM of cultured material. Figs 21, 22. Whole valve and central part of *P. delicatissima*. Figs 23, 24. Central part and whole valve of *P. dolorosa*.

Pseudo-nitzschia fraudulenta (Cleve) Hasle (Figs 25-28)

In colonies, the cells overlap by 1/5 of the cell length. The valves are lanceolate, sometimes fusiform, with pointed ends and a central interspace (Figs 25, 27). The cells are 54-86 µm long and 4-6 µm wide. The density of the striae (22-24 in 10 µm) and the fibulae (20-22 in 10 µm) is about the same (Figs 26-27). The striae are perforated by two or three rows of poroids closely compacted (6 in 1 µm). The cingular bands (valvocopula) have striae perforated by two irregularly rows of small poroids close to the interstriae. Interstriae in the cingular bands are large, almost the size of the striae (Fig. 28).

In culture, the reduction in cell length was 42% (34.1 µm) over 10 months. *Pseudo-nitzschia fraudulenta* was found in August 2000 and again in February and March 2004 (Fig. 2).

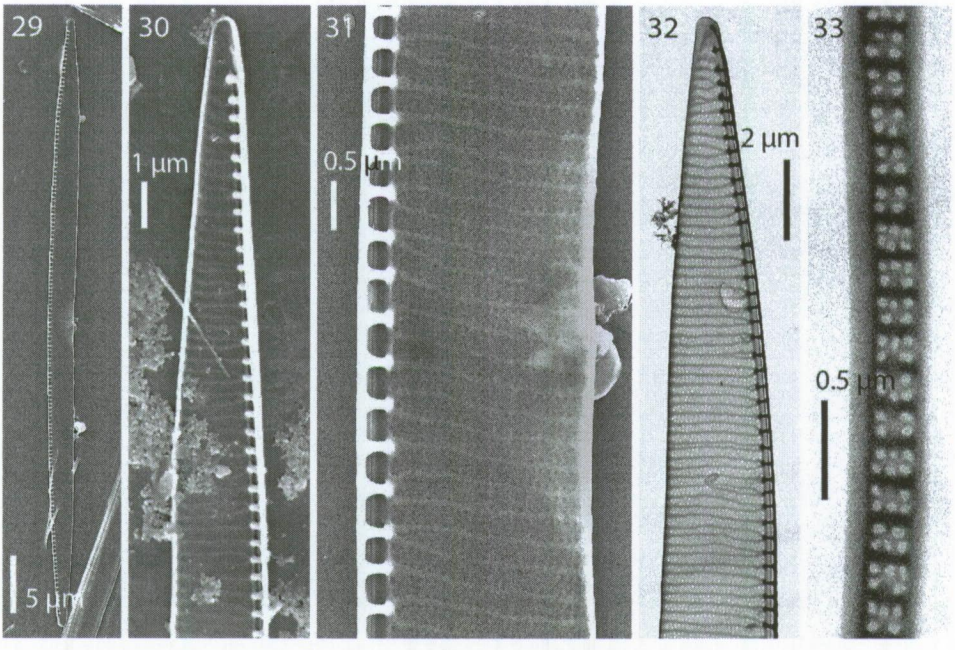


Figs 25-28. *Pseudo-nitzschia fraudulenta,* SEM. Fig. 25. Internal view of whole valve. Fig. 26. Valve end, internal view. Fig. 27. Middle part of valve, internal view. Fig. 28. Girdle band from cultured material.

Pseudo-nitzschia multistriata (Takano) Takano (Figs 3, 29-33)

Under the light microscope, the cells were recognisably different from other *Pseudo-nitzschia* species in Aveiro coastal waters. The cells are sigmoid in girdle view (Fig. 3) and in valve view, they are asymmetrical with one side almost straight and the other more convex (Fig. 29). The longer cells may appear linear in the middle. The apices are more or less pointed (Figs 29-30, 32). In colonies, the cells overlap by 1/8 of the cell length (Fig. 3). The length of the valve is 51-70 µm and the width 2.7-3.0 µm. The densities of striae and fibulae in 10 µm are 36-39 and 24-26, respectively. Each stria contains two rows of small poroids (10-11 in 1 µm). A central nodule is absent (Fig. 31). The only type of cingular band observed had striae perforated by two rows of two or three poroids (Fig. 33).

Pseudo-nitzschia multistriata survived well in culture but the sigmoid shape became less defined in older cells. In culture, there was no significant size reduction over 10 months. It was found in October 2003 (~5000-25,000 cells L^{-1}) and again in October 2004 (Fig. 2).

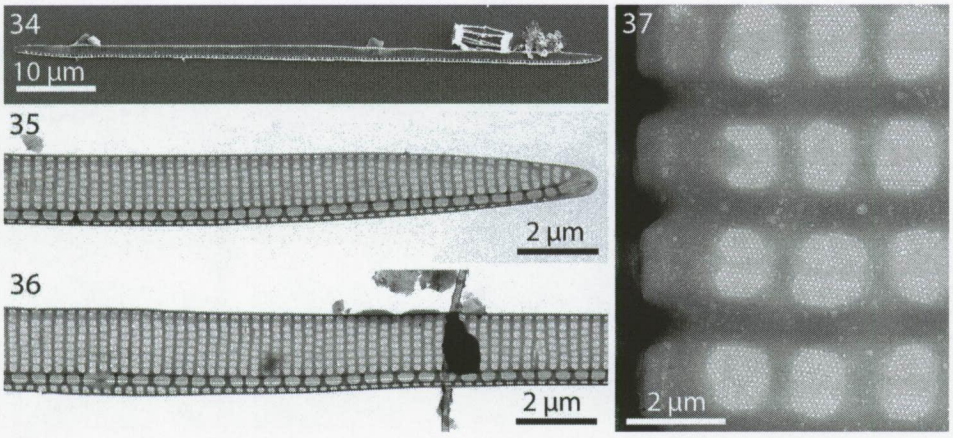


Figs 29-33. *Pseudo-nitzschia multistriata*. Cultured material. Fig. 29. Whole valve, SEM. Figs 30, 31. Internal view of valve tip and middle part of valve, SEM. Fig. 32. Tip of valve showing fusion of interstriae and fibulae, TEM. Fig. 33. Girdle band, TEM.

Pseudo-nitzschia pseudodelicatissima (Hasle) Hasle *emend.* Lundholm, Hasle et Moestrup (Figs 34-37)

In colonies, the cells overlap by 1/5 to 1/6 of their length. The cells are linear and symmetrical in girdle and valve view (Fig. 34) with a needle-like appearance. The apices are sharply rounded (Figs 34-35). The cell length ranges from 75 to 82 µm and cell width from 1.2 to 1.6 µm. A central nodule is present (Fig. 36). The fibulae (22-26 in 10 µm) are less numerous than the striae (38-41 in 10 µm). Each stria contains one row of large squared to oval poroids (5 in 1 µm). The hymen of the poroids is divided into two parts and it may hence resemble striae with two rows of poroids (Fig. 37). The cingular bands we observed contain one row of squared poroids (not shown).

Pseudo-nitzschia pseudodelicatissima appeared throughout the sampling period of 2000 (except in March and October) and in September and October 2003 (Fig. 2).



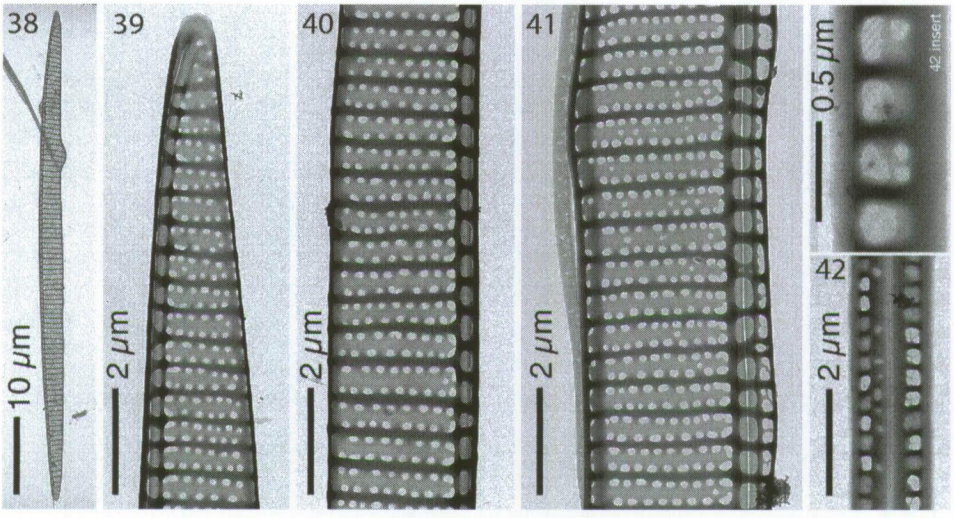
Figs 34-37. *Pseudo-nitzschia pseudodelicatissima*. Fig. 34. Internal view of whole valve, SEM of field material. Figs 35-36. Tip and middle part of valve, respectively, TEM. Field material. Fig. 37. Poroid structure, TEM. Field material.

A new variety of *Pseudo-nitzschia pungens* (Figs 38-42)

The colonies may sometimes be very long, up to 12 cells that overlap by 1/5 to 1/6 of the cell length. The cells are symmetrical and linear to lanceolate in both valve (Fig. 38) and girdle view, the apices being more or less pointed (Figs 38-39). The cell length is 47-100 µm and the valve width is 2.7-3.7 µm. In culture, initial cells resulting from sexual reproduction reached a length of 188 µm with an average of 167.7±8.5 µm. The valves are strongly silicified, and it is possible to see the interstriae and fibulae even in live

material. The striae are as dense as fibulae (13-16 in 10 μm). A central nodule is absent (Fig. 38). Most of the valves have striae with two rows of poroids (Fig. 40), but sometimes a third row, more or less fully formed, is seen (Fig. 41). Two types of cingular bands were observed (Fig. 42): one type with only one row of oval poroids, the other type with squared poroids being split into two to three parts (Fig. 42, insert). In the cingular bands, the poroid density is 21-25 in 10 μm.

Pseudo-nitzschia pungens grew well in culture and smaller cells were more fusiform. In culture, the reduction in length was 35% (28.7 μm) over 10 months. This species was always present throughout both sampling periods except in one sample (October 2000). It was often one of the most abundant *Pseudo-nitzschia* species, second only to *P. australis* (Fig. 2).



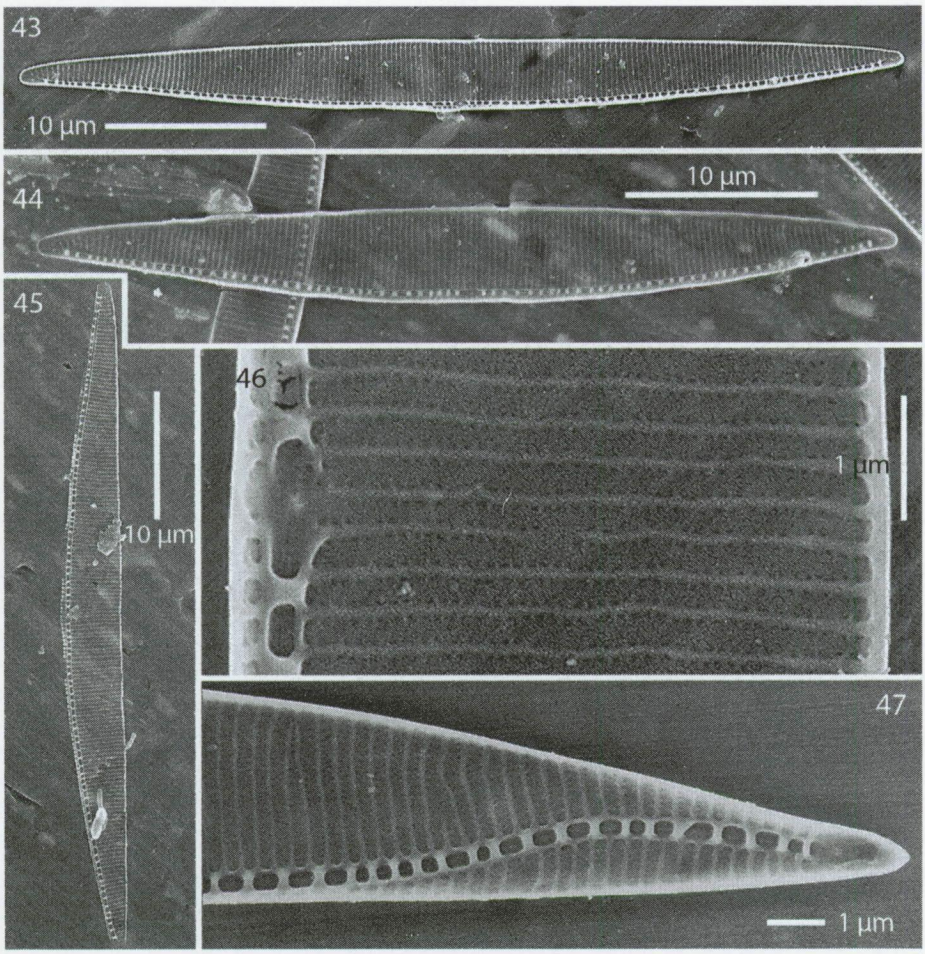
Figs 38-42. *Pseudo-nitzschia pungens* var. *aveirensis* var. nov., TEM. Cultured material. Fig. 38. Whole valve. Figs 39-40. Tip and middle part of valve illustrating striae with only two rows of poroids. Fig. 41. Middle part of valve showing mantle and a tendency for three rows of poroids in the striae. Fig. 42 and Fig. 42 insert (a magnification of Fig. 42). Girdle bands (first two girdle bands) and the insert showing a magnification of the valvocopula. Note a tendency for each stria consisting of several poroids.

Pseudo-nitzschia subpacifica (Hasle) Hasle (Figs 43-47)

The cells are asymmetric in valve view, with one side convex and the other varying from somewhat convex to nearly straight (Figs 43-45). In colonies, the cells overlap by 1/5 to 1/6 of their length. The valves are 37-58 μm long and 4.1-5.5 μm wide. The density of striae (28-32 in 10 μm) is larger than the density of fibulae (16-20 in 10 μm). A central

nodule is present (Fig. 46). The striae contain two rows of small poroids (7-10 in 1 µm). A deformation consisting of a shift in the position of the raphe end was common in the cultures (Fig. 47).

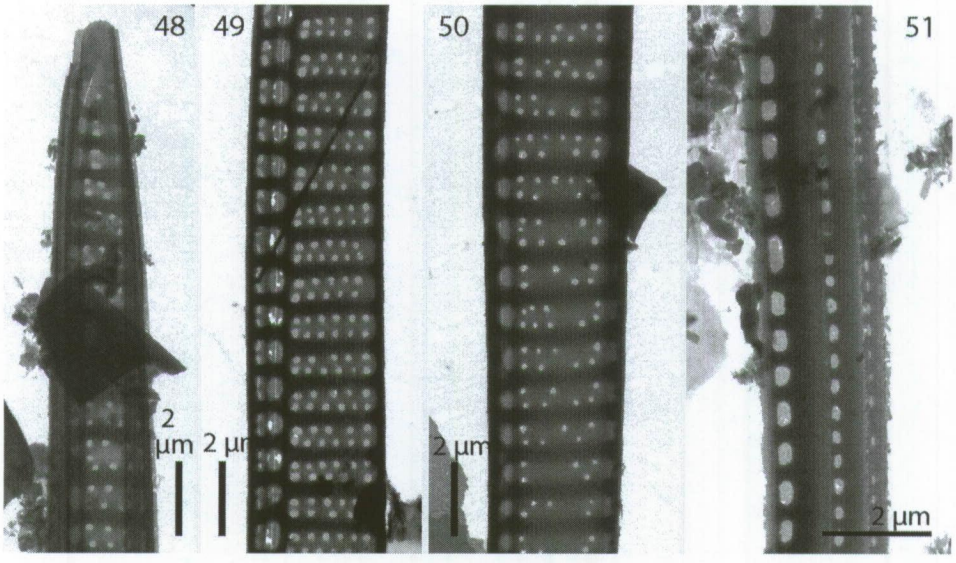
Pseudo-nitzschia subpacifica appeared in spring and summer of 2000 (March to August) but was not found in 2003/04 (Fig. 2).



Figs 43-47. *Pseudo-nitzschia subpacifica,* SEM. Cultured material. Figs 43-45. Internal view of whole valves, showing variation in morphology. Fig. 46. Middle part of valve with central nodule. Fig. 47. Valve end, showing culture-induced deformation affecting raphe position.

Type material of *P. pungens* var. *pungens*

The lectotype material examined (figs 48-51) revealed symmetrical valves with a width of 2.7-3.5 µm and a similar density of fibulae and striae of 8.6-11.6 in 10 µm. The striae contained two rows of poroids with a poroid density of 2-3 in 1 µm. A few extra poroids of an absent third row was found near the interstriae (Fig. 50). The three copulae all contained one row of poroids, which decreased in size in the more abvalvar bands. In the valvocopula the density of band striae was 11.6-18.9 in 10 µm. These measurements are in agreement with measurements on a light micrograph of the lectotype (Hasle 1995, fig. 1), which showed a density of striae and fibulae of 10-11 in 10 µm, a valve width of 3.3 µm and furthermore a valve length of 110 µm.



Figs 48-51. *Pseudo-nitzschia pungens*, TEM. Lectotype material. Yeddo Bay, Japan. Fig. 48. Tip of valve. Fig. 49. Middle of valve. Fig. 50. Middle of valve, note a few extra poroids apart from the two rows. Fig. 51. Three girdle bands with one row of poroids.

Toxicity

Domoic acid was not detected in the cultures of *P. australis*, *P. americana*, *P. fraudulenta*, *P. multistriata* and the new variety of *P. pungens* established during the present study.

Phylogenetic analyses

The phylogenetic analyses supported the morphological identification of *P. australis, P. fraudulenta, P. multistriata* and *P. pungens* as all strains appeared together with similar strains of the respective species (see Lundholm et al. 2006 for which strains) with 100% bootstrap support (tree not shown). The Portuguese *P. australis* was identical to the other strain of *P. australis* included in the phylogenetic analyses (see Lundholm et al. 2006), *P. fraudulenta* differed by two transitions from the other strain, and *P. multistriata* differed by two transitions, one transversion and two insertions. The phylogenetic analyses of the alignment supported the division of *P. pungens* into at least three clades, as identified by Casteleyn et al. (2008) (Fig. 52). These clades were supported in all three analyses (NJ, MP and ML). The sequences of the ten Portuguese *P. pungens* strains (Table 1) were all identical and appeared in the third clade. Clade III was split into two sub-clades (IIIa and IIIb). Clade IIIa was supported by bootstrap values >96% in all analyses and consisted of strains from Portugal and Mexico. Clade IIIb was not supported by bootstrap values above 50% in the parsimony analyses; it consisted of Vietnamese strains. Folding of ITS2 showed no compensatory base changes (CBC) and one hemiCBC (HCBC) between clade IIIa and IIIb.

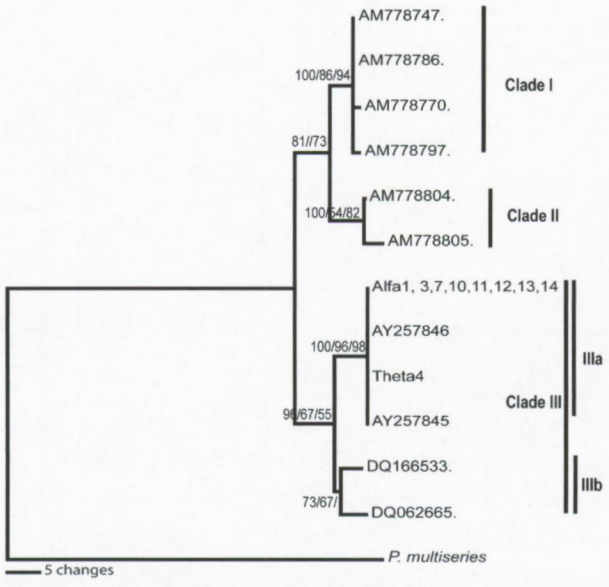


Fig. 52. Phylogenetic tree (neighbour joining, NJ) of *P. pungens* rooted with *P. multiseries*. Bootstrap values are shown as NJ before slash, maximum likelihood (ML) between slashes and maximum parsimony (MP) after slashes.

Mating experiments

The mating experiments showed reproduction among the Portuguese *P. pungens* strains (strain alfa10theta4 represent a daughter strain) (Table 3) and also mating of the Ukranian strains with the American strains (belonging to clade I, unpublished results), but not all strains did mate even though strains of both mating types were present and below the upper size threshold for sexual reproduction (Chepurnov et al. 2005). Mating was never observed between the strains from Portugal and those from Ukraine/USA (Table 3).

Table 3. Results of mating experiments among strains from Portugal, Ukraine and USA. (–) indicates no sexual reproduction, (++) indicates vigorous sexual reproduction.

		Alfa7	Alfa10	Alfa14	Theta4	DO212-B	DO212-F	DO242-C3	DO214-7	DO218-5	NWFSC094
Clade IIIa	Alfa7										
Clade IIIa	Alfa10	++									
Clade IIIa	Alfa14	-	++								
Clade IIIa	Theta4	-	++	-							
Clade I	DO212-B	-	-	-	-						
Clade I	DO212-F	-	-	-	-	++					
Clade I	DO242-C3	-	-	-	-	-	-				
Clade I	DO214-7	-	-	-	-	++	-	-			
Clade I	DO218-5	-	-	-	-	-	-	-	++		
Clade I	NWFSC094	-	-	-	-	++	-	-	-	-	

Comparison between morphological and ITS variation patterns in *P. pungens*

Ordination of the valve measurements showed three groups of strains, corresponding with the three molecular clades (Fig. 53). One-way ANOVAs for each measurement type showed that there was an overall significant difference between means of the three ITS clades (Table 5). Post hoc Tukey HSD tests pointed out that clade III showed a significantly higher striae and fibulae density than the other 2 clades. On the basis of poroid and band striae densities, clade III clones are significantly different from clade I clones but not from clade II clones. Clade III and clade I have a similar valve width but are both significantly narrower than clade II.

The structure of the band striae, especially of the valvocopula, was also different between the three clades. The band striae of clade III consist of partly split poroids (Fig. 42 insert), whereas clade I has a single row of poroids and clade II has two rows of two to three poroids (Table 4).

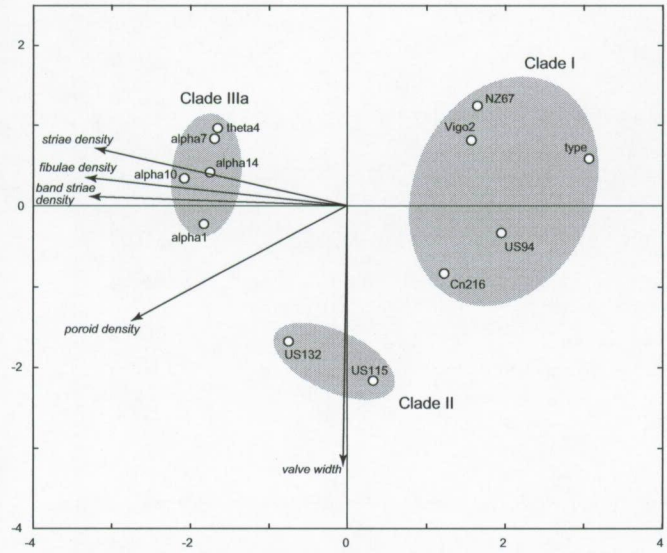


Fig. 53. PCA of morphological measurements for 12 *P. pungens* strains with indication of the three ITS clades. The first two axes are shown, explaining respectively 67% and 23% of the total variation.

Table 4. Morphology of the three clades of *P. pungens*. Data on type material of var. *pungens* and on clade III are from the present study, data on clades I and II are from Casteleyn et al. (2008), data from the original of var. *cingulata* is from Villac & Fryxell (1998). Mean values and standard deviations were added where appropriate in the lower line.

Clade	Apical length (µm)	Valve width (µm)	Striae (in 10 µm)	Fibulae (in 10 µm)	Poroids (in 1 µm)	Band striae (in 10 µm)	Valvocopula striae/poroids
Type material of var. *pungens* Yeddo Bay, Japan	110	2.7-3.5 3.1±0.3	8.6-11.6 10.1±0.9	8.6-11.6 10.1±0.9	2-3 2.3±0.4	11.6-18.9 14.9±3.6	1 poroid
Clade I (var. *pungens*)	24.4-121.0 79.1±8.7	2.4-4.2 3.2±0.4	9-13 11.1±0.5	10-14 11.9±0.3	2-4 3.0±0.5	nd	1 poroid
Description of var. *cingulata* (Villac & Fryxell 1998)	71-140	2.8-4.5	10-14	10-14	3-4.5	20-24	2x2-3 poroids
Clade II (var. *cingulata*)	87.9-110.8 101.5±27.2	3.5-4.7 3.9±0.1	10-13 11.6±0.3	11-15 12.7±0.2	3-5 4.2±0.5	nd	2x2-3 poroids
Clade III (var. *aveirensis* var. nov.)	47.5-100 67.7±14.8	2.7-3.7 (4.0) 3.3±0.6	13-16 14.8±0.7	13-16 14.9±0.7	3-5 4.0±0.0	21-25 23.0±1.1	1 split poroid

Table 5. Univariate ANOVAs and Tukey HSD tests for morphometric trait variation by ITS clades (Fig 52).

	clade I	clade II	clade III	ANOVA	Probabilities for Tukey HSD-tests		
	mean ± SD	mean ± SD	mean ± SD	P value	between clade I and II	between clade I and III	between clade II and III
Width (µm)	3.1 ± 0.4	3.9 ± 0.1	3.1 ± 0.2	0.001	0.002	1.0	0.003
Striae density (in 10 µm)	11.0 ± 0.6	11.6 ± 0.3	14.8 ± 0.2	0.0000	0.08	0.0002	0.0002
Fibulae density (in 10 µm)	11.7 ± 0.7	12.7 ± 0.2	15.0 ± 0.2	0.0000	0.02	0.0002	0.0002
Poroid density (in 1 µm)	2.9 ± 0.4	4.2 ± 0.3	3.7 ± 0.3	0.001	0.003	0.006	0.3
Band striae density (in 10 µm)	15.4 ± 0.9	20.4 ± 4.0	23.1 ± 0.6	0.0001	0.009	0.0002	0.1

Discussion

Pseudo-nitzschia diversity, density and spatial distribution

Pseudo-nitzschia species are rather common in Portuguese coastal waters. During summer, *Pseudo-nitzschia* has been reported to dominate the plankton community, making up a maximum of 86% of the total phytoplankton off the coast of Portugal (Abrantes & Moita 1999). The most common species found during the present study were the new variety of *P. pungens* (present in all the samples but one, i.e. during the whole sampling period except in October 2000) and *P. australis* (present in all samples except three). The same dominance was found by Costa & Garrido (2004) when looking at *Pseudo-nitzschia* species in the stomach contents of sardines collected off the coast south of Aveiro. Five species: *Pseudo-nitzschia australis*, *P. delicatissima*, *P. fraudulenta*, *P. pseudodelicatissima* and the new variety of *P. pungens* were found in both 2000 and 2003/04 surveys. Three species: *P. americana*, *P. calliantha* and *P. multistriata* were found only in 2003/4 and three others only in 2000: *Pseudo-nitzschia cuspidata*, *P. dolorosa* and *P. subpacifica*. In addition, *P. inflatula* (Hasle) Hasle has been identified in samples from Aveiro (Lundholm unpublished data) and Hasle et al. (1996) reported *P. subfraudulenta* off the Portuguese coast. With the present surveys, the number of *Pseudo-nitzschia* species recorded from Portugal coastal waters is hence 13. For comparison, studies from Irish waters (Cusack et al. 2004), from the Bay of Fundy, Canada (Kaczmarska et al. 2005), from coastal waters of Washington (Stehr et al. 2002), from Californian waters (Miller & Scholin 1998) and from shelf waters in

Argentina (Almandoz et al. 2007) each recorded 7-8 different species. The species diversity in coastal waters near Aveiro is therefore considered reasonably high. One of the possible explanations could be that the variable environment, e.g. regarding temperature, salinity and nutrient concentrations, in connection with frequent upwelling events (Moita 1993, Estrada 1995), gives the opportunistic *Pseudo-nitzschia* species possibilities to thrive and gives room for different species occupying slightly different ecological niches. Another explanation could be that some not frequently occurring species have been overlooked at some of the other locations.

The Aveiro coastal lagoon has a considerably large longitudinal salinity and temperature gradient: the water mass has high salinity and low temperatures at the mouth (Barra) of the lagoon and salinity and temperature decreases and increases, respectively, when the distance to the mouth increases inward in the lagoon (Dias et al. 1999). The ocean and freshwater inflows are the most important factors causing the salinity variations from 35 to 2 psu through the lagoon (Dias et al. 1999). This gradient in salinity reflected the variation in the abundance and species diversity of *Pseudo-nitzschia*, both always being higher at the mouth of the lagoon and decreasing inward along the two southern channels. This is in accordance with Thessen et al. (2005), who found that in the Louisiana coast (Gulf of Mexico) *Pseudo-nitzschia* were present in 90% of the samples with salinities higher than 35 psu but only in ~30% of the samples with salinities between 10 and 20 psu. In addition, culture experiments have shown that salinities below ~10 psu either reduced growth of *P. pungens* (Jackson et al. 1992) or completely inhibited growth of *P. calliantha* (as *P. pseudodelicatissima*) (Lundholm et al. 1997). Temperature also affects growth rate. Culture experiments have shown *P. multiseries* and *P. calliantha* to grow with increasing growth rates from 5 to 25 ºC (Lewis et al. 1993, Lundholm et al. 1997). Considering that the temperature gradient in the lagoon was comparatively small: 12-19 ºC, we do not regard temperature but rather salinity as one of the factors causing the observed changes in species diversity and cell density.

The highest cell density of *Pseudo-nitzschia* spp. (106.000 cells L^{-1}) was found in March 2004 and *P. australis* accounted for approximately 75% of this bloom. *Pseudo-nitzschia australis* has previously been reported as producing DA in European countries like Spain, Ireland and Scotland (Cusack et al. 2002, Fehling et al. 2004, Fraga et al. 1998) and it is recognised as one of the strong producers of DA. It has caused accumulation of DA in several different shellfish, crabs, squid, anchovies, mackerel, tuna, sardines, and probably a number of other fish as well as in pelicans, cormorants and sea lions during blooms all around the world (Trainer et al. 2008). Cell densities found in March 2004 are similar to those found during previous toxic blooms of *P.*

australis (Buck et al. 1992, Trainer et al. 2000). It is hence not unexpected that DA contamination has been reported previously in shellfish from the Aveiro lagoon, mainly in spring (Vale et al. 1998, 2008, Vale & Sampayo 2001). *Pseudo-nitzschia australis* is a likely candidate for having produced the DA, as it is usually the most abundant species in the lagoon, also in spring. The other abundant species, *P. pungens*, has also previously been reported to produce DA on the west coast of USA and in New Zealand, but only in low amounts of DA per cell and hence higher cell densities are needed before a bloom will cause accumulation of DA. Several other strains tested have been found not to produce DA (Lundholm & Moestrup 2007).

Toxin production

Of the 11 species found in Aveiro during the present study, *P. australis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. multistriata* and *P. pungens* have previously been reported as toxic elsewhere (Rhodes 1998, Trainer et al. 1998, Cusack et al. 2002, Orsini et al. 2002, Stehr et al. 2002, Lundholm et al. 2003). The toxicity tests were, however, all negative for all the species tested: *P. americana*, *P. australis*, *P. fraudulenta*, *P. multistriata* and the new variety of *P. pungens*. All the cultures used in the analyses were more than one year old. It is hence possible that some of the cultures had been toxic at the time of isolation but that toxicity had declined to below detection limits, as it is commonly known that *Pseudo-nitzschia* cultures tend to lose toxicity over time (Bates 1998, Pan et al. 1998). The production of DA is furthermore dependent on environmental conditions (Pan et al. 1998). Hence, if a species under study is not producing DA, it does not necessarily mean that DA production is not possible under other conditions than those tested (Parsons et al. 1999). It is possible that the isolates tested were non-toxic, as non toxic and toxic strains have been reported elsewhere for most of the species tested (Lundholm et al. 1994, Bates et al. 1998, Fraga et al. 1998, Rhodes et al. 2000, Orsini et al. 2002, Fehling et al. 2005). Domoic acid tests on cultures of *P. australis* from Aveiro using UV-DAD HPLC have previously showed production of the toxin (Skov & Lundholm unpubl. results).

Taxonomy

Pseudo-nitzschia americana is easily differentiated from other *Pseudo-nitzschia* species by being shorter and more rectangular in valve view and having broadly rounded ends. It may, however, previously have been overlooked due to its small size and its potential epiphytic lifestyle (Lundholm et al. 2002). *Pseudo-nitzschia americana* has been observed as single cells and attached to the setae of *Chaetoceros* spp. or in short chains when free-floating in the water column (Rhodes 1998, Lundholm et al. 2002, Orlova &

Shevchenko 2002, Kaczmarska et al. 2005). During the present study, it was not found as epiphytic. It formed short stepped colonies at the time of isolation into culture and colonies were seen during the exponential growth phase. It tended, however, quickly to lose the ability to form colonies, similar to *P. galaxiae* (Lundholm & Moestrup 2002).

Pseudo-nitzschia calliantha is a recently described species that belongs to the *P. pseudodelicatissima* complex (Lundholm et al. 2003). It has probably been present in the area for a long time without getting noticed, possibly being identified as the similar *P. pseudodelicatissima*. *Pseudo-nitzschia calliantha* has previously been identified in the relatively close Black Sea coast of Turkey (Bargu et al. 2002), the Mediterranean Sea coast of Italy (Caroppo et al. 2005) and Scandinavian waters, Adriatic waters and off Spain (Lundholm et al. 2003). Similarly *P. cuspidata*, also being a part of the *P. pseudodelicatissima* complex may have been misidentified as *P. pseudodelicatissima*. However, of the three species, *P. pseudodelicatissima* is by far the most frequently observed species in Aveiro lagoon.

Pseudo-nitzschia dolorosa is a very recently described species morphologically close to *P. delicatissima* (Lundholm et al. 2006). The type locality is actually the mouth of the Aveiro lagoon, where we found it only during the survey in 2000. Neither *P. dolorosa* nor *P. delicatissima* were among the most abundant or frequently observed species.

Pseudo-nitzschia multistriata has been reported several times in the Pacific and in the Mediterranean Sea (Rhodes et al. 2000, Hasle 2002, Orsini et al. 2002). It was found for the first time in Atlantic coastal waters of Morocco (Akallal et al. 2002) and more recently in the Gulf of Mexico (Thessen et al. 2005). Because it is not easily confused with other species, even by light microscopy, due to the sigmoid shape of the valve, it may represent a recent addition to the Atlantic coast of Portugal, although the possibility that it occurred previously in low numbers but was overlooked cannot be excluded.

Most of the species identified during the present study were morphologically in agreement with descriptions in the literature and the identification of *P. australis, P. fraudulenta* and *P. multistriata* was furthermore supported by the molecular data. *Pseudo-nitzschia subpacifica* was, however, slightly narrower (4.1-5.5 µm) than described by Hasle (1965) (5-7 µm).

The Portuguese specimens of *P. pungens* showed occasionally an incomplete third row of poroids in the striae. A third row of poroids on valvar striae has previously been observed by Hasle et al. (1996), Skov et al. (1999) and Casteleyn et al. (2008). The presence of a third row could suggest a similarity with *P. pungens* var. *cingulata*. When Villac & Fryxell (1998) erected the variety, the third row of poroids was, however, not the only morphological difference from the nominal form. Casteleyn et al. (2008) found

a third row of poroids also in the nominal variety. *Pseudo-nitzschia pungens* var. *cingulata* was described as having two types of cingular bands, one type with one row of poroids (striae) and the other type with 2 rows of 2-3 poroids in each stria (Villac & Fryxell 1998). *Pseudo-nitzschia pungens* var. *pungens* has three cingular bands, all with one transversal row of oval poroids (striae) (Hasle 1995, Hasle et al. 1996, Skov et al. 1999, present study). Furthermore, the band stria density is higher in var. *cingulata* (20-24 in 10 µm; Villac & Fryxell 1998) than in the nominal variety (11.6-18.9 in 10 µm; present study). In our specimens, the cingular bands were neither similar to *P. pungens* var. *cingulata* nor *P. pungens* var. *pungens.* Rather we found band striae with split poroids on especially the valvocopula but also on the second band. The density of band striae is, however, similar to *P. pungens* var. *cingulata* and higher than in the nominal variety. Cells resulting from sexual reproduction between Portuguese strains of *P. pungens* showed valves with as well as valves without traces of a third stria. They had cingular bands identical to the parent strains.

Casteleyn et al. (2008) found in a study of many clones of *P. pungens* a division into three molecular clades (I, II and III), where clade II corresponds to *P. pungens* var. *cingulata* and clade I to the nominal variety of *P. pungens*. Our phylogenetic study supports the three clades identified by Casteleyn et al. (2008) and place the Portuguese strains in clade III. The split of clade III into two minor clades, IIIa (containing the Portuguese strains) and IIIb, was not supported in all analyses. In our study, we found that morphologically, clade III differs significantly from the other two clades. We were not able to determine whether clades IIIa and IIIb differ morphologically as we do not have access to strains of clade IIIb.

Casteleyn et al. (2008) observed mating between clades I and II, but live clade III strains were not available to test their mating capability. In the mating experiments, we found mating among the Portuguese strains (clade III) and among the Ukrainian/American strains (that belonged to clade I, unpublished data) but we found no mating between clade I and clade III in spite of several trials. The ITS sequence divergence between clade III and the other two clades was 2.8-3.7%. This agrees with the similar value in Casteleyn et al. (2008) being 2.7-4.4%; a divergence that corresponds to the divergence between *P. australis* and *P. seriata.* This supports the finding that there was no mating between clade I and III. The sequence divergence between clade IIIa and IIIb was low (1.2-1.3%) similar to the divergence between clade I and II (1.3%) in Casteleyn et al. (2008) which could indicate that the clades would be able to mate.

Compensatory base changes (CBC) have previously been shown to be absent if two strains can mate, but the opposite is not always true (Coleman 2000, Amato et al. 2007).

Casteleyn et al. (2008) found no compensatory base changes between clade I and II but one CBC and one hemiCBC (HCBC) between clade III and the other clades, indicating that these strains do not reproduce (gene flow has stopped). The ITS2 sequences of the Portuguese strains were identical to the Mexican strain (Mex18; AY257846) included in the analyses of Casteleyn et al. (2008). Hence the CBC and one hemiCBC between clade III and the other two clades is in agreement with the present study. We did not find any CBC between clade IIIa and IIIb, only one HCBC.

The demarcation of clade III from the other two clades in our phylogeny and the presence of a CBC, along with morphological differences and mating incompatibility fulfil the requirements for a biological, morphological and phylogenetic species designation and hence for recognition of a separate species. However, it is difficult to know whether negative results in mating studies are actually due to mating incompatibilities, as it has previously been shown that some strains sometimes do not mate even though they are known to be reproductively compatible (unpublished results, Casteleyn et al. 2008). This lack of mating could e.g. be due to the effect of factors like the presence of an internal clock, presence of bacteria or other environmental factors on the induction of sexual reproduction. Clade IIIa and IIIb only differed by a single HCBC and not all the phylogenetic analyses supported clade IIIb. We therefore regard these two sub-clades as representing intraspecific variations of the species.

A description of clade III as a new species would cause a lot of confusion and practical problems when identifying cells that previously — without major problems — could be identified as *P. pungens*. As all three clades form a monophyletic clade, a pragmatic solution would be to describe clade III as a new variety of *P. pungens*, keeping the species designation for the clade comprising all three clades. Even though we regard clade III as making up a separate species we hence decided to take a conservative approach and describe it as a variety.

Pseudo-nitzschia pungens var. ***aveirensis*** Lundholm, Churro, Carreira, Calado var. nov. Figs 38-42, Table 4.

Diagnosis: *Cellulae symmetricae, lineares ad lanceolatae aspectu et valvari et cingulari. Apices plus minusve acuminatae. Longitudo cellularum 47.5-100-(188) µm, latitudo valvarum 2.7-3.7 µm. Valvae valde silificatae. Interstriae et fibulae in LM visibiles. Densitas striarum et fibularum 13-16 per 10 µm. Nodulus centralis absens. Striae fere duabus seriebus pororum, nonnumquam etiam serie tertia incompleta. Duae formae copularum cinguli. Altera serie una pororum ovalium, altera poris quadratis in duas vel tres partes divisa. In valvocopula densitas pororum 21-25 per 10 µm.*

Cells symmetrical and linear to lanceolate in valve and girdle view. Apices more or less pointed. Cell length is 47.5-100-(188) µm, valve width is 2.7-3.7 µm. The valves are strongly silicified. Interstriae and fibulae visible in LM. Density of striae and fibulae is 13-16 in 10 µm. A central nodule is absent. Striae most often with two rows of poroids; sometimes a partly formed third row. Two types of cingular bands: one type with one row of oval poroids, the other type with squared poroids split into two to three parts. In valvocopulae, the poroid density is 21-25 in 10 µm.

Holotype: Fixed material of culture theta 4, isolated from Barra, Portugal on 13 October 2003, deposited at the herbarium of the National History Museum of Copenhagen registered as C-A-T-2394.

Isotypes: Rinsed material deposited at the herbarium of the National History Museum of Copenhagen (C-A-T-2395) and the University of Aveiro Herbarium (AVE-A-T-1).

Type locality: Aveiro coastal lagoon.

Etymology: referring to the place where it was found: Aveiro coastal lagoon.

Geographically, *P. pungens* var. *aveirensis* is presently recorded from Portugal, Vietnam and Mexico and therefore probably has a worldwide distribution. However, studies including several strains from different areas are needed to confirm this. Inclusion of many clones of *Pseudo-nitzschia pungens* var. *pungens* from geographically widespread areas have shown it to be globally distributed in temperate areas, whereas *P. pungens* var. *cingulata* so far has only been recorded from the North East Pacific off USA (Casteleyn et al. 2008).

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Summary

It is a long-held belief that marine microbial plankton communities consist of a limited number of ubiquitously distributed species that lack biogeographic structuring (Palumbi 1994, Finlay 2002, Carr et al. 2003). This is not only attributed to the fact that oceans are fluid environments, lacking obvious barriers to gene flow, but also to the enormous population sizes and (supposedly) high passive dispersal potential of planktonic micro-organisms, which would effectively dampen diversification and speciation through allopatric mechanisms. This belief however, which is largely based on morphology-based taxon delineations, has recently been challenged by molecular-genetic investigations which are transforming our understanding of marine biodiversity, particularly regarding micro-organisms. Many taxa, which were considered single species on the basis of morphological criteria, have been shown to contain high levels of genetic diversity. As a result, several taxa that were believed to be cosmopolitan are now recognized as complexes of (semi)cryptic species, some of which have restricted geographic distributions (e.g. Kooistra et al. 2008). A variety of evolutionary processes, both allopatric and sympatric, has been proposed to explain the presence of such genetic diversity in the marine environment (de Vargas et al. 1999, 2002, Darling et al. 2004, Goetze 2005, Darling & Wade 2008).

Diatoms are one of the most abundant and diverse groups of eukaryotic planktonic micro-organisms. Among them, the pennate diatom genus *Pseudo-nitzschia* has recently received much attention as some of its representatives are able to produce domoic acid, a neurotoxin responsible for amnesic shellfish poisoning (Bates and Trainer 2006). *Pseudo-nitzschia* species occur in coastal and open-ocean waters worldwide and, based on morphological criteria, most appear to have a cosmopolitan distribution (Hasle 2002). Recent studies however suggest that intraspecific genetic variation may be considerable in *Pseudo-nitzschia* species. For example, molecular probes designed for identification of certain species (Scholin et al. 2003) appeared to be successful only in certain regions and not in others (e.g. Orsini *et al.* 2002, Parsons et al. 1999). In addition, differences in toxicity have been observed between strains belonging to the same morphospecies and conflicting data on the toxicity of species have been reported (Bates et al. 1998). The occurrence of (pseudo)cryptic variation within *Pseudo-nitzschia* species would not only have important implications for harmful algal bloom monitoring, but may also affect our

perception of geographical or temporal variation patterns within established species (Parsons *et al.* 1999).

Pseudo-nitzschia pungens (Grunow *ex* Cleve) Hasle is one of the most commonly reported, potentially toxic representatives of the genus worldwide, and is also one of the most common species in the North Sea (Vrieling *et al.* 1996, Hasle 2002). Interestingly, *P. pungens* isolates from various geographic areas have been shown to exhibit different abilities to produce domoic acid (until now toxic clones have only been reported from New Zealand and the Pacific coast of the USA), but the underlying causes of this variation remained unclear.

The present study was designed to obtain a better understanding of the species structure and biogeography of *Pseudo-nitzschia pungens*. More specifically, we wanted to find out (1) whether *P. pungens* is a single species or comprises multiple (semi)cryptic species, by analyzing variation patterns in selected molecular markers, sexual compatibility and morphological characteristics on local, regional and global scales; (2) whether the distribution of *P. pungens* is truly cosmopolitan or whether potential (semi)cryptic entities show different, possible restricted, geographic distributions; and (3) whether intraspecific, geographical genetic structuring could be detected by investigating population genetic diversity and differentiation both regional and global scales.

The first part of the thesis (chapters 2 and 3) deals with a detailed analysis of morphological, reproductive and molecular-genetic characteristics and variation patterns of regional (North Sea) and global isolates of *P. pungens*.

Chapter 2 reports on the basic cell and life cycle features, and more specifically sexual reproduction, auxosporulation and mating system of a series of monoclonal *P. pungens* isolates from various localities in the southern part of 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. Clones were shown to be heterothallic and sexual reproduction involved physiological anisogamy. 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. Auxospore development was accompanied by deposition of a transverse and then a longitudinal perizonium. 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 and described.

In **chapter 3,** genetic, reproductive and morphological variation patterns were studied in 193 global strains (six geographically distant areas in the North Sea, Atlantic and Pacific Oceans) of *P. pungens* to assess potential intraspecific variation and biogeographic distribution patterns. Genetic differentiation between allo- and sympatric strains was investigated using the ITS1 – 5.8S – ITS2 rDNA region. Phylogenetic analyses revealed three strongly supported ITS clades (I-III). All available clones were involved in crossing experiments to assess mating barriers. Clones of opposite mating type were sexually compatible within clades I and II, and viable F1 hybrid offspring were produced in crosses between them. The three ITS clades showed slight but consistent morphological differences. Strains of clade I were found to morphologically correspond to the nominate *P. pungens* variety while strains of clade II fit the circumscription of *P. pungens* var. *cingulata*, described by Villac and Fryxell in 1998. Members of the third clade have been described as a new variety, *P. pungens* var. *aveirensis*, based on morphological features and evidence for reproductive isolation (Churro et al., in press, **appendix**). The three ITS clades showed different geographic distributions. Clade II was restricted to the NE Pacific, whereas clones belonging to clade III originated from geographically widely separated but predominantly (sub)tropical-warm temperate areas (Vietnam, China, Mexico and Portugal). ITS clade I was recovered in all locations studied: the North Sea (Belgium, The Netherlands, France), the eastern and western N Atlantic (Spain, Canada), the NW and S Pacific (Japan, New Zealand) and the NE Pacific (Washington State). Clade I thus appears to be globally distributed in temperate coastal areas and provided the first strong evidence for the global distribution of a biologically, genetically and morphologically defined diatom species.

Hybridization between genetically distinguishable taxa provides opportunities for investigating speciation. While hybridization is a common phenomenon in various macro-organisms, natural hybridization among micro-eukaryotes has only rarely been documented. In **chapter 4** we used a nuclear and a chloroplast molecular marker to demonstrate the presence of natural hybrids of two genetically and morphologically distinct varieties of the marine planktonic diatom *Pseudo-nitzschia pungens* (clades I and II, corresponding to the vars. *pungens* and *cingulata*) in a contact zone in the northeast Pacific. Cloning and sequencing of the rDNA internal transcribed spacer region revealed strains containing ribotypes from both varieties, indicating hybridization. Both varieties were found to also have different chloroplast encoded *rbc*L sequences. Hybrid strains were either hetero- or homoplastidial, as demonstrated by Denaturing Gradient Gel Electrophoresis, which is in accordance with expectations based on the mode of

chloroplast inheritance in *Pseudo-nitzschia*. While most hybrids are probably first generation, there are also indications for further hybridization. Morphologically, the hybrids resembled var. *pungens* for most characters rather than having an intermediate morphology. Despite the occurrence of occasional hybridization between clades I and II, both clades appear to persist, even in sympatry, and can therefore be regarded as two independently evolving lineages.

In the second part of this thesis we studied genetic structure at the population level to increase our understanding of the underlying mechanisms that may have caused geographically structured variation patterns in the biodiversity of marine holoplanktonic organisms in general, and in *P. pungens* in particular. We focused on *P. pungens* var. *pungens*, which was found to have a widespread distribution in southern and northern temperate areas in both the Atlantic and the Pacific Oceans.

In **chapter 5** the spatial and temporal variation patterns in population genetic structure of *P. pungens* var. *pungens* populations in heterogeneous water masses of the Southern Bight of the North Sea was investigated using six microsatellite markers. We isolated 310 cells from contrasting coastal environments (sea, estuary and non-tidal saline lagoon) and with different degrees of connectivity (from complete isolation to supposedly free exchange between environments) on a scale of ca. 100 km. No evidence for genetic differentiation was found, indicating that different environmental conditions or partial isolation does not restrict gene flow between populations at a local to regional scale. Furthermore, broadening of our sampling area by the inclusion of isolates from the German, French and the Dutch parts of the North Sea (ca. 650 km), suggest the presence of homogenous population structure for this species in large parts of the North Sea.

In **chapter 6** global patterns of population structure and gene flow were investigated in *P. pungens* var. *pungens* to find out whether *P. pungens* var. *pungens* consists of a single homogenous global population or if genetic structure could be detected, providing clues for barriers to gene flow in global marine species. 242 isolates from different localities in the Atlantic and the Pacific were genotyped using six polymorphic microsatellite loci. Population genetic analyses indicate that even within a globally distributed lineage, significant population differentiation exist, suggesting restricted gene-flow on a global scale. Our results provide indications that allopatric processes may play an important role in the diversification of potentially high-dispersal marine plankton, such as diatoms. Our study is the first to investigate population genetic structure of a marine planktonic diatom on a global scale using microsatellite markers, and is a first step in understanding genetic structure at the species level and in determining the biogeographic extent of planktonic micro-organisms.

Samenvatting

Gedurende lange tijd werd algemeen aangenomen dat gemeenschappen van planktonische micro-organismen[1] wereldwijd slechts uit een beperkt aantal soorten bestonden, en dat mariene micro-organismen bijgevolg geen typische biogeografische verspreidingspatronen vertonen zoals we die kennen bij dieren en hogere planten (Palumbi 1994, Carr et al. 2003). Dit werd vooral toegeschreven aan het ontbreken van significante barrières in mariene milieus,wat samen met de enorme populatiegroottes en het potentieel hoge (passieve) verspreidingsvermogen van micro-organismen soortsvorming door allopatrische processen[2] verhinderde. Deze veronderstelling, die grotendeels gebaseerd was op een morfologisch soortsconcept, wordt de laatste jaren echter meer en meer in twijfel getrokken. Moleculair-genetische studies hebben ons inzicht in mariene microbiële diversiteit drastisch veranderd. Binnen heel wat morfologisch afgebakende soorten werd immers een hoge mate van genetische diversiteit waargenomen. Talrijke soorten die voordien als kosmopolieten werden beschouwd, worden nu erkend als complexen van (semi)cryptische soorten[3], vaak met beperkte geografische verspreidingsgebieden (bv Kooistra et al. 2008). Verschillende hypotheses werden voorgesteld betreffende de evolutionaire processen (waaronder allo- en sympatrische) die aan de basis liggen van deze hoge genetische diversiteit in mariene milieus (de Vargas et al. 1999, 2002, Darling et al. 2004, Goetze 2005, Darling & Wade 2008).

Diatomeëen[4] vormen één van de meest soortenrijke en ecologisch succesvolle groepen in het marien planktonisch milieu. *Pseudo-nitzschia* is een relatief klein genus, maar kreeg de laatste jaren veel wetenschappelijke aandacht omdat enkele soorten het neurotoxine "domoic acid" produceren, dat verantwoordelijk is voor "amnesic shellfish poisoning" (Bates and Trainer 2006). Deze ketenvormende, planktonische diatomeeën komen algemeen voor in oceanisch en kustgebonden plankton van polaire tot tropische gebieden, en de meeste soorten zijn kosmopoliet (Hasle 2002). Recent onderzoek heeft echter een hoge mate van intraspecifieke genetische variatie aangetoond bij verscheidene soorten. Zo kon men vaststellen dat moleculaire probes, ontworpen voor identificatie van bepaalde soorten (Scholin et al. 2003), enkel doeltreffend waren binnen bepaalde geografische regio's (e.g. Orsini *et al.* 2002, Parsons et al. 1999). Ook werden verschillen in toxiciteit waargenomen binnen eenzelfde morfologische soort (Bates et al. 1998). Cryptische diversiteit binnen *Pseudo-nitzschia* soorten heeft niet alleen

belangrijke implicaties voor de monitoring van plaagalgenbloeien ("harmful algal blooms"), maar is ook belangrijk om de geografische en temporele variatie binnen soorten beter te begrijpen (Parsons et al. 1999).

Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle is een van de meest algemene, potentieel toxische vertegenwoordigers van het genus wereldwijd, en komt ook voor in de Noordzee (Vrieling et al. 1996, Hasle 2002). *P. pungens* stammen uit verschillende geografische regio's verschillen in "domoic acid" productie (toxische stammen werden tot nu toe enkel aangetroffen in Nieuw-Zeeland en de Westkust van Noord-Amerika), maar de werkelijke oorzaak van deze variatie is niet gekend.

De doelstelling van deze thesis bestond erin om een beter begrip te verkrijgen van de soortstructuur en de biogeografie van *Pseudo-nitzschia pungens*. Meer specifiek wilden we (1) aan de hand van een studie van moleculaire merkers, seksuele compatibiliteit en morfologische kenmerken op lokale, regionale en globale schaal onderzoeken of *P. pungens* uit één dan wel meerdere (semi)cryptische soorten bestaat,; (2) nagaan of *P. pungens* werkelijk een wereldwijde verspreiding kent of dat eventuele (semi)cryptische entiteiten meer beperkte geografische verspreidingspatronen vertonen; en (3) onderzoeken of geografisch gestructureerde variatie kon waargenomen worden binnen de soorten, door populatiegenetische diversiteit en differentiatie te bestuderen op regionale en globale schaal.

In het eerste deel van deze thesis (hoofdstukken 2 en 3) werden morfologische, reproductieve en moleculair-genetische kenmerken en variatie patronen bestudeerd in regionale (Noordzee) en globale isolaten van *P. pungens*.

In **hoofdstuk 2** worden cellulaire en levenscycluskenmerken van monoklonale culturen van *P. pungens* isolaten uit de Noordzee in detail beschreven. Bijzondere aandacht ging hierbij uit naar seksuele reproductie, auxosporulatie en "mating system". Er werd aangetoond dat *P. pungens,* net zoals andere *Pseudo-nitzchia* soorten, gekenmerkt wordt door een heterothallisch mating systeem. Dit betekent dat seksuele reproductie enkel mogelijk is tussen twee genetisch verschillende, seksueel compatibele partners van een tegengestelde mating type (wat zelfbevruchting onmogelijk maakt). Dit kenmerk stond ons toe om seksuele compatibiliteit te bestuderen door middel van kruisingsproeven.

Hoofdstuk 3 omvat een studie van genetische, reproductieve en morfologische variatiepatronen in 193 *P. pungens* isolaten van verschillende gebieden in de Noordzee,

de Atlantische en de Stille Oceaan, met als doel potentiële intraspecifieke variatie en biogeografische patronen na te gaan. Genetische differentiatie tussen allo- en sympatrische isolaten werd onderzocht aan de hand van "internal transcribed spacer" (ITS regio's uit het ribosomale cistron) sequenties. Fylogenetische analyses resulteerden in drie duidelijke ITS clades (I-III). Kruisingsproeven werden uitgevoerd om seksuele compatibiliteit na te gaan tussen de isolaten/genotypes. Stammen van tegengestelde mating type waren seksueel compatibel binnen clades I en II, en levensvatbare F1 hybride nakomelingen tussen beide konden worden verkregen. De drie ITS clades vertoonden kleine maar consistente morfologische verschillen. Op basis van deze morfologische kenmerken kon clade I geïdentificeerd worden als *P. pungens* var. *pungens*, terwijl clade II overeen bleek te komen met *P. pungens* var. *cingulata* (Villac & Fryxell 1998). Vertegenwoordigers van de derde clade werden recent beschreven als een nieuwe variëteit, *P. pungens* var. *aveirensis*, op basis van duidelijke morfologische verschillen en reproductieve isolatie (Churro et al., in druk, **appendix**). De drie ITS clades hebben een verschillende geografische distributie. Clade II komt enkel voor in the noordoostelijke Stille Oceaan, terwijl clade III een disjunct verspreidingspatroon vertoont in tropische tot warm-gematigde zeeën (Vietnam, China, Mexico and Portugal). ITS clade I werd aangetroffen in de Noordzee (België, Nederland en Frankrijk), de noordoostelijke en noordwestelijke Atlantische Oceaan (Spanje en Canada), de noordwestelijke en zuidelijke Stille Oceaan (Japan, Nieuw-Zeeland) en de noordoostelijke Stille Oceaan (Washington). ITS clade I (*P. pungens* var. *pungens*) blijkt dus wereldwijd verspreid te zijn in gematigde gebieden. Deze studie vormt het eerste overtuigende bewijs voor een kosmopoliete verspreiding van een biologisch, genetisch en morfologisch omschreven soort.

Onderzoek naar hybridisatie tussen genetisch verschillende taxa kan inzicht verschaffen in soortvorming. Hybridisatie is goed bestudeerd in verschillende macro-organismen (voornamelijk in planten), maar in micro-eukaryoten is het fenomeen slecht gekend. In **hoofdstuk 4** toonden we het bestaan aan van natuurlijke hybriden tussen twee op genetisch en morfologisch vlak verschillende *P. pungens* variëteiten (vars. *pungens* and *cingulata*, clades I en II) in een contact zone in the noordoostelijke Stille Oceaan. Deze hybriden konden geïdentificeerd worden aan de hand van nucleaire (ITS) en chloroplast (*rbc*L) moleculaire merkers. Door middel van klonering en DNA sequentie bepaling van het ITS kon aangetoond worden dat bepaalde stammen ITS types van beide variëteiten bezaten. Beide variëteiten konden ook onderscheiden worden op basis van verschillen in chloroplast-gecodeerde *rbc*L sequenties. Aan de hand van "Denaturing Gradient Gel Electrophoresis" kon worden aangetoond dat de twee chloroplasten in hybriden ofwel afkomstig waren van beide ouders of van één van de twee, wat verwacht werd op basis van de gekende manier van chloroplastovererving in

Pseudo-nitzschia. De meeste hybriden waren naar alle waarschijnlijkheid eerste generatie hybriden, maar er waren ook aanwijzingen voor verdere hybridisatie. Morfologisch leken de hybriden het meest op var. *pungens*, eerder dan intermediaire kenmerken te vertonen. Ondanks het voorkomen van natuurlijke hybridisatie tussen clades I en II, blijven beide clades standhouden in eenzelfde regio, en kunnen dus aanzien worden als onafhankelijk evoluerende evolutielijnen.

In het tweede deel van deze thesis werd binnen de wijdverspreide *P. pungens* var. *pungens* de genetische structuur op populatieniveau onderzocht. Dit had tot doel om een beter inzicht te verwerven in mogelijke onderliggende mechanismen die geografisch gestructureerde variatie in mariene holoplanktonische organismen kunnen verklaren.

Hoofdstuk 5 bestudeert ruimtelijke en temporele variatiepatronen in de populatiegenetische structuur bij *P. pungens* var. *pungens* uit heterogene watermassa's in de zuidelijke Noordzee, aan de hand van zes microsatellietmerkers. Uit verschillende mariene milieus (open zee, estuarium en een niet aan getijdenwerking onderhevige kustlagune) met verschillende graden van connectiviteit (gaande van volledige afscheiding tot volledige verbinding), en allen gelegen binnen een straal van ongeveer 100 km, werden 310 cellen geïsoleerd. Microsatellietanalyses toonden geen genetische differentiatie aan, wat erop wijst dat "gene flow"[5] binnen het studiegebied niet beperkt wordt door verschillen in milieuomstandigheden tussen de staalnamelokaties of door isolatie op een lokale tot regionale schaal. Bovendien werd ook op een grotere geografische schaal (stalen uit de Nederlandse, Duitse en Franse Noordzee, op ca. 650 km afstand gelegen) eveneens een homogene populatiestructuur aangetoond.

In **hoofdstuk 6** werden populatiestructuur en "gene flow" in *P. pungens* var. *pungens* bestudeerd op een globale schaal. Meer bepaald wilden we nagaan of *P. pungens* var. *pungens* uit één enkele homogene, globale populatie bestaat dan wel of er toch genetische structuur aanwezig is, wat zou kunnen wijzen op het bestaan van "gene flow" barrières in globaal verspreide mariene soorten. 242 isolaten uit verschillende locaties in de Atlantische en Stille Oceaan werden gegenotypeerd aan de hand van zes polymorfe microsatellietmerkers. Deze populatiegenetische analysen toonden duidelijk populatiedifferentiatie aan, wat wijst op beperkte "gene flow" op wereldwijde schaal. Onze resultaten verschaffen belangrijke aanwijzingen voor het belang van allopatrische processen in diversificatie van mariene planktonische organismen met hoge dispersiecapaciteit, zoals diatomeëen. Deze pionierstudie van mariene planktonische diatomeëen is een eerste stap naar een beter inzicht in de genetische structuur en biogeografische verspreiding van soorten op wereldschaal.

[1] Planktonische micro-organismen is een verzamelnaam voor microscopisch kleine organismen die drijvend in het water leven, en zodoende voor hun verplaatsing voornamelijk afhankelijk zijn van de heersende stromingen. Hieronder vallen ondermeer bacteriën, protozoa en eencellige algen (zoals diatomeëen).

[2] Allopatrische speciatie (soortsvorming) is het ontstaan van dochtersoorten uit een ancestrale soort als gevolg van een geografische scheiding tussen dochterpopulaties. Door die scheiding kunnen de dochterpopulaties evolueren tot aparte soorten.

[3] Cryptische soorten zijn soorten die genetisch duidelijk van elkaar verschillen zijn maar die zo sterk op elkaar lijken dat je ze niet op basis van morfologische eigenschappen kunt onderscheiden. Semicryptische soorten lijken eveneens erg goed op elkaar maar kunnen op basis van minieme morfologische verschillen van elkaar onderscheiden worden.

[4] Diatomeëen (of kiezelwieren) zijn eukaryote algen met een typisch extern skelet van silica (glazen schaaltjes). De meeste diatomeëen zijn eencellig maar sommigen vormen kolonies in verschillende vormen en groottes. Zo bestaat het genus *Pseudo-nitzschia* uit lange ketenvormige kolonies, opgebouwd uit langwerpige cellen.

[5] "Gene flow" of genmigratie is een cruciaal begrip binnen de evolutie en het wil zeggen dat de genen van een soort zich verspreiden van de ene populatie naar omringende of ver afgelegen andere populaties van die soort. Een nieuwe variant van een gen blijft dan dus niet alleen op de plek waar zij ontstaan is maar reist mee naar nieuwe oorden door dispersie.

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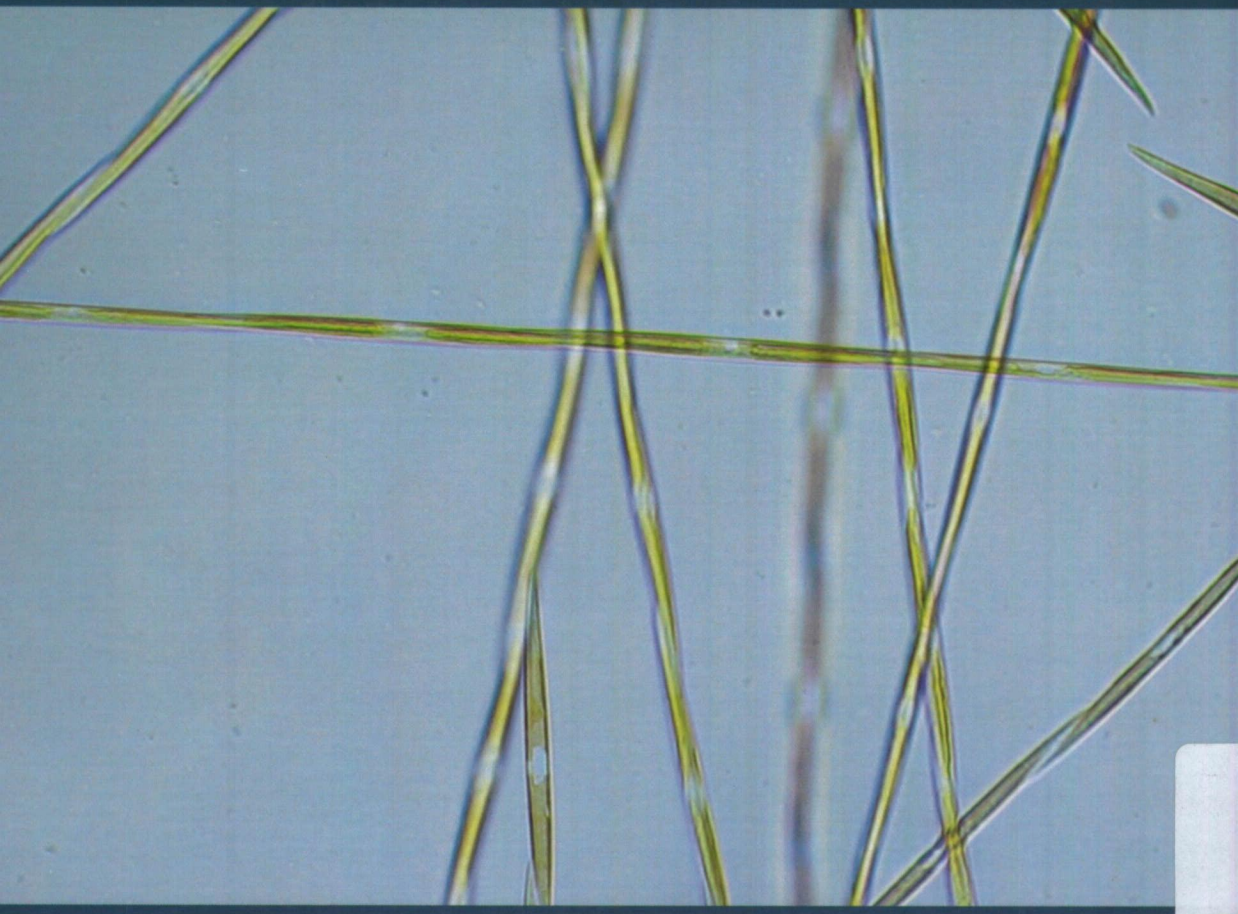

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