Biogeography, species diversity and stress tolerance of aquatic and terrestrial diatoms

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In science we resemble children collecting a few pebbles at the beach of knowledge, while the wide ocean of the unknown unfolds itself in front of us.

Isaac Newton
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1. General introduction

1.1 Biogeography
1.1.1 General biogeographical processes
Biogeography studies the geographical distribution of species over space and time. While before the 20th century biogeography was part of natural history and mainly a descriptive research field assessing “where” and “when” organisms live and had lived (e.g. Linnaeus, 1781; de Candolle, 1820; Wallace, 1876), it changed throughout the 20th century into a more explanatory research area, trying to answer the questions “why” a particular organism lives or had lived at a particular place, and “why not”. This change was stimulated by the findings that species richness could be predicted by e.g. area, immigration and extinction rate (Arrhenius, 1921; MacArthur & Wilson, 1963) and the discovery of plate tectonics (Wegener, 1924), supplying explanatory mechanisms for the observed historical and present distribution patterns of organisms. Since then, much has been done to further understand the processes underlying biogeographical patterns. Simplifying the whole process, the biogeographical range of a species starts with its formation in a suitable habitat in a certain region, dispersal into and eventually colonization of new regions resulting in an expansion of its biogeographical range, further range contractions and expansions under influence of large-scale environmental changes and biotic interactions, and ultimately ends with the extinction of the species or further evolution and speciation. It is generally accepted that two groups of factors affect the distribution of an organism, namely historical factors and local environmental factors (Lomolino, 2010).

Historical factors
Historical factors include on one hand the spatial configuration of suitable habitat patches in a region (influencing dispersal rates among these patches), the geographical position and isolation of regions and larger geographical areas, and historical changes in
these configurations due to climate change (influencing glaciation and sea level changes) and plate tectonics. On the other hand, historical factors also include features of the species itself such as its area of origin, its evolutionary background and the resulting phylogenetic constraints on the characteristics of the species (stress tolerance levels, adaptive and competitive potential, environmental niche, etc.) which will affect its dispersal and colonization abilities. The dispersal ability of a species depends on a whole range of factors, including its morphological and physiological characteristics (such as active locomotion or features enhancing passive motility, dormancy and tolerance levels for adverse conditions during dispersal), and the spatial configuration and characteristics of the medium or habitat they have to bridge during dispersal (physical barriers, heterogeneity, environmental factors, biotic interactions). The colonization ability of a species depends on its competitive strength, niche breadth and adaptive abilities. The combined action of the intrinsic and extrinsic historical factors will ultimately affect the dispersal capacities and environmental niche of the species and thus its realized biogeographical range, but can also result in subsequent speciation or in extinction (Coyne & Orr, 2004).

Local environmental factors
Local environmental factors comprise the present biotic and abiotic conditions in a habitat patch, including positive and negative interactions with other organisms and the chemical characteristics of the environment. Selection imposed by these biotic and abiotic factors in a local patch is called “species sorting” (Leibold et al., 2004) and can result in the local extinction or unsuccessful colonization of species for which the habitat patch is not suitable.

The interplay between local environmental factors (species sorting) and the spatial configuration of habitat patches on species’ occurrences and community structure is explicitly incorporated in the metacommunity concept (Leibold et al., 2004). A metacommunity can be defined as a set of local communities that are linked by dispersal of multiple potentially interacting species, which are available in the regional species pool. Using this concept, several studies have recently tried to estimate the relative contribution of local environmental factors and the spatial configuration of habitat patches in determining contemporary local community composition, over a range of spatial scales and organism groups (e.g. Potapova &
General introduction

Speciation and extinction
Speciation and extinction are two leading processes in biogeography. Speciation is the evolution of a distinct genetic lineage (De Queiroz, 2007), and occurs when gene flow between populations of a single species is reduced or prevented by a geographical, reproductive and/or ecological barrier (Cook, 1906, 1908; Coyne & Orr, 2004). The geographical distance between populations or the occurrence of a physical barrier (any unsuitable habitat type such as oceans, continents, rivers or mountains) can reduce dispersal and thus gene flow between populations. When gene flow is sufficiently reduced, random genetic drift and/or ecological adaptation will occur and eventually result in the formation of genetically separated lineages, which will become reproductive isolated over time (allopatric and peripatric speciation, Coyne & Orr, 2004). Reduction of gene flow can also occur inside a single area when populations get ecologically and reproductively separated, resulting in a barrier for gene flow independent of geography, with subsequent genetic differentiation (sympatric speciation, Cook, 1908; Coyne & Orr, 2004). Because of the crucial position of dispersal in allopatric speciation, which is supposed to be the main mode of speciation (Coyne and Orr, 2004), the level of dispersal could also affect total species diversity. Taken together, dispersal has an important influence on a large range of processes including biogeography (Macarthur & Wilson, 1963), population dynamics (Johst & Brandl, 1997) and genetics (Hanski, 1999), metacommunity dynamics (Leibold et al., 2004), and speciation (Coyne & Orr, 2004).

1.1.2 Microbial biogeography
Knowledge of the general processes determining the biogeographical range of species mainly comes from studies on macroorganisms (see for example the classic studies compiled in Lomolino et al., 2004). For microorganisms, much less is known on their biogeographies, mainly because their small sizes require the use of microscopes, culturing or molecular techniques to detect them and because it was assumed that microorganisms wouldn’t show a biogeographical structure anyway (Martiny et al., 2006). The combination of high local population abundances (and thus a higher chance to get dispersed) and small sizes (and thus low weight, increasing the
chance and distance of passive transport) was believed to increase
the dispersal probabilities of microorganisms to such an extent that
no geographical barriers would exist and only species sorting would
affect their distribution (Beijerinck, 1913; Baas-Becking, 1934).

Meanwhile, the rise of several technologies including DNA
sequencing and electron microscopy has allowed getting a better
understanding of the ecology and evolution of microorganisms.
Based on genetic data, studies do reveal a geographical pattern
in several eukaryotic (e.g. Fawley et al., 2004; Green et al., 2004;
Pringle et al., 2005; Boenigk et al., 2006; Taylor et al., 2006; Evans
et al., 2009; Boo et al., 2010; Casteleyn et al., 2010) and prokaryotic
microorganisms (e.g. Cho & Tiedje, 2000; Papke et al., 2003; Whitaker
et al., 2003), while other species turn out to have cosmopolitan
distributions (e.g. Glockner et al., 2000; Brandao et al., 2002; Ward
& O’mullan, 2002; Scheckenbach et al., 2005; Boo et al., 2010). This
indicates that microbial biogeographies are very likely shaped by
the same processes as assessed for macroorganisms (Martiny et al.,
2006), being history and dispersal limitation, next to species sorting.

Problems related to studying microbial biogeography
Besides the geographical component, these recent studies also
highlight the different factors that prevented the detection of
these geographical patterns and have biased our interpretation of
microbial biogeography towards a pattern uniquely determined by
species sorting. First and most importantly, the taxonomic resolution
used in microbial studies is usually much lower compared to the
studies on macroorganisms (Martiny et al., 2006). Indeed, leading
genetic markers in microbial geographic studies are SSU rDNA (e.g.
Fawley et al., 2004; Boenigk et al., 2006) or the even coarser DNA
fingerprint techniques on these markers (e.g. Green et al., 2004),
while it has been reported that for example for diatoms SSU rDNA is
too conservative to distinguish closely related species (Behnke et al.,
2004). Furthermore, identification of species based on morphological
characteristics is commonly used in eukaryotic microorganisms.
However, many studies have reported within single morphospecies
the presence of distinct genetic lineages or species without or with
only very subtle morphological differences between them (cryptic
and pseudocryptic species, respectively; e.g. Slapeta et al., 2006;
Beszteri et al., 2007; Simon et al., 2008). The oldest lineages of the
flagellated green algal morphospecies Micromonas pusilla (Butcher)
Manton & Parke appeared to have an age of no less than 65 million
years (Slapeta et al., 2006). This further biases our understanding of microbial biogeography. Secondly, there is a lack of knowledge on the dispersal abilities of microorganisms (Martiny et al., 2006). The dispersal rates and distances of an organism depend on one hand on intrinsic factors such as the number of cells dispersing (and thus population sizes), their morphology (weight and special structures promoting transport) and their physiological tolerance to the adverse conditions encountered during transport and colonization; and on the other hand on habitat-related factors such as the degree of habitat fragmentation which will influence the chance to be picked up by a dispersal vector and the chance to land in a suitable habitat patch. Apart from that, successful dispersal also depends on organism characteristics allowing it to compete with the local communities and populations already established in a patch (such as competitive ability, dormancy, adaptive potential). Of all these factors, however, little is known for microorganisms. First, while microorganisms certainly can have extremely large population sizes, this is most probably not the case for all microbial species (see for example Jones & Lennon, 2010). Secondly, it is not certain that the dispersal probabilities of all microorganisms are high enough to overcome genetic divergence between populations (Martiny et al., 2006). Many bacteria and eukaryotic microorganisms do form dormant stages (Hutchinson, 1967; Hargraves & French, 1983; Nicholson et al., 2000; Anderson, 2010), but this is for several taxa still unknown and there are not always data available on the tolerance of these stages for dispersal-related stresses. In addition, variation in niche breadth, competitiveness and adaptive capacities have neither been studied in much detail. Nevertheless, it is clear that interspecific variation does exist for dispersal-related traits (e.g. Evans, 1958; Elster et al., 2008; Müller et al., 2010), suggesting that taxa will also vary in their dispersal probabilities, dispersal rates and realized biogeographies (Martiny et al., 2006). This would fit the range of distribution patterns reported in microorganism and the range of scales on which geographical structure has been detected, and would further corroborate the current opinion that microbial biogeography is dictated by the same processes as macroorganisms (Green & Bohannan, 2006; Martiny et al., 2006).
Dispersal mechanisms and dispersal barriers of microorganisms

Dispersal is the transport of an organism from one geographical location to another, away from its parental population. An individual of a species can disperse from its parental population into another population, or disperse from its parental population towards an empty patch, colonize this patch and start a new population (if propagating asexually) or await a second individual of opposite mating type and start a new population (for sexual organisms). For biogeography, dispersal has both an influence when the movement is towards an alternative population (thus providing gene flow between populations and adding stability in a meta-population system) and when the movement is towards a new patch (thus expanding a biogeographical range or enhancing the number of local populations). Importantly, an effective dispersal event requires subsequent reproduction and the onset of a population.

Two main types of dispersal mechanisms are generally recognized, being active and passive dispersal. Active dispersal will depend on local factors (local population size, resource limitation, habitat size, etc.) forcing organisms to actively move out of a specific patch (Bowler & Benton, 2005). When passively dispersed, however, organisms depend on the kinetic energy occurring in the environment (animal and plant vectors, wind, water currents, gravity) for their movement (Maguire, 1963). For microorganisms, the active movement of a single individual will be spatially restricted and unlikely to bridge the gap between populations. Therefore, passive dispersal is the main dispersal type of microorganisms. Microorganisms are thus dispersed through animal and plant vectors, wind and water currents.

Dispersal barriers prevent the movement of a particular organism and include geographical barriers and physiological barriers. Both are difficult to disentangle, as geographical barriers (rivers, oceans, land barriers, mountains, etc.) only preclude the dispersal of an individual because of physiological restrictions. Therefore, dispersal barriers are species-specific (and will also vary between individuals). During dispersal, several more or less hostile conditions will be encountered, ranging from among others an adverse dispersal medium (animals’ gut, air or water for example), adverse environmental conditions in the crossed habitat patches, to biotic interactions. Tolerance levels for dispersal-related factors will therefore influence the spatial scale of dispersal and the probability of surviving a dispersal event. Besides this, the availability
of individuals (and thus population size) and morphological characteristics will further influence the chance of a species to be dispersed passively, while the availability of suitable habitat patches and colonization potential will affect the probability to arrive in a suitable habitat patch and colonize it. However, little is known on the extent in which dispersal limitation occurs in microorganisms and on the stress tolerance for dispersal-related factors.

1.2 Organisms under study: benthic freshwater and terrestrial diatoms

1.2.1 Diatoms: general introduction
Diatoms (Bacillariophyceae, Heterokontophyta) are a group of unicellular microalgae characterized by a siliceous cell wall (the frustule) consisting of two “valves” connected by girdle elements (Fig. 1.1), and a diplontic life cycle involving gradual cell size reduction by vegetative divisions followed by cell size restitution through sexual reproduction (Round et al., 1990; Chepurnov et al., 2004). Diatoms are best known for their intricately ornamented siliceous cell walls, which have traditionally been used to delineate species (Round et al., 1990). Moreover, as this silica wall is relatively resistant to corrosion, diatoms have an extensive fossil record, going back to the Jurassic, 190 million years ago (Rothpletz, 1896, 1900 in Sims et al., 2006). Within the more than 200,000 extant species estimated to exist (Mann & Droop, 1996), two large morphological groups are traditionally delineated (Fig. 1.1), being the “centric” diatoms with radially organized valves and the “ pennate” diatoms with bilaterally organized valves. A subgroup of pennate diatoms has evolved a longitudinal slit along the apical axis of the valve, called the raphe (Fig. 1.1), through which polysaccharide strands can be extruded onto the substrate, allowing the movement over a substrate (Drum & Hopkins, 1966). While centric and araphid pennate diatoms (having no raphe) are immotile and live suspended as plankton or attached to surfaces, the raphid pennate diatoms are motile and live mostly benthic in sediments and on substrates. Since their origin more than 70 Ma ago (Witt, 1886; Pantoceks, 1889; Chacon-Baca et al., 2002; Singh et al., 2006), raphid diatoms have diversified enormously in benthic environments and now account for the vast majority of the extant diatom species, indicative of the
Being photoautotrophic, diatoms thrive worldwide in the photic zone of the marine and freshwater plankton and benthos (Round et al., 1990). They are ecologically very important as primary producers, and are estimated to account for 20-25 % of the global primary production in oceans (Werner, 1977). To a lesser extent, diatoms also occur in terrestrial habitats and live in and on soils and on wet rocks, mosses, tree barks and other humid substrates (Round et al., 1990). However, not much is known about the ecology of terrestrial diatoms, as most ecological, physiological, biochemical and molecular studies have focused on marine or freshwater diatoms. Concerning biogeography and dispersal, several studies have been performed and some general trends and work hypotheses can be deduced from them (see section 1.2.2). Nevertheless, the specific mechanisms controlling diatom distributions are still unknown, and many questions remain. Moreover, similar to other microbial taxa,
several species complexes containing (pseudo)cryptic species have been reported (e.g. Sarno et al., 2005; Beszteri et al., 2007; Mann & Evans, 2007; Vanormelingen et al., 2008; Trobajo et al., 2009). Because cryptic diversity hampers the interpretation of geographical ranges, this issue has to be taken into account when reading previous morphology-based biogeographical studies and when devising new ones.

1.2.2 Diatom biogeography: an interplay of species sorting and dispersal limitation?
Several recent studies have tackled the question whether the geographical distribution of diatom species is only determined by species sorting, or by an interaction between local ecological factors and historical factors (Potapova & Charles, 2002; Soininen et al., 2004; Telford et al., 2006; Vyverman et al., 2007; Verleyen et al., 2009). Studies on marine planktonic and lacustrine benthic diatoms reveal that their geographical distributions are explained by local environmental variables and spatial variables, indicating that both species sorting and dispersal limitation account for the observed geographical structure. This result is further supported by reports of endemic diatom species and genera [e.g. species of Muelleria (Frenguelli) Frenguelli (Van De Vijver et al., 2010), and the genus Eunophora Vyverman, Sabbe & Mann (Vyverman et al., 1998)] and by microsatellite studies revealing restricted gene flow between populations of the marine planktonic species Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle (Casteleyn et al., 2010), Skeletonema marinoi Sarno & Zingone (Godhe & Harnström, 2010) and Ditylum brightwellii (West) Grunow (in which the species boundaries were however unclear) (Rynearson et al., 2009), and of the freshwater species Sellaphora capitata Mann & McDonald (Evans et al., 2009).

There are no reliable data available on the geographical distribution of terrestrial diatom species, but it is hypothesized that the biogeographical ranges of terrestrial diatoms would be larger than those of lacustrine diatoms (Spaulding et al., 2010). Compared to lakes which are islands of suitable habitat within a hostile matrix, terrestrial habitats are presumably less fragmented on a regional scale, increasing the dispersal probabilities and dispersal rates of terrestrial species. Moreover, because terrestrial habitats are characterized by larger daily fluctuations in temperature and moisture content (Starks et al., 1981; Gao et al., 2008) compared to
permanent lakes, it is assumed that terrestrial species are adapted to overcome these more hostile conditions [analogous to green algae (Zoe et al., 2008)], which would also increase their tolerance to dispersal-related stresses such as desiccation. Finally, because dryer sediment is more susceptible to be picked up by air currents (Chepil, 1956), terrestrial diatoms will be more susceptible to dispersal by wind compared to lacustrine diatoms. The combination of the less fragmented terrestrial habitat, higher susceptibility for wind dispersal and higher tolerance for desiccation would enhance the dispersal probabilities of terrestrial diatoms compared to aquatic diatoms (Schlichting, 1969; Ehresmann & Hatch, 1975). Dependent of the dispersal rate, a higher gene flow in terrestrial diatoms could also result in lower speciation rates, and it is thus expected that terrestrial diatoms are less diverse than aquatic diatoms. As said, no data are yet available to confirm or refute these hypotheses.

While it has been indicated that dispersal limitation does play a role in at least lacustrine and marine diatom distributions, it remains unknown on which scale and at what rate dispersal and gene flow occurs, and how fast speciation occurs [but see the rapid morphological evolution of *Stephanodiscus niagarae* Ehrenberg to *S. yellowstonensis* Theriot & Stoermer taking ca. 4,000 years (Theriot et al., 2006)]. It is neither clear how large the interspecific variations in these parameters are, nor if differences in habitat fragmentation would indeed lead to differences in dispersal probabilities. Furthermore, while there are some clues on the general tolerances of diatoms during transport by air currents and animals (see section 1.4), there is almost no knowledge on the range of interspecific differences in dispersal tolerances.

To study the spatial scale and rates of gene flow between populations of a single species, two methods are currently available based: phylogeographical studies and microsatellite analysis. These are based on the genetic information stored in individual genomes, and on the fact that, independently of environmental selection, neutral mutations will accumulate with time in the genomes (called “genetic drift”). Dispersal events between populations will counteract this genetic drift through the exchange of genetic material. By studying the frequency of alleles (different versions of the same gene) within and between populations one will gather an approximation of the amount of dispersal between these populations. When analyzing this in an explicit geographical framework as done in phylogeographical studies, one will gather information on both the amount and
geographical scale of dispersal of the different alleles. To further investigate the amount and rate of gene flow between geographical populations of a single species, frequencies of microsatellites (non-coding sequences of 1 to 6 base pairs repeated 10 to 100 times in tandem) or SNPs (Single Nucleotide Polymorphisms, variations in a single nucleotide in DNA fragments recovered over the genome) have to be analyzed.

**Phylogeographies**

On shorter time-scales, going back to the last glacial cycles, phylogeographical studies will evaluate more recent divergences between and within species in an explicit geographical framework using data from different geographical locations (Avise, 2000). By doing this, species and population diversifications can be assessed over spatial scales by using allele frequencies in the different populations. Such analyses have for example been used to retrace the colonization routes after the last glacial maximum 10,000 years ago from the glacial refugia to the present locations (e.g. King & Ferris, 1998; Grivet & Petit, 2002; Magri et al., 2006). For diatoms, phylogeographical studies are scarce and limited to the marine planktonic diatoms *Skeletonema* Greville (Kooistra et al., 2005) and *Thalassiosira weissflogii* (Grunow) Fryxell & Hasle (Sorhannus et al., 2010).

**Microsatellite studies**

To investigate rates of gene flow between geographically separated populations, microsatellite frequencies can be analyzed. For diatoms, different studies on marine planktonic species [*Pseudo-nitzschia pungens* (Casteleyn et al., 2010), *Skeletonema marinoi* (Godhe & Harnström, 2010), and also *Ditylum brightwellii* (Rynearson et al., 2009) in which the species boundaries were however unclear] and in a single freshwater species [(*Sellaphora capitata* (Evans et al., 2009))] reveal that gene exchange is restricted in more or lesser extent between geographically separated populations on even small geographical scales, while a study on *P. pungens* on smaller scale revealed no genetic differentiation and high gene flow between populations (Casteleyn et al., 2009).
1.3 Species diversity and speciation

1.3.1 Species as fundamental units of biology
In the most diverse fields of biology, including biogeography, species are used as basic biological study unit. A species (or lineage) is a group of individuals structured as a metapopulation that evolves separately from other groups of individuals (de Queiroz, 2007). During speciation a lineage splits into two (or more) new lineages (Fig. 1.2). During this split, different properties will be acquired by these evolving lineages: the lineages will become morphologically distinct, ecologically differentiated, reproductively incompatible, and genetically reciprocally monophyletic, amongst others. These properties are used as different lines of evidence (or operational criteria) for species delimitation, and were traditionally incorporated in different alternative species concepts, including the morphological, ecological, biological and phylogenetic species concepts. It is important to clearly distinguish between the theoretical concept of species (i.e. separately evolving metapopulation lineages) and the operational criteria (morphology, ecology, genetics) used to empirically detect species boundaries. Because these different properties are acquired at different time points through time, there is a grey zone (Fig. 1.2) between the initial lineage separation and the fully completed lineage separation during which alternative species concepts can give conflicting indications on whether two or a single species are present. On each outer side of this grey zone, there will be unanimous agreement on the number of species. For these reason, combining different lines of evidence, for example morphological, genetic and physiological data, will enhance the probability to delineate the species correctly.

In diatom taxonomy, valve morphology is traditionally the main basis on which species are delineated, to a lesser extent supplemented with cytological data (Round et al., 1990). Consistently morphologically differentiated groups of individuals are gathered into “morphospecies” as an approximation of the real species (as done by C. Linnaeus in his Systema Naturae). In diatom research, this morphological or typological concept is predominantly used in several study areas, including ecology and biogeography. Recently, the accessibility of molecular-genetic techniques has enabled the use of nucleotide information as additional line of evidence for species delimitation. Phylogenetic analyses of this genetic information will group strains into lineages depending on the nucleotide composition.
Fig. 1.2 Diagram visualizing lineage separation and divergence (speciation) and species concepts (after de Queiroz, 2007). A single lineage (species) splits to form two lineages (species). The horizontal lines labeled SC (species criterion) 1 to 9 represent the times at which the lineages acquire different properties (reciprocally monophyletic, reproductively incompatible, ecologically distinct, etc.). In the grey zone, alternative species concepts come into conflict. On either side of the grey zone, there will be unanimous agreement about the number of species.
and visualize this on a phylogenetic tree in which branch lengths are proportional to the number of nucleotide substitutions (Felsenstein, 2004). Individuals with minor variation in nucleotide composition will cluster together, while individuals which vary in nucleotide composition will be placed on separated branches, the branch lengths corresponding to the amount of divergence. When similar patterns occur using independent sources of genetic data (for example chloroplast genes vs. nuclear or mitochondrial genes, or different genes from a single genome source), the probability to correctly delineate species increases, and the same holds for morphological data.

1.3.2 Diatom diversity and cryptic species

Diatoms are one of the most species-rich groups of microalgae, estimated to include 200,000 extant species (Mann & Droop, 1996). They thrive in a wide range of habitats and ecological conditions, which all contain a whole range of species resulting in a high overall species diversity. The unique characteristics of the diatom genome, combining genes present in bacteria, higher plants and higher animals next to their own set of genes, is hypothesized to result in a high adaptive potential (Bowler et al., 2008), which might facilitate ecological adaptation and subsequent speciation. While the causal relationship between ecological change and speciation is still uncertain, it has been shown that closely related (pseudo) cryptic diatom species can be ecologically diverged, as seen for example for differences in salinity tolerances in the Navicula phyllepta Kützing species complex (Vanelslander et al., 2009) and differences in temperature optima in the Cylindrotheca closterium (Ehrenberg) Reimann & Lewin complex (Vanelslander et al., unpubl.). The morphological evolution from Stephanodiscus niagarae Ehrenberg to the endemic S. yellowstonensis Theriot & Stoermer was correlated with progressive warming (Theriot et al., 2006). Ecological niche differentiation could thus be a mechanism for speciation in diatoms and further account for the high diversity.

The high number of estimated diatom species is partly a result of the reports of cryptic species (Mann & Droop, 1996). The last decades, the use of DNA sequencing for species delineation has revealed that several diatom strains which at first sight did not differ in (observed) valve morphologies were genetically differentiated [e.g. Skeletonema costatum (Greville) Cleve (Sarno et al., 2005), Cyclotella meneghiniana Kützing (Beszteri et al., 2007), Sellaphora pupula
1.3.3 Speciation over time and space

As discussed for macrobiota (see section 1.2.1), speciation and extinction are two leading processes in biogeography. Speciation rates, periods and locations will therefore give further insight into the biogeographical processes at work. Geological and climatic events such as glacial cycles, tectonic movements and sea level fluctuations are often very well dated and can therefore be linked to dated speciation events. Concerning diatoms, there is a well-documented fossil sequence showing the abrupt morphological evolution from *Stephanodiscus niagarae* Ehrenberg to the endemic *S. yellowstonensis* Theriot & Stoermer over a time period of 4,000 years and correlated with progressive warming following the retreat of the glaciers 10,000 years ago (Theriot et al., 2006). Because the discovery of such well-preserved sequences is extremely rare and thus not generally applicable, other methods have been devised to estimate the time of lineage splitting using the DNA information of extant species. Neutral genetic markers are sequences of nucleotide information, often coding for very conservative proteins or having no function in protein coding, which are assumed not to be under ecological selection and thus only vary due to neutral mutations. Such markers will evolve over time and will further diverge between species due to genetic drift, resulting in less variation between recently evolved species and more variation between phylogenetically distant species, while the constant gene flow within species will prevent (or drastically reduce) this divergence.

Depending on the mutation rates of the used genetic markers, these can evolve slowly, thus giving more information on the deeper and older divergences (e.g. 18S rDNA for raphid diatoms) for example between larger groups of species or genera; or evolve faster and being of no use for deeper divergences but only for more recent speciation events (e.g. 28S rDNA, *rbcL*, *cox*1, ITS). When genetic markers evolve even faster and point mutations occur also within species (e.g. ITS or sometimes *rbcL*), these can be used to
trace variations between geographically separated populations of a single species. That said, depending on the time-scale one wants to investigate, a different choice of genetic markers is needed. To really investigate the amount and rate of gene flow between geographical populations of a single species, frequencies of microsatellites (non-coding sequences of 1 to 6 base pairs repeated 10 to 100 times in tandem) or SNPs (Single Nucleotide Polymorphisms, variations in a single nucleotide in DNA fragments recovered over the genome) have to be analyzed (see section 1.2.2).

**Time-calibrated molecular phylogenies**

On geological time-scales, the time of divergence between species or between larger groups of species can be investigated by combining genetic data with fossil calibration points into time-calibrated molecular phylogenies. Such time-calibrated phylogenies serve as a starting point to interpret species divergences in a geological framework (Donoghue & Benton, 2007). For example, for the marine planktonic foraminifer *Neogloboquadrina* Bandy, Frerichs & Vincent, a time-calibrated phylogeny based on strains isolated from different locations showed that lineage divergences between cryptic species in the Arctic and Antarctic were correlated with changes in ice masses, but that exchange between the two poles still occurred within the last 200,000 years (Darling & Wade, 2008). For diatoms, such explicit assessments of geological timeframes have only been done for the larger taxonomic groups of diatoms (Kooistra & Medlin, 1996; Medlin *et al.*, 1997; Sorhannus, 2007; Brown & Sorhannus, 2010) trying to estimate the geological processes triggering the diversification of diatoms or the centrics as a whole, and for a single marine planktonic species [*Pseudo-nitzschia pungens* (Casteleyn *et al.*, 2010)] estimating the divergence within the *P. pungens* species complex during the Pleistocene glacial cycles.

### 1.4 Stress tolerance and dispersal limitation

As summarized in section 1.1, dispersal is a key process controlling biogeographical ranges and knowledge on dispersal probabilities of organisms is thus indispensable to correctly interpret observed biogeographical patterns. The dispersal probability of an organism will depend on the spatial configuration and characteristics of the medium or habitat they have to bridge (physical barriers,
heterogeneity, environmental factors, biotic interactions) but also on its morphological and physiological characteristics such as active locomotion or features enhancing passive motility, dormancy and tolerance levels for adverse conditions during dispersal. The stress tolerance of an organism is therefore an important factor determining its dispersal potential and subsequently its potential geographical range. However, as reviewed by Martiny et al. (2006), knowledge on stress tolerance for dispersal-related stress and knowledge on interspecific variation in these tolerance levels is almost completely lacking for microorganisms, and the same holds for diatoms. Before being able to examine dispersal-related stress tolerance, it is important to have a good knowledge on the dispersal mechanisms diatoms can use and subsequently deduce the problems diatoms encounter during dispersal. Based on the available data, some first hypotheses can be put forward on dispersal tolerances of freshwater and terrestrial diatoms.

1.4.1 Freshwater diatom dispersal

Due to their small size, diatoms live on a different scale than macroorganisms, and this has to be taken into account. Most diatoms do not move actively. However, the raphid diatoms do move on smaller scales, but this movement is aimed to leave less suitable patches and search randomly for a more suitable patch with higher nutrient concentrations (pers. comm. J. Gillard), lower or higher light conditions (Admiraal et al., 1984; Perkins et al., 2010), or available mating partners (pers. comm. J. Gillard), among others. By doing this, they can move at speeds of 1-25 μm per second (Round, 1971; Edgar & Pickett-Heaps, 1984). However, this active, small-scale dispersal of cells will not affect the geographical structure. Of course, over multiple generations a diatom species could eventually extend its range over larger scales by active movement, but by that time it is likely that genetic divergence from the source population has occurred, except if a second, faster dispersal mechanism has allowed sufficient gene flow between these populations. Active movement is thus not likely to be the key dispersal mechanism of diatoms to extent their geographical range and this will mainly be achieved by passive dispersal.

During passive dispersal, the organism is transported by a supportive medium (air currents, water currents or another organism) without consuming itself additional energy for movement. However, the dispersed organism is entirely dependent
on the transport medium for the rate, direction, distance and point of time of dispersal (Isard & Gage, 2001). From the point of view of the passively dispersed organism the transport is random, but in some cases the movement of the medium is less random than expected, such as for dominant air currents, dominant water flows or migration routes of animals, and will largely determine the main dispersal routes of the passively dispersed organisms. As such, the distribution of some passively dispersed zooplankton species in North America matched the dominant migration routes of waterfowl (Green & Figuerola, 2005). Meanwhile, at more local scales, passive dispersal will be random. Traditionally, short and long distance dispersal has been distinguished, using a more or less arbitrary distance as boundary, for example 10 km as boundary for aquatic invertebrates (Green & Figuerola, 2005), or defining it as respectively dispersal inside a habitat type and dispersal over different habitats (Isard & Gage, 2001). However, nothing is known on the distances over which diatoms are transported generally, nor on the heterogeneity encountered by a diatom cell inside a habitat type, and both types of definitions remain thus quite subjective for diatom dispersal.

1.4.2 Dispersal vectors and dispersal-related stress factors
Three types of transport vectors account for the passive dispersal of diatoms, being water currents, air currents and living organisms (Kristiansen, 1996).

Dispersal by water currents
Freshwater currents in rivers or as ground water, and oceanic currents, the thermohaline circulation and other water currents all transport algae passively, including diatoms (Finlay & Esteban, 2004). A colonization experiment using an artificial water sink connected to an existing pond showed that over short time periods the same species occurred as in the source pond, while new species, transported over land, only occurred after several years (Atkinson, 1988). Water currents are the fastest way of dispersal for algae, but are constrained by the position and availability of sources and destinations (Kristiansen, 1996). Of course – and this applies to all dispersal mechanisms – effective dispersal is only acquired when a
propagule arrives alive in a suitable patch and is able to divide and start a new population.

**Dispersal by organisms**

Diverse types of organisms can potentially transport diatoms. Macroalgae and macrophytes will transport the cells during their passive dispersal through water currents and animals. Actively moving animals including fish, insects, mammals and birds can transport the cells internally (endozoochory) or externally (epizoochory). For freshwater habitats several organisms have been shown to contain living diatoms in or on their bodies. Fish guts can contain living diatoms (Velasques, 1940), but such dispersal is restricted to the watercourse. Less restricted but still over short distances is external transport on water beetles (Migula, 1888), while transport over longer distances (up to 850 km) can occur internally and externally via dragon flies (Parsons et al., 1966) and aquatic mammals (Irénéé-Marie, 1938). However, the most important dispersers of freshwater algae are waterfowl (Kristiansen, 1996; Green & Figuerola, 2005) which move generally in a periphery of 20-30 km but can migrate over several hundreds of km (Green & Figuerola, 2005). Externally, some living diatoms have been found on feet, bills and feathers of ducks (Schlichting, 1960; De Guerne (1888) in Kristiansen, 1996), but algal cells having slime sheets or forming spores or cysts are quantitatively much in favour (Schlichting, 1960) because desiccation during external transport limits the survival of transported cells (Figuerola & Green, 2002). The presence of humid sediment will mitigate this desiccation (Evans, 1959), but most diatoms already die when the moisture content of the surrounding sediment decreases to 50% (Evans, 1959), while the mud remains rarely longer than 30 minutes on the feet of ducks, decreasing the probability of long distance dispersal by this way (Schlichting, 1960).

For effective internal transport by waterfowl, the passage through the guts should be survived, which depends on the physical, chemical and bacterial components of the birds gut, but also on the thickness and composition of the cell wall of the dispersed cell (Kristiansen, 1996; Charalambidou & Santamaria, 2002; Green & Figuerola, 2005). The amount of living algae present in faeces and guts lies however much lower than the amount of algae carried externally (Schlichting, 1960) and out of five diatom species tested only a single survived digestion experiments (Atkinson, 1971). However, internal transport could occur over longer distances than
external transport (Proctor, 1966), and living *Asterionella* Hassall has been found in faeces two hours after feeding, corresponding to a flight of 220 km (Atkinson, 1980).

Also humans disperse microalgae and diatoms between ponds through the introduction of exotic fishes and plants (Cofey & Miller, 1988) and through scientific activities and the use of unsterilized material (Talling, 1951). Conclusively, there are enough indications that diatoms are internally and externally transported by various organisms, but knowledge on the frequency and distance of transport (Figuerola *et al.*, 2005), and on the dispersed species and tolerance levels of different species (Charalambidou & Santamaria, 2002) is extremely scarce.

**Dispersal by air currents**

In general it is assumed that wind transport plays an important role for the dispersal of microalgae (Round, 1981; Chalmers *et al.*, 1996; Kristiansen, 1996). However, the general pattern is that predominantly terrestrial algae, including cyanobacteria, green algae and to lesser extent terrestrial diatoms, are transported through the air, while there is uncertainty on the frequency, distance and extent of aerial transport. Concerning diatoms, it is already longer known that their empty frustules are present in wind currents (Darwin, 1845; Meier & Lindbergh, 1935; Maynard, 1968; Chalmers *et al.*, 1996), but also living diatoms have been reported from air samples. Van Overeem (1937), Schlichting (1961, 1964), Brown *et al.* (1964) and Roy-Ocotla & Carrera (1993) assessed the presence of living microalgae and found predominantly cyanobacteria and coccoid green algae, besides some sporadic diatom cells from the genera *Navicula* Bory de Saint-Vincent, *Hantzschia* Grunow, *Nitzschia* Hassall and *Melosira* Agardh. Their conclusions are that principally green algae such as *Chlorococcum* Meneghini, *Chlorella* Beijerinck and *Klebsormidium* Silva, Mattox & Blackwell are transported by wind while diatoms are less often encountered (Van Overeem, 1937; Schlichting, 1961; Brown *et al.*, 1964; Schlichting, 1964; Roy-Ocotla & Carrera, 1993), and that mainly terrestrial taxa are recovered (Brown *et al.*, 1964; Roy-Ocotla & Carrera, 1993). However, Geissler & Gerloff (1966) found, besides the typical green algae, a large amount of living diatom species in their air traps, qualitatively and quantitatively corresponding to the nearby situated river Havel. These data contradict the previous opinion that diatoms are not common in air currents, and that only a limited amount of taxa are found, but no data are given on the
set-up, the exact location of the traps and the weather conditions. Despite this last study, on average around 1 to 2 microalgal cells per m³ are found, which is not much compared to the on average 142,3 pollen per m³ (Brown et al., 1964; Tormo et al., 2001).

Several factors play a role in the occurrence of wind dispersal (Isard & Gage, 2001). Meteorological conditions such as relative humidity, wind velocity and wind shearing, air temperature, atmospheric pressure and the occurrence of turbulences or eddies will determine the takeoff of organisms from the substrate, the period and distance it will remain in the air and move, and the moment of landing into a new patch. As dry soils are more wind erodible than wetter soils (Chepil, 1956), it is understandable that predominantly terrestrial algae are found in the air, and that the highest abundances of dispersed algae are found in dryer periods (Van Overeem, 1937; Tormo et al., 2001). Besides meteorological conditions, the intrinsic features of the transported organism will determine the ease to be picked up and transported by air currents (such as size, morphological characteristics, local population abundances and habitat) and the capacity to survive the stresses experienced during aerial transport such as UV-radiation, desiccation and extreme temperatures (Schlichting, 1961; Aylor, 1986; Lighthart & Mohr, 1987; Wynn-Williams, 1991). Physiological, morphological and cytological characteristics will enhance survival probabilities, such as the presence of slime sheets (Shephard, 1987), the increase in concentrations of pigments (Imshenetsky & Kondrateva, 1987) or compatible compounds (Welsh, 2000), or the formation of resting stages (Hargraves & French, 1975; Hoffmann, 1996).

1.4.3 Dispersal rates and probabilities
The influence of dispersal on biogeographical ranges depends not only on its prevalence, but also on its rate. To be able to overcome a speciation event in a population, gene flow between populations must be high enough to overcome genetic drift and selection. It is thus possible that very low levels of dispersal are not able to prevent population differentiation. This level will depend on the local population abundances in the colonized patch, but also on the colonization and competition abilities of the colonizing cells. Concerning diatoms, no data are available on rates of effective dispersal. However, based on the literature we can estimate their probabilities of dispersal, giving a first, rough indication of the effective dispersal rate.
First, terrestrial habitats are assumed to be less fragmented compared to aquatic habitats, enhancing the probability of effective dispersal for terrestrial diatoms. Second, mainly terrestrial diatoms are transported by air currents, whereas lacustrine diatoms will be transported by waterfowl, but the presence of living diatoms is for both vectors quite low. Based on the knowledge that diatoms are not very commonly transported by waterfowl and air currents (see above), we can assume that their dispersal rates will in general not be extremely high. Third, dispersal probabilities decrease with distance, and short-distance dispersal of diatoms will therefore be more probable than long-distance dispersal. Combining these assumptions, we can hypothesize that for both lacustrine and terrestrial diatoms long-distance dispersal will not be sufficiently enough to overcome genetic differentiation between populations and prevent the formation of geographical structure, while dispersal over short distances does very likely occur. For terrestrial diatoms which are transported by air-currents, we hypothesize that they will disperse farther away than lacustrine diatoms due to the less fragmented terrestrial habitat and the occurrence of aerial dispersal, albeit not on a world wide scale, creating thus geographical structure on a somewhat broader scale.

1.5 Aims and thesis outline

The overall aim of this study is to improve our understanding of the role of dispersal limitation for generating geographical patterns in lacustrine and terrestrial diatoms. This is done by using multiple lines of evidence, including the analysis of large and regional scale biodiversity patterns, time-calibrated phylogenies and fossil evidence, and ecophysiological studies. Our primary study region comprises Antarctica and the sub-Antarctic islands. Because of the relatively species-poor diatom flora and strong degree of geographical isolation of many habitats, this region is amenable to such studies, making it possible to undertake comprehensive, region-wide studies of biodiversity patterns and dynamics within a region with a relatively well-documented geological and climatological history for most of the Cenozoic period [65.5 million year (Ma) ago till present] during which the pennate diatoms radiated.
The thesis is organized in an introduction, three parts with our main research results, and a general discussion:

**INTRODUCTION**

◊ **Chapter 1** gives a general introduction on biogeography and microbial biogeography, and focuses further on diatom biogeography, species diversity and the interaction of dispersal probabilities and stress tolerance.

**PART 1: PATTERNS IN DIATOM DISTRIBUTION**

◊ In **Chapter 2** we combine analyses of biogeographical patterns of Antarctic and Arctic diatoms, fossil data and a time-calibrated molecular phylogeny to provide evidence that adaptive radiations through allopatric speciation, survival in glacial refugia, and regional extinction operate in microorganisms at similar spatial and temporal scales as observed in macroscopic organisms. These findings categorically reset the current paradigm in diatom biogeography that diatoms are not limited in their dispersal and consequently do not have biogeographies.

◊ In **Chapter 3** we examine if terrestrial and lacustrine diatoms show different patterns in geographical structure at the regional scale, using distance decay analysis of community similarities and turnover of diatom assemblages between the sub-Antarctic islands Crozet and Kerguelen situated in the South Indian Ocean, and South Georgia situated in the South Atlantic Ocean. Community similarities decrease over space for both terrestrial and aquatic habitats, but no significant differences are found between them. Only at smaller spatial scales, within islands, terrestrial diatoms have a significantly lower turnover between sites, suggesting higher dispersal rates and/or broader niches for terrestrial diatoms.

**PART 2: SPECIATION IN TIME AND SPACE**

◊ To further explore the importance of time in speciation events, divergence and thus biogeographical patterning, we construct in **Chapter 4** a multi-locus phylogeny of the diatom genus *Pinnularia* Ehrenberg, and calibrate it in time using fossil material. Our analysis suggests that diversification of the genus started *ca.* 60 Ma ago, 10 to 30 Ma earlier than previously thought. The genus diversified into 3 major clades and several sub-clades, in some cases congruent with observable morphological and cytological innovations, and all major clades have dispersed successfully worldwide.
The multi-locus phylogeny of the genus *Pinnularia* from chapter 4 allows us to investigate the evolution of morphological characters and to compare the phylogenetic signal present in genetic and morphological data in Chapter 5. This is done by using ancestral state reconstruction of the morphological data and by performing cladistic analyses on the morphological data. While some morphological character states evolved several times over different clades, other character states were restricted to a certain clade. The morphological data allows us to reconstruct some interspecific relationships, but only the genetic data are able to fully resolve these relationships. However, similar clades are recovered, indicating that a similar phylogenetic signal is present in both datasets.

In Chapter 6 we select the cosmopolitan terrestrial morphospecies *Pinnularia borealis* Ehrenberg and *Hantzschia amphioxys* (Ehrenberg) Grunow and determine the degree of genetic differentiation between Antarctic and non-Antarctic strains using the plastid marker *rbcL* and the nuclear marker 28S rDNA. We place the divergences of *P. borealis* in a geological time-frame using the time-calibration of the genus *Pinnularia*. We also assess differences in thermal adaptation between genetically differentiated lineages for both taxa. The results indicate that in both taxa the Antarctic strains were genetically and ecologically differentiated, suggesting the occurrence of allopatric speciation on the Antarctic.

**PART 3: TOLERANCE LEVELS FOR DISPERSAL-RELATED STRESSES**

In Chapter 7 we investigate for a selection of benthic freshwater and terrestrial diatom strains the tolerance levels of vegetative cells for two dispersal-related stress factors, namely desiccation and extreme temperatures. We observe a very high sensitivity of both aquatic and terrestrial diatoms for desiccation, while terrestrial diatoms are more tolerant for extreme temperatures.

Because resting stages are in many organisms the dominant dispersing stages, we analyze in Chapter 8 the tolerance levels of diatom resting cells for desiccation and freezing, and this for a selection of taxa occurring over a gradient from permanent aquatic habitats to terrestrial soils. While all taxa formed resting stages, only those from species that occurred mainly or exclusively outside water bodies were tolerant for desiccation and freezing, showing that terrestrial diatoms are adapted to their environment, and increasing their dispersal probabilities compared to aquatic diatoms.
GENERAL DISCUSSION

In Chapter 9 the results of the previous chapters are discussed in the general framework of biogeography and dispersal probabilities.
Part 1.
Patterns in diatom distribution
2. Poles apart: Interhemispheric contrasts in diatom diversity driven by climate and tectonics

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Submitted manuscript
Contribution of CS: part of the statistical analysis and molecular phylogeny
Abstract

Arctic and Antarctic floras and faunas differ strongly in their overall composition and diversity (Abbott & Brochmann, 2003; Convey & Stevens, 2007), reflecting interhemispheric differences in climate history and landmass connectivity (Barnes et al., 2006). In contrast, prevailing microbial biogeographic theory (Finlay, 2002) predicts that Arctic and Antarctic ecosystems should harbour the same microbial communities, with allopatric speciation being rare as a result of the ubiquitous dispersal of microbes. Here we show that, contrary to this paradigm, patterns of biodiversity and endemism in the diatom floras of Arctic and Antarctic lakes are strongly divergent. Antarctic communities are impoverished and imbalanced relative to their Arctic counterparts, and are characterized by high levels of endemism, the absence of key functional groups such as planktonic taxa, an overrepresentation of terrestrial lineages, and a general paucity of globally successful genera. Comparison of contemporary Antarctic floras with fossil Miocene assemblages and a molecular clock analysis of diversification patterns point to high rates of local extinction during Neogene and Quaternary glacial maxima, in combination with radiations through allopatric speciation in glacial refugia. We propose that processes controlling the distribution and diversification of microorganisms can operate at similar spatial and temporal scales as those for macroscopic organisms, leading to strikingly congruent biogeographic patterns.
2.1 Introduction

Spatial patterns of global biodiversity are determined by phylogenetic diversification and extinction and dispersal of lineages in relation to tectonic processes and climate dynamics (Lomolino, 2010). This is particularly evident in polar terrestrial biota, where interhemispheric differences in glacial history (Zachos et al., 2001) and in the spatial configuration of landmasses are reflected in contrasting patterns of contemporary plant and animal biodiversity (Barnes et al., 2006). Most Arctic biota are thought to have evolved in the high mountain ranges of temperate regions and colonized the high latitudes to form an extensive tundra biome (Abbott et al., 2000) shortly after the onset of the late Miocene glaciations about 3 Ma ago (Zachos et al., 2001). Other species originated during the Holocene, or, alternatively, are descendents from the forest biota that occupied the Arctic during the late Tertiary (Abbott & Brochmann, 2003). During the Pleistocene ice ages, many of these cold-adapted species probably proliferated in vast tundra populations further south or in high latitude glacial refugia (Hultén, 1937), followed by postglacial recolonisation of ice-free terrain during interglacials, which prevented a significant loss of genetic diversity (Brochmann & Brysting, 2008). As a result of the high connectivity between landmasses of the Northern Hemisphere and the lack of strong dispersal barriers, most taxa have a circum-Arctic distribution (Abbott & Brochmann, 2003).

In the Southern Hemisphere, by contrast, the isolated and fragmented nature of landmasses prevented such large-scale latitudinal shifts of cold-adapted species. Molecular clock analyses have revealed that taxa started to diverge into endemic lineages through allopatric speciation shortly after the isolation of Antarctica from the other continents of the southern hemisphere (Convey et al., 2008). Many biota were driven to extinction after the mid-Miocene cooling event (ca. 14 Ma), when the East Antarctic Ice Sheet advanced and full polar climate conditions were established (Lewis et al., 2008). This resulted in a highly impoverished and imbalanced flora and fauna on the Antarctic continent (Convey & Stevens, 2007) as restrictions placed on dispersal by continental isolation prevented recolonization of Antarctica from the mid-southern latitudes during interglacial periods.

In contrast to the multicellular flora and fauna, the importance of dispersal limitation, allopatric speciation, and long-term tectonic and climate dynamics have been considered to be of minor relevance in shaping contemporary polar microbial communities.
Indeed, prevailing theory predicts that similar habitats at both poles should share the same microbial communities as a result of their unlimited dispersal capacities (Finlay, 2002). Local environmental factors are therefore believed to act as the major process structuring their metacommunities through lineage sorting (Van der Gucht et al., 2007) while biogeographical patterns reflecting past climate changes and tectonic processes are predicted to be absent (Martiny et al., 2006). Recent population genetic studies have challenged this idea and revealed that dispersal limitation may lead to allopatric divergence of populations, which may ultimately result in the formation of new microbial species (Evans et al., 2009; Casteleyn et al., 2010). Dispersal limitation and allopatric speciation can also explain the importance of spatial factors in determining patterns in diversity (Telford et al., 2006; Vyverman et al., 2007), metacommunity structure (Verleyen et al., 2009) and the presence of endemism in various microbial lineages (Petz et al., 2007; Pommier et al., 2007). Here we test if speciation promoted by geographical isolation is also a driving force in the diversification of diatoms in Arctic and Antarctic lakes. Specifically, we predict that under a scenario of predominant allopatric speciation, the greater degree of geographic isolation in the Southern Hemisphere would be reflected in (i) an imbalanced Antarctic diatom flora compared with similar habitats in the Arctic region, and (ii) higher levels of endemism in the most isolated areas.

2.2 Results and discussion

In our comparison of Arctic and Antarctic floras we strictly controlled for possible variation in diatom assemblages due to limnological differences between the studied regions (Fig. S2.1). In accordance with our first prediction, the Antarctic diatom flora is highly imbalanced and impoverished (123 species in 83 lakes) compared with its Arctic counterpart (223 species in 56 lakes, Fig. 2.1). The Arctic flora is most speciose in freshwater genera that are widespread and abundantly present in the majority of the freshwater systems worldwide. The Antarctic flora is rich in endemic species belonging to typically terrestrial genera and globally successful genera are underrepresented. The present-day Antarctic diatom flora is also strikingly different from the ca. 14 Ma old fossilized assemblages preserved in McMurdo Dry Valley deposits (Lewis et al., 2008),
Fig. 2.1 Number of species per diatom genus in Arctic and Antarctic lakes sharing the same limnological properties, showing the highly impoverished and disharmonic nature of the Antarctic diatom flora, which is relatively rich in endemic species belonging to terrestrial genera. The Arctic flora is more balanced and dominated by globally widespread and successful genera.
which were characterized by planktonic taxa entirely absent from contemporary and Late Quaternary Antarctic floras.

The incidence of endemism in the Antarctic diatom flora was assessed using a dataset of 392 Antarctic and sub-Antarctic freshwater lakes encompassing the three main biogeographical provinces in the Southern Ocean (Fig. 2.2) recognized for macroscopic organisms (Chown & Convey, 2007). Multivariate analysis revealed that the geographical structuring of diatom biodiversity is similar to that of macroscopic organisms (Fig. 2.3). Moreover, despite the low overall diversity (Fig. S2.2 and Fig. S2.3) each of the three biogeographical provinces showed a high degree of endemism (i.e. 43%, 35% and 51%, respectively, for Sub-Antarctica, maritime Antarctica and continental Antarctica). This is strikingly congruent with patterns found in multicellular organisms (Chown & Convey, 2007), where approximately 50% of the lichen, tardigrade and dipteran species are endemic to Antarctica, as are the majority of mites and springtails, and possibly all nematodes (Convey et al., 2008). The role of isolation in the diversification of the Antarctic diatom flora is further evidenced by the significant correlation between isolation and the proportion of endemic taxa ($R^2=0.5998; p=0.002, n=13$; Fig. S2.4), in accordance with our second prediction, and by a time-calibrated phylogeny of the globally distributed morphospecies *Pinnularia borealis*. The molecular phylogeny demonstrates that continental Antarctic strains belong to a separate lineage that diverged about 7.67 Ma ago from its closest relatives (Fig. 2.4). This has at least two important consequences. First, our morphology-based estimates of endemism are conservative and will likely further increase when molecular-phylogenetic analyses are applied to other presumably ubiquitous taxa (Beszteri et al., 2007; Evans et al., 2009). Secondly, the time of diversification in this species complex is similar to that observed in Antarctic springtails (Stevens et al., 2006) and mites (Mortimer et al., 2011), but later than divergence times in midges (Allegrucci et al., 2006). These findings suggest that macroevolutionary events may have occurred simultaneously in non-related groups of Antarctic organisms, in response to tectonic and climate change and increasing habitat fragmentation resulting from the expanding ice sheets.

The imbalanced nature of the Antarctic diatom flora, the high levels of endemism and the timing of speciation all point to an ancient evolutionary history in geographic isolation, characterized by regional extinction of key functional groups and freshwater genera, and selective survival of taxa in glacial refugia since the
Fig. 2.2 Map of the Southern Hemisphere showing the different biogeographical provinces in the Southern Ocean (dotted lines) and pie charts showing the number of ubiquitous versus endemic taxa. Maritime Antarctic species occur on the western side of the Antarctic Peninsula down to ca. 72°S and on the eastern side north of 65°S, as well as in the South Shetland and South Orkney islands. Continental Antarctic endemics are restricted to all other areas and islands of Antarctica south of 65°S. Sub-Antarctic endemics occur on the islands or island groups between 46° and 55°S. Antarctic endemics s.l. are restricted to the Southern Ocean region but occur in at least two provinces.

Fig. 2.3 Biplot of a principle component analysis of Hellinger transformed species data showing the difference between the diatom communities of 392 lakes and the congruence with the biogeographical zonation observed in multicellular taxa. The eigenvalues (EV) are given next to the axes.
Fig. 2.4 Time-calibrated molecular phylogeny of the diatom morphospecies Pinnularia borealis showing a divergence of the continental Antarctic lineage (in **bold**) from a western European lineage around 7.67 Ma [1.95-14.77 Ma, 95% Highest Posterior Probability interval (HPD)], based on concatenated rbcL and LSU rDNA (D1-D2 region) sequence data. Triangles represent the different phylogenetic lineages. The number of sequenced clones per lineage is given between brackets. Bars represent the 95% HPD intervals. The grey encircled nodes were time-constrained during the analysis.
mid-Miocene cooling event, followed by widespread allopatric speciation. Moreover, we have shown that these processes operate at similar spatial and temporal scales as in macroscopic organisms. The dominance of terrestrial taxa is likely related to the lack of lacustrine habitats during glacial maxima, when the sparse lakes that escaped glacial overriding were probably permanently covered with lake ice (Hodgson et al., 2005), precluding the survival of obligate planktonic diatoms. Our results thus impose a reconsideration of the current paradigm in microbial biogeography (Finlay, 2002) and have important implications for the response of polar microbial communities in the face of the accelerated rates of warming, which have already impacted on the primary productivity and functioning of lacustrine ecosystems (Quayle et al., 2002). As endemic species are particularly prone to extinction because of their restricted distribution in relatively small geographic regions, stringent measures to preserve ecosystem integrity of Antarctic lakes and to protect them against the introduction of alien species is highly justified.

2.3 Materials and methods

Sample collection and diatom analysis

In deep lakes, surface sediment cores were extracted from the deepest part using a gravity corer. Diatom samples were collected from the uppermost 5 mm. In shallow lakes, which have no or only little ice cover around the edges during summer, sediment samples were collected between 0.5 - 1m depth in the littoral zone. Subsamples for diatom analysis were digested with H2O2 (30 %) and CH3COOH (95%). For light microscopy, a subsample was dried onto a glass coverslip, mounted in Naphrax® and studied using a microscope equipped with differential interference contrast. For scanning electron microscopy, oxidized sample material was directly air-dried onto specimen stubs and sputter-coated, and examined with a Jeol JSM-840 operated at 15 kV. For transmission electron microscopy, 10 μl aliquots of oxidized material were placed on formvar-coated copper slot grids. Grids were examined with a Jeol JEM 1010 operating at 60 kV.

The dataset was developed by combining newly obtained diatom analyses with existing datasets (see Table S2.1). The primary diatom data consisted of enumerations of species relative abundances in surface sediment samples, each of which typically represents a
record of diatom growth in the lake from both pelagic and benthic habitats spanning several years. Between 400 and 600 specimens were counted per sample. Taxonomic consistency between datasets was obtained through slide or sample exchange between the different analysts. When the identity of a species could not be determined with certainty it was considered to be a non-endemic. Hence the incidence of endemism is a conservative measure and likely to be an underestimation. The geographic distribution of the diatom species was based on literature data provided with unambiguous illustrations and/or descriptions and our own observations. The endemic species were subdivided according to the biogeographical province in which they occur.

For the bipolar comparison *Eunotia* and *Gomphonema* were excluded from the analysis because these genera are in need of taxonomic revision.

**Time-calibrated molecular phylogeny**

To develop a time-calibrated molecular phylogeny for *Pinnularia borealis*, 51 strains from 7 regions were isolated, cultured, and sequences of the nuclear D1-D2 region of the 28S rDNA (LSU) and plastid gene rbcL were obtained as described in Souffreau *et al.* (submitted). RbcL was partitioned into codon position (1st +2nd combined); LSU was used as a single partition. Calibration of the molecular clock was based on the 95% highest posterior density (HPD) resulting from the preferred time-calibration of the *Pinnularia* phylogeny which was constrained by the fossil record (Souffreau *et al.*, submitted). Three nodes were constrained (gray circles in Fig. 2.4) using each a uniform distribution of their recovered 95% HPD. The outgroup consists of representatives of the most closely related *Pinnularia* lineages as recovered by a five-marker molecular phylogeny for the genus *Pinnularia* (Souffreau *et al.*, submitted).

Metropolis-coupled Monte Carlo Markov Chains were run by BEAST v1.5.4 (Drummond & Rambaut, 2007) under a GTR + Γ (4) model for each partition and a relaxed, uncorrelated lognormal clock model and Yule tree prior. Three independent analyses, each started by a UPGMA tree, were run for 100 million generations and sampled every 1,000th generation. Burn-in values of 30 to 40% were assessed using Tracer v.1.5 (Rambaut & Drummond, 2007). All post-burn in trees were combined after which the maximum clade credibility chronogram with mean node heights was calculated using TreeAnnotator v.1.5.4.
Statistical analysis
A principal component analysis with standardized and centered environmental variables (pH, conductivity and major ions) was used to select Arctic and Antarctic lakes with similar environmental conditions (Fig. S2.1). For the bipolar comparison we retained lakes with overlapping scores for the first and the second ordination analysis which resulted in the selection of 83 Antarctic and 56 Arctic freshwater lakes. A principal component analysis of Hellinger transformed relative abundance data was used to assess the biogeographical zoning in the diatom floras of the Southern Ocean. All ordinations were performed in Canoco 4.5 for windows (Ter Braak, 2002).

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The Australian Antarctic Division, the British Antarctic Survey, the French Institute for Polar Research, the Japanese Institute for Polar Research, the Polar Continental Shelf Program and the South African National Antarctic Program provided logistic support. Louis Beyens, Niek Gremmen, Justine D. Shaw, Andrew McMinn, Steve J. Roberts, James A. Smith, Sakae Kudoh, and Tamotsu Hoshino are thanked for providing samples and/or help during sampling campaigns. Vivienne J. Jones, Donna Roberts, Mieke Sterken and Ines Tavernier provided diatom data. Pete Convey is thanked for providing the map. EV, CS, and PV are funded by the Fund for Scientific Research Flanders (FWO), Belgium. This work was funded by the BelSPO projects HOLANT and AMBIO and the FWO project G.0533.07N.
Supplementary figures

Fig. S2.1 Principal component analysis of standardized and centred environmental variables (conductivity, pH, Na+, K+, Ca2+, Cl−, silicate) in high Arctic (red) and Antarctic (blue) oligotrophic freshwater lakes. The selected lakes used to assess the floristic differences between the polar regions (Fig. 2.1) are indicated by the rectangle and characterized by site scores on the first axis between -1 and 0.8, and on the second axis between 0.7 and -0.5.

Fig. S2.2 Species accumulation curves showing the total number of species in (A) maritime Antarctic, and (B) continental Antarctic lakes.
Fig. S2.3 Species accumulation curves showing the total number of species in (A) Sub-Antarctic, and (B) Arctic lakes.

Fig. S2.4 Scatter plot showing the proportion of endemic species in relation to the shortest distance to the nearest continent (Antarctica excluded).

Interhemispheric contrasts in diatom diversity
### Supplementary table

**Table S1 References to the diatom datasets used.**

*Indicate datasets which were newly developed for the present study
†Indicate datasets for which the taxonomy was revised

<table>
<thead>
<tr>
<th>Island/oasis</th>
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<th>Province</th>
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</tbody>
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Supplementary references of Table S1


3. Distance decay and species turnover in terrestrial and aquatic diatom communities across multiple spatial scales using three sub-Antarctic islands: an exploratory study

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Unpublished manuscript
Abstract

The decrease in community similarity with geographical distance or "distance decay" is the result of various processes, including environmental species sorting and dispersal limitation. The relative importance of these processes will affect the rate of this decrease. For example, organisms with higher dispersal capacities will display shallower slopes. Terrestrial diatoms are assumed to have a higher dispersal potential than lacustrine diatoms due to the higher susceptibility to be picked up by air currents, the higher connectivity in their habitat, and/or their higher tolerances for desiccation. We tested this hypothesis for both habitats using a taxonomically intercalibrated dataset covering two South Indian Ocean archipelagos (Kerguelen and Crozet) and a South Atlantic Ocean island (South Georgia). Both terrestrial and aquatic diatom community similarities decrease with distance over intermediate (ca. 1,500 km) and large (ca. 6,000 km) spatial scales, but this decay was only significant at the largest scale for the terrestrial communities. The rate of distance decay in terrestrial communities was not significantly different from that in lacustrine communities at none of the spatial scales, but at the intermediate scale the trends towards flatter slopes and higher community similarities in terrestrial communities and the significant lower species turnover suggest higher dispersal rates and/or broader niches for terrestrial diatoms. Within islands, species turnover (β-diversity) was relatively high for the two communities compared to the within-site (α) diversity, possibly reflecting small-scale environmental differentiation and species sorting or strong priority effects.
3.1 Introduction

Community similarity decreases with geographic distance. This spatial pattern has been widely observed in macroorganisms (e.g. Nekola & White, 1999; Condit et al., 2002; Tuomisto et al., 2003; Wiersma & Urban, 2005), eukaryote microorganisms (e.g. Hillebrand et al., 2001; Green et al., 2004; Shurin et al., 2009) and even bacteria (e.g. Cho & Tiedje, 2000; Whitaker et al., 2003; Horner-Devine et al., 2004). The decrease in similarity of communities with increasing distance reflects the change or turnover in species composition among sites, termed beta-diversity (Whittaker, 1960, 1972; Legendre et al., 2005), and is accounted for by at least three mechanisms. First, environmental characteristics change over distance causing a decrease in biotic similarity due to species sorting (Nekola & White, 1999; Leibold et al., 2004). Second, limited dispersal capacity will result in a decrease in community similarity with distance (Hubbell, 2001). Third, dispersal rates are affected by the spatial configuration of the landscape between patches, and the presence of dispersal barriers will result in a decrease in community similarity (Garcillan & Ezcurra, 2003). These three mechanisms are not mutually exclusive, and various data indicate that in most communities they jointly control distance decay (Tuomisto et al., 2003; Cottenie, 2005).

The rate (or slope) of spatial decrease in community similarity depends on several factors, including organismal features such as type of dispersal (passive vs. active) (Soininen et al., 2007), size (Hillebrand et al., 2001; Soininen et al., 2007), dispersal tolerance and formation of resting stages (Martiny et al., 2006) and dispersal ability (Ozinga et al., 2005), but also includes the spatial scale under study (Nekola & White, 1999; Soininen et al., 2007), sampling effort and taxonomic resolution (Green & Bohannan, 2006; Soininen et al., 2007), and habitat type (terrestrial, marine or freshwater) due to differences in spatial configuration (Soininen et al., 2007). Microorganisms are shown to have lower rates of distant decay than macroorganisms (Hillebrand et al., 2001; Green et al., 2004), but the lower taxonomic resolution used for microbial studies such as SSU rDNA sequences might have a large effect and bias this pattern towards flatter slopes (Green & Bohannan, 2006; Martiny et al., 2006). Similarly, terrestrial organisms have lower rates of distance decay than freshwater organisms, plausibly due to the more fragmented configuration of lakes, and passive dispersers have flatter slopes than active dispersers indicating the effectiveness of passive dispersal (Soininen et al., 2007). Because of the importance of organism-related
characteristics, significant among-species differences are expected; however, knowledge on dispersal ability and niche is, in particular for microorganisms, mostly lacking (Martiny et al., 2006). Because distance decay unites several ecological phenomena, it is valuable for visualizing spatial turnover across sites and a starting point to interpret the mechanisms behind the pattern (Soininen et al., 2007).

Lacustrine diatom community similarities are known to show distance decay (Hillebrand et al., 2001; Azovsky, 2002), and this fits with results from multivariate studies showing that both spatial and local environmental factors shape these communities (Telford et al., 2006; Vyverman et al., 2007; Verleyen et al., 2009). Furthermore, recent studies on diatom biogeography revealed a high degree of endemism (Verleyen et al., submitted) and limited gene flow (Evans et al., 2009), indicating that dispersal limitation does play a role in shaping the distribution of lacustrine diatoms. This idea is further strengthened by reports that lacustrine diatoms are very sensitive to desiccation and extreme temperatures (Souffreau et al., 2010; Souffreau et al., unpubl.), very likely decreasing their dispersal potential. All these data indicate that the biogeographies of lacustrine diatoms are shaped by the same factors as higher organisms, namely a combination of historical factors (climatic and environmental history, geological processes, evolutionary background, isolation and dispersal limitation) and local ecological factors (Lomolino, 2010).

For terrestrial diatoms, however, much less is known. Terrestrial diatoms thrive in soils and on mosses, rocks, tree barks and other substrates (Round et al., 1990) and compared with lacustrine habitats such as ponds and lakes, which form islands in a matrix of unsuitable habitats, the availability of terrestrial habitats seems much higher and less fragmented (however, this could not apply to very specific patches such as bare soil) while uptake of the drier sediment by wind currents is more likely (Chepil, 1956). Furthermore, soil-inhabiting diatoms are shown to be more tolerant for temperature extremes than lacustrine diatoms (Souffreau et al., 2010) while their resting stages are able to survive desiccation (Souffreau et al., unpubl. b), both potentially enhancing their dispersal capacity. Indeed, the small amount of viable diatom species reported from air traps are predominantly terrestrial taxa (Van Overeem, 1937; Schlichting, 1961; Brown et al., 1964; Schlichting, 1964; Roy-Ocotla & Carrera, 1993) while aquatic diatoms are only rarely encountered (but see Geissler & Gerloff, 1966). The combination of a less fragmented habitat, a
higher susceptibility to aerial dispersal, and a higher tolerance to desiccation raises the probability that terrestrial diatoms have higher gene flow, less speciation and thus larger geographic distributions than lacustrine diatoms (Spaulding et al., 2010). Despite these indications, no data are available on the dispersal and biogeography of terrestrial diatoms and no studies have yet explicitly compared differences in biogeographical patterns of terrestrial and aquatic diatoms.

The only dataset available covering both lacustrine and terrestrial diatom communities is a survey from B. Van de Vijver & N.J.M. Gremmen on multiple sub-Antarctic islands. The sub-Antarctic region has had a minor human historical impact compared to other geographically less remote regions (Frenot et al., 2001), being the rationale behind studying this area. Using oceanic islands for our study has the drawback that both the terrestrial and lacustrine habitats are equally fragmented on intermediate spatial scale. However, within each island the fragmentation level of terrestrial and lacustrine habitats still differs, while between islands differences in distance decay could still be interpreted in the framework of environmental variation or dispersal limitation. From this large dataset, we selected the data of the best sampled islands, being the archipelagos of Kerguelen, Crozet and South Georgia, to analyze the distance decay of terrestrial and aquatic diatom communities (Fig. 3.1). While the three archipelagos all lay between 55° and 46° S (South Georgia: 54°S; Îles Crozet: 46°S; Îles Kerguelen: 49°S; spanning a latitude comparable from mid-France to South Denmark), they do differ in several characteristics. South Georgia, lying in the South Atlantic Ocean, is of continental origin and belongs to the Scotia island arch (related to the subduction of the South American Plate to its east), is geologically characterized by a gneiss and schist bedrock, and is colder than the two other islands (average annual temperature 1.85°C at Grytviken). The archipelagos of Crozet and Kerguelen are situated in the South Indian Ocean, are both of volcanic (hotspot) origin resulting in a basalt bedrock, and have an average annual air temperature of 5.56°C and 4.79°C (at Alfred Faure and Port-aux-Prince), respectively. South Georgia is the oldest island (min. 120 Ma), while Kerguelen (min. 40 Ma) and Crozet (starting 8.7 Ma) are much younger.

The South Atlantic Ocean and South Indian Ocean form two distinct biogeographical provinces, based on floral and faunal discontinuities (Skottsberg, 1960; Lewis Smith, 1984; Van Der Putten
et al., 2010). Situated 1,300 km (South Georgia), 2,500 km (Îles Crozet) and 3,900 km (Îles Kerguelen) from the nearest continent, these three islands are very isolated. The archipelagos of Kerguelen and Crozet, separated by 1,420 km ocean, are more similar in geological, climatological and environmental characteristics, but they do differ in rainfall levels (Crozet: 2,400 mm/year; Kerguelen: >3,200 mm/year on the west side, <800 mm/year on the east side) (Frenot et al., 1998), in area (Crozet: a fragmented 147+130 km²; Kerguelen: 7,200 km²), in maximum elevation (Crozet: 1,050 m; Kerguelen: 1,850 m) and therefore also in present ice cover (Crozet: 0%; Kerguelen: 12%) (summarized in Van Der Putten et al., 2010). However, they both have a volcanic history with several outbreaks, and during the Last Glacial Maxima the lower parts of both archipelagos remained ice-free (reviewed in Van Der Putten et al., 2010). A thorough study comparing the environmental variables on both islands is thus necessary before we can use these two archipelagos as a case-study to explore differences in distance decay of soil-inhabiting and lacustrine diatom communities at intermediate scale while assuming the availability of similar habitat types. Meanwhile, we can assume that the environmental conditions of Îles Crozet and Îles Kerguelen are more alike compared to South Georgia, which would result in more comparable diatom communities. The distance

Fig. 3.1 Map of the South Polar Region including the Antarctic and sub-Antarctic. The island groups of South Georgia, Crozet and Kerguelen are indicated by grey boxes.
between these South Indian Ocean Islands and South Georgia (5,900 and 6,600 km from Crozet and Kerguelen, respectively) forms an interesting setting to assess distance decay of diatom communities on even larger scales.

Following the assumption that soil-inhabiting diatoms (termed “terrestrial” below) have larger dispersal abilities than lacustrine diatoms (termed “aquatic” below) we hypothesize that the terrestrial communities will be characterized by lower species turnover and higher community similarities than the aquatic communities 1) within islands; and 2) between Crozet and Kerguelen. However, due to the larger environmental variations and geographical distance between the South Indian and the South Atlantic Ocean, we predict that 3) both terrestrial and aquatic communities will display pronounced distance decays at this larger scale.

### 3.2 Materials & Methods

#### Sampling procedure

Samples were taken in such a way as to assure that all habitat types were included, across a wide range of altitudes, moisture levels and intensities of sea spray and manuring and animal trampling. In waterbodies such as lakes, pools and rivers, very wet mires and drainage lines, the bottom sediment and/or water squeezed from bryophytes was collected in PVC bottles and fixed with 3% formaldehyde. A few moss plants were included in the sample. In drier habitats, bryophytes (or where these were absent, the top soil layer) were collected and air-dried in paper bags. In the case of seepage areas on rocks the algal mat was collected and air-dried. More information regarding the sampling can be found in Van de Vijver & Beyens (1997), Van de Vijver et al. (2002; 2004b) and Van de Vijver & Mataloni (2008).

#### Slide preparation and counting

Diatom samples for observation by light microscopy (LM) were prepared following the method described in Van der Werff (1955). Small parts of the samples were cleaned by adding 37% H2O2 and heating to 80° C for about 1 h. The reaction was completed by addition of KMnO4. Following digestion and centrifugation (three times for 10 minutes at 3,700 x g), cleaned material was diluted with
distilled water to avoid excessive concentrations of diatom valves on the slides. Cleaned diatom material was mounted in Naphrax®. The samples and slides are stored at the Department of Bryophytes and Thallophytes, National Botanic Garden of Belgium. In each sample, 500 diatom valves were identified and enumerated on random transects at x1,000 magnification under oil immersion using an Olympus BX51 microscope, equipped with Differential Interference Contrast (Nomarski) and Colorview I Soft Imaging System. In some samples, it proved difficult to obtain large counts. Even after scanning several entire slides at x1,000, some samples yielded less than 250 valves. Considering the extreme environments involved, these numbers seemed acceptable (Van de Vijver & Beyens, 1997). Diatom identifications were based on frustule morphology, and all samples were counted by the same person ensuring that identification was consistent across all datasets. Identifications of Antarctic species are based on descriptions by Bourrelly & Manguin (1954), Le Cohu & Maillard (1983, 1986), Schmidt et al. (1990), Oppenheim (1994), Van de Vijver et al. (2002; 2004a) and Le Cohu (2005). Nomenclature follows Bukhtiyarova & Round (1996), Round & Bukhtiyarova (1996), Lange-Bertalot (2001) and Krammer (2000, 2002).

**Data analysis**

Based on the samples of Crozet, Kerguelen and South Georgia, a species list was compiled using presence-absence and relative abundance data. Sites were subdivided into “aquatic”, “terrestrial” and “semi-terrestrial” habitat classes. The aquatic class contained all permanent lakes. The terrestrial class contained all dry soil samples and thus the true soil-inhabiting diatom communities. All other sites were classified as semi-terrestrial, covering very diverse habitat types including peats and bogs, seepages and mosses. While aquatic sites are the least susceptible to dry out, semi-terrestrial habitats are intermediate susceptible to dry out, and terrestrial habitats are the most likely to undergo desiccation. Datasets of aquatic, terrestrial and semi-terrestrial sites were compiled for each island and analyzed separately.

**Community similarity and distance decay**

For each habitat class, pairwise community similarities of sites were calculated between sites 1) within islands, and 2) among the three different islands in all three pairwise combinations. Similarities
Species turnover of diatom communities were based on the Jaccard Index, using presence-absence data as we are mainly interested in dispersal limitation and therefore in the absence or presence of species. Similarities were calculated in R using the vegan package (Oksanen et al., 2011) after which the median, first and third quartile and the minimal and maximal values were calculated using Excel (Microsoft Office). Differences in similarity between terrestrial and aquatic datasets were statistically assessed using t-tests (Statistica 7, Statsoft). For the within-island comparisons, the median similarity values of the three islands were taken as replicates. For the among-island comparisons, we only compared between oceans (i.e. largest spatial scale); the median similarity values of the pairwise comparisons of Crozet vs. South Georgia and Kerguelen vs. South Georgia were taken as replicates.

To analyze the distance decay per habitat class, similarities were plotted against the distance between the compared sites. The median within-island similarities were all plotted at a site distance of 1 km because we had no data yet on the geographical coordinates of the individual sites. The median between-island similarities were plotted at the shortest distance between each island pair. The slopes of the straight lines connecting the median similarity values (per habitat class) for all between-island pairs were calculated using linear regression in Excel (Microsoft Office 2010) on logarithmically transformed similarity and distance data. For the statistical analyses, the distance decay slopes for the data points of Kerguelen and Crozet were taken as replicates per distance comparison (intermediate and large scale). Significance of the differences between the slopes of the aquatic and terrestrial datasets was analyzed using t-tests in Statistica 7 (Statsoft).

**Diversity partitioning**

Diversity partitioning is a tool to partition the total diversity of a dataset or region (i.e. γ-diversity) into its two components, being the average local (sample/site) diversity (i.e. α-diversity) and the average turnover in diversity between localities (samples/sites) (i.e. β-diversity). Gamma and alpha-diversities can be calculated from the dataset using a diversity index of choice. We used two different diversity indices: species richness (Q=0) and Shannon diversity (Q=1). Based on the gamma and alpha-diversities, beta-diversity is calculated as $\beta=\gamma-\alpha$ (for additive species richness) or as $\beta=\gamma/\alpha$ (for multiplicative species richness and for Shannon diversity) (Jost, 2007). This diversity partitioning was performed for the terrestrial
Analyses were performed for each habitat class separately based on relative abundances using the software PARTITION v3 (Veech & Christ, 2009). Samples were not weighted, and randomization was individual-based using 1,000 randomizations. The “among islands” and “among oceans” analyses were hierarchically analyzed, such that the total beta-diversity was decomposed into the beta-diversity between sites within each island or within each ocean (β1), and the beta-diversity among the islands or among the oceans (β2). Species richness was partitioned in the additive way, with γ=α+β1+β2 (Lande, 1996). Additive partitioning has the advantage over multiplicate partitioning of species richness that the different components can be compared with each other and with the components of other communities (Lande, 1996; Veech et al., 2002). Shannon diversity can only be partitioned multiplicatively, and to make the alpha, beta and gamma components relate additively, we transformed these components into Shannon entropies using the natural logarithm (Jost, 2007).

### 3.3 Results

**Dataset properties**

In total 556 samples were analyzed, containing a total of 339 species. The number of samples and number of species per island is given in Table 3.1. South Georgia was less intensively sampled, and we therefore predominantly focus on the island groups of Kerguelen and Crozet. Note that distance decay depends on sampling effort, and lower efforts will result in higher similarities (Soininen et al., 2007).

**Similarities and distance decay of similarity**

Median similarities were higher within islands than between oceans (Fig. 3.2; Fig. 3.3) showing a clear distance decay, but this difference was only statistically significant for the terrestrial datasets (Fig. 3.2 B, p=0.0127). At the smallest spatial scale, within islands, the aquatic sites showed a lower median similarity compared with the terrestrial and semi-terrestrial sites when measured per island (Fig. 3.2 A;
Table 3.1 Dataset characteristics indicating the number of sites and species per island group and per habitat on each island group. The number of species occurring only in that particular habitat is given between brackets.

<table>
<thead>
<tr>
<th>Island group</th>
<th>Total # sites</th>
<th>Total # species</th>
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<th>Terrestrial</th>
<th>Semi-terrestrial</th>
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<td></td>
<td># sites</td>
<td># species</td>
<td># sites</td>
<td># species</td>
<td># sites</td>
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<td>181</td>
<td>220</td>
<td>42</td>
<td>128 (14)</td>
<td>13</td>
</tr>
<tr>
<td>Crozet</td>
<td>304</td>
<td>246</td>
<td>97</td>
<td>174 (20)</td>
<td>91</td>
</tr>
<tr>
<td>South Georgia</td>
<td>71</td>
<td>166</td>
<td>35</td>
<td>115 (59)</td>
<td>4</td>
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</table>

Table 3.2 Summary of the slopes of the distance decays. Linear regressions were calculated between distance (km) and similarities (as fraction of 1) within the basis island (A) and similarities between the basis island (A) and the compared island (B). Distance and similarities were both logarithmically transformed.

<table>
<thead>
<tr>
<th>Basis island (A)</th>
<th>Compared island (B)</th>
<th>Distance</th>
<th>Slopes of distance decay</th>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>Crozet</td>
<td>1,420 km</td>
<td>-0.093</td>
</tr>
<tr>
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<td>South Georgia</td>
<td>5,900 km</td>
<td>-0.0913</td>
</tr>
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<td>Kerguelen</td>
<td>South Georgia</td>
<td>6,600 km</td>
<td>-0.115</td>
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</table>
Fig. 3.2 Percentages of community similarity (Jaccard Index) per habitat type summarized A) per island or island combination, and B) as box plots using the three different islands as replicates (within island similarities) or the pairwise similarities between South Georgia and Kerguelen respectively Crozet as replicates (between oceans similarities). * indicates statistical differences in community similarity percentages (p<0.05).
Fig. 3.3). However, the differences between terrestrial and aquatic community similarities were not statistically significant ($p>0.05$) (Fig. 3.2 B).

At intermediate scale (i.e. when comparing Crozet and Kerguelen; ca. 1,500 km), the aquatic communities had again a lower similarity compared with the terrestrial and semi-terrestrial communities (Fig. 3.2 A; Fig. 3.3) but because no replicate island combinations were available at that scale significance could not be assessed. When plotted against distance (Fig. 3.3), terrestrial and semi-terrestrial communities showed a flatter decay in similarity, while the aquatic communities decreased stronger in similarity, but the slopes of the linear regressions between similarity and distance (Table 3.2) did not differ significantly between habitat types ($p>0.05$).

At the largest spatial scale when comparing the South Indian Ocean islands (Crozet and Kerguelen) with the South Atlantic Ocean island South Georgia (ca. 6,000 km), similarities were very low.
for all habitat types (Fig. 3.2; Fig. 3.3). Among oceans, similarities decreased drastically for all habitat types, but more slowly for the aquatic communities (Table 3.2; Fig. 3.3), as their initial within-island similarities were lower. Again, no significant differences between slopes were found ($p>0.05$).

**Beta-diversity within islands**
Within islands, the average diversity within sites ($\alpha$-diversity) was significantly lower than the average diversity between sites ($\beta$-diversity, turnover) when calculated as species richness (aquatic datasets: $p=0.00001$; terrestrial datasets: $p=0.0168$) (Fig. 3.4 A-B, Fig. 3.5 A, C), indicating that, compared to the total diversity ($\gamma$-diversity) available, individual sites contained a low number of species, but sites differed greatly in the species present. This pattern was opposite when calculated as Shannon diversity (Fig. 3.4 C, Fig. 3.5 B, D). The $\alpha$-diversity of terrestrial communities was then significantly higher than the $\beta$-diversity ($p=0.0256$), but no significant effect was assessed for the aquatic dataset. The Shannon Index also accounts for abundances and evenness in the communities and can be interpreted as the chance that the next species is identical to the previous one. The opposite results based on species richness and the Shannon Index suggests that the large turnover in species based on species richness is mainly due to variations in absence or presence of rare species between the different sites. Terrestrial communities had a lower $\beta$-diversity or species turnover than aquatic communities (Fig. 3.4 B-C; Fig. 3.5 C-D), but this was not significant (Fig. 3.5 B, D; $p>0.05$).

**Beta-diversity among islands on intermediate scale (ca. 1,500 km)**
Focusing on Crozet and Kerguelen, a larger turnover in species is observed within the single islands ($\beta1$) than between the two island groups ($\beta2$) (Fig. 3.6 A-C, left) both using species richness and Shannon diversity. The total diversity of the islands ($\gamma$-diversity) was thus more determined by the single islands, meaning that a large part of the diversity was already present on both islands. The terrestrial community had a lower turnover within islands ($\beta1$) than aquatic communities, as previously observed. However, it had larger among-island turnover than the aquatic community when using species richness as diversity index (Fig. 3.6 B, left), and
lower among-island turnover when using Shannon diversity (Fig. 3.6 C, left), which indicates that species turnover in the terrestrial communities was mainly due to changes in the rare taxa. However, because no replicate islands are available on this intermediate scale no statistical significance can be calculated.

Beta-diversity among islands on larger scale (ca. 6,000 km)
When looking individually at the turnover between South Georgia and Crozet respectively Kerguelen (Fig. 3.6 A-C, middle and right graphs), there was a larger turnover (β2) at this largest distance than between Kerguelen and Crozet (first three graph columns) for all habitat types. Between the sites within each island (β1), terrestrial communities had a lower species turnover between sites than aquatic communities (Fig. 3.6 A-B), which was significant when using species richness (species richness $p=0.0222$, Shannon Index $p=0.0545$). However, contrary to our expectations, the species turnover between Crozet or Kerguelen and South Georgia (β2) was larger in terrestrial communities compared to aquatic communities (Fig. 3.7 C-D) and significant when using species richness ($p=0.0182$) but not significant when using the Shannon Index. The fact that this pattern was only significant for species richness could indicate that the large turnover observed for terrestrial communities was mainly due to variations in rare species. When giving more weight to abundances and evenness by using the Shannon Index, aquatic and terrestrial communities had similar species turnover (Fig. 3.7 D). Furthermore, over all habitat types, β2-diversity based on species richness (Fig. 3.6 B; Fig. 3.7 C) was larger than when based on Shannon Index (Fig. 3.6 C; Fig. 3.7 D), indicating that in general the species turnover among islands was predominantly due to differences in rare species.

Beta-diversity among the South Atlantic and South Indian Ocean
When performing a hierarchical partitioning over the two oceans (Fig. 3.8 A-C), the same pattern occurs as between the individual islands, but no replicate ocean combinations were available and thus no statistical analyses could be performed. The β2-diversity, the turnover in diversity between the oceans, tended to be larger for terrestrial communities than for aquatic communities and again
Fig. 3.4 Diversity partitioning within islands for each habitat type shown per island as absolute values of additive species richness (A) and percentages of the $\gamma$-diversity based on additive species richness (B) and Shannon Index (C). Total diversity consists of the $\alpha$-diversity or average diversity within sites, and $\beta$-diversity or average diversity between sites within the island. $\text{Aq} = \text{aquatic communities}, \quad \text{Ter} = \text{terrestrial communities}, \quad \text{Semi} = \text{semi-terrestrial communities}$.
Fig. 3.5 Box plots of α-diversity or average within-site diversity (A, B) and β-diversity or average between-sites diversity (C, D) for the aquatic and terrestrial communities using the three islands Crozet, Kerguelen and South Georgia as replicates. Diversity was partitioned using species richness (A, C) and using Shannon Index (B, D).
Fig. 3.6 Diversity partitioning among islands in the three pairwise combinations, shown per combination as absolute values of additive species richness (A) and percentages of the γ-diversity based on additive species richness (B) and Shannon Index (C) for the three habitat types. Total diversity consists of the α1-diversity or average within-site diversity per island, β1-diversity or average among-site diversity within each island, and β2-diversity or average between-island diversity. Aq = aquatic communities, Ter = terrestrial communities, Semi = semi-terrestrial communities.
Fig. 3.7 Box plots of β1-diversity or average within-island diversity (A, B) and β2-diversity or average between-island diversity (C, D) for the aquatic and terrestrial communities using Kerguelen/Crozet versus South Georgia as replicates. Diversity was partitioned using species richness (A, C) and using Shannon Index (B, D). * indicates statistically different datasets (p<0.05).
Species turnover of diatom communities

Fig. 3.8 Diversity partitioning among the South Atlantic and South Indian oceans shown for the three habitat types as absolute values of additive species richness (A) and percentages of the $\gamma$-diversity based on additive species richness (B) and Shannon Index (C). Total diversity consists of the $\alpha_1$-diversity or average within-site diversity per ocean, $\beta_1$-diversity or average among-site diversity within each ocean, and $\beta_2$-diversity or average between-ocean diversity.
larger when using species richness compared to the Shannon Index. The β1-diversity, the turnover between samples within each ocean, was lower for the terrestrial habitat than for the aquatic habitat, and again lower when using Shannon Index.

### 3.4 Discussion

Our results only partially confirmed the three hypotheses we set out to test, and due to the lack of replicate data we were not able to statistically evaluate some observed trends. First, within the studied islands, terrestrial diatom communities had higher similarities and lower turnover between sites than aquatic communities. While this was not significant when analyzing the islands separately (Fig. 3.5 C-D), the within-island diversity (β1) of terrestrial communities was significantly higher when analyzing Crozet/Kerguelen versus South Georgia (Fig. 3.7 A-B). These higher similarities and lower species turnover of terrestrial communities within islands could be a result of the higher connectivity between the sites, lower environmental variation between the sites, broader niches of terrestrial diatoms, a higher dispersal ability of terrestrial diatoms over short distances, or a combination of these. While we were not able to disentangle these mechanisms using our data, this pattern is in general agreement with results of a meta-analysis by Soininen et al. (2007) revealing that the smallest scale similarities were highest in the terrestrial realm and lowest for freshwater communities.

This higher similarity between terrestrial communities within islands is most likely related to the less fragmented terrestrial habitat matrix (Soininen et al., 2007), causing among others higher dispersal and colonization probabilities and higher prevalence of terrestrial species. We can assume that this is also the case for diatoms because there are no *a priori* reasons to expect that terrestrial diatoms would have broader niches, or soils would be less variable with respect to the abiotic environment than lake sediments. The extent of variation in environmental conditions of dry soils and lakes could be assessed, however, using environmental data. Moreover, the significantly higher turnover (β-diversity) between the terrestrial sites compared to the local diversity within sites (α-diversity) suggests the presence of species sorting and environmental small-scale heterogeneity, but this could also be caused by priority effects (*i.e.* first arrivals have an advantage and dominate the community) (Drake, 1991). However, strong priority effects would not lead to higher similarity
between sites, except if a small number of species are dominant, more abundant and would therefore have higher dispersal rates and a higher chance to first colonize a patch. The lower turnover in terrestrial communities could thus indicate higher dispersal rates and/or stronger priority effects compared to aquatic communities. For all habitats, the turnover was higher when based on species richness than when based on the Shannon Index. Because species richness uses presence-absence data, it gives a disproportionally higher weight to rare species, while Shannon diversity also accounts for relative abundances and evenness of communities. The higher turnover based on species richness indicates that the rare species contribute most to the variations between sites.

In a second hypothesis we predicted that terrestrial communities would have lower turnover and higher similarities at intermediate spatial scales (ca. 1,500 km) between the island groups Kerguelen and Crozet when compared to the aquatic communities. Indeed, lower similarities were observed for the aquatic communities, but species turnover (β2-diversity) between these islands was only lower for the terrestrial communities when using Shannon diversity and actually higher when using species richness (Fig. 3.6). Because no replicate island combinations were available at this intermediate scale the statistical significance of this trend could not be assessed. Similar to the patterns observed within single islands, the lower similarity between the aquatic sites could be the result of environmental constraints, dispersal limitation or priority effects, while the higher similarities of terrestrial communities could be due to larger dispersal capacities, less environmental differences between sites on the two islands, or a more generalist life-style of terrestrial diatoms. Given the high geological, floristic and faunal similarity between the archipelagos of Kerguelen and Crozet, it is possible that these island groups share similar terrestrial and aquatic habitat types (but as noted in the introduction, this should be further analyzed). Assumed that environmental variations among islands would both affect the aquatic and terrestrial sediments in a similar extent (as assumed in Hillebrand et al., 2001), we can hypothesize that these differences in similarity level between the aquatic and terrestrial communities could be a result of higher dispersal capacities of terrestrial diatoms due to higher habitat availability (or higher chance to arrive in a suitable patch), higher susceptibility to dispersal (Schlichting, 1961; Brown et al., 1964; Schlichting, 1964; Roy-Ocotla & Carrera, 1993) and higher tolerance to desiccation (Souffreau et al., unpubl. b).
However, this should be further analyzed using environmental and community composition data.

The opposite pattern in species turnover based on species richness and Shannon diversity among the terrestrial and aquatic communities of Crozet and Kerguelen needs to be interpreted in the light of what both indices account for. As stated above, species richness gives a disproportionally higher weight to rare species. The lower turnover in terrestrial communities based on the Shannon diversity indicates that the dominant, abundant species in terrestrial environments are present in the different sites on the islands, while it are mainly the rare species which account for the higher turnover between the islands detected using species richness. For aquatic communities, the opposite is true, and variation in community composition is thus mainly determined by the more abundant species. This could indicate that the distribution of aquatic species are more influenced by species sorting, as species will locally reach high abundances at sites where they find suitable conditions and not at other sites. Alternatively, this could indicate that aquatic diatoms are more dispersal limited, and species that arrived by single, unlikely dispersal events will thrive at that location, but will not be able to be easily dispersed to a next, suitable lake, or that priority effects are stronger for the aquatic communities.

For the terrestrial communities, abundant species could be the more generalist species, thriving in different environmental habitats while the rare species are restricted to some very specific locations. Alternatively, the abundant terrestrial species, by having larger population densities, are more likely to successfully disperse over the two islands, creating a low turnover in these dominant species, while the rare, less abundant species are less likely to successfully disperse. Alternatively, it is possible that the rare terrestrial species are species that do not have high tolerances to dispersal, while the dominant species do. Because patterns in turnover cover a whole range of possible mechanisms, it is not easy to interpret these and further study is needed to reveal the processes at work. However, these patterns should first be tested statistically using data of additional isolated islands.

In agreement with our last hypothesis, we observed a low community similarity between the South Indian Ocean and South Atlantic Ocean islands (ca. 6,000 km) and high distance decay for both terrestrial and lacustrine communities, which was only significant for the terrestrial communities (Fig. 3.2 B). This is in
agreement with similar discontinuities over these oceans reported for higher plants (Van Der Putten et al., 2010), Coleoptera (Morrone, 1998) and insects and birds (Greve et al., 2005), and the distinction of these oceanic basins into two biogeographic provinces (Skottsberg, 1960; Lewis Smith, 1984; Van Der Putten et al., 2010). The distribution of macroorganisms on the sub-Antarctic islands is determined by environmental variables such as temperature and geological features including island age and area, but also distance to the nearest continent and thus dispersal limitation (Chown et al., 1998). While we did not assess the relative influence of environmental and spatial variation on the distribution of terrestrial and aquatic diatoms yet (Borcard et al., 1992; Borcard & Legendre, 1994), recent studies on lacustrine diatom biogeography underscore the importance of both environmental and spatial variables, stressing the influence of dispersal limitation on diatom distribution (Telford et al., 2006; Vyverman et al., 2007; Verleyen et al., 2009), and our data suggest that this also applies for the sub-Antarctic diatom communities.

It is clear that many questions still remain and need further study using additional replicate locations to enable statistical testing of the observed patterns, and using environmental and geographical data. While we focused on very isolated islands, it would be interesting to compare the patterns between terrestrial and aquatic communities in a more continuous landscape. Furthermore, the influence of environmental variation on both terrestrial and aquatic communities should be assessed to elucidate to what degree the observed distance-decay relationship is related to environmental factors and species sorting or to differences in dispersal limitation; and many aspects of the ecology of soil-inhabiting diatoms are still unknown. As a first step, our results show that for both terrestrial and lacustrine diatoms, distance does play a role in their distribution on isolated islands, and that terrestrial and aquatic communities indeed differ in this respect.

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Part 2.  
Speciation in time and space
4. Time-calibrated multi-gene phylogeny of the diatom genus *Pinnularia*

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Abstract

*Pinnularia* is an ecologically important and species-rich genus of freshwater diatoms (Bacillariophyceae) having considerable variation in frustule morphology. Interspecific evolutionary relationships were inferred for 36 *Pinnularia* taxa using a five-locus dataset. A range of fossil taxa, including newly discovered Middle Eocene forms of *Pinnularia*, was used to calibrate a relaxed molecular clock analysis and investigate the temporal aspects of the genus’ diversification. The multi-gene approach resulted in a well-resolved phylogeny of three major clades and several subclades that were frequently, but not universally, delimited by valve morphology. The genus *Caloneis* was not recovered as monophyletic, confirming that, as currently delimited, this genus is not evolutionarily meaningful and should be merged with *Pinnularia*. The *Pinnularia-Caloneis* complex is estimated to have diverged between the Upper Cretaceous and the early Eocene, implying a ghost range of at least 10 million year (Ma) in the fossil record. This result predicts an earlier origin for the raphid diatoms, 10-35 Ma before that inferred by other published molecular clock studies.

**Key words:** Molecular phylogenetics, relaxed molecular clock, fossil record, raphid diatoms, *Pinnularia*, Bacillariophyceae, Eocene
4.1 Introduction

Diatoms are an extremely diverse group of unicellular algae that are uniquely characterized by a siliceous cell wall (the frustule) consisting of two valves (Round et al., 1990) and a diplontic life cycle involving gradual size reduction during vegetative divisions and rapid size restitution usually through sexual reproduction (Chepurnov et al., 2004). In the so-called pennate diatoms the valve pattern is organized bilaterally around the longitudinal axis, and in most cases the valve is elongate. Raphid pennate diatoms possess a longitudinal slit along the apical axis (the raphe) (Fig. 4.1), from which extracellular polymeric substances are exuded and used in locomotion and adhesion to the substratum (Round et al., 1990). The raphe is considered a derived character state that distinguishes the raphid diatoms from the more ancestral araphid pennate forms that lack this structure and the oldest known forms, the radially organized “centric” taxa (Sims et al., 2006). Based on fossil remains, the araphid pennates first appear in the Upper Cretaceous (ca. 75 Ma; Chambers, 1966; Hajós & Stradner, 1975), and the raphe-bearing forms soon thereafter, around 70.6–55.8 Ma (Witt, 1886; Pantocsek, 1889; Chacon-Baca et al., 2002; Singh et al., 2006). Monophyly of pennate diatoms as a whole, as well as the raphid pennates, has been documented using SSU rDNA and rbcL sequences (e.g. Kooistra et al., 2003; Sorhannus, 2004, 2007). Since their origin, raphid pennate diatoms have diversified enormously and account for the majority of the over 200,000 extant species estimated to exist (Mann & Droop, 1996), indicating the evolutionary advantages conferred by the raphe (Sims et al., 2006).

Despite the diversity and ecological success of raphid pennate diatoms, relatively few detailed molecular phylogenetic reconstructions exist. Phylogenies applied at genus to ordinal levels have yielded partly unsupported results (e.g. Bruder & Medlin, 2008; Trobajo et al., 2009), in part due to very limited taxon sampling and the use of a limited number of genetic markers (Mann & Evans, 2007). In addition, molecular phylogenies of individual genera have focused largely on the identification of cryptic diversity rather than the elucidation of evolutionary relationships between lineages (e.g. Lundholm et al., 2006; Beszteri et al., 2007; Evans et al., 2008). Except for a few studies of diatoms as a whole, or the wider heterokont group (Kooistra & Medlin, 1996; Medlin et al., 1997; Sorhannus, 2007; Brown & Sorhannus, 2010), or even eukaryotes (Berney & Pawlowski, 2006), there are no explicit time-calibrated phylogenies and few
Time-calibrated phylogeny of *Pinnularia*
analyses formalize the evolutionary associations between the timing of lineage splitting and ecological, morphological, physiological and/or reproductive strategies, life cycles and geographical distributions (but see Casteleyn et al., 2010). Furthermore, despite important recent micropaleontological discoveries, some of which confirm that raphid diatoms may be older than previously suspected (Siver & Wolfe, 2007; Siver et al., 2010), numerous gaps remain in the fossil record. As a result, the overall course of evolution in raphid pennate diatoms is not known and the relationships between many groups remain uncertain (Mann & Evans, 2007).

Pinnularia Ehrenberg (1843) is one of the most species-rich genera of raphid pennate diatoms, with 2462 taxon names present in Algaebase of which 412 are currently accepted (Guiry & Guiry, 2011). It occurs globally in freshwater habitats of varying pH and trophic status, and to a lesser extent in moist soils, peatlands, spring seeps and marine coastal environments (Round et al., 1990; Krammer, 2000). Members of Pinnularia and the closely related genus Caloneis Cleve (1894) have linear-lanceolate, occasionally capitate valves with a central raphe system (Fig. 4.1) that terminates internally

Fig. 4.1 Morphological variation in Pinnularia and representative illustrations of strains included in the multi-gene phylogeny. The valve construction of typical frustules belonging to the P. divergens group [strain (Tor7)c] is shown by scanning electron micrographs [outside (a), inside (b) and side (= girdle) view (c)]. Cultured and sequenced strains are illustrated by a series of light micrographs (d–x), divided, where appropriate, into subclades recovered in the phylogeny with vertical dashed lines.

Clade A (d–f) includes Caloneis lauta (d) as well as P. divergens grade representatives (Tor7)c in (e) and (Tor1)b in (f). Clade B (g–p) includes the “grunowii” subclade (g–l) with Pinnularia sp. (Tor4)i in (g), P. subanglica Pin650 in (h), and P. cf. marchica (Ecrins4) a in (i); the “nodosa” subclade (j–k) with P. acrosphaeria (Val1)b in (j) and P. nodosa Pin885 in (k); and the “subgibba” subclade (l–p) represented by P. parvuillissima Pin887 in (l), Pinnularia sp. “gibba-group” (Tor7)f in (m), P. subcapitata var. elongata (Wie)c in (n), P. sp. (Tor4)r in (o), and Pinnularia sp. “gibba-group” (Tor8)b in (p).

Clade C (q–x) comprises the “viridis” subclade (q–r) represented by P. neglectiformis Pin706 in (q) and P. viridiformis (Enc2)a in (r); with its sister P. acuminata Pin876 in (s); the “subcommutata” subclade (t) represented by P. subcommutata var. nonfasciata Corsea10 in (t); forms that do not readily fit into well-defined subclades represented by P. sp (Wie)c in (u); the “borealis-microstauron” subclade (v–w) including P. cf. microstauron (B2)c in (v) and P. borealis Alka1 in (w); and subclade C1 (x) represented by P. cf. altiplanensis (Tor11)b in (x).

Live cells of Pinnularia sensu lato (i.e. including Caloneis) also show two distinct plastid arrangements, which are illustrated by light (y, a’, b’) and laser-scanning confocal microscopy of the autofluorescent organelles (z, c’). For example, representatives of the P. subcommutata and gibba taxa have parallel plastids on either side of the apical axis (y, z), while Caloneis silicula (a’) and P. grunowii (b’, c’) have plastids that are joined by a central bridge. See text for details. All scale bars are 10 μm, and images (d–x) are reproduced at the same magnification to facilitate size comparisons between taxa.
Time-calibrated phylogeny of *Pinnularia*
in helictoglossae at the poles (Fig. 4.1, b; Fig. 4.2, b, d, f, h). They possess two plate-like plastids (Fig. 4.1, y-z) or a single H-shaped plastid (Fig. 4.1, a'-c'). *Pinnularia* is characterized by the presence of a chambered, double-walled valve structure in which the outer surface is ornamented by multiple rows of small pores forming a multiseriate stria (detail in Fig. 4.2, c), while the inner wall of each chamber or alveolus is perforated by a large transapically elongate aperture (Fig. 4.1, b; Fig. 4.2, b,d,f,h) (Round et al., 1990). *Caloneis* is similar, except that the inner aperture is smaller and often circular, and sometimes there are two apertures per stria. Despite their species richness, morphological diversity and ecological significance in freshwater and terrestrial ecosystems, the evolutionary relationships among *Pinnularia* and *Caloneis* species are poorly known. Bruder et al. (2008) constructed a molecular phylogeny of *Pinnularia* and *Caloneis* using 18S, 28S and rbcL genes for 15 species. However, this resulted in an overall low support for the clades, suggesting that a more exhaustive sampling with respect to both taxa and genetic markers is needed to produce a well-resolved phylogeny.

The temporal aspects of the diversification of *Pinnularia* are also poorly documented, despite the fact that numerous fossils have been reported for the genus. To date, the oldest known diatoms reliably assigned to *Pinnularia* originate from the Wagon Gap Formation of Wyoming, U.S.A. (Lohman & Andrews, 1968). The age of these sediments is not known precisely because diatom-containing sediment clasts have been redeposited in a carbonate conglomerate, but their age is situated between the Late Eocene and Early Oligocene (35-32 Ma). Dating from the same period, the Oamaru diatomite deposits also contain two species of *Pinnularia* (Desikachary & Sreelatha, 1989). From the Early Miocene on, diverse morphological types of *Pinnularia* are reported from freshwater...

**Fig. 4.2** Supporting SEM micrographs showing the shape of the central raphe endings (left column: a, c, e, g) and the extension of the alveolar opening (right column: b, d, f, h). Central raphe endings can be linear (a, e), drop-like (c) or round (g). Linear raphe endings occur in *Caloneis silicula* (a), the subcommutata clade (e), *P. acuminata*, (Wie)a and *P*. cf. altiplanensis. Drop-like endings are present in clade B (c), the borealis-microstauron clade and the divergens group; while round endings occur in the viridiformis clade (g). The alveolar openings can be small (b), large (d, h) or intermediate (f). Small openings are typical for *Caloneis silicula* (b) but also occur in *P*. acrosphaeria; intermediate openings (f) occur in the subcommutata and viridiformis clades and *P*. acuminata; while large openings (d, h) are present in all other species, including the divergens group, clade B (without *P*. acrosphaeria), the borealis-microstauron clade with *P*. borealis (h) and the isolates (Wie)a and *P*. cf. altiplanensis. Scale bars represent 2 μm (a, b, c, e, g) and 5 μm (d, f, h).
deposits or as freshwater inwash in marine deposits (for Miocene deposits including *Pinnularia* see for e.g. Pantocsek, 1886; Héribaud, 1902; Hajós, 1986; Servant-Vildary *et al.*, 1988; Vanlandingham, 1991; Ognjanova-Rumenova & Vass, 1998; Saint Martin & Saint Martin, 2005; Yang *et al.*, 2007; Lewis *et al.*, 2008; Li *et al.*, 2010). The origin of *Pinnularia* thus predates the Late Eocene, and the occurrence of several taxa within the Wagon Gap material suggests an even earlier origin for the genus. As such, despite the well-established fossil record dating from after the diversification of *Pinnularia* into different morphological types, the earlier fossil record is very scarce and too fragmentary to provide detailed information about the timing of early diversification events. With this in mind, and given that ghost lineages are anticipated to be common in algae in general and diatoms in particular (Brown & Sorhannus, 2010), it is particularly relevant to produce a well-resolved, time-calibrated phylogeny for this genus.

The goal of the present study is to reconstruct a molecular phylogeny for a representative selection of *Pinnularia* taxa spanning the morphological variability of the genus, and to infer a time-calibrated phylogeny constrained by accurately dated fossil representatives. To achieve this, we sequenced two nuclear markers (18S rDNA, 28S rDNA), two plastid markers (*rbcL, psbA*) and a mitochondrial marker (*cox1*) from 36 species of the genus and inferred phylogenies using partitioned models in a likelihood framework. We present new *Pinnularia* fossils from the Middle Eocene of Canada that are included as constraints in the relaxed molecular clock analyses.

### 4.2 Material and Methods

**Taxon sampling**

We selected strains belonging to 36 *Pinnularia* taxa (Tables 4.1-2), covering the range of morphological variation within the genus (Fig. 4.1). Because the relationship of *Pinnularia* and *Caloneis* remains ambiguous (Cox, 1988; Round *et al.*, 1990; Mann, 2001; Bruder & Medlin, 2008; Bruder *et al.*, 2008), we added three sequences of *Caloneis*. Five taxa from the genera *Sellaphora*, *Eolimna* and *Mayamaea* were selected as outgroups based on their apparent phylogenetic relatedness (Kooistra *et al.*, 2003; Bruder & Medlin, 2008; Bruder *et al.*, 2008; Evans *et al.*, 2008). A list of the cultured and sequenced taxa is provided in Table 4.2, together with their geographical origin...
and morphometric data. Summary photomicrographs of the strains considered are shown in Fig. 4.1, and more detailed morphological descriptions of living and oxidized material are presented elsewhere (Souffreau et al., unpubl. a). Identifications are based on Krammer (2000) for *Pinnularia* and Krammer and Lange-Bertalot (1986) for *Caloneis*. Voucher slides of oxidized material of all natural samples and cultures and samples of extracted and purified DNA are held in the Laboratory of Protistology & Aquatic Ecology (Ghent University) and are available upon request.

**DNA extraction, amplification and sequencing**

DNA was extracted from centrifuged diatom cultures following Zwart et al. (1998) using a bead-beating method with phenol extraction and ethanol precipitation. After extraction, DNA was purified with a Wizard® DNA Clean-up system (Promega). Sequences of the nuclear 18S and the D1-D2 region of 28S, the two plastid genes *rbcL* and *psbA*, and the mitochondrial gene *cox1* were amplified using standard PCR primers and protocols (Scholin et al., 1994; Daugbjerg & Andersen, 1997; Guillou et al., 1999; Van Hannen et al., 1999; Yoon et al., 2002; Evans et al., 2007; Bittner et al., 2008). PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The sequencing reaction was performed by cycle sequencing (initial step of 1 min at 96°C, 30 cycles of 10 s at 96°C, 10 s at 50°C and 1 min 15 s at 60°C) using the ABI Prism BigDye V 3.1 Terminator Cycle Sequencing kit (Applied Biosystems). The resulting sequencing reaction products were analysed on a Perkin-Elmer ABI Prism 3100 automated DNA sequencer (Applied Biosystems). Primer sequences of both PCR and sequencing reactions and PCR temperatures are listed in Appendix A.4. All newly generated sequences have been deposited in GenBank (Table 4.1).

**Sequence alignment**

The sequences of 18S, 28S, *rbcL*, *psbA* and *cox1* were edited separately and automatically aligned using ClustalW (Thompson et al., 1994), as implemented in BioEdit 7.0.3 (Hall, 1999). Plastid and mitochondrial markers aligned unambiguously without any gaps. The 18S and 28S alignments were corrected manually using the secondary structure of *Toxarium undulatum* (Alverson et al., 2006) and *Apedinella radians* (Ben Ali et al., 2001), respectively, after which ambiguously aligned regions were removed.
Table 4.1: Taxon list with Genbank accession numbers. Missing sequences are indicated with a dash. Data taken from GenBank are indicated in **bold**.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Taxon</th>
<th>18S</th>
<th>28S</th>
<th>psbA</th>
<th>rbcL</th>
<th>cox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal 890 TM</td>
<td><em>Caloneis budensis</em> (Grunow) Krammer</td>
<td>AM502003</td>
<td>AM710559</td>
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<td>AM710470</td>
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<td><em>Caloneis lauta</em> Carter &amp; Bailey-Watts</td>
<td>AM502039</td>
<td>AM710595</td>
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<td>AM710506</td>
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<td>Cal 890 TM</td>
<td><em>Caloneis silicula</em> (Ehrenberg) Cleve</td>
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<td>(Wes2)f</td>
<td><em>Eolimna minima</em> (Grunow) Lange-Bertalot</td>
<td>AM501962</td>
<td>AM710516</td>
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<td>AM710427</td>
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<td>Pin 876 TM</td>
<td><em>Mayamaea atomus</em> var. <em>permitis</em> (Hustedt) Lange-Bertalot</td>
<td>AM501969</td>
<td>AM710524</td>
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<td>AM710435</td>
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<td>(Tor12)d</td>
<td><em>P. borealis</em> Ehrenberg cf. var. <em>subislandica</em> Krammer</td>
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<td><em>P. cf. altiplanensis</em> Lange-Bertalot</td>
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<td>Cal 878 TM</td>
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<td><em>P. cf. marchica</em> Ilka Schönfelder</td>
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<td><em>P. cf. microstauron</em> (Ehrenberg) Cleve (“southern microstauron”)</td>
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<td>AM710461</td>
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<td><em>P. nodosa</em> (Ehrenberg) W. Smith</td>
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<td><em>P. sp.</em></td>
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<td>(Wie)a</td>
<td><em>P. sp.</em></td>
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<td><em>P. sp.</em> (divergens-group)</td>
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<td><em>P. sp.</em> (gibba-group)</td>
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<td><em>P. australogibba var. subcapitata</em> Van de Vijver, Cahttová &amp; Metzeltin</td>
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<td>Pin 650 K</td>
<td><em>P. subanglica</em> Krammer</td>
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<td>(Wie)c</td>
<td><em>P. subcapitata var. elongata</em> Krammer</td>
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<td>Corsea 10</td>
<td><em>P. subcommutata var. nonfasciata</em> Krammer</td>
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<td><em>P. viridiiformis</em> Krammer</td>
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<td><em>P. viridiiformis</em> Krammer</td>
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<td><strong>P. viridis</strong> <em>(Nitzsch)</em> <strong>Ehrenberg</strong></td>
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<td><em>Pinnularia acrosphaeria</em> W. Smith</td>
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<td>(Bfp5x8)F1-3</td>
<td><em>Sellaphora blackfordensis</em> D.G. Mann &amp; S. Droop</td>
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<tr>
<td>Strain</td>
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<td>Collector</td>
<td>Length (μm)</td>
<td>Width (μm)</td>
<td># stria per 10 μm</td>
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<td>Cal 890 TM</td>
<td><em>Caloneis silicula</em> (Ehrenberg) Cleve</td>
<td>Threipmuir Resr. (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>48.8 ± 3.7</td>
<td>13.0 ± 0.9</td>
<td>17.4 ± 0.5</td>
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<td>(Wes2)f</td>
<td><em>Mayarnea atomus var. permitis</em> (Hustedt) Lange-Bertalot</td>
<td>De Panne (Belgium) – 03.III.2008</td>
<td>C. Souffreau</td>
<td>8.7 ± 0.1</td>
<td>3.9 ± 0.2</td>
<td>22.4 ± 1.9</td>
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<tr>
<td>(Enc2)b</td>
<td><em>Pinnularia acrosphaeria</em> W. Smith</td>
<td>Ovalle (Chile) – 22.I.2007</td>
<td>C. Souffreau</td>
<td>22.8 ± 1.3</td>
<td>10.6 ± 0.4</td>
<td>12.1 ± 0.3</td>
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<tr>
<td>Pin 876 TM</td>
<td><em>P. acuminata</em> W. Smith</td>
<td>Threipmuir Resr. (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>38.9 ± 2.6</td>
<td>11.5 ± 0.8</td>
<td>10.0 ± 0.0</td>
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<td>Alka 1</td>
<td><em>P. borealis</em> Ehrenberg</td>
<td>Podkova (Czech Republic) – 19.IV.2006</td>
<td>A. Poulicková</td>
<td>37.4 ± 1.3</td>
<td>8.2 ± 0.6</td>
<td>4.6 ± 0.3</td>
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<tr>
<td>(Tor12)d</td>
<td><em>P. borealis</em> Ehrenberg d. var. <em>subislandica</em> Krammer</td>
<td>Seno Otway (Chile) – 02.II.2007</td>
<td>C. Souffreau</td>
<td>36.8 ± 1.6</td>
<td>9.5 ± 0.5</td>
<td>5.0 ± 0.0</td>
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<td>(Ecrins7)a</td>
<td><em>P. borealis</em> Ehrenberg var. <em>borealis</em> Krammer</td>
<td>Allofroide (France) – 23.IX.2006</td>
<td>C. Souffreau</td>
<td>33.0 ± 1.7</td>
<td>8.1 ± 0.3</td>
<td>5.3 ± 0.4</td>
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<tr>
<td>(Tor3)a</td>
<td><em>P. borealis</em> Ehrenberg var. <em>subislandica</em> Krammer</td>
<td>Torres del Paine (Chile) – 30.I.2007</td>
<td>C. Souffreau</td>
<td>27.3 ± 0.6</td>
<td>9.6 ± 0.3</td>
<td>6.0 ± 0.0</td>
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<tr>
<td>(Tor11)b</td>
<td><em>P. cf. altiplanensis</em> Lange-Bertalot</td>
<td>Torres del Paine (Chile) – 01.II.2007</td>
<td>C. Souffreau</td>
<td>17.9 ± 0.5</td>
<td>4.5 ± 0.3</td>
<td>20.0 ± 0.0</td>
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<tr>
<td>Cal 878 TM</td>
<td><em>P. cf. iseliana</em> K. Krammer</td>
<td>Threipmuir Resr. (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>33.4 ± 1.1</td>
<td>7.4 ± 0.3</td>
<td>12.1 ± 0.3</td>
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<tr>
<td>(Ecrins4)a</td>
<td><em>P. cf. marchica</em> Ilka Schönfelder</td>
<td>Allofroide (France) – 22.IX.2006</td>
<td>C. Souffreau</td>
<td>27.3 ± 0.4</td>
<td>5.4 ± 0.4</td>
<td>15.6 ± 0.7</td>
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<tr>
<td>(B2)c</td>
<td><em>P. cf. microstauron</em> (Ehrenberg) Cleve (“southern microstauron”)</td>
<td>Beak Island (Antarctic Peninsula) – 1.I.2006</td>
<td>E. Verleyen</td>
<td>38.9 ± 1.4</td>
<td>9.9 ± 0.3</td>
<td>14.0 ± 0.4</td>
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<tr>
<td>Pin 889 MG</td>
<td><em>P. grunowii</em> Krammer</td>
<td>Balerno (Scotland) – 02.II.2008</td>
<td>D.G. Mann</td>
<td>41.9 ± 0.8</td>
<td>7.9 ± 0.2</td>
<td>13.0 ± 0.8</td>
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<tr>
<td>Pin 706 F</td>
<td><em>P. neglectiformis</em> Krammer</td>
<td>Figgate Loch (Scotland) – 23.I.2008</td>
<td>D.G. Mann</td>
<td>105.3 ± 3.9</td>
<td>18.5 ± 0.5</td>
<td>8.1 ± 0.2</td>
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<tr>
<td>(Tor1)a</td>
<td><em>P. neomajor</em> Krammer</td>
<td>Torres del Paine (Chile) – 30.I.2007</td>
<td>C. Souffreau</td>
<td>151.4 ± 3.6</td>
<td>24.9 ± 0.4</td>
<td>8.2 ± 0.4</td>
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<tr>
<td>Corsea 2</td>
<td><em>P. neomajor</em> Krammer</td>
<td>Glen Corse Resr. (Scotland) – 06.IV.2008</td>
<td>P. Vanormelingen</td>
<td>169.2 ± 1.9</td>
<td>28.4 ± 0.5</td>
<td>7.8 ± 0.3</td>
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<tr>
<td>Pin 885 TM</td>
<td><em>P. nodosa</em> (Ehrenberg) W. Smith</td>
<td>Threipmuir Resr. (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>42.1 ± 1.2</td>
<td>6.7 ± 0.2</td>
<td>10.0 ± 0.0</td>
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<tr>
<td>Pin 877 TM</td>
<td><em>P. parvulissima</em> Krammer</td>
<td>Threipmuir Resr. (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>58.3 ± 1.5</td>
<td>9.8 ± 0.3</td>
<td>10.0 ± 0.0</td>
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<td>Locality and date of collection</td>
<td>Collector</td>
<td>Length (μm)</td>
<td>Width (μm)</td>
<td># stria per 10 μm</td>
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<td>(Tor4)i</td>
<td>P. sp.</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>33.3 ± 1.3</td>
<td>9.4 ± 0.5</td>
<td>14.0 ± 0.0</td>
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<td>(Tor4)r</td>
<td>P. sp.</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>42.6 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td>12.4 ± 0.5</td>
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<tr>
<td>(Wie)a</td>
<td>P. sp.</td>
<td>De Wieden (The Netherlands) – 26.11.2007</td>
<td>P. Vanormelingen</td>
<td>46.7 ± 0.9</td>
<td>9.7 ± 0.2</td>
<td>12.1 ± 0.2</td>
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<tr>
<td>Pin 873 TM</td>
<td>P. sp.</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>42.6 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td>12.4 ± 0.5</td>
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<td>PinnC7</td>
<td>P. sp.</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>42.6 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td>12.4 ± 0.5</td>
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<tr>
<td>(Tor1)b</td>
<td>P. sp. (divergens-group)</td>
<td>Torres del Paine (Chile) – 30.1.2007</td>
<td>C. Souffreau</td>
<td>51.5 ± 2.1</td>
<td>10.4 ± 0.3</td>
<td>10.8 ± 0.8</td>
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<td>(Tor7)c</td>
<td>P. sp. (divergens-group)</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>88.4 ± 1.7</td>
<td>12.5 ± 0.4</td>
<td>10.4 ± 0.5</td>
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<td>(Tor7)f</td>
<td>P. sp. (gibba-group)</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>61.2 ± 1.6</td>
<td>8.9 ± 0.2</td>
<td>10.1 ± 0.5</td>
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<td>(Tor8)b</td>
<td>P. sp. (gibba-group)</td>
<td>Torres del Paine (Chile) – 31.1.2007</td>
<td>C. Souffreau</td>
<td>40.0 ± 0.5</td>
<td>5.9 ± 0.3</td>
<td>11.8 ± 0.4</td>
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<td>(W045)b</td>
<td>P. australigibba var. subcapitata Van de Vijver, Cahttová &amp; Metzeltin Amsterdam Island – 06.XII.2007</td>
<td>B. Van de Vijver</td>
<td>37.1 ± 0.8</td>
<td>8.7 ± 0.2</td>
<td>11.2 ± 0.6</td>
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<tr>
<td>Pin 649 K</td>
<td>P. sp. (subcommutata-group)</td>
<td>Kew Billabong (Australia) – 02.XII.2007</td>
<td>K.M. Evans</td>
<td>53.0 ± 3.2</td>
<td>11.7 ± 0.3</td>
<td>10.1 ± 0.2</td>
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<tr>
<td>Pin 883 TM</td>
<td>P. sp. (subcommutata-group)</td>
<td>Threipmuir Resr. (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>63.1 ± 2.8</td>
<td>14.1 ± 0.1</td>
<td>10.3 ± 0.5</td>
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<tr>
<td>Pin 650 K</td>
<td>P. subanglica Krammer</td>
<td>Kew Billabong (Australia) – 02.XII.2007</td>
<td>K.M. Evans</td>
<td>46.2 ± 0.3</td>
<td>8.2 ± 0.2</td>
<td>11.1 ± 0.7</td>
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<tr>
<td>(Wie)c</td>
<td>P. subcapitata var. elongata Krammer</td>
<td>De Wieden (The Netherlands) – 26.11.2007</td>
<td>P. Vanormelingen</td>
<td>53.3 ± 0.6</td>
<td>6.3 ± 0.3</td>
<td>11.9 ± 0.3</td>
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<tr>
<td>Corsea 10</td>
<td>P. subcommutata var. nonfasciata Krammer</td>
<td>Glen Corse Resr. (Scotland) – 06.IV.2008</td>
<td>P. Vanormelingen</td>
<td>50.1 ± 1.8</td>
<td>10.7 ± 0.1</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>(Enc2)a</td>
<td>P. viridiformis Krammer</td>
<td>Ovalle (Chile) – 22.1.2007</td>
<td>C. Souffreau</td>
<td>74.9 ± 1.8</td>
<td>17.7 ± 0.5</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>Pin 870 MG</td>
<td>P. viridiformis Krammer</td>
<td>Balerno (Scotland) – 02.III.2008</td>
<td>D.G. Mann</td>
<td>80.5 ± 2.3</td>
<td>16.7 ± 0.3</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>(Bfp04)02</td>
<td>Sellaphora blackfordensis D.G. Mann &amp; S. Droop</td>
<td>Culture collection BCCM/DCG</td>
<td>-</td>
<td>38.1 ± 13.6</td>
<td>9.0 ± 1.4</td>
<td>20.2 ± 1.1</td>
</tr>
<tr>
<td>(Bfp5x8)</td>
<td>Sellaphora blackfordensis D.G. Mann &amp; S. Droop</td>
<td>Culture collection BCCM/DCG</td>
<td>-</td>
<td>n.m.</td>
<td>n.m</td>
<td>n.m.</td>
</tr>
</tbody>
</table>
Model testing and phylogenetic analyses

Nine alternative partitioning strategies were tested: partitioning into genes, codon positions, stem and loop regions of rDNA regions, functionality and some combinations of these. Selection of a suitable partitioning strategy and models for the different partitions was based on the Bayesian Information Criterion (BIC, Schwarz, 1978). For each partitioning strategy six substitution models were optimized (JC, F81, K80, HKY, SYM, GTR) with or without a proportion of invariable sites and/or a gamma distribution to accommodate rate variation across sites. All parameters were unlinked between partitions. The preferred model and partitioning strategy was a GTR + $\Gamma$4 in which 18S and 28S each formed a separate rDNA partition, while plastid and mitochondrial genes were separated by genome and were both partitioned into three codon positions.

Single genes and the complete concatenated dataset were analysed by maximum likelihood phylogenetic inference using RAxML 7.2.6 (Stamatakis, 2006) under the preferred model and partition strategy with 10,000 independent tree searches from randomized MP starting trees. Maximum likelihood bootstrap analyses (Felsenstein, 1985) consisted of 1,000 replicates. Bayesian phylogenetic inference was carried out with MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) under the same model and partitioning scheme. Using default settings, two independent runs with four incrementally heated Metropolis-coupled Monte-Carlo Markov Chains were run for five million generations for individual genes and 70 million generations for the concatenated dataset. Runs were sampled every 1,000th generation, and convergence and stationarity of the log-likelihood and parameter values was assessed using Tracer v.1.5 (Rambaut & Drummond, 2007). The first million generations and 10 million generations were discarded as burn-in for the single-gene and multi-gene analyses, respectively. Runs for psbA converged only when using a heating factor of 0.1. We therefore also analysed the concatenated dataset with a heating factor of both 0.2 and 0.1 but no differences in topology, likelihood or posterior probabilities were detected. The post-burn-in trees from the different runs were summarized and posterior probabilities (PPs) were calculated in MrBayes using the sumt command. All analyses were performed using the computer cluster Bioportal (Kumar et al., 2009).
Fossil *Pinnularia*

In order to assist with the temporal calibration of the multi-gene phylogeny, we document a new fossil occurrence of the genus *Pinnularia* from Middle Eocene lacustrine facies in northwestern Canada. The Giraffe kimberlite fossil locality (64°48’N, 110°04’W) contains unpermineralized diatom-rich organic sediments, which constitute the post-eruptive infilling of the site’s maar crater. These sediments are dated between 40 and 48 Ma (Lutetian Stage), and have already revealed first occurrences for a number of freshwater diatom lineages (Siver & Wolfe, 2007; Wolfe & Siver, 2009; Siver et al., 2010). The excellent preservation of Giraffe *Pinnularia* specimens is illustrated by a range of light and scanning electron micrographs (Fig. 4.3).

Relaxed molecular clock analysis

A time-calibrated phylogeny was inferred using a relaxed molecular clock method as implemented in BEAST v.1.5.4 (Drummond & Rambaut, 2007). An uncorrelated lognormal clock model and Yule tree prior were specified along with the same partitioning scheme and models of sequence evolution used for the phylogenetic reconstruction. Different calibration strategies based on the fossil record were carried out to assess congruence between calibration points. The root node calibration prior was varied (uniform, gamma and truncated normal probability distribution), and all internal calibration points had a uniform prior (Ho & Phillips, 2009). All calibration points and strategies are summarized in Table 4.3 and fossil constraints are explained below. For each calibration strategy, three independent runs were carried out, starting from a user-defined, linearized time-constrained starting tree constructed in r8s (Sanderson, 2003) based on the ML tree of the phylogenetic analysis. Markov chains were run for 50 million generations and sampled every 1,000 generations. Convergence and stationarity of log-likelihood and parameter values were assessed using Tracer v.1.5 (Rambaut & Drummond, 2007) and 10% of the generations were discarded as burn-in. The post-burn-in trees of three independent runs were combined, after which a maximum clade credibility chronogram with mean node heights was calculated with TreeAnnotator v.1.5.4.

Fossil diatoms were used to calibrate the tree in geological time using the following constraints. The root node of *Pinnularia* was given a minimum age of 40 Ma following the recovery of *Pinnularia*.
in sediments from the Giraffe locality (Fig. 4.3). Two different maximum age scenarios were considered. A first maximum age of the root node was set at 100 Ma (Albian, Lower Cretaceous), based on the occurrence of rich diatom floras in southern hemisphere ocean sediments containing a range of centric morphologies but neither raphid nor araphid pennate taxa (Gersonde & Harwood, 1990; Harwood & Gersonde, 1990). This date can be assumed to be highly conservative, and therefore we also considered an alternate upper boundary for *Pinnularia* at 75 Ma (Campanian, Upper Cretaceous), the period for which the earliest araphid pennate diatoms have been confirmed (Chambers, 1966; Hajós & Stradner, 1975). Because these fossils are abundant but not diverse, it is believed that araphid pennate forms had evolved only recently by this time (Sims et al., 2006). Following this reasoning, we assume that raphid pennates had not yet evolved.

Four internal calibration points were also used (Fig. 4.4), based on first-appearance dates for characteristic morphological types or Baupläne recovered from late-Oligocene to mid-Miocene freshwater deposits. The following morphological types were used: diatoms morphologically similar to extant *P. viridis* (11.7 Ma; Saint Martin & Saint Martin, 2005), *P. mesolepta* (11.7 Ma; Saint Martin & Saint Martin, 2005), *P. nodosa* (14.5 Ma; pers. comm. A. Menicucci) and *P. borealis* (13.0 Ma; Servant-Vildary et al., 1988). These fossil occurrences were used as minimum estimates for the

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**Table 4.3**: List of calibration points and alternative calibration schemes used to date the phylogenetic tree, with indication of the minimum (min) and maximum (max) age constraints. Numbers refer to the calibration points on the phylogenetic tree (Fig. 4.4).

<table>
<thead>
<tr>
<th>node</th>
<th>Calibration scheme with age Constraints (Ma)</th>
<th>Bauplan</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>root scheme</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>root node</td>
<td>root</td>
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<td></td>
<td>min</td>
<td>40</td>
<td>40</td>
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<tr>
<td></td>
<td>max</td>
<td>75</td>
<td>75</td>
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<tr>
<td>2</td>
<td>neomajor-neglectiformis clades</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>-</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>max</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>nodosa acrospaeria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>-</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>max</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>grunowii mesolepta</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>max</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>borealis microstauron</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>max</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 4.3 Middle Eocene Pinnularia diatoms from the Giraffe kimberlite pipe fossil locality with a minimum age of 40 Ma. In light microscopy, valve (a-c) and girdle (d) views illustrate morphologies consistent with the genus as currently circumscribed. Under scanning electron microscopy, the valve overview (e), close-ups of proximal (f) and distal external raphe endings (g), as well as details of the outer (h-j) and inner (k) valve surfaces all confirm considerable affinity with extant forms. The alveolate striae of the Eocene diatom (k) are directly comparable with numerous modern congeners, here shown is a representatives of the P. subgibba subclade [(Tor4)r] in (l). Scale bars are 10 μm (a-d), 20 μm (e), 5 μm (f,i,j), and 2 μm (g,h,k,l).
Fig. 4.4 Phylogenetic relationships within the genus Pinnularia inferred from a five-locus DNA alignment using maximum likelihood under a partitioned model. Numbers at nodes indicate statistical support, ML bootstrap proportions - BI posterior probabilities (both given as percentages). Encircled numbers represent nodes constrained in the relaxed molecular clock analysis (see Table 4.3).
corresponding Bauplan, thus constraining the MRCA (most recent common ancestor) of clades containing species within each of these (Fig. 4.4). The maximum age for these different Baupläne was set at 75 Ma when using the conservative calibration scheme, and 40 Ma for the less conservative calibration strategy. Although specimens originating from the Wagon Gap Formation clearly belong to *Pinnularia* (Lohman & Andrews, 1968), in the absence of electron microscopical observations they cannot be assigned with confidence to any of the modern morphological types used in our phylogeny. Linked to the uncertain dating of this locality, Wagon Gap *Pinnularia* were not used as an explicit constraint for our phylogeny.

### 4.3 Results

**Dataset properties**

Our 44-taxon, five-locus dataset is 87% filled (192 sequences; Table 4.1) and includes 4852 sites of which 1012 (21%) are parsimony-informative. The most complete markers are 28S rDNA and *rbcL* (0 of 44 missing), followed by 18S rDNA (4 sequences or 9% missing), *psbA* (8 or 18% missing) and *cox1* (16 or 36% missing). The 18S rDNA sequences provided most characters (1706) followed by *rbcL* (1386), *psbA* (762), *cox1* (615) and 28S rDNA (383). The percentage of parsimony-informative characters varied between genes, with the highest percentages for 28S rDNA (42%) and *cox1* (37%), the lowest for *psbA* (10%), and 18S rDNA (18%) and *rbcL* (17%) being intermediate.

**Phylogenetic relationships**

Phylogenies of individual genes revealed no strong conflicts; therefore we show only the results of the concatenated analysis. Similarly, ML and BI analyses of the concatenated dataset produced identical topologies so we show only the ML phylogeny with both ML bootstrap support (BS) and BI posterior probabilities (PP) (Fig. 4.4). Based on our concatenated dataset, taxa representing *Pinnularia* and *Caloneis* form a monophyletic group comprising three robustly supported clades that each contain several well-supported subclades. Clade A comprises *Caloneis silicula*, *C. lauta*, and two species of *Pinnularia cf. divergens*. No apparent morphological synapomorphies unite these taxa, *Caloneis silicula* being characterized by linear external central raphe endings (Fig. 4.2, a), small alveolar apertures...
(Fig. 4.2, b) and parallel striae (Fig. 4.1, d), while the divergens-group has drop-like external central raphe endings (Fig. 4.2, c), large alveolar apertures (Fig. 4.2, d) and oblique striae (Fig. 4.1, e, f).

Clade B includes small, linear Pinnularia species (Fig. 4.1, g–p) that have drop-like external central raphe endings (Fig. 4.2, c). Clade B is subdivided into three well-supported subclades: the grunowii, nodosa and subgibba subclades. The grunowii subclade contains P. grunowii, P. subanglica and P. marchica. Species in this group contain a single H-shaped plastid with two pyrenoids (Fig. 4.1, b’-c’), whereas, with the exception of some species (see Souffreau et al., unpubl. a, for detailed information) all other clades of the tree are characterized by two linear, strip-like plastids (Fig. 4.1, y–z). The nodosa subclade includes P. nodosa and P. acrosphaeria, which are both characterized by irregular wart-like structures on the external valve face (Fig. 4.1, j, k; detail in Fig. 2, c). The subgibba subclade contains different species resembling P. subgibba, P. parvulissima, and P. subcapitata, all characterized by a combination of an elongated shape often with ghost striae (striae that are partially filled in with silica) and a broad, non-porous, central area (fascia) (Fig. 4.1, l–p).

Clade C is composed of two subclades. Subclade C1 contains Caloneis budensis and Pinnularia cf. altiplanensis (Fig. 4.1, x), but is poorly supported (BS=56; PP=87). Subclade C2 is further subdivided into moderately to highly supported groups, all of which have characteristic morphologies. The borealis-microstauron group includes the P. borealis species complex, which is easily recognizable by its relatively broad cells and coarse striae (Fig. 4.1, w), and P. microstauron with its relatively robust shape and wedge-shaped ends (Fig. 4.1, v). The remaining groups (the subcommutata and viridiformis clades) contain large, elongated-elliptical species, with almost parallel striae and small central areas (Fig. 4.1, q-t), undulate external raphe fissures, intermediate-sized alveolar openings (Fig. 4.2, f), and plastids without pyrenoids. The subcommutata group contains Pinnularia species with linear central raphe endings (Fig. 4.2, e) while species of the viridiformis group have round central raphe endings (Fig. 4.2, g) and a complex raphe system, visible as three parallel (Fig. 4.1, q) or twisting lines (Fig. 4.1, r) with light microscopy (Round et al., 1990; Krammer, 1992, 2000). Sister to the viridiformis and subcommutata groups is the isolate (Wie)a (Fig. 4.1, u), an undescribed Pinnularia species morphologically similar to P. microstauron. In the single-gene trees, (Wie)a is always retrieved in clade C, but with a closer (unsupported) relationship to the viridiformis-group based on
18S and 28S rDNA, or with a sister relationship to *P. cf. divergens* and *P. borealis* based on *rbcL* and *psbA*.

**Newly-observed fossil *Pinnularia* specimens**

*Pinnularia* specimens from the Middle Eocene Giraffe kimberlite locality show considerable affinity to modern representatives of the genus. While they are never abundant in this material, sufficient numbers have been observed to document their morphology in light and scanning electron microscopy (Fig. 4.3). The specimens are characterized by an alveolate valve structure (Fig. 4.3, k), placing them confidently in the genus *Pinnularia*. Moreover, the Giraffe *Pinnularia* forms have large alveolar openings (Fig. 3, k), identical to the *Pinnularia* species from clades A, B, C1 and the *microstauron-borealis* group of clade C (e.g. Fig. 4.3, l; Fig. 4.2, d,h). However, the particular combination of morphological characters of the fossil specimens, including wart-like structures on the valves (similar to *P. acrosphaeria*), the very wide axial area (similar to *P. acuminata*) and the distinctly lanceolate or “naviculoid” shape (not encountered in our species) meant that we were not able to place these specimens within a known *Pinnularia* Bauplan in the present phylogeny nor, to our knowledge, with any currently described species. Their distribution in samples spanning tens of meters of core, typically associated with chrysophyte and euglyphid siliceous microfossils and benthic eunotioid diatoms, and their occurrence within the mudstone matrix discounts the possibility that they are contaminants. A more complete analysis of the authenticity of Giraffe microfossils is given elsewhere (Wolfe & Siver, 2009). Being ≥40 Ma in age, these *Pinnularia* specimens represent the earliest known forms of *Pinnularia*, providing us with a fossil calibration point to constrain the basal node of our phylogeny temporally.

**Time-calibrated phylogeny**

The different calibration schemes analyzed in BEAST all recover identical relationships to the RAxML and MrBayes analyses with comparable posterior probabilities. The average node ages and 95% HPD (Highest Posterior Density) resulting from the different calibration schemes are given in Fig. 4.5. Estimates using the internal calibration points based on the frustule morphologies of *viridis*, *nodosa* and *borealis* were consistent with estimates using only a root calibration. However, adding the internal calibration point based on
Fig. 4.5 Resulting mean node ages (indicated
as “x”; in Ma) and 95% HPD (error bars;
in Ma) for nine of the alternative calibration
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The preferred calibration scheme used for
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were only constrained on the root. Scheme
C used a less conservative calibration, with
a maximum of 75 Ma and all internal
calibration points. Scheme E used a very
conservative calibration with a maximum
of 100 Ma and all internal calibration
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on the grunowii-mesolepta Bauplan is
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estimations based on “root” and “scheme C”
or “scheme E”.

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Time-calibrated phylogeny of Pinnularia


grunowii-mesolepta resulted in larger 95% HPD intervals and higher average node ages (plus 5–12 Ma) at all nodes. Using only the root constraint, the grunowii-mesolepta node is estimated as being 3.5–12.6 Ma. Constraining this node to have a minimum age of 11.7 Ma therefore pushes its own time-estimate, and that of all other nodes, back in time (Fig. 4.5).

Using the conservative maximum root age of 100 Ma resulted in a 7–11 Ma higher average root node age and a 12–20 Ma higher 95% HPD maximum root age compared with the alternative 75 Ma maximum age boundary, but for the internal nodes, changes in mean age and 95% HPD were only 1–6 Ma. Furthermore, the use of different probability distributions on the root calibration (uniform, gamma or truncated normal) resulted in predictable differences in average node ages and 95% HPD up to 6 Ma for the less-conservative 75 Ma schemes and up to 10 Ma for the conservative 100 Ma scheme. Because the Eocene fossil record is so fragmentary, we chose the conservative uniform prior probability distribution as the preferred calibration strategy.

Fig. 4.6 presents our preferred relaxed molecular clock as applied to the Pinnularia phylogeny, constrained by all four internal calibration points (scheme C = viridis, borealis, nodosa and mesolepta), with the root age constrained between 40 and 75 Ma using a uniform distribution. This estimate places the origin of Pinnularia around 64 Ma, between the Campanian and Early Eocene [75–50]. The Pinnularia complex began to diversify between the Maastrichtian and Middle Eocene 60 Ma [73–45] and continued through to the Pliocene, with no obvious evidence for increased rates of diversification over any time period. Clades A and C diversified mostly during the Paleocene–Eocene (respectively, 49 Ma [65–33] and 52 Ma [64–38]), while clade B started to diverge between the Eocene and Oligocene (39 Ma [50–28]).

4.4 Discussion

Relationships within Pinnularia

The current study presents a multi-gene phylogeny of the genus Pinnularia and extends the sampling of Bruder et al. (2008) threefold. Our five-locus analysis yielded a well-supported phylogenetic tree, with Pinnularia and Caloneis resolved in a single monophyletic clade, in accordance with the results of Bruder and Medlin (2008) and Bruder et al. (2008). Our phylogeny shows that Caloneis (as
Fig. 4.6 Chronogram of the genus Pinnularia from a Bayesian relaxed molecular clock analysis performed with BEAST (Drummond and Rambaut, 2007), time-constrained by four internal fossil calibration points (viridis, borealis, nodosa and mesolepta) and a 40 Ma minimum and 75 Ma maximum root age with uniform probability distribution (scheme C) based on the fossil data from the Giraffe kimberlite Pipe. Values at nodes are mean node ages and grey bars represent 95% HPD (highest probability density) intervals.
currently defined) is not monophyletic, but rather that its species were recovered in various places among the *Pinnularia* species. This finding supports the work of Cox (1988), Round *et al.* (1990) and Mann (2001) who concluded that the current separation of *Caloneis* from *Pinnularia* was not supported by plastid and frustule structure. Cleve (1894) delimited *Caloneis* from *Pinnularia* using light microscopy, but he admitted that their discrimination, based on stria orientation, the path of the terminal raphe fissures, plastids and the presence of “longitudinal lines” (the margins of the alveolar apertures on the inner side of the valve) was not consistent. While Krammer & Lange-Bertalot (Krammer & Lange-Bertalot, 1986; Krammer, 1992) argued that *Caloneis* can indeed be distinguished from *Pinnularia* based on a specific combination of characters, Round *et al.* (1990) suggested that, although *Pinnularia–Caloneis* is a natural group characterized by the double-walled, chambered (alveolated) valve structure, any split of the *Pinnularia–Caloneis* complex would not follow the traditional boundary between the two genera. Despite the fact that our molecular phylogeny contained only three *Caloneis* species, there is no apparent genetic boundary between *Pinnularia* and *Caloneis*. Nevertheless, ultrastructural characters, e.g. the size of the alveoli, and/or plastid characters can apparently be used, alongside molecular synapomorphies, to delimit groups or genera within this complex. However, much further genetic and morphological investigation of the *Pinnularia–Caloneis* complex is required before any attempt is made to formally subdivide it. Alternatively, subdivision could be considered unnecessary, given the highly characteristic general morphology of the complex.

*Pinnularia* is a morphologically diverse genus and the interspecific relationships based on our five-locus dataset generally follow morphological patterns. Our dataset revealed three large, well-supported clades that were also recovered with lower or no support in the concatenated (18S, 28S, *rbcL*) dataset of Bruder *et al.* (2008). With the exception of clade A, which contains two *Caloneis* species and the *Pinnularia divergens* group, the remaining clades are morphologically well-defined. Clade B includes *Pinnularia* taxa of intermediate size that have a fascia, and is subdivided into three highly supported and morphologically well-defined subclades. The structural similarities between *P. acrosphaeria* and *P. nodosa* have been described by Cleve (1895) and more recently by Krammer & Lange-Bertalot (1986). The *grunowii* and *subgibba* subclades are also well-defined by H-shaped plastids and a wide fascia, respectively.
Clade C contains the larger and more robust forms of *Pinnularia*, and is subdivided into well-supported subclades, each with a distinct morphology. The combined *viridiformis* and *subcommutata* group, characterized by intermediate alveolar openings (Fig. 4.2, f), was previously described by Cleve (1895) as the Maiores and Complexae, and more recently as a single group by Krammer & Lange-Bertalot (1986).

Besides these well-defined and well-supported clades, some clusters are less easily interpreted. Some enigmatic relationships within clade A can only be further resolved and understood through additional taxon sampling. This is also the case for the positions of the undescribed species (Wie)a and *P. cf. altiplanensis*, and the relationship between *P. microstauron* and *P. borealis*. Because only a small subset of the described *Pinnularia* species were included in this analysis, it is plausible that they represent more elaborate clusters. Adding taxa in those parts of the tree could therefore improve our understanding of the evolution of morphological features.

**Evolution of the genus *Pinnularia***

Based on our preferred time-calibration, *Pinnularia* originated between the Campanian (Late Cretaceous, *ca.* 75 Ma) and Early Eocene (*ca.* 50 Ma). This estimate is 10 to 35 Ma older than the *ca.* 40 Ma origin deduced from the chronogram of Sorhannus (2007). However, the goal of the latter study was to elucidate deeper divergences within major diatom lineages, and furthermore only two *Pinnularia* taxa were included and analyzed with a single genetic marker (nuclear-encoded SSU rDNA). More comprehensive taxon sampling (Sanderson, 1990), the use of multiple genes (Rodríguez-Trelles et al., 2003), and full integration of the fossil record (Donoghue & Benton, 2007) have all been shown to improve the accuracy of molecular dating. In the case of *Pinnularia*, the ≥40 Ma Giraffe specimens convincingly support the older range of our estimated ages for the origination of the genus (*i.e.* Late Cretaceous). Given the morphological similarity of Giraffe *Pinnularia* forms to extant taxa, we are confident that such older forms exist. While the Giraffe specimens are, so far, the oldest representatives from well-dated sediments, they do not inform the search for morphologically primitive *Pinnularia*, or its immediate ancestor.

The new estimate for the origin of *Pinnularia* implies a considerable gap in the fossil record of freshwater diatoms. The magnitude of this ghost lineage (*ca.* 10-35 Ma) should not be
surprising given that freshwater deposits are underrepresented and understudied in the palaeontological record. Similar gaps apply to nearly all extant raphid diatom lineages, and the scarcity of fossil raphid diatoms of Late Cretaceous to Paleocene age has resulted in a lack of consensus on plausible minimum ages for most genera (but see Singh et al., 2006). It is therefore difficult to place the proposed age for *Pinnularia* in a broader context, although generally our observations are consistent with the preferred model of Berney & Pawlowski (2006), in which pennate diatoms arose some 98 (110–77) Ma ago, leaving the entire Late Cretaceous for innovations such as the raphe. Apart from *Pinnularia*, no raphid diatom genera have yet been dated by phylogenetic methods, but the time-calibrated phylogeny of Sorhannus (2007) suggests that several raphid genera are as old as, or even older than, our estimate for *Pinnularia*.

Given the available fossil and phylogenetic records, our estimated timing of 60 Ma for the origin of *Pinnularia* seems plausible. Since its origin, *Pinnularia* has diversified widely into a large number of species and a range of morphologies. The evolution of particular morphological innovations appears to have sparked the appearance of new lineages, such as the evolution of the “complex” raphe, which may have allowed the development of larger cells, as seen in the *viridiformis* group, or the evolution of H-shaped plastids in the *grunowii* group. Further research will be needed to integrate the ecological, morphological, genetic and evolutionary processes underlying the diversification of diatom genera such as *Pinnularia*. Our time-calibrated multi-gene phylogeny of *Pinnularia* forms a first important step towards this goal by providing a temporal context in which to interpret the diversification of the genus, and has only been possible by integrating molecular and palaeontological information.

**Acknowledgments**

We thank Aloisie Poulíčková for providing cultures of *Pinnularia borealis* and Elie Verleyen for providing live environmental samples. Alex Ball enthusiastically assisted with confocal microscopy. Sequencing was done by Andy Vierstrate. Funding was provided by the Fund for Scientific Research-Flanders (FWO-Flanders; PhD fellowship to CS, post-doctoral fellowships to HV and PV, and research grant 3G/0533/07). Research on fossil diatoms is partially supported by the U.S. National Science Foundation (DEB-0716606 to PAS) and the Natural Sciences and Engineering Council of Canada.
(APW). Morphological analyses at the Natural History Museum, London, were supported by an EU framework 7 SYNTHESYS grant (GB-TAF-77) to CS.
## Appendix 4.A

DNA regions, primer sequences and PCR conditions. F= forward, R= reverse.

<table>
<thead>
<tr>
<th>DNA region</th>
<th>Primer sequence</th>
<th>PCR protocol (°C, min:sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rDNA</td>
<td>P2F: CTG GTT GAT TCT GCC AGT</td>
<td>94°C, 3:00 + 40 x (94°C, 1:00 - 55°C, 1:00 - 72°C, 1:50) + 72°C, 10:00</td>
</tr>
<tr>
<td></td>
<td>P4R : TGA TCC TCC YGC AGG TCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P12: CGG CCA TGCACC ACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P14: CGG TAA TGC CAG CTC C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P15 13: TTT GAC TCA ACA CGG G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P16: GWA TTA CCG CGG CKG CTG</td>
<td></td>
</tr>
<tr>
<td>28S rDNA</td>
<td>DIR-F: ACC CGC TGA ATT TAA GCA TA</td>
<td>95°C, 5:00 + 35 x (94°C, 1:00 - 45°C, 1:00 - 74°C, 1:00) + 72°C, 10:00</td>
</tr>
<tr>
<td></td>
<td>D2C-R: CCT TGG TCC GTG TTT CAA GA</td>
<td></td>
</tr>
<tr>
<td>rbcl</td>
<td>DPrbcl1F: AAG GAG AAA THA ATG TCT</td>
<td>94°C, 3:00 + 40 x (94°C, 1:00 - 55°C, 1:00 - 72°C, 1:50) + 72°C, 5:00</td>
</tr>
<tr>
<td></td>
<td>DPrbcl7R: AAC AAC CTT GTG TAA GTC TC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rbcL13F: CGT TTA GAA GAT ATG CGT ATT C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDrbcL6F: GTA AAT GGA TGC GTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDrbcL12R: GCA CCT AAT AAT GG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15R: ACA CCA GAC ATA GGC ATC CA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17R: TGA CCA ATT GTA CCA CC</td>
<td></td>
</tr>
<tr>
<td>psbA</td>
<td>psbAF: ATG ACT GCT ACT TTA GAA AGA CG</td>
<td>94°C, 3:00 + 35 x (94°C, 1:00 - 46°C, 1:00 - 72°C, 1:00) + 72°C, 10:00</td>
</tr>
<tr>
<td></td>
<td>psbAR: GCT AAA TCT ARW GGG AAG TGG TGG</td>
<td></td>
</tr>
<tr>
<td>cox1</td>
<td>GazF2: CAA CCA YAA AGA TAT WGG TAC</td>
<td>95°C, 3:00 + 35 x (95°C, 0:30 - 50°C, 1:00 - 72°C, 1:50) + 72°C, 5:00</td>
</tr>
<tr>
<td></td>
<td>KedtmR: AAA CTT CWG GRT GAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAA AAA</td>
<td></td>
</tr>
</tbody>
</table>

Time-calibrated phylogeny of *Pinnularia*  101
5. Phylogenetic signals in the valve and plastid morphology of the diatom genus *Pinnularia*

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Unpublished manuscript
Abstract

The diatom genus *Pinnularia* is characterized by broad variation in frustule and plastid morphology, making it an interesting genus in which to analyze the phylogenetic signal of these characters with respect to interspecific relationships. We performed a cladistic analysis on 48 strains using 17 frustule and 3 plastid characters and made a comparison with a multi-gene phylogeny of the same taxa. Independent phylogenetic analyses of molecular and morphological data generated similar clades, and Mantel tests found a significant correlation between morphological and molecular distances. However, while phylogenograms based on five molecular markers containing in total 1012 parsimony-informative sites were fully resolved, the lower number of phylogenetically informative morphological characters inevitably resulted in fewer clades being recovered, and we were never able to fully resolve the interspecific relationships of *Pinnularia* based on morphology. The best resolution for morphology-based species differentiation was acquired by analyzing all morphological characters simultaneously. Mainly “non-structural” frustule characters were found to be homoplasious while “structural” characters (such as central raphe endings, raphe complexity and alveolus openings) and one plastid character (number of pyrenoids) were informative for reconstructing the phylogenetic relationships within the genus. Some problems of homology were encountered and are discussed.

**Key words:** ancestral state reconstruction, *Caloneis*, cladistics, nuclear genes, *Pinnularia*, phylogeny, plastid genes, plastid morphology, valve morphology
5.1 Introduction

Diatom taxonomy and classification are still largely based on the species-specific shape and ornamentation of the silica cell wall, revealed by light and scanning electron microscopy (Round et al., 1990). To a lesser extent, cytological information, such as chloroplast morphology and organelle location, has been incorporated into diatom systematics (Mereschkowsky, 1902; Cox, 1981; Round et al., 1990; Cox & Williams, 2006). Genetic information in the form of nucleotide sequences from (parts of) faster and slower evolving genes are increasingly being used to infer relationships in this microalgal group (reviewed in Alverston, 2008), but as with morphology and cytology, there is the risk that some level of parallel evolution and convergence in base composition obscures the real relationships.

For DNA nucleotides, the knowledge on their biological function (i.e. the coding of amino acids used to construct proteins) and chemical properties (e.g. the stronger hydrogen bound between guanine and cytosine than between adenine and thymine) allowed to model their evolution using mathematical language. By applying such models on a larger number of nucleotides originating from different taxa, the phylogenetic relationships between these taxa (including probability levels) can be estimated (Felsenstein, 2004).

In contrast, morphological and cytological features are expected to be generated by the coordinated action of multiple genes (by analogy with higher plants, e.g. Mathur, 2006; Qian et al., 2009) making the selection of the used characters and character states, the weighting of different sets of characters, and the probabilities of gains and losses of character states more ambiguous, and thus more prone to interpretational (e.g. are these different character states of a single character, or different characters) and analytical mistakes. Moreover, the low number of morphological characters commonly used to infer relationships (see for example Edgar & Theriot, 2004; Bleidorn et al., 2009; Kooistra et al., 2010 and compare the number of morphological characters with the number of nucleotides) enhances the impact of potentially misleading characters on the outcome, making the reconstruction of phylogenetic relationships based on morphological datasets very challenging.

Meanwhile, cladistic analyses have provided useful results at higher (intergeneric) (e.g. Kociolek & Stoermer, 1986; Cox & Williams, 2000; Marivaux et al., 2004; Cox & Williams, 2006; Ohl & Spahn, 2010) and lower (intragenic) taxonomic levels (e.g. Julius & Tanimura, 2001; Uriz & Carballo, 2001; Edgar & Theriot, 2004; Kooistra et al.,
In addition, phylogenetic analysis of morphological datasets can provide insights into the phylogenetic signal and resolution of different character types for inferring relationships (Cox & Williams, 2000; Maximino, 2008), reveal the evolution of morphological stages (Maximino, 2008) and the selective pressure behind morphological evolution (Stark & O’grady, 2010). Morphology is also the link that allows the integration of fossil taxa and large molecular datasets (Edgar & Theriot, 2004; Smith & Turner, 2005; Giribet, 2010), and thus the interpretation of evolutionary patterns across time (Marivaux et al., 2004). Morphology may complement the patterns derived from molecular data (e.g. Jacobs et al., 2008; Mizuno, 2008; Ivanovic et al., 2009) and, interestingly, molecular-phylogenetic analyses of diatom genera often support previous morphological observations (Lundholm et al., 2002; 2003; Edgar & Theriot, 2004; Lundholm et al., 2006; Chen et al., 2007; Sarno et al., 2007). There is also evidence from different organisms that the combined analysis of molecular and morphological datasets can enhance the resolution (Wortley & Scotland, 2006) and overall probability of the phylogenetic signal (Lee & Camens, 2009). For all these reasons morphological, cytological, biochemical, ecological and developmental data remain important components for the inference of evolutionary relationships and understanding of evolutionary processes.

Despite the recognized importance of using only phylogenetically informative data when reconstructing relationships, there is a lack of knowledge on the phylogenetic value of morphological characters in diatoms. Morphological data are mostly analyzed using cladistics, a technique that describes evolutionary history based on shared (homologous), derived characters (Hennig, 1965, 1966), but identifying homologous characters and defining character states can be fairly subjective. It is important to use positional, structural and developmental information to interpret potential phylogenetically informative characters (Cox, 2010) because similar features may be formed in different ways (Cox, 2001) while contrasting features may share developmental pathways (Cox, 2002). Even if the systematics and cladistic analysis of diatoms have been dominated by frustule characters, cytological features such as shape and number of chloroplasts have also been used to infer relationships (Mereschkowsky, 1902; Kociolek & Stoermer, 1986; Round et al., 1990; Cox & Williams, 2000; Cox & Reid, 2004; Cox & Williams, 2006). Partitioned analyses of protoplast and frustule data have shown that both datasets are informative at
different levels (Cox & Williams, 2000). Because the genus *Pinnularia* exhibits a broad variety of plastid morphology potentially useful for subgeneric groupings (Cox, 1988), as well as large variation in frustule characters (Round *et al.*, 1990; Krammer, 2000), it is an ideal genus with which to analyze the phylogenetic signal of both valve and plastid characters at the infrageneric level using a cladistic approach. Moreover, the availability of a molecular-phylogenetic tree of the genus based on five molecular markers (Souffreau *et al.*, submitted) allows us to compare the morphological signal with the molecular one.

The genus *Pinnularia* Ehrenberg contains biraphid, benthic diatoms and occurs world-wide, mainly in freshwater habitats. It was described by Ehrenberg (1843) based on *Pinnularia viridis* (Nitzsch) Ehrenberg and is one of the most speciose diatom genera. At the time of writing, Algaebase (Guiry & Guiry, 2011) includes 2462 species names and varieties of which 413 are currently accepted taxonomically, but in the light of recent discoveries of pseudocryptic species (Sarno *et al.*, 2005; Mann & Evans, 2007; Vanormelingen *et al.*, 2008) it is believed that the worldwide species diversity of *Pinnularia* could exceed this last number by almost ten-fold (pers. comm. Bart Van de Vijver). The fossil record of the genus dates back to the Middle-Eocene (ca. 40 Ma ago) (Lohman & Andrews, 1968; Souffreau *et al.*, submitted) placing *Pinnularia* among the earliest freshwater diatoms yet recovered, and a fossil-calibrated molecular clock estimates its origin at around 60 Ma (Souffreau *et al.*, submitted).

*Pinnularia* is morphologically characterized by the possession of alveolate or “chambered” valves in which the striae (as seen using light microscopy) correspond to separate alveoli (chambers) (Round *et al.*, 1990; Krammer, 2000). The outer wall of each chamber is a porous plate with many rows of small round poroids (occluded by hymenes), while the inner wall consists of a plain plate perforated by one (large or small) aperture. The raphe system is central and the terminal fissures are long hooks. *Pinnularia* is readily distinguished from other diatom genera by these features, including the most closely related genera *Sellaphora* and *Eolinna* (Round *et al.*, 1990; Bruder & Medlin, 2008; Evans *et al.*, 2008), but not from *Caloneis* Cleve, which also has an alveolated valve (Round *et al.*, 1990; Krammer, 1992, 2000). When first describing *Caloneis*, Cleve (1894) noted the similarities with *Pinnularia*. The controversy still exists, and while Cox (1988), Round *et al.* (1990) and Mann (2001) consider it no longer possible to make a clear distinction along the traditional
boundary between these two genera, Krammer & Lange-Bertalot (1985, 1986) and Krammer (1992, 2000) consider that the combination of a lanceolate valve outline, parallel or radial orientation of the striae at the apices and marginal longitudinal lines separate Caloneis from Pinnularia. In molecular phylogenies (Bruder & Medlin, 2008; Bruder et al., 2008) Pinnularia and Caloneis were recovered as a single clade, but with low bootstrap support. The present study does not aim to resolve this issue, but we have added some strains of Caloneis to complete the sampling.

Given its distinguishing morphological characteristics, Pinnularia exhibits considerable morphological diversity, including very small to very large forms (Round et al., 1990; Krammer, 2000). Valve shape varies from linear to undulate, apices can be rounded to capitate, and the raphe system can be simple and linear or very complex with a tongue and groove system visible as multiple parallel or twisting lines in LM (Round et al., 1990; Krammer, 1992, 2000). Plastid morphology varies from two girdle-appressed, linear chloroplasts per cell to a single H-shaped plastid with varying numbers of pyrenoids (Cox, 1988). Relationships within Pinnularia have never been resolved based on morphology. A number of authors have recognized different morphological groups within the genus based on valve size and outline and stria orientation (Cleve, 1895; Krammer & Lange-Bertalot, 1985, 1986), but these have not been used in a classification [except in Patrick & Reimer (1966)] and were only intended to group the broad variety of morphologies for identification purposes. A molecular-phylogenetic analysis based on 44 taxa (Souffreau et al., submitted) revealed three large clades, each of them containing different, statistically supported subclades, which were frequently but not always morphologically defined.

The aim of this study was to analyze the utility of different valve and plastid characters for inferring the interspecific relationships in the genus Pinnularia and to compare this with the information retrieved from the five genetic markers in Souffreau et al. (submitted). First, we conducted a cladistic analysis on both the valve and plastid morphology to reconstruct evolutionary relationships in the genus Pinnularia and to identify which characters contribute most to the reconstruction of these relationships. Secondly, the congruence between the morphological and multi-gene phylogeny (Souffreau et al., submitted) was determined by Mantel tests (Mantel, 1967; Mantel & Valand, 1970). Finally, we performed ancestral state reconstructions of the morphological characters based on the multi-
gene phylogeny of Souffreau et al. (submitted) and compared our results with the available fossil record.

5.2 Materials & Methods

Strain isolation and identification
Strains used for this study were isolated by micropipette or by the lens-tissue technique from environmental samples of aquatic sediments and soils collected from different regions in the world between 2006 and 2008 (Table 5.1). All cultures were kept in liquid WC-medium [(Guillard & Lorenzen, 1972) but without addition of vitamins] under standard conditions of $18^\circ\text{C} \pm 2^\circ\text{C}$, 25-30 $\mu$mol ph m$^{-2}$ s$^{-1}$ light intensity and a 12:12 hours light:dark period. Cultures were re-inoculated in fresh medium when reaching late exponential phase. For morphological and genetic characterization, cells were harvested in exponential phase and concentrated by centrifugation. Pellets for DNA characterization were conserved at -20$^\circ\text{C}$ and processed as described in Souffreau et al. (submitted); pellets for morphological study by light microscopy (LM) were instantly oxidized in hydrogen peroxide and, after several washing steps, embedded in Naphrax®. Pictures of the cleaned valves were taken using a Zeiss Axioplan 2 microscope equipped with an AxioCam Mrm camera. Valve length, width and stria density of 10 valves per strain were measured using ImageJ 1.37v software. Identification of the cleaned valves was based on Krammer (2000) for *Pinnularia*, and Krammer & Lange-Bertalot (1986) for *Caloneis*. Voucher slides of all natural samples and cultures are kept in the Laboratory of Protistology & Aquatic Ecology and are available upon request. To cover the wide range of morphological variation of the genus *Pinnularia*, a total of 48 morphologically dissimilar strains were selected for further analysis.

Morphological characterization
The morphology of cleaned frustules was examined and photographed using both LM and scanning electron microscopy (SEM). For SEM, the oxidized material was coated with gold for 60 sec with a JEOL JFC-1200 Fine Coater and examined using a JEOL JSM-5600LV SEM. Cytological observations of plastids and pyrenoids were made on living cells of non-synchronized, exponentially growing strains using a Zeiss Axioplan 2 microscope equipped with
an AxioCam Mrm camera. To improve the visibility of the plastid shape and pyrenoids, Leica Confocal Laser Scanning Microscopy (CLSM) was used to visualize the autofluorescence of the plastids of both living and 2% glutaraldehyde-fixed cells. Pyrenoids were stained as explained in Ettl (1976) with acidified Azocarmine-G solution (Sigma, Germany) prepared following Gerlach (1977).

Character coding
Frustule and cytological characters were chosen based on five criteria: characters must (i) show minimal variation within a taxon, (ii) be variable between taxa, (iii) be independent, (iv) be discrete, (v) be heritable (Pimentel & Riggins, 1987; Farris, 1990). Table 5.2 gives an overview of the 17 frustule and 3 plastid characters and their respective character states. Descriptions of the different characters are given with comments and micrographs in Appendix 5.1-3 and terminology is based on Ross et al. (1979), Cox & Ross (1981) and Round et al. (1990). Character states were coded from simple to complex. To assign the primitive states we did not use the outgroup method (Kociolek, 1986) because the intergeneric relationships of *Pinnularia* and *Caloneis* are not clear and character states of the non-alveolate genera *Sellaphora*, *Eolimna* or *Mayamaea* (see molecular outgroups in Souffreau et al., submitted) are too dissimilar from those of *Pinnularia* to be workable. The ancestral states were thus based on the earliest known fossil material of *Pinnularia* described by Lohman & Andrews (1968), and the same character states apply for the Middle Eocene *Pinnularia* species reported in (Souffreau et al., submitted) apart from the absence of surface irregularities and the cuneate apices. The taxon/character matrix is given in Table 5.3.

Cladistic analysis
Frustule characters included both “structural” features, such as type of raphe slit, extent and position of alveolus opening and girdle band areolae, which are expected to have a genetic background and thus phylogenetic value, and “non-structural” characters, such as valve outlines, shapes of hyaline areas and stria orientation, which depend on the arrangement of other (first formed and genetically constrained) valve components but are independent of the type of these valve components (e.g. stria orientation is independent of the type of stria and pore construction, but will be constrained by the ontogenetic sequence of silica deposition at specific places) (Cox,
### Table 5.1 Overview of the strains used in the morphological and molecular phylogenetic analyses with their identification, origin and morphometric characteristics.

1 Geographic location in case the cultures were isolated by the authors from environmental samples; in the other case, the person who provided the culture is mentioned.

2 Morphometric measurements were performed on 10 oxidized specimens per culture, and are given as average ± the standard deviation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Taxon</th>
<th>Origin1</th>
<th>Length (µm)²</th>
<th>Width (µm)³</th>
<th># striae per 10 µm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B2)c</td>
<td><em>P. cf. microstauron</em></td>
<td>Antarctic Peninsula - Beak Island</td>
<td>38.9 ± 1.4</td>
<td>9.9 ± 0.3</td>
<td>14.0 ± 0.4</td>
</tr>
<tr>
<td>(B2)g10</td>
<td><em>P. cf. microstauron</em></td>
<td>Antarctic Peninsula - Beak Island</td>
<td>40.8 ± 1.2</td>
<td>10.7 ± 0.3</td>
<td>14.1 ± 0.5</td>
</tr>
<tr>
<td>(Ecrins4)a</td>
<td><em>P. cf. marchica</em></td>
<td>France – Les Ecrins</td>
<td>27.3 ± 0.4</td>
<td>5.4 ± 0.4</td>
<td>15.6 ± 0.7</td>
</tr>
<tr>
<td>(Ecrins4)d</td>
<td><em>Caloneis silicula</em></td>
<td>France – Les Ecrins</td>
<td>24.2 ± 2.1</td>
<td>8.3 ± 0.7</td>
<td>20.7 ± 1.0</td>
</tr>
<tr>
<td>(Enc2)a</td>
<td><em>P. viridiformis</em> Ehrenberg</td>
<td>Chile - Valle de Encanto</td>
<td>74.9 ± 1.8</td>
<td>17.7 ± 0.5</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>(Enc2)b</td>
<td><em>P. acrophaeria</em> W. Smith</td>
<td>Chile - Valle de Encanto</td>
<td>22.8 ± 1.3</td>
<td>10.6 ± 0.4</td>
<td>12.1 ± 0.3</td>
</tr>
<tr>
<td>(No5)j</td>
<td><em>P. isselana</em> Krammer</td>
<td>Norway - Bergen</td>
<td>31.2 ± 8.4</td>
<td>8.3 ± 0.4</td>
<td>12.0 ± 0.0</td>
</tr>
<tr>
<td>(Pinn C-Noel)7</td>
<td><em>P. sp.</em></td>
<td>Sub-Antarctic - Iles Crozet</td>
<td>11.8 ± 0.8</td>
<td>6.3 ± 0.3</td>
<td>13.8 ± 0.6</td>
</tr>
<tr>
<td>(Tor1)a</td>
<td><em>P. neomajor</em> Krammer</td>
<td>Chile - Torres del Paine</td>
<td>151.4 ± 3.6</td>
<td>24.9 ± 0.4</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>(Tor1)b</td>
<td><em>P. sp.</em> (divergens-group)</td>
<td>Chile - Torres del Paine</td>
<td>51.5 ± 2.1</td>
<td>10.4 ± 0.3</td>
<td>10.8 ± 0.8</td>
</tr>
<tr>
<td>(Tor1)g</td>
<td><em>P. neomajor</em> Krammer</td>
<td>Chile - Torres del Paine</td>
<td>102.8 ± 2.6</td>
<td>24.7 ± 1.3</td>
<td>8.3 ± 0.5</td>
</tr>
<tr>
<td>(Tor11)b</td>
<td><em>P. cf. altiplanensis</em></td>
<td>Chile - Torres del Paine</td>
<td>17.9 ± 0.5</td>
<td>4.5 ± 0.3</td>
<td>20.0 ± 0.0</td>
</tr>
<tr>
<td>(Tor12)d</td>
<td><em>P. borealis</em> Ehrenberg</td>
<td>Chile - Torres del Paine</td>
<td>36.8 ± 1.6</td>
<td>9.5 ± 0.5</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>(Tor3)a</td>
<td><em>P. borealis</em> Ehrenberg</td>
<td>Chile - Torres del Paine</td>
<td>27.3 ± 0.6</td>
<td>9.6 ± 0.3</td>
<td>6.0 ± 0.0</td>
</tr>
<tr>
<td>(Tor4)i</td>
<td><em>P. sp.</em></td>
<td>Chile - Torres del Paine</td>
<td>33.3 ± 1.3</td>
<td>9.4 ± 0.5</td>
<td>14.0 ± 0.0</td>
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<tr>
<td>(Tor4)r</td>
<td><em>P. sp.</em></td>
<td>Chile - Torres del Paine</td>
<td>42.6 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td>12.4 ± 0.5</td>
</tr>
<tr>
<td>(Tor7)c</td>
<td><em>P. sp.</em> (divergens-group)</td>
<td>Chile - Torres del Paine</td>
<td>88.4 ± 1.7</td>
<td>12.5 ± 0.4</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>(Tor7)f</td>
<td><em>P. sp.</em> (gibba-group)</td>
<td>Chile - Torres del Paine</td>
<td>61.2 ± 1.6</td>
<td>8.9 ± 0.2</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td>(Tor8)b</td>
<td><em>P. sp.</em> (gibba-group)</td>
<td>Chile - Torres del Paine</td>
<td>40.0 ± 0.5</td>
<td>5.9 ± 0.3</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>(Tor8)d</td>
<td><em>P. sp.</em> (gibba-group)</td>
<td>Chile - Torres del Paine</td>
<td>28.2 ± 0.5</td>
<td>5.6 ± 0.2</td>
<td>12.2 ± 0.6</td>
</tr>
</tbody>
</table>

Morphology of *Pinnularia*
<table>
<thead>
<tr>
<th>Strain</th>
<th>Taxon</th>
<th>Origin</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th># striae per 10 μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Val1)b</td>
<td><em>P. acrophaeria</em> W. Smith</td>
<td>Chile – Valdivia</td>
<td>45.7 ± 1.3</td>
<td>10.4 ± 0.3</td>
<td>11.3 ± 0.5</td>
</tr>
<tr>
<td>(W045)b</td>
<td><em>P. sp.</em> (gibba-group)</td>
<td>Sub-Antarctica - Amsterdam Island</td>
<td>37.1 ± 0.8</td>
<td>8.7 ± 0.2</td>
<td>11.2 ± 0.6</td>
</tr>
<tr>
<td>(W123)a</td>
<td><em>P. sp.</em></td>
<td>Sub-Antarctica - Amsterdam Island</td>
<td>23.5 ± 1.2</td>
<td>6.8 ± 0.1</td>
<td>13.7 ± 0.3</td>
</tr>
<tr>
<td>(Wie)a</td>
<td><em>P. sp.</em></td>
<td>The Netherlands – De Wieden</td>
<td>46.7 ± 0.9</td>
<td>9.7 ± 0.2</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>(Wie)c</td>
<td><em>P. subcapitata var. elongata</em> Krammer</td>
<td>The Netherlands – De Wieden</td>
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<td>6.3 ± 0.3</td>
<td>11.9 ± 0.3</td>
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<td><em>P. subcommutata</em> Krammer</td>
<td>Belgium – Ter Yde</td>
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<td>11.4 ± 0.4</td>
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<td><em>P. borealis</em> Ehrenberg</td>
<td>Prof. A. Poulíková</td>
<td>37.4 ± 1.3</td>
<td>8.2 ± 0.6</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Cal 769 B</td>
<td><em>Caloneis silicula</em> (Ehrenberg) Cleve</td>
<td>Prof. D.G. Mann</td>
<td>27.5 ± 2.9</td>
<td>10.5 ± 0.5</td>
<td>18.6 ± 0.5</td>
</tr>
<tr>
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<td><em>Caloneis silicula</em> (Ehrenberg) Cleve</td>
<td>Prof. D.G. Mann</td>
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<td>19.4 ± 0.5</td>
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<tr>
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<td>7.4 ± 0.3</td>
<td>12.1 ± 0.3</td>
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<tr>
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<td><em>P. subcommutata var. nonfasciata</em> Krammer</td>
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<td>50.1 ± 1.8</td>
<td>10.7 ± 0.1</td>
<td>11.8 ± 0.4</td>
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<td><em>P. subcommutata var. nonfasciata</em> Krammer</td>
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<td>11.8 ± 0.4</td>
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<td>28.4 ± 0.5</td>
<td>7.8 ± 0.3</td>
</tr>
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<td>11.5 ± 0.5</td>
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<tr>
<td>Pin 12 TM</td>
<td><em>P. parvulissima</em> Krammer</td>
<td>Prof. D.G. Mann</td>
<td>33.2 ± 2.4</td>
<td>8.9 ± 0.2</td>
<td>11.4 ± 0.5</td>
</tr>
<tr>
<td>Pin 649 K</td>
<td><em>P. sp.</em> (subcommutata-group)</td>
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<td>Pin 867 inv</td>
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<tr>
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<td>Pin 876 TM</td>
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<td>Prof. D.G. Mann</td>
<td>38.9 ± 2.6</td>
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<tr>
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<td>13.0 ± 0.8</td>
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Table 5.2 Summary of the 17 frustule and 3 plastid characters and their respective character states and codes used for the cladistic analyses.

* Structural, non-structural and plastid characters were selected. Characters of the frustule can be structural or non-structural. See the text for further details.

* Descriptions of the characters and character states can be found in Appendix 5.1.

* Coding refers to the code of the character states used in the cladistic analyses and mentioned in Table 5.3.

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<th>Character states</th>
<th>Coding</th>
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<td>Cuneate</td>
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<td>Path of external raphe slit</td>
<td>Curved</td>
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<td>Elongated drop-like</td>
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<td>Broad: &gt; 1/4</td>
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<td>Valve width: 1/1</td>
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<td>Radiate</td>
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<td>Transverse</td>
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<td>Convergent</td>
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<td>Transverse</td>
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<td>Alveolar opening length: proportion of half valve width</td>
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<td>2/3 - 1/4</td>
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<td>&lt; 1/4</td>
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Table 5.3 Taxon/character matrix of the 48 strains. The a priori determined ancestral states are given in the last line (“ancestral”).

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<td>(B2)g10</td>
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<td>(Ecrins4)d</td>
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</table>

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2010). Based on this, the morphological dataset was subdivided into three subsets: all frustule characters (characters 1-17 in Table 5.2), only structural frustule characters (3-6, 11-12, 15-17), and plastid characters (19-20), which were analysed separately and in combination. Cladistic analyses were performed in PAUP* 4.0b10 (Swofford, 2002) using a Maximum Parsimony (MP) heuristic search with branch swapping and tree bisection-reconnection (TBR) rearrangement followed by bootstrap analysis with 1,000 replicates. All characters were unordered and unweighted. Because a large number of most parsimonious trees was recovered in all analyses, strict consensus trees were computed, together with the consistency index (CI) and retention index (RI) of these trees and the single characters. The CI measures the relative amount of homoplasy in a character or cladogram (with CI=1 for non-homoplasious characters/cladograms), while the RI measures the amount of synapomorphy expected from a data set which is retained as synapomorphy on a cladogram (with RI=1 for strongly informative characters/cladograms).

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Additionally, to check the effect of exclusion of non-informative characters, we selected for every analysis the characters with a CI ≥ 5 or if the CI of that character was smaller than 5, an RI ≥ 5 as “informative” and used them subsequently in a second cladistic analysis. Afterwards, tree length, CI and RI were compared for both analyses (all characters/only informative characters) of the different character subsets. In addition, the CI and RI of the morphological characters were also computed based on a molecular tree constructed using five molecular markers as described in Souffreau et al. (submitted).

**Mantel tests**
To evaluate whether morphological and molecular distances between strains are significantly correlated we used Mantel tests (Mantel, 1967; Mantel & Valand, 1970). Mantel tests were computed using the ZT software tool (Bonnet & Van De Peer, 2002) with 100,000 permutations. The genetic distances of the concatenated molecular data set were derived from the most likely molecular tree by Treefinder (Jobb et al., 2004). The morphological distances between the strains were calculated as total distance by PAUP* 4.0b10 (Swofford, 2002) based on the taxon/character matrix (Table 5.3) for all subsets (all characters, and frustule, structural frustule and plastid characters separately and in pairwise combinations). The significance level was $p=0.05$.

**Ancestral state reconstruction**
Ancestral state estimations were performed by Mesquite 2.73 (Maddison & Maddison, 2010). As a backbone we used the most likely molecular tree recovered by the concatenated DNA analyses described in Souffreau et al. (submitted) incorporating all morphologically dissimilar strains per species (see Table 5.1). The tree was first pruned to delete outgroups and sequences from GenBank. In a phylogenetic tree, branch lengths are proportional to the amount of molecular evolution in the genes used to build the tree. Over the different branches, variations in the mutation rate will occur. Because the genes we used in our study are not assumed to be linked to the morphological characters we investigate, this rate variation was smoothed out by constructing an ultrametric tree in which branch lengths are roughly proportional to evolutionary time instead of to amounts of molecular evolution. The original
phylogenetic tree was therefore smoothed into an ultrametric tree using penalized likelihood (Sanderson, 2002, 2003). The same character coding was used as for the cladistic analyses, and ancestral state reconstructions were using both maximum parsimony (MP) and maximum likelihood (ML) methods with default settings.

5.3 Results

Strain selection
Analyses were performed on 48 morphologically different strains, 44 of which were identified as Pinnularia and 4 as Caloneis silicula (Table 5.1, Figs 5.1-50). A total of 31 morphological species (not including varieties) were recognized, of which only 15 could be assigned to a described species. Of the remaining 16 taxa, 11 could not even be assigned to a morphologically closely resembling taxon and can be considered as undescribed species. Table 5.1 gives an overview of the selected strains and their identification, origin and morphometric characteristics. Oxidized material of all strains is shown in Figs. 5.1-50.

Morphological characters
Table 5.2 gives an overview of the 20 selected characters and their character states. In total 17 frustule characters (Table 5.2; 1-17) and 3 cytological characters (Table 5.2; 18-20) were used and most characters had more than two character states (Table 5.2). For frustule characters, we discriminated between structural and non-structural wall characters as described above (see Materials and methods).

Cladistic analyses
All cladistic analyses resulted in a large number of most parsimonious trees, and therefore strict consensus trees (SCTs) were constructed based on these most parsimonious trees to examine the resulting patterns of relationships. Bootstrap values are given for the recovered clades but are in general very low. An overview of the recovered clades from the different analyses is given in Table 5.4. Length of the trees, CI and RI of SCTs and characters are summarized in Table 5.5.

Cladistic analysis of all characters revealed four clades in addition to a cluster containing the two stains of P. cf. microstauron (Fig. 5.51; Table 5.4). Three of the four clades corresponded closely to the clades recovered in the molecular phylogeny (Souffreau et
Figs 5.1-34 Oxidized material of the strains used for the cladistic analyses and reconstruction of the molecular-phylogenetic tree. Figs 1-6 show strains of Clade A. Figs 7-25 show strains of Clade B, which is subdivided into the “grunowii”-clade (Figs 7-11), the “nodosa”-clade (Figs 12-15) and the “subgibba”-clade (Figs 17-25). Figs 28-34 show one part of the strains of Clade C, belonging to the “borealis-microstauron”-clade (Figs 29-33) and two single lineages (Figs 28, 34). Figs 26-27 are drawings of the valve and plastid characters of the ancestral Pinnularia resulting from ancestral state reconstruction (ASR) (see text).

Fig. 1. Pinnularia sp. (divergens-group) (Tor7)c. Fig. 2. Pinnularia sp. (divergens-group) (Tor1)b. Fig. 3. Caloneis silicula (Cal 890). Fig. 4. Caloneis silicula (Cal 769). Fig. 5. Caloneis silicula (Cal 856). Fig. 6. Caloneis silicula (E4)d. Fig. 7. Pinnularia subanglica (Pin 650). Fig. 8. Pinnularia sp. (Tor4)i. Fig. 9. Pinnularia cf. marchica (Ecrins4)a. Fig. 10. Pinnularia grunowii (Pin 867). Fig. 11. Pinnularia grunowii (Pin 889). Fig. 12. Pinnularia acrosphaeira (Enc2)b. Fig. 13. Pinnularia acrosphaeira (Val1) b. Fig. 14. Pinnularia nodosa (Pin 885). Fig. 15. Pinnularia nodosa (Pin 888). Fig. 16. Pinnularia subcapitata var. elongate (Wie)c. Fig. 17. Pinnularia sp. (Tor4)r. Fig. 18. Pinnularia sp. (Pinn C-Noel)7. Fig. 19. Pinnularia sp. (W123)a. Fig. 20. Pinnularia sp. (subgibba-group) (Tor8)b. Fig. 21. Pinnularia sp. (subgibba-group) (Tor8)d. Fig. 22. Pinnularia sp. (subgibba-group) (Tor7)f. Fig. 23. Pinnularia sp. (subgibba-group) (W045)b. Fig. 24. Pinnularia sp. (subgibba-group) (Pin 12). Fig. 25. Pinnularia sp. (subgibba-group) (Pin 877). Fig. 26. Drawing of the valve characters of a hypothetical ancestral Pinnularia, as estimated by ASR. Fig. 27. Drawing of the plastid characters of a hypothetical ancestral Pinnularia, as estimated by ASR. Fig. 28. Pinnularia cf. altiplanensis (Tor11)b. Fig. 29. Pinnularia cf. microstauron (B2)c. Fig. 30. Pinnularia cf. microstauron (B2)g10. Fig. 31. Pinnularia borealis (Tor3)a. Fig. 32. Pinnularia borealis (Ecrins7)a. Fig. 33. Pinnularia borealis (Tor12)d. Fig. 34. Pinnularia sp. (Wie) a. Scale bar represents 10μm.
Morphology of *Pinnularia*
Figs 5.35-50 Oxidized material of the strains used for the cladistic analyses and reconstruction of the molecular-phylogenetic tree, showing the strains belonging to Clade C. Figs 35-41 show strains from the subclade “subcommutata”, Fig. 42 shows P. acuminata, and Figs 43-50 show strains from the subclade “viridiformis”. Fig. 35. Pinnularia sp. (Pin 873). Fig. 36. Pinnularia cf. isselana (Cal 878). Fig. 37. Pinnularia subcommutata var. nonfasciata (Corsea 10). Fig. 38. Pinnularia subcommutata var. nonfasciata (Corsea 11). Fig. 39. Pinnularia subcommutata var. nonfasciata (Corsea 6). Fig. 40. Pinnularia isselana (No5)). Fig. 41. Pinnularia subcommutata (Yde1)a. Fig. 42. Pinnularia acuminata (Pin 876). Fig. 43. Pinnularia sp. (subcommutata-group) (Pin 883). Fig. 44. Pinnularia sp. (subcommutata-group) (Pin 649). Fig. 45. Pinnularia neglectiformis (Pin 706). Fig. 46. Pinnularia viridiformis (Pin 870). Fig. 47. Pinnularia viridiformis (Enc2)a. Fig. 48. Pinnularia neomajor (Tor1)g. Fig. 49. Pinnularia neomajor (Tor1)a. Fig. 50. Pinnularia neomajor (Corsea 2). Scale bars represent 10 μm.
Table 5.4 Overview of the clades recovered in the strict consensus trees resulting from the MP heuristic analyses based on the different morphological subsets (all characters, frustule characters, plastid characters, structural frustule characters, structural frustule + plastid characters) when analyzing all selected characters (All char.) or only the “informative” characters (Inform.). The morphological clades are compared with the molecular clades of Souffreau et al. (submitted). An “x” designates the presence of all taxa (shaded in dark grey). Presence of additional strains (“x +”) and absence of strains (“x −”) compared to the molecular clades is indicated by light grey shading.

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al., submitted) and were termed the “grunowii”, “subcommutata-acuminata-viridiformis” and “subgibba” clade, respectively. The “grunowii” clade contained smaller strains with H-shaped plastids while the “subcommutata-acuminata-viridiformis” clade contained the more robust Pinnularia’s. The “subgibba” clade contained the “subgibba”-like Pinnularia’s, smaller Pinnularia’s characterized by ghost striae, together with (Tor7)c and P. nodosa which were placed separately in the molecular phylogeny. The “silicula-acrosphaeridia” clade contained Caloneis silicula and P. acrosphaeria and was never recovered as a single clade in the molecular phylogeny.

When plastid characters were left out of the analysis (Fig. 5.52; Table 5.4), the “grunowii” and “subgibba” clades were not recovered, and (Wie)a was included in the “subcommutata-acuminata-viridiformis” clade. Analysis of structural characters only (Fig. 5.53; Table 5.4) resulted in the “silicula-acrosphaeridia” and “subcommutata-acuminata-viridiformis” clade, but without Pin870 and Pin873. Interestingly, a “viridiformis”-subclade (identical to the molecular “viridiformis” subclade) was found inside this clade, separating the “viridiformis” strains from the “subcommutata” strains. Analysis of the structural and plastid characters only revealed the “silicula-acrosphaeridia” clade, and no clades were formed when using the three plastid characters only (Figs not shown).

**Characters supporting the morphological clades**

The “grunowii” clade [without (Tor4)i compared to the molecular clade] was supported by a bilateral fascia, a diamond-shaped central area and H-shaped plastids with two pyrenoids, none of which were synapomorphies. This clade disappears when plastid characters are discarded, indicating the importance of these characters to recover the relationship. The same applies to the “subgibba” clade (+ (Tor7)c and P. nodosa compared to the molecular clade) which was partly supported by a bilateral fascia, but also by linear plastids having each a single pyrenoid.

The two other clades were supported by structural characters and were therefore also recovered when plastid or non-structural characters were omitted. The “silicula-acrosphaeridia” clade was characterized by a transverse orientation of the apical striae and the synapomorphic small, distal opening of the alveoli. The characters supporting the “subcommutata-acuminata-viridiformis” clade were the undulate external raphe fissure, the non-drop-like central raphe endings, the intermediate length of the alveolar opening, and the
Fig. 5.51 Strict consensus tree (SCT) from the most parsimonious trees of the cladistic analysis based on all 20 morphological characters, with indication of the resolved clades. The presence of additional strains, or absence of strains, compared to the molecular clades is noted on the right of the cladogram and indicated by a star (*) on the tree.
Fig. 5.52 Strict consensus tree (SCT) from the most parsimonious trees of the cladistic analysis based on the 17 frustule characters, with indication of the resolved clades. The presence of additional strains, or absence of strains, compared to the molecular clades is noted on the right of the cladogram and indicated by a star (*) on the tree.
Fig. 5.53 Strict consensus tree (SCT) from the most parsimonious trees of the cladistic analysis based on the 9 structural frustule characters, with indication of the resolved clades. The presence of additional strains, or absence of strains, compared to the molecular clades is noted on the right of the cladogram and indicated by a star (*) on the tree.
absence of pyrenoids. Because all these characters, except for pyrenoids, were structural characters, this clade is also supported in the cladistic analysis of those characters. Support for the “viridiformis” subclade in the structural character analysis resulted from the synapomorphic round central raphe endings, undulate external raphe fissures and complex raphes or raphes with 3 visible lines.

Effect of deleting non-informative characters
Table 5.5 summarizes the CI and RI of the different characters from all analyses. Because none of the plastid characters was recovered as “informative” in the first analysis based on the CI and RI, a subsequent analysis including only “informative” characters was impossible. In all analyses the most informative characters (CI=1, RI=1) were the helictoglossa, surface irregularities, alveolus opening and location, and girdle band areolae, all structural frustule characters. Stria orientation and the number of pyrenoids had a CI of 0.5. The CI of all other characters was lower than 0.5. However, selecting only the more “informative” characters based on the CI and RI had a negative influence on the number of clades obtained (Table 5.4), probably partly due to the even lower number of characters included in the analysis. This shows that characters with low CI and RI can still make a valuable contribution to the recovery of relationships.

When analyzing the morphological character matrix using the topology of the molecular tree, characters with a CI≥0.5 were again the helictoglossa, surface irregularities, alveolus opening and location, girdle band areolae and number of pyrenoids, but additionally also central raphe endings, raphe complexity and fasciae. Again, most of these features are structural characters. Note that stria orientation had a low CI when based on the molecular tree topology, and that the molecular topology was much less parsimonious (see the higher tree length in Table 5.5) and thus contained many more changes in character states of the morphological characters over the tree, compared to the MPTs resulting from the maximally parsimonious cladistic analyses (Table 5.5).

Ancestral state reconstruction
Fig. 5.54 shows ultrametric molecular phylogenies on which the character states for the different morphological characters are plotted, together with the probable states (ML) of the ancestral
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Table 5.5 Summary of the CI and RI values of the morphological characters based on the strict consensus trees, and CI, RI and length of the strict consensus tree (SCT) and most parsimonious trees (MPT’s) for all cladistic analyses, subsets of characters and using all characters (all char.) or only the informative characters (informative char.), and based on the topology of the molecular phylogeny (molecular tree). Values ≥ 0.5 are given in black and bold, values < 0.5 are given in grey. Open spaces designate characters that were not used in the respective subsets. Light-grey shading indicates characters that were not informative and were excluded during subsequent analyses using informative characters only.

\(^{1}\) See Table 5.2 and Appendix 5.1 for more information on the characters.
<table>
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<th>Structural frustule characters</th>
<th>Plastid characters</th>
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<td>Inf.&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>RI</td>
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1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  |

0.5  | 0.9  | 0.5  | 0.9  | 0.7  | 1.0  | 0.1  | 0.2  | 0.7  | 0.6  | 0.1  | 0.2  |

1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 0.5  | 0.8  | 0.5  | 0.8  | 1.0  | 1.0  | 0.5  | 0.4  |

1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  |

0.1  | 0.0  | 0.2  | 0.7  | 0.1  | 0.2  | 0.1  | 0.0  | 0.3  | 0.3  | 0.1  | 0.1  |

0.1  | 0.0  | 0.3  | 0.8  | 0.1  | 0.1  | 0.3  | 0.3  | 0.7  | 0.6  | 0.1  | 0.1  |

0.4  | 0.8  | 0.5  | 0.9  | -  | -  | 0.1  | 0.0  | -  | -  | 0.1  | 0.2  |

0.8  | 1.0  | 0.8  | 1.0  | 0.5  | 0.8  | 0.8  | 1.0  | 0.4  | 0.8  | 0.6  | 0.9  | 1.0  | 1.0  | 0.4  | 0.8  |

| 32  | 21  | -  | 44  | -  | 116 | 4  | -  |

| 15  | 13  | 25  | 5  | 11  | 26  | 4  | 36  |

<sup>7</sup> All= All characters belonging to the above-mentioned character subset were included during the cladistic analysis

<sup>8</sup> Inf.= Informative characters; only the informative characters (based on the CI and RI of the analysis with all characters of the specific subset) were included during the cladistic analysis

<sup>9</sup> Mol.= Molecular tree; the topology of the molecular tree of Souffreau et al. (submitted) was used to determine the CI and RI values of the different morphological characters

<sup>10</sup> SCT= Strict consensus tree, <sup>11</sup> MPT= Most parsimonious tree
nodes. Based on the ancestral state reconstruction (ASR) using both MP and LM, ancestral *Pinnularia* (see Figs 5.26-27) had valves with convex margins and rounded apices, a simple raphe with curved external path with elongated central raphe endings, an intermediate width axial area mid-branch, but narrow axial area by the central raphe endings. The central area was elliptical and a bilateral fascia was present, without ghost striae or surface irregularities. Striae were radiate at the centre, convergent near the apices, with large internal alveolar openings. The helictoglossa was simple, tongue-like and the girdle bands had elongated areolae. Plastids would have been linear with broad extensions and one pyrenoid per plastid (Fig. 5.27). These recovered ancestral states were also estimated a priori by us as being the primitive frustule character states (see Table 5.2 and 5.3) based on the morphology of the fossils reported by Lohman & Andrews (1968), except for the rounded apices and the presence of a fascia. Most of the fossil species of Lohman & Andrews (1968) had cuneate apices rather than rounded ones and no fascia, however, both alternative character states were also present in a single species. The fossil *Pinnularia* reported as being the earliest representative lacked a fascia and even had rostrate rather than rounded apices, and surface irregularities.

Based on this ASR, most of the character states evolved multiple times during the evolution of *Pinnularia*, while other character states evolved only once, thereby often characterizing a single molecular clade. Of the 20 characters studied, only 8 contained character states which evolved once in the present phylogeny (central raphe endings, helictoglossae, girdle band areolae, surface irregularities, raphe complexity, length and location of alveolus openings, and pyrenoids). Round central raphe endings were synapomorphic for the "viridiformis" clade (Fig. 5.54, character 4), while linear central raphe endings evolved at the base of the cluster containing the "subcommutata-acuminata-viridiformis" clade, (Wie)a and (Tor11)b. *Caloneis silicula* also has linear central raphe endings, but note that this linearity contrasts with the first mentioned linear raphe endings (SEM photographs: Appendix 2, n-n´). Hooked helictoglossae and round girdle band areolae were synapomorphic for *Caloneis silicula*, while the presence of surface irregularities only evolved in *P. acrosphaeria* (Figs not shown).

Raphes with three parallel lines visible in LM (Appendix 5.2, r) or three twisting lines (complex raphe, Appendix 5.2, s) evolved
once at the base of the “viridiformis” clade (Fig. 5.54, character 6). However, the complex, twisting raphe structure evolved multiple times within this clade. Because we also observed raphes with a complex, twisting structure and with three parallel lines within monoclonal strains, those two characters states are probably part of a continuum and should be regarded as a single character. Intermediate alveolus openings were synapomorphic for the “subcommutata-acuminata-viridiformis” clade (Fig. 5.54, character 15), while small and distantly located alveolar openings occurred in both *P. acrosphaeria* and *C. silicula*. Also the absence of pyrenoids evolved at the base of the “subcommutata-acuminata-viridiformis” clade (Fig. 5.54, characters 20).

**Mantel tests**

Mantel tests between the morphological pairwise distance matrices and the molecular distance matrices revealed significant correlations between the datasets (*p*=0.00001) for all morphological subsets.

**5.4 Discussion**

Independent phylogenetic analyses of molecular and morphological data from 48 morphologically different strains of the genera *Pinnularia* and *Caloneis* recovered similar clades, and morphological and molecular distances were significantly correlated. However, while phylograms based on five molecular markers were almost fully resolved (Souffreau *et al.*, submitted), morphological data generated fewer clades, due to the lower number of phylogenetically informative characters compared to the molecular data, and was never able to fully resolve the interspecific relationships of *Pinnularia*. The phylograms give insight into the phylogenetic patterns of the different characters states and, while predominantly non-structural frustule characters were found to be homoplasious, several structural and plastid characters showed a phylogenetic pattern and were therefore informative in reconstructing the interspecific relationships. However, excluding the characters with low CI and RI in a subsequent analysis did not improve the resolution of the phylogeny. Inferences of the most likely ancestral states of characters in the different clades of *Pinnularia* are fairly congruent with the currently known sequence of appearances in the fossil record.

Our molecular and morphological datasets supported similar clades. Most of the clades retrieved by the morphological data were
Fig. 5.54 Ultrametric trees of the molecular-phylogenetic tree constructed as described in Souffreau et al. (submitted) using 18S, 28S, rbcL, psbA and cox1, with indication of the ancestral state reconstruction of 18 of the 20 morphological characters using maximum likelihood. Likelihood of each characters state at each node is given proportionally by a shaded circle. Shading legend is given for each tree separately. Bootstrap support – Posterior probability is given for the first tree (character 1).
Morphology of *Pinnularia*
Morphology of *Pinnularia*

Char. 7: Axial area: relative width at mid-branch

Char. 8: Axial area: relative width at central raphe endings

Char. 9: Fascia

Char. 10: Central area: shape
Morphology of *Pinnularia*
Morphology of Pinnularia

Char. 16: Opening of alveoli: location
Char. 18: Plastid: shape
Char. 19: Plastid: extension under valve surface
Char. 20: Pyrenoids: number per plastid
also recovered by molecular-genetic analyses: the “grunowii” and “subgibba” clades, and the “viridiformis” and “subcommutata” clades; and congruence was emphasized by the significant correlation of genetic and morphological distances using Mantel tests. Congruence between morphological and molecular data has been encountered in a wide variety of organisms (e.g. Marvaldi et al., 2002; Scotland et al., 2003; Edgar & Theriot, 2004; Lundholm et al., 2006; Jacobs et al., 2008) and retrieving independent support for clades strengthens the plausibility of the phylogenetic hypotheses (Patterson, 1982). However, as in our study, most comparative studies find lower resolution in morphological data compared to molecular sequences (Wortley & Scotland, 2006). Morphological analyses could not assign all taxa to a clade, and different clades retrieved in the molecular analysis could not be recovered with the morphological dataset, e.g. the kinship of the P. borealis strains and the affinity between P. nodosa and P. acrosphaeria, nor were the deeper relations between the clades resolved. Moreover, bootstrap support was never high. The low resolution and support of morphological phylogenies is likely caused by the low number of morphological characters (Scotland et al., 2003; Giribet, 2010) and, because of the low character/taxon ratio, bootstrap percentages of morphological studies are inherently low (Bremer et al., 1999). Meanwhile, difficulties are also encountered with molecular data when seeking to recover relationships with a small number of nucleotides or non-informative markers (Rokas et al., 2003). For the current molecular phylogeny of Pinularia five different markers (covering 4852 nucleotides, including 1012 parsimony-informative sites) were needed to obtain support at the deeper levels (Souffreau et al., submitted).

In this study we used 20 morphological characters, mostly with two or three character states, which is low compared to the 48 taxa studied. Only discrete variables were used, but incorporating continuous data for example for size measurements and shape could increase the number of characters and enhance the phylogenetic resolution. Morphometric data are known to improve phylogenetic estimates (Edgar & Theriot, 2004) and new algorithms have been devised to analyze continuous data (Wiens, 2001; Goloboff et al., 2006; Gonzalez-Jose et al., 2008). Nevertheless, at the intrageneric level relatively low variation in morphology is to be expected since diatom genera are delimited based on morphological similarity and homogeneity (Kociolek, 1997). Cladistic analyses are mostly successful at intergeneric levels (Kociolek & Stoermer, 1986; Cox...
Morphology of Pinnularia (Marivaux et al., 2000; Cox & Williams, 2006; Ohl & Spahn, 2010), while using only morphological data to estimate interspecific relationships can be quite problematic (Bleidorn et al., 2009; Denk & Grimm, 2009; Ohl & Spahn, 2010) as is further demonstrated here. It has been argued that incorporating as much data as possible from different sources, including morphological, biochemical, ecological and molecular ones, best approximates the true phylogeny (Wiens, 1998). Combining different datasets - be it different genetic markers or morphological and molecular data - improves the resolution and support of phylogenies due to amplification of shared historical signals (Marvaldi et al., 2002; Edgar & Theriot, 2004; Wortley & Scotland, 2006; Lee & Camens, 2009), while examining data partitions independently gives insight into the differences in their phylogenetic histories (Wiens, 1998) and the phylogenetic informativeness of different subsets of characters (Cox & Williams, 2006).

We analyzed three different subsets of morphological characters: plastid, frustule appearance (LM) and frustule structure (SEM). Partitioning of the morphological characters affected the number of resolved clades. Frustule structures contained most of the phylogenetically informative characters (external raphe path, central raphe endings, raphe structure, alveolus opening). However, excluding plastid characters prevented the resolution of the “subgibba” and “grunowii” clades, supporting the phylogenetic value of plastid characters emphasized by Cox (1988) and Cox & Williams (2000). The highest number of resolved clades was obtained by analyzing all characters simultaneously, confirming the positive effects of combining datasets.

Based on the cladistic analyses, the most informative characters (CI=1, RI=1) for Pinnularia were the helictoglossa, girdle band areolae, surface irregularities, and alveolus opening and location. The first three features are apomorphic for Caloneis silicula and P. acrosphaeria and were not informative for resolving other interspecific relationships. There was however an interesting phylogenetic pattern in alveolus opening as the “subcommutata-acuminata-viridiformis” clade is characterized by a synapomorphic intermediate alveolus opening length. Krammer & Lange-Bertalot (1985) used the alveolus openings to distinguish three groups within the Pinnularia/Caloneis complex: open alveoli (in our terminology having a length of > 1/3 valve width), half-closed alveoli (1/8 < x < 1/3; intermediate length), and almost completely closed alveoli (< 1/8...
valve width), seen in LM as more central or marginal longitudinal bands (Krammer, 2000). The clade characterized by intermediate alveolus opening length in our phylogenies corresponded to their group of “majores & complexae, P. gibba, C. amphisbaena, C. latiuscula”. The two other groups were not recovered. Of course, we did not sample all *Pinnularia* and *Caloneis* species and further taxon sampling is needed to support this preliminary result.

Based on both the morphological and molecular analyses, homoplasious characters include valve margins, apical shape, stria orientation (both central and apical), all non-structural characters, and plastid extension. All other characters showed some phylogenetic pattern. Some non-structural features were important for the delineation of phylogenetic groups, such as the axial area and presence of fascia for the “subgibba” clade, but on the whole structural characters were more informative. The number of pyrenoids also showed an interesting pattern. Absence of pyrenoids in the *P. viridis* group had already been noted by Schmid (2001) and we retrieved this in our “subcommutata-acuminata-viridiformis” clade. Cox (1988) had indicated the possible phylogenetic significance of pyrenoids in this genus, and also remarked the great variety in plastid shape. Similarly, we found taxa with linear and H-shaped plastids. However, it was not always clear whether H-shaped plastids were truly single chloroplasts, or the result of integration of the outer plastid membranes (pers. comm. David Mann). This can only be resolved by thorough examination of the complete cell-cycle and/or TEM observations. Therefore, it is not clear whether the H-shaped plastids characteristic of the “grunowii” clade are homologous to the H-shaped plastid of *C. silicula* or *P. microstauron* (see Figs in Appendix 2, q-q’). It should be noted that the plastids of the “grunowii” clade and *P. cf. microstauron* have two pyrenoids, compared to one pyrenoid in *C. silicula*, which would indeed suggest a two-plastid origin for the former.

Problems in establishing homology were also encountered with respect to the elongated central raphe endings of the “viridiformis” clade and *C. silicula* (compare Appendix 5.2, n and n’), and in the short alveolar openings of *P. acrosphaeria* and *C. silicula*. Determination of homology of morphological characters is one of the most problematic issues in cladistics (Pimentel & Riggins, 1987; Scotland & Pennington, 2000; Wagner, 2001; Rieppel & Kearney, 2002) and requires a careful examination of developmental patterns (Maximino, 2008; Cox, 2010) and an understanding of the processes
underlying morphological evolution (Scotland et al., 2003). However, in diatoms developmental pathways have not been investigated for most frustule and plastid characters (but see Cox, 1999).

The patterns derived from phylogenetic analyses can also be used to reconstruct hypotheses of historical evolutionary patterns (Pagel, 1997). Ancestral state reconstruction (ASR) is a statistical method using MP or ML to estimate the character states of ancestral nodes based on the present distributions (Cunningham et al., 1998). In our analysis, the most parsimonious and most likely ancestral states for Pinnularia were mostly congruent with the morphology of the earliest known fossils (Lohman & Andrews, 1968; Souffreau et al., submitted), except for the round apices and bilateral fasciae, although a single fossil species (P. spatula Lohman & Andrews) did have a fascia. Complex raphe systems and intermediate alveolar openings, which are characteristic for the “subcommutata-acuminata-viridiformis” clade, are all derived character states for the genus, and this is in agreement with the presently known fossil sequence. Based on the fossil record, the larger viridis-like taxa are suggested to have evolved between the late Eocene and middle Miocene (Lohman & Andrews, 1968; Ognjanova-Rumenova & Vass, 1998). It is important to remember that phylogenies are only hypotheses of genealogies, and consequently inaccuracies in phylogenies will influence the reliability of the ASR (Pagel, 1997). The most problematic drawback of the present phylogeny of Pinnularia is the low taxon sampling. However, it provides a first estimate of the interspecific relationships and broader clades within this large genus, and further taxon sampling of Pinnularia and Caloneis will undoubtedly improve these preliminary phylogenetic estimates.

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Appendix 5.1
Description of and comments on the selected frustule and plastid characters

Frustule outline
1. Margins: convex (0); parallel (1), undulate (2); constricted in the middle (3); inflated in the middle (4).
   The margins of the frustules in valve view were delimited from halfway along one raphe branch, beside the central area, to halfway along the second raphe branch. The character, margin, therefore describes the central portion of the valve. The relationship between the opposite margins can be convex (App. 2, a), parallel (App. 2, b), undulate (App. 2, c), constricted in the middle (App. 2, d) or inflated in the middle (App. 2, e).
2. Apices: cuneate (0); rounded (1); rostrate (2); capitate (3).
   The apex is defined as the apical part of the valve, from halfway along each raphe branch. In valve view, the outline of the apices can be abruptly narrowing toward the tip, like a wedge (cuneate; App. 2, f) or smoothly rounded (rounded; App. 2, g). Valve apices that have undulating margins towards the apex can be divided into two groups: the valve may narrow, but never become narrower than the apex itself (rostrate; App. 2, h), or it may narrow and then widen again towards the apex itself, forming a distinct off-set distal part or “head” (capitates; App. 2, i).

Raphe characters
3. Path of the external raphe fissure: curved (0); undulate (1).
   The line of the raphe slit which is visible on the outer side of the valve using SEM is termed the external raphe fissure. This fissure can be continuously curved to one side of the valve only (curved; App. 2, j), or can switch its course between the two sides of the valve, and undulate over the valve (undulate; App. 2, k).
4. Central raphe endings: elongated drop-like (0); round (1), linear (2).
   The central raphe endings were observed using SEM and can be elongated and drop-like (App. 2, l), round (App. 2, m) or linear (App. 2, n-n’).
5. Helictoglossa: tongue-like (0); hooked (1).
   The helictoglossa (internal, apical raphe ending) was observed using SEM and can have an elongated, tongue-like form (tongue-
Morphology of *Pinnularia*

6. **Raphe complexity**: simple (0); three lines visible (1); complex (2).

In cross section, the raphe slit has a parallel “groove” and a “tongue” (see Krammer 1992 Plate 2 figs 1-4), which fit one in the other (App. 2, t). The edge of the tongue and the two edges (external and internal) of the groove are visible in LM as thin, longitudinal lines. The number of visible lines depends on the relative positions of these margins. Usually, the outer and inner margins of the groove are superimposed and visible as a single line. In this case, two thin lines are visible: the groove margins and the tongue edge. Using LM, the course of the groove margin can be compared with the straight course of the tongue margin. If the groove margin follows the course of the tongue margin without crossing it, the raphe is termed “simple” and two thin, parallel lines are visible (App. 2, q). If the groove margin crosses the straight tongue margin at different points along the course of the raphe, the raphe is classified as “complex” (App. 2, s). A special case of the simple raphe occurs when the two margins of the groove are not superimposed but are visible as separate lines. In LM, three non-crossing, thin lines are then visible (two lines of the groove and one line of the tongue). Because of the underlying morphological difference, we classified this as a separate character state (three thin lines; App. 2, r).

**Axial area**

7. **Axial area**: relative width at mid-branch: intermediate: 1/4 – 1/6 (0); narrow: < 1/6 (1); broad: > 1/4 (2).

The relative width of the axial area was determined halfway along the raphe branches in LM and compared with the total width of the valve at the same position.

8. **Axial area**: relative width at central raphe endings: narrow: < 1/2 (0); broad: between 1/2 and 1/1 (1); valve width: 1/1 (2).

The relative width of the axial area was determined at the junction between the central raphe ending and the raphe branch in LM, and compared with the total width of the valve at the same position.

**Central area**

9. **Fascia**: absent (0); unilateral (1); bilateral (2).

The central striae can be infilled (solid), thus forming a fascia. A fascia can be absent (App. 3, a), only present on one side of a valve.
14. Morphology of Pinnularia

(unilateral; App. 3, b), or present at both sides of a valve (bilateral; App. 3, c). If both unilateral and bilateral fasciae were present on different valves of a single strain, we classified this strain as having a “bilateral” fascia.

10. Central area: shape: elliptical (0); diamond (1); rectangular (2).

We delimited the central area by the junctions between the central pores and the raphe branches, thus including the central pores. The shape of the central area is determined by the arrangement of the adjacent striae. The striae can lengthen very gradually towards the apices, forming a convex edge and creating an elliptical central area (App. 3, d), or can lengthen more abruptly, forming a concave rim and creating a diamond-shaped central area (App. 3, e). If striae are absent or no variation in stria length is observed, the central area is rectangular (App. 3, f).

Surface

11. Ghost striae: absent (0); present (1).

Ghost striae are partially silicified striae that have been incompletely filled-in during the ontogenesis. No areolae are present, but the outlines of the striae are more or less visible and create parallel darker shadows. Ghost striae are for example typical for the gibba-group of Pinnularia and can be seen as four spots in the central area (indicated by arrows in App. 3, g).

12. Surface irregularities: absent (0); present (1).

On the outside of the valve, the central and axial areas can be covered with irregular silicified wrats (App. 3, h).

Striae

13. Stria orientation: centre: radiate (0); transverse (1).

The striae at the centre of the valve can be oriented parallel to the transapical axis of the valve (transverse; App. 3, j) or oblique to the transapical axis of the valve, the proximal (to the raphe) ends of the striae being closer to the valve centre than the distal ends (radiate; App. 3, i).

14. Stria orientation: apex: convergent (0); radiate (1); transverse (2).

The striae near the valve apices can be oriented parallel to the transapical axis of the valve (transverse; App. 3, k). They can also be oblique to the transapical axis of the valve, distal ends of the striae being closer to the valve centre than the proximal ends (convergent; App. 3, i), or the proximal (to the raphe) ends of the striae being
closer to the valve centre than the distal ends (radiate; App. 3, l).

Alveoli

15. **Alveolus opening length:** proportion of half valve width: > 2/3 (0); 2/3 – 1/4 (1); < 1/4 (2).

The alveoli open on the inner side of the valves and are visible using SEM as openings in the chamber roofs above the striae. The length of the alveolus opening was determined halfway along the raphe branches and was calculated relative to the distance between the raphe slit and the valve margin at the same location. These proportions could be > 2/3 (App. 3, m); 2/3 – 1/4 (App. 3, n); < 1/4 (App. 3, o)

16. **Alveolus opening:** location: central (0); distal (1).

Alveolus openings located halfway between the margin and the raphe are termed “central” (App. 3, m-n), while alveolus openings located adjacent to the valve margins are termed “distal” (App. 3, o).

Girdle bands

17. **Girdle bands:** shape of areolae: elongated (0); round (1).

The girdle bands were visualized using SEM and bear a single row of areolae. Areolae can be elongated or roundish in shape.

Plastids

18. **Plastid: shape:** linear (0); H-shaped (1).

Plastids were observed in non-synchronized, exponentially growing cultures using LM and confocal laser scanning microscopy (CLSM). Cells could contain two large, linear plastids lying along each side of the girdle and extending under the valves (linear; App. 3, p-p’’), or a single plastid, comprising two linear plates lying along the girdle and extending under the valves, connected by a narrow central bridge against one valve face, above the nucleus (H-shaped; App. 3, q-q’’). As this bridge can be difficult to see using conventional LM, CLSM was used to confirm its presence. Strains were categorized as having an H-shaped plastid if a bridge was observed in all examined cells.

19. **Plastid:** extension under valve face: narrow (0); broad (1).

Both linear and H-shaped plastids have margins that extend under the valve face. The degree to which the margins extended was described as “narrow” if it was less than or equal to 1/4 valve width,
and described as “broad” if it occupied more than 1/4 of the valve width.

20. **Pyrenoids:** number per plastid: one (0); two (1); absent (2).

Pyrenoids are proteinaceous bodies located in the plastid, which contain high levels of the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase, which mediates CO2-fixation. No storage material is present around the pyrenoids in diatoms. Using regular LM or CLSM, pyrenoids were visible as pale (LM) or non-fluorescent (CLSM) regions in the plastid (indicated by arrows in App. 3, p-q’). We used additional staining with Azocarmine G to improve visualization of the pyrenoids in LM. If no staining was observed, the strains were classified as lacking pyrenoids (absent).
Appendix 5.2

Supporting figures for the description of the morphological characters.
Appendix 5.3

Supporting figures for the description of the morphological characters.
6. Molecular evidence for the existence of distinct Antarctic lineages in the cosmopolitan terrestrial diatoms *Pinnularia borealis* and *Hantzschia amphioxys*

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Abstract
Recent studies based on morphology revealed that the Antarctic continent has a high percentage of endemic freshwater diatom species, besides a considerable percentage of presumed cosmopolitan species. Given the widespread (pseudo)cryptic species diversity in diatoms, we used two of these cosmopolitan taxa, *Pinnularia borealis* and *Hantzschia amphioxys*, to assess the molecular divergence between Antarctic strains and strains from other locations. Molecular phylogenies based on the plastid gene *rbcL* and the nuclear 28S rDNA (D1-D2 region) revealed that *P. borealis* and *H. amphioxys* are two species complexes consisting of multiple genetically distinct lineages, each including a distinct Antarctic lineage. A molecular time-calibration estimates the origin of the species complex *P. borealis* 35.6 million years (Ma) ago, and the first extant lineage splitting at 22.0 Ma, making this the oldest known diatom species complex. The Antarctic *P. borealis* lineage is estimated to have diverged 7.7 (15-2) Ma ago from a European sister lineage, largely after the final opening of the Drake Passage which resulted in geographical isolation of the continent, and after the start of the Mid-Miocene cooling event which resulted in the formation of a permanent ice sheet and full polar conditions. In addition, the Antarctic lineages of both *P. borealis* and *H. amphioxys* diverged physiologically from most lineages from more temperate regions and tend to have a higher relative growth rate at low temperature, a lower optimal temperature and lower lethal upper temperature, indicating niche differentiation. Taken together, these data indicate that many of the presumed cosmopolitan Antarctic diatom species are in fact species complexes, possibly containing Antarctic endemic species.

Key words: thermal adaptation, *rbcL*, LSU rDNA, cryptic diversity, diatoms, biogeography, Antarctica, dispersal limitation, allopatric speciation
6.1 Introduction

The Antarctic continent is a geographically and thermally isolated area. Since the breakup of Gondwana 180 Ma ago it gradually drifted away from other land masses, resulting in a strong geographical isolation. During the Eocene (50-32 Ma), the gradual opening of the ocean between Antarctica and Australia respectively South America triggered the establishment of circumpolar winds and ocean currents which blocked heat transfer from the tropics. This resulted in the thermal isolation of the Antarctic continent and the formation of a continental ice sheet (Lawver & Gahagan, 2003; Barker & Thomas, 2004; Brown et al., 2006). Since at least 14 Ma, the Antarctic continent is therefore a cold and windy desert, characterized by extreme climatic conditions constituting an ecological challenge for any resident or colonizing organism (Block et al., 2009). The remaining ice-free regions are small and highly scattered, especially in continental Antarctica, and strongly influenced by glacial cycles, resulting in high regional extinction risks and low colonization rates (Pugh & Convey, 2008). As a result, the Antarctic region, and especially continental Antarctica, is characterized by a highly impoverished flora and fauna (Lewis Smith, 1984) and a high incidence of endemism in both freshwater and terrestrial habitats for macroscopic organisms such as dipterans (Currie & Adler, 2008), pycnogonids (Munilla & Membrives, 2009) and Collembola and Oribatida (Greenslade, 1995; Marshall & Pugh, 1996). Based on discontinuities in species distributions for several of the above taxonomic groups, the continent is subdivided into two biogeographic regions, namely the continental and maritime Antarctic regions, which are separated by the so-called Gressitt line situated in the South of the Antarctic Peninsula (Chown & Convey, 2007).

For microalgae, inferring species’ geographic distributions was until recently hampered by undersampling and taxonomical problems due to the limited number of available morphological characters and the uncertain interpretation of small morphological differences. For terrestrial algae, Broady (1996) concluded that the Antarctic diversity is low, and lower in continental than in maritime Antarctica, with only a small element of endemic species. This conclusion is now contradicted by molecular phylogenies with separate Antarctic lineages of cyanobacteria and green algae, suggesting a long history of isolation on the continent (Taton et al., 2003; Taton et al., 2006; De Wever et al., 2009). For lacustrine diatoms, a substantial body of work in the (sub-)Antarctic based on careful
morphological analysis showed (1) a highly impoverished diatom flora at high latitudes of the Southern hemisphere (Vyverman et al., 2007), (2) an overrepresentation of typical terrestrial genera and poverty of globally successful genera and planktonic taxa (Verleyen et al., submitted), (3) a clear subdivision into three biogeographic provinces, being the sub-, maritime, and continental Antarctic (Verleyen et al., submitted), and (4) a high percentage of (sub-) Antarctic endemic species (27-63%, Verleyen et al., submitted) and little overlap with the Arctic flora (Van de Vijver et al., 2005). Together, this suggests that colonization is limited on both a regional and continental scale, and that allopatric speciation, or lineage divergence under influence of genetic drift and/or divergent selection during long-term geographic isolation (after rare long-term dispersal events and/or habitat fragmentation due to climate change or continental drift) (Mayr, 1963; Coyne, 1992; Rice & Hostert, 1993), is responsible for the diversification of both Antarctic macro- and microorganisms, contrary to the hypothesis that microorganisms have unlimited dispersal probabilities (Beijerinck, 1913; Finlay, 2002; Finlay et al., 2002).

However, besides this high number of endemic morphospecies, there also appears to be a substantial fraction of cosmopolitan diatom morphospecies on the Antarctic continent (Verleyen et al., submitted). How this should be interpreted is unclear, given the prevalence of (pseudo)cryptic species diversity in diatoms (e.g. Sarno et al., 2005; Evans et al., 2008; Trobajo et al., 2009). They might be truly generalistic, cosmopolitan species which have (recently) colonized the Antarctic, or it might concern cryptic species complexes containing genetic lineages with potentially restricted geographic distributions (Boo et al., 2010) and ecological niches (Vanelslander et al., 2009; Vanelslander et al., unpubl.). In that respect, it is well-known that many Antarctic algae have evolved special ecophysiological properties in response to the extreme climatological conditions (Kirst & Wiencke, 1995). For instance, several marine diatoms are obligately psychrophilic: optimum temperatures are at 3°-5° C and temperatures above 6°-8° C are lethal (Fiala & Oriol, 1990). Cryptic lineages (or species, de Queiroz, 2007) are often identified using molecular phylogenies based on species-level molecular markers (e.g. De Vargas et al., 1999; Beszteri et al., 2007; Boo et al., 2010). When sufficiently resolved, such phylogenies can be time-calibrated using the fossil record, feasible for diatoms given their resistant siliceous cell walls (Sims et al., 2006), and combined with geographical distributions and niche
characterization to obtain a better understanding of the evolutionary history of these (cryptic) lineages (see e.g. Darling et al., 2007; Verbruggen et al., 2009).

The diatom morphospecies *Pinnularia borealis* Ehrenberg and *Hantzschia amphioxys* (Ehrenberg) Grunow are two common taxa in mainly terrestrial habitats on all continents, including Antarctica (Krammer & Lange-Bertalot, 1986, 1988). For both taxa, some morphological variants have been described as varieties, but there is morphological overlap and as a result their taxonomic status is uncertain (Cleve-Euler, 1952; Krammer & Lange-Bertalot, 1986; Krammer, 2000). In this study, we (1) test whether both taxa are species complexes with a distinct Antarctic lineage or not, (2) estimate divergence times for any such lineage(s) in *P. borealis* based on a time-calibrated *Pinnularia* phylogeny (Souffreau et al., submitted), and (3) determine the temperature preferences of Antarctic strains in comparison to their more temperate counterparts. To this end, we isolated monoclonal strains of *P. borealis* and *H. amphioxys* from continental Antarctica and (cold) temperate regions in Europe, the Americas and Asia. These were used to construct molecular phylogenies based on *rbcL* and the D1-D2 region of 28S rDNA, calculate lineage divergence times using molecular-clock methods for *P. borealis*, and assess strain growth rates over a temperature gradient from 3°-32°C.

### 6.2 Materials & Methods

#### Taxon sampling

We used 52 monoclonal strains of *Pinnularia borealis* (Table 6.1) and 31 of *Hantzschia*, including *H. amphioxys*, *H. abundans* and *Hantzschia sp.* (Table 6.2) isolated from different continents, including the Schirmacher Oasis on continental Antarctica. Geographic origin of the strains is listed in Tables 6.1 and 6.2. In the future, more detailed information about sampling dates and localities will be available in the barcode database (www.boldsystems.org) in which all used strains will be included. Per location, multiple environmental samples were used. Environmental samples were taken by scraping the upper half cm of bare soil using a sterile falcon tube.

For the non-Antarctic samples, a small amount of soil was incubated upon arrival in the lab in liquid WC medium (Guillard & Lorenzen, 1972, but without pH adjustment and vitamin addition) at standard culture conditions of 18°C, 25-30 μmol ph m⁻² s⁻¹ light.
Table 6.1 List of strains of *Pinnularia borealis* used in this study, along with the identification, geographic origin, simple morphometrics (± standard deviation), and availability (x) of the molecular markers *rbc*L and LSU. Missing genetic data are indicated by a dash. Strains used for temperature preference experiments are indicated in the last column.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identification</th>
<th>Origin</th>
<th>Morphometric data (± stdv.)</th>
<th>Temp. exp.</th>
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<td>Length (μm)</td>
<td>Width (μm)</td>
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<td>(Ban1)a</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Canada – Banff</td>
<td>31.6±0.8</td>
<td>7.1±0.3</td>
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<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>E13</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>P8</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>P10</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>P12</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>P14</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>P17</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>S18</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>V2</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>V4</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>V15</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>V18</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>V20</td>
<td><em>P. borealis</em> var. <em>borealis</em> Ehrenberg</td>
<td>Antarctica - Schirmacher Oasis</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
Table 6.2 List of strains of Hantzschia sp. used in this study, along with the identification, geographic origin, simple morphometrics (± standard deviation), and availability (x) of the molecular markers rbcl and LSU. Missing genetic data are indicated by a dash. Strains used for temperature preference experiments are indicated in the last column.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identification</th>
<th>Origin</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Striae density (#/10 μm)</th>
<th>Fibulae density (#/10 μm)</th>
<th>rbcl</th>
<th>LSU</th>
<th>Temp. exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ban1)b</td>
<td>H. sp. 1</td>
<td>Canada - Banff</td>
<td>39.2±1.1</td>
<td>7.5±0.4</td>
<td>21.7±0.7</td>
<td>8.3±0.9</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Ban1)d</td>
<td>H. sp. 1</td>
<td>Canada - Banff</td>
<td>37.7±0.7</td>
<td>6.3±1.0</td>
<td>21.9±0.6</td>
<td>7.9±0.7</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Ban1)e</td>
<td>H. sp. 1</td>
<td>Canada - Banff</td>
<td>38.1±1.1</td>
<td>7.9±0.6</td>
<td>21.4±0.7</td>
<td>6.8±1.2</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Ban1)f</td>
<td>H. sp. 1</td>
<td>Canada - Banff</td>
<td>43.6±0.8</td>
<td>7.7±0.4</td>
<td>21.1±0.9</td>
<td>7.9±0.4</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Ban1)h</td>
<td>H. sp. 1</td>
<td>Canada - Banff</td>
<td>40.4±0.5</td>
<td>7.8±0.3</td>
<td>21.0±1.1</td>
<td>7.2±0.9</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Mo1)a</td>
<td>H. sp. 2</td>
<td>Mongolia - Kangai-Nuruu</td>
<td>85.2±2.6</td>
<td>8.8±0.3</td>
<td>20.0±0.8</td>
<td>6.9±1.0</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Mo1)e</td>
<td>H. sp. 2</td>
<td>Mongolia - Kangai-Nuruu</td>
<td>86.4±3.1</td>
<td>8.3±0.5</td>
<td>20.2±1.2</td>
<td>7.2±0.8</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Mo1)h</td>
<td>H. sp. 2</td>
<td>Mongolia - Kangai-Nuruu</td>
<td>88.4±0.5</td>
<td>8.5±0.5</td>
<td>19.4±0.7</td>
<td>7.1±0.3</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Mo1)i</td>
<td>H. sp. 2</td>
<td>Mongolia - Kangai-Nuruu</td>
<td>87.6±1.3</td>
<td>8.7±0.6</td>
<td>19.9±0.5</td>
<td>7.2±0.5</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(Mo1)m</td>
<td>H. sp. 2</td>
<td>Mongolia - Kangai-Nuruu</td>
<td>87.9±4.5</td>
<td>8.9±0.5</td>
<td>19.8±0.8</td>
<td>7.8±0.6</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(St1)e</td>
<td>H. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>42.9±0.4</td>
<td>6.2±0.1</td>
<td>25.2±0.9</td>
<td>1.1±0.7</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St1)f</td>
<td>H. cf. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>48.3±0.9</td>
<td>9.0±0.4</td>
<td>21.6±0.7</td>
<td>6.9±0.9</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(St1)h</td>
<td>H. cf. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>36.8±1.8</td>
<td>8.7±0.3</td>
<td>21.2±0.9</td>
<td>6.9±0.7</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(St2)d</td>
<td>H. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(St3)a</td>
<td>H. cf. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>36.8±0.5</td>
<td>6.6±0.5</td>
<td>23.7±1.0</td>
<td>9.2±0.7</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St3)c</td>
<td>H. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>42.8±0.6</td>
<td>6.4±0.2</td>
<td>24.7±1.1</td>
<td>7.7±0.7</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>(St4)a</td>
<td>H. amphioxys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>36.0±0.6</td>
<td>6.0±0.4</td>
<td>25.8±0.4</td>
<td>8.0±0.9</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St4)b</td>
<td>H. amphibrixys (Ehr.) Grunow</td>
<td>Belgium - Gent</td>
<td>38.1±0.8</td>
<td>6.0±0.3</td>
<td>24.1±0.9</td>
<td>9.3±0.7</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Strain</td>
<td>Identification</td>
<td>Origin</td>
<td>Morphometric data (± stdv)</td>
<td>rbcL</td>
<td>LSU</td>
<td>Temp. exp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Length (μm)</td>
<td>Width (μm)</td>
<td>Striae density (#/10 μm)</td>
<td>Fibulae density (#/10 μm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(St5)c</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Belgium – Gent</td>
<td>68.1±2.8</td>
<td>9.7±0.4</td>
<td>17.1±1.0</td>
<td>6.6±0.5</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St6)b</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Belgium – Gent</td>
<td>68.1±0.9</td>
<td>8.1±0.5</td>
<td>18.8±1.4</td>
<td>7.2±1.2</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St6)e</td>
<td><em>H. sp.</em> 3</td>
<td>Belgium – Gent</td>
<td>52.3±0.5</td>
<td>7.3±0.4</td>
<td>19.3±0.7</td>
<td>6.8±0.7</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St6)f</td>
<td><em>H. sp.</em> 3</td>
<td>Belgium – Gent</td>
<td>52.3±0.4</td>
<td>7.3±0.4</td>
<td>18.8±1.0</td>
<td>7.9±1.0</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(St8)c</td>
<td><em>H. cf. amphioxys</em> (Ehr.) Grunow</td>
<td>Belgium – Gent</td>
<td>46.8±0.7</td>
<td>8.2±0.3</td>
<td>20.3±1.3</td>
<td>7.0±0.7</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(Tor3)c</td>
<td><em>H. abundans</em> Lange-Bertalot</td>
<td>Chile – Torres del Paine</td>
<td>68.1±0.9</td>
<td>8.1±0.5</td>
<td>18.8±1.4</td>
<td>7.2±1.2</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(Wes1)b</td>
<td><em>H. abundans</em> Lange-Bertalot</td>
<td>Belgium – De Panne</td>
<td>56.1±0.2</td>
<td>7.1±0.6</td>
<td>20.4±0.5</td>
<td>6.8±0.4</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>(Wes3)b</td>
<td><em>H. abundans</em> Lange-Bertalot</td>
<td>Belgium – De Panne</td>
<td>51.8±0.2</td>
<td>7.3±0.3</td>
<td>19.6±0.9</td>
<td>7.6±0.9</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>S10</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Antarctica – Schirmacher Oasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>S11</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Antarctica – Schirmacher Oasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>S13</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Antarctica – Schirmacher Oasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>S15</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Antarctica – Schirmacher Oasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>S16</td>
<td><em>H. amphioxys</em> (Ehr.) Grunow</td>
<td>Antarctica – Schirmacher Oasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
intensity and a 12:12 hours light:dark period. Within the next week, cells resembling \textit{P. borealis} and \textit{H. amphioxyys} were isolated under a binocular using a needle and micropipette. Monoclonal strains were maintained under standard culture conditions and re-inoculated when reaching late exponential phase. Antarctic samples were kept cold during transport and were incubated in WC medium at 5°C and low light intensities (ca. 5 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)) to prevent the death of possibly psychrophilic diatoms. Isolations of cells using needle and micropipette were performed within the next 10 days. Monoclonal strains were first maintained at 8°C and later at 15°C after initial tests demonstrated that the Antarctic cultures tolerated this temperature.

Morphological observations
Cultures were harvested for morphological observations in late exponential phase, oxidized using hydrogen peroxide and embedded in Naphrax®. Pictures were taken using a Zeiss Axioplan 2 microscope equipped with an AxioCam Mrm camera. Valve length, width and stria density of 10 valves per strain were measured using ImageJ 1.37v software. Identiifications were based on Kramer (2000) for \textit{Pinnularia borealis} and Lange-Bertalot (1993) for \textit{Hantzschia}. Identifications and morphometric data are listed in Tables 6.1 and 6.2, respectively. Voucher material is kept in the Laboratory of Protistology & Aquatic Ecology (Gent University, Belgium) and is available upon request.

Molecular-genetic analyses
Cultures were harvested for genetic analyses in exponential phase, and DNA was extracted from centrifuged diatom cultures following Zwart et al. (1998) using a bead-beating method with phenol extraction and ethanol precipitation. After extraction, DNA was purified with a Wizard® DNA Clean-up system (Promega). Sequences of the D1-D2 region of the nuclear 28S rDNA and of the plastid gene \textit{rbcL} were amplified following standard PCR primers and protocols (Scholin et al., 1994; Daugbjerg & Andersen, 1997; Jones et al., 2005). Primer sequences, PCR conditions and sequencing protocol are described in detail in Souffreau et al. (submitted).

Outgroups of \textit{P. borealis} were based on a genus phylogeny of \textit{Pinnularia} (Souffreau et al., submitted) and include \textit{P. cf. microstauron} (B2)c, \textit{P. sp.} (Wie)a, \textit{P. sp. Pin873}, \textit{P. subcommutata var. elongata} Krammer Corsea10, \textit{P. acuminata} W. Smith Pin876, \textit{P. neomajor} Krammer

Genetic divergence in \textit{P. borealis} and \textit{H. amphioxyys} 159
Corsea 2, P. neglectiformis Krammer Pin706. For H. amphioxys, all available strains of Hantzschia identified as not being H. amphioxys were used as outgroup (Table 6.2). Sequences were separately edited and automatically aligned using ClustalW (Thompson et al., 1994) implemented in BioEdit 7.0.3 (Hall, 1999). Plastid sequences aligned unambiguously without any gaps. The alignment of 28S was corrected manually using the secondary structure of Apedinella radians (Ben Ali et al., 2001) after which ambiguously aligned regions were removed. For P. borealis, sequence lengths of 1,428 and 511 sites were used for rbcL and 28S rDNA, respectively. For Hantzschia this was 1,457 and 525 sites, respectively. The rbcL alignment was partitioned into the first two, slower evolving codon positions and a separate third codon position. The 28S was analyzed as a single partition. Datasets of Pinnularia borealis and Hantzschia amphioxys were analyzed separately. Model selection was performed using TreeFinder (Jobb et al., 2004) on the concatenated dataset using a three-partition strategy (28S, and two codon positions of rbcL) and on the single genes, and based on the Bayesian Information Criterion (BIC, Schwarz, 1978) as a selection criterion.

Individual genes and concatenated datasets were analysed by maximum likelihood phylogenetic inference using RAxML 7.2.6 (Stamatakis, 2006) under the preferred GTR+Γ4 model with 10,000 independent tree searches from randomized MP starting trees. Maximum likelihood bootstrap analyses (Felsenstein, 1985) consisted of 1,000 replicates. Bayesian inference was applied using MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) with the same models and partition schemes. Using default settings, two independent runs with four incrementally heated Metropolis-coupled Monte-Carlo Markov Chains were run for ten million generations for the individual genes and 30 million generations for the concatenated datasets. Runs were sampled every 1,000th generation and convergence and stationarity of the log-likelihood and parameter values was assessed using Tracer v.1.5 (Rambaut & Drummond, 2007). The first 10% generations were discarded as burn-in for the individual gene and multi-gene analyses. The post-burn-in trees from the different runs were summarized and posterior probabilities (PPs) were calculated in MrBayes using the sumt command. All analyses were performed using the publicly available computer resource Bioportal (Kumar et al., 2009).
Molecular time-calibration
Based on the molecular dataset, the phylogenetic relationships of *P. borealis* were placed in a time frame by constructing a time-calibrated phylogeny. Calibration of the molecular clock was based on the 95% highest posterior desities (HPD) resulting from the preferred time-calibration of the *Pinnularia* genus phylogeny in Souffreau et al. (submitted) which was constrained by the fossil record. Three nodes of the phylogeny of *P. borealis* were constrained (gray circles in Fig. 6.2) using each a uniform distribution of their recovered 95% HPD. *RbcL* was partitioned into codon position (1st + 2nd combined), LSU was used as a single partition. Metropolis-coupled Monte-Carlo Markov Chains were run by BEAST v1.5.4 (Drummond & Rambaut, 2007) under a GTR + Γ4 model for each partition and a relaxed, uncorrelated lognormal clock model and Yule tree prior. Three independent analyses, each started by a UPGMA tree, were run for 100 million generations and sampled every 1,000th generation. Burn-in values of 30 till 40% were assessed using Tracer v.1.5 (Rambaut & Drummond, 2007). All post-burn-in trees were combined after which the maximum clade credibility chronogram with mean node heights was calculated using TreeAnnotator v.1.5.4.

Temperature preference experiments
For *P. borealis*, we selected per environmental sample two genetically characterized strains for the experiments, giving in total 22 strains (Table 6.1). The cells of the cultures from the Chilean samples Tor12 and Tor3 were too small to be used. For *Hantzschia*, we selected their genetically nearest sister taxon (St3)a. All Antarctic strains and 1 or 2 strains per lineage were analyzed (depending on the availability), giving in total 11 strains (Table 6.2). Before experimental treatment, all strains were grown in standard culture conditions at 15°C during at least 2 months. The growth rate of the strains was assessed over a temperature range from +2.9°C to +32.4°C using a temperature gradient table (Labio, Ltd.) and fluorescence measurements of dark-adapted cells (F0) as a proxy for density (Consalvey et al., 2005). Before fluorescence measurements, cells were dark-adapted during 15 minutes at 18°C, and F0 was measured daily during 7 days by Pulse Amplitude Modulated (PAM) fluorescence using a Waltz MAXI Imaging-PAM (M-series) with default settings, intensity of 12, gain of 8 and damping of 1. The experiments were carried out in
triplicate using wells of 24-well plates and the table was filled with 9 series of 4 plates. Each of the four rows of wells in a series of plates corresponded to a specific temperature, while a total of 24 wells (4 plates in a series, 6 wells per plate) were available per temperature in a single experiment. In total 12 different experimental temperatures were selected, spread over the 9 series of plates. Per temperature, strains were randomly inoculated in the wells. Replicates were spread over different experiments to avoid any possible effects of experiment.

To ensure that all differences among strains would be due to genetic effects and not to mere differences in physiological condition, all strains were prior to the experiment inoculated at the same cell density corresponding with a F0 PAM fluorescence value of 0.050 using the above-mentioned settings and grown during 5 days in standard culture conditions in 6-well plates. Next, all strains were again re-inoculated at an F0-value of 0.050 and grown for 5 days during which the culture medium was refreshed daily to keep them in exponential phase. Together, this treatment ensures growth in early to mid-exponential phase for all strains for in total approximately 5 generations. The strains were harvested by micropipette and inoculated in the well plates at a starting density of 0.025 (F0-value), after which they were placed on the gradient table. Culture densities were measured daily by PAM fluorescence as described above. After measurement, the different well plates from each temperature series were placed back in random order at their appropriate temperature on the table. The temperature of each well was measured using a calibrated precision thermometer (Ebro) at the end of each experiment, and average temperature was calculated for each temperature series and finally averaged over all replicate experiments per assessed temperature. To calculate the growth rate for each temperature per strain, F0-values were log 2 transformed, the seven data points per replicate were plotted against time (in hour), and the slope of the exponential growth curve was estimated using linear regression in Excel 2007 (Microsoft Office).

6.3 Results

Taxon sampling

In total 52 strains of *P. borealis* were studied, including the morphological varieties *borealis*, *islandica* and *scalaris* (Table 6.1). For *Hantzschia*, from the 31 strains only 11 strains could be
morphologically assigned to *Hantzschia amphioxys* (stria density of 20-29/10 µm), while some of the other strains were identified as *H. abundans* (stria density of 15-20/10 µm) (Table 6.2). Several strains could not be identified to a known morphological species, and were reported as *Hantzschia sp.1, sp. 2 and sp. 3* or *H. cf. amphioxys*.

**Molecular-phylogenetic analyses of**

*P. borealis*

Analyses using ML and BI gave identical results based on the individual genes and concatenated dataset. Therefore only the ML phylogeny of the concatenated analysis is shown in Fig. 6.1. As shown previously (Souffreau *et al.*, submitted), *P. borealis* was recovered as monophyletic. Within the *P. borealis* lineage, eight highly supported lineages can be clearly delineated (indicated by grey boxes in Fig. 6.1). These same lineages were all resolved with high support in the individual gene phylogenies, enhancing the probability that these lineages correspond to different species. Three of these lineages contained strains from different locations [Belgium + French Alps; Czech Republic + Canada (1); Belgium + Canada (2)]. There appears to be some (phylo)geographic signal within these lineages since strains from different locations (Canada vs. Europe, and Belgium vs. French Alps) had different sequences. At a single site in Belgium (St = Gent, Sterre) three different lineages were detected and a single sediment sample (St6) contained two lineages, showing that at least some of the lineages occur sympatrically. Only a single morphological variety, var. *islandica*, is restricted to a single lineage (Fig. 6.1), the two other varieties are scattered throughout the phylogeny indicating that these varieties as currently defined have no evolutionary meaning. Based on our taxon sampling, the *P. borealis* species complex started to diversify 22 Ma ago [15.5-27.2 Ma 95% highest posterior densities (HPD)] (Fig. 6.2). The different lineages diverged on average between 11.9 and 5.0 Ma ago, with a minimum and maximum 95% HPD of 0.45 and 17.91 Ma over all lineages.

**Molecular-phylogenetic analyses of**

*H. amphioxys*

Again, we only show the ML phylogeny based on the concatenated dataset since both ML and BI and the two individual genes gave
Fig. 6.1 Maximum likelihood phylogeny based on rbcL and the D1-D2 region of 28S rDNA for P. borealis with indication of bootstrap values - posterior probabilities. Lineages are indicated by grey boxes, with dark grey boxes containing lineages that could potentially be further divided.
identical results (Fig. 6.3). All Hantzschia amphioxys strains were recovered as a monophyletic clade. In total 5 H. amphioxys lineages can be delineated (indicated by grey boxes in Fig. 6.3), of which 4 were isolated from a single site in Belgium (St = Gent, Sterre). Compared to the P. borealis phylogeny, the branch lengths between these lineages are much shorter. Outside the H. amphioxys clade, three lineages contained strains which superficially resembled H. amphioxys (H. cf. amphioxys), two of which were placed in a large clade containing most other Hantzschia strains, another one as sister to the H. amphioxys clade (Fig. 6.3). Also for other strains, e.g. those identified as H. abundans or H. sp. 1, the identifications based on morphology in LM often didn’t match the separation in lineages in the phylogeny.

Thermal adaptation in P. borealis
Average growth rate per lineage versus temperature is shown in Fig. 6.4. The optimal growth temperature (i.e. the temperature at which the highest growth rate is observed) of the Antarctic lineage lays around 15°C, while for most other lineages this is situated around 20-24°C. The Antarctic lineage is thus clearly adapted to colder temperatures. Note however that the lineage containing the Mongolian strains shows a similar growth rate profile as the Antarctic strains. Also the upper lethal temperature is situated around 24°C for the Antarctic and Mongolian strains, while this lies between 25 and 29°C for the other lineages, indicating that the Antarctic strains have lost the capacity to survive at higher temperatures. The lineage Canada(1)-Czech Republic and the lineage from the French Alps are intermediate concerning their upper lethal temperature. Meanwhile, because the strains of the French Alps underwent cell size enlargement in culture (the mechanism was not observed), these results should be interpreted with caution. Despite this, the two colder-adapted lineages (Mongolia and Antarctica) belong to a first large subclade of P. borealis (Fig. 6.1, upper clade), while the higher temperature tolerant lineages all form a second subclade (Fig. 6.1, lower clade).

Thermal adaptation in H. amphioxys
Similar to the P. borealis species complex, the optimal and upper lethal temperatures of the Antarctic lineage are lower compared to the other lineages (Fig. 6.5), but to a lesser extent than in the P. borealis species complex (Fig. 6.4). The optimal temperature lays
Fig. 6.2 Time calibrated molecular phylogeny of P. borealis based on rbCL and the D1-D2 region of 28S rDNA, with indication of mean ages in black and posterior probabilities in grey. The three grey circles indicate the constrained nodes using the 95% HPD results of the time-calibrated phylogeny of the genus Pinnularia in Souffreau et al. (submitted) based on fossil constraints. Grey bars represent the 95% HPD intervals. The number of sequenced strains per lineage is given between brackets.
around 20°C for the Antarctic lineage, and around 23°C for the other lineages. The temperature preference of the single *H. cf. amphioxyx* strain (St3) a sister to the *H. amphioxyx* clade was similar to that of the temperate *H. amphioxyx* strains.

6.4 Discussion

Based on the molecular phylogenies, *P. borealis* and *H. amphioxyx* consist of multiple genetically diverged lineages, including a distinct Antarctic lineage. Based on a molecular clock estimate, the species complex *P. borealis* originated between 30 and 47 Ma ago, while the Antarctic lineage of *P. borealis* is estimated to have diverged around 7.7 Ma ago from its sister lineage. In addition, the Antarctic lineages of both *P. borealis* and *H. amphioxyx* diverged in optimal temperature and in upper lethal temperature from the lineages from more temperate regions. Taken together, despite our doubtlessly non-exhaustive taxon sampling, this indicates that the high percentage of endemics found on the Antarctic continent as reported by Verleyen *et al.* (submitted) is an underestimation and raises questions about the reality of the other presumed cosmopolitan species in the Antarctic.

(Pseudo)cryptic speciation has been reported in a variety of diatom genera (Sarno *et al.*, 2005; Mann & Evans, 2007; Vanormelingen *et al.*, 2008; Trobajo *et al.*, 2009; Vanselslander *et al.*, 2009) and we gathered genetic indications that *Pinnularia borealis* and *Hantzschia amphioxyx* can be added to this list. The branching pattern in the molecular phylogenies is composed of groups of strains with few nucleotide substitutions (*i.e.* lineages) compared to the large number of substitutions separating them, which is typical for the species level (*e.g.* Jargeat *et al.*, 2010; Tronholm *et al.*, 2010). The fact that identical lineages were recovered by two different, independent markers (*in casu* rbcL and 28S rDNA), even when it concerned sympatric strains, reinforces this conclusion. In both species complexes, the different lineages have no clearly distinct morphologies, and only correspond partially to the identifications based on valve morphology. We doubt whether further morphological examination could reveal (a combination of) characters allowing to confidently identifying the different lineages. AlgaeBase (Guiry & Guiry, 2011) cites 45 varieties of *P. borealis*, while the 7 varieties mentioned in Krammer (2000) already overlap in described morphological characteristics. Concerning *H. amphioxyx*, 25 varieties are mentioned in AlgaeBase (Guiry & Guiry, 2011), and Cleve-Euler (1952) characterized ca. 30
Fig. 6.3 Maximum likelihood phylogeny based on rbcL and the D1-D2 region of 28S rDNA for H. amphioxys with indication of bootstrap values - posterior probabilities. Lineages are indicated by grey boxes.
intraspecific taxa within Scandinavian *H. amphioxys*, while Krammer & Lange-Bertalot (1988) recognized the extreme morphological diversity in *H. amphioxys* but estimated the disentanglement of this complex beyond reach. On the other hand, advanced morphometrics has been useful for separating pseudocryptic species in a few of the more diverse diatom species complexes (Droop et al., 2000; Mann et al., 2004). Despite this, routine identifications based on valve morphology alone probably will always be difficult to impossible. DNA barcoding, a diagnostic technique in which short characteristic DNA sequence(s) are used for species identification (Hebert et al., 2003; Savolainen et al., 2005), could offer a solution (Mann et al., 2010).

The genetic divergence between the Antarctic and other *P. borealis* and *H. amphioxys* lineages indicates that dispersal is limited enough to allow lineage divergence or speciation on the Antarctic continent, even for such abundant terrestrial diatoms. While the sampling of strains from the non-Antarctic locations is less exhaustive, we similarly detect genetically differentiated lineages in *P. borealis* sampled from different regions such as Chile, Mongolia and Belgium. Geographically limited gene flow is also suggested by the observation that strains within the same lineage but from different geographic locations possess some variable point mutations, indicating that there is phylogeographic structure within lineages between and perhaps even within continents. However, this should be investigated in more detail by increasing the number of sampled locations and by increasing the number of individual strains sampled per population. Furthermore, a high number of sequenced individuals per meta-population (*i.e.* per species) is necessary to assess the genetic variability within a specific lineage. That way, algorithmic sequence-based species delimitation which determines the boundary between species and populations can be performed (Pons et al., 2006; Monaghan et al., 2009). On a chronogram, the transition from the species-level processes (speciation and extinction) to population-level processes (coalescence) is association with a sudden increase in branching rate (*i.e.* a sudden increase in number of differentiated genotypes).

Compared with freshwater habitats, soils are characterized by a higher spatial availability and continuity, while the dryer sediment is more susceptible to be picked up by air currents (Chepil, 1956). Combined with the relatively high tolerance of soil-inhabiting diatoms, including *P. borealis* and *H. amphioxys*, compared to their
Fig. 6.4 Temperature-dependent growth rate of P. borealis. The graph represents the growth rate per assessed temperature based on a total of 22 P. borealis strains, averaged per lineage with standard deviations.

Fig. 6.5 Temperature-dependent growth rate of H. amphioxys. The graph represents the growth rates per assessed temperature based on a total of 11 H. amphioxys strains, averaged per genetic lineage with standard deviations.
aquatic counterparts to temperature extremes (Souffreau et al., 2010) and even desiccation (Souffreau et al., unpubl. b), this will enhance the dispersal potential and thus colonization chances of these diatoms. Living cells of Hantzschia amphioxys have been encountered in air traps at unknown height (Geissler & Gerloff, 1966) and during a colonization experiment on the roof of a university building (C. Souffreau, unpubl. data). Little is known about (wind) dispersal rates across different spatial scales, but living diatoms cells are mostly encountered at low heights, close to the soil (Van Overeem, 1937; Schlichting, 1961; Brown et al., 1964), suggesting that short distance dispersal over a few metres is definitely possible while long-distance dispersal might be associated with much higher mortalities. In any case, if any diatom species had a high chance of being cosmopolitan, it would have been P. borealis and H. amphioxys, making it likely that many of the other so-called cosmopolitan species on (sub-)Antarctica are also species complexes with a distinct (sub-)Antarctic lineage.

The Antarctic continent is highly isolated, and we can realistically assume that the surrounding oceans are a relatively strong dispersal barrier for diatom cells. The age of the species complex P. borealis, being between 30 and 47 Ma, has allowed it to colonize all continents, including the Antarctic. Based on our taxon sampling the current Antarctic lineage of P. borealis present in the Schirmacher Oasis is estimated to have diverged between 15 and 2 Ma ago from its sister lineage, which is fairly recent compared to the age of the species complex, but most likely situated after the final opening of the Drake Passage between the Antarctic Peninsula and South America around 14 Ma (Lagabrielle et al., 2009) and after the start of the Mid-Miocene cooling event of the Antarctic continent, which resulted in the increase of the East Antarctic ice sheet and the establishment of full polar climate conditions (Flower & Kennett, 1994). This corresponds to divergent dates of Antarctic mites and springtails from the sub-Antarctic lineages (Stevens et al., 2006; Mortimer et al., 2011). These time estimates indicate the establishment of P. borealis on the geographically isolated continent through long-distance dispersal, and subsequent lineage divergence. It is yet unclear whether any P. borealis inhabiting the continent before the Mid-Miocene cooling went extinct, or if there still is a second more ancient P. borealis lineage on the continent, which started diverging after opening of the Drake passage but is currently regionally absent from the Schirmacher Oasis. Alternatively, for P. borealis, the upper limit of divergence for the Antarctic lineage (15 Ma) is correct and
the speciation event of the Antarctic clade is associated with the final opening of the Drake passage or the Mid Miocene cooling event triggering allopatric speciation.

The growth rate experiments show that the Antarctic lineages of *P. borealis* and *H. amphioxys* have a relatively low optimal growth temperature and low upper temperature limit compared to lineages from more temperate areas. However, they are not psychrophilic, in contrast to a range of marine planktonic diatoms which die at temperatures no higher than 12-15°C (Bunt et al., 1966; Van Baalen & Odonnell, 1983; Fiala & Oriol, 1990; Thomas et al., 1992; Smith et al., 1994). This might be related to the fact that their habitat is less buffered against temperature changes. In sheltered sun-exposed shallow water and wet soils or seepages, temperatures can be as high as 15°C even on continental Antarctica. In the Antarctic *H. amphioxys*, there is a modest shift in temperature preference with the most closely related temperate lineages, implying the loss of the ability to grow well at temperatures well above 20°C. For Antarctic *P. borealis*, the most closely related lineages from the French Alps and the Mongolian Khangai mountain range have quite similar above 20°C group into another lineage suggesting that there might be some phylogenetic signal in temperature preference in *P. borealis*. Species from one clade might be adapted to warmer climates, while species from the other clade, including the Antarctic lineage, might inhabit colder climates. Thermal adaptation related to climatic zones has also been shown for the heteroflagellate *Spumella* (Boenigk et al., 2007) and the marine diatom *Cylindrotheca* (Vanelslender et al., unpubl.), and our results contribute to the idea that protist species are not temperature generalists but instead inhabit specific climatic zones.

Here, we have shown for two cosmopolitan diatom morphospecies that they consist of different lineages with a distinct continental Antarctic lineage which has diverged in its temperature preference from more temperate counterparts. However, many questions remain about their diversification and biogeography. Further research should therefore focus on assessing the geographic distribution of *P. borealis* and *H. amphioxys* lineages, especially in the (sub-)Antarctic and Arctic, to further unravel the evolutionary history of (polar) diatom lineages. More complete molecular phylogenies through more extensive taxon sampling, in combination with niche modelling, should also allow determining phylogenetic
Genetic divergence in *P. borealis* and *H. amphioxys*

signals in temperature preference. Shorter-term regional extinction-colonization dynamics due to climate change in the Antarctic should be investigated using phylogeographic markers such as SNPs, or with microsatellites. The latter can also give insight into contemporary patterns of gene flow among populations and thus of the dispersal rates and distances. However, the correct interpretation of such studies requires a decent knowledge on species boundaries, and by doing so, this study lays a basis for future molecular research on the biogeography of polar diatoms.

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Part 3.
Tolerance levels for dispersal-related stresses
7.
Tolerance of benthic diatoms from temperate aquatic and terrestrial habitats to experimental desiccation and temperature stress

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Abstract
Soils differ from aquatic sediments in environmental characteristics such as moisture availability and temperature fluctuations, and it is therefore believed that soil-inhabiting diatoms have a broader tolerance range to these stresses than aquatic diatoms. To test this hypothesis, we assessed the survival capacities of vegetative cells of 34 benthic diatom species from terrestrial and aquatic habitats in Belgium when exposed to desiccation and temperature stress. Six different stress conditions were studied: gradual heating up to +30°C and +40°C, abrupt heating to +40°C, freezing to -20°C, and desiccation with and without preconditioning at +30°C. All six conditions resulted in a significantly decreased survival of cells compared to control conditions. Desiccation killed all tested strains, freezing was survived by only three species, and abrupt heating was significantly more lethal than gradual heating, suggesting a generally high sensitivity of vegetative diatom cells to these three stress factors. While tolerance to temperature extremes (+40°C and -20°C) was to a large extent species-specific, habitat-specific differences in survival were also detected. Only terrestrial species survived freezing and aquatic diatoms were less tolerant to gradual heating to +40°C, both pointing at a higher tolerance of terrestrial diatoms to temperature extremes. Moreover, in two species with both aquatic and terrestrial isolates only the terrestrial strains survived +40°C. We conclude that vegetative cells of benthic diatoms (1) are very sensitive to desiccation, freezing and abrupt heating, and (2) have a habitat-dependent tolerance to temperature extremes. The consequences of these observations for the dispersal capacities and the subsequent biogeographical patterns of diatoms are discussed.

Key words: aquatic, benthic diatoms, desiccation, dispersal capacity, stress tolerance, temperature extremes, terrestrial
7.1 Introduction

The majority of diatom species are confined to aquatic environments (Patrick, 1977). However, various species belonging to several genera can be found in terrestrial habitats such as soils and humid rocks (Patrick, 1977), where they grow together with cyanobacteria and green algae (Ettl & Gärtner, 1995). Terrestrial microalgal assemblages are characterized by specialized species (Ettl & Gärtner, 1995; Johansen, 1999; Van Kerckvoorde et al., 2000; Van de Vijver et al., 2004). In unicellular green algae, phylogenetic studies have shown the separate evolution of terrestrial and aquatic lineages (Lewis & Flechtner, 2004; Zoe et al., 2008). The main reasons for this lineage divergence are probably the specific adaptations required in terrestrial environments (Zoe et al., 2008). Diatoms inhabiting soils, for instance, must typically withstand large diurnal fluctuations in temperature, pH and moisture availability in the near-surface layer (Starks et al., 1981; Gao et al., 2008).

In order to survive locally, terrestrial algae have to be adapted to these extreme fluctuations in environmental conditions. Some terrestrial cyanobacteria and green algae are known for their enhanced tolerance to various habitat-related stress conditions such as desiccation (Dodds et al., 1995; Potts, 1999; Sabacka & Elster, 2006; Gray et al., 2007), osmotic stress (Tamaru et al., 2005), freezing (Sabacka & Elster, 2006) and heating (Renaud et al., 2002). This is achieved by diverse protection mechanisms including extracellular polysaccharide layers (Potts, 1999; Tamaru et al., 2005), thick cell walls (Evans, 1958, 1959) and morphologically distinct resting stages (Evans, 1958, 1959; Paerl, 1988; Hoffmann, 1996). For terrestrial diatoms however, data on their tolerance to temperature extremes and drought are scarce. Freezing tolerance has been reported for a single terrestrial species (Hostetter & Hoshaw, 1970), and two desiccation experiments on soil samples from temporary ponds both revealed species-specific survival and a decrease in the number of surviving diatom species with decreasing moisture content (Evans, 1958, 1959; Hostetter & Hoshaw, 1970). In planktonic freshwater diatoms from temperate climates, lethal heating temperatures range from +30°C to +40°C (Suzuki & Takahashi, 1995; Butterwick et al., 2005).

Stress protection mechanisms in terrestrial diatoms are poorly known. Both the accumulation of oil in the cytoplasm and vertical movements in the sediment layer were observed in benthic diatoms from drying ponds (Evans, 1958, 1959). Morphologically discernable
resting spores are commonly formed in marine centric species during nutrient depletion and are made to survive cold and dark winter conditions [reviewed in Hargraves & French (1983) and McQuoid & Hobson (1996)], but only a single experiment gave evidence for a partly enhanced tolerance for desiccation (Hargraves & French, 1975). In benthic freshwater diatom species resting spores have thus far only been observed in seven species and are all associated with the formation of internal valves (Getler, 1953, 1971; Schmid, 1979; Von Stosch & Fecher, 1979). Interestingly one of these species, Craticula cuspidata (Kützing) D.G. Mann, forms resting stages in case of desiccation and recovers after rehydration (Schmid, 2009). However, most freshwater species are not known to form similar morphologically distinct spores and there is no evidence yet that soil diatom communities bridge harsh environmental conditions in this way. Another possibility is the formation of physiological resting cells (McQuoid & Hobson, 1996), but no information on their stress tolerance is available.

Apart from the importance of stress tolerance for local population persistence, aerial dispersal success also depends on tolerance for stress factors such as desiccation, UV-irradiance and abrupt temperature changes (Isard & Gage, 2001; Figuerola & Green, 2002; Sharma et al., 2007). Of various dispersal mechanisms (reviewed by Kristiansen, 1996), waterfowl is considered to be the most important vector for aquatic microalgae (Schlichting, 1960; Kristiansen, 1996; Figuerola & Green, 2002). Survival of the attached cells is mainly limited by desiccation (Figuerola & Green, 2002) given that most of the green algae externally transported on birds formed protection mechanisms such as slime sheets, resting spores or cysts. The dominance of terrestrial cyanobacteria and green algae found in air traps (Van Overeem, 1937; Schlichting, 1961; Brown et al., 1964; Schlichting, 1964; Roy-Ocotla & Carrera, 1993; Marshall & Chalmers, 1997) suggests that terrestrial microalgae are more easily transported by air currents (Brown et al., 1964; Schlichting, 1964; Marshall & Chalmers, 1997). In both waterfowl- and wind-mediated dispersal pathways, living diatoms are rarely encountered. Although this may be explained by their lower abundance in terrestrial habitats compared to cyanobacteria and green algae (Davey, 1991; Zancan et al., 2006), this could also be due to a lower tolerance of diatoms for the adverse environmental conditions encountered during transport. Regardless, differences in stress tolerance between algal groups or between algae from different habitats [e.g. aquatic versus terrestrial
habitats, as seen in green algae (Gray et al., 2007)] may result in differential dispersal rates, and therefore pronounced differences in population structure and the geographical distribution of species.

A better understanding of the stress tolerance and associated dispersal abilities of diatoms could thus provide insight on their biogeography. Due to undersampling and controversy regarding taxonomic resolution and species delineation, the biogeographical distribution patterns of diatom species remain poorly known (Vanormelingen et al., 2008b). In contrast to the hypothesis that diatoms are dispersed on a broad spatial scale (Finlay et al., 2002), recent analyses on lacustrine diatoms have demonstrated the importance of historical factors such as isolation (and thus dispersal limitation) for diversity patterns (Telford et al., 2006; Vyverman et al., 2007). This is also supported by reports of endemism in some regions (Vanormelingen et al., 2008b). One of the possible mechanisms behind these restricted distribution patterns is the supposed sensitivity of diatoms to stress factors associated with dispersal. Furthermore, a different tolerance level between terrestrial and aquatic diatoms might have repercussions for their distribution patterns.

In this experimental study, we broaden the knowledge on the stress tolerance of diatoms by investigating in culture experiments (1) to what extent vegetative cells of terrestrial and benthic freshwater diatoms tolerate desiccation and extreme temperatures, and (2) whether terrestrial diatoms survive these harsh conditions better than aquatic taxa.

### 7.2 Materials & Methods

#### Sampling

Samples of dry, moist and aquatic sediments were taken at five different locations in Belgium: “Campus Sterre” (St) in Gent, nature reserve “De Westhoek” (Wes) in De Panne, nature reserve “Ter Yde” (Yde) in Oostduinkerke, nature reserve “De Fonteintjes” (Fon) in Blankenberge, and lake “Kraenepoel” (Kra) in Aalter. St is situated in the centre of Belgium where the soil consists of sandy loam; Wes, Yde and Fon are situated at the Belgian coast and have a sandy soil; Kra is located slightly further inland and is characterized by a humus-rich sandy soil (Geo-Vlaanderen, 2001). Table 7.1 gives an overview of the different samples from each location with the geographical coordinates taken by GPS, and habitat, sediment and
vegetation characteristics. The St-samples were taken in autumn on 21 November 2007; all other samples on 3 March 2008.

**Cultures**

A total of 20 samples from soils (15) and ponds (5) was incubated on the day of sampling in liquid WC-medium (Guillard & Lorenzen, 1972) in standard conditions of 18°C ± 2°C, 25-30 μmol ph m⁻² s⁻¹ light intensity and a 12:12 hours light:dark period. After one week of incubation, single diatom cells were isolated by micropipette and grown as monoclonal cultures (strains) at the above conditions. We isolated diatom cells from all different sizes and shapes present in the sample, in an attempt to culture all species present, without *a priori* species selection. Where feasible, at least two clones were isolated per species. Cultures were re-inoculated in fresh medium when they reached late exponential phase. For morphological analysis, cultures were oxidized in hydrogen peroxide and embedded in Naphrax®. Pictures were taken using a Zeiss Axioplan 2 microscope equipped with an AxioCam Mrm camera. Valve length, width and stria density of 10 valves per strain were measured using ImageJ 1.37v software. Identiﬁcations were based on Krammer & Lange-Bertalot (1986, 1988, 1991a, b) and Krammer (2000) for the genus *Pinnularia* Ehrenberg. For *Hantzschia amphioxys* (Ehrenberg) Grunow, *Rhopalodia gibba* (Ehrenberg) O. Müller and *Cymbella subaequalis* Grunow, clear discontinuities in valve morphology (width, striae density, overall shape) were observed between the isolates. As it has become clear recently that subtle, discontinuous variations in morphology in diatom morphospecies are generally correlated with differences in reproductive, molecular, physiological and ecological characters (*e.g.* Mann, 1999; Behnke *et al.*, 2004; Lundholm *et al.*, 2006; Vanormelingen *et al.*, 2008a), these isolates were divided in different morphotypes (Table 7.2) and treated as separate species. Their taxonomy needs further study, however. Voucher slides of all natural samples and cultures are kept in the Laboratory of Protistology & Aquatic Ecology and are available upon request. Sixty-nine strains from aquatic or terrestrial habitat were selected for the stress tolerance experiments. Pictures of oxidized material from the different species taken using light microscopy are presented in Figs 7.1-35.
Table 7.1 Characteristics of the environmental samples from which the tested diatom strains were isolated.

1 T stands for terrestrial samples, A for aquatic samples.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample</th>
<th>Geographical coordinates</th>
<th>Type</th>
<th>Habitat description</th>
<th>Sediment type</th>
<th>Vegetation type &amp; cover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterre</td>
<td>St1</td>
<td>51°01'28.0&quot;N 03°42'45.2&quot;E</td>
<td>T</td>
<td>soil filled fissure between two concrete slabs, moist</td>
<td>sandy-silt with humus</td>
<td>moss 5%</td>
</tr>
<tr>
<td>Sterre</td>
<td>St2</td>
<td>51°01'33.3&quot;N 03°42'52.8&quot;E</td>
<td>T</td>
<td>wet car track in meadow with grasses and Betulas</td>
<td>silt</td>
<td>none</td>
</tr>
<tr>
<td>Sterre</td>
<td>St3</td>
<td>51°01'36.6&quot;N 03°42'58.4&quot;E</td>
<td>T</td>
<td>bare soil under Quercus in meadow, moist</td>
<td>sandy-silt with humus</td>
<td>moss 5%</td>
</tr>
<tr>
<td>Sterre</td>
<td>St4</td>
<td>51°01'40.3&quot;N 03°43'04.6&quot;E</td>
<td>T</td>
<td>bare soil under Chamaecypares, moist</td>
<td>sandy-silt with humus</td>
<td>microbial biofilm</td>
</tr>
<tr>
<td>Sterre</td>
<td>St5</td>
<td>51°01'44.5&quot;N 03°43'06.2&quot;E</td>
<td>T</td>
<td>bare soil under Taxus moist</td>
<td>sandy</td>
<td>microbial biofilm, moss 3%</td>
</tr>
<tr>
<td>Sterre</td>
<td>St6</td>
<td>51°01'30.8&quot;N 03°42'55.2&quot;E</td>
<td>T</td>
<td>concrete pavement with biofilm, moist</td>
<td>sandy-silt with humus</td>
<td>moss 5%</td>
</tr>
<tr>
<td>Sterre</td>
<td>St8</td>
<td>51°01'26.9&quot;N 03°42'50.2&quot;E</td>
<td>T</td>
<td>grooves between pavement tiles, moist</td>
<td>sandy-silt</td>
<td>moss 5%</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes1</td>
<td>51°05'49.9&quot;N 02°34'26.7&quot;E</td>
<td>T</td>
<td>dry sand and moss on dune</td>
<td>sand</td>
<td>moss 100%</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes2</td>
<td>51°04'96.8&quot;N 02°34'43.4&quot;E</td>
<td>T</td>
<td>humus-rich moist soil in dune shrub (Hippophae rhamnoides)</td>
<td>humus-rich sand</td>
<td>Claytonia perfoliata 75%</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes3</td>
<td>51°05'33.7&quot;N 02°34'65.3&quot;E</td>
<td>T</td>
<td>dry sand and moss on dune</td>
<td>sand</td>
<td>moss 30%</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes4</td>
<td>51°05'28.6&quot;N 02°34'59.2&quot;E</td>
<td>A</td>
<td>sandy sediment of shallow lake in dune slack, aquatic 200 m²</td>
<td>sand</td>
<td>Phragmites australis 10%</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes5</td>
<td>51°05'27.2&quot;N 02°34'57.3&quot;E</td>
<td>T</td>
<td>wet sand with biofilm in dune slack</td>
<td>sand + biofilm</td>
<td>microbial biofilm, moss 5%, Carex arenaria 10%</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes6</td>
<td>51°05'43.9&quot;N 02°34'37.5&quot;E</td>
<td>A</td>
<td>sandy sediment of temporary puddle in dune slack, aquatic 40 m²</td>
<td>sand + fine organic material</td>
<td>Chara 5%</td>
</tr>
<tr>
<td>Location</td>
<td>Sample</td>
<td>Geographical coordinates</td>
<td>Type</td>
<td>Habitat description</td>
<td>Sediment type</td>
<td>Vegetation type &amp; cover</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
<td>-----------------------------------</td>
<td>------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>Westhoek</td>
<td>Wes7</td>
<td>51°05'44.4&quot;N 02°34'38.3&quot;E</td>
<td>T</td>
<td>wet sand in dune slack with biofilm</td>
<td>sand</td>
<td>Carex arenaria 10%</td>
</tr>
<tr>
<td>Ter Yde</td>
<td>Yde1</td>
<td>51°08'18.4&quot;N 02°41'86.1&quot;E</td>
<td>T</td>
<td>moist sample of moss from moss-covered plain in dune slack</td>
<td>none (small amount of sand)</td>
<td>moss 100%</td>
</tr>
<tr>
<td>Ter Yde</td>
<td>Yde2</td>
<td>51°08'18.4&quot;N 02°41'86.1&quot;E</td>
<td>T</td>
<td>moist till wet sample of humid sand from moss-covered plain in dune slack</td>
<td>sand</td>
<td>moss 50%</td>
</tr>
<tr>
<td>Ter Yde</td>
<td>Yde3</td>
<td>51°08'18.4&quot;N 02°41'86.1&quot;E</td>
<td>T</td>
<td>wet sample of wet sand from moss-covered plain in dune slack</td>
<td>sand</td>
<td>moss 30% (none in depression)</td>
</tr>
<tr>
<td>Fonteintjes</td>
<td>Fon1</td>
<td>51°19'31.7&quot;N 03°09'38.7&quot;E</td>
<td>A</td>
<td>sediment sample of fishpond, aquatic 100 m²</td>
<td>sand + organic material</td>
<td>Chara, Phragmites, Ceratophyllum; total 20%</td>
</tr>
<tr>
<td>Fonteintjes</td>
<td>Fon3</td>
<td>51°19'50.1&quot;N 03°09'93.4&quot;E</td>
<td>A</td>
<td>sediment sample of puddle in meadow, aquatic 5m²</td>
<td>sand + organic material</td>
<td>moss, Carex, Luzula; total 75%</td>
</tr>
<tr>
<td>Kraenepoel</td>
<td>Kra1</td>
<td>51°04'53.8&quot;N 03°28'93.9&quot;E</td>
<td>A</td>
<td>sediment sample of lake, aquatic</td>
<td>organic material, little sand</td>
<td>Phragmites, Luzula, submersed macrophytes</td>
</tr>
</tbody>
</table>
Table 7.2 Overview of the examined diatom strains with morphometric features and results of the stress tolerance treatments.
A stands for aquatic, T for terrestrial habitat of origin.
1 Morph. means morphotype.
2 Values are given as average ± standard deviation, N=10.
3 The range of %VC is given based on triplicate treatments. Cases are shaded in function of the minimum %VC: minimum 0% (no shading), 1-24% (10% shading), 25-49% (25% shading), 50-74% (50% shading) and 75-100% (75% shading). Desic. means desiccation.
4 Invisible in LM.
5 Growth observed in a single replicate.
6 Number of fibulae.
7 This might be another species than Navicula radiosa.

<table>
<thead>
<tr>
<th>Habitat¹</th>
<th>Taxon²</th>
<th>Origin</th>
<th>Length³ (µm)</th>
<th>Width³ (µm)</th>
<th># striae/10 µm</th>
<th>Minimal - maximal % of viable cells after treatment⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>Achnanthes coarcta (Brébisson) Grunow</td>
<td>(Wes3)f</td>
<td>28.7 ± 1.2</td>
<td>7.0 ± 0.5</td>
<td>16.4 ± 1.1</td>
<td>98 – 99 94 – 96 65 – 70 60 – 74 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Achnanthes minutissimum (Kützing) Czarnecki</td>
<td>(Yde2)d</td>
<td>10.4 ± 0.3</td>
<td>3.0 ± 0.0</td>
<td>28.0 ± 1.2</td>
<td>89 – 93 80 – 90 39 – 48 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Caloneis molaris (Grunow) Krammer</td>
<td>(Yde2)a</td>
<td>36.0 ± 0.6</td>
<td>7.3 ± 0.2</td>
<td>20.4 ± 0.5</td>
<td>84 – 93 65 – 79 83 – 91 72 – 87 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Caloneis molaris (Grunow) Krammer</td>
<td>(Yde2)b</td>
<td>35.0 ± 0.8</td>
<td>7.5 ± 0.2</td>
<td>21.2 ± 0.4</td>
<td>88 – 94 65 – 79 54 – 71 72 – 87 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Cricula cf. halophila (Grunow in Van Heurck) D.G. Mann</td>
<td>(Wes7)b</td>
<td>35.5 ± 0.7</td>
<td>8.2 ± 0.4</td>
<td>18.0 ± 0.0</td>
<td>97 – 99 96 – 99 65 – 69 64 – 72 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Cymbella subaequalis Grunow morph.1</td>
<td>(Yde2)e</td>
<td>24.9 ± 1.2</td>
<td>7.8 ± 0.3</td>
<td>13.4 ± 0.9</td>
<td>91 – 95 94 – 98 2 – 7 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph.1</td>
<td>(St1)e</td>
<td>42.9 ± 0.4</td>
<td>6.2 ± 0.1</td>
<td>25.2 ± 0.9</td>
<td>98 – 99 99 85 – 88 91 – 97 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph.1</td>
<td>(St3)a</td>
<td>36.8 ± 0.5</td>
<td>6.6 ± 0.5</td>
<td>23.7 ± 1.0</td>
<td>87 – 94 86 – 91 89 – 93 85 – 90 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph.1</td>
<td>(S3)c</td>
<td>42.8 ± 0.6</td>
<td>6.4 ± 0.2</td>
<td>24.7 ± 1.1</td>
<td>98 – 99 98 98 – 99 91 – 95 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph.1</td>
<td>(St4)a</td>
<td>36.0 ± 0.6</td>
<td>6.0 ± 0.4</td>
<td>25.8 ± 0.4</td>
<td>71 – 76 74 – 82 73 – 77 79 – 86 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph.1</td>
<td>(St4)b</td>
<td>38.1 ± 0.8</td>
<td>6.0 ± 0.3</td>
<td>24.1 ± 0.9</td>
<td>93 – 96 82 – 92 94 – 95 1 0 0 0</td>
</tr>
<tr>
<td>Habitat¹</td>
<td>Taxon²</td>
<td>Origin</td>
<td>Length³ (µm)</td>
<td>Width³ (µm)</td>
<td># striae⁴/10 µm</td>
<td>Minimal – maximal % of viable cells after treatment⁴</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>--------</td>
<td>--------------</td>
<td>-------------</td>
<td>----------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow</td>
<td>(St5)a</td>
<td>40.5 ± 0.5 5.6 ± 0.2</td>
<td>24.0 ± 0.0</td>
<td>98 – 99</td>
<td>Control: 98 – 99, 30°C: 96 – 99, 40°C: 99 – 97 – 98</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 1</td>
<td>(Wes2)a</td>
<td>39.5 ± 0.7 6.0 ± 0.3</td>
<td>24.0 ± 0.7</td>
<td>73 – 79</td>
<td>Control: 96 – 98, 30°C: 96 – 97, 40°C: 96 – 98</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 1</td>
<td>(Wes2)b</td>
<td>40.4 ± 0.9 6.2 ± 0.2</td>
<td>24.6 ± 0.9</td>
<td>96 – 98</td>
<td>Control: 96 – 97, 30°C: 96 – 98, 40°C: 96 – 91 – 95</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 2</td>
<td>(St1)f</td>
<td>48.3 ± 0.9 9.0 ± 0.4</td>
<td>21.6 ± 0.7</td>
<td>97 – 98</td>
<td>Control: 97 – 97 – 98, 30°C: 47 – 64, 40°C: 41 – 45</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 2</td>
<td>(St8)c</td>
<td>46.8 ± 0.7 8.2 ± 0.3</td>
<td>20.3 ± 1.3</td>
<td>62 – 80</td>
<td>Control: 60 – 62, 30°C: 80 – 86, 40°C: 30 – 49</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 3</td>
<td>(St6)e</td>
<td>52.3 ± 0.5 7.2 ± 0.4</td>
<td>19.7 ± 0.7</td>
<td>85 – 89</td>
<td>Control: 85 – 89, 30°C: 81 – 88, 40°C: 80 – 88</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 3</td>
<td>(St6)f</td>
<td>52.3 ± 0.4 7.3 ± 0.4</td>
<td>18.8 ± 1.0</td>
<td>99 – 100</td>
<td>Control: 99 – 100, 30°C: 97 – 99, 40°C: 99 – 97 – 95</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 4</td>
<td>(Wes1)b</td>
<td>56.6 ± 0.8 7.2 ± 0.4</td>
<td>20.0 ± 0.0</td>
<td>89 – 92</td>
<td>Control: 89 – 92, 30°C: 81 – 83, 40°C: 5 – 44</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 4</td>
<td>(Wes1)b</td>
<td>56.1 ± 0.2 7.1 ± 0.6</td>
<td>20.4 ± 0.5</td>
<td>97 – 98</td>
<td>Control: 97 – 98, 30°C: 91 – 96, 40°C: 93 – 96</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 4</td>
<td>(Wes3)a</td>
<td>52.6 ± 0.7 7.3 ± 0.4</td>
<td>20.0 ± 0.0</td>
<td>98 – 99</td>
<td>Control: 98 – 99, 30°C: 100, 40°C: 76 – 78</td>
</tr>
<tr>
<td>T</td>
<td>Hantzschia amphioxys (Ehrenberg) Grunow morph. 4</td>
<td>(Wes3)b</td>
<td>51.8 ± 0.2 7.3 ± 0.3</td>
<td>19.6 ± 0.9</td>
<td>99 – 100</td>
<td>Control: 99 – 100, 30°C: 97 – 99, 40°C: 88 – 90</td>
</tr>
<tr>
<td>T</td>
<td>Mayamaea atomus (Kützing) Lange-Bertalot var. permits (Hustedt)</td>
<td>(Wes2)e</td>
<td>8.6 ± 0.3 3.8 ± 0.2</td>
<td>20.4 ± 0.9</td>
<td>100</td>
<td>Control: 100, 30°C: 49 – 85, 40°C: 51 – 76</td>
</tr>
<tr>
<td>T</td>
<td>Mayamaea atomus (Kützing) Lange-Bertalot var. permits (Hustedt)</td>
<td>(Wes2)f</td>
<td>8.7 ± 0.1 3.9 ± 0.2</td>
<td>22.4 ± 1.9</td>
<td>100</td>
<td>Control: 100, 30°C: 31 – 43, 40°C: 19 – 54</td>
</tr>
<tr>
<td>T</td>
<td>Navicula cryptotenella Lange-Bertalot</td>
<td>(Wes5)c</td>
<td>22.6 ± 0.4 5.4 ± 0.2</td>
<td>15.0 ± 0.7</td>
<td>82 – 87</td>
<td>Control: 82 – 87, 30°C: 77 – 81, 40°C: 75 – 80</td>
</tr>
<tr>
<td>T</td>
<td>Navicula libonensis Schoeman</td>
<td>(Wes3)e</td>
<td>32.3 ± 0.4 6.6 ± 0.2</td>
<td>13.0 ± 0.0</td>
<td>97 – 98</td>
<td>Control: 97 – 98, 30°C: 92 – 93, 40°C: 89 – 91</td>
</tr>
<tr>
<td>T</td>
<td>Navicula radiosa</td>
<td>(Wes3)d</td>
<td>45.0 ± 1.1 9.6 ± 0.2</td>
<td>11.3 ± 0.4</td>
<td>100</td>
<td>Control: 100, 30°C: 99 – 100, 40°C: 88 – 92</td>
</tr>
<tr>
<td>T</td>
<td>Navicula radiosa Kützing 8</td>
<td>(Wes5)a</td>
<td>41.5 ± 1.5 9.7 ± 0.2</td>
<td>12.0 ± 0.0</td>
<td>98 – 99</td>
<td>Control: 98 – 99, 30°C: 96 – 97, 40°C: 97 – 98</td>
</tr>
</tbody>
</table>

¹ Stress tolerance of vegetative diatom cells
² Habitat
³ Taxon
⁴ Origin
⁵ Length
⁶ Width
⁷ # striae/10 µm
⁸ Minimal – maximal % of viable cells after treatment
⁹ Control
¹⁰ + 30°C
¹¹ + 40°C gradual
¹² + 40°C abrupt
¹³ - 20°C desic.
¹⁴ 10 min.
¹⁵ + desic.
<table>
<thead>
<tr>
<th>Habitat</th>
<th>Taxon</th>
<th>Origin</th>
<th>Length$^1$ (μm)</th>
<th>Width$^1$ (μm)</th>
<th># striae$^1$/10 μm</th>
<th>Minimal – maximal % of viable cells after treatment$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>Navicula radiosa Kützing 8</td>
<td>(Wes5)b</td>
<td>4.0 ± 1.6</td>
<td>9.8 ± 0.2</td>
<td>11.7 ± 0.4</td>
<td>96 – 97 95 – 97 0 0 0 0 0 – 84$^6$</td>
</tr>
<tr>
<td>T</td>
<td>Navicula veneta Kützing</td>
<td>(Yde3)f</td>
<td>22.2 ± 0.2</td>
<td>22.2 ± 0.2</td>
<td>15.6 ± 0.5</td>
<td>79 – 80 71 – 85 69 – 88 76 – 80 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Navicula cf. wiesneri Lange-Bertalot</td>
<td>(Yde1)h</td>
<td>16.2 ± 0.4</td>
<td>4.9 ± 0.1</td>
<td>13.0 ± 0.7</td>
<td>90 – 92 81 – 96 31 – 59 6 – 24 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Nitzschia australis Hustedt</td>
<td>(Yde1)c</td>
<td>18.0 ± 0.5</td>
<td>2.9 ± 0.1</td>
<td>28.0 ± 0.7</td>
<td>64 – 73 73 – 78 72 – 78 79 – 85 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Nitzschia palea (Kützing) W. Smith</td>
<td>(Wes7)e</td>
<td>44.0 ± 0.4</td>
<td>4.3 ± 0.5</td>
<td>invisible 5</td>
<td>91 – 95 92 – 94 71 – 82 86 – 88 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Nitzschia perminuta (Grunow) M. Peragallo</td>
<td>(St8)g</td>
<td>32.7 ± 0.7</td>
<td>2.9 ± 0.2</td>
<td>29.7 ± 0.5</td>
<td>74 – 85 64 – 74 62 – 86 66 – 80 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St1)b</td>
<td>39.6 ± 1.2</td>
<td>7.6 ± 0.3</td>
<td>6.2 ± 0.4</td>
<td>65 – 82 74 – 77 48 – 63 63 – 73 14 – 23 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St6)c</td>
<td>37.1 ± 0.8</td>
<td>8.0 ± 0.5</td>
<td>6.3 ± 0.4</td>
<td>67 – 75 77 – 82 62 – 70 62 – 70 2 – 5 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St6)g</td>
<td>28.5 ± 1.4</td>
<td>6.9 ± 0.3</td>
<td>6.6 ± 0.6</td>
<td>81 – 85 85 – 88 91 – 93 86 – 90 3 – 12 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St8)a</td>
<td>36.8 ± 1.1</td>
<td>7.4 ± 0.3</td>
<td>6.2 ± 0.4</td>
<td>80 – 85 85 – 92 81 – 87 68 – 82 15 – 21 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St8)d</td>
<td>42.4 ± 0.6</td>
<td>8.9 ± 0.4</td>
<td>5.0 ± 0.0</td>
<td>76 – 86 80 – 86 89 – 91 67 – 71 22 – 47 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St1)d</td>
<td>40.9 ± 0.6</td>
<td>8.1 ± 0.3</td>
<td>6.0 ± 0.2</td>
<td>56 – 75 45 – 48 22 – 31 33 – 47 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St1)i</td>
<td>35.9 ± 0.6</td>
<td>8.2 ± 0.3</td>
<td>5.8 ± 0.4</td>
<td>45 – 52 20 – 37 1 – 8 1 – 12 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St1)k</td>
<td>33.7 ± 0.8</td>
<td>7.6 ± 0.3</td>
<td>6.0 ± 0.0</td>
<td>46 – 55 26 – 28 1 – 20 2 – 8 0 0 – 10$^f$ 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St5)c</td>
<td>40.7 ± 0.6</td>
<td>7.9 ± 0.5</td>
<td>6.0 ± 0.0</td>
<td>65 – 84 56 – 68 64 – 74 47 – 65 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>(St8)a</td>
<td>34.6 ± 0.7</td>
<td>7.2 ± 0.4</td>
<td>6.7 ± 0.5</td>
<td>87 – 90 84 – 88 81 – 82 62 – 70 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia divergentissima (Grunow) Cleve</td>
<td>(St5)b</td>
<td>23.0 ± 0.7</td>
<td>3.6 ± 0.2</td>
<td>14.1 ± 0.7</td>
<td>89 – 93 62 – 81 1 – 3 0 – 1.6 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia isseana Krammer</td>
<td>(St2)d</td>
<td>27.1 ± 1.7</td>
<td>7.1 ± 0.4</td>
<td>12.8 ± 0.4</td>
<td>90 – 92 88 – 89 85 – 94 89 – 91 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia marchica Ilka Schönfelder</td>
<td>(St2)g</td>
<td>28.0 ± 0.6</td>
<td>5.4 ± 0.3</td>
<td>14.5 ± 0.5</td>
<td>94 – 98 91 – 98 93 – 97 96 – 96 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia obscurea Kraske</td>
<td>(Wes2)c</td>
<td>18.0 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>13.2 ± 0.8</td>
<td>46 – 63 100 53 – 66 5 – 32 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia subcommutata Krammer var.</td>
<td>(Yde1)a</td>
<td>62.3 ± 2.0</td>
<td>11.4 ± 0.4</td>
<td>10.1 ± 0.2</td>
<td>97 – 98 98 – 99 94 – 98 44 – 60 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Pinnularia subcommutata Krammer var.</td>
<td>(Yde1)f</td>
<td>65.8 ± 0.7</td>
<td>10.9 ± 0.3</td>
<td>10.3 ± 0.3</td>
<td>98 – 99 96 – 96 15 – 38 3 – 16 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Rhopalodia gibba (Ehrenberg) O. Müller morph. 1</td>
<td>(Yde1)d</td>
<td>41.4 ± 0.7</td>
<td>8.1 ± 0.6</td>
<td>7.5 ± 0.7</td>
<td>87 – 92 90 – 92 21 – 33 34 – 61 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>T</td>
<td>Rhopalodia gibba (Ehrenberg) O. Müller morph. 1</td>
<td>(Yde1)e</td>
<td>42.2 ± 0.8</td>
<td>8.4 ± 0.5</td>
<td>7.6 ± 0.5</td>
<td>97 – 98 98 – 99 63 – 94 78 – 83 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Amphora pediculus (Kützing) Grunow</td>
<td>(Fon1)d</td>
<td>16.4 ± 1.7</td>
<td>3.3 ± 0.2</td>
<td>20.2 ± 0.4</td>
<td>95 – 98 96 – 99 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

$^1$ Habitat: T = Tolerant, A = Arlington

$^2$ Taxon: Origin (Yde1), (Wes7), (St5), (St6), (Yde3)

$^3$ Length (μm), Width (μm), Minimal ± Maximal

$^4$ % of viable cells after treatment: 0 = 0% (dead cells)

$^5$ Minimal – maximal

$^6$ Gradual: 0°C per minute, Abrupt: 0°C at end

$^7$ 0°C desiccation: 10 min., 30°C desiccation

$^8$ + 30°C

$^9$ + 40°C gradual

$^10$ + 40°C abrupt

$^11$ Temperature: 20°C

$^12$ + 30°C
<table>
<thead>
<tr>
<th>Habitat</th>
<th>Taxon</th>
<th>Origin</th>
<th>Length$^3$ (μm)</th>
<th>Width$^3$ (μm)</th>
<th># striae$^3$/10 μm</th>
<th>Minimal - maximal % of viable cells after treatment$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control + 30°C + 40°C + 40°C abrupt + 20°C desic. + 30°C</td>
</tr>
<tr>
<td>A</td>
<td>Cymbella subaequalis Grunow morph.2 (Wes6)c</td>
<td>24.3 ± 0.9 6.4 ± 0.2</td>
<td>13.7 ± 0.4</td>
<td>85 – 91 86 – 91</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Eunotia bilunaris (Brenberk) Schaarschmidt (Fon3)g</td>
<td>56.3 ± 1.6 4.3 ± 0.5</td>
<td>15.4 ± 0.9</td>
<td>70 – 75 28 – 42</td>
<td>49 – 63 15 – 40</td>
<td>0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Eunotia bilunaris (Brenberk) Schaarschmidt (Fon3)i</td>
<td>56.3 ± 1.4 4.5 ± 0.3</td>
<td>16.0 ± 1.6</td>
<td>79 – 82 45 – 66</td>
<td>40 – 61 30 – 45</td>
<td>0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Eunotia implicata Nörpel et al. (Kra1)b</td>
<td>26.7 ± 0.6 4.4 ± 0.2</td>
<td>14.4 ± 0.9</td>
<td>96 – 97 89 – 97</td>
<td>87 – 90 46 – 67</td>
<td>0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Eunotia implicata Nörpel et al. (Kra1)c</td>
<td>25.5 ± 0.9 4.2 ± 0.3</td>
<td>15.0 ± 1.0</td>
<td>96 – 98 90 – 95</td>
<td>86 – 95 58 – 65</td>
<td>0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Navicula ibonensis Schoeman (Wes4)e</td>
<td>32.1 ± 0.1 6.6 ± 0.1</td>
<td>13.0 ± 0.0</td>
<td>88 – 91 84 – 91</td>
<td>84 – 90 86 – 92</td>
<td>0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Navicula radiosa Kützing (Wes4)a</td>
<td>68.9 ± 0.5 10.9 ± 0.2</td>
<td>11.1 ± 0.2</td>
<td>88 – 91 80 – 90</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Navicula radiosa Kützing (Wes4)d</td>
<td>44.4 ± 0.7 9.5 ± 0.3</td>
<td>11.8 ± 0.4</td>
<td>94 – 95 92 – 98</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Navicula radiosa Kützing (Fon3)e</td>
<td>64.3 ± 1.4 10.3 ± 0.4</td>
<td>11.0 ± 0.6</td>
<td>92 – 96 85 – 91</td>
<td>0 0 0 0 – 74 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Navicula radiosa Kützing (Fon3)f</td>
<td>66.1 ± 1.0 10.3 ± 0.3</td>
<td>10.9 ± 0.7</td>
<td>91 – 93 85 – 91</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Navicula radiosa Kützing (Kra1)a</td>
<td>50.3 ± 0.9 10.3 ± 0.1</td>
<td>11.0 ± 0.0</td>
<td>99 – 100 98</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Navicula veneta Kützing (Wes6)d</td>
<td>21.5 ± 0.4 5.7 ± 0.1</td>
<td>16.0 ± 0.0</td>
<td>99 – 99 98</td>
<td>97 – 97</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Pinnularia viridiformis Krammer (Fon3)a</td>
<td>86.4 ± 2.1 18.6 ± 0.7</td>
<td>8.9 ± 0.2</td>
<td>77 – 84 65 – 74</td>
<td>81 – 90 59 – 80</td>
<td>0 0 0</td>
</tr>
<tr>
<td>A</td>
<td>Rhopalodia gibba (Ehrenberg) O. Müller morph.2 (Fon1)a</td>
<td>53.9 ± 0.9 9.1 ± 0.3</td>
<td>8.8 ± 0.8</td>
<td>71 – 77 69 – 80</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Rhopalodia gibba (Ehrenberg) O. Müller morph.2 (Fon1)b</td>
<td>52.0 ± 1.6 9.1 ± 0.4</td>
<td>8.6 ± 0.5</td>
<td>90 – 92 85 – 93</td>
<td>0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>
Experimental set-up

Seven different treatments were carried out in triplicate for each strain: (1) control (standard growth conditions); (2) gradual heating to +30°C; (3) gradual heating to +40°C; (4) abrupt heating to +40°C; (5) freezing at -20°C; (6) desiccation during 10 minutes; and (7) preconditioning by gradual heating to +30°C followed by desiccation during 10 minutes (Table 7.3). The temperatures 30°C and 40°C were chosen because the lethal temperature for most diatoms is situated between these two values (Suzuki & Takahashi, 1995; Butterwick et al., 2005). Gradual heating and preconditioning were applied because acclimatization increases the tolerance to unfavourable conditions in various organisms (e.g. Bierkens et al., 1998; Bayley et al., 2001; Sung et al., 2003; Dunlap et al., 2007), including diatoms (after 8h, Rousch et al., 2004). Because periods of dryness coincide with elevated temperatures, we chose to precondition the cells to desiccation by gradual heating.

The experiments were conducted in 24-well plates. A separate well plate was used for each of the seven treatments, and for every treatment three wells per strain were used as replicates. Not all strains could be tested at the same moment. Every day six or seven randomly chosen strains were subjected to the seven treatments. Each well was filled with 1.5 ml of fresh WC medium and cells from exponentially growing cultures were inoculated in the experimental wells three days before the treatment. We used different starting densities for the different species (ranging from around 120 to 185,000 cells per well) because cell densities varied widely between species due to differences in growth rates.

Table 7.3 Overview of the protocols of the different stress tolerance treatments. Dark conditions occurred at the same temperatures as stated in the middle column, except if further specified between brackets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature treatment (duration)</th>
<th>Duration of dark condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>18°C</td>
<td>15h + 3h</td>
</tr>
<tr>
<td>+30°C gradual</td>
<td>25°C (15h) + 28°C (1h30min) + 30°C (2h)</td>
<td>15h + 1h30min + 2h</td>
</tr>
<tr>
<td>+40°C gradual</td>
<td>25°C (15h) + 28°C (1h30min) + 30°C (2h) + 40°C (3h15)</td>
<td>15h + 1h30min + 2h + 3h15min</td>
</tr>
<tr>
<td>+40°C abrupt freezing -20°C</td>
<td>-20°C (4h45min)</td>
<td>15h (18°C) + 3h15min (40°C)</td>
</tr>
<tr>
<td>desiccation 10 minutes</td>
<td>air-dry desiccation (10min)</td>
<td>15h (18°C) + 3h (18°C) + 10min</td>
</tr>
<tr>
<td>+30°C + desiccation 10 minutes</td>
<td>25°C (15h) + 28°C (1h30min) + 30°C (2h) + desiccation (0h10min)</td>
<td>15h + 1h30min + 2h + 10min</td>
</tr>
</tbody>
</table>
The control treatment was placed in the dark at 18°C for 15h overnight plus an additional 3h to approximate the length of stress treatments. The four heating treatments were conducted in a temperature-controlled incubator, and air temperature was verified with a mercury thermometer. Details of exposure time to the different temperatures are listed in Table 7.3. For the freezing treatment, the plates were placed in a freezer at -20°C for 4h45min. For the desiccation treatment, the culture medium was removed with a Pasteur pipette, after which each well was carefully observed with a binocular until the whole film of water surrounding the cells and in the edge of the well had evaporated. After an additional 10 minutes, 1.5 ml of fresh WC-medium was added. Preconditioning before desiccation was achieved by gradual heating of the plates to +30°C for 18h30min (Table 7.3). All treatments were conducted in the dark to avoid photo-oxidative stress. After treatment, the plates were returned to standard conditions.

Immediately after treatment (t0) an average of 200 cells per well was counted using a Zeiss Axiovert inverted microscope and densities (number of cells per mm²) were calculated. At t0 total cell densities were determined. After 14 days (t14) separate cell counts were performed of i) dead cells (shrivelled and colourless cell content or empty frustule) and ii) all cells (dead and alive together). Again, we counted an average of 200 cells per well in both t14 cell counts. However, if the number of dead cells was too low for this, we counted the exact number of dead cells present in the entire well. An increase in the total cell density in a well between t0 and t14 validated “growth” and thus survival of the replicate. A strain was considered “tolerant” for a treatment if at least two out of three replicates showed positive growth. A species was designated as “tolerant” for a treatment if at least one of its strains was considered tolerant. The percentage of viable cells (uncorrected %VC) equalled 100 x [1 - (number of dead cells per mm² at t14 / total number of cells per mm² at t0)]. In an attempt to correct for possible effects of differences between strains in “background mortality” (i.e. % of dead cells in control conditions) on the stress treatment mortality (which is a combination of background mortality and the stress mortality), we calculated for each strain a “corrected %VC” by adding the average background mortality to the average uncorrected %VC after treatment. Both uncorrected and corrected %VC were further analyzed.
Statistical analysis

For each treatment, statistical analyses were performed on two measured variables: (i) the tolerance of a species (presence/absence of growth), and (ii) the survival rate, measured as the average percentage of viable cells of a species (%VC). We performed the analyses on both the uncorrected and corrected %VC. As our data were not normally distributed, we used non-parametric statistical tests. Habitat types were subdivided in two categories (terrestrial and aquatic). Strains of the same species occurring in different habitat types were seen as different ecological entities and treated as separate species. All statistical analyses were performed with Statistica 7.0 (StatSoft, Inc., Tulsa, Oklahoma, USA), and the significance level was set at \( p = 0.05 \).

First, we tested for each of the six stress treatments whether the treatment resulted in a lower average %VC of the different species compared to the control treatment using non-parametric Wilcoxon matched pairs tests (Sokal & Rohl, 2000). Likewise, Wilcoxon matched pairs tests were used to test for differences in %VC between the different stress treatments. Based on environmental samples, it has been noted that the average valve length of populations of the terrestrial diatom species *Pinnularia borealis* Ehrenberg and *Hantzschia amphioxys* decreases with a decreasing soil moisture content (Van de Vijver & Beyens, 1997; Van Kerckvoorde et al., 2000). Therefore, we additionally calculated the Pearson Product-Moment correlations between length and %VC for each treatment. This was done for all species together and for the strains of *Pinnularia borealis* and *Hantzschia amphioxys* separately.

Secondly, we determined for each stress treatment whether the tolerance (presence/absence of growth) of species differed significantly between habitat types. For each treatment, a contingency table with the numbers of tolerant and intolerant species per habitat type was made and analyzed with the non-parametric Pearson’s chi square test (Sokal & Rohl, 2000) to test for effects of habitat type. This test works with observed frequencies and takes into account the asymmetrical set-up.

Finally, for each treatment we tested whether the average %VC of species varied between habitat types using the non-parametric Mann-Whitney U test (Sokal & Rohl, 2000).
7.3 Results

Diatom strains
In total 69 strains of 34 species (25 terrestrial and 9 aquatic) were used, belonging to 13 genera (Table 7.2 and Figs 7.1-35). The terrestrial samples revealed taxa that are often encountered in terrestrial habitats, such as *Pinnularia borealis*, *Hantzschia amphioxys*, *Nitzschia austriaca* Hustedt, *N. perminutum* (Grunow) M. Peragallo, *N. palea* (Kützing) W. Smith, and *Mayamaea atomus* (Kützing) Grunow var. *permitis* (Hustedt) Lange-Bertalot (formerly known as *Navicula atomus*) (Ettl & Gärtner, 1995). Especially the first two species are among the most frequently reported terrestrial diatoms (Patrick, 1977; Flechtner *et al.*, 1998; Van Kerckvoorde *et al.*, 2000; Van de Vijver *et al.*, 2004). The aquatic strains belonged to aquatic species such as *Amphora pediculus* (Kützing) Grunow and *Eunotia implicata* Nörpel *et al.*. Strains of five species were isolated from both habitats. The terrestrial and aquatic isolates from *Navicula radiosa* Kützing (Figs 7.15-17), *N. libonensis* Schoeman (Figs 7.12-13) and *N. veneta* Kützing (Figs 7.9, 7.11) were morphologically identical, while those from *Cymbella subaequalis* (Figs 7.31-32) and *Rhopalodia gibba* (Figs 7.28-29) differed in valve width, and in outline and fibula density, respectively (Table 7.2).

Survival in control conditions
Most of our tested strains had a %VC of more than 90% in the control conditions. However, some strains – for example three strains of *Hantzschia amphioxys*, six strains of *P. borealis*, *P. obscura* and *Nitzschia austriaca* had %VC of less than 70%. These strains were probably in poor health. In the case of *N. austriaca*, *P. obscura* Krasske, one strain of *P. borealis*, and one strain of *H. amphioxys* morphotypes 1 and 2, heating improved the survival percentage compared to the control survival. The uncorrected and corrected %VC in the control condition was not significantly different between habitat types (*p*=0.78 for both the uncorrected and corrected dataset).

Tolerance to heating
Heating was tolerated by the majority of the species (Table 7.2), but all four temperature treatments had a significantly lower %VC compared to the control conditions (*p < 0.001*) (Fig. 7.36). There were also differences in %VC between the different heating
Figs 7.1-17 LM pictures from oxidized culture material of a selection of the strains used in the experiments with the corresponding voucher slide identification and habitat type. Scale bar = 10 μm. A designates aquatic habitat types of origin, T terrestrial habitat types.

Fig. 7.1 Pinnularia viridiformis (Fon3)a; A. Fig. 7.2 Pinnularia subcommutata var. nonfasciata (Yde1)a; T. Fig. 7.3 Pinnularia borealis (St1)b; T. Fig. 7.4 Caloneis molaris (Yde2)a; T. Fig. 7.5 Pinnularia isseliana (St2)d; T. Fig. 7.6 Pinnularia marchica (St2)a; T. Fig. 7.7 Pinnularia divergentissima (St5)b; T. Fig. 7.8 Pinnularia obscura (Wes2)c; T. Fig. 7.9 Navicula veneta (Wes6)c; A. Fig. 7.10 Navicula cryptotenella (Wes5)c; T. Fig. 7.11 Navicula veneta (Yde3)f; T. Fig. 7.12 Navicula libonensis (Wes3)c; T. Fig. 7.13 Navicula libonensis (Wes4)c; A. Fig. 7.14 Craticula cf. halophila (Wes7)b; T. Fig. 7.15 Navicula radios (Wes5)a; T. Fig. 7.16 Navicula radios (Wes3)d; T. Fig. 7.17 Navicula radios (Wes4)a; A.
Figs 7.18-35 LM pictures from oxidized culture material of a selection of the strains used in the experiments with the corresponding voucher slide identification and habitat type. Scale bar = 10 μm. A designates aquatic habitat types of origin, T terrestrial habitat types.

Fig. 7.18 Hantzschia amphioxys morphotype 4 (Wes1)a; T. Fig. 7.19 Hantzschia amphioxys morphotype 3 (St6)f; T. Fig. 7.20 Hantzschia amphioxys morphotype 2 (St8)c; T. Fig. 7.21 Hantzschia amphioxys morphotype 1 (St1)e; T. Fig. 7.22 Nitzschia palea (Wes7)e; T. Fig. 7.23 Nitzschia perminutum (St8)e; T. Fig. 7.24 Nitzschia austriaca (Yde1)c; T. Fig. 7.25 Amphora pediculus (Fon1)d; A. Fig. 7.26 Navicula cf. wiesneri (Yde1)h; T. Fig. 7.27 Eunotia bilunaris (Fon3)i; A. Fig. 7.28 Rhopalodia gibba morphotype 2 (Fon1)a; A. Fig. 7.29 Rhopalodia gibba morphotype 1 (Yde1)e; T. Fig. 7.30 Achnanthes coarctata (Wes3)f; T. Fig. 7.31 Cymbella subaequalis morphotype 1 (Yde2)e; T. Fig. 7.32 Cymbella subaequalis morphotype 2 (Wes6)c; A. Fig. 7.33 Eunotia implicata (Kra1)c; A. Fig. 7.34 Achnanthidium minutissimum (Yde2)d; T. Fig. 7.35 Mayamaea atomus var. permitis (Wes2)e; T.
Fig. 7.36 Box plots of the percentages of viable cells (%VC) of the diatom species for the different temperature treatments without separation in habitat type. N=34 for each treatment.

Fig. 7.37 Percentages of tolerant species (representing growth in at least two out of three replicates of at least one strain) per habitat type for each treatment. White bars represent aquatic taxa, black bars represent terrestrial taxa. For terrestrial taxa N=25, for aquatic taxa N=9.
treatments ($p<0.05$ for both uncorrected and corrected %VC) (Fig. 7.36). All strains survived gradual heating to +30°C (Fig. 7.37) with no significant difference in %VC between habitat types (Fig. 7.38).

Gradual heating to +40°C was survived by a significantly lower number of aquatic species compared to terrestrial species ($p=0.013$) (Fig. 7.37). The aquatic *Amphora pediculus*, strains of *Navicula radiosa* from both habitats and the aquatic strains of *Cymbella subaequalis* and *Rhopalodia gibba* were killed by the gradual heating (Table 7.2). In contrast, the terrestrial strains of *Cymbella subaequalis* and *Rhopalodia gibba* survived gradual heating to 40°C. The %VC was highly variable among species of the same habitat with extremes between 0 and 98% (Table 7.2 and Fig. 7.38), while no significant differences were detected between habitats.

Abrupt heating to +40°C was more lethal than the gradual heating as three more strains isolated from terrestrial habitat were intolerant: *Achnanthidium minutissimum* (Kützing) Czarnecki, *Pinnularia divergentissima* (Grunow) Cleve and *Cymbella subaequalis* (Table 7.2). Survival frequencies were not dependent on habitat type ($p=0.142$) (Fig. 7.37) and, similar to the gradual heating, the %VC was highly variable and no significant differences were detected between habitat types (Fig. 7.38). For both habitat types together, a significantly lower %VC was present after abrupt heating compared to gradual heating to +40°C ($p=0.017$ for both uncorrected and corrected %VC) (Fig. 7.36).

**Tolerance to freezing**

Freezing at -20°C was lethal for most of the isolates (Table 7.2). None of the aquatic strains survived. Only some strains of three terrestrial species tolerated the treatment, being *Pinnularia borealis* (5 out of 10 strains), *Mayamaea atomus* var. *permitis* (both tested strains) and *Hantschiae amphioxys* morphotype 2 (1 out of 4) and morphotype 4 (1 out of 2) (Table 7.2). The %VC fluctuated greatly between replicates, from 2 to 64% (Table 7.2). No significant differences were observed between habitat types based on survival frequencies ($p=0.217$) (Fig. 7.37) or on %VC ($p>0.05$ for both uncorrected and corrected %VC) (Fig. 7.38).

**Tolerance to desiccation**

None of the species consistently tolerated desiccation, with or without preconditioning (Table 7.2 and Fig. 7.37). Growth was recorded in a
single replicate of five strains from three different species, *Pinnularia borealis* (2 strains), *Hantzschia amphioxys* morphotype 4 (1 strain) and *Navicula radiosa* (2 strains). The first two are typical terrestrial species while resistant strains of the third species were isolated from an aquatic and a terrestrial sample (Table 7.2). %VC in these replicates varied between 5 and 84% (Table 7.2).

**Influence of valve length**

Similarly, valve length appeared unimportant in explaining %VC of all species analyzed together (R² not higher than 0.08, p>0.05), nor the %VC of the strains of *P. borealis* and *H. amphioxys* separately.
7.4 Discussion

All temperature and desiccation treatments conducted in this study clearly represented adverse conditions for vegetative diatom cells, as the percentage viable cells (%VC) significantly decreased in all six treatments compared to the control condition. Furthermore, our results indicate a high sensitivity of freshwater diatoms to abrupt heating, freezing and desiccation. Forty percent of the tested species died after abrupt heating to +40°C, only three species survived freezing, and not a single species consistently survived desiccation. Our results agree with published data for freshwater planktonic diatoms which report tolerance to high temperatures only up to 30-40°C (Suzuki & Takahashi, 1995; Butterwick et al., 2005), and no survival when exposed to desiccation (Jaworski & Lund, 1970). They are in stark contrast however with stress tolerances reported for vegetative cells of terrestrial and aquatic cyanobacteria and green algae, with some representatives being able to survive desiccation periods ranging from one day to several weeks (Potts, 1999; Sabacka & Elster, 2006; Gray et al., 2007), and freezing temperatures ranging from -40°C to liquid nitrogen (Tamaru et al., 2005; Sabacka & Elster, 2006).

While desiccation and freezing both result in the dehydration of cells (Welsh, 2000), our results revealed an even lower survival for desiccation compared to freezing. This agrees with observations that desiccation is a more injurious stress than freezing (Oldenhof et al., 2006; Sabacka & Elster, 2006). Interestingly, some strains of the terrestrial species H. amphioxys and P. borealis did survive freezing or desiccation in a single replicate. As the percentages of surviving cells were very low, this may have been due to the combination of a low survival probability and a low number of tested cells per well (between 300 and 1000). This means that some cells of these species are capable of surviving freezing or desiccation, possibly by means of differential expression of universal molecular mechanisms such as osmolytes (Welsh, 2000), stress proteins (Bierkens et al., 1998; Rousch et al., 2004), antioxidants or fatty acid composition (Holmstrup et al., 2002; Rousch et al., 2003; Vicre et al., 2004; Dunlap et al., 2007), or by their cell cycle phase (Hodgson et al., 1992). In addition, two strains of N. radiosa showed survival percentages for desiccation of 74 and 84% in a single replicate. Possibly, the cells were clustering during desiccation, or were in a different culture growth phase, despite the fact that we attempted to keep all cultures in exponential phase. Also, a mechanism involving intercellular communication
of stressful conditions and a subsequent response of the individual cells could be hypothesized. While these observations indicate that cells of at least some (terrestrial or habitat-unspecific) diatom species can be in a physiological state in which they can survive desiccation or freezing, the reasons for this rare survival are not clear and should be further investigated.

In contrast to desiccation to the air, previous experiments with sediments show that most terrestrial species survive in sediment with over 50% moisture content (Evans, 1959). Soils show a large vertical gradient in water content in the shallow surface layer (Gao et al., 2008) and the observed vertical migration of semi-terrestrial diatoms and cyanobacteria in drying ponds (Evans, 1959) could thus be their main mechanism to avoid desiccation. Moreover, in natural conditions, the slower desiccation rate in sediments allows more species to survive (Evans, 1959; Hostetter & Hoshaw, 1970), possibly due to the longer time interval during which cells could initiate protective mechanisms (Oldenhof et al., 2006) or form physiological resting stages (McQuoid & Hobson, 1996). In our study, preconditioning by heating the cells to +30°C did not enhance their tolerance to desiccation, despite the known positive influence of pre-treatment on stress tolerance by the initiation of protection mechanisms in various organisms (Bierkens et al., 1998; Bayley et al., 2001; Sung et al., 2003; Dunlap et al., 2007). However, in the heating treatments there was an effect of preconditioning, as several species survived gradual but not abrupt heating to 40°C. Moreover, abrupt heating had in general a more negative influence on %VC than gradual heating. On the other hand, some strains of for instance Rhopalodia gibba, Nitzschia and H. amphioxys did have a higher survival after the abrupt heating compared to the gradual heating, a fact that could be due do the longer duration of stress during the gradual heating.

The sensitivity of freshwater diatoms to freezing, desiccation and abrupt heating may influence their dispersal capacities and consequently rates of allopatic speciation. Diatom speciation has been generally tied to evolution within ancient water bodies or landscapes (Kociolek & Spaulding, 2000; Rossiter & Kawanabe, 2000), whereas terrestrial diatoms are thought to be the least likely to become reproductively isolated and undergo speciation due to higher dispersal abilities (Spaulding et al., 2010). Nevertheless, our results clearly indicate that vegetative cells of most taxa do not survive harsh adverse conditions, implying that most diatoms –
terrestrial species included – are likely to be limited in their dispersal capacities. This is also in agreement with the rare occurrence of living diatoms in the air (Van Overeem, 1937; Schlichting, 1961; Brown et al., 1964; Schlichting, 1964; Roy-Ocotla & Carrera, 1993) or on waterfowl (Schlichting, 1961). We therefore argue that physically unprotected vegetative diatom cells will not survive long-distance dispersal by wind or birds. This was already suggested by the high differentiation for microsatellite markers between populations of the benthic freshwater diatom *Sellaphora capitata*, even when located only some 100s of kilometres from each other (Evans et al., 2009). A limited dispersal is also in agreement with recent taxonomic revisions, which led to the discovery of a large number of regionally endemic species in isolated areas, for example in the genera *Diadesmis* Kützing (Van de Vijver et al., 2002), *Luticola* D.G. Mann (Van de Vijver & Mataloni, 2008), *Muelleria* (Frenguelli) Frenguelli (Spaulding et al., 1999), and *Stauroneis* Ehrenberg (Van de Vijver et al., 2005). However, the occasional survival of a single replicate from our experiments and the rare occurrence of single, living diatom cells on waterfowl and in air traps indicate that diatom cells can indeed be occasionally dispersed. For instance, two years after the volcanic activity had ceased on Surtsey Island, 33 km of the coast of Iceland, 69 different diatom species were reported, mainly terrestrial species also occurring in Iceland (Behre & Schwabe, 1970 in Kristiansen, 1996). Therefore, we conclude that, although occasional dispersion events indeed may be successful, vegetative diatom cells will not be massively dispersed by wind and waterfowl.

Besides the high sensitivity of both terrestrial and aquatic species to our treatments, our results do not reveal a marked and consistently higher tolerance of terrestrial diatom species compared to aquatic species. All species, regardless of habitat, were very sensitive to desiccation, and, apart from three species, to freezing. The very broad ranges of %VC between species within the two habitat types for the other treatments - ranging from 0 to 100% - indicate highly species-specific tolerances, which were for a large part independent of habitat. Similar species-specific stress tolerances were already reported in other microalgae (Butterwick et al., 2005; Gray et al., 2007).

As we were mainly interested in the general survival capacities of vegetative diatom cells and habitat-dependent survival differences among species, we did not explicitly test for intraspecific variation in %VC. Although the experimental design was not standardized
enough to draw firm conclusions on this matter, and some strains were obviously in a less healthy physiological state than others, there seem to be some differences in stress tolerance between strains of the same species. For instance, one strain of *H. amphioxys* morph. 4 survived freezing much better than the other strains. The same was true for *P. borealis*, in which cells of half of the strains tolerated freezing. Also in the heating to +40°C treatments, differences in %VC between strains were obvious for several species. Future experiments should focus on determining the extent of intraspecific genetic variation for these traits.

Nevertheless, apart from these inter- and intraspecific differences, the habitat-dependent tolerances for gradual heating to +40°C and freezing do point at a better adaptation of vegetative cells of the terrestrial species to their more extreme habitat. First, a higher number of terrestrial species survived gradual heating to +40°C compared to aquatic species, which is probably an adaptation to the extreme diurnal variations in temperature in the upper soil layer (Gao et al., 2008). The positive responses of the terrestrial strains of *Rhopalodia gibba* and *Cymbella subaequalis* to this treatment compared to the aquatic strains underscore this habitat-specific difference. Interestingly, the aquatic and terrestrial strains of both species also differed morphologically. These different ecological entities might represent different species, similar to the ecophysiological and genetically separated aquatic and terrestrial lineages of green algae (Lewis & Lewis, 2005; Gray et al., 2007; Zoe et al., 2008). In diatoms, it has recently become clear that a high (pseudo)cryptic species diversity exists (Sarno et al., 2005; Mann & Evans, 2007; Vanormelingen et al., 2008a), and closely related species can occupy different niches (Vanelslander et al., 2009). Alternatively, it may concern different locally adapted populations, although it is unclear in that case why there is a correlation with valve morphology. In contrast, Navicula radiosa strains from both habitat types responded in exactly the same way to our tolerance experiments and showed (in our eyes) no morphological differences. As such, *N. radiosa* may be a truly generalist species occurring in both habitats. However, it might be that the terrestrial strains are not identical to *Navicula radiosa*. This needs further study, however.

A second clue for a habitat-specific adaptation of diatoms – although not statistically underpinned – resulted from the freezing experiment. The nine surviving strains all belonged to the typical terrestrial taxa *Hantzschia amphioxys*, *Pinnularia borealis* and *Mayamaea*.
atomus var. permitis (Ettl & Gärtner, 1995). Similarly, the only diatom reported in literature to tolerate freezing is the terrestrial Stauroneis anceps Ehrenberg originating from temperate desert crusts (Hostetter & Hoshaw, 1970). The higher tolerance to freezing may have arisen as an adaptation to the more extreme variations in temperature in terrestrial habitats (Gao et al., 2008).

Interesting to note is the possible influence of climatic conditions on the observed stress tolerance differences between aquatic and terrestrial diatoms. All our strains were isolated from warm temperate, fully humid areas with warm summers (Kottek et al., 2006). Various organisms from more extreme climates have a wider tolerance range for temperature and drought compared to their counterparts from milder climates (e.g. Sinclair et al., 2003; Tomanek, 2008; Xiao et al., 2008; Dong & Somero, 2009). As such, the relatively small difference in stress tolerance between terrestrial and aquatic diatoms in our study could result from the temperate climate they inhabit, and may be larger in more extreme climates.

In conclusion, in the present study we detected a high sensitivity of vegetative cells of benthic freshwater diatoms to desiccation, freezing and abrupt heating. This is in agreement with the high population differentiation observed in a freshwater benthic diatom and may explain the widespread endemism observed in freshwater diatoms. Secondly, vegetative cells of terrestrial diatoms are more tolerant to temperature extremes than their aquatic counterparts, probably as an adaptation to the more extreme terrestrial environment. Additional studies should address the influence of climate on diatom stress tolerance, the molecular mechanisms underlying stress tolerance, and the evolutionary divergence of terrestrial and aquatic diatom lineages. Ultimately, linking differential levels of stress tolerance among species to population differentiation and the geographical distribution of species will reveal how stress tolerance influences dispersal and colonization and thus shapes diatom diversity and distribution.

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diatom communities, and the FWO-project 3G/0419/08 on thermal adaptation.
8.

Resistance of freshwater benthic diatom resting stages to experimental desiccation and freezing depends on the temporality of their habitat

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Unpublished manuscript
Abstract

Lakes, temporary ponds and terrestrial soils vary widely in the extent and periodicity of fluctuations in moisture content and temperature. While these habitat types are characterized by different diatom communities, it is unclear how terrestrial diatoms overcome regularly encountered stress factors such as desiccation and freezing, and whether diatom stress tolerance is habitat-dependent. We experimentally assessed the tolerance for desiccation and freezing of diatom resting cells, in comparison to vegetative cells, for 17 benthic freshwater morphospecies occurring over a gradient from permanent aquatic habitats to terrestrial soils, divided into five moisture categories. Resting cell formation was initiated by nitrogen depletion in dark conditions. Vegetative cells were in general highly sensitive to desiccation and freezing. Only strains of three terrestrial taxa survived freezing in vegetative stage while only a single strain survived 5 minutes of desiccation. In contrast, resting cells showed a higher tolerance for desiccation, especially when preceded by a heat treatment, and more strains and taxa survived freezing, albeit often with low survival percentages and a large interclonal variation. Strikingly, only the resting cells of terrestrial taxa, i.e. those occurring mainly in wet and moist or temporarily dry places (moisture category 4) or nearly exclusively outside water bodies (category 5) survived desiccation and freezing, indicating the presence of habitat-specific adaptations to these adverse conditions. Possible implications of the observed (lack of) stress tolerance for dispersal, population structure, geographic distributions and allopatric speciation of diatoms are discussed.

Key words: benthic diatoms, terrestrial, freshwater, resting cells, desiccation, freezing, stress tolerance, dispersal
8.1 Introduction

Diatoms thrive in a range of aquatic habitats, including permanent water bodies, but also temporary habitats such as semi-permanent to ephemeral pools and seepages and a variety of wet or moist soils (Round et al., 1990). These temporary habitats frequently dry out completely and undergo large diurnal and seasonal fluctuations in temperature due to the (near-)absence of a buffering water column (Starks et al., 1981; Gao et al., 2008). A gradient exists from permanent over semi-permanent and ephemeral water bodies to temporarily wet soils, entailing variations in other physical and chemical parameters (Scholnik, 1994). Over this gradient, there is a turnover of benthic diatom assemblages with a number of diatom morphospecies largely confined to moist or wet soils, classified as “terrestrial” diatoms (Petersen, 1935; Ettl & Gärtner, 1995), including for instance *Achnanthes coarctata* (Brébisson) Grunow, *Luticola spp.* (Mann), *Hantzschia amphioxys* (Ehrenberg) Grunow, *Pinnularia borealis* Ehrenberg and *Mayamaec atomus* (Kützing) Lange-Bertalot (Ettl & Gärtner, 1995; Johansen, 1999; Van Kerckvoorde et al., 2000; Van de Vijver et al., 2004; Villanueva, 2006). Van Dam et al. (1994) outlined five moisture categories and assigned the benthic freshwater diatoms of The Netherlands to a category based on how often they can be found outside water bodies.

Aquatic organisms inhabiting temporary habitats have to be able to cope with large environmental fluctuations, but also bridge unfavourable periods in which the habitat is not suitable for growth. It is as yet unclear how terrestrial diatoms deal with desiccation and temperature extremes. Different mutually non-exclusive strategies are possible. First, vegetative cells could have protection mechanisms reported to enhance stress tolerance in other microalgae, such as an EPS-layer (Potts, 1999; Tamaru et al., 2005) or the accumulation of various metabolites (Welsh, 2000; Rousch et al., 2004; Hayward et al., 2007; Rajendran et al., 2007). However, vegetative cells of terrestrial diatoms are only slightly more tolerant for temperature extremes than their aquatic counterparts and do not survive desiccation (Souffreau et al., 2010), corresponding to the general high sensitivity of vegetative diatom cells to desiccation (Evans, 1958, 1959; Hostetter & Hoshaw, 1970; Suzuki & Takahashi, 1995; Butterwick et al., 2005). Desiccation stress might to a certain extent be mitigated by sediment, but passing a threshold of 50% moisture content most diatom cells die (Evans, 1958, 1959). Secondly, terrestrial diatoms might avoid stress conditions by migrating deeper into the sediment. This is
plausible given that marine intertidal diatoms are well-known to avoid light stress by vertical migration (Perkins et al., 2010). Thirdly, terrestrial communities could be part of a metacommunity involving local extinction during adverse conditions and (re)colonization from nearby patches when the patch becomes suitable again. However, such extinction-colonization dynamics do imply a minimal tolerance of adverse conditions encountered during wind or animal-mediated dispersal (Kristiansen, 1996; Isard & Gage, 2001). A final possibility is the formation of tolerant resting stages, which would not only allow terrestrial species to persist locally but also to colonize suitable new habitat patches.

Resting stages are by definition stages of the life cycle of an organism during which growth and development are stopped and cells don’t divide. They are characterized by a reduced metabolic activity, and are typically formed to survive adverse environmental conditions. Resting stages are widespread among aquatic organisms, including bacterial endospores (e.g. Nicholson et al., 2000), akinetes of cyanobacteria (e.g. Sutherland et al., 1979), and cysts and spores of various protists (e.g. Anderson, 1975; Alve & Goldstein, 2010; Anderson, 2010; Müller et al., 2010) but also zooplankton resting eggs (e.g. Slobodkin, 1954; Hutchinson, 1967; Gilbert & Schreiber, 1995), dormant stages of insects (e.g. Levins, 1969; Taylor, 1980; Bradford & Roff, 1993) and seeds of higher plants (e.g. Vleeshouwers et al., 1995). Also diatoms form resting stages. Two types of resting stages are reported; resting spores have a distinct morphology (reviewed in McQuoid & Hobson, 1996), while resting cells are morphologically identical to the vegetative stages (Lund, 1953; Anderson, 1975; Sicko-Goad et al., 1986). Resting cells are more flexible because they form and germinate faster and don’t require silica for their formation, while resting spores survive longer in species forming both types (Kuwata & Takahashi, 1990; Kuwata et al., 1993). The typical cell content of both types of resting stages can consist of condensed plastids, larger vacuoles or more lipid droplets and a granular cytoplasm, but this varies among the different taxa studied (Anderson, 1975; Hargraves & French, 1975; Hoban et al., 1980; Sicko-Goad et al., 1986). Resting spores are mainly reported in centric diatoms, with some notable exceptions (reviewed in Hargraves & French, 1975; McQuoid & Hobson, 1996; Schmid, 2009), while resting cells prevail in the pennate diatoms (Sicko-Goad et al., 1986; McQuoid & Hobson, 1996) which constitute the bulk of benthic diatom communities.

In various aquatic diatom populations, resting cells act as a
seed bank in the sediment (Lund, 1953; McQuoid & Hobson, 1995; Schelske et al., 1995; Persson, 2002; Jewson et al., 2006; Poulíčková et al., 2008), which may explain the fast and dense spring blooms of planktonic diatoms (Gran, 1912; Lund, 1953). Resting cells in sediment seed banks have to be tolerant to dark conditions, but also to nutrient depletion or anoxia, which was experimentally demonstrated for a few species (e.g. Hargraves & French, 1975; Hollibaugh et al., 1981; Kuwata et al., 1993; McQuoid & Hobson, 1995; Jewson et al., 2006). In temporary aquatic habitats, however, the main stress factors are different, being desiccation and temperature extremes. While it has been suggested that resting cells of terrestrial diatoms might be important for surviving desiccation (Evans, 1958, 1959), it is unknown to what extent (terrestrial) diatom resting cells are tolerant to these stress factors. In this study we therefore experimentally tested the following hypotheses; (1) resting cells of terrestrial diatoms are tolerant to desiccation and freezing, in contrast to vegetative cells, and (2) they are more tolerant to desiccation and freezing than the resting cells of freshwater benthic diatoms, given the different stress factors encountered by diatoms from both habitat types. Resting cells were induced by cold dark conditions and nitrogen depletion (McQuoid & Hobson, 1995) for 50 strains from 17 morphospecies of pennate benthic diatoms, divided over the five moisture categories of Van Dam et al. (1994). Next, their tolerance to freezing and desiccation (with or without preceding gradual heating) was determined and compared to the tolerance of vegetative cells of the same strains.

8.2 Materials & Methods

Taxon sampling and identification
In total 50 clonal strains of 17 benthic diatom morphospecies were used (Table 8.1), divided over the five moisture categories outlined by Van Dam et al. (1994) (Table 8.2). Only morphospecies for which at least two strains were available were included, to be able to at least in part account for trait variation within the taxa studied. Details on the environmental sampling, strain isolation, morphological characterization and identification can be found in Souffreau et al. (2010; submitted), Trobajo et al. (2006), Evans et al. (2009), Vanormelingen et al. (2008) and Trobajo et al. (2009) (see footnotes in Table 8.1). The strains (M070)b and (W043)b were isolated as described in Souffreau et al. (2010) from aquatic samples of
Table 8.1 Strain list with indication of moisture category (M.C., see Table 8.2), identification, origin and percentage of living cells (minimum-maximum) in the different treatments for both resting cells (RC) and vegetative cells (VC). A single * designates one replicate without survival, two ** designate two replicates without survival. Light shading indicates a maximal survival percentage below 1.0%; intermediate shading between 1.0 and 10.0%; dark shadings above 10.0%. References to publications in which morphological characteristics and oxidized material are shown are given in footnotes.

*a Souffreau et al. (submitted) Phylogenetic signals in the valve and plastid morphology of the diatom genus Pinnularia.

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Note: All values are given as percentages unless otherwise specified.
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<th>RC</th>
<th>VC</th>
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<td>0-0</td>
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<td>0.3-1.6</td>
<td>0-0.8*</td>
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<td>(St8)d</td>
<td>Pinnularia borealis Ehrenberg</td>
<td>Belgium – Gent</td>
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<td>0-0</td>
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Table 8.2 Moisture categories of Van Dam et al. (1994) with the description of the categories, the number of listed morphospecies belonging to each category and the number of taxa from each category used for this study. The total taxon number in the list by Van Dam et al. (1994) was 1,309, of which 729 were assigned to a moisture category.

<table>
<thead>
<tr>
<th>Moisture category</th>
<th>Description</th>
<th># of taxa in the list</th>
<th># of taxa in this study</th>
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<td>1</td>
<td>never, or only very rarely, occurring outside water bodies</td>
<td>167</td>
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<td>2</td>
<td>mainly occurring in water bodies, sometimes on wet places</td>
<td>148</td>
<td>2</td>
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<td>3</td>
<td>mainly occurring in water bodies, also rather regularly on</td>
<td>282</td>
<td>6</td>
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<tr>
<td>4</td>
<td>mainly occurring on wet and moist or temporarily dry places</td>
<td>116</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>nearly exclusively occurring outside water bodies</td>
<td>16</td>
<td>1</td>
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Amsterdam Island, sampled in December 2007, and were identified using Krammer & Lange-Bertalot (1988). Strain (Ban2)b was isolated using the same technique from a soil sample from Banff (Canada) sampled in May 2008, and identified using Krammer & Lange-Bertalot (1991). Strains were maintained in WC medium (Guillard & Lorenzen, 1972; but without pH adjustment or vitamin addition) in standard culture conditions of 18°C ± 2°C, 25-30 μmol ph m—2 s-1 and a 12:12 hours light:dark period, and reinoculated every few weeks when reaching late exponential phase.

Experimental set-up
The experiments consisted of four different treatments to which the vegetative and resting cells of the clonal strains were exposed: (1) control (standard growth conditions); (2) freezing at -20°C during 4h45; (3) desiccation during 5 minutes; and (4) preconditioning by gradual heating to +30°C followed by desiccation during 5 minutes. A detailed description of these treatments can be found in Souffreau et al. (2010), except that cells were desiccated during 5 minutes instead of 10. The rationale behind the preconditioning is given in Souffreau et al. (2010). The duration of desiccation was based on a preceding experiment testing desiccation periods ranging from 1 minute to 6 hours (unpublished data). Stress exposure of both resting cells and vegetative cells was conducted in triplicate in 24-well plates and in the dark to avoid photo-oxidative stress.

Induction of resting cells was based on McQuoid & Hobson (1995), who used a combination of nitrogen depletion and cold dark conditions for 10 days, but we used a dark period of 14 days. The
formation of resting cells was triggered by a combination of nitrogen-free WC medium and a dark and cold (5°C) environment. To avoid disturbance of the resting cells due to cell transfer, resting cells were induced directly in the experimental wells. Cell densities were determined by Pulse Amplitude Modulated (PAM) fluorescence using a Waltz MAXI Imaging-PAM (M-series) with default settings, intensity 6, gain 4 and damping 1. The basal fluorescence or F0 following a 15 minutes dark-adaptation was used as a proxy for cell density (Consalvey et al., 2005). To ensure that all strains were healthy and in the same physiological condition prior to the experiment, cultures were reinoculated twice at a starting density of F0=0.030 each time followed by exponential growth for 4 days. Exponentially growing cultures were harvested, washed twice with nitrogen-free medium, and inoculated in the experimental wells at an initial density of F0=0.030 per well in 2 ml of nitrogen-free medium. The well plates were placed in standard culture conditions during seven days to allow the cells to exhaust all remaining nitrogen and become stationary, which is clearly visible on inverted microscope at 200 times magnification due to the presence of a dense cytoplasm. The stationary phase cells were placed in the dark at 5°C for two weeks to induce the formation of resting cells (McQuoid & Hobson, 1995).

After two weeks, the experiments on the resting stages were immediately performed following a slightly modified protocol of Souffreau et al. (2010). Because the different experimental treatments could not take place simultaneously and durations of experimental handling varied, all well plates were kept in the dark at 5°C until all treatments were finalized. All treatments were carried out in nitrogen-free medium. After termination of the different stress treatments, the nitrogen-free medium was replaced for all plates by normal WC medium and the plates were transferred to standard culture conditions at 18°C.

Cultures for vegetative cells were prepared for the experiments by a double re-inoculation in the same way as the cultures for resting stages. Similarly, per treatment triplicate wells of 24-well plates were filled with 2 ml culture suspension at a starting density of F0=0.030. Plates were placed in standard culture conditions for two days to allow the cells to recover from potential stress during re-inoculation, after which the same treatments were performed as with the resting cells. Cultures were kept in the dark at 18°C during the period that the different treatments were carried out, and were only placed in the light at standard culture conditions.
after the last treatment was terminated and the medium of all wells was replaced by fresh WC medium, similar as with the resting cells.

The proportion of surviving cells in each well was determined by counting the densities of living and dead cells on an inverted microscope on the day of the experiment (t0) and after 7 days of growth (t7). Treated cell densities varied between 2,300 and 136,000 cells per well, with on average 33,000 cells per well treated at t0. If many cells survived, as was the case for the control conditions, we counted at least 200 cells per well. In the other cases the very low survival percentages forced us to carefully count all living cells present over the whole surface of the single wells. Also the absence of growth was only recorded after careful examination of the whole well. The proportion of viable cells at t0 was calculated as 100 x [1 - (number of dead cells per mm² at t7 / number of treated cells per mm² at t0)].

8.3 Results

Strain selection

Three morphospecies were analysed for moisture category 1 (never, or only very rarely, occurring outside water bodies), two for category 2 (mainly occurring in water bodies, sometimes on wet places), six for category 3 (mainly occurring in water bodies, also rather regularly on wet and moist places), five for category 4 (mainly occurring on wet and moist or temporarily dry places) and a single species for category 5 (nearly exclusively occurring outside water bodies). The fact that we only were able to sample a single species of category 5 is due to the low number of species in that category (16; Table 8.1). Nevertheless, several of the most abundant terrestrial diatoms, such as Hantzschia amphioxys or Pinnularia borealis, were included in the study.

Cytological changes in resting cells and survival of resting cell induction

After one week in nitrogen-depleted medium in light conditions, cells of most strains contained few large or multiple small oil droplets. After the subsequent two weeks in the dark at 5°C, species differed cytologically (Fig. 8.1 a-h). The cytoplasm was in multiple cases more granular, but never contracted. Vacuoles were much enlarged or two small or larger oil droplets were present (Fig. 8.1).
Fig. 8.1 Photographs by inverted microscope of resting stages at t0. a) Pinnularia grunowii (Pin 889), M.C.=1; b) Navicula cryptotenella (Pan2)d, M.C.=2; c) Sellaphora capitata (Mf38), M.C.=2; d) Eunotia bilunaris (DM22-17), M.C.=3; e) Nitzschia palea (02)7D, M.C.=3; f) Nitzschia frustulum (Yde1), M.C.=3; g) Pinnularia borealis (St8)a, M.C.=4; h) Achnanthes coarctata (Ban2)b, M.C.=5.
Chloroplasts were contracted, but strongly contracted, rounded plastids were never observed; and plastids could be darker or paler than their vegetative counterparts. We did not observe consistent differences in cytological changes between aquatic and terrestrial diatoms, or between desiccation and freezing tolerant and sensitive terrestrial diatom strains. Survival percentages of resting cells in control conditions were close to 100 for all strains, indicating that the combined dark conditions and nitrogen limitation did cause almost no cell death (Table 8.1).

**Tolerance to desiccation**
Vegetative cells of all strains, with the exception of one strain of *Achnanthes coarctata* (moisture category 5), did not survive desiccation for 5 minutes, whether preceded by a heat treatment or not (Fig. 8.2 A-B). The same was true for resting cells of morphospecies belonging to moisture categories 1-3; none survived desiccation (preceded by a heat treatment) (Fig. 8.2 A-B). In stark contrast, resting cells of at least some strains of each morphospecies from moisture categories 4 and 5, except for the two *Nitzschia communis* Grunow strains, survived desiccation preceded by a heat treatment (Fig. 8.2 B). Without heat treatment, fewer strains survived desiccation, including both *Hantzschia* Grunow sp. strains. There was a large between-strain variation in desiccation survival of resting cells for both *H. amphioxys* and *P. borealis* (Table 8.1). In general, however, resting cell survival percentages to desiccation (preceded by a heat treatment) were low, and only reached higher values (>25%) for four of *Hantzschia amphioxys* (Table 8.1). Survival percentages were generally similar or lower without preceding heat treatment, except for two *H. amphioxys* strains which showed the reverse pattern (Table 8.1).

**Tolerance to freezing**
Vegetative or resting cells of strains from morphospecies belonging to moisture categories 1-3 did not survive freezing (Fig. 8.2 C). In stark contrast, freezing was survived by vegetative cells of at least one strain from three of the morphospecies belonging to moisture categories 4 and 5. As resting cells, more strains from these moisture classes survived freezing, to the extent that at least a single strain of each morphospecies survived freezing, again except for the two *Nitzschia communis* strains. Survival percentages were mostly low, with as most notable exception *A. coarctata* for which the vast
majority of cells of both strains survived freezing (on average 93%). There was a large between-strain variation in freezing survival for both vegetative and resting cells of *H. amphioxys*, *P. borealis*, and vegetative cells of *A. coarctata* (Table 8.1). Survival percentages were not necessarily higher for resting cells than for vegetative cells (Table 8.1), since the *P. borealis* and *Mayamaea atomus* strains that survived freezing quite well as vegetative cells had very low percentages of surviving resting cells.

### 8.4 Discussion

This study assessed the tolerance for desiccation and freezing of resting cells, in comparison to vegetative cells, from 17 benthic diatom species occurring over a gradient from permanent freshwater aquatic habitats to terrestrial soils. Three main results, discussed below, emerged. First, resting cell formation of benthic freshwater diatoms could indeed be initiated by nitrogen depletion in cold dark conditions, although cytological changes varied widely between species. Secondly, resting cells generally showed a higher tolerance than vegetative cells for desiccation and freezing, albeit often with very low survival percentages. Moreover, some strains survived freezing better as vegetative cells. Thirdly, only resting cells of species of moisture categories 4 (mainly in wet and moist or temporarily dry places) and 5 (occurring nearly exclusively outside water bodies) survived desiccation and freezing, indicating the presence of habitat-specific adaptations to these adverse conditions.

The observed cytological changes in response to the imposed growth arrest due to the combination of nitrogen limitation, low temperature and darkness, applied previously by McQuoid & Hobson (1995), suggest that resting cell formation was successful. Moreover, almost no mortality occurred during the 14 days of cold darkness and nitrogen depletion, indicating that the cells were able to overcome these metabolically adverse conditions by dormancy. Cell cycle arrest in diatoms already occurs after 1 to 2 days during prolonged dark conditions (Vaulot et al., 1986; Brzezinski et al., 1990; Gillard et al., 2008), by nitrogen-depletion (Olson et al., 1986) and by the combination of both (Huysman et al., 2010). How fast and in response to which factor(s) diatom cells undergo cytological changes transforming them into resting cells is less clear. Resting cells used in this study were cytologically always clearly differentiated from their vegetative counterparts, but differences were taxon-dependent.
Fig. 8.2 Graphs showing the average % survival per morphospecies for both resting stages (black) and vegetative cells (grey), subdivided into the five moisture categories of Van Dam et al. (1994). A) desiccation; B) desiccation with preceding gradual heating; C) freezing. Values behind the bars are the average % survival. Errors bars represent one standard deviation. Note the logarithmic scale for both average % survival and error bars.
with variation in the extent of vacuole enlargement, cytoplasm granularity, size and number of oil droplets and chloroplast pigmentation. Different taxa thus undergo different cytological changes when forming resting cells, which might be linked to distinct physiological strategies in storing energy in the form of fatty acids or sugars and protecting the cell content from various stress factors. However, no consistent difference in cytological changes between aquatic and terrestrial diatoms, or between desiccation and freezing tolerant and sensitive terrestrial diatom strains, was observed.

Vegetative cells were highly sensitive to both desiccation and freezing. Only a single strain of *Achnanthes coarctata* survived desiccation (with preceding heat treatment) as vegetative cells, but strains of three taxa from moisture categories 4 and 5 did tolerate freezing as vegetative cells with low to moderate survival percentages. Freezing tolerance of vegetative cells of some of the strains used here was also tested by Souffreau et al. (2010). Results were comparable since the same strains survived (or died) and 4 out of 6 surviving common strains had highly similar survival percentages. Survival percentages of the two remaining strains (*P. borealis* (St8)a and (St8)d) were much lower in this study, however. This shows the repeatability of observations and confirms that vegetative cells of some terrestrial diatoms have an intrinsic protection against freezing
which is absent in aquatic diatoms.

Compared to vegetative cells, resting cells of terrestrial diatoms showed an enhanced resistance for desiccation (preceded by a heat treatment) since more strains and taxa survived desiccation as resting cells, 4 (5) taxa out of 6 compared to the vegetative stage (one taxon). This was to a lesser extent the case for freezing, since three out of five tolerant taxa were also resistant as vegetative cells. However, while only a limited number of strains survived freezing as vegetative cells, almost all strains had viable cells as resting cells. Resting cells of the single species of moisture category 5, *A. coarctata* of which one strain survived freezing as vegetative cells, had a highly increased freezing survival percentage, up to 96%. On the other hand, the *P. borealis*, and *M. atomus* strains surviving freezing as vegetative cells had a (much) lower resting cell survival, suggesting that they might have a different strategy for surviving freezing. Enhanced tolerance levels of resting stages for desiccation and/or freezing have been shown previously for resting spores of diatoms (Hargraves & French, 1975; Schmid, 2009) and resting stages of various organisms such as the akinetes of cyanobacteria (Sutherland *et al.*, 1979; Baker & Bellifemine, 2000; Hori *et al.*, 2003), bryozoans statoblasts (Bushnell & Rao, 1974), zooplankton resting eggs (Gilbert & Schreiber, 1995; Fryer, 1996) and ciliate cysts (Müller *et al.*, 2010). Note however that all these resting stages are physically protected by thick cell walls or protective layers, which is not the case for diatom resting cells.

While resting cells of terrestrial diatoms generally had an enhanced stress tolerance compared to vegetative cells, survival percentages were often very low, and there was a large variation in survival between terrestrial morphospecies and between strains of the same morphospecies. In most cases, the percentage of viable cells was not higher than 5% and could be as low as 0.001%. Detection of such low percentages is only possible when having high cell numbers, and we took care to have as many cells as possible in both the vegetative and resting cell cultures. This low survival is in contrast to the high survival levels of vegetative stages of for example terrestrial cyanobacteria (Gupta & Agrawal, 2006; Sabacka & Elster, 2006) or some green algae (Elster *et al.*, 2008). This emphasizes that in general not only vegetative cells of diatoms but also the resting cells are still quite sensitive, although high resting cell survival was observed for some strains. The large interspecific differences in survival percentage are in agreement with several other studies on
microalgae (Butterwick et al., 2005; Sabacka & Elster, 2006; Gray et al., 2007). Interpretation of the large variation among strains of the same terrestrial morphospecies is less straightforward. Many diatom morphospecies are in fact species complexes (Sarno et al., 2005; Mann & Evans, 2007; Vanormelingen et al., 2008), including *P. borealis* and *H. amphioxys* (Souffreau et al., unpubl. c). As a result it is not very clear to what extent it concerns intraspecific rather than interspecific variation. In the first case, there is a large standing genetic variation for stress tolerance within a single species, and thus a large potential for rapid microevolution and population persistence in response to environmental change. In the second case, terrestrial diatom species might be specialists with relatively limited intraspecific genetic variation confined to certain terrestrial habitats. A large part of the variation encountered during this study might be intraspecific, given that there is variation for desiccation resistance after heat treatment among two strains of both *Hantzschia* *sp.* and *H. amphioxys* [(St1)e and (St3)c] belonging to a single lineage (Souffreau et al., unpubl. c) and in both freezing and desiccation resistance for three *P. borealis* strains from the same lineage [(St1)b, (St1)i and (St6)a]. This should be investigated further. Finally, there was no trade-off in our strains between desiccation and freezing tolerance, probably because these are both osmotic stresses and require quite similar (but not identical) protection mechanisms (Welsh, 2000; Oldenhof et al., 2006).

It is striking that in all conditions and both as vegetative or resting cells, only strains survived that belong to taxa of moisture categories 4 or 5 (following Van Dam et al., 1994), occurring mainly in wet and moist or temporarily dry places or nearly exclusively outside water bodies. A single taxon of category 4, *Nitzschia communis*, did not show any tolerance, which is surprising given that all other terrestrial taxa did. Given the prevalence of (pseudo) cryptic speciation in diatoms (Sarno et al., 2005; Mann & Evans, 2007; Vanormelingen et al., 2008a) and the possibility of niche differentiation between closely related species (Vanelislander et al., 2009), these strains isolated from the subtropical Amsterdam Island in the Indian Ocean might not belong to the *N. communis* species of The Netherlands for which Van Dam et al. (1994) assigned a moisture category. Adding to this hypothesis is the fact that both *N. communis* strains were isolated from a permanent lake. Nevertheless, the tolerance of resting cells for desiccation and freezing is clearly habitat-dependent which indicates that diatom species occurring in terrestrial habitats are adapted to the specific stress factors they
encounter there. Diatoms inhabiting permanent water bodies don’t have to invest energy in the production of protective metabolites, which would result in lower freezing and desiccation tolerance.

Besides allowing temporal persistence of terrestrial diatom populations, the presence of stress tolerant resting cells probably has large consequences for patterns of dispersal, and consequently for population structuring (Figuerola et al., 2005) and geographic distributions (Alve & Goldstein, 2010), since during dispersal by air currents or on animals low moisture levels are likely to be encountered (Kristiansen, 1996). As some (resting) cells of terrestrial diatom populations are able to survive at least short periods of desiccation, dispersal over (at least) short distances (Brown et al., 1964) or protected inside a lump of crusted sediment (Evans, 1958, 1959) should be possible, which is demonstrated by the reports of viable diatom cells of terrestrial taxa found in air traps (Van Overeem, 1937; Schlichting, 1961; Brown et al., 1964; Schlichting, 1964; Roy-Ocotla & Carrera, 1993). Living cells of aquatic diatom species are generally absent from such air traps, which might be related to both a lower chance of being picked up by wind and by their higher sensitivity to desiccation. These higher dispersal rates among terrestrial diatom populations should result in lower differentiation for neutral markers between populations of terrestrial diatoms than for aquatic diatoms over similar spatial scales. Furthermore, Spaulding et al. (2010) hypothesized that this would also result in large geographic ranges of terrestrial species and lower levels of allopatric speciation. However, depending on the geographical scale of dispersal, the higher possibility for terrestrial diatoms to undergo uncommon long distance dispersal events could result in even higher levels of allopatric speciation [called founder speciation when based on a small number of arriving individuals (Templeton, 2008)] compared to lacustrine diatoms. It would therefore be highly interesting to check if patterns of population structure and geographical distributions match the observed differentiation in stress tolerance between terrestrial and aquatic benthic diatoms.
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9. General discussion

9.1 Diatom biogeography

9.1.1 Geographical distribution patterns

Given the controversial importance of historical factors for microbial distributions (reviewed in Martiny et al., 2006), several recent studies based on careful morphological investigations have attempted to disentangle the relative importance of historical factors and local factors on freshwater benthic diatom community structure and diversity (reviewed in Vanormelingen et al., 2008b). These showed that, on large spatial scales (hundreds to thousands of km), distance explains a substantial fraction of the variation in local community structure independent of environmental factors (Potapova & Charles, 2002; Soininen et al., 2004; Verleyen et al., 2009). This is mainly due to species with restricted distributions which are not readily explainable by environmental limitations. Moreover, local diversity is limited by habitat availability (Telford et al., 2006), and even regional genus diversity can be strongly limited in isolated regions, such as those found at high latitudes of the Southern hemisphere (Vyverman et al., 2007). This indicates that dispersal limitation is an important factor restricting diatom distributions on different spatial levels, from incomplete local recruitment from the regional species pool to restrictions on geographical species ranges, and shows that reports of endemic species cannot solely be attributed to the geographically restricted occurrence of suitable habitat.

In this thesis we gathered further evidence for the importance of historical factors for diatom distributions and the occurrence of endemic species in the (sub-)Antarctic based on morphological surveys.

1. First, we show that there is a high level of endemicity in lacustrine diatoms (27-63%, chapter 2), similar to the levels found for macroorganisms in the (sub-)Antarctic (Allegrucci et al., 2006; Stevens et al., 2006; Convey et al., 2008). Moreover, the finding of cryptic continental Antarctic lineages
using molecular phylogenies in two of the cosmopolitan morphospecies (chapter 6) suggests that the percentage of (sub-)Antarctic endemic species is in fact (much) higher than the above-mentioned figures. Also the main biogeographic regions (the sub-, maritime and continental Antarctic, Chown & Convey, 2007) can be distinguished for lacustrine diatoms (chapter 2). Together, these strikingly congruent biogeographical patterns show that biogeographical processes generating the distribution and diversification of diatoms in the (sub-) Antarctic operate at similar spatial and temporal scales as in macroscopic organisms.

2. Secondly, regional lacustrine diatom diversity in the Southern hemisphere is not only reduced (Vyverman et al., 2007) but is also imbalanced with an overrepresentation of predominantly terrestrial lineages and a general poverty of globally successful genera and planktonic lineages (chapter 2). This can only be explained by the presence of historical limitations in diatom lineage dispersal into the (sub-)Antarctic.

3. Finally, the low community similarity for both lacustrine and terrestrial diatoms among the sub-Antarctic island groups Crozet and Kerguelen as compared to the within-island similarities (chapter 3) indicate the presence of a geographic signal in diatom distributions, even within the sub-Antarctic. This is congruent with previous reports on species endemic to single islands or groups of islands in the sub-Antarctic (Van de Vijver et al., 2002; Van de Vijver et al., 2004; Van de Vijver & Mataloni, 2008; Van de Vijver et al., 2010), but it is still unclear if these community differentiations are due to environmental differences between the two archipelagos and species sorting, or to other factors including dispersal limitation and priority effects.

9.1.2 Terrestrial vs. lacustrine diatoms
As mentioned in the introduction, we hypothesized that terrestrial diatoms have wider geographical ranges compared to their aquatic counterparts resulting from higher dispersal probabilities due to the less fragmented terrestrial habitat (Spaulding et al., 2010), a higher probability of being picked up by wind, and their assumed higher tolerance for desiccation and temperature extremes than aquatic taxa (Schlichting, 1969; Ehresmann & Hatch, 1975), which was confirmed by our experiments (chapters 7 and 8). In line with our
expectations, community similarity analyses on a dataset covering the sub-Antarctic island groups Crozet and Kerguelen indicated that terrestrial diatom communities were more similar within and between islands than the lacustrine communities on an intermediate scale (ca. 1,500 km) (chapter 3), indicating higher dispersal rates within and between islands for terrestrial taxa. However, while these two islands are similar in terms of climate and geology, we did not explicitly assess the influence of environmental variables on the observed patterns, and it therefore remains possible that the higher community similarities found for terrestrial communities were a result of higher dispersal, wider realized niches for terrestrial species or less environmental variability in the terrestrial habitat.

It is a general pattern that community similarities of terrestrial organisms show less distance decay than those of aquatic organisms, and this has been attributed to the less fragmented configuration of terrestrial habitats (Soininen et al., 2007). However, also higher dispersal capacities and wider realized niches could contribute to this pattern, and more in-depth studies separating the influences of environmental and spatial variables are needed to fully interpret the observed decreases in community similarity with distance (Legendre et al., 2005; Soininen et al., 2007). At the same time, even terrestrial diatoms do show strong geographical patterns over large spatial scales. First, terrestrial community similarities decreased significantly over the South Indian Ocean and South Atlantic Ocean (chapter 3). Secondly, the Antarctic strains of Pinnularia borealis and Hantzschia amphioxys were genetically and thermally differentiated from the non-Antarctic strains (chapter 6). While lacustrine and terrestrial diatoms thus are suggested to have different geographical patterns at smaller spatial scales (up to 1,500 km), they are similar at larger scales. These patterns are discussed in the light of dispersal probabilities and stress tolerance further below in section 9.3.

9.2 Species diversity and speciation in the (sub-)Antarctic

(Pseudo)cryptic species diversity is widespread in diatoms, with sometimes tens of species discovered in a single species complex (Behnke et al., 2004; Kooistra et al., 2005; Sarno et al., 2005; Beszteri et al., 2007; Mann & Evans, 2007; Vanormelingen et al., 2008a; Evans et al., 2009; Trobajo et al., 2009; Vaneelslander et al., 2009), and confirms the predictions of Mann & Droop (1996). Almost at the same time as these
findings, a trend to attribute subtle morphological variation patterns to phenotypic plasticity has reversed and thousands of new species have been described including lots of potential endemics, many of which would previously be regarded to be part of a morphological continuum within species (reviewed in Vanormelingen et al., 2008b). The current study on P. borealis and H. amphioxys (chapter 6) shows that species diversity is still underestimated even when applying a fine-grained morphological species concept (cf. chapter 2). The P. borealis lineage has an estimated age of no less than 42 Ma, and the oldest reported lineage splitting occurred at least 22 Ma ago, making it the oldest known cryptic diatom species complex known to date. Estimated lineage divergence times for the marine diatom Pseudo-nitzschia pungens were 0.5-0.9 Ma (Casteleyn et al., 2010) and the age of the Sellaphora pupula-bacillum complex is at least 12 Ma (Evans et al., 2008). The high species diversity encountered calls for studies into diatom speciation. It is currently not well known what the contribution of various historical and ecological processes is for diatom diversification, while also other factors might play a role. In birds for instance, more diverse bird families had a significantly higher synonymous mutation rates compared to the less diverse families based on phylogenetic analyses, indicating that there could be a causal link between mutation rates and net diversification (i.e. speciation minus extinction) (Lanfear et al., 2010). One recent study on polyploidy speciation in a marine planktonic diatom highlighted the potential importance of polyploidization (Koester et al., 2010), known to be involved in 15% of plant speciation and 31% of fern speciation events (Wood et al., 2009).

The high percentage of diatom species endemic to (parts of) the (sub-)Antarctic suggests that there is ample opportunity for allopatric speciation in the region (chapters 2 and 6). Molecular phylogenies are not only useful for (cryptic) species delimitation but can, when sufficiently resolved and time-calibrated using fossil data, provide an idea of the frequency, timing and order of lineage splitting. When combined with data on ecological niches and geographical distributions, they allow to reconstruct the evolutionary history of taxa and thus get an idea of the driving (historical and ecological) forces behind lineage splits (see e.g. Darling et al., 2007; Verbruggen et al., 2009; Casteleyn et al., 2010). Darling et al. (2007), for instance, reconstructed the evolutionary history of the polar planktonic foraminifera species complex Neogloboquadrida pachyderma sinistral using time-calibrated molecular phylogenies and geographic
distributions of the different lineages. This showed a stepwise progression of diversification starting with the allopatric isolation of Atlantic Arctic and Antarctic populations 1.5 - 1.8 Ma ago. Further diversification in the Southern Hemisphere, including the evolution of an extreme polar lineage capable of living in the sea ice and a currently isolated lineage in the Benguela upwelling system, could be linked to glacial-interglacial climate dynamics. Together with other such studies, a rather complete picture is emerging on the biogeography of planktonic foraminifers (reviewed in Darling & Wade, 2008).

While it is clear that we are still far from achieving such a goal for freshwater or terrestrial diatoms in general and more specifically in the (sub-)Antarctic, some important progress has been made towards achieving this goal with this PhD-thesis. These are (1) the reconstruction of a time-calibrated molecular phylogeny of the globally important diatom genus *Pinnularia*, allowing to date lineage splits for any sampled taxon in this genus (chapter 4 and chapter 5), (2) the construction of a molecular phylogeny for the globally distributed diatom *Pinnularia borealis* (chapter 6) showing the presence of a number of distinct lineages including a continental Antarctic lineage with an estimated split from a northern temperate lineage 7.7 Ma ago, remarkably similar to divergence times of Antarctic lineages of some other organism groups (Stevens *et al.*, 2006; Mortimer *et al.*, 2011), and (3) an assessment of temperature preferences of these lineages showing that Antarctic *P. borealis* has a preference for relatively low temperatures but not to an extreme extent, similar to the Antarctic lineage of *H. amphioxys* (chapter 6). Further efforts should be directed at lineage discovery in other geographic areas (especially in the (sub-)Antarctic), at the same time assessing the geographic distribution of these lineages, and further niche characterization. Together, this will allow a reconstruction of the evolutionary history of *P. borealis* and potentially other *Pinnularia* lineages in the Southern Hemisphere, including a view on the driving forces behind freshwater diatom diversification.

9.3 Stress tolerance and consequences for dispersal and biogeography

To effectively disperse and colonize a new patch, propagules have to survive the transport. The survival probability of dispersal depends on the tolerance levels of the species and individual cells
for the adverse conditions encountered during transport. This critical point in dispersal is not easy to quantify. First, the conditions during transport fluctuate in time and space and are determined by several factors (Isard & Gage, 2001). For example, desiccation rates during external transport by birds or by wind will depend on the relative humidity and temperature of the air, the wind rate and solar incidence, next to the presence of mud around the cells and the presence of protecting grooves in the feet epidermis of ducks. It is therefore not easy to set up experimental conditions that cover the range of possible conditions, while at the same time covering a whole range of taxa. Second, to quantify propagule viability in natural transport conditions, knowledge on starting densities are needed and, as stated earlier, viability will vary under different natural conditions. Third, because the interspecific variations in viabilities are equally important as the viability level itself, it is important to assess the tolerance of as many species as possible. In our experiments (chapters 7 and 8), we decided to put more weight on the number of taxa instead of the number of conditions, mainly because it is very difficult to cover the whole range of potential conditions while interspecific and intraspecific variations will still be detected using a low number of stress conditions, and because our principal aim was to retrieve differences in tolerance level between terrestrial and aquatic species.

Two main results came out of our stress tolerance experiments on lacustrine and terrestrial diatoms. First, vegetative cells are very sensitive for desiccation and extreme temperatures (chapter 7). Second, also resting stages are very sensitive to these stresses, except the resting stages of typical terrestrial diatoms which can survive short-term (5 minutes) desiccation, albeit generally with low survival percentages (chapter 8). This has important consequences for diatom dispersal rates, and consequently also for population structures, speciation and geographical distributions. First, freshwater diatoms inhabit aquatic “islands” in an “ocean of inhospitable land”, and dispersal among such “islands” might not be very common given the general low desiccation tolerance of both vegetative and resting diatom cells and the resulting extremely low probabilities of transport of viable cells by air. Even if the conditions encountered during natural transport differ somewhat from the experimental conditions we used (chapters 7 and 8), desiccation is known to be a key factor during both wind and bird transport (Schlichting, 1960; Ehresmann & Hatch, 1975; Kristiansen, 1996; Figuerola & Green,
The absence of resistant dispersal stages in aquatic diatoms is in agreement with the single microsatellite study on freshwater diatoms (and even microalgae), showing that populations separated by no more than some hundreds of kilometers differ strongly in microsatellite allele frequencies, indicative of a very low connectivity between populations (Evans et al., 2009). Current microsatellite studies on freshwater diatoms are directed at pinpointing at exactly how small the spatial scale is at which connectivity breaks down (pers. comm. P. Vanormelingen). Interestingly, marine diatom populations show on the same spatial scale no or much lower differentiation (Casteleyn et al., 2009; Casteleyn et al., 2010; Godhe & Harnström, 2010), but additional studies are necessary to assess the generality of this pattern. Moreover, given the difference in desiccation tolerance between aquatic and terrestrial diatoms it can be hypothesized that terrestrial diatom populations show less population differentiation than aquatic diatoms at the same spatial scale.

Secondly, dessication and freezing tolerant resting stages were only present in terrestrial and not in aquatic taxa (chapter 8) showing that diatoms are specifically adapted to their habitat. This pattern is also found in other taxa with both terrestrial and aquatic representatives, such as in green algae (Zoe et al., 2008). Furthermore, the tolerance of these terrestrial resting stages to desiccation will enhance their survival probabilities of dispersal by animals and through the air, indicating that terrestrial taxa are better adapted to dispersal by wind than aquatic taxa. It should be noted however that it is not clear how desiccation survival depends on the desiccation period, since cultures were only desiccated for 5 minutes, which is of course critical for determining the distances over which dispersal can take place. In any case, the tolerance of only terrestrial diatoms to desiccation agrees with the findings that predominantly terrestrial microalgae are retrieved alive in air traps (Van Overeem, 1937; Schlichting, 1961; Brown et al., 1964; Roy-Ocotla & Carrera, 1993), albeit this is most likely a combination of the higher susceptibility of terrestrial material to get suspended in the air, and the desiccation survival of terrestrial diatom resting stages. The observed higher presence of terrestrial diatoms in the air also corresponds to the observation that terrestrial diatom community similarities might decrease less over intermediate distance (within islands and up to 1,500 km) compared to the aquatic communities (chapter 3, Soininen et al., 2007). However, this general pattern could be a result of the combination of three mutually non-exclusive factors: the less
fragmented terrestrial habitat, the higher susceptibility of terrestrial organisms to get picked up by air currents or to disperse over land, and the adaptation of terrestrial organisms to a terrestrial life-style with its requirements of tolerance to at least desiccation.

Thirdly, the desiccation tolerance of terrestrial diatom resting cells (chapter 8) emphasizes the importance of resting stages for dispersal. Resting stages are widely known to be the dominant dispersal stages in several organism groups (Hutchinson, 1967; Levins, 1969; Sutherland et al., 1979; Vleeshouwers et al., 1995; Nicholson et al., 2000; Anderson, 2010) and often have specific adaptations enhancing dispersal, such as wings or sticky hairs on the seeds of higher plants, a protective outer ectocarp, or the thick-walled protective ephippium enclosing the resting eggs of zooplankton. Besides for dispersal through space, resting stages are also very important as “seed bank” to overcome adverse conditions in time (Vleeshouwers et al., 1995; Figuerola et al., 2003; Poulícková et al., 2008; Jones & Lennon, 2010), and they therefore fulfill a key role in the long-term persistence of populations and species (Jones & Lennon, 2010). For marine and freshwater planktonic diatom populations the importance of resting cells and spores to overcome seasonally unfavorable light, nutrient and temperature conditions is well-established (Gran, 1912; Hargraves & French, 1975, 1983; McQuoid & Hobson, 1995, 1996). Terrestrial habitats are subjected to large daily and seasonal fluctuations in temperature and humidity (Starks et al., 1981; Gao et al., 2008), and the overall higher stress tolerance of terrestrial diatom resting cells compared to vegetative cells (chapter 8) indicates that they are important for population persistence under unfavorable conditions. However, there are no data on the population dynamics of terrestrial diatoms, and it is therefore not known whether terrestrial diatom populations show a metapopulation structure with frequent extinctions and recolonizations from other populations in the metapopulation once the habitat patch becomes suitable again, or whether a local seed bank guarantees long-term population survival. The presence of resistant resting stages, however, does indicate that both a metapopulation structure with regular exchange between patches and the presence of a local seed bank are possible.
9.4 Biogeographical patterns of terrestrial and aquatic diatoms

In line with their higher tolerance to desiccation compared to aquatic taxa (chapter 8), our first results comparing the spatial turnover of diatom communities in aquatic and terrestrial habitats indicate that terrestrial diatom communities tend to have a lower decrease in community similarity over distances up to 1,500 km (chapter 3), and a significantly lower species turnover within isolated islands than aquatic communities (chapter 3). At larger spatial scales this community similarity decreases strongly in a way similar to the aquatic communities (chapter 3). We therefore hypothesize that terrestrial diatoms are better dispersers than aquatic diatoms over shorter distances due to a certain degree of desiccation tolerance (chapter 8), a higher susceptibility for wind dispersal and a less fragmented terrestrial habitat on a within-island scale, but that this difference between terrestrial and aquatic diatoms fades over longer distances. This might be due to the fact that such long-distance dispersal is extremely rare also for terrestrial diatoms, resulting in very large community dissimilarities. However, further research is needed on this differentiation between aquatic and terrestrial diatom communities. While differences in environmental conditions seem similar for both types of habitats within and between the sub-Antarctic islands studied, it can’t be excluded that terrestrial habitats might be less differentiated in environmental variables compared to lakes, or that terrestrial diatoms have wider niches.

9.5 studying dispersal

During this thesis, we gathered data indicating that diatom distributions are similar to those recorded for macroorganisms (chapter 2), that presumed cosmopolitan diatom species may not be cosmopolitan after all (chapter 6), and that both vegetative and resting cells of diatoms are very sensitive to desiccation (chapters 7 and 8). However, while the initial subject of this thesis was “dispersal” of diatoms, we did not estimate dispersal rates between natural populations. We therefore present three more or less feasible approaches to further study natural diatom dispersal, each with its strengths and weaknesses. Ideally, they are combined to provide a complete view on dispersal.
1. Finding dispersing cells. By sampling and quantifying the living diatom flora present in or on dispersal vectors such as animals and air currents, we can further improve our knowledge on dispersed taxa and abundances. Previous knowledge has been summarized in section 1.4.2, but we still lack quantitative information on species-level. It should be stressed that directly oxidizing the transported diatom material renders it useless since it doesn’t allow to distinguish living cells from dead frustules, which are well-known to be easily taken up by air and water currents and deposited away from their origin. Therefore, culture-based methods or decent fixation methods are needed. While this method can provide an idea of dispersal rates through different vectors when dispersal is common, it is difficult to impossible to detect the rare long-distance dispersal events which might be responsible for the colonization of new habitats.

2. Colonization and transplant experiments. The only way to prove (but not disprove) dispersal limitation in diatoms is a “transplant” experiment. This involves placing diatoms from one area to a geographically separated, ecologically similar, area and assess if the transplanted diatoms can establish populations in that new area, which would imply that the only factor previously preventing those diatoms from occurring in the other area was dispersal limitation. While this is ethically not always justifiable, there are already some accidental transfers of diatoms which could have been better studied, or could be better monitored in the future [see e.g. the transfer of organisms with ballast water of oceanic ships, or the transfer of commercial macrophytes or fish (Hallegraeff & Bolch, 1992; Klein et al. 2010)]. In a colonization experiment, a new habitat is created and the build-up of diversity is followed. This way, it can be determined how long it takes before new communities are saturated with species, and thus how long dispersal limitation plays a role in these communities. It should be noted however that decent control communities are necessary in which dispersal limitation is prevented by artificial introductions.

3. Studies of population differentiation. Differences between populations in allele frequencies for neutral molecular markers (e.g. microsatellites) can be used to infer the current level of gene flow between these populations. This is because, when an
equilibrium between drift and gene flow is reached, the level of differentiation between populations is directly dependent on the number of effective migrating individuals between those populations (Griffiths et al., 1996). For such interpretations to be made, it is absolutely necessary that neutral markers are used and that these are not linked to genes under selection (which would be the case when more than one, cryptic, species is included in the study). In such a case, natural selection (species sorting) will contribute to the observed “population” differentiation and no inference can be made about dispersal rates. One other potential explanation for observed population differentiation for neutral markers are persistent founder effects (the equilibrium between drift and gene flow is not yet reached), which may be quite common in various freshwater organisms (see De Meester et al., 2002). However, when one is sufficiently aware of the potential complications, this is a highly valuable method to get an idea of the actual dispersal rates and dispersal scales of diatoms.

9.6 Final thoughts

While enough data are available now to confirm that diatom biogeographies are shaped by both species sorting and historical factors, many questions remain. First of all, as stated by Green & Bohannan (2006) we can shift from the question “is microbial biogeography fundamentally differently regulated than those from macroorganisms”, towards the question “on which spatial scale and using which taxonomical resolution microbial biogeography approaches this of macroorganisms”. Quantification of spatial turnover will thus be important in the future, and this also applies for diatom research. Local abundances of (living) terrestrial diatoms and seasonal dynamics of population density are unknown. Dispersal rates and scales, and speciation rates and mechanisms are all needed to infer the threshold levels at which speciation will occur between populations. Microsatellite studies and the frequencies of neutral variation between populations offer first estimates of effective dispersal rates between populations and the scale at which dispersal occurs, provided that a correct single genetic lineage is used (i.e. a single species) and that distinctions are made within species complexes [see for example the problems related with Ditylum brightwellii (Rynearson et al., 2009)]. While only a single microsatellite
study is available for a freshwater diatom [*Sellaphora capitata* (Evans et al., 2009)] and a second and third are under way (pers. comm. P. Vanormelingen), terrestrial diatoms remain unexplored.

Compared to aquatic diatoms, much less is known about the ecology of terrestrial diatoms, but their life style and population dynamics are as much intriguing. Several questions concerning terrestrial diatoms have been put forward in the previous sections. Population-level studies are needed to better understand the needs of terrestrial diatoms to overcome adverse conditions and the mechanisms they use, besides the importance of resting stages. Despite the fact that terrestrial diatoms have been thriving under our feet since we evolved, they are in many perspectives still a closed book.
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Summary

Until recently, the geographical distribution of microorganisms was assumed to be only controlled by local ecological factors (species sorting); their high local abundances and small size would increase their dispersal probabilities sufficiently to overcome all geographical barriers (Finlay et al., 2002). Since then, several studies have recently suggested and shown that when using a higher taxonomical resolution, microorganisms do have geographically restricted distributions affected by historical factors (reviewed in Green & Bohannan, 2006; Martiny et al. 2006). Also for diatoms, recent studies using different techniques have shown that marine and lacustrine diatom distributions are shaped by both environmental factors and dispersal limitation (Potapova & Charles, 2002; Soininen et al., 2004; Telford et al., 2006; Vyverman et al., 2007; Verleyen et al., 2009; Evans et al., 2009; Casteleyn et al., 2010).

During this thesis, we aimed to improve our understanding of the role of dispersal limitation for generating geographical patterns in lacustrine and terrestrial diatoms by analyzing global and regional scale biodiversity patterns, time-calibrated phylogenies and fossil evidence, and by ecophysiological studies examining the stress tolerance for dispersal-related factors. Our primary focus was the (sub-)Antarctic region because of the relatively species-poor diatom flora and strong degree of geographical isolation on different spatial scales.

In chapter 2, we analyzed the biogeographical patterns of lacustrine diatoms in the Antarctic and Arctic and combined these results with fossil data and a time-calibrated molecular phylogeny. Compared to the Arctic communities, Antarctic diatom communities are impoverished and imbalanced, and are characterized by high levels of endemism, the absence of key functional groups such as planktonic taxa, an overrepresentation of terrestrial lineages, and a general paucity of globally successful genera. A comparison of contemporary Antarctic floras with fossil Miocene assemblages
and a molecular clock analysis of diversification patterns point to high rates of local extinction during Neogene and Quaternary glacial maxima, in combination with radiations through allopatric speciation in glacial refugia. This indicates that processes controlling the distribution and diversification of diatoms can operate at similar spatial and temporal scales as those for macroscopic organisms, resulting in congruent biogeographical patterns.

In **chapter 3**, we examined if terrestrial and lacustrine diatoms show different patterns in geographical structure at different spatial scales, using distance decay analysis of community similarities of diatom assemblages on the sub-Antarctic islands Crozet and Kerguelen, situated in the Southern Indian Ocean, and South Georgia, situated in the Southern Atlantic Ocean. We observed that within islands species turnover (β-diversity) was relatively high for the two communities compared to the within-site (α) diversity, possibly reflecting small-scale environmental differentiation and species sorting or strong priority effects. Meanwhile, terrestrial diatoms had a significantly lower turnover within islands compared to aquatic communities, suggesting higher dispersal rates and/or broader niches or less environmental variation between the sites. At an intermediate scale, within the South Indian Ocean region (ca. 1,500 km), there was a non-significant trend towards higher community similarities and lower turnover for terrestrial communities, while at the largest spatial, among the two oceanic basins (ca. 6,000 km), both communities showed a strong decrease in community similarity, albeit only significant for the terrestrial dataset. These first results suggest that terrestrial and aquatic diatoms could differ in their dispersal probabilities at small to intermediate scales, but further research should control for a possible influence of environmental variation.

In **chapter 4** we constructed a fossil-calibrated time-scaled phylogeny of the diatom genus *Pinnularia* to explore the importance of time in speciation events. Using a five-locus dataset we inferred the interspecific evolutionary relationships for 36 *Pinnularia* taxa. A range of fossil taxa, including newly-discovered Middle Eocene forms of *Pinnularia*, were used to calibrate a relaxed molecular clock analysis and investigate the temporal aspects of the genus’ diversification. The multi-gene approach resulted in a well-resolved phylogeny consisting of three major clades and several subclades that were frequently, but not universally, delimited by valve morphology. Diversification of the genus is estimated to have started
ca. 60 Ma ago, 10 to 30 Ma earlier than the fossil record suggests, and most larger clades diverged in the Miocene, allowing them to successfully disperse worldwide.

In chapter 5 we used the molecular phylogeny created in chapter 4 to compare the phylogenetic signal of molecular and morphological data. We performed a cladistic analysis on 48 strains using 17 frustule and 3 plastid characters. Independent phylogenetic analyses of molecular and morphological data generated similar clades, and Mantel tests found a significant correlation between morphological and molecular distances. However, while phylograms based on five molecular markers containing in total 1,012 parsimony-informative sites were fully resolved, the lower number of phylogenetically informative morphological characters inevitably resulted in fewer clades being recovered, and we were never able to fully resolve the interspecific relationships of *Pinnularia* based on morphology. The best resolution for morphology-based species differentiation was acquired by analyzing all morphological characters simultaneously. Mainly “structural” characters (such as central raphe endings, raphe complexity and alveolus openings) and one plastid character (number of pyrenoids) were informative for reconstructing the phylogenetic relationships within the genus.

In chapter 6, we used two presumed cosmopolitan taxa, *Pinnularia borealis* and *Hantzschia amphioxys*, to assess the molecular divergence between Antarctic strains and strains from other locations. Molecular phylogenies based on the plastid gene *rbcL* and the 28S rDNA D1-D2 region revealed that *P. borealis* and *H. amphioxys* are two species complexes consisting of multiple genetically diverged lineages, each including a distinct Antarctic lineage. A molecular time-calibration estimates the origin of the species complex *P. borealis* at 35.6 million years (Ma) ago, and the first lineage splitting based on extant taxa at 22.0 Ma, making this the oldest known diatom species complex. The Antarctic *P. borealis* lineage is estimated to have diverged 7.7 (15-2) Ma ago from its sister lineage, after the final opening of the Drake Passage, suggesting the occurrence of a long-distance dispersal event followed by allopatric speciation. In addition, the Antarctic lineages of both *P. borealis* and *H. amphioxys* had a higher relative growth rate at low temperature, a lower optimal temperature and lethal upper temperature than most lineages from more temperate regions, indicating thermal niche differentiation. These data indicate that many of the presumed cosmopolitan Antarctic diatom species are in fact species complexes,
possibly containing Antarctic endemic lineages.

In chapter 7, we conducted a laboratory experiment using 34 benthic freshwater and terrestrial diatom strains to investigate the tolerance levels of vegetative cells for two dispersal-related stress factors, namely desiccation and extreme temperatures. Six different stress conditions were studied: gradual heating up to +30°C and +40°C, abrupt heating to +40°C, freezing to -20°C, and desiccation with and without preconditioning at +30°C. Our results showed that vegetative diatom cells are very sensitive for desiccation, as none of the strains survived this treatment. Tolerance to temperature extremes (+40°C and -20°C) was to a large extent species-specific. However, only terrestrial species survived freezing and aquatic diatoms were less tolerant to gradual heating to +40°C, both pointing at a higher tolerance of terrestrial diatoms to temperature extremes, and indicating that terrestrial diatoms are adapted to their habitat.

In chapter 8, we experimentally assessed the tolerance for desiccation and freezing of diatom resting cells, in comparison to vegetative cells, for 17 benthic freshwater morphospecies occurring over a gradient from permanent aquatic habitats to terrestrial soils, divided into five moisture categories. Resting cell formation was initiated by nitrogen depletion in dark conditions. As observed previously, vegetative cells were in general highly sensitive to desiccation and freezing. Only strains of three terrestrial taxa survived freezing as vegetative cells while a single strain survived 5 minutes of desiccation. In contrast, resting cells showed a higher tolerance for desiccation, especially when preceded by a heat treatment, and more strains and taxa survived freezing, albeit often with low survival percentages and a large interclonal variation. Strikingly, only the resting cells of terrestrial taxa, i.e. those occurring mainly in wet and moist or temporarily dry places (moisture category 4) or nearly exclusively outside water bodies (category 5) survived desiccation and freezing, indicating the presence of habitat-specific adaptations to these adverse conditions.

From the results gathered in this thesis, we can conclude that diatom biogeographies are shaped by the same processes operating for macrobiota. Biogeographical patterns at large spatial scales indicate that historical factors and dispersal limitation are at work, while a too low taxonomical resolution and the prevalence of cryptic species still mask some of the geographical patterns. The high sensitivity of vegetative and most resting cells of diatoms to desiccation drastically decreases the previously assumed
high dispersal probabilities of diatoms, despite their high local abundances. At intermediate and smaller spatial scales, we observed a tendency for terrestrial diatoms to have lower species turnover compared to lacustrine diatom communities, which suggests higher dispersal rates for the former and fits the desiccation tolerance of their resting cells.
Samenvatting

Tot voor kort werd verondersteld dat de geografische verspreiding van micro-organismen alleen wordt bepaald door lokale ecologische factoren (species sorting); hun hoge lokale abundanties en geringe afmeting zouden hun dispersiekansen dermate verhogen dat geografische barrières van geen tel zouden zijn (Finlay et al., 2002). Echter, verschillende studies hebben onlangs gesuggereerd en aangetoond dat, gebruik makend van een hogere taxonomische resolutie, micro-organismen wel degelijk geografisch beperkte verspreidingen kunnen hebben onder invloed van historische factoren (Green & Bohannan, 2006; Martiny et al. 2006). Met betrekking tot diatomeeën vond een gelijkaardige evolutie plaats. Recent studies hebben met behulp van verschillende technieken aangetoond dat de verspreidingen van mariene en lacustriene diatomeeën bepaald worden door zowel omgevingsvariabelen als dispersie limitatie (Potapova & Charles, 2002; Soininen et al., 2004; Telford et al., 2006; Vyverman et al., 2007; Verleyen et al., 2009; Evans et al., 2009; Casteleyn et al., 2010).

Dit proefschrift had tot doel de rol van dispersielimitatie in het tot stand komen van geografische patronen van lacustriene en terrestrische diatomeeën beter te begrijpen, via de analyse van biodiversiteitspatronen op globale en regionale schaal, tijdsgekalibreerde fylogenieën en fossiel bewijsmateriaal, en via ecofysiologische studies naar de stress tolerantie voor dispersiegerelateerde factoren. Er werd een primaire focus op Antarctica en de sub-Antarctische eilanden gelegd vanwege de relatief soortenarme diatomeeënflora en de sterke geografische isolatie van de habitats op verschillende ruimtelijke schalen.

In hoofdstuk 2 analyseerden we de biogeografische patronen van lacustriene diatomeeën in Antarctica en Arctica en combineerden we deze resultaten met fossiele gegevens en een tijdsgekalibreerde moleculaire fylogenie. Ten opzichte van de Arctische gemeenschappen zijn de Antarctische diatomeeëngemeenschappen verarmd en onevenwichtig, en worden ze gekenmerkt door een hoge mate van endemiciteit, het ontbreken van belangrijke functionele groepen zoals planktonische taxa, een oververtegenwoordiging van terrestrische genera, en een algemene schaarste aan wereldwijd succesvolle genera. Een vergelijking van de hedendaagse Antarctische flora met fossiele assemblages uit het Mioceen en een moleculaire klok-analyse op diversificatiepatronen wijzen op een hoge graad van lokale extinctie tijdens de Neogene en Kwartaire.

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glaciale maxima, in combinatie met radiaties door allopatrische soortvorming in glaciale refugia. Dit wijst erop dat de processen die de verspreiding en de diversificatie van diatomeeën controleren op vergelijkbare ruimtelijke en temporele schalen werken als deze voor macroscopische organismen, hetgeen resulteert in congruente biogeografische patronen.

In hoofdstuk 3 onderzochten we of terrestrische en lacustriene diatomeeën een verschillend patroon kennen in hun geografische structuur op regionale schaal met behulp van “distance decay” of het verval in gemeenschapsovereenkomsten over afstand en de “turnover” van diatomeëngemeenschappen tussen de sub-Antarctische eilanden Crozet en Kerguelen gelegen in de Zuid-Indische Oceaan, en South Georgia gelegen in de Zuid-Atlantische Oceaan. Binnen eilanden was voor de twee habitats de turnover (β-diversiteit) relatief hoog in vergelijking met de binnen-site (α) diversiteit, hetgeen kan wijzen op milieudifferentiatie op kleine schaal en species sorting, of op sterke prioriteitseffecten. Terrestrische diatomeeën hadden een significant lagere turnover binnen eilanden ten opzichte van aquatische gemeenschappen, wat wijst op hogere dispersiesnelheden en/of bredere niches, of op een homogener habitat. Op intermediaire schaal, binnen de Zuid-Indische Oceaan (ca. 1500 km), was er slechts een niet-significante trend naar hogere gemeenschapssimilariteiten en lagere turnover voor terrestrische gemeenschappen, terwijl op de grootste ruimtelijke schaal, tussen de twee oceaanbekkens (ca. 6000 km), voor beide habitattypes een sterke daling in gemeenschapssimilariteiten waargenomen werd die echter alleen significant was voor de terrestrische dataset. Deze eerste resultaten suggereren dat terrestrische en aquatische diatomeeën inderdaad zouden verschillen in hun dispersiewaarschijnlijkheden op kleine en intermediaire ruimtelijke schalen, maar verder onderzoek moet voor de invloed van variatie in omgevingsfactoren controleren.

In hoofdstuk 4 construeerden we een fossiel-gekalibreerde, tijdsge Chancellor de fylogenie voor het genus *Pinnularia* om het belang van tijd in soortvorming te verkennen. Met behulp van een vijf-locus dataset maakten we de interspecifieke evolutionaire relaties op voor 36 *Pinnularia* taxa. Een reeks van fossiele taxa, inclusief een nieuw ontdekte vorm uit het Midden-Eoceen, werd gebruikt om de moleculaire klok analyse te kalibreren en de temporele aspecten van de diversificatie van het genus te onderzoeken. De multi-gen aanpak resulteerde in een goed opgeloste fylogenie, bestaande uit drie grote
clades en diverse subclades die vaak, maar niet universeel, begrensd werden door valve morfologie. Diversificatie van het geslacht is naar schatting ca. 60 Ma geleden begonnen, 10 tot 30 Ma eerder dan wat de fossiele record uitwijst. De meeste grotere clades divergierden gedurende het Mioceen, waardoor ze wereldwijd succesvol konden verspreiden.

In hoofdstuk 5 gebruikten we de moleculaire fylogenie uit hoofdstuk 4 om het fylogenetisch signaal van morfologische en genetische data te vergelijken. We voerden op 48 clones een cladistische analyse uit met 17 frustule- en 3 chloroplastenmerken. Onafhankelijke analyses van de moleculaire en morfologische data genereerde gelijkaardige clades, en Mantel tests gaven een significante correlatie tussen morfologische en genetische afstanden. Echter, terwijl het fylogram op basis van 5 genetische merkers met een totaal van 1012 parsimonie-informatieve sites volledig opgelost was, resulteerde het lagere aantal fylogenetisch informatieve morfologische kenmerken onvermijdelijk in een lager aantal opgeloste clades. Op basis van de morfologische data konden de interspecifieke relaties van *Pinnularia* nooit volledig opgelost worden. De beste resolutie voor de morfologisch gebaseerde soortenonderscheiding was bij een analyse van alle kenmerken samen. Voornamelijk “structurele” frustule kenmerken (zoals centrale raphe uiteinden, raphe complexiteit en alveolus opening) en één chloroplastenmerk (aantal pyrenoiden) waren informatief in de reconstructie van de fylogenetische relaties in het genus.

In hoofdstuk 6 gebruikten we twee vermeerende kosmopolitische taxa, *Pinnularia borealis* en *Hantzschia amphioxys*, om de moleculaire divergentie te beoordelen tussen Antarctische stammen en stammen afkomstig van andere locaties. Uit moleculaire fylogenieën gebaseerd op de chloroplast merker *rbcL* en de nucleaire 28S *rDNA* D1-D2 regio is gebleken dat *P. borealis* en *H. amphioxys* twee soortscomplexen zijn bestaande uit meerdere genetisch gedivergeerde lineages, elk met een aparte Antarctische lineage. Een moleculaire tijdskalibratie raamde de herkomst van het soortscomplex *P. borealis* op 35,6 miljoen jaar (Ma), en de eerste divergentie op 22,0 Ma hetgeen dit het oudst gekende diatomeeënsoortscomplex maakt. De Antarctische *P. borealis* lineage is naar schatting 7,7 (15-2) Ma geleden gedivergeerd van haar zusterlineage, na de definitieve opening van de Drake Passage, hetgeen wijst op een lange-afstand dispersie evenement gevolgd door allopatrische soortvorming. Bovendien zijn de Antarctische lineages van zowel *P. borealis* als *H. amphioxys* genetisch
afwijkend van de meeste lineages uit meer gematigde streken en neigen ze beide naar een hogere relatie groei bij lage temperatuur, een lagere optimale temperatuur en een lagere lethale maximum temperatuur, wat wijst op niche-differentiatie. Deze gegevens wijzen erop dat veel van de vermoedelijk kosmopolitische Antarctische diatomeeënsoorten soortscomplexen kunnen zijn, eventueel met endemische Antarctische lineages.

In hoofdstuk 7 voerden we een laboratoriumexperiment uit op 34 benthische zoetwater en terrestriese diatomeeën stammen om de toleranties van de vegetatieve cellen te onderzoeken voor twee dispersiegerelateerde stressfactoren, namelijk uitdroging en extreme temperaturen. Zes verschillende stresscondities werden bestudeerd: geleidelijke opwarming tot +30°C en +40°C, abrupte opwarming op +40°C, bevriezing op -20°C, en uitdroging met en zonder voorbehandeling bij +30°C. Onze resultaten toonden aan dat de vegetatieve cellen van diatomeeën zeer gevoelig zijn voor uitdroging, aangezien geen van de stammen deze behandeling overleefde. Tolerantie voor extreme temperaturen (+40°C en -20°C) was in grote mate soortsspecifiek. Echter, alleen terrestriese soorten overleefden bevriezing en aquatische diatomeeën waren minder tolerant voor geleidelijke verhitting tot +40°C, hetgeen wijst op een hogere tolerantie van terrestriese diatomeeën aan extreme temperaturen, en verder aangeeft dat terrestriese diatomeeën aangepast zijn aan hun habitat.

In hoofdstuk 8 onderzochten we experimenteel de tolerantie van rustcellen van diatomeeën voor uitdroging en bevriezing in vergelijking met hun vegetatieve cellen, en dit voor 17 benthische morfosoorten over een gradiënt van permanent aquatische habitats tot terrestriese bodems, onderverdeeld in vijf vochtighedscategorieën. Rustcelvorming werd ingeleid door nutriëntenlimitatie in donkere omstandigheden. Zoals eerder waargenomen waren vegetatieve cellen in het algemeen zeer gevoelig voor uitdroging en bevriezing. Alleen stammen van drie terrestriese taxa overleefden bevriezing in de vegetatieve fase, terwijl slechts één stam 5 minuten uitdroging overleefde. Rustcellen echter vertoonden een hogere tolerantie voor uitdroging, vooral wanneer dit voorafgegaan werd door een warmtebehandeling; en meer stammen en taxa overleefden bevriezing, zij het vaak met lage overlevingpercentages en een grote interclonale variatie. Opvallend was dat alleen de rustcellen van terrestriese taxa, d.w.z taxa die hoofdzakelijk in natte en vochtige of tijdelijk droge plekken (vochtighedsklasse 4) of bijna uitsluitend
buiten watermassa’s (vochtigheidsklasse 5) voorkomen, uitdroging en bevriezing overleefden, hetgeen wijst op habitatspecifieke aanpassingen aan deze ongunstige omstandigheden.

Uit de resultaten verzameld in dit proefschrift kunnen we concluderen dat de biogeografie van diatomeën gecontroleerd wordt door dezelfde processen als bij macrobiota. De biogeografische patronen op wereldschaal tonen aan dat historische factoren en dispersielimitatie inderdaad van toepassing zijn, terwijl cryptische soorten en een te lage taxonoomische resolutie nog steeds een aantal van de geografische patronen maskeren. De hoge gevoeligheid voor uitdroging van vegetatieve cellen en de meeste rustcellen kan de dispersiekansen van diatomeën drastisch verminderen, ondanks hun hoge lokale abundanties. Op intermediaire en kleinere schaal, observeerden we een tendens voor lagere turnover in terrestrische gemeenschappen ten opzichte van lacustriene gemeenschappen, hetgeen een gevolg zou kunnen zijn van hogere dispersiekansen door een betere droogtetolerantie van hun rustcellen.
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Caroline Souffreau
Gent, 15 May 2011

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