Microbial evolution: patterns of diversity in aquatic protists

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Owing to this struggle, variations, however, slight and from whatever cause proceeding, if they be in any degree profitable to the individuals of a species, in their infinitely complex relation to other organic beings and to their physical conditions of life, will tend to the preservation of such individuals, and will generally be inherited by the offspring. The offspring, also, will thus have a better chance of surviving, for, of the many individuals of any species which are periodically born, a small number can survive. I have called this principle, by which each slight variation, if useful, is preserved, by the term natural selection, in order to mark its relation to man's power of selection.

The Origin of Species, Charles Darwin, 1859

"Nothing in biology makes sense except in the light of evolution"

Theodosius Dobzhansky, Ukrainian geneticists and evolutionary biologist (1900–1975)

La ciencia se compone de errores, que, a su vez, son los pasos hacia la verdad.

Julio Cortázar, Argentine writer (1914–1984)

Those are my principles, and if you don't like them... well, I have others.

Groucho Marx, US comedian with Marx Brothers (1890–1977)

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in ms).

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Introduction

A natural bias

We are interested in organisms which are familiar to us. During our evolution, we have interacted with several other relatively big organisms, such as animals, plants, and fungi. Since our own survival depended upon the correct interaction with those organisms, there was an early interest to classify them. Thus, most ancient cultures have generated (many times independently) classifications systems. For instance, in one of his books, the Argentine writer Jorge Luis Borges (1899–1986) describes a certain Chinese Encyclopedia, called "The Celestial Emporium of Benevolent Knowledge", in which animals are divided into:

1. Those that belong to the Emperor,
2. Embalmed ones,
3. Those that are trained,
4. Suckling pigs,
5. Mermaids,
6. Fabulous ones,
7. Stray dogs,
8. Those included in the present classification,
9. Those that tremble as if they were mad,
10. Innumerable ones,
11. Those drawn with a very fine camelhair brush,
12. Others,
13. Those that have just broken a flower vase,
14. Those that from a long way off look like flies.

An array of classification systems coexisted until Carl von Linné (1707–1778) entered the scene. He dedicated an important part of his life to find out what he considered the patterns of God's creation. In his *Systema Naturae* (1735), Linné provided a description of the species known during his days and also introduced a classification scheme. The hierarchical classification method introduced by Linné is the standard classification system used today. For classification purposes, Linné ignored characteristics that he considered unimportant and used the "important" features of organisms to reveal what he believed to be God's creative plans (Futuyma, 1998). After *The origin of species* of Darwin (1809 – 1882) the classification system achieved a radically different significance, and was now supposed to reflect evolution from common ancestors rather than discovering any mystical essence. Thus, species in the same genera are more similar because they come from a relatively recent ancestor, while genera in the same family have departed further from a common ancestor, and so on in a hierarchical scheme.

Even before Linné was born, Robert Hooke (1635–1703) in England and Anton Van Leeuwenhoek (1632–1723) in Holland were making significant progress into an instrument which would allow people to see things that had never been seen before: the microscope. Around 1674, Van Leeuwenhoek told the world about the existence of bacteria, protists, sperm cells, blood cells, etc. (Woodruff, 1939). After the initial descriptions by Van Leeuwenhoek, subsequent taxono-

mists applied the Linné classification system to microbes. Thus, several Phyla, Classes, Genera and Species of microbes were coined based on morphological characteristics.

For several years, the classification of organisms proposed by Linné as well as the concept of evolution introduced by Darwin, were applied for cataloging the biodiversity of the world. Despite the initial success, it soon became clear that this classification system had its limitations. Specifically, microbes proved to be difficult to classify. Early researchers noted that it was difficult to classify "little green balls" (green algae) or minute pellet-like cells based on morphological features alone. They questioned whether they were different species, genera, families, or even different phyla? Situations like these started to appear relatively often among microbial taxonomists, troubling their apparent determination to classify every organism.

The fact that the Linné classification scheme showed limitations is not a surprise. We like to classify things, make simplifications of the world which are useful to us, but which might not be totally accurate in describing reality. In Richard Feynman's (1918–1988) words *"here in wine is found the great generalization: all life is fermentation. Nobody can discover the chemistry of wine without discovering, as did Louis Pasteur, the cause of much disease. If our small minds, for some convenience, divide this glass of wine, this universe, into parts — physics, biology, geology, astronomy, psychology, and so on — remember that Nature does not know it! So let us put it all back together, not forgetting ultimately what it is for. Let it give us one more final pleasure: drink it and forget it all!"*

Species

One of the main problems that arose among early taxonomists was how to delineate species based on morphological characters; e.g. are two plants which differ only in the length of their leaves different or the same species? Or, can two types of beetles be different species based only their different types of wings? It is clear that depending on human ad hoc definitions, a group of organisms can be classified as 10 species for one taxonomist or 100 for his/her colleague. This illustrates that it is difficult to ascertain which morphological characters indicate different species and which ones are just population or phenotypic variation. Moreover, it is challenging to determine which differences were involved in the speciation process, and which ones arose after speciation. Overall, species definitions based on morphological characters harbor many ambiguities, which become much more evident in microbial studies.

During 1930–40 the Biological Species Concept (BSC) emerged, which is based on reproductive characteristics of organisms (mate choice) in order to delineate species. The BSC, mostly attributed to Ernst Mayr (1904–2005), states that "Species are groups of interbreeding natural populations that are reproductively isolated from other such groups" (see Coyne and Orr, 2004). This species definition gained great popularity and is the most commonly used today. Nevertheless, this definition also has its limitations. There are several organisms which are considered different species, and still hybridize keeping their own identity (see review in Schilthuizen, 2001). Likewise, there are several organisms (e.g. bacteria, certain algae, rotifers, etc) that do not appear to reproduce sexually, and for which the BSC cannot be applied. These inconsistencies promoted other researchers to propose several new species concepts based on genetic/phenotypic cohesion, evolutionary cohesion and evolutionary history (Coyne and Orr, 2004). Today, there are more than a dozen of species concepts, each definition with its own applicability, but so far, if there is such a thing as a universal species concept, it has not been found. Back to Darwin, in *The origin of species*, he mentions that *"I look at the term species as one arbitrarily given, for the sake of convenience, to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms".* The conclusion that Darwin appears to have reached is that species are real and that there are gaps between them, but that it is impossible to define a single criteria that tells what makes a species a species (see Schilthuizen, 2001).

Speciation

If delineating species is a complex business, understanding the mechanisms by which species

arise in nature represent a challenge of equal or higher magnitude. In short, natural selection, sexual selection as well as chance events (genetic drift) have been recognized as the major mechanisms promoting speciation (see Futuyma, 1998). These mechanisms can act on geographically separated (allopatric), adjacent (parapatric) or within the same (sympatric) population. Species arising in allopatry, parapatry and sympatry are considered to be product of allopatric, parapatric and sympatric speciation respectively. Allopatric speciation gained popularity and has been widely accepted by the scientific community (Coyne and Orr, 2004). Allopatric and parapatric speciation mechanisms assume that the geographical separation between the populations will diminish the gene-flow, and consequently the populations will take different evolutionary directions. The action of natural selection can promote this divergence process if it is acting differentially in the different populations. On the other hand, the occurrence of sympatric speciation is still one of the most contentious topics in evolutionary biology. Sympatric speciation requires that the evolutionary forces that cause the interbreeding population to diverge to be stronger than recombination, a cohesive force which causes populations to fuse. Overall, sympatric speciation has been supported by theoretical (Doebeli et al., 2005), laboratory (Rainey and Travisano, 1998) and field studies (Coyne and Orr, 2004).

At this point, it is worth mentioning that most of the knowledge on speciation comes from the study of multicellular organisms. So far, in the study of speciation, microbes have been mostly ignored. In my opinion, this may introduce a strong bias into what we know as species and speciation mechanisms. After all, microbes constitute the major part of the biodiversity and probably biomass on the Earth (see Torsvik and Ovreas, 2002; Torsvik et al., 2002; see Fig.1). Most biological innovation at the metabolic and genomic level is microbial. Moreover, microbes existed on the Earth for about 3 billion years before the mul-

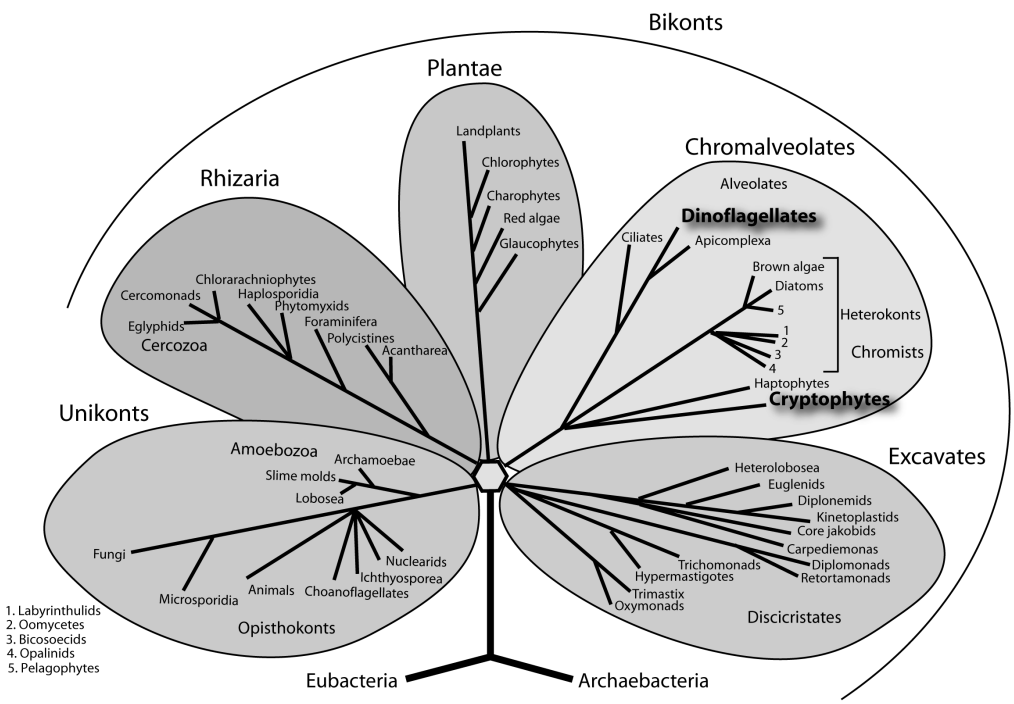


Figure 1. Tree of eukaryote diversity redrawn from Keeling (2004) by Minge (2005). The position of the organisms used in this thesis (Dinoflagellates and Cryptophytes) appears in **bold**. Note that most of the groups are unicellular and therefore considered as protists (i.e. unicellular eukaryotes).

ticellular organisms evolved. If we think in numbers, around 75 % of the time the process of biological evolution has been running, it has been occurring at microbial sizes. Therefore, microbial speciation has been predominant during most of the evolution of life on Earth, and still, very little is know on how microbes diversify and eventually speciate. Thus, when we normally refer to speciation and, to some extent, evolution as a whole, we should consider that we are talking about multicellular evolution and thus, we may be seeing only one fraction of the whole story.

Microbial diversification and speciation

An important question within evolutionary biology is how microbial populations diversify and ultimately speciate in nature. More specifically, do the recognized mechanisms promoting evolutionary diversification in multicellular organisms (e.g. natural selection and genetic drift) have the same importance in microbial evolution? A number of differences between microbes and multicellular organisms suggest that the role of natural selection and genetic drift might not be equivalent in promoting the diversification of these types of organisms. In principle, there are at least three main differences between microbes and most multicellular organisms which could affect their tempo and mode of evolutionary divergence: (1) population size, (2) reproductive rate (sexual and asexual), (3) potential for long distance dispersal.

Population size and genetic diversity in microbes

The sizes of microbial populations appear to be several orders of magnitude higher than most populations of multicellular organisms (see e.g. Snoke et al., 2006). In the first place, big population sizes suggest that the action of genetic drift is more restrained in microbes. Moreover, the huge population sizes mean that there are many individuals where mutations, the ultimate source of diversity, can occur. Furthermore, every microbial cell can potentially pass genetic information to the next generations, sexually or asexually. Altogether, the normally huge population sizes and the potential capability of virtually every cell to pass information to the next generations suggest that microbial populations could harbor high genetic diversity.

Studies in marine microbial species have shown high genetic diversity within populations (e.g. Bolch et al., 1999b; Bolch et al., 1999a; Medlin et al., 2000; Rynearson and Armbrust, 2000; De Bruin et al., 2004; Shankle et al., 2004; Evans et al., 2005; Wilson et al., 2005; Iglesias-Rodriguez et al., 2006). In marine environments, there are no obvious geographical barriers for microbial dispersal, and therefore, these high genetic diversities could reflect in part high dispersal rates. In contrast, lakes and ponds represent patches of favorable environments separated by unfavorable areas. These characteristics could affect the gene-flow of microbes among the lake-populations and therefore their patterns of genetic diversity. Moreover, if lakes provide more stable environmental conditions than marine habitats, then it is possible that one or a few well-adapted genotypes would dominate the lake-populations. However, the limited available data indicate that microbial lake-populations harbor high genetic diversities (e.g. De Bruin et al., 2004; Muller et al., 2005; Zhang et al., 2006), but more studies in different microbial taxa are needed to ascertain if this is a general phenomenon for all microbial lake-populations.

To further investigate this subject, I have studied the genetic diversity within five dinoflagellate (protist) species present predominantly in lakes and ponds (**Paper 5**). Dinoflagellates are an ancestrally marine unicellular eukaryote lineage, which is present in both marine and fresh waters. Dinoflagellates appear to have diverged from ciliates and apicomplexans around 900 million years ago [MYA] (Escalante and Ayala, 1995; see Fig. 1). At the beginning of the Mesozoic (~250 MYA), dinoflagellates showed a tremendous evolutionary radiation (Fensome et al., 1996; Fensome et al., 1999). Dinoflagellates have an enormous diversity of life strategies, with symbionts, parasites, free living planktonic or sessile photosynthesizers-heterothrophs and mixothrophs (Hackett et al., 2004). Usually, dinoflagellates spend most of their life cycles as haploid cells that proliferate by mitotic division (Von Stosch, 1973). Sexuality can be induced by endo- and exogenous factors,

and in many cases, results in a diploid resting cyst with environmental resistance and dispersal functions (Pfiester and Anderson, 1987). Last but not least, dinoflagellates have key roles in the functioning of marine and freshwater ecosystems, and several species are well known toxin producers (e.g. Hallegraeff, 1993).

Overall, my results indicated that lacustrine environments harbor a multitude of dinoflagellate genotypes, instead of a few ones. We have analyzed 68 dinoflagellate strains and we have not found identical genotypes. Instead, we detected an array of genotypes ranging from little to highly differentiated. Our data also indicated that different species could harbor different levels of genetic diversity. For instance, we found that a population of the lacustrine brackish *Peridiniopsis borgei* harbored a relatively high level of genetic diversity, whereas, the polar *Polarella glacialis* appeared to have lower levels of diversity across its distributional range (**Paper 5**).

Due to the relatively high levels of genetic diversity that we found, I hypothesized that there could be genetic structure (i.e. genetically differentiated populations) within a single lake. In higher animals, where the structuring patterns of the genetic diversity have been studied much more extensively, there are a variety of examples of genetically differentiated populations of fish within a single lake (e.g. Dynes et al., 1999; Wilson et al., 2004). In contrast, the possibility that genetically differentiated but sexually compatible microbial populations can coexist within the same lake has been little explored. A number of studies have found ecophysiological evidence indicating that genetic populations of protists can coexist within one lake (see Weisse, 2002; Weisse and Rammer, 2006). Interestingly, we found evidence that different genetic populations are present within single lake populations in *Peridinium aciculiferum* (lake Brodammen), *Peridiniopsis borgei* (lake Stora Kalkbrottsdammen) and possibly *Peridinium cinctum* (Gyllebosjön) [**Paper 5**]. Among these dinoflagellate species, sexuality has only been confirmed in *P. cinctum* (Pfiester, 1975), but likely occurs in the other species since resting cysts, which are mostly associated to sex, have been observed in both of them (Rengefors and Anderson, 1998; Boltovskoy, 1999). However, there are also examples where resting cyst production is not necessarily associated to sex (Kremp and Parrow, 2006), at least in the laboratory. Nevertheless, it is likely that in the studied lakes some mechanisms might prevent the genetic homogenization of strains from different genetic populations. For instance, mechanisms of gamete recognition (e.g. Starr et al., 1995), where cells from the same genetic population tend to recombine; temporal or spatial separation of strains from different genetic populations or selection against recombinant genotypes.

Overall, ours (**Paper 5**) and others' (De Bruin et al., 2004; Muller et al., 2005; Zhang et al., 2006) results, suggest that microbial lake-populations are genetically diverse, instead of being dominated by a few well-adapted genotypes. Thus, it has become increasingly apparent that marine and lacustrine microbial populations harbor high genetic diversity, despite the different nature of these environments. Moreover, our results suggest that genetically different and sexually recombining microbial populations can coexist within a single lake. Future studies in other microbial taxa are needed to confirm these patterns.

High reproductive rates and the potential for rapid divergence in microbes

The reproductive rates in microbes are much higher than in most macroorganisms. While the generation time for humans is around 20 years, the generation time for bacteria can be 20 minutes, around 500,000 times faster. Such differences in generation times are translated into differences in the rates of evolution between microbes and macroorganisms. There are a variety of laboratory studies showing rapid evolutionary diversification in Bacteria (e.g. Rainey and Travisano, 1998). However, there are few studies in which the recent diversification of microbes in the wild has been investigated. In **Papers 2, 3** and in part of **5**, I investigated the recent diversification of a dinoflagellate lineage. This lineage consists of the species /strains *Scrippsiella hangoei*, *Peridinium aciculiferum*, and the bipolar *Scrippsiella* aff. *hangoei*, which inhabit marine and lacustrine environments with very different salinities (~ 0–32). Moreover, they are distributed across a wide geographical area (Antarctica, Arctic, and Northern-Central Europe) [Fig. 2]. In **Paper 2**, we show

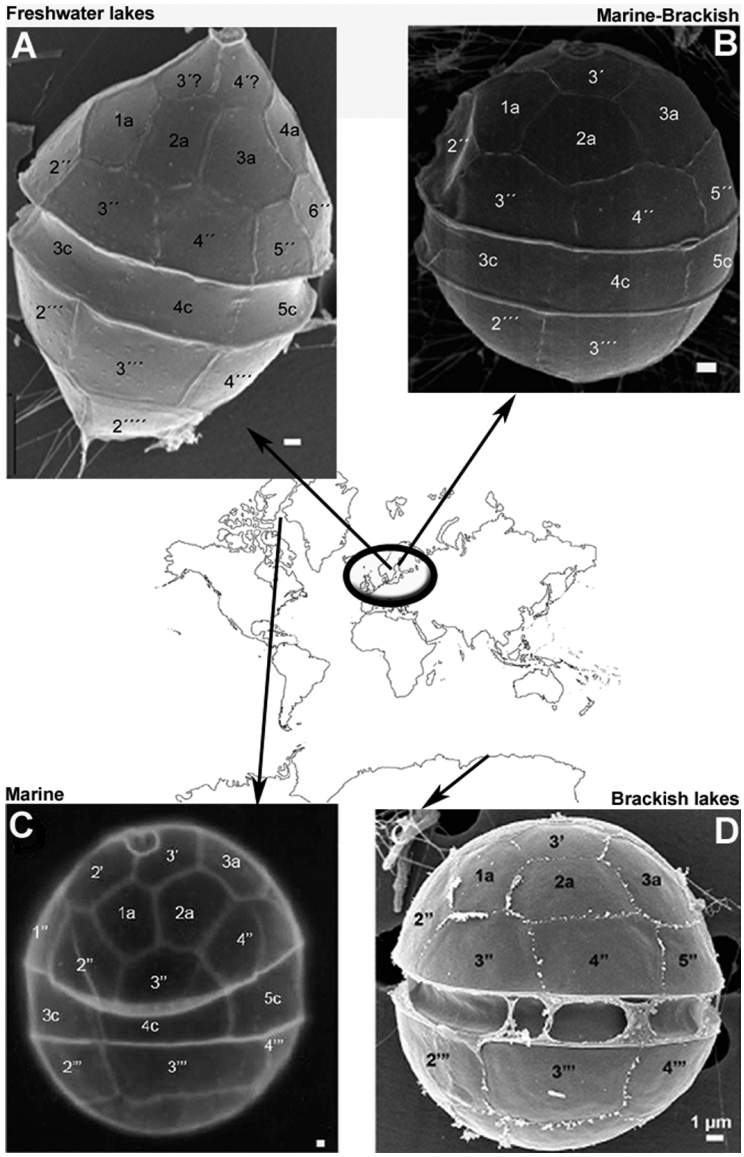


Figure 2. A recently diverged protist lineage distributed in marine and lacustrine environments across a wide geographic range. A= *Peridinium aciculiferum*: freshwater lakes in Northern-Central Europe; B= *Scrippsiella hangoei*: Marine-brackish, Baltic Sea; C= *Scrippsiella* aff. *hangoei*: Marine, Arctic; D= *Scrippsiella* aff. *hangoei*, brackish Antarctic lakes. The scalebar = 1µm. The numbers over the cell armor correspond to the dinoflagellate Kofoidian tabulation. Note the difference in general morphology between *Peridinium* (A) and *Scrippsiella* (B–D).

that *P. aciculiferum* and *S. hangoei* are adapted to different salinities and have morphological differences (Figs. 2A–B, 3). Despite these phenotypic differences, *P. aciculiferum* and *S. hangoei* were found to have identical sequences in the Internal Transcribed Spacer (ITS) nuclear ribosomal DNA (nrDNA), as well as SSU and LSU nrDNA (see Box 1 if this does not mean anything to you).

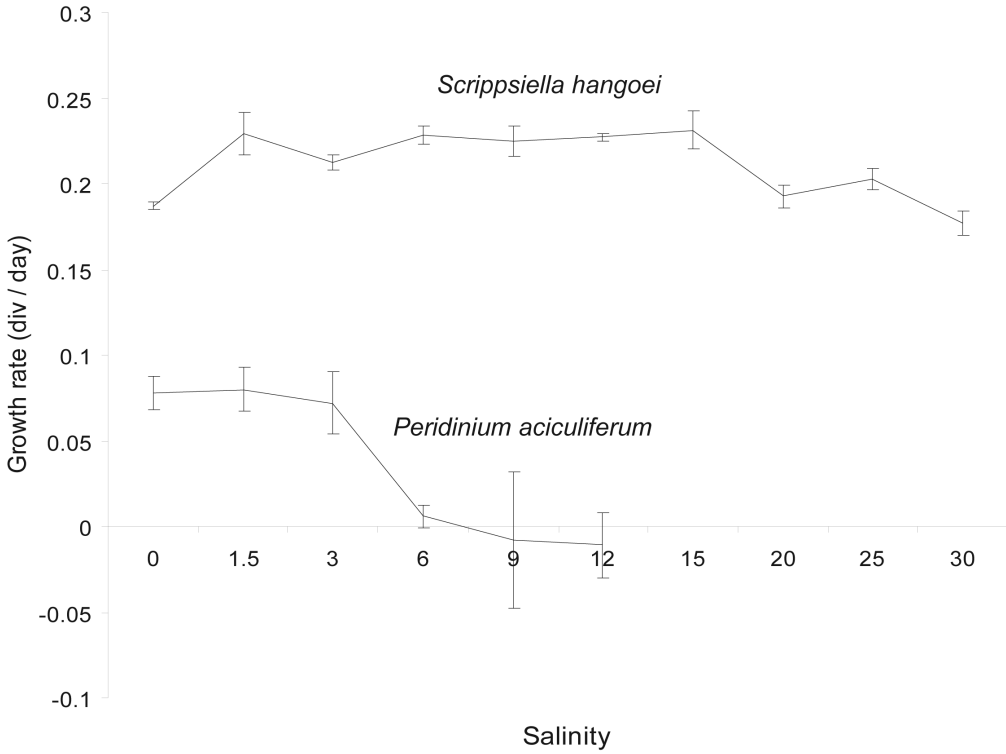


Figure 3. Growth rates of *Scrippsiella hangoei* and *Peridinium aciculiferum* at different salinities. Data points refer to treatment means ± 1 SE. Note that *S. hangoei* was grown at 150 µmol photons $m^{-2}s^{-1}$ and 14:10 h light-dark while *P. aciculiferum* was grown at 20 µmol photons $m^{-2}s^{-1}$ and 12:12 h light-dark, precluding direct comparison

Finding identical ITS sequences in these species was surprising, since the ITS is a rapidly evolving marker in dinoflagellates (Litaker et al., 2007). Moreover, relatively high levels of ITS differentiation were previously found among dinoflagellate strains with identical morphologies (e.g. Montresor et al., 2003b; Kim et al., 2004). Despite sharing identical ITS, *P. aciculiferum* and *S. hangoei*, were found to be clearly differentiated when analyzed by a genome-screening technique, Amplified Fragment Length Polymorphism (AFLP) [see **Paper 2**]. Thus, I proposed that *P. aciculiferum* and *S. hangoei* have diverged rapidly (in evolutionary times), possibly due to a marine-freshwater transition that exposed the same ancestral species to different natural selection regimes.

When analyzing other species from polar regions, we found out that *P. aciculiferum* and *S. hangoei* were part of a larger dinoflagellate lineage which has diversified recently (Fig. 2.; **Paper 3**). Overall, *P. aciculiferum*, *S. hangoei*, and the bipolar *Scrippsiella* aff. *hangoei* turned out to have a very low ITS differentiation among each other (ranging between 0 – 1.43 %; the term aff.(= affinis) which means "similar to" was applied since there is not a formal description for this species yet). Such low differentiation was a clear indicator that these species have diverged relatively recently, especially since the ITS differentiation among most dinoflagellate species is above 4 % (Litaker et al., 2007). It still needs to be confirmed if *P. aciculiferum*, *S. hangoei*, and the bipolar *S.* aff. *hangoei* are different species or they can still interbreed. If they can, they would be one species according to the Biological Species Concept.

To further test the cohesion among the strains/species in this recently evolved lineage, I sequenced the mitochondrial gene Cytochrome b (COB; see Box 1). Since the COB is a slowly evolving gene in dinoflagellates (Zhang et al.,

2005), I expected a very low differentiation among the sequences from the different strains/species. However, the COB differentiation was much higher than I expected in comparison with the low ITS differentiation (see Fig. 4; **Paper 3**). Since the ITS is a rapidly evolving marker in dinoflagellates, I had anticipated a very low COB differentiation. Instead, I proposed that the COB sequences present in *P. aciculiferum*, *S. hangoei* and the bipolar *S.* aff. *hangoei* are the product of a COB diversification which started before the diversification of these strains/species. The COB mtDNA does not recombine and therefore, each specific COB sequence evolves clonally among and within the populations. Since dinoflagellates have enormous population sizes (compared to multicellular organisms), it is possible that ancient COB variants have been retained in the population.

We have also investigated if rapid evolutionary diversification has taken place among strains of the dinoflagellate species *Scrippsiella* aff. *hangoei* and *Polarella glacialis* present in a variety of Antarctic marine-derived lakes (**Paper 5**). The studied Antarctic lakes emerged from the sea around 6,000 years ago by isostatic uplift (Zwartz et al., 1998; Fig. 5). During that period, some of the lakes suffered drastic changes in salinity, thus exposing the ancestrally marine phytoplankton communities to a variety of new natural selection regimes. There is evidence from multicellular organisms that salinity can be an important selective force (Lee and Bell, 1999). More recent evidence indicates that salinity is also an important selective force among microbes (Alverson et al., 2007, **Papers 6, 7**). Thus, the saline Antarctic lakes represent a natural experiment on microbial rapid evolution. Our results (**Paper 5**) showed that marine and lacustrine *Polarella glacialis* were strongly separated in the AFLP trees, suggesting that genetic differentiation has occurred between the marine and lacustrine *Polarella* populations. Such genetic differentiation should have occurred within the last 6,000 years, and this could be considered a case of rapid evolution. Among the lakes, no significant clusters were detected, although the AFLP analyses showed that some strains from the same lake displayed a tendency to cluster together, suggesting an incipient among-lake genetic diversification (**Paper 5**). The most likely explanation for the inconclusive results regarding the presence of genetically differentiated lake populations was the low sample sizes.

Potential for long distance dispersal

Another difference between microbes and multicellular organisms is related to long-distance dispersal. Despite that there seem to be microbes which disperse over short geographical distances only, many others appear to be able to disperse over very large areas (see Hughes Martiny et al., 2006). In multicellular organisms, there are relatively few examples of species which can travel long distances (e.g. some birds, whales, fish, etc.). In **Papers 4**, **5** I present data that confirm previous evidence of long-distance dispersal in *Polarella glacialis* (see Montresor et al., 2003a) using SSU, ITS and AFLP. Bipolar *Pa. glacialis* strains were found to have identical ITS sequences and a relatively low AFLP differentiation. These data suggest that dispersal has occurred relatively recently between the Arctic and Antarctic. In **Paper 3,** I found a low ITS differentiation among Arctic and Antarctic *S.* aff. *hangoei* strains (Fig. 2 C, D), also indicating a relatively recent bipolar dispersal.

According to some researchers, long-distance or unrestricted dispersal should have a considerable effect on microbial diversification by reducing the chances for allopatric speciation (see review in **Paper 1**). The allegedly reduced speciation rates due to unrestricted dispersal were used to explain the apparently low diversity of microbial species (e.g. Finlay, 2002). However, this pattern was mainly based on morphological analyses. During the past 15 years, the use of molecular techniques has unveiled that there is a relatively high amount of diversity hidden under similar or identical cellular morphologies (i.e. cryptic diversity) [see review in **Paper 1;** Huber et al., 2007]. This clearly indicates that microbial diversification has been occurring at a much higher rate than previously thought. Consequently, if unrestricted dispersal is occurring in microbes, it does not appear to significantly have restrained their diversification. Other mechanisms, like sympatric diversification and speciation could be taking place in microbes. For instance, we have found evidence that differ-

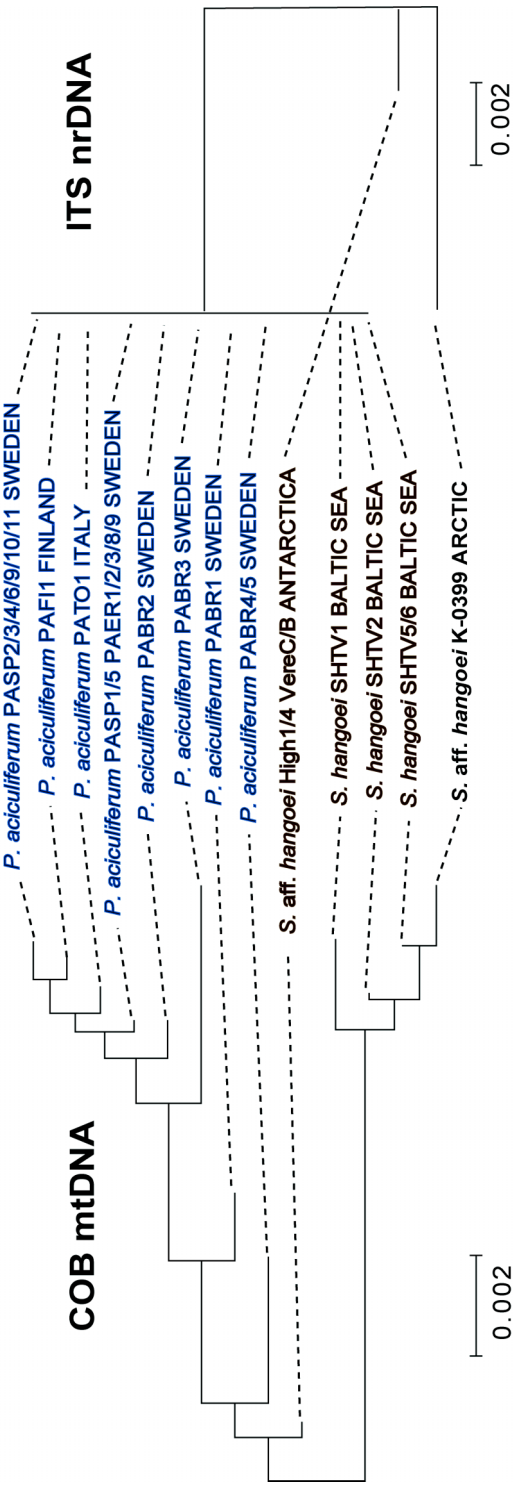


Figure 4. COB mtDNA variation vs. ITS nrDNA homogeneity (description of these markers in Box 1). These results were not expected since the COB evolves much more slowly than the ITS. The figure shows contrasting Neighbor-Joining trees based on uncorrected genetics distances (*p*) between COB haplotypes (left) and the corresponding ITS sequences (right). Note that several strains with the same ITS presented variation in their COB sequences. Freshwater lacustrine strains/species appear in light blue, brackish in green and marine in black. *p* was calculated using 836 COB and 558 ITS nucleotides. PABR= *Peridinium aciculiferum* Brodammen, Sweden. PASP= *P. aciculiferum* Stora Pildammen, Sweden. PAFI= *P. aciculiferum* Lake Österträsk, Finland. PATO= *P. aciculiferum*, Lake Tovel, Italy. SHTV= *Scrippsiella hangoei*, Tvärminne, Baltic Sea. K-0399= *Scrippsiella* aff. *hangoei*, Arctic. High1/4 and VereC/B = *S.* aff. *hangoei* from Highway and Vereteno lakes, Antarctica. The scalebar indicates *p*.

Figure 5. Some of the sampled marine-derived Antarctic lakes in the Vestfold Hills. A = Pendant Lake during December 2004. B = Ace Lake during January 2005. C = Ace Lake during December 2004. D = Hand Lake during December 2004.

ent genetic populations are present within small lakes (**Paper 5**), suggesting that diversification can occur in sympatry.

The interplay of high dispersal, high genetic diversity and huge population sizes in the microbial colonizations of new environments

From an evolutionary perspective, oceans can be considered as continents and lakes as islands for aquatic organisms. However, there is an important difference to take into account in this analogy. Marine and freshwater lakes are two environments which differ in their general physicochemical characteristics, and that has no parallel when continents and islands are compared. For the majority of aquatic animals, the differences in osmotic pressure and ionic concentrations between marine and fresh waters represent a strong barrier that cannot be crossed by most species, which are normally adapted to one environment or the other, but not to both (Lee and Bell, 1999). It is unclear, however, to what extent the physicochemical differences affect the exchange of microbes between marine and freshwater lakes. As a consequence of high dispersal and enormous population sizes, one would expect that there is a constant migration of microbes between marine and fresh waters. Moreover, since several microbial populations harbor relatively high genetic diversities (e.g. Bolch et al., 1999b; Bolch et al., 1999a; Medlin et al., 2000; Rynearson and Armbrust, 2000; De Bruin et al., 2004; Shankle et al., 2004; Evans et al., 2005; Wilson et al., 2005; Iglesias-Rodriguez et al., 2006), it would be expected that sooner or later, new colonizing genotypes will be established at either environment. A similar reasoning could be applied for microbial transitions between marine and saline lakes. Even though salinity might not be the most important difference in this case, marine and saline-lacustrine environments most probably differ in several other aspects (ecology, physicochemical conditions, etc.). These differences could be translated into differ-

ent natural selection regimes operating in marine and saline-lacustrine environments.

If microbes were able to migrate frequently between marine and lacustrine environments, then we would expect both closely and distantly related marine and lacustrine taxa when analyzing the phylogenies (i.e. evolutionary history) of microbial lineages present in both environments. However, since early on, morphological phylogenies have suggested that several protist (phytoplankton) lineages are segregated into predominantly marine and freshwater groups (e.g. Taylor, 1987; Popovsky and Pfiester, 1990; Graham and Wilcox, 2000). Nevertheless, the ambiguities of morphological phylogenies precluded further investigations on the evolutionary relationships between marine and freshwater taxa.

Today, molecular phylogenies open a new opportunity for studying the number and timing of marine-lacustrine transitions during the evolutionary history of a microbial lineage. For instance, the presence of a few monophyletic (i.e. sharing a common ancestor) clusters of lacustrine species that are distantly related to all other lacustrine species within an ancestral marine lineage, would indicate that lacustrine colonizations are rare and probably not recent events (Fig. 6 B). On the other hand, the presence of many closely related marine and lacustrine species or strains would suggest that marine-lacustrine transitions have been occurring more frequently (Fig. 6 A). Also, the presence of marine species nested within freshwater clades would indicate marine recolonization events (Fig. 6 C).

Until now, very few studies have used phylogenies as tools to investigate marine-lacustrine transitions. The idea though, has been developing in the scientific community, especially since several molecular studies with microbes indicated that marine and freshwater species are normally not closely related (Methe et al., 1998; Zwart et al., 1998; Zwart et al., 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Saldarriaga et al., 2004; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; von der Heyden and Cavalier-Smith, 2005; Figueroa and Rengefors, 2006; Sims et al., 2006; Lefevre et al., 2007). These studies, however, were mostly focused on other questions than marine-freshwater transitions.

In the **Papers 6** and 7, we have analyzed the historical marine-freshwater transitions in two protist lineages: Dinophyceae and Cryptophyceae. The general characteristics of dinoflagellates have already been mentioned. As for the cryptomonads, this is a relatively small and easily recognized group of predominantly photoautotrophic protists with around 100–200 species (see Hoef-Emden and Melkonian, 2003) [Fig. 1]. This group has a cosmopolitan distribution and species are found in both oceans and in most types of freshwater habitats (Klaveness, 1988, 1989). In **Papers 6** and 7 we have constructed extensive 18S (SSU) and 28S (LSU, D1/D2; dinoflagellates only) phylogenies [see Box 1] (Figs. 7, 8).

Our most important results regarding marine-freshwater transitions for dinoflagellates and cryptomonads were:

a) marine and freshwater species are usually not closely related, b) several freshwater species cluster into monophyletic groups, c) most marine-freshwater transitions do not seem to have occurred recently, d) only a small fraction of the marine lineages appear to have colonized fresh waters and e), only a small number of freshwater species seem to have re-colonized marine environments. Thus, it becomes apparent that the marine-freshwater boundary has acted as a major barrier during the evolutionary diversification of dinoflagellates and cryptomonads.

At the same time that **Paper 6** was accepted for publication, a very similar study with another protist group, Diatoms (photoautotrophic phytoplankton; Fig. 1), was published (Alverson et al., 2007). The conclusions from this work were similar to ours: marine-freshwater transitions do not appear as common phenomena in diatoms. Moreover, this completely independent study gave extra support to the use of phylogenies for inferring past colonization events. Thus, it is becoming increasingly apparent that the marine-freshwater boundary represents a major barrier for microbes in general. Future studies will need to investigate if the marine-freshwater barrier is generated only by salinity gradients or whether it is caused also by other factors, such as competitive exclusion by adapted residents (De Meester et al., 2002) or high extinction rates in small limnic habitats (island biogeography theory).

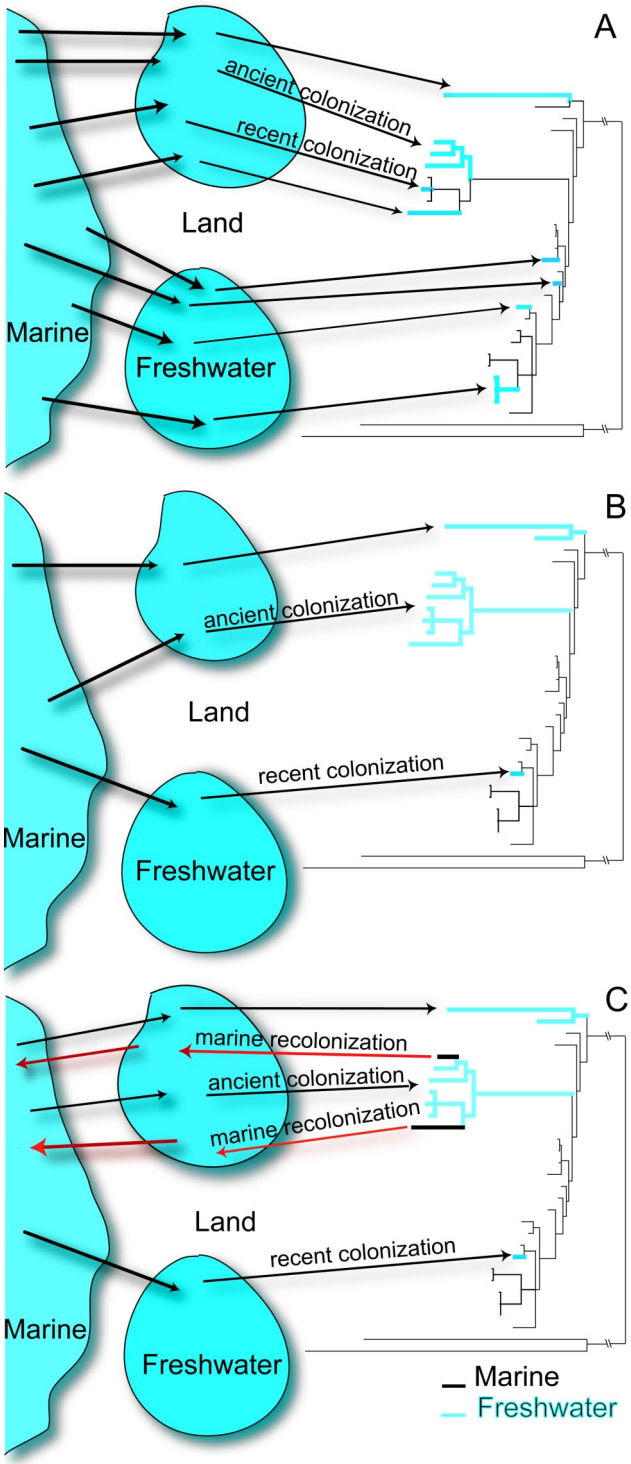


Figure 6. Three possible marine-freshwater transition scenarios in a lineage inferred from phylogenies. **A** = marine-freshwater transitions were common during the diversification of a lineage. Several closely related marine (black) and freshwater (light-blue) species are present in different parts of the tree (i.e. freshwater colonization occurred in parallel in different lineages). **B** = marine-freshwater transitions were rare during the diversification of a lineage. Few closely related marine and freshwater species are observed. Several freshwater species cluster into monophyletic groups, indicating that they derive from a single freshwater colonization event and a posterior diversification in fresh waters. **C** = marine recolonization by a freshwater lineage. Marine species appear nested within freshwater lineages.

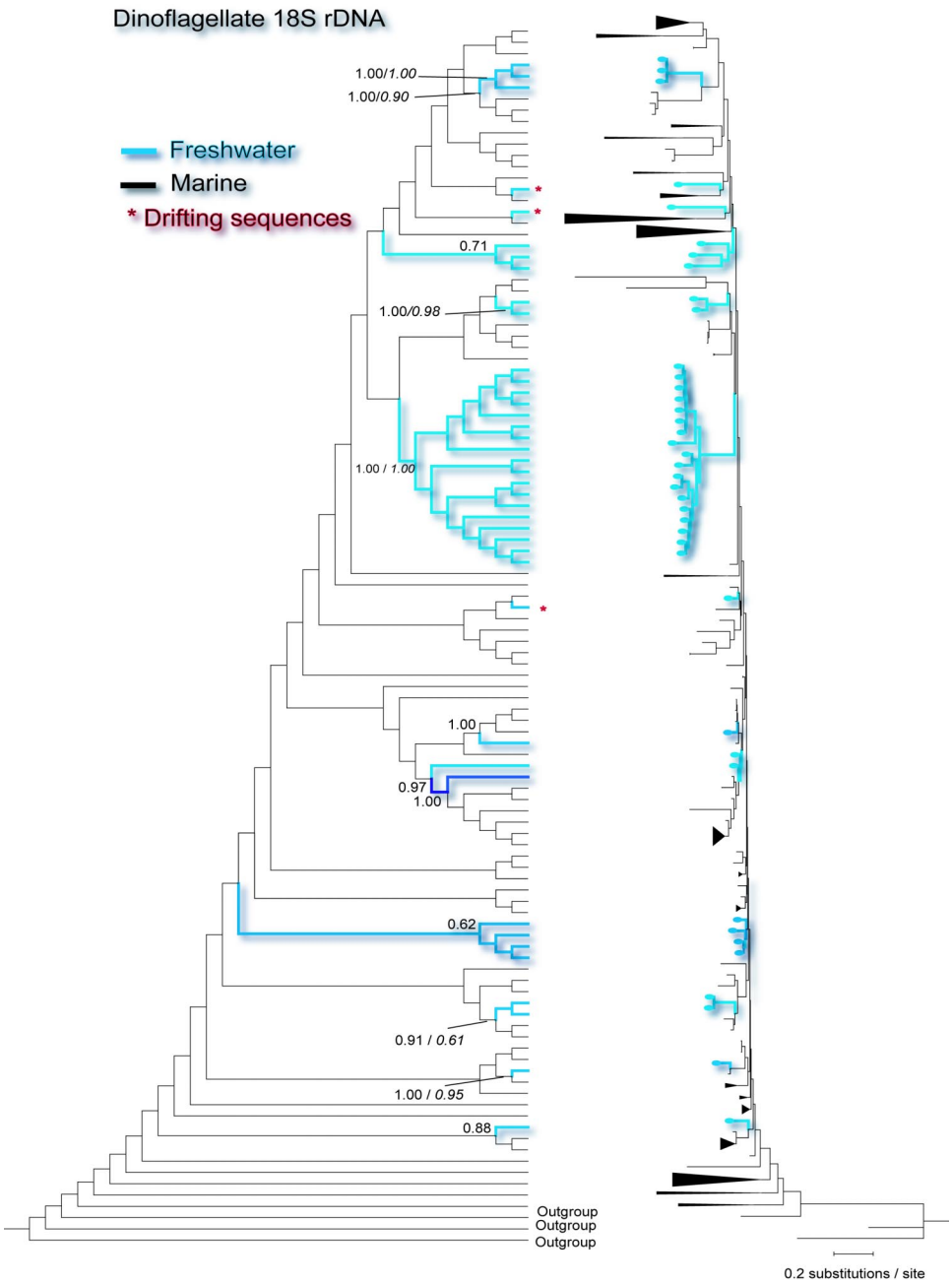
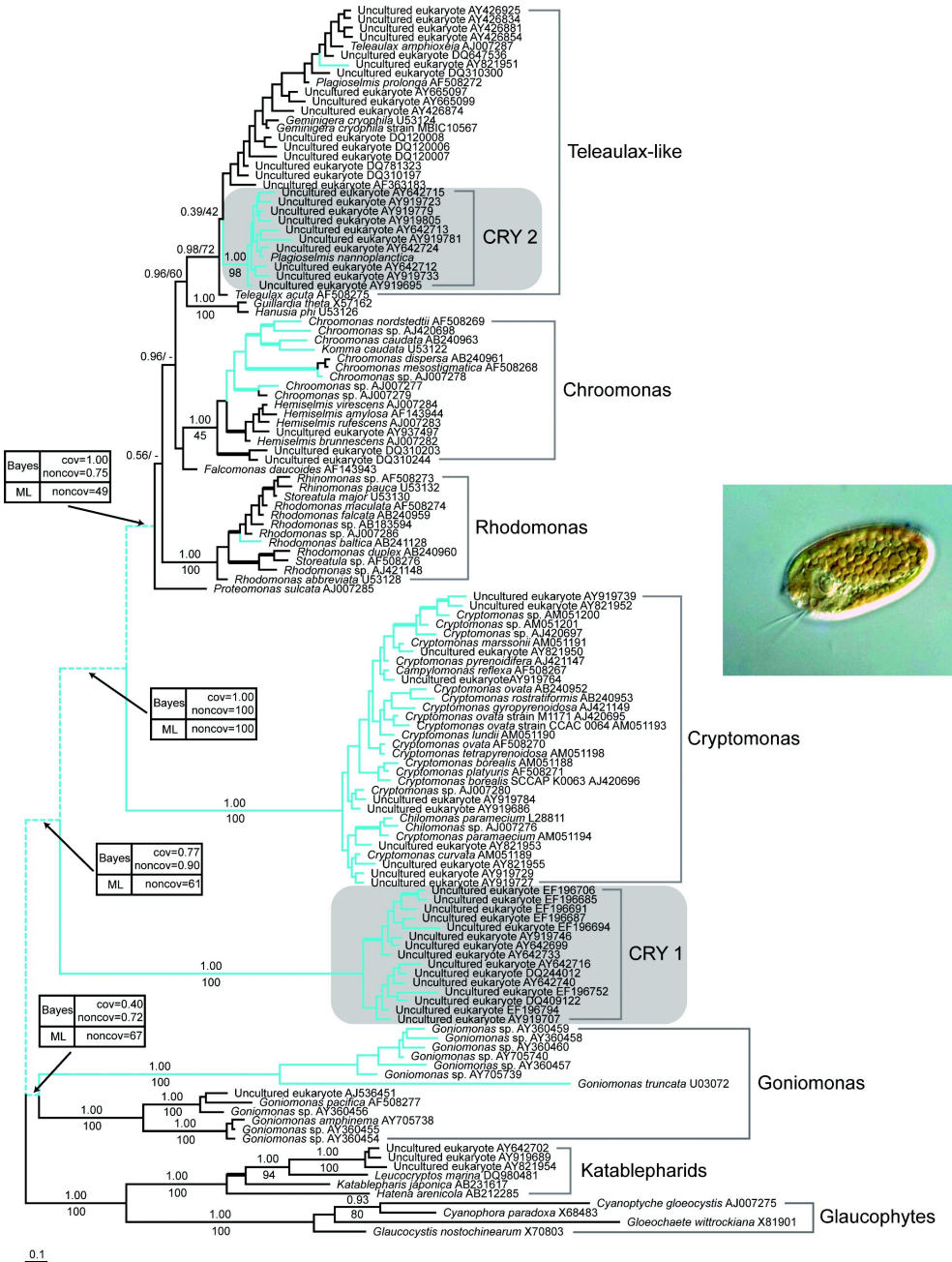


Figure 7. Separation between marine and freshwater species in dinoflagellates according to a 18S phylogeny (species names not shown). The slow rate of evolution of the 18S allows comparing evolutionary distantly related species (more information on this marker in Box1). The tree was constructed using Bayesian inference (see **paper 6**). The tree on the left shows the topology, and the tree on the right shows the genetic distances. Posterior probability and bootstrap support (*Italics*) values > 0.50 are shown for some clades. Drifting sequences are those which appeared in very different positions each time a tree was constructed with the same dataset.


Teleaulax-like
CRY 2
Chroomonas
Rhodomonas
Cryptomonas
CRY 1
Goniomonas
Katablepharids
Glaucophytes
Bayes cov=1.00 noncov=0.75
ML noncov=49
Bayes cov=1.00 noncov=100
ML noncov=100
Bayes cov=0.77 noncov=0.90
ML noncov=61
Bayes cov=0.40 noncov=0.72
ML noncov=67
0.1

In our analyses of marine-freshwater transitions in dinoflagellates, we detected very few closely related marine and freshwater species (**Paper 6**). Nevertheless, we investigated in-depth a pair of very closely related marine-brackish and freshwater species (*Peridinium aciculiferum* and *Scrippsiella hangoei*). Our results indicate that *P. aciculiferum* has diverged from marine ancestors and colonized fresh waters relatively recently (**Paper 2**). As already mentioned, this was indicated by the identical ITS sequences between *P. aciculiferum* and the marine-brackish *S. hangoei*. Moreover, our phylogenetic studies using a variety of markers indicated that *P. aciculiferum* originated from an ancestrally marine lineage (**Papers 2, 3, 6**). In particular, in **Paper 2,** we show that *P. aciculiferum* and *S. hangoei* have evolved different salinity tolerances (Fig. 3). I proposed that the observed phenotypic divergence between *P. aciculiferum* and *S. hangoei* (Fig. 2) is the product of very different selective regimes in marine and fresh waters (**Papers 2, 3**).

Some of the species studied in the **Papers 3, 4, 5** have colonized saline lacustrine environments recently. In **Paper 4**, we describe, for some species the first time, the presence of several marine dinoflagellate species in saline Antarctic lakes. As previously indicated, these lakes have evolved from original marine conditions to brackish and hypersaline in the last 6,000 years (Zwartz et al., 1998; Fig. 5). Little is known on how the original marine phytoplankton community evolved during that period of time in these newly formed and geographically relatively isolated lakes. For that reason, it was of paramount interest to describe the community composition and annual dynamics of the dinoflagellate species present in saline Antarctic lakes. Our results indicated that the ancestral marine dinoflagellate community has undergone a radical simplification in the lakes, leaving behind a few well-adapted species (**Paper 4**). This contrasts to the species rich Southern Ocean. Moreover, we found that dinoflagellates occur year-round in the studied lakes, despite the extremely low light levels during the winter. In particular, the bipolar marine species *Polarella glacialis* was for the first time described for lacustrine habitats, being an important phototrophic component in the higher salinity lakes. In the brackish lakes we described *Scrippsiella* aff. *hangoei*, a species already studied in **Papers 2, 3.** In **Paper 3,** we show that *S.* aff. *hangoei* belongs to a protist lineage which has diverged recently in evolutionary terms, encompassing the marine arctic *S.* aff. *hangoei*, as well as *S. hangoei* and *P. aciculiferum*.

Next (**Paper 5**), we explored the genetic diversity patterns within *Pa. glacialis* and *S. hangoei* in the Antarctic lakes. Our main results have been discussed in previous sections. However, I would like to remind the reader, that the relatively low diversification found in the saline Antarctic lake populations may reflect a relatively recent colonization (~ 6,000 years ago) event by a few strains. Moreover, the relatively low diversity of dinoflagellates in the lakes when compared to the Ocean indicates that most species were not able to colonize the lakes, despite being originally there. This indicates that during the last 6,000 years, the saline lakes have developed new natural selection regimes which were not tolerated by most marine dinoflagellate species. Overall, this suggests that marine-lacustrine (either saline or freshwater) transitions are rare events in dinoflagellates.

Figure 8. Separation between marine and freshwater species in cryptomonads. Note the potential marine recolonization cases within *Chroomonas*. This Bayesian phylogeny was inferred using the 18S nrDNA (see **Paper 7**). New freshwater groups CRY1 and CRY2 identified from GenBank are marked with grey boxes. Freshwater and marine cryptomonad lineages are marked with blue and black lines, respectively. Lines with unclear marine or freshwater origin are indicated with dashed lines. Groups with posterior probability > 0.90 and bootstrap support > 75 % are marked with thick branches. Values at internal nodes represent posterior probability values and maximum likelihood bootstrap analysis. Thick lines indicate posterior probability > 0.95% and bootstrap > 85%. Some of the mentioned groups are also indicated in Figure 1. The picture corresponds to the freshwater *Cryptomonas platyuris* (cell about 40 μm long) and was obtained from the Natural History Museum, London, AlgaeVision project (http://www.nhm.ac.uk/botany/algaevision)

Box 1: General introduction to the molecular techniques used in this thesis

If you do not have a clue about molecular genetics, this Box if for you, otherwise please read the methods in each of the papers. In the first place, what is the advantage of using molecular data? Is it just fashion? Well, in part it may be. In some of the corridor talks, it was said that if genetic studies had been the first to be carried out we would now have been interested in studying phenotypic variation. Nevertheless, genetic data represent an extra type of data, no better, no worse than, for instance, phenotypic or physiological data. Overall, all types of data should be considered to understand an organisms or a pattern as a whole.

The current interest in molecular studies is due to the present availability of molecular tools which were not available 20 or 10 years ago. Similar to informatics and the computer industry, the biotechnological industry is growing at an incredible speed. Fifteen years ago, you would not have thought about paying your bills, calling to another continent, listening music, or booking a plane ticket through Internet. In the same way, fifteen years ago sequencing genes was still a very laborious and expensive work, and you probably would have been happy if you could get a short DNA sequence. Today, several **genomes** (i.e. the whole genetic information within an organism) have been sequenced, comprising several million nucleotides each. Moreover, DNA sequencing has become more affordable and now it is the standard in several ecological laboratories. As for October 2007, the crest of the wave in molecular ecology and evolution appears to have moved farther, to gene expression, genome and environmental sequencing. It is difficult to predict which molecular techniques will be the standard in twenty years from now.

Genetic markers and techniques

In this thesis, most of the research was carried out by analyzing patterns of genetic variation. Such variation occurred at a) the nucleotide level in homologous genes (i.e. genes with the same ancestor) or b) at the genome level. For studies at the nucleotide level, I have sequenced fragments associated to the **ribosomal and mitochondrial DNA**. The ribosomes are the structures in charge of protein synthesis, while the mitochondria produce energy for the cells. The ribosomal DNA is located within the cell nucleus and the mitochondrial DNA is within the mitochondria. Within a eukaryote cell, many copies of ribosomal genes are distributed along the genome. Each ribosomal DNA copy (rDNA) consists of three conserved fragments (i.e. they change very slowly with time). These are, the **18S** (small subunit, SSU), the **28S** (large subunit, LSU), and the **5.8S** (Fig. 9). Separating the 18S, 5.8S and 28S, are two spacers, the internal transcribed spacer one (**ITS1**) and two (**ITS2**) [Fig. 9]. The nucleotide composition of the ITS1/2 changes much faster than the 18S, 5.8S and 28S. The reason for this variability in the rates of change is that the 18S, 5.8 and 28S have important roles in the assembly of ribosomes (therefore, their nucleotide variation is much more restrained by natural selection), and that is not the case with the ITS1/2, which have more freedom to change (see Hillis and Dixon, 1991). The ribosomal DNA has been extensively used in phylogenetic and population genetic studies since it has many advantages over other markers. All eukaryotes have ribosomes, there are many copies in the genome (plenty of template for genetic analyses) coding for ribosomes, and the different rates of evolution of the different fragments (i.e. 18S, 28S, 5.8S, and ITS1/2) provide information at different evolutionary levels. The slow rate of change in the 18S makes possible to compare many species that have started to diverge far back in time. On the other hand, the rapid rate of change in the ITS1/2 allows comparing organisms that have diverged more recently.

There are also advantages of using mitochondrial genes. Many mitochondria are present within the cells and therefore there is abundant template for genetic analyses. Moreover, mitochondrial DNA

Figure 9. Description of the different areas of the ribosomal DNA. 18S=Small Subunit Ribosomal DNA. 28S= Large Subunit Ribosomal DNA. ITS=Internal Transcribed Spacer. Many copies of this ribosomal set of fragments are distributed across all eukaryote genomes. The 18S, 28S and 5.8S have slow or moderate rates of evolution, while the ITS1/2 evolve much faster.

does not recombine, and this is an advantage since recombination can complicate the reconstruction of the evolutionary relationships (Avise, 2000). In this thesis, I have used the mitochondrial gene **cytochrome b (COB)**, which has been used in several population genetic and phylogenetic (i.e. evolutionary history) studies (e.g. Avise, 2000, 2004). In dinoflagellates, the COB evolves very slowly (Zhang et al., 2005), and therefore it can provide information about evolutionary events that occurred long time ago.

So far, I have mentioned how I have worked with the nucleotide variation occurring in one or a few genes. For analyzing the genetic variation at the genome level (without looking at any gene in particular) I have used the **Amplified Fragment Length Polymorphism (AFLP)** technique. I will not go into the details of this technique, which utility in ecology has been analyzed in several recent papers (e.g. Bensch and Akesson, 2005). What you need to know for reading this introduction is that AFLP sample the genetic variation in the whole genome. Thus, a more general idea of the overall genetic variation can be obtained than when a few genes are used. However, AFLP is very sensitive, and you use this technique when investigating closely related species, populations, or even individuals. Sequencing genes is a more reasonable approach when you are dealing with species that are not very closely related.

Analyses of the genetic variation: population genetics and phylogenetics

When you are investigating the genetic variation among populations or closely related species you are doing **population genetics**. When you investigate species that have diverged farther back in time, you are doing **phylogenetics**. To analyze AFLP and DNA sequence data, several complex algorithms are used. Their descriptions go far beyond this introduction, and there are several introductory textbooks on this topic (e.g. Avise, 2004). Basically, what these algorithms do is to try to reconstruct the evolution of a group of species with the genetic data you have provided. What you need to know for reading this thesis introduction is that the evolutionary relationships between species are normally depicted as trees. Similar to family genealogies, two species which are very near in the tip of a tree-branch are more closely related (e.g. siblings in a family genealogy, who share the same parents). In a similar manner, when two species are more separated in the trees, this means that the common ancestor between them occurred farther back in time (e.g. cousins in a family tree, who share a common grandfather or more distant ancestors). Thus, trees are a simple way to illustrate hypothetical reconstructions of the evolutionary history of a **lineage** (i.e. all the individuals with a common ancestor). Note that lineage can then be a population or a whole kingdom.

Conclusions

The general conclusions of this thesis are:

a) The marine-freshwater boundary constitutes a barrier for dinoflagellate and cryptomonad transitions between these environments
Because microbes have huge populations, high reproductive rates and the alleged ability for long-distance dispersal, I had expected that aquatic microbes would be able to colonize, sooner or later, most marine and fresh waters. However, we found that relatively few lineages of dinoflagellates and cryptomonads have been able to make successful transitions between these environments. Overall, this suggests that the marine-freshwater boundary could constitute a barrier for most microbes.

b) A wide diversity of dinoflagellate genotypes can coexist within lacustrine environments
My results indicate that dinoflagellate lake-populations are not dominated by one or a few genotypes. Variable or different environmental conditions could promote the coexistence of many genotypes within a single lake.

c) Genetically different dinoflagellate populations can coexist within the same lake
Within a single lake, all strains would be expected to interbreed, thus homogenizing the lake gene-pool. The evidence that different genetic populations can be present within a single lake indicates that there are interbreeding restrictions.

d) Morphologically and physiologically different dinoflagellate morphospecies can be evolutionary very closely related
This result, together with contrasting studies demonstrating extensive dinoflagellate cryptic diversity shows that morphology has to be used with care for inferring evolutionary relationships in protists.

e) Microbial diversification does not appear to be an uncommon process
Some researchers have indicated that the high microbial dispersal would restrain their evolutionary diversification. Here, we found a case of recent diversification in a protist lineage inhabiting distant locations around the world (**Paper 3**). Moreover, we found evidence of different genetic populations even within single lakes, indicating that microbial diversification could occur in sympatry. These and others' results suggest that high dispersal does not necessarily restrain microbial diversification.

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I

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Debate

Does the global microbiota consist of a few cosmopolitan species?

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[Keywords: cosmopolitanisms, endemics, microbes]

[Palabras clave: cosmopolitanismo, endémicos, microbios]

Biogeography studies the distribution of biodiversity over space and time. Currently, there is a strong debate on the biodiversity and biogeography of free-living microorganisms. For several years, morphological studies have promoted the idea that, at the global level, there is a relatively small number of cosmopolitan microbial species (Baas-Becking 1934; Finlay 2002, 2004; Fenchel 2005). This view has been the most popular until recently, when molecular data started to unveil a much more complex scenario. Within the realm of this controversy, the objectives of this work are twofold: a) to review the traditional viewpoints on microbial biodiversity and biogeography and, b) to present and discuss new molecular data which are challenging previous ideas.

Since the number of species relies on the chosen species definition, any discussion on biodiversity and biogeography needs to mention upon which species concept conclusions are based. So far, the Morphological Species Concept (MSC; species are groups of morphologically identical or very similar organisms (Futuyma 1998)) has been the most popular definition in studies on microbial biodiversity and biogeography. In a few cases, the Biological Species Concept (BSC; species are groups of interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1942)) has been applied to microeukaryotes where sexual reproduction is known (Andersen 1998). In a more controversial approach, a group of researchers working on prokaryotes have defined that organisms having more than 70% of DNA-DNA similarity belong to the same species (Wayne et al. 1987).

For years, the use of the MSC in microbial taxonomy has promoted the view that the global microbiota consists of a relatively small number of cosmopolitan species; '*the cosmopolitan view*' (Finlay 2002, 2004). The most popular hypothesis for explaining this putative pattern claims that due to their small sizes and huge abundances, microbes have no geographical barriers for their dispersal (Fenchel 2005). As a consequence, there is little opportunity for genetic diversification and therefore, a low number of cosmopolitan species should be expected. In agreement with these ideas, Griffin et al. (2002) indicate that between 10^{18} and 10^{20} microorganisms are transported annually through the atmosphere, making it difficult to imagine how topographic features of the Earth's surface could act as barriers for their dispersal. Morphological studies support the 'cosmopolitan hypothesis' by indicating that there would be less than 5.000 species of microbes, a number quite low in comparison with the estimated 750.000 species of insects or 280.000 of all other animals (Papke & Ward 2004).

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A lack of biogeographic patterns and a low global diversity for free-living microbes has been the most accepted view until the advent of molecular studies. Interestingly, some molecular data support the 'cosmopolitan hypothesis' by showing a low genetic differentiation between populations separated by continental distances (e.g. Darling et al. 2000; Montresor et al. 2003a). However, there are mounting data revealing a very different picture, as it will be described and discussed in the following sections.

Is there a relatively low number of microbial species?

The technical limitations during the times of the early taxonomists were probably an important factor in generating a false impression of a low number of microbial species. For instance, before the arrival of the electron microscope there were 12 algal classes, while today more than 30 are recognized (Andersen 1998). Although more advanced microscopes have contributed significantly to improve our knowledge, they still have a limit of resolution. This limit is not only technological: it is widely recognized that there are different microbial species and/or genetically isolated strains that are virtually indistinguishable using morphological characters (cryptic species/strains).

It has been proposed that cryptic species/strains are the product of natural selection exerting an important stabilizing force on particular morphologies that represent adaptive peaks (Potter et al. 1997; Finlay 2004). However, selection could be disruptive or absent on other areas of the genome. Thus, it would not be surprising if it is revealed that several well established microbial species defined on a morphological basis (morphospecies) are actually an agglomeration of a range of genetic and physiological strains/species that are morphologically undistinguishable. An increasing number of molecular studies are clearly pointing in this direction (e.g. Pace 1997; Potter et al. 1997; Daugbjerg et al. 2000; Coleman 1996, 2001a, 2001b; Casamatta et al. 2003; Kim et al. 2004; Wilson et al. 2005). Altogether, they are showing that several morphospecies are in fact composed of genetically divergent populations or cryptic species. For instance, Montresor et al. (2003b) carried out morphological and genetic studies on different populations of the cosmopolitan marine dinoflagellate *Scrippsiella trochoidea* and found that within the same morphotype, there are strains which show a genetic differentiation that is in the order of what is normally found between other dinoflagellate species. In the light of their data, it seems likely that the morphotype known as *Scrippsiella trochoidea* is actually a complex of cryptic species.

A good example of how similar morphologies can mask biodiversity comes from coccoid picoplanktonic algae, which are mere tiny (<5 µm) balls that are present in most oceans. According to Potter et al. (1997), the morphology of these microeukaryotes provides so little information that it is not possible to go lower than the taxonomical 'Class' level. Moreover, sexual reproduction was rarely observed, so the BSC could not be used to ascertain their species status. In order to investigate the biodiversity of these picoplankters, Potter et al. (1997) carried out genetic studies on the 'brown-type tiny balls', which were morphologically indistinguishable between each other. Interestingly, they found that, lumped together as 'brown tiny balls', there are members of three distinct eukaryotic groups: the heterokont, the haptophyte and the green algae.

Another factor that may have contributed to an underestimation of the number of microbial species is that most studies have been carried out on cultivable organisms. Today it is estimated that > 99 % of microorganisms are not cultivable using standard techniques (Aman et al. 1995; Oren 2004). During the last decade, phylogenetic data from ribosomal DNA (rDNA) sequences directly amplified from environmental samples (environmental DNA surveys), revealed an unexpected diversity of prokaryotes (Giovannoni et al. 1990; Fuhrman et al. 1992; Hugenholtz et al. 1998). More recently, the same approach was used on marine microeukaryotes, revealing not only an unsuspected high diversity, but also the presence of several novel organisms with unknown close relatives (López-Garcia et al.

2001; Moreira & López-Garcia 2002). For instance, Moon-van der Staay et al. (2001) used the environmental DNA approach to investigate the eukaryotic diversity in marine picoplankton. They took one plankton sample (<3 µm) at a depth of 75 m in the equatorial Pacific Ocean and sequenced the small-subunit (SSU) rDNAs present in the whole sample. They obtained 35 SSU rDNA sequences, most of which belong to previously unknown members of the phyla prasinophytes, haptophytes, dinoflagellates, stramenopiles, choanoflagellates and acantharians. Moreover, six sequences were forming a clade that cannot be assigned to any known eukaryotic taxonomic group. In another study, Dawson and Pace (2002) used the environmental DNA approach for investigating the microeukaryotic biodiversity in anoxic sediments. By sequencing the SSU rDNA from marine and lake samples, they detected many previously unrecognized eukaryotes, including representatives of seven lineages that are not related to any known organism. Using the 'whole-genome shotgun sequencing' technique to microbial plankton from the Sargasso Sea, Venter et al. (2004) found an unexpected high diversity of oceanic prokaryotes and microeukaryotes.

Considering these results, it becomes apparent that the hypothesis proposing a low global diversity for microbes does not agree with nature. Thus, assuming that the real microbial diversity is much higher than previously recognized, the next natural step is to investigate if there are patterns in the distribution of that diversity.

Are microbial species cosmopolitan?

The 'cosmopolitan hypothesis' predicts that microbes will be present in all environments where they can live due to unrestricted dispersal capabilities. Despite some molecular data support this hypothesis (e.g. Darling et al. 2000; Montresor et al. 2003a), multiple lines of evidence indicate that there are microbial species/strains with restricted geographical distributions (Vincent 2000; Wilson et al. 2005; Martiny et al. 2006). For instance, Whitaker et al. (2003) showed that, on a global scale, populations of the hyperthermophilic prokaryote, *Sulfolobus*, are geographically isolated from one another. In other words, thousands of kilometers of separation have left a genetic imprint in *Sulfolobus*. Additionally, high levels of endemicity were also found in soil bacteria (Fulthorpe et al. 1998) and in microbial eukaryotes. For example, in a study of testate amoeba, Wilkinson (2001) found that many species, with body sizes around 100 µm, are restricted either to the Arctic or the Antarctic. Several researchers indicate the existence of endemic lacustrine microalgae strains/species (Tyler 1996; Vincent 2000; Coleman 2001a; Casamatta et al. 2003; Taton et al. 2003; Wilson et al. 2005). Shayler and Siver (2004) mention the unique microalgal flora that is present in the Ocala National Forest (Florida, USA). Recent studies also show the existence of several genetically differentiated ecotypes within a marine planktonic cyanobacterium morphospecies (Coleman et al. 2006; Johnson et al. 2006).

Implications of new molecular data

Altogether, new molecular data indicate that: a) the diversity of free-living microbes is much higher than previously recognized and b) there are endemic as well as cosmopolitan species (e.g. Pace 1997; Fulthorpe et al. 1998; Glöckner et al. 2000; Massana et al. 2000; Sabbe et al. 2001, 2003; Wilkinson 2001; Taton et al. 2003; Whitaker et al. 2003; Oren 2004; Papke & Ward 2004; Martiny et al. 2006). This new knowledge has profound implications for the understanding of microbial diversification and biogeography. In particular, the discrepancy *per se* between molecular data and traditional concepts on the diversity and distribution of microbes, points to a very clear fact: we still know very little about the factors that promote the evolutionary diversification of microbes. The knowledge gained from approximately seven decades of evolutionary studies in macroorganisms is not enough to understand microbial diversification. This is due in large part to the fact that microbes normally differ from macroorganisms in having high dispersal, high reproductive rates and enormous individual abundances (Finlay 2002, 2004), all of which probably affect the tempo and mode of their evolution.

Geographical isolation has traditionally been regarded as the prevailing agent of microbial divergence (Papke & Ward 2004). However, molecular data suggest that local adaptation plays a significant role in microbial diversification (e.g. Rynearson & Armbrust 2000, 2004; Casamatta et al. 2003; Saez et al. 2003; Kim et al. 2004; Wilson et al. 2005; Coleman et al. 2006; Johnson et al. 2006). The role of natural selection in the divergence and eventual speciation of organisms in general has been recently reconsidered, receiving support from natural (Hendry et al. 2000; Rundle et al. 2000), laboratory (Rainey & Travisano 1998; Rainey et al. 2000) and theoretical (Doebeli et al. 2005) studies. In particular, there is increasing evidence that natural selection can generate exceptionally rapid divergences, as illustrated by the diverse macroorganisms that have colonized islands or lakes (Orr & Smith 1998; Coyne & Orr 2004). Evidence also indicates that natural selection can generate rapid divergences in microbes (Leblond et al. 2006; Logares et al. 2006).

Molecular data indicate that microbial dispersal can be restricted by distance and/or geographical features (e.g. Wilkinson 2001; Whitaker et al. 2003; Martiny et al. 2006). Following the classical speciation theory, dispersal restrictions will diminish gene flow between populations, which will then further diverge with time (by genetic drift and /or natural selection) to eventually form new strain/species. However, even if there were microbial species which had no geographical barriers for their dispersal, gene flow between their populations would not necessarily have to be high. Local adaptation of different populations to particular environmental conditions can constitute a considerable barrier to gene flow by lowering the fitness of immigrants from other populations (De Meester et al. 2002). Thus, high dispersal would be compatible with the emergence and permanence of genetically differentiated populations/strains and eventually endemic species. The most controversial new evidence indicates that microbial diversification can occur in the presence of gene flow (Rainey & Travisano 1998; Rainey et al. 2000; Friesen et al. 2004). The genetic mechanisms underlying this type of divergence are still poorly understood.

To date, molecular techniques have been highly successful in the investigation of microbial biodiversity and biogeography, but new studies including more taxa from different locations are needed to gain a better understanding of microbial diversification, biodiversity and biogeography. It should always be remembered that most of the diversity of life is microscopic, thus, studying microbial diversification is necessary for understanding the process of evolution as a whole. There are also practical reasons for investigating microbial diversity and biogeography. For instance, in the search for novel drugs or compounds of commercial importance as well as in the fight against microbial diseases (like malaria) and microbial species that produce enormous economical loses (e.g. dinoflagellates during blooms).

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II

Microbial Ecology

Phenotypically Different Microalgal Morphospecies with Identical Ribosomal DNA: A Case of Rapid Adaptive Evolution?

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Abstract

The agents driving the divergence and speciation of free-living microbial populations are still largely unknown. We investigated the dinoflagellate morphospecies *Scrippsiella hangoei* and *Peridinium aciculiferum*, which abound in the Baltic Sea and in northern temperate lakes, respectively. Electron microscopy analyses showed significant interspecific differences in the external cellular morphology, but a similar plate pattern in the characteristic dinoflagellate armor. Experimentally, *S. hangoei* grew in a wide range of salinities (0–30), whereas *P. aciculiferum* only grew in low salinities (0–3). Despite these phenotypic differences and the habitat segregation, molecular analyses showed identical ribosomal DNA sequences (ITS1, ITS2, 5.8S, SSU, and partial LSU) for both morphospecies. Yet, a strong interspecific genetic isolation was indicated by amplified fragment length polymorphism (F_{ST} = 0.76) and cytochrome b (*cob*) sequence divergence (~1.90%). Phylogenetic reconstructions based on ribosomal (SSU, LSU) and mitochondrial (*cob*) DNA indicated a recent marine ancestor for *P. aciculiferum*. In conclusion, we suggest that the lacustrine *P. aciculiferum* and the marine-brackish *S. hangoei* diverged very recently, after a marine–freshwater transition that exposed the ancestral populations to different selective pressures. This hypothetical scenario agrees with mounting data indicating a significant role of natural selection in the divergence of free-living microbes, despite their virtually unrestricted dispersal capabilities. Finally, our results indicate that identical ITS rDNA sequences do not necessarily imply the same microbial species, as commonly assumed.

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Introduction

The diversity and biogeography of free-living microorganisms is presently a subject of general debate. Traditional studies based on morphological variation have promoted the view that most microbial species have cosmopolitan distributions [4, 20]. The high dispersal and huge individual abundances of microbes were identified as the main causes of such apparent lack of distributional patterns [21, 22]. This hypothetical absence of barriers for microorganisms dispersal has an interesting evolutionary prediction: unimpeded gene flow will diminish the speciation rate, and therefore the global number of microorganisms species will be relatively small [19, 22]. The prevailing view consisted of a lack of biogeographical patterns and a low global biodiversity in free-living microbes, until molecular data started to unveil a more complex reality. On one hand, there is evidence of genetic cohesion between microbial populations separated by continental distances [14, 45, 52]. On the other hand, numerous molecular studies indicate a much higher microbial diversity than previously estimated, with abundant examples of cryptic and endemic species [10, 11, 34, 42, 55, 61, 64, 86, 94].

The discrepancy between new molecular results and traditional ideas on the diversity and distribution of free-living microbes shows how little is still known about the

factors that drive the evolutionary divergence of microorganisms. The knowledge gained from macroorganisms is not enough for us to understand microbial divergence and speciation, because microbes normally differ from large plants and animals in at least three fundamental ways: high dispersal, high reproductive rates, and enormous individual abundances [20, 21]. Although geographical isolation has traditionally been considered as the prevail suggest that natural selection is a significant divergence force [10, 34, 75–77]. The role of selection in the divergence of organisms in general has been recently reconsidered, receiving support from natural [28, 74], laboratory [69, 70], and theoretical [16] studies. In particular, there is increasing evidence that natural selection produced by environmental change can generate exceptionally rapid divergence, as illustrated by diverse macroorganisms that have colonized islands or lakes [13, 59].

After the last Pleistocene glaciations (~10,000 years BP), numerous freshwater lakes came into existence in both hemispheres [92]. Only a relatively small number of marine species colonized these postglacial lakes, which indicates that the boundary between marine and freshwater environments constitutes a formidable adaptive barrier for most organisms [39]. In many marine animals that recently invaded freshwaters, the new physical and ecological conditions seem to have promoted the divergence of morphological, physiological, and life history traits [39, 47]. For microorganisms, there are studies that investigated the ancient divergence between marine and freshwater lineages [89], although there are virtually no data regarding recent freshwater invasions by marine microbial lineages. Because microorganisms have huge individual numbers, and high dispersal and high reproductive rates, the tempo and mode of their evolution after a marine–freshwater transition could potentially differ from what is known for animals and plants.

We have studied a marine-brackish and a freshwater dinoflagellate morphospecies that proved to have identical ribosomal DNA sequences, but at the same time a significant genetic isolation and phenotypic divergence. Dinoflagellates are important components of marine and freshwater microbial communities. Many marine and freshwater morphospecies are considered to have cosmopolitan distributions [67, 83], although there is mounting molecular evidence of endemicity and cryptic diversity [34, 53, 65]. Some dinoflagellate morphospecies produce highly toxic compounds, with potential damaging effects for humans and fish during the so-called red tides [81]. Usually, dinoflagellates spend most of their life cycles as haploid cells that proliferate by mitotic division [90]. Sexuality can be induced by endo- and exogenous factors, and in many cases, results in a resting cyst with high environmental resistance and dispersal functions [66].

Scrippsiella hangoei (Schiller) Larsen is a dinoflagellate endemic from the Baltic Sea [37] and *Peridinium aciculiferum* Lemmermann is normally found in several north temperate postglacial lakes [60, 67]. Both species normally grow in cold, ice-covered waters [37, 71] and may dominate the winter phytoplankton community. In this study, we indicate that *P. aciculiferum* and *S. hangoei* are evolutionarily very closely related, despite a substantial phenotypic differentiation, genetic isolation, and habitat segregation. Moreover, our results suggest that the lacustrine *P. aciculiferum* has diverged recently from marine ancestors, most probably as a result of different selective regimes experienced by populations in freshwater and marine-brackish habitats.

Methods

Collection Sites and Cultures. *S. hangoei* was collected in the Baltic Sea, near the entrance of the Gulf of Finland (59°50′N, 23°15′E). At the collection site, the seawater normally has a salinity of 5–8 (the Practical Salinity Scale was used to determine all the salinities; therefore salinity values are presented without units) and the region remains superficially ice-covered during approximately 2 months per year [58]. *P. aciculiferum* was collected in Lake Erken, Sweden (59°25′N, 18°15′E). Lake Erken was formed by isostatic uplift ~2000 years BP [18], and it is presently located ~15 km from the Baltic Sea at 11.1 m asl. Its surface covers 23.7 km^2, with a salinity of ~0 and a mean depth of 9 m. The lake is normally ice-covered for 3–5 months per year [93].

Four clonal culture strains of *S. hangoei* (SHTV-1/2/5/6) and five of *P. aciculiferum* (PAER-1/2/3/8/9) were used. Strains SHTV-1/2/5/6 were isolated in 2002 from germinated cysts collected in 2001. The PAER-1 strain was isolated in 1995 from a germinated cyst, whereas PAER-2/3/8/9 were isolated in 2004 from different plankton samples. *S. hangoei* was cultured in F/2 medium [26] (salinity, 6.5) prepared with sterile filtered seawater from the Baltic Sea. *P. aciculiferum* was cultured in modified Woods Hole medium (salinity, 0) [25] prepared with Milli-Q (Millipore Corp., Bedford, MA, USA). Cultures were kept in an incubator at 3 ± 1°C, 20 μmol photons m^{-2} s^{-1}, and 12:12 h light–dark cycle.

Molecular Analyses

DNA Sequencing and Phylogeny Construction. DNA was extracted from *S. hangoei* (SHTV-1/2/5/6) and *P. aciculiferum* (PAER-1/2/3/8/9) clonal culture strains following a protocol described by Adachi *et al.* [1]. The Internal Transcribed Spacer (ITS) 1 and 2, 5.8S, Small Subunit Ribosomal DNA (SSU rDNA), and two hypervariable domains of the Large Subunit Ribosomal DNA (LSU rDNA; D1/D2 domains) of the nuclear ribosomal DNA cistron were sequenced. The mitochondrial (mt) gene Cytochrome b (*cob*) was sequenced as well. The SSU is a

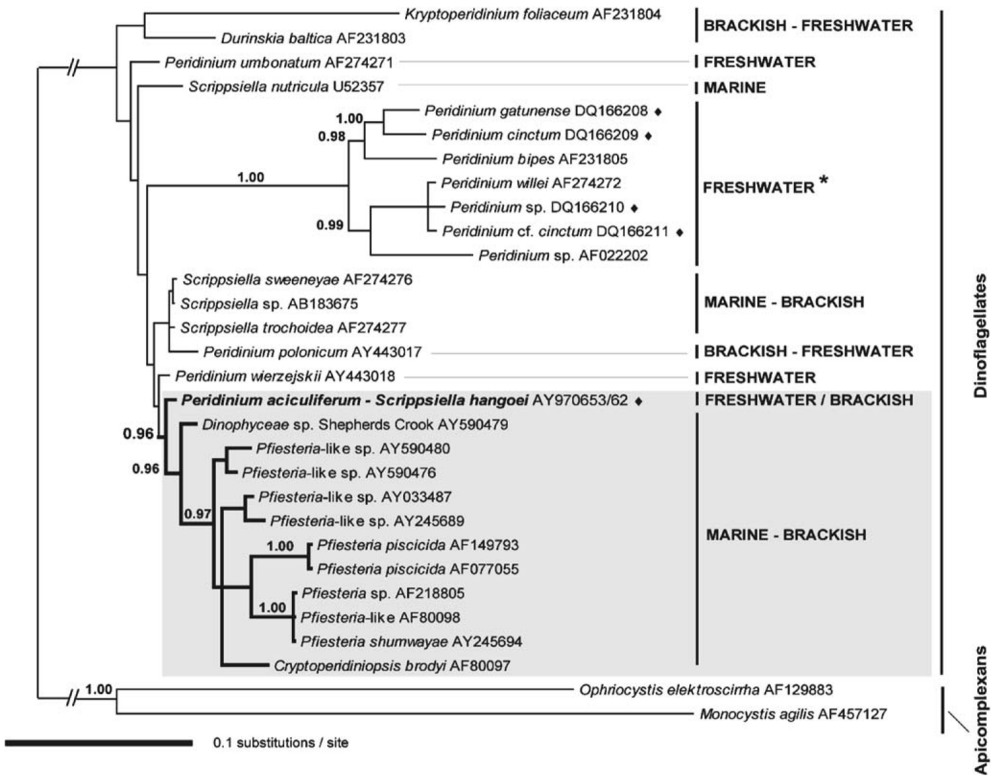

Figure 1. Bayesian SSU rDNA phylogram of 30 sequences based on 1749 nucleotides (GTR + G model; ran for 4×10^6 generations). The gray box indicates the relationship of *Peridinium aciculiferum* and *Scrippsiella hangoei* with other marine/estuarine species. Bayesian posterior probability branch support values ≥ 0.95 are shown (calculated from 3×10^4 trees after the log-likelihood stabilization). Dinoflagellate habitats were obtained from [67, 83] and species/strain data from Genbank. *Monophyletic freshwater *Peridinium* clade. ♦Sequences obtained within this study.

highly conserved region normally used for phylogenies between distant taxa (e.g., [78]). The D1/D2 LSU domains are usually used for phylogenies of dinoflagellates at the generic and species level (e.g., [65, 80]). The ITS region is highly variable and most useful for studies of divergence between populations or closely related species [17, 34, 53]. The *cob* gene appears to be relatively conserved in dinoflagellates [96].

Ribosomal DNA polymerase chain reaction (PCR) amplifications were carried out by using 25 ng of template genomic DNA, 0.05 mM of each nucleotide, 3.0 mM $MgCl_2$, 1× PCR buffer, 0.1 μM of each primer, and 0.5 U of *Taq* DNA polymerase (AmpliTaq®, Applied Biosystem) in 100 μL total volume. For the ITS/5.8S, the primers ITS1 (forward) 5′-TCCGTAGGTGAACCTGC GG-3′ and ITS4 (reverse) 5′-TCCTCCGCTTATTGATAT GC-3′ were used. The ITS PCR temperature profile consisted of an initial denaturing step of 5 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 45°C, 1 min at 72°C, and ended with 10 min at 72°C. For the SSU we used the combination of the primers 4616 (forward) 5′-AAC CTGGTTGATCCTGCCAG-3′ and 4618 (reverse) 5′- TG ATCCTTCTGCAGGTTCACCTAC-3′. The SSU PCR started with 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1.5 min at 55°C, 1 min at 72°C, and ended with 7 min at 72°C. We used the primers DinFi (forward) 5′-GCATATAAGTAMGYGGWGG-3′ and DinRi (reverse) 5′- CCGTGTTTCAAGACGGGTC-3′for the LSU. The LSU temperature profile was equivalent to the SSU, except that it consisted of 30 cycles with an annealing temperature of 50°C. The *cob* PCR reactions (final volume, 25 μL) consisted in 25 ng of total genomic

DNA, 0.125 mM of each nucleotide, 1.5 mM MgCl2, 1× PCR buffer, 0.4 μM of each primer, and 0.1 U *Taq* DNA. We used the primers Dinocob1F (forward), 5′-ATGAA ATCTCATTTACAW WCATATCCTTGTCC-3′, and Dinocob1R (reverse), 5′-TCTCTTGAGGKAATTGWKM ACCTATCCA-3′. The *cob* PCR temperature profile consisted of 1 min at 95°C, followed by 40 cycles of 20 s at 94°C, 30 s at 55°C, and 40 s at 72°C, finished by 10 min at 72°C. All PCR products were cleaned with PCR-M™ Clean-Up System (Viogene). Amplified rDNA and mitochondrial DNA (mtDNA) fragments were directly sequenced from both ends using BigDye (v1.1, Applied Biosystems) in an ABI Prism 3100 sequencer (Applied Biosystems). The sequencing primers were the same used in PCR, except for the SSU where the primers 516F (forward) 5′-CACA TCTAAGGAAGGCAGCA-3′ and 1416R (reverse) 5′-TTCAGCCTTGCGACCATACTC-3′ were also used. Sequences were deposited in GenBank under the accession numbers DQ094821–DQ094829, AY970649–AY970662, DQ022927, DQ022928, and DQ 166208–DQ166211.

The sequences were edited with Bioedit (v7.0.4.1; [27]) and aligned with ClustalX (v1.8; [85]). All clones of *P. aciculiferum* and *S. hangoei* were included in the alignments of the ITS1–2 /5.8S rDNA [564 nucleotides (nt)] and the *cob* mtDNA (845 nt). At least one clone of each species was included in the LSU (526 nt) and SSU (1717 nt) rDNA alignments. The software DnaSP (v4.10.3; [73]) was used to analyze the genetic polymorphism of mtDNA sequences and to perform the McDonald–Kreitman test of neutrality [46]. Additional sequences downloaded from GenBank were included in the alignments used for phylogeny construction. The program ModelTest (v3.7; [68]) was used to select the most appropriate model of nucleotide substitution for our data. SSU, LSU, and *cob* phylogenies were estimated by using a Bayesian inference approach as implemented in MrBayes (v3.0B4; [32]) under the general time reversible (GTR) substitution model with a gamma (G) distributed rate of variation across sites (Fig. 1). All Bayesian analyses were run with four Markov chains for 4×10^6 generations and the chain was sampled every 100 generations, which resulted in 4×10^4 sampled trees. Each analysis was repeated at least four times from independent starting trees and the obtained posterior probability (PP; estimate of branch support) values for the branching pattern as well as likelihood scores for the trees were compared to ensure convergent tree reconstruction. Bayesian PPs were calculated from the 3×10^4 trees after the log-likelihood stabilization (burn-in phase) (Fig. 2). The trees generated with MrBayes were visualized in TreeView (v1.6.6; [62]).

Amplified Fragment Length Polymorphisms. We analyzed the amplified fragment length polymorphism (AFLP) variability between four clonal strains of *S. hangoei* (SHTV-1/2/5/6) and five of *P. aciculiferum* (PAER-1/2/3/8/9) following a fluorescein protocol based on Vos *et al.* [91]. DNA (250 ng) from each clone was digested during 1 h at 37°C using 2.5 U *Eco*RI (Amersham Pharmacia), 2.5 U TruI (Fermentas), 1 μg BSA, and 1× TA buffer in each reaction (final volume, 20 μL). Ligation of adaptors was carried out for 3 h at 37°C using 0.5 μM of E adaptor, 5 μM of M adaptor, 0.5 U of T4 ligase (USB®), and 1× ligation buffer in a 5.0-μL reaction. The ligation product was diluted 10 times and subsequently used as a template for the preamplification step. The preamplification reaction (final volume, 20 μL) consisted of 10 μL of the ligation product, 0.4 U *Taq* DNA polymerase (AmpliTaq, Applied Biosystems), 0.3 μM of E primer (5′-GACTGCGTACCAATTCT-3′), and 0.3 μM of M primer (5′-GATGAGTCCTGAGTA AC-3′), 0.2 mM dNTPs, 1× PCR buffer, and 2.5 mM $MgCl_2$. The preamplification thermal profile included an initial denaturing step of 2 min at 94°C, 20 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C, followed by a final step of 10 min at 72°C. The preamplification product was diluted 10 times and used as a template for the selective amplification.

The selective amplification was carried out using four primer pairs: E_{TCT}–M_{CAC}, E_{TCG}–M_{CGG}, $E_{T\ AG}$–M_{CGA}, E_{TCG}–M_{CGA}. Selective amplifications reactions (final volume, 10 μL) included 2.5 μL preamplification product, 0.04 U *Taq* DNA polymerase (AmpliTaq, Applied Biosystems), 0.2 mM dNTP, 0.6 μM of each selective primer, 2.5 mM $MgCl_2$, and 1× PCR buffer. The temperature profile consisted of an initial denaturing step of 2 min at 94°C, 12 cycles of 30 s at 94°C, 30 s at 65°C $^{-}$0.7°C/cycle, 60 s at 72°C, continued by 23 cycles of 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, followed by a final step of 10 min at 72°C. After the incubation, 10 μL formamide dye (100% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) was added to the reactions and then products were stored overnight at 4°C before further analysis.

Selective amplification products were denatured (3 min at 95°C) and then 3.5 μL was loaded onto 6% polyacrylamide gels. AFLP fragments were separated using 30 W during 80–90 min, and detected by the fluorescein-labeled E primers in a FluorImager (Vistra Fluorescens, Molecular Dynamics Inc., Sunnyvale, CA, USA). Each strain was amplified at least twice for each primer combination. Reproducible, polymorphic bands were scored as 1 (presence) or 0 (absence) for the 156 surveyed loci. *P. aciculiferum* and *S. hangoei* were considered as two populations. Indices of genetic diversity (H_T, D_{ST}, and H_S) and differentiation among populations (F_{ST}) were calculated by using the Lynch and Milligan [44] approach for dominant loci as implemented in AFLP-SURV (v1.0; [88]). The program TFPGA (v1.3; [49]) was used to create an UPGMA

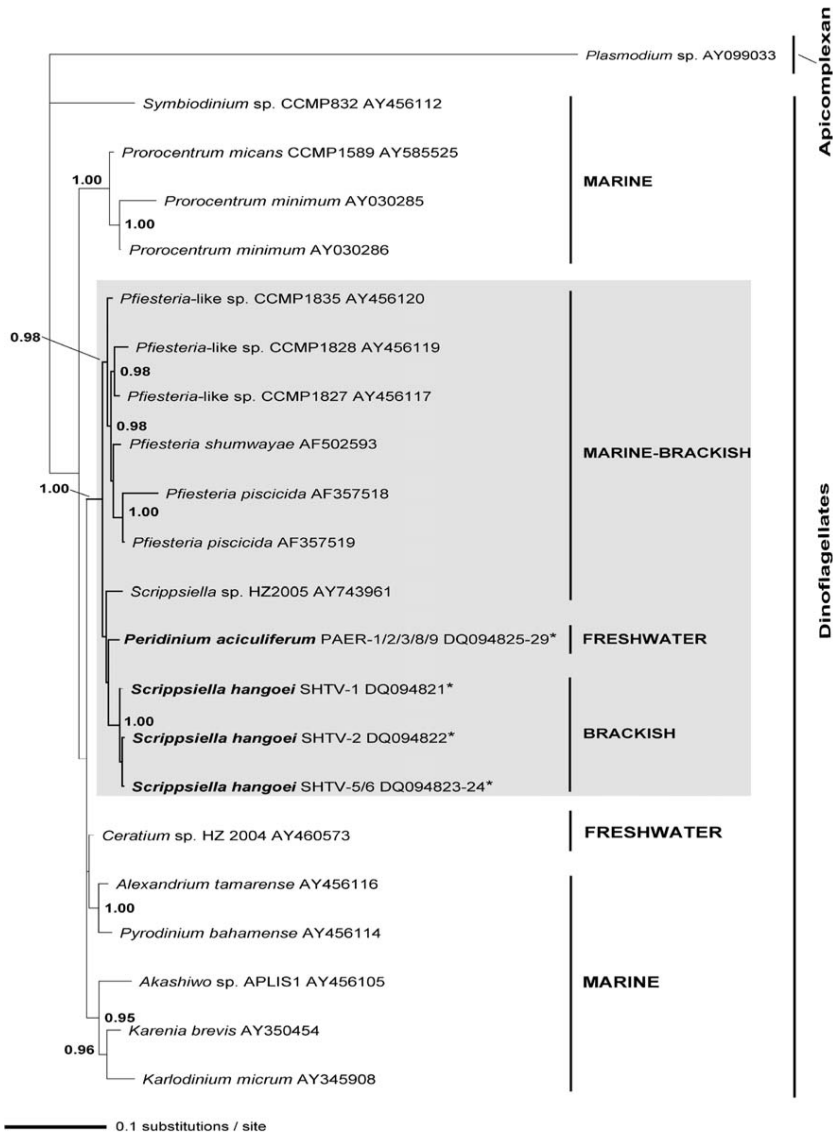

Figure 2. Bayesian *cob* mtDNA phylogram of 22 sequences using 826 nucleotides (GTR + G model; ran for 4×10^6 generations). The gray box indicates the relationship of *P. aciculiferum* and *S. hangoei* with other marine/estuarine species. Bayesian posterior probability branch support values ≥ 0.95 are shown (obtained from 3×10^4 trees after the log-likelihood stabilization). Dinoflagellate habitats were obtained from [67, 83], and species/strain data from Genbank. *Sequences obtained within this study.

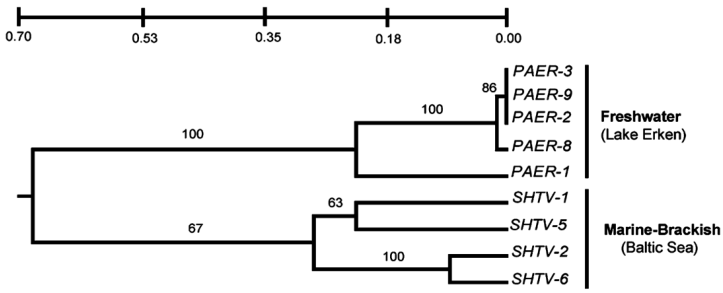

Figure 3. UPGMA phylogram based on Nei's minimum distances for the 156-screened AFLP loci. Five clonal cultures were used for *P. aciculiferum* and four for *S. hangoei*. PAER: *P. aciculiferum*; SHTV: *S. hangoei*. Values over branches indicate bootstrap support (1000 pseudoreplicates).

phylogram based on Nei's minimum distances [57]. Branch support for the phylogram was estimated by 1000 bootstrap pseudoreplicates.

Identity and Purity of the Cultures. The *S. hangoei* cultures (SHTV-1/2/5/6) and the *P. aciculiferum* culture PAER-1 were independently isolated and identified in Finland and Sweden, respectively. The strains PAER-1 and SHTV-1 were independently sent from Sweden and Finland to Germany for DNA analyses (carried out by M. Gottschling), which showed that both morphospecies shared identical ITS rDNA sequences. After these preliminary results, we isolated the strains PAER-2/3/8/9 and started in Sweden the DNA analyses with the whole set of strains, which were carefully examined with optical microscope before DNA extraction. For each strain, we used the same extracted DNA as a template for amplifying the SSU, LSU, ITS, *cob* mtDNA, and for AFLP. Thus, if cross-contamination were the case, the DNA templates giving identical interspecific rDNA sequences should have also given identical *cob* sequences and AFLP band patters, which was not observed. Regarding the identity of each strain, it was confirmed by electron microscope analyses.

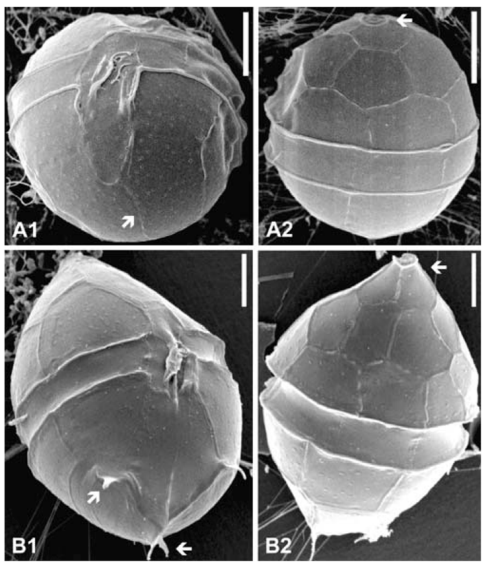

Figure 4. SEM micrographs showing the morphological differences between egg-shaped *S. hangoei* (A1, 2; Baltic Sea) and subspherical *P. aciculiferum* (B1, 2; Lake Erken). The presence of spines in *P. aciculiferum* and its absence in *S. hangoei* is shown in A1 and B1 (*arrows*). Differences in the apical zone (*arrows*) are shown in A2 and B2. Scale bars = 5 μm.

Scanning Electron Microscopy and Morphological Measurements. *P. aciculiferum* cells were fixed in 2% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2) for at least 2 h at 4°C. Fixed cells were collected on a TMTP polycarbonate filter (5 μm pore size, 13 mm diameter; Millipore) with a syringe. Cells were subsequently rinsed with 0.1 M sodium cacodylate buffer, dehydrated using ethanol series, critical point dried, and sputter-coated with gold–palladium alloy. Samples were imaged on a JSM 6400 Scanning Microscope (Jeol) at 5 kV.

S. hangoei cultures were mixed 1:1 with a 0.2% solution of Triton X-100™ (Sigma-Aldrich) prepared in culture media. The mix was gently agitated by using a Pasteur pipette several times over 10 min and cells were subsequently collected onto a 5-μm PTFE filter (Millipore). Specimens were fixed in 2% glutaraldehyde, rinsed with Milli-Q water, and dehydrated using ethanol and Freon series. Cells were critical point dried, sputter-coated with gold–palladium

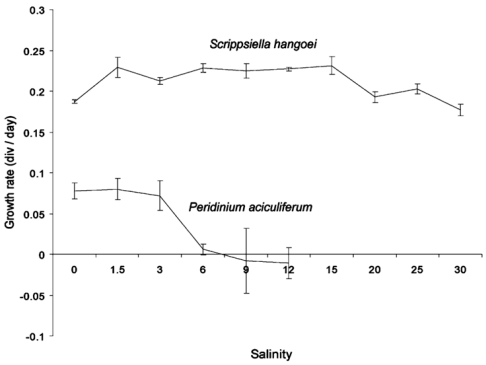

Figure 5. Growth of *S. hangoei* and *P. aciculiferum* in different salinity media. Data points refer to treatment means ± 1 SE. Note that *S. hangoei* was grown at 150 µmol photons m^{-2} s^{-1} and 14:10 h light–dark, whereas *P. aciculiferum* was grown at 20 µmol photons m^{-2} s^{-1} and 12:12 h light–dark, precluding direct comparison.

alloy, and imaged on a Stereoscan 240 scanning electron microscope (Cambridge Instruments) at 15 kV.

Cell length and width of *S. hangoei* and *P. aciculiferum* were determined from measurements of 20 cells of each species fixed in Lugol's iodine, by using a Nikon Eclipse (TS100) light microscope at 400× magnification. In *P. aciculiferum* measurements, spines were not taken into account.

Salinity Tolerance. The range of salinity tolerance of *P. aciculiferum* and *S. hangoei* was assessed by studying their growth in culture media of different salinities. For *P. aciculiferum*, the salinity treatments were 0 (control), 1.5, 3, 6, 9, and 12 (each treatment with four replicates). These treatments were prepared in culture flasks (Nunc), with 50 mL of MWC media [25] prepared with an autoclaved mix of NaCl and Milli-Q that was adjusted to each treatment salinity. For *S. hangoei*, the treatment salinities were 0, 3, 6.5 (control), 9, 12, 15, 20, 25, and 30. Treatments were conducted in replicates (three) in 50-mL tissue culture flasks (Nunc) containing 30 mL F/2 enriched culture medium [26]. To obtain salinities lower than 6.5, sterile filtered seawater from the Baltic was diluted with Milli-Q or MWC freshwater medium. For treatment salinities above 6.5 and below 20, sterile filtered seawater from the Baltic was evaporated. For salinities above 20, full saline seawater from Trondheimsfjord, Norway, was used. Nutrients and vitamins were added after salinities had been adjusted according to Guillard and Ryther [26].

Initially, a culture of *P. aciculiferum* growing exponentially in MWC at 0 salinity was used to simultaneously inoculate the 0, 1.5, and 3 treatments to attain initial treatment densities of ~500 cells mL^{-1}. In the same manner, a *S. hangoei* culture growing exponentially in F/2 at salinity 6.5 was used to simultaneously inoculate the 3 and 9 treatments with initial densities of ~500 cells mL^{-1}. Each culture was adapted to the new salinities for at least 1 week before samples were collected for initial cell counts. The cell density in each treatment was determined weekly by using a Sedgewick-Rafter counting chamber and a Nikon Eclipse (TS100) light microscope at 100× magnification. Before cell counts, we gently homogenized the cultures and took subsamples (1.5 mL) that were fixed in Lugol's iodine (2.5%). From each count (comprising at least 400 cells), growth rates were measured according to Schmidt and Hansen [79]. When treatment cultures reached exponential growth, cells were transferred to the next salinity treatment to initial densities of ~500 cells mL^{-1}. All *P. aciculiferum* treatment cultures were maintained at 3 ± 1°C, 20 µmol photons m^{-2} s^{-1}, under a 12:12 h light–dark cycle. *S. hangoei* treatment cultures were maintained at 3 ± 1°C, 150 µmol photons m^{-2} s^{-1} under a 14:10 h light– dark cycle.

Results

Nuclear DNA Sequences. The sequenced rDNA fragments (ITS1 and 2, 5.8S, LSU [D1/D2], and SSU) from *P. aciculiferum* and *S. hangoei* were identical. Close examination of the sequencing chromatograms did not reveal any evidence of intraclonal rDNA polymorphism, which indicates a high degree of concerted evolution between multiple copies of the rDNA cistron.

Mitochondrial DNA Sequences. Three *cob* haplotypes were identified among *S. hangoei* and one among *P. aciculiferum* clonal strains, none of which was shared between the morphospecies. The *cob* haplotype sequences between *P. aciculiferum* and *S. hangoei* differed by ~1.90%, whereas the haplotype divergence among *S. hangoei* strains was about 10 times lower (~0.23%). Selection acting on the *cob* gene was not evidenced by the McDonald-Kreitman test ($P > 0.05$). We did not identify any case of size polymorphism or heteroplasmy.

Using *cob* sequences from the dinoflagellates *Pfiesteria piscicida* (AF357518, AF357519), *P. shumwayae* (AF502593), *Prorocentrum minimum* (AY030285, AY030286), and *P. micans* (AY585525), we estimated intra- and interspecific haplotype divergences. For *Pfiesteria piscicida* and *Prorocentrum minimum*, intraspecific haplotypes differed between 3.31% and 3.51%. *Pfiesteria piscicida* and *P. shumwayae* haplotypes differed ~4.64%, whereas haplotypes of *Prorocentrum minimum* and *P. micans* differed ~4.61%.

Amplified Fragment Length Polymorphisms. AFLP showed a strong genetic isolation between *P. aciculiferum* and *S. hangoei* (F_{ST} = 0.76, SD = 0.025, $P < 0.001$; H_T = 0.64, D_{ST} = 0.49, H_S = 0.14; Fig. 3). For the 156 loci screened, *S. hangoei* displayed 40% polymorphism whereas *P. aciculiferum* displayed 21.3%. Within *P. aciculiferum*, the strain PAER-1 (isolated in 1995) displayed a higher divergence in comparison with the strains PAER-2/3/8/9 (isolated in 2004) (Fig. 3).

Molecular Phylogenies. The SSU (1717 nts alignment) and *cob* (826 nt) phylogenies clustered *P. aciculiferum* and *S. hangoei* with estuarine/marine species of the genera *Pfiesteria* and *Scrippsiella* (Figs. 1 and 2). The LSU phylogeny (526 nt) gave virtually the same results as the SSU, and is therefore not shown.

Morphology. *P. aciculiferum* and *S. hangoei* differed in their overall morphology. Cells of *P. aciculiferum* were significantly longer (mean = 38.41 μm; $P < 0.001$, *t*-test) and wider (mean = 28.63 μm; $P < 0.001$) than *S. hangoei* cells (mean length = 23.00 μm; mean width = 20.24 μm). Moreover, *P. aciculiferum* was more elongated (mean length/width ratio = 1.35; $P < 0.001$) than *S. hangoei* (mean length/width ratio = 1.14). *P. aciculiferum* displayed a distinctively elongated apical zone (Fig. 4B1, 2) that was absent in *S. hangoei* (Fig. 4A1, 2). In addition, *P. aciculiferum* normally displayed three to four spines in the antapical region (Fig. 4B1, 2) that were absent in *S. hangoei* (Fig. 4A1, 2). Despite these differences in the general morphology, the plate pattern of both species was very similar (*S. hangoei* Po, X, 4′, 3a, 7″, 6c, 7s, 5‴, 2⁗; from this study and Larsen *et al.* [37] and *P. aciculiferum* Po, X, 4′, 3a, 7″, 6c, ?5s, 5‴, 2⁗; from this study and Bourrelly [9]).

Growth Under Different Salinities. *S. hangoei* grew under a wide range of salinities, whereas *P. aciculiferum* only grew at low salinities (Fig. 5). *P. aciculiferum* cells grew exponentially in salinities up to 3. In the treatments with salinities higher than 3 cells did not grow, and except for a few cells in the salinity 6 treatments, no movement was observed. Deformed cells were common in the *P. aciculiferum* treatments with salinities higher than 3, although this deformation did not follow any pattern. The *P. aciculiferum* treatments with salinities higher than 3 were maintained for more than 1 year, and during this period we never detected any growth.

Discussion

In this work we present one of the first cases where two microbial morphospecies revealed identical ribosomal DNA (rDNA) sequences, but at the same time a significant phenotypic differentiation and genetic isolation. Similar discrepancies between phenotypic and genetic data regarding species recognition have been investigated in other organisms. In general, these studies involve cases of cryptic genetic diversity, where genetic divergence is not reflected in morphological and physiological differentiation (e.g., [6, 10, 34, 53]). On the contrary, there are cases where physiological differentiation (assumed to have a genetic basis) between morphologically identical clones is not reflected in the variation of rapidly evolving areas of the rDNA, like the ITS-1/2 [12, 43, 84]. However, when more sensitive techniques were used (such as DNA fingerprinting), morphologically identical strains with identical ITS sequences were found to harbor genetically differentiated subgroups [2, 33]. To our knowledge, there is only one case (in nematodes) where two species, with slightly different morphology and biology, share identical rDNA sequences [50]. In summary, these data indicate that organisms sharing identical rDNA sequences do not necessarily belong to the same species.

The rDNA homogeneity found between the lacustrine dinoflagellate *P. aciculiferum* and the marine-brackish *S. hangoei* indicates that they are evolutionarily very closely related. In particular, it is remarkable that both morphospecies revealed identical ITS rDNA sequences, as diverse microbial morphospecies, including dinoflagellates, show substantial intraspecific polymorphisms in this rapidly evolving neutral marker [34, 53].

Mitochondrial DNA and morphological traits also indicated a close evolutionary relationship between *P. aciculiferum* and *S. hangoei.* The estimated differentiation between *P. aciculiferum* and *S. hangoei cob* mtDNA haplotypes (~1.90% sequence divergence) is significantly lower than the intra- and interspecific differentiation (~3.30% and ~4.60%, respectively) we calculated for this gene in other closely related dinoflagellate morphospecies.

Despite the clear differences between the general morphology of *P. aciculiferum* and *S. hangoei* cells (Fig. 4) evidenced by electron and light microscopy, the armor plate pattern (traditionally used to infer phylogenetic relationships in dinoflagellates) of the two morphospecies were virtually identical, indicating that both organisms are evolutionarily closely related. According to its plate pattern, *P. aciculiferum* is much closer to the genus *Scrippsiella* than to *Peridinium sensu stricto* (which has five cingular plates instead of six) and the remainder freshwater *Peridinium* (e.g., groups Umbonatum, Elpatiewsky, and Cunningtonii, with reduced number of plates in the intercalary and/or precingular series) [40]. In particular, both *S. hangoei* and *P. aciculiferum* have a pentagonal second intercalary (2a) plate, whereas most *Scrippsiella* species have a hexagonal 2a plate. Moreover, although in most *Scrippsiella* species the posterior sulcal plate (Sp) is in contact with the cingular area, in *S. hangoei*

and *P. aciculiferum* the plate Sp does not contact the cingulum due to a division in the plate [5, 23, 82]. Even though sharing these characteristics also suggests a close phylogenetic relationship between *P. aciculiferum* and *S. hangoei*, there are heterogeneities within the genus *Scrippsiella* in relation with the shape and disposition of the intercalary and sulcal plates [5, 23, 31, 51, 54, 82]. Regarding the morphology of the cysts (dinoflagellate resting stage), a round, transparent, and smooth-walled cyst was described for *S. hangoei* [36]. The cysts of *P. aciculiferum* are also transparent and smooth-walled, but their general morphology resembles a peanut [71]. Another similarity between the cysts of *S. hangoei* and *P. aciculiferum* is that they contain an orange-red accumulation body [36, 71]. Contrary to most *Scrippsiella* species, the cysts of *S. hangoei* (and *P. aciculiferum*) do not have a calcareous external layer. However, a number of noncalcareous cysts were reported for the genus *Scrippsiella* [37].

Biochemical analyses also support a close evolutionary relationship between *P. aciculiferum* and *S. hangoei*. Profiles of sterol production from both species were virtually identical (Leblond et al., unpublished data), indicating that the two morphospecies diverged recently [38].

Marine Ancestry. Molecular phylogenies based on nuclear (SSU, LSU rDNA) and mitochondrial (*cob*) markers indicated that *P. aciculiferum* and *S. hangoei* are evolutionarily related to the estuarine/marine dinoflagellate genera *Pfiesteria* and *Scrippsiella* (Figs. 1 and 2) [7, 83]. Other phylogenies, using the ITS rDNA as a marker, show similar results [24]. Thus, it becomes evident that the lacustrine *P. aciculiferum* is unrelated to the lineage of freshwater dinoflagellates (i.e., the true monophyletic *Peridinium*) to which it was assigned based on its morphology, and must have evolved from marine ancestors during an independent marine–freshwater transition. Nevertheless, because the present article is focused on evolutionary processes and not taxonomical nomenclature, the discussion on renaming *P. aciculiferum* (and perhaps *S. hangoei*) will not be addressed.

Whether or not *S. hangoei* has always been marine or a is product of a very recent marine recolonization, our data suggest that the marine–freshwater transition undergone by *P. aciculiferum* has occurred relatively recently, most probably within the last 30 million years. This was indicated by the sequence divergence (~2%) between the SSU of *P. aciculiferum*/*S. hangoei* and the SSUs of several other estuarine/marine species of the genera *Pfiesteria* and *Scrippsiella* (according to the SSU molecular clock calibration made by Uwe *et al.* [87]). As a comparison, the marine–freshwater transition undergone by the lineage of freshwater *Peridinium* seems to have occurred more than 100 million year ago [87], and some species within this genus show an SSU sequence divergence of ~4%.

The *cob* mtDNA gave similar results, a relatively low sequence divergence between the pair *P. aciculiferum*/*S. hangoei* and species of *Scrippsiella* and *Pfiesteria* (Fig. 2), although divergence dates cannot be estimated because there are no molecular clock calibrations for the *cob* of dinoflagellates.

If *S. hangoei* had always been marine, then the marine–freshwater transition of *P. aciculiferum* should have occurred much more recently (i.e., during or after the last glaciations), as would be indicated by the rDNA and *cob* sequence similarity between both species. A relatively ancient colonization of freshwaters by *S. hangoei* ancestors with a subsequent marine recolonization is less likely, because such a scenario would require two marine–freshwater transitions. The high similarity between the *cob* sequence of *S. hangoei* and the *cob* of a *Scrippsiella* species (Fig. 2; strain HZ2005) isolated from the estuarine area of Long Island Sound in North America, indicates that *S. hangoei* is primarily marine-brackish (i.e., not derived from a marine recolonization). Furthermore, the presence of only one *cob* mtDNA haplotype among the five *P. aciculiferum* strains (isolated in 1995 and 2004) and three haplotypes among the four clones of *S. hangoei* (isolated from the same area) suggests that the colonization came from the sea, because colonizers typically display a reduced genetic diversity [29]. AFLP results gave a similar pattern, a higher genetic polymorphism in *S. hangoei* (40% polymorphic loci) than in *P. aciculiferum* (21.3%). In other marine organisms that have recently colonized freshwaters, a wide salinity tolerance in the marine ancestral populations/species and a narrow tolerance in the freshwater invaders have been documented [39]. This also suggests that the wide-salinity tolerant *S. hangoei* (or a related ancestral species) colonized freshwaters and evolved into the freshwater form *P. aciculiferum*, and not vice versa. Moreover, marine–freshwater transitions are not phenomena that seem to occur back and forth in short evolutionary periods [39, 89], which make marine recolonization a less likely scenario.

Genetic Divergence and the Marine–Freshwater Transition. The freshwater *P. aciculiferum* and the marine-brackish *S. hangoei* showed a strong genetic isolation (AFLP F_{ST} = 0.76; absence of shared *cob* mtDNA haplotypes) despite sharing identical rDNA sequences. Such genetic segregation was most probably caused by one or both of the most common divergence agents: natural selection and geographical isolation. The transition between freshwater and marine habitats represents a considerable shift between adaptive zones [39] that is expected to promote the adaptive divergence of lineages occurring in both habitats. The differential salinity tolerances of *P. aciculiferum* and *S. hangoei* indicate the action of disruptive natural selection in marine and freshwater habitats, and suggest that other interspecific differences might have evolved as a result of

the same selective forces. A strong adaptation to low salinity, along with adaptive differences in morphological and life history traits, is characteristic of several marine animals that have recently colonized freshwaters [39]. Studies involving free-living aquatic microorganisms indicate that even small environmental differences can drive and maintain the genetic differentiation of connected or semiconnected populations [34, 53, 76, 77].

Geographical isolation most probably played a minor role in the divergence of *P. aciculiferum* and *S. hangoei*. Foremost, because Lake Erken (source of the studied *P. aciculiferum* population) has been connected to the Baltic Sea (source of the studied *S. hangoei* population) through a network of flowing waters throughout its history [18]. Consequently, gene flow should have been possible (at least in the direction from the lake to the sea) between the diverging freshwater and marine lineages. So far, there are very few cases, typically involving continental distances, where geographical isolation alone has been proposed as the main divergence agent between populations or strains of a given free-living microbial lineage [63, 94]. Alternatively, there are many cases where local adaptation explains better the genetic differences between microbial populations or strains [34, 76].

Because the molecular clock for the *cob* gene is not calibrated for dinoflagellates, and the evolution of this marker varies considerably among taxa [3], we cannot make an estimation of when *P. aciculiferum* and *S. hangoei* started to diverge. In a preliminary evaluation, an interspecific *cob* sequence divergence of ~1.90% contradicts a scenario of rapid postglacial divergence, as the time involved would be too short to assume that most substitutions arose separately in each lineage without the action of selection (as indicated by McDonald–Kreitman test) [96]. Still, we calculated intraspecific *cob* divergences of ~3.30% for other dinoflagellates, suggesting that part of the divergence measured between *P. aciculiferum* and *S. hangoei* could be the product of a shared ancient polymorphism and a subsequent sorting of haplotypes in each lineage during a marine–freshwater transition.

Identical rDNAs: Rapid Divergence or Historical Hybridization? The identical rDNA sequences revealed by *P. aciculiferum* and *S. hangoei* can be the outcome of two processes: a rapid divergence that did not provide enough time for neutral mutations to be fixed in the rapidly evolving ITS rDNA of each lineage (e.g., [56]), or an extensive introgressive hybridization along with concerted evolution [72]. Natural selection caused by environmental change can produce extraordinarily fast divergence [39, 59]. Several examples of rapid divergence and speciation come from large animals that recently invaded postglacial lakes or islands [13, 15, 48]. These organisms are often characterized by a considerable morphological and ecological differentiation but a high similarity in neutral genetic markers [59]. Alternatively, most common hybridization cases deal with species that have diverged in isolation and then contacted each other after a range expansion [13, 29, 30].

A rapid divergence between *P. aciculiferum* and *S. hangoei* is the most parsimonious explanation for their identical rDNA sequences. We envisage a scenario in which *P. aciculiferum* marine ancestors were landlocked or invaded newly formed lakes after the last ice age, and diverged rapidly as a result of a strong selection caused by the new ecological and physical conditions found in freshwater environments. The substantial phenotypic differentiation and genetic isolation measured between *P. aciculiferum* and *S. hangoei*, together with their sequence homogeneity in the rapidly evolving neutral ITS rDNA marker, resemble patterns observed in macroorganisms that underwent selection-driven fast divergences [59]. For instance, several species of marine fish that invaded postglacial lakes in Eurasia and North America and evolved significant morphological and ecological differences in less than 15,000 years [13].

We cannot discard extensive introgressive hybridization between *S. hangoei* and *P. aciculiferum* as the source of their identical rDNA sequences, although this hypothesis seems unlikely. The strong genetic isolation between *P. aciculiferum* and *S. hangoei* shown by AFLP, and the absence of shared *cob* haplotypes indicate no recent interspecific gene flow, even though a few *P. aciculiferum* individuals may reach the Baltic [35, 67, 95]. Thus, the measured genetic isolation of *P. aciculiferum* and *S. hangoei* indicates that the putative hybridization should have occurred further back in time, and therefore there should have been enough time for neutral mutations to be fixed in the rapidly evolving ITS rDNA of each lineage.

Another problem with the hypothesis of introgressive hybridization is the lack of evidence for this process in other microorganisms. Because of their high dispersal, hybridization between closely related microbial species should be a relatively common phenomenon. However, molecular data show an increasing number of cases where there is a substantial isolation between genetic lineages of the same morphospecies [6, 34, 53, 76, 77].

Conclusions and Implications. This work suggests that the divergence between the two studied microeukaryote morphospecies was promoted by different selective regimes in marine-brackish and freshwater habitats. Moreover, our results indicate that the divergence occurred rapidly, probably after the last glaciations (~10,000 years BP), with not enough time for neutral mutations to be fixed in the rDNA of each lineage. Thus, although the rDNA sequence homogeneity indicates that *P. aciculiferum* and *S. hangoei* are the same species, AFLP as well as physiological and morphological data indicate that they are two different evolutionary lineages and can

therefore, to our judgment, be regarded as two different species.

Our work has two main implications for microbial biodiversity and evolution:

(1) Opposite to what has been reported for several microbial morphospecies (e.g., [34, 53, 89]), we have found that two microeukaryote morphospecies, with significant morphological and physiological differentiation, can have identical rDNA sequences. This implies that identical ITS rDNA sequences do not necessarily mean the same microbial species, as generally assumed (e.g., [12]).
(2) The suggestion that natural selection has been the agent of divergence between the studied marine and freshwater microbial morphospecies. So far, most examples of adaptive divergence involve large animals and plants [13], and little is known about the occurrence of this phenomenon in free-living microbes.

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III

Recent evolutionary diversification of a protist lineage

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Little is known on the diversity and distributions of microbial populations and closely related species. Here, we investigate a group of cold-water protists (dinoflagellates) present in marine and lacustrine habitats, which are distributed along a broad range of salinities (0–32) and geographic distances (0–18,000 KM), and which present some morphological variability. Altogether, we analyzed 30 strains, generating 55 new DNA sequences. The nuclear ribosomal DNA (nrDNA) sequences (including rapidly evolving introns) were very similar or identical among all the analyzed isolates. This very low nrDNA differentiation was contrasted by a relatively high cytochrome b (COB) mitochondrial DNA (mtDNA) polymorphism, even though the COB evolves very slowly in dinoflagellates. The 16 Maximum Likelihood and Bayesian phylogenies constructed using nr/mtDNA indicated that the studied cold water dinoflagellates constitute a monophyletic group (supported also by the morphological analyses), which appears to be evolutionary related to marine-brackish and sometimes toxic *Pfiesteria* species. We conclude that the studied dinoflagellates belong to a lineage which has diversified recently and spread, sometimes over long distances, across low-temperature environments which differ markedly in ecology (marine vs. lacustrine communities) and salinity. Probably, this diversification was promoted by the variety of natural selection regimes encountered in the different environments.

Introduction

The diversity and geographic distribution of many microbial lineages are poorly known, as well as the mechanisms which promote their evolutionary diversification and determine their spatial distributions. In multicellular organisms, the mechanisms which promote diversification and the formation of biogeographic patterns have been widely studied (e.g. natural selection, genetic drift, geographic/climatic barriers, etc; see e.g. Futuyma, 1998; Coyne and Orr, 2004). The same mechanisms are assumed to be governing

the diversification and formation of geographic patterns in microbes. However, in contrast to multicellular organisms, microbes normally have huge effective population sizes, high reproductive rates, as well as small sizes, which allegedly leads to long-distance dispersal capabilities (see Finlay, 2002; Lynch and Conery, 2003; Snoke et al., 2006). These characteristics most probably affect the tempo and mode of diversification and formation of spatial-distribution patterns in microbes. For instance, the huge population sizes as well as the apparently absence of barriers for microbial dispersal have been used to support a view indicating that the global microbiota is composed of relatively few cosmopolitan species (see Finlay, 2002, 2004; Finlay et al., 2006; see Logares, 2006 for a review). This pattern would be generated by an unrestricted dispersal and gene flow which would restrain the opportunities for microbial diversification. Furthermore, the relative importance of some divergence mechanisms in microbes is under discussion. For example, the action of genetic drift becomes apparent in small populations, and the huge population sizes that microbes usually have indicate that this divergence mechanism probably does not have similar effects in microbes as in multicellular organisms. Thus, it is likely that the relative importance of the processes which govern the diversification and formation of spatial-distribution patterns in microbes and in multicellular organisms are not equivalent.

During the last 15 years, molecular studies have been revealing new data and patterns which have improved our understanding on microbial diversity and biogeography. For example, several molecular studies indicate that the microbial diversity is much higher than previously estimated, that evolutionary diversification can occur without geographic isolation, that there are geographically restricted microbial taxa, that not all microbes are long-distance dispersers, and that not all dispersers are able to colonize all environments available to them (see Hughes Martiny et al., 2006).

Investigating microbial lineages which have diversified recently can shed some light on the process of diversification as well as the distribution of microbial diversity over space. Previous work, suggested the existence of a lineage of closely related protists (Logares et al., 2007b; Rengefors et al., 2007). Here, we have identified and investigated that lineage, which consists of a group of dinoflagellate strains/species which are present in low-temperature marine and lacustrine habitats along wide ranges of salinity (0–32) and geographical distance (0–18,000 KM). Dinoflagellates are ubiquitous unicellular eukaryotes with important ecological roles in marine and freshwater ecosystems. Dinoflagellates have a high diversity of life strategies, with symbionts, parasites, photosynthesizers, heterotrophs and mixotrophs (Hackett et al., 2004). Free swimming dinoflagellates are normally haploid (Von Stosch, 1973) and reproduce asexually. Sexuality can, however, be induced by endo- and exogenous stimuli, resulting in many cases in a diploid resting cyst with environmental resistance and dispersal functions (Pfiester and Anderson, 1987). Some dinoflagellate species can produce potent toxins during red tides, thus representing an important concern for human and ecosystem health as well as local economies (Hallegraeff, 1993). In this study, we analyzed the patterns of ribosomal and mitochondrial DNA variation among 30 cold-water dinoflagellate strains (55 new sequences), as well as their evolutionary relationships with other dinoflagellates. We conclude that the studied cold-water dinoflagellates belong to a lineage (i.e. common evolutionary origin) which has diversified recently and spread widely in geographic terms, colonizing environments which differ markedly in ecology (marine vs. lacustrine communities) and salinity. Probably, the diversification of this group of dinoflagellates was promoted by the variety of natural selection regimes that populations encountered in the different environments. These results are in contradiction with theories proposing that the diversification of microbes is normally restrained by high gene flow (Finlay, 2002).

Materials and Methods

Dinoflagellate cultures and morphospecies identification

All dinoflagellate clonal cultures were obtained by isolating single cells from plankton samples, except when specified otherwise. *Peridinium acicu-*

liferum Lemmermann isolate PATO-1 was obtained from Lake Tovel by G. Flaim during the winter 2002/3. Lake Tovel is located at 1178 m above sea level (a.s.l.) in the Italian Alps (46° 15′ 40′′N, 10° 49′ 40′′E) and was formed after the last glaciations [~ 15,000 years ago] (Kulbe et al., 2005). *P. aciculiferum* isolates PASP-1/2/3/4/5/6/9/10/11 were obtained during March 2006 from Stora Pildammen, an approximately 100 year old artificial pond located in the city of Malmö, Southern Sweden (55° 35′ 21′′N, 12° 59′ 44′′E). *P. aciculiferum* isolates PABR-1/2/3/4/5 were obtained during March 2006 from Brodammen, a small artificial pond ~ 10 year old located near the city of Malmö (55° 32′ 44′′N, 12° 58′ 50′′E). Both St. Pildammen and Brodammen are located ~ 20 m a.s.l. and remain ice-covered from December to March approximately. *P. aciculiferum* isolates PAER-1/2/3/8/9 were obtained from Lake Erken, Eastern-Central Sweden (59° 51′ N, 18° 36′ E), located ~ 10 KM from the Baltic Sea. Lake Erken was formed by isostatic rebound, and emerged from the sea ~ 3000 years ago (Ekman and Fries, 1970). The isolate PAER-1 was obtained from a cyst in 1995, while the other isolates were obtained as vegetative cells from an under-ice bloom during the winter of 2004. *P. aciculiferum* PAFI-1 was isolated during March 2006 from Lake Österträsk, Åland, Finland (60° 21′ N, 20° 00′ E). As many other lakes in Scandinavia, Lake Österträsk originated within the last 10,000 years, after the last glaciations (Bjorck, 1995). *Scrippsiella hangoei* isolates from the Baltic Sea were obtained from germinated resting cysts. Sediment samples containing cysts of *S. hangoei* were collected from the Tvärminne area in the South West coast of Finland (59° 50′ N, 23° 15′ E) during 2002. The Baltic Sea is a semi enclosed brackish Sea with a salinity gradient ranging from 1 to 25. The postglacial history of the Baltic Sea started about 8,500 years ago when the preceding freshwater lake opened to the North Sea and the inflow of marine waters caused a rise in salinity. The northern parts of the Baltic are ice covered for approximately 2 month per year. The *Scrippsiella* aff. *hangoei* isolates High-1/4 and Vere-B/C were obtained from Highway Lake (68° 27′ 48′′ S, 78° 13′ 24′′ E; 8 m a.s.l.) and Vereteno Lake (68° 30′ 55′′S, 78° 24′ 51′′E; 0.3 m a.s.l.) respectively during the summer of 2004/5. Both lakes are in the Vestfold Hills, an ice-free coastal area in Princess Elizabeth Land, Antarctica , that was formed by isostatic uplift after the last glaciations ~10,000 year ago (Zwartz et al., 1998). Highway and Vereteno lakes are brackish (both with ~ 4–5 salinity) and are usually ice-free for around four weeks each year. Plankton samples were collected through holes in the ice-cover. The Arctic isolate of *Scrippsiella* aff. *hangoei* was obtained from a plankton sample collected through a lead in the sea ice in the vicinity of Igloolik Island, northern Foxe Basin, Canadian Arctic (69° 23′ N; 81° 45′W) during June 1989. The salinity in the upper 30 m water column ranged from 0 to 32.

Scrippsiella aff. *hangoei* isolates from the Antarctic were cultured in F/2 medium (Guillard and Ryther, 1962) prepared with sterile filtered water from Highway Lake (~5 salinity). *P. aciculiferum* was cultured in modified Woods Hole medium (Guillard and Lorenzen, 1972; MWC, 0 salinity) prepared with MilliQ water (Millipore Corp., Bedford, USA). *Scrippsiella* aff. *hangoei* from the Arctic was cultured in seawater based Throndsen medium (Throndsen, 1978). This isolate (K-0399) has died due to an incubator failure. Cultures were kept in an incubator at 3 ± 1 °C, 20 µmol photons $m^{-2}s^{-1}$ and 12:12 h light-dark cycle.

For morphological taxonomic identification, armor plate patterns were analyzed by light microscopy. The *P. aciculiferum* cultures (PATO, PAFI, PASP, PABR) were fixed in formaldehyde 5 %. Dinoflagellate plate detachment between slide and cover slip was carried out with the aid of diluted sodium hypochlorite instillation. Squashed empty thecae and detached plates were observed under a Standard 14 Zeiss optical microscope with Nomarsky interference contrast illumination. Live cells of *Scrippsiella* aff. *hangoei* K-0399 were photographed with differential inference contrast. In order to study the plate pattern of this isolate, live cells were stained with CalcoFlour White (Fritz and Triemer, 1985) and viewed with a filter arrangement for violet excitation (400 – 410 nm) using a BH-2 Olympus microscope. Morphological data for the remaining cultures is presented in Rengefors and Legrand (2001); Logares *et al.* (2007b); and Rengefors et al., [2007] (see also Fig. 1).

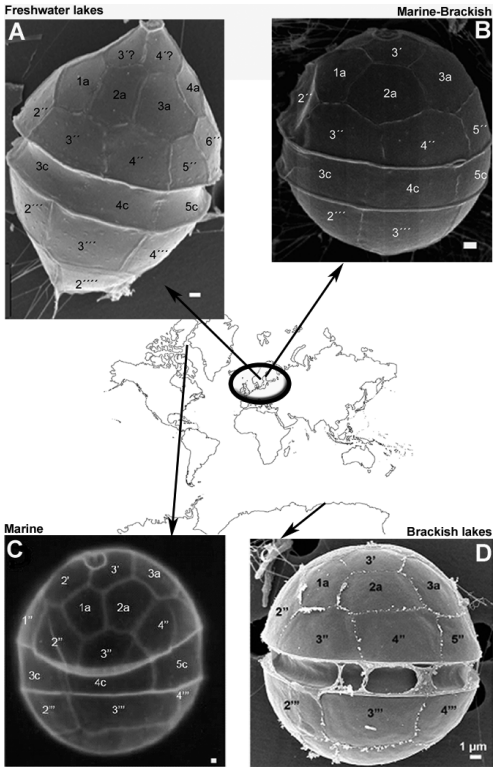

Figure 1. Morphology and geographic source of the investigated dinoflagellate strains/species. A= *Peridinium aciculiferum*: freshwater lakes in Northern-Central Europe (picture from Logares et al., 2007b); B= *Scrippsiella hangoei*: Marine-brackish, Baltic Sea (picture from Logares et al., 2007b); C= *Scrippsiella* aff. *hangoei*: Marine, Arctic (picture from this study; detailed morphological description in Fig. S1, Supplementary Materials); D= *Scrippsiella* aff. *hangoei*, brackish Antarctic lakes (picture from Rengefors et al., 2007). The scale-bar = 1µm. The numbers over the cell armor correspond to the dinoflagellate Kofoidian tabulation. Note the difference in general morphology between *Peridinium* (A) and *Scrippsiella* (B–D).

DNA extraction, PCR and sequencing

DNA was extracted following Adachi *et al.* (1994). For the Arctic *Scrippsiella* aff. *hangoei* K-0399, DNA was extracted from 200 µl of frozen pelleted culture using a GENERATION Capture Column Kit (Gentra Systems, Minneapolis).

For this work, we used different areas of the nuclear ribosomal DNA (nrDNA) molecule as well as the mitochondrial (mt) gene Cytochrome b (COB). The amplified and sequenced nrDNA fragments were: Internal Transcribed Spacer 1 and 2 (ITS1/2), 5.8S, Small Subunit (SSU) and the D1/D2 domains of the Large Subunit (LSU). The different rates of evolution in the different nrDNA regions provide evolutionary information at different taxonomic levels (Hillis and Dixon, 1991). The COB mtDNA is a highly conserved marker in dinoflagellates (Zhang et al., 2005).

Nuclear ribosomal and COB mitochondrial DNA PCR amplifications were done using 25 ng of template genomic DNA, 0.125 mM of each nucleotide, 1.5 (3.0 for SSU) mM of $MgCl_2$, 1X PCR buffer, 0.4 µM of each primer and 0.5 u of Taq DNA Polymerase (AmpliTaq, Applied Biosystems) in 25 µl total volume reactions. For the ITS1/2 and 5.8S, the primers ITS1 (forward) 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 (reverse) 5′-TCCTCCGCTTATTGATATGC-3′ were used. The ITS PCR temperature profile consisted of an initial denaturing step of 5 min at 95 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 45 °C, 1 min at 72 °C, and ended with 10 min at 72 °C. For the SSU PCR we used the combination of the universal primers 4616 (forward) 5′-AACCTGGTTGATCCTGCCAG-3′ and 4618 (reverse) 5′-TGATCCTTCTGCAGGTTCAC-CTAC-3′. The SSU PCR started with 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C, 2 min at 72 °C and ended with 7 min at 72 °C. For the domains D1/D2 of the LSU nrDNA we used the primers DinFi (forward) 5′-GCATATAAGTAMGYGGWGG-3′ and DinRi (reverse) 5′-CCGTGTTTCAA-GACGGGTC-3′. The LSU temperature profile differed from the SSU in that it consisted of 30 amplification cycles with a primer annealing temperature of 50 °C and only 1 min at 72 °C. For the COB PCR we used the primers Dinocob1F (forward), 5′- ATGAAATCTCATTTACAWW-CATATCCTTGTCC-3′, and Dinocob1R (reverse), 5′-TCTCTTGAGGKAATTGWK-MACCTATCCA-3′ (Zhang et al., 2005). The COB PCR temperature profile consisted of 1 min at 95 °C, followed by 40 cycles of 20 s at 94 °C, 30 s at 55 °C, and 40 s at 72 °C, finished by 10 min at 72 °C.

All PCR amplicons were cleaned using PCR-M™ Clean-Up System (Viogene, Taiwan). ITS,

LSU and COB fragments were directly sequenced from both sides using the same PCR primers. SSU amplicons were directly sequenced using the PCR primer 4616, plus the sequencing primers 516F 5′-CACATCTAAGGAAGGCAGCA-3′, 528F 5′-CGGTAATTCCAGCTCC-3′, 690F 5′-CAGAGGTGAAATTCT-3′ and 1055F 5′-GGTGGTGCATGGCCG-3′ (Edvardsen et al., 2003). The sequencing reaction was carried out using BigDye (v1.1, Applied Biosystems) chemistry and the products were precipitated following the manufacturer instructions and then loaded into an ABI Prism 3100 sequencer (Applied Biosystems). The obtained sequences were edited and assembled by analyzing carefully the chromatograms using Bioedit (v7.0.4.1; Hall, 1999). Sequences were deposited in Genbank (accession numbers shown in Table 1). The SSU sequencing of the *Scrippsiella* aff. *hangoei* K-0399 was unsuccessful, however the lack of this sequence is not pivotal to this work.

Alignments and phylogenetic analyses

ITS, SSU, LSU nrDNA and COB mtDNA sequences from several dinoflagellate taxa were downloaded from Genbank and used for constructing alignments along with our sequences. In particular, we included all sequences reported for *Scrippsiella hangoei* and *Peridinium aciculiferum* in Logares et al. (2007b). The sequences were aligned using ClustalX (v1.8; Thompson et al., 1997). Ambiguously aligned positions and divergent regions were excluded from the alignment using the program Gblocks (v0.91b; Castresana, 2000) and visual examination. The alignment datasets used for this work are described in Table S1 (Supplementary materials; alignments available upon request). The construction of one general nrDNA alignment concatenating the SSU, LSU and ITS was precluded by the unavailability, in several cases, of the three markers for the same strains or species.

Phylogenies were constructed using Maximum Likelihood (ML) and Bayesian inference (BI) as implemented in GARLI (serial version, v0.951; Zwickl, 2006) and MrBayes (v3.1.2 parallel version [MPI]; Metropolis-coupled Markov Chain Monte Carlo model [MCMC] approach for approximation of Bayesian posterior probabilities [PPs]; Huelsenbeck and Ronquist, 2001; Altekar et al., 2004). The program ModelTest (v3.7; Posada and Crandall, 1998) indicated that the General Time Reversible (GTR) model of nucleotide substitution, with a Gamma (G) distributed rate of variation across sites and a proportion of invariable sites (I) was the most appropriate evolutionary model for our ITS, LSU, SSU nrDNA and COB mtDNA datasets. In ML and BI analyses, the shape parameter (α) of the Gamma distribution and the proportion of invariable sites (I) were estimated from the datasets using default options.

All Bayesian MCMC analyses were run with seven Markov chains (six heated, one cold) for 5.10^6 generations and the trees were sampled every 100 generations, which resulted in 5.10^4 sampled trees. Each analysis used default (flat) priors and was repeated at least twice from independent starting trees. Bayesian analyses with the COB mtDNA were carried out using the evolutionary model GTR+G+I. The evolutionary model used in nrDNA Bayesian analyses consisted in the GTR+G+COV. The Covarion Model (COV) allows substitution rates to change across positions through time [Miyamoto and Fitch, 1995; Huelsenbeck, 2002]. The covarion model was used since previous phylogenetic analyses with dinoflagellate nrDNA (Shalchian-Tabrizi et al., 2006), along with studies in other taxa (Galtier, 2001; Huelsenbeck, 2002), indicate that this model gives a better explanation of nrDNA data. The obtained posterior probability (PP) values for the branching pattern as well as the likelihood scores for the trees were compared to ensure convergent tree reconstruction. Consensus trees were constructed using the 3.10^4 trees after the log-likelihood stabilization.

ML analyses in GARLI were run with 1000 bootstrap pseudoreplicates (Felsenstein, 1985). All parameters were used in default options, except the number of generations that the program should run with no significant improvements in the scoring of the topology, which was set to 5000. All analyses in GARLI were run under the GTR+G+I model, since the covarion model is not implemented. Consensus trees from the bootstrap output were generated using MrBayes. Phylogenetic analyses with MrBayes and GARLI

Table 1. Analyzed strains, obtained sequences, and other important sequences for this work.

						Genbank Accession Numbers			
Morphospecies	**Isolate**	**Collection site**	**Coordinates**	**~Salinity**	**Isolation**	**LSU**	**SSU**	**ITS**	**COB**
Scrippsiella hangoei	SHTV-1	Baltic Sea, near Tvärminne	59° 50′ N, 23° 15′ E	8	2002	AY970658	AY970662	AY970654	DQ094821
Scrippsiella hangoei	SHTV-2	Baltic Sea, near Tvärminne	59° 50′ N, 23° 15′ E	8	2002	AY970659	–	AY970655	DQ094822
Scrippsiella hangoei	SHTV-5	Baltic Sea, near Tvärminne	59° 50′ N, 23° 15′ E	8	2002	AY970660	–	AY970656	DQ094823
Scrippsiella hangoei	SHTV-6	Baltic Sea, near Tvärminne	59° 50′ N, 23° 15′ E	8	2002	AY970661	**EF417316***	AY970657	DQ094824
Peridinium aciculiferum	PAER-1	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	1995	AY970652	AY970653	AY970649	DQ094825
Peridinium aciculiferum	PAER-2	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	2004	**EF417308***	**EF417314***	DQ022927	DQ094826
Peridinium aciculiferum	PAER-3	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	2004	–	–	DQ022928	DQ094827
Peridinium aciculiferum	PAER-8	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	2004	–	–	AY970650	DQ094828
Peridinium aciculiferum	PAER-9	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	2004	–	–	AY970651	DQ094829
Peridinium aciculiferum	PASP-1	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417292***	**EF417324***
Peridinium aciculiferum	PASP-2	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417293***	**EF417325***
Peridinium aciculiferum	PASP-3	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417294***	**EF417326***
Peridinium aciculiferum	PASP-4	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417295***	**EF417327***
Peridinium aciculiferum	PASP-5	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417296***	**EF417328***
Peridinium aciculiferum	PASP-6	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417297***	**EF417329***
Peridinium aciculiferum	PASP-9	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	–	**EF417298***	**EF417330***
Peridinium aciculiferum	PASP-10	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	**EF417311***	–	**EF417299***	**EF417331***
Peridinium aciculiferum	PASP-11	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	2006	–	**EF417315***	**EF417300***	**EF417332***
Peridinium aciculiferum	PABR-1	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	2006	–	–	**EF417287***	**EF417319***
Peridinium aciculiferum	PABR-2	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	2006	–	–	**EF417288***	**EF417320***
Peridinium aciculiferum	PABR-3	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	2006	**EF417309***	**EF417313***	**EF417289***	**EF417321***
Peridinium aciculiferum	PABR-4	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	2006	–	–	**EF417290***	**EF417322***
Peridinium aciculiferum	PABR-5	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	2006	–	–	**EF417291***	**EF417323***
Peridinium aciculiferum	PAFI-1	Lake Österträsk, Finland	60° 16′ N, 20° 06′ E	0	2006	**EF417310***	–	**EF417286***	**EF417333***
Peridinium aciculiferum	PATO-1	Lake Tovel, Italy	46° 15′ N, 10° 49′ E	0	2003	**EF417312***	–	**EF417285***	**EF417334***
Scrippsiella aff. *hangoei*	K-0399	Foxe Basin, Arctic	69° 23′ N, 81° 45′ W	0–32	1989	AF260392	–	**EF506568***	**EF506569***
Scrippsiella aff. *hangoei*	High-1	Highway Lake, Antarctica	68° 14′ S, 78° 28′ E	5	2005	EF058275	EF417318	**EF417301***	**EF417335***
Scrippsiella aff. *hangoei*	High-4	Highway Lake, Antarctica	68° 14′ S, 78° 28′ E	5	2005	–	–	**EF417302***	**EF417336***
Scrippsiella aff. *hangoei*	Vere-B	Vereteno Lake, Antarctica	68° 30′ S, 78° 24′ E	4	2005	–	–	**EF417306***	**EF417337***
Scrippsiella aff. *hangoei*	Vere-C	Vereteno Lake, Antarctica	68° 30′ S, 78° 24′ E	4	2005	–	–	**EF417307***	**EF417338***
Peridiniopsis borgei	PBSK-A	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	2005	EF058261	**EF058241***	–	**EF417339***
Peridinium centenniale	CCAC0002	Cornwall, England	–	0	–	EF058254	EF058236	–	**EF417340***
Polarella glacialis	CCMP1383	Antarctica	–	Marine	1991	–	EF417317	–	**EF417341***

* (bold) sequences obtained for this work.

were run at the University of Oslo Bioportal (http://www.bioportal.uio.no/). The trees generated with MrBayes and GARLI were visualized in TreeView (v1.6.6; Page, 1996).

Genetic differentiation

The software DnaSP (v4.10.9, Rozas et al., 2003) and Mega MEGA (v 3.1, Kumar et al., 2004) were used to analyze the genetic polymorphism of the mtDNA sequences. The COB reading frame was obtained by analyzing the sequences at the ExPASy Proteomics Server (http://www.expasy.org/). The protozoan mitochondrial genetic code was used to translate the COB sequences.

Results

Phylogenetic relationships

Across the 16 constructed nrDNA and COB mtDNA phylogenies using Bayesian Inference (BI) and Maximum Likelihood (ML), *Peridinium aciculiferum* from European freshwater lakes, *Scrippsiella hangoei* from the Baltic Sea, *Scrippsiella* aff. *hangoei* from Antarctic lakes and *S.* aff. *hangoei* from the Arctic (Fig. 1) clustered together (=PASH cluster) with a support that ranged from moderate to high (0.55 <PPs / BVs< 0.98; Fig. 2; Table S1 in Supplementary Materials) [PPs =Posterior Probabilities; BVs =Bootstrap Values]. In the nrDNA and COB mtDNA phylogenies, PASH clustered with *Pfiesteria* and *Pfiesteria*-like species (PASH+PFIE cluster) [Fig. 2]. Other species included within PASH+PFIE depending on the alignment datasets (i.e. alignments included different species depending on their availability in GenBank) were, for the nrDNA, *Cryptoperidiniopsis* spp., *Leonella granifera*, *Thoracosphaera heimii*, *Amyloodinium ocellatum*, *Paulsenella vonstoschii* (Fig. 2). In the COB phylogenies, the sequences *Scrippsiella* sp. HZ2005 (AY743961) and *Peridinium centenniale* CCAC0002 (EF417340) clustered within PASH+PFIE (Fig. 2 COB). In the nrDNA phylogenies, the PFIE+PASH cluster received variable support by the BI and ML analyses (0.25 < PPs / BVs < 0.99; Fig. 2 nrDNA; Table S1, Supplementary Materials). The support given by COB BI and ML phylogenies to PFIE+PASH ranged from low to moderate (0.51 <PPs / BVs< 0.71; Fig. 2 COB; Table S1, Supplementary Materials).

nrDNA homogeneity vs. COB mtDNA differentiation

All the *Peridinium aciculiferum* isolates from lakes in Sweden, Finland and Italy, as well as the *Scrippsiella hangoei* isolates from the Baltic Sea shared **identical** nrDNA sequences (Fig. 3). The *Scrippsiella* aff. *hangoei* isolates from the Antarctic Vereteno and Highway lakes also shared the same nrDNA among themselves (Fig. 3). Among the pair *P. aciculiferum* – *S. hangoei* and the bipolar *S.* aff. *hangoei* isolates, there was a very low nrDNA differentiation. The sequences comprising the ITS1/2 [=ITS] (ITS1/2 are the most variable areas within nrDNA) differed less than 1.43 % among the 30 analyzed strains (Fig. 3). The ITS differentiation between the bipolar *S.* aff. *hangoei* was ~0.90 % (Fig. 3). The ITS differentiation was also ~0.90 % between *P. aciculiferum-S. hangoei* and the Antarctic *S.* aff. *hangoei*, while the ITS differentiation between *P. aciculiferum-S. hangoei* and the Arctic *S.* aff. *hangoei* was ~1.43 % (Fig. 3). A total of 558 ITS nucleotides were analyzed to obtain the percentages. The D1/D2 LSU sequence from the Arctic *S.* aff. *hangoei* differed ~0.57 % from *P. aciculiferum* - *S. hangoei* and ~0.95 % from the Antarctic *S.* aff. *hangoei*. The D1/D2 LSU differentiation between *P. aciculiferum-S. hangoei* and the Antarctic *S.* aff. *hangoei* was ~0.76 %, based on a total of 541 nucleotides. The SSU differentiation between *P. aciculiferum-S. hangoei* and the Antarctic *S.* aff. *hangoei* was ~0.16 %, calculated from a total of 1232 nucleotides. The SSU of the Arctic *S.* aff. *hangoei* could not be obtained.

A total of 13 COB mtDNA haplotypes, with a genetic differentiation ranging between 0.12 – 2.40 %, were identified among the 30 analyzed strains (Fig. 3). Within the 21 *Peridinium aciculiferum* isolates from five lakes, eight haplotypes with a differentiation ranging between 0.12 – 1.32 % were detected (Fig. 3). The three haplotypes that were detected within the four *Scrippsiella hangoei* isolates from the Baltic had a differentiation ranging between 0.12 – 0.30 % (Fig. 3). Only one haplotype was detected within the four

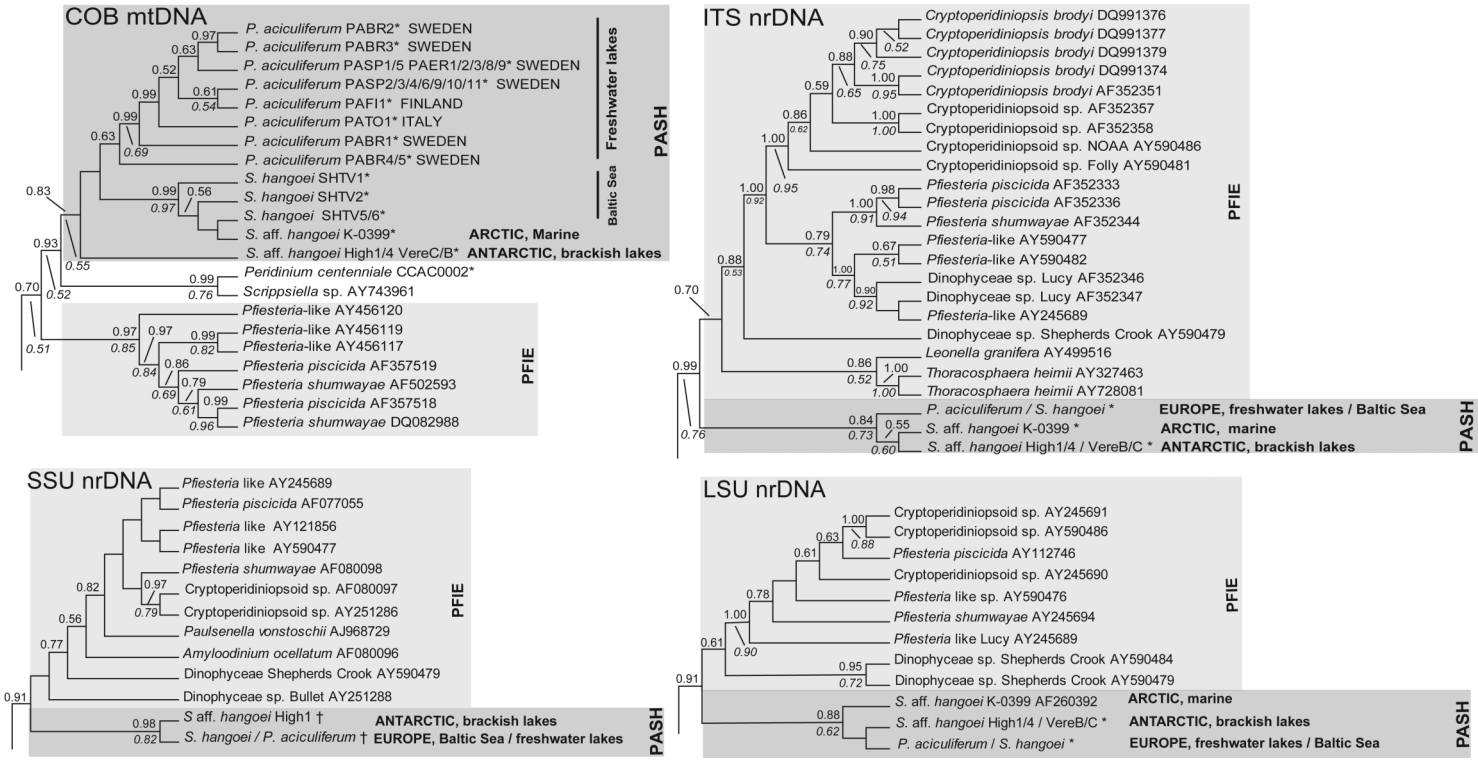

Figure 2. Bayesian Inference (BI) and Maximum Likelihood (ML) consensus phylogenies constructed with the COB mtDNA and ITS, SSU, LSU nrDNA. The BI and ML phylogenies were constructed under the GTR+G+I (COB) or the GTR+G+COV (nrDNA) models. Posterior Probabilities (PPs) and Bootstrap support values (BVs) > 0.5 are shown. PPs appear in normal print above the nodes, and BVs are shown in *italics* below the nodes. PPs were calculated from the 3.10^4 trees obtained after the log-likelihood stabilization, while BVs were calculated from 1000 bootstrap pseudoreplicates. The frames indicate the PASH and PFIE clusters (phylogenies including the complete sequence datasets are available upon request). * Sequences obtained for this work (see Table 1; strains sharing the same sequence appear together). **COB mtDNA:** consensus COB tree constructed from an 818-character alignment comprising 43 sequences; corresponds to trees 13 and 15, Table S1, supplementary materials). **ITS nrDNA:** consensus ITS tree constructed from a 287-character alignment comprising 53 sequences; corresponds to trees 2 and 3, Table S1. **SSU nrDNA:** consensus SSU tree constructed from a 1047-character alignment comprising 57 sequences; corresponds to trees 9 and 12, Table S1. † Accession numbers given in Table 1. **LSU nrDNA:** consensus LSU (D1/D2 domains) tree constructed from a 476-character alignment comprising 51 sequences; corresponds to trees 6 and 8, Table S1.

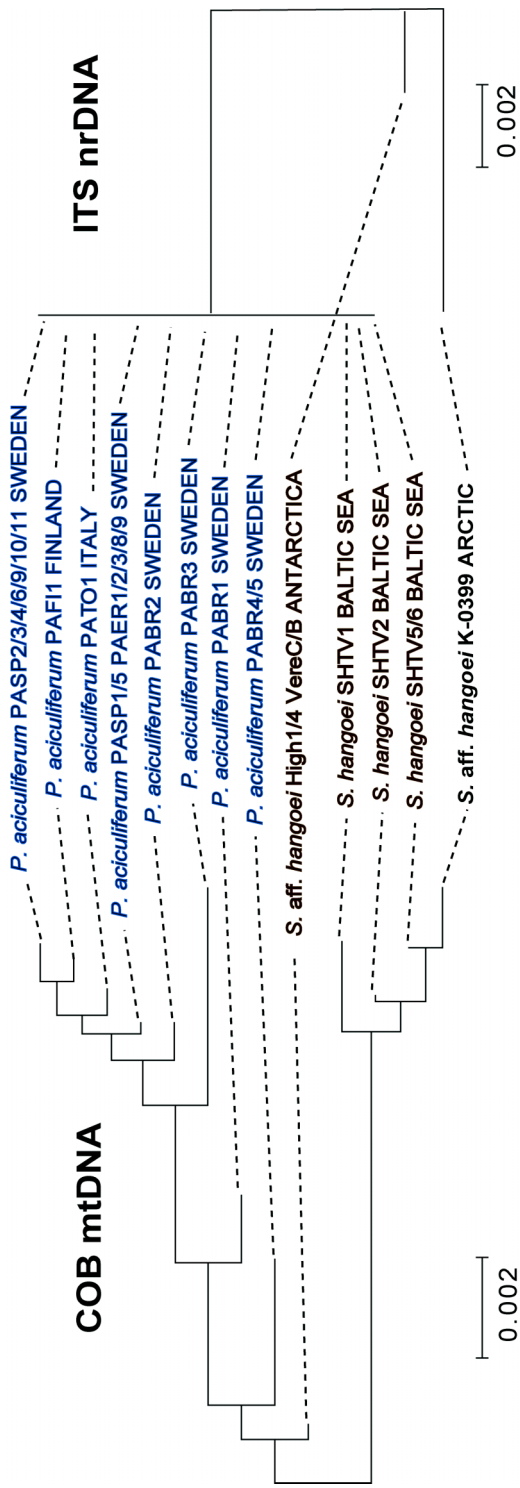

Figure 3. COB mtDNA variation vs. ITS nrDNA homogeneity. Contrasting Neighbor-Joining phylograms presenting the uncorrected genetics distances (*p*) between COB haplotypes (left) and the corresponding ITS sequences (right). Freshwater lacustrine strains/species appear in light blue, brackish in green and marine in black. *p* was calculated using 836 COB and 558 ITS nucleotides. PABR= *Peridinium aciculiferum* Brodammen, Sweden. PASP= *P. aciculiferum* Stora Pildammen, Sweden. PAFI= *P. aciculiferum* Lake Österträsk, Finland. PATO= *P. aciculiferum*, Lake Tovel, Italy. SHTV= *Scrippsiella hangoei*, Tvärminne, Baltic Sea. K-0399= *Scrippsiella* aff. *hangoei*, Arctic. High1/4 and VereC/B = *S.* aff. *hangoei* from Highway and Vereteno lakes, Antarctica. The scalebar indicates *p*.

S. aff. *hangoei* isolates from the two Antarctic lakes. The haplotype from the Arctic *S.* aff. *hangoei* presented only a difference of one nucleotide (~0.12 %) from the haplotype found in the *S. hangoei* SHTV-5/6 from the Baltic. All percentages were calculated using 836 COB nucleotides. There were no shared haplotypes between *P. aciculiferum*, *S. hangoei*, and the bipolar *S.* aff. *hangoei* among our samples. In all cases, a clear chromatogram was obtained from each dinoflagellate strain, indicating no mtDNA heteroplasmy.

A total of 23 variable COB sites were identified, out of 836 analyzed, among the 13 recognized haplotypes. These 23 variable sites accounted for 24 mutations, of which only six generate amino acid replacements, resulting in a total of eight different amino-acid haplotypes (Table S2, Supplementary Materials).

Morphological analyses

The optical microscopy analyses confirmed the morphospecies identity of all the *P. aciculiferum* isolates. However, the isolates from St. Pildammen (PASP) and Brodammen (PABR) did not present the typical antapical spines. The plate pattern of the bipolar *S.* aff. *hangoei* was virtually identical to the plate pattern of *S. hangoei* as originally described by Schiller and subsequently redescribed by Larsen *et al.* (1995) [Fig. 1, S1 in Supplementary Materials; see also Rengefors et al., 2007]. Moreover, the general cell morphology of the bipolar *S.* aff. *hangoei* was very similar to *S. hangoei* (Fig. 1). Even though the *Scrippsiella* populations/species shared a very similar plate pattern with *Peridinium aciculiferum*, the general morphology between them was different (Fig. 1 and Logares et al., 2007b).

Discussion

The current study has identified a group of cold-water protists (dinoflagellates) which share a common ancestor and has diversified recently. This diversification appears to have occurred in parallel with transitions between environments with very different ecologies (marine vs. lacustrine communities) and salinities, implying in some cases the dispersal across great geographic distances. The presence of the studied lineage in such variety of environments suggests that natural selection could have had a major role in promoting diversification.

Recent evolutionary diversification

The low nrDNA differentiation between *Peridinium aciculiferum, Scrippsiella hangoei* and the bipolar *Scrippsiella* aff. *hangoei* indicates a recent divergence between these strains/species. In particular, the ITS (=ITS1/2-5.8s), which harbor two rapidly evolving introns (ITS1/2) normally used for investigating the genetic differentiation between populations and closely related dinoflagellate species (Litaker et al., 2007), showed a remarkably low variation (0 – 1.43 %, [p <0.0143]; p= uncorrected genetic distances). Significantly higher levels of ITS variation have been reported within single dinoflagellate morphospecies that do not present detectable morphological variation. For instance, morphologically identical isolates of the freshwater *Peridinium limbatum*, inhabiting neighboring lakes, have shown much higher levels of ITS differentiation [8.50 – 11.00 % ; 0.085 < p < 0.11] (Kim et al., 2004). A number of morphologically identical isolates of the marine *Scrippsiella trochoidea* obtained from the same geographical area (Gulf of Naples, Mediterranean Sea) were found to have a range of ITS variation (0.18 – 1.46 %; 0.0018 < p < 0.0146; Montresor et al., 2003) which was very similar to the range we have found in the cold water dinoflagellates . Nevertheless, there are also cases where no ITS variation was observed among populations of morphologically identical dinoflagellates living in similar environments (e.g. Loret et al., 2002; Tengs et al., 2003), which would represent cases of high intraspecific gene-flow. Here, the low nrDNA differentiation among the studied strains/species contrasts with detectable morphological and physiological variability (Fig. 1; Logares et al., 2007b; Rengefors et al., 2007), which do not appear to be produced by different patterns of gene expression (Logares et al., 2007b; Rengefors et al., 2007). Thus, despite the close evolutionary relationship among the studied cold-water dinoflagellates, they do not appear to constitute one global panmictic population.

In a recent survey of dinoflagellate ITS sequence variation, including 81 species from 14

genera, it was indicated that $p \geq 0.04$ delineate most free-living dinoflagellate species, with the exception of recently evolved ones and species with slow evolutionary rates (Litaker et al., 2007). Thus, according to this proposition, the level of ITS variation among the studied cold water dinoflagellates ($p < 0.0143$) would place them into the same biological species; unless it is assumed that they have evolved recently or have slow rates of evolution. The genetic and phenotypic differences among most of the strains/species studied here do not suggest that they belong to the same biological species (see Logares et al., 2007b; Rengefors et al., 2007).

A fast recent divergence appears to have occurred between *P. aciculiferum* and *S. hangoei*, which share identical ITS sequences but present clear differences at the genome level (Logares et al., 2007b). Other organisms which are known to have diversified recently also present identical or very similar sequences for particular rapidly evolving neutral markers, but differentiation in other areas of the genome (see Orr and Smith, 1998; Muir et al., 2000, 2001). This pattern can occur, for instance, as a result of strong disruptive natural selection acting over certain areas of the genome of different populations. These selected areas can diverge even faster than rapidly evolving neutral markers, and therefore the variation of those neutral markers might not reflect the rapid diversification process (see Orr and Smith, 1998). Despite the nrDNA similarity between the studied dinoflagellates does not appear to be due to slow evolutionary rates, we can not totally dismiss this possibility. It has been proposed that organisms living at low temperatures will have slower rates of evolution than counterparts living at higher temperatures (Rohde, 1978; Rohde, 1992; Bromham and Cardillo, 2003). However, no clear supporting evidence for this hypothesis has been reported so far for any taxa (Bromham and Cardillo, 2003; Bromham and Penny, 2003). In addition, if the evolution of *P. aciculiferum* and *S. hangoei* was slow, lower levels of multilocus differentiation would have been expected between these strains/species which share identical ITS. As a comparison, strains of *Alexandrium tamarense* presenting a range of variability in the ITS, were found to have a lower multilocus differentiation among themselves, than the differentiation found between *P. aciculiferum* and *S. hangoei* (as measured by AFLP; $F_{st} < 0.64$ among *A. tamarense* [John et al., 2004], $F_{st} \sim 0.75$ between *P. aciculiferum-S. hangoei* [Logares et al., 2007b]).

The ITS divergence could potentially be used to estimate the divergence times among the cold-water dinoflagellates. However, no consensus calibration has been proposed so far for this marker in dinoflagellates. Nevertheless, for the ITS2 of symbiotic *Symbiodinium* dinoflagellates, LaJeunesse (2005) estimated a range of clock rates between 0.75–1.3 million years per change and/or difference. Using this estimation, the ITS2 differentiation between the northern hemisphere *S. hangoei* and the Antarctic *S.* aff. *hangoei* indicates that their divergence could have occurred between 4 – 2 million years ago (MYA). On the other hand, the divergence between the bipolar *S.* aff. *hangoei* could have occurred between 1.5 – 0.5 MYA (these divergence dates were calculated using 204 ITS2 nucleotides and are used as rough estimations).

Distribution across ecologically and physicochemically diverse low-temperature habitats

Altogether, the variety of environments that the studied cold water dinoflagellates inhabit could probably explain part of their diversification, since differential natural selection regimes can be a strong motor of divergence (e.g. Orr and Smith, 1998). The diversification of the studied cold water dinoflagellates appear to have occurred in parallel with transitions between environments with different salinity (freshwater, brackish, marine) and ecologies (marine vs. lacustrine communities), which sometimes are separated by large distances. This suggests a high capacity for dispersal and adaptability to new environments in the studied lineage of dinoflagellates. Resting cysts have been identified in the strains/species *S. hangoei, S.* aff. *hangoei* and *P. aciculiferum* (Rengefors et al., 1998; Kremp and Parrow, 2006; Rengefors et al., 2007). The cysts could allow the dispersal over long distances, eventually across the equatorial warm-water belt in some strains/species. Despite the presence of the studied dinoflagellates in a variety of habitats, in all cases these habitats were characterized by low, permanent or

seasonal, temperatures. Neither of the studied species or close relatives were so far identified (morphologically or phylogenetically through SSU BLAST searches) in environments with permanent warm temperatures. In addition, field or laboratory studies/observations indicate that at least three of the studied species form cysts when temperature increases (Rengefors et al., 1998; Kremp and Parrow, 2006; Rengefors et al., 2007). Thus, despite the apparently high adaptability of this lineage to different environments, it appears that the vegetative stages (i.e. free-swimming) have been restricted to low-temperature habitats. Rengefors et al. (1998) suggested that the restriction of *P. aciculiferum* to cold waters could be partially related with an ecological strategy to avoid intensive grazing by zooplankton.

The pathways through which the different strains/species colonized the habitats in which they are currently present are still unclear. Nevertheless, we can propose a few scenarios. The presence of *S.* aff. *hangoei* in Antarctic coastal lakes with less than 10,000 years is most probably the outcome of a colonization from the sea (the bipolar lacustrine and marine strain/species are very closely related at the ITS level), even though *S.* aff. *hangoei* has not yet been confirmed for marine Antarctic waters (see Rengefors et al., 2007). Most likely, the divergence between *P. aciculiferum* and *S. hangoei* (and the colonization of fresh waters by *P. aciculiferum*) occurred very recently, since these morphospecies still share identical ITS nrDNA, despite presenting genetic differentiation at the genome level (Logares et al., 2007b). *S. hangoei* is supposed to have been always marine-brackish and not the product of a marine recolonization by a freshwater species (see Logares et al., 2007b). The Baltic Sea used to be a freshwater lake that opened to the North Sea ~ 8500 years ago, and the ancestral *S. hangoei* probably entered to this sea carried by the influx of marine water. Little can be said about the origin of the Arctic *Scrippsiella* based on the available data. Overall, the analysis of 30 strains allowed us to identify the cold-water dinoflagellate lineage and investigate general patterns of genetic differentiation among strains/species, as well as phylogenetic relationships between this lineage and other dinoflagellates. Future work including more samples is needed to address more precise questions on strains/species diversity and phylogeography.

Phylogenetic relationships

Altogether, our nrDNA and COB mtDNA phylogenetic results support a common evolutionary origin for the studied cold water dinoflagellates (PASH clade; see Fig. 2). These results are the summary of a total of 16 constructed phylogenies using three nuclear and one mitochondrial marker from 30 analyzed strains. The Maximum Likelihood (ML) bootstrap values (BVs) and Bayesian Inference (BI) posterior probabilities (PPs) for the PASH clade using the SSU, LSU, ITS and COB ranged from moderate (~ 0.60) to high (> 0.90) [see Table S1, Supplementary Materials]. PPs were normally higher than BVs for the PASH clade using the nrDNA and COB markers, and this most likely reflects the fact that BVs are normally more conservative than PPs (see Cummings et al., 2003; Simmons et al., 2004). The common evolutionary origin of the cold-water dinoflagellates was also supported by morphological analyses. All the studied strains/species shared virtually the same armor plate-pattern [a phylogenetically informative character in dinoflagellates] (see Fig. 1). In addition, the external morphology among the *Scrippsiella* strains/species was very similar (Fig. 1 [more detailed morphological description of *S.* aff. *hangoei* in Fig. S1, Supplementary Materials]; see also Rengefors et al., 2007).

The clustering of the cold-water dinoflagellates (PASH clade) with *Pfiesteria* and *Pfiesteria*-like species (PFIE+PASH cluster) received variable support across the 16 nrDNA and COB phylogenies (Table S1, Supplementary Materials). *Pfiesteria* and *Pfiesteria*-like dinoflagellates are marine-brackish and in several cases toxin producers (e.g. Steidinger et al., 1996; Marshall et al., 2000; Steidinger et al., 2001; Burkholder et al., 2005). The ITS nrDNA gave significant (> 0.76) BV and PP support for the clustering of PFIE+PASH, a result which agrees with other works (e.g. Gottschling et al., 2005; Marshall et al., 2006). In the ML and BI phylogenies using LSU, SSU and COB, the clade PASH+PFIE normally obtained significant PPs (> 0.70) and non-significant BVs (< 0.70). The reason of this incongruence is unclear. Altogether, our phylogenetic

results indicate that the studied cold-water dinoflagellate lineage is evolutionary related to *Pfiesteria* and *Pfiesteria*-like species, which agrees with previous findings (Gottschling et al., 2005; Marshall et al., 2006).

Does mitochondrial COB diversification predate nrDNA divergence?

Unexpectedly, the high nrDNA similarity among the studied cold-water dinoflagellates was contrasted by a relatively high COB mtDNA differentiation (see Fig. 3). Since the COB mtDNA is a much more conserved marker than the ITS nrDNA in dinoflagellates (Zhang et al., 2005; Litaker et al., 2007), we initially expected a very low COB polymorphism. Even though all the examined isolates of the Antarctic *S.* aff. *hangoei* had the same COB haplotype, the amount of haplotype differentiation ranged from low to relatively high within *S. hangoei* (0.12 – 0.36 %) and *P. aciculiferum* (0.12 – 1.32 %). The opposite and expected situation (ITS differentiation vs. COB similarity) was only observed between the strains/species *S.* aff. *hangoei* K-0399 from the Arctic and *S. hangoei* SHTV-5/6 from the Baltic (Fig. 3). This could be the product of introgression or simply an ancient shared polymorphisms; more samples are needed to ascertain the relationships between these two particular COB haplotypes.

A number of other studies, involving multicellular organisms mostly, have also reported high mitochondrial haplotypic diversities contrasting with low or null ITS nrDNA differentiation (e.g. Navajas et al., 1998; Mukabayire et al., 1999; Navajas and Boursot, 2003). However, in some cases (e.g. Mukabayire et al., 1999), this pattern seems to be simply the outcome of higher evolutionary rates for the mtDNA in comparison with the ITS nrDNA, which is not the case in dinoflagellates (Zhang et al., 2005; Litaker et al., 2007). Within the studied cold-water dinoflagellates, the observed COB polymorphism could be the outcome of a COB diversification which started before the diversification of the nrDNA. The retention of this putative ancestral COB polymorphism could be a consequence of the massive effective population sizes that dinoflagellates normally have, which would allow several selectively neutral COB haplotypes to persist during long periods of time within lineages, due to a mild genetic drift (see Avise, 2000). In the cold-water dinoflagellates investigated in this study, ~ 75 % of the detected COB DNA polymorphism does not generate amino acid replacements (described in Table S2, Supplementary Materials) and therefore, that polymorphism could potentially persist in the large populations for long periods of time, as a consequence of a reduced action of selection and genetic drift. Nevertheless, Ho and Larson (2006) pointed out that in lineages that have diverged recently, the observed polymorphisms can give the false impression of an ancient divergence, since short-term mutation rates are interpreted as long-term substitution rates. The given explanation is that part of these polymorphisms will not persist during long evolutionary times due to their removal by purifying selection and genetic drift (Ho and Larson, 2006). However, genetic drift most probably does not have substantial effects in large microbial populations, and therefore, some of the neutral polymorphisms in the cold-water dinoflagellates could be product of a relatively old divergence.

Concluding remarks

Our identification of a protist lineage which has diversified recently contributes to a growing body of evidence indicating that microbial diversification is probably not an uncommon process as some theories have proposed (see Finlay, 2002). The presence of the studied lineage across different environments suggests that natural selection might have had an important role in promoting diversification. In particular, the different salinities of the environments where members of this lineage are present have probably exerted a strong disruptive selection over different populations (see Lee and Bell, 1999; Logares et al., 2007a). The role of genetic drift during this diversification is unclear, although the huge population sizes that dinoflagellates normally have indicate that it has probably been negligible. The presence of several COB haplotypes which appear to have diverged before the nrDNA divergence suggests that a considerable amount of ancestral mtDNA polymorphisms could be maintained within large microbial populations.

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Supplementary Material

Table S1. The 16 constructed phylogenies and resulting parameters. Support values for the clades PASH, PASH+PFIE across the phylogenies are shown.

	Alignment								Clade PP/BV support♦	
#	Marker	Method	Seq	Char	Model	–lnL	α	pinvar	PASH	PFIE+PASH
1	ITS	BIa	53	287	GTR+G+COV	3213.3	0.33	–	0.84	0.99
2	ITS	BIb	53	287	GTR+G+COV	3211.5	0.33	–	0.84	0.99
3	ITS	MLa	53	287	GTR+G+I	2487.4	0.52	0.02	*0.73*	*0.76*
4	ITS	MLb	53	287	GTR+G+I	2535.2	0.48	0.01	*0.72*	*0.78*
5	LSU	BIa	51	476	GTR+G+COV	7400.2	0.69	–	0.88	0.91
6	LSU	BIb	51	476	GTR+G+COV	7399.5	0.68	–	0.89	0.87
7	LSU	MLa	51	476	GTR+G+I	6512.7	0.74	0.01	*0.62*	*0.25*
8	LSU	MLb	51	476	GTR+G+I	6504.4	0.72	0.03	*0.62*	*0.29*
9	SSU	BIa	57	1047	GTR+G+COV	7361.5	0.17	–	0.98*	0.91*
10	SSU	BIb	57	1047	GTR+G+COV	7361.8	0.17	–	0.98*	0.94*
11	SSU	MLa	57	1047	GTR+G+I	6396.1	0.59	0.43	*0.82**	*0.28**
12	SSU	MLb	57	1047	GTR+G+I	6250.4	0.56	0.36	*0.79**	*0.28**
13	COB	BIa	43	818	GTR+G+I	5294.1	0.81	0.16	0.83	0.70
14	COB	BIb	43	818	GTR+G+I	5303.7	0.81	0.16	0.82	0.71
15	COB	MLa	43	818	GTR+G+I	4499.4	0.75	0.24	*0.55*	*0.51*
16	COB	MLb	43	818	GTR+G+I	4627.9	1.26	0.27	*0.57*	*0.52*

Seq= number of sequences in the alignment. Char= number of characters in the alignment. –lnL = –log-likelihood. α= estimated shape parameter of the Gamma distribution. Pinvar = estimated proportion of invariable sites. ITS= ITS1/2, 5.8s nrDNA. LSU= D1/D2 domains, Large SubUnit nrDNA. SSU= Small SubUnit nrDNA. COB= cytochrome b, mtDNA. BI= Bayesian Inference. ML= Maximum Likelihood. GTR= General Time Reversible model. G= Gamma distributed rate of variation across sites model. I= proportion of invariable sites model. COV= covarion model. For ML analyses, the –lnL, α and pinvar correspond to the highest –lnL phylogeny found during 1000 bootstraps. In BI analyses, –lnL (harmonic mean), α (mean) and, when corresponding, the pinvar (mean), were estimated from the 3.10^4 trees after the log-likelihood stabilization (i.e. after the burn-in phase). PASH= clade comprising *Scrippsiella hangoei-Peridinium aciculiferum*, *Scrippsiella* aff. *hangoei* (bipolar). PFIE= clade comprising *Pfiesteria* spp., *Pfiesteria*-like spp. and related species.

♦ BI Posterior Probabilities (PP) appear in normal print and ML Bootstrap Values (BV) appear in *Italics*.

[a/b] Replicates of each run

* Here PASH does not include the *Scrippsiella* aff. *hangoei* sequence from the Arctic.

Table S2. Description of the DNA and Amino Acid polymorphisms among the analyzed COB haplotypes.

		Position of the variable sites within the Cytochrome b DNA																						
	Haplotypes	102	192	243	309	330	339	342	354	426	463	492	525	543	570	618	627	633	665	675	708	762	783	811
DNA	**1** = PABR-1	A	A	A	A	A	A	T	G	T	A	T	A	T	C	C	C	C	G	T	T	C	T	C
	2 = PABR-2	T	A	A	A	A	A	T	G	T	G	T	A	T	A	C	A	C	G	C	T	C	T	A
	3 = PABR-3	T	A	A	A	A	A	C	G	T	G	T	T	T	A	C	A	A	G	C	A	C	T	A
	4 = PABR-4/5	A	A	A	A	A	A	C	T	T	A	T	T	T	C	C	T	A	G	T	A	C	T	C
	5 = PASP-2/3/4/6/9/10/11	A	G	A	A	A	A	T	G	T	G	T	A	T	A	C	A	C	G	C	T	C	T	C
	6 = PAFI-1	A	G	A	A	A	A	T	G	T	G	T	A	T	A	C	A	C	G	C	T	C	T	A
	7 = PATO-1	A	A	A	A	A	A	T	G	T	G	T	A	T	A	C	A	C	G	C	T	C	T	C
	8 = PASP-1/5 & PAER-1/2/3/8/9	A	A	A	A	A	A	T	G	T	G	T	A	T	A	C	A	C	G	C	T	C	T	A
	9 = SHTV-1	T	A	T	G	C	T	C	T	T	A	C	A	C	C	T	T	C	C	T	A	C	A	C
	10 = SHTV-2	T	A	T	G	C	T	C	T	T	A	C	A	T	C	T	T	C	C	T	A	T	T	C
	11 = SHTV-5/6	T	A	T	G	C	T	C	T	T	A	C	A	C	C	T	T	C	C	T	A	T	T	C
	12 = High-1/4 & Vere C/B	T	A	A	A	A	A	T	T	T	A	T	A	T	C	C	T	C	G	T	A	C	T	C
	13 = K-0399	T	A	T	G	C	T	C	T	C	A	C	A	C	C	T	T	C	C	T	A	T	T	C
AA	**A** = PABR-1	L	-	-	-	-	-	-	-	-	R	-	-	-	F	-	-	F	G	-	-	-	-	L
	B = PABR-2	F	-	-	-	-	-	-	-	-	G	-	-	-	L	-	-	F	G	-	-	-	-	I
	C = PABR-3	F	-	-	-	-	-	-	-	-	G	-	-	-	L	-	-	L	G	-	-	-	-	I
	D = PABR-4/5	L	-	-	-	-	-	-	-	-	R	-	-	-	F	-	-	L	G	-	-	-	-	L
	E = PASP-2/3/4/6/9/10/11 & PATO-1	L	-	-	-	-	-	-	-	-	G	-	-	-	L	-	-	F	G	-	-	-	-	L
	F = PASP-1/5 & PAER-1/2/3/8/9 & PAFI-1	L	-	-	-	-	-	-	-	-	G	-	-	-	L	-	-	F	G	-	-	-	-	I
	G = SHTV-1-2-5-6 & K-0399	F	-	-	-	-	-	-	-	-	R	-	-	-	F	-	-	F	A	-	-	-	-	L
	H = High-1/4 & Vere C/B	F	-	-	-	-	-	-	-	-	R	-	-	-	F	-	-	F	G	-	-	-	-	L

Each row indicates one haplotype. The position numbers refers to an 836-nucleotide alignment; the position 102 is around 200 nucleotides apart from the beginning of the COB gene. AA= amino acids. The AA codes are: A=Alanine, F=Phenylalanine, G=Glycine, I=Isoleucine, L=Leucine, R=Arginine. "–" indicates synonymous mutations. PABR= *Peridinium aciculiferum* Brodammen, Sweden. PASP= *P. aciculiferum* St. Pildammen, Sweden. PAFI= *P. aciculiferum* Lake Österträsk, Finland. PATO= *P. aciculiferum*, Lake Tovel, Italy. SHTV= *Scrippsiella hangoei*, Tvärminne, Baltic Sea. K-0399= *Scrippsiella* aff. *hangoei*, Canadian Arctic. High and Vere = *S.* aff. *hangoei* from Highway and Vereteno lakes, Antarctica.

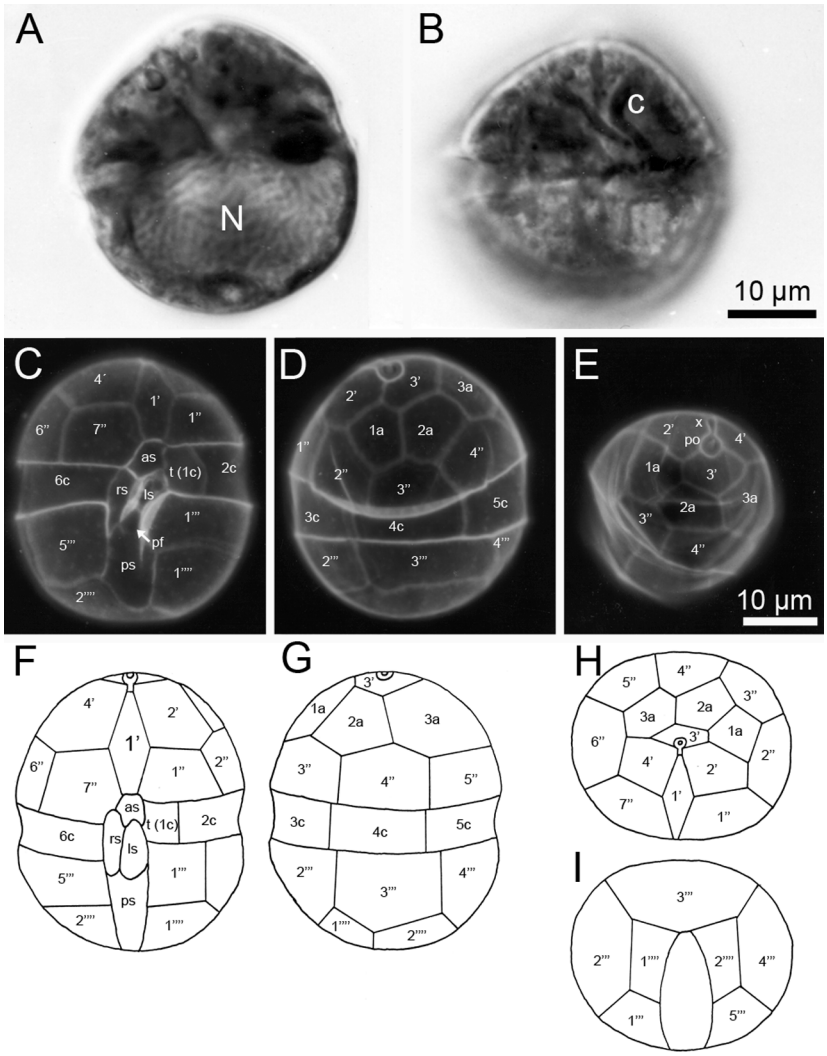

Figure S1. Light micrographs and schematic diagrams of *Scrippsiella* aff. *hangoei* from the Canadian Arctic, isolate K-0399. Fig. A–B: Differential interference contrast, C–E: Flourescence microscopy of CalcoFlour White stained cells. F-I: Schematic diagram of the thecal plates deduced from flourescence microscopy. A. Ventral view showing the large posterior nucleus (N). B. Sausage-shaped chloroplasts (c) located in the cell perimeter. C. Ventral view, note the slightly displaced cingulum. D. Dorsal view. E. Oblique apical view. The individual thecal plates are labeled according to the Kofoidian plate formula in C-I. The plate tabulation for the Arctic *Scrippsiella* aff. *hangoei* is po, x, 4', 3a, 7", 6c, (t+5c), 6s, 5"', 2"".

IV

Marine-derived dinoflagellates in Antarctic saline lakes: community composition and annual dynamics

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The saline lakes of the Vestfold Hills in Antarctica offer a remarkable natural laboratory where the adaptation of planktonic protists to a range of evolving physico-chemical conditions can be studied. The present study illustrates how an ancestral marine community has undergone radical simplification leaving a small number of well-adapted species. Our objective was to investigate the species composition and annual dynamics of dinoflagellate communities in three saline Antarctic lakes. We found that dinoflagellates occur year-round despite extremely low PAR during the southern winter, which suggests significant mixotrophic or heterotrophic activity. Only a handful dominant dinoflagellate species were found in each lake, in contrast to the species rich Southern Ocean from which the lake communities are believed to be derived. We verified that the lake species were representatives of the marine polar dinoflagellate community, and not freshwater species. *Polarella glacialis* Montresor et al., a bipolar marine species, was for the first time described in a lake habitat, and was an important phototrophic component in the higher salinity lakes. In the brackish lakes, we found a new sibling species to the brackish-water species *Scrippsiella hangoei* (Schiller) Larsen, previously observed only in the Baltic.

Introduction

The microbial communities of Antarctic lakes are of great scientific interest for at least two fundamental reasons. First, they offer a system for studying microbial evolution and diversity in a geographically isolated region. The separation of the Antarctic from the other southern continents and the formation of the polar front occurred over ten million years ago (Vincent 2000). Consequently, organisms on the Antarctic continent have either been isolated for millions of years, or have colonized the continent from the sea or through long distance dispersal (Marshall 1996). Secondly, the Antarctic lake communities make it possible to study adaptations to an extreme environment, including very low temperatures and high variability in salinity conditions, in combination with long periods of little or no light availability. Ex-

treme environmental conditions are likely to exert considerable abiotic selection pressure on the organisms present. In particular, due to the different physicochemical characteristics of the lakes, selection will be driven in different directions from lake to lake. Thus, colonizing populations that previously experienced a broadly similar, less extreme environment would be exposed to an array of new selective conditions.

The Vestfold Hills in Eastern Antarctica, contains many marine-derived chloride-dominated lakes ranging in salinity from slightly brackish to hypersaline (Ferris et al. 1988). The saline lakes were formed by isostatic uplift leaving behind pockets of marine water, which have subsequently undergone salinity changes due to dilution from snow melt or concentration by evaporation (Adamson and Pickard 1986). Consequently, some lake waters have declined in salinity, becoming brackish or even non saline, while others have increased in salinity, by up to ten times that of seawater, becoming hypersaline (Pickard 1986). There is good paleolimnological evidence to show that periods of marine incursions and successive periods of meromixis have occurred, for example in Ace Lake (Cromer et al. 2005, Roberts and Mcminn 1999). In these instances, marine incursions are attributed to phases when eustatic sea level changes occurred more rapidly than isostatic uplift (Hodson et al. 2004). It is proposed that most lakes were isolated from the sea about 6,500–5,500 yr BP (Zwartz et al. 1998). Consequently, many of these lakes have become marine relics with a biota consisting of a subset of the marine microbial species (Rankin et al. 1999). The planktonic food webs are simple and fishless, with few or no zooplankton taxa (Laybourn-Parry 1997). Some of the brackish lakes have been invaded by species from the many freshwater lakes in the Vestfold Hills, for example rotifers, diatoms, and the cladoceran *Daphniopsis studeri* Riihe (Laybourn-Parry and Marchant 1992, Swadling et al. 2001).

The phytoplankton communities of the saline lakes in the Vestfold hills are dominated by small phototrophic flagellates, including prymnesiophytes and cryptophytes (Parker et al. 1982, Perriss and Laybourn-Parry 1997). However, dinoflagellates are also present, and were found in 14 out of 16 lakes in a previous study (Perriss and Laybourn-Parry 1997). Dinoflagellates are of particular interest since they are both diverse and abundant in the coastal waters surrounding Antarctica (e. g. Gast et al. 2006, Mckenzie and Cox 1991, Stoecker et al. 1992). Dinoflagellates include species with a range of nutritional modes spanning from obligate autotrophs to mixotrophic and completely heterotrophic (Stoecker 1999). They contain chloroplasts from a range of different origins, permanent as well as kleptoplastids (e. g. Fields and Rhodes 1991, Gast et al. 2007, Tengs et al. 2000). In addition, dinoflagellates have a number of other adaptive strategies including diel vertical migration (Heaney and Furnass 1980), cyst formation (Anderson and Wall 1978), and toxin production (e. g. Van Dolah 2000), to deal with competition, predation, and adverse environmental conditions. These characteristics probably allowed dinoflagellates to survive and proliferate in the extreme environmental conditions of the polar regions. Thus, we have turned our focus on the dinoflagellate community of the saline lakes.

The objective of the present study was to investigate the species composition and annual dynamics of the dinoflagellate communities in three saline Antarctic lakes. Annual dynamics have not previously been described for the individual dinoflagellate species in these Antarctic lakes, nor have the species identities been confirmed by SEM or DNA sequence analyses. Here, we present data on dinoflagellate dynamics in relation to environmental conditions, as well as an attempt to unravel the identity of dominant species in three brackish-saline Vestfold Hills lakes.

Materials and Methods

Three lakes in the Vestfold Hills (68° S, 78° E) (Fig. 1), Eastern Antarctica, were sampled from November 2002 to December 2003. The lakes included slightly brackish Highway Lake (salinity of 5) of 0.2 km^2 and a maximum depth of 15 m. Brackish Pendant Lake (salinity 16 – 20, hereafter referred to as brackish-saline) with a maximum depth of 12 m and an area of 0.16 km^2, and the saline and meromictic Ace Lake (salinity 18 in the upper mixolimnion), with a maximum depth of 20 m, and an area of 0.16 km^2. In Ace Lake the chemocline occurred between 8–10 m. All three

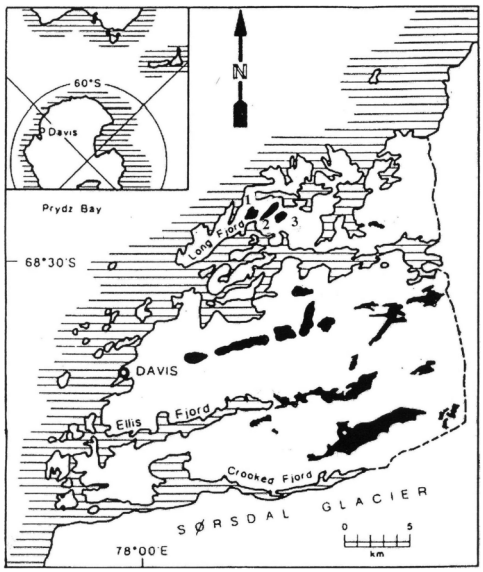

Figure 1. A map of the Vestfold Hills showing the larger freshwater and saline lakes. The study lakes are situated on Long Peninsula as follows: 1 – Ace Lake, 2 – Highway Lake, 3 – Pendant Lake.

lakes are ice-covered for at least 10 months per year, but are usually partially or completely ice-free during late summer.

Annual Sampling. The three lakes were sampled on a biweekly or monthly basis, except for a period during March and April when the lakes were not accessible due to logistic and sea ice conditions. Except when lakes were ice-free, a hole was drilled at the deepest point in each lake with a Jiffy drill. The water column was sampled at 2 m (immediately under the ice), 4 m, 6 m, 8 m depth in all three lakes. Ace Lake was also sampled at 10 and 12 m depth. Samples of 2.5 L were collected using a Kemmerer sampler. Subsamples of 1 L were preserved with Lugol's solution for dinoflagellate counts, concentrated by settling and counted in a Sedgewick-Rafter counting chamber under DIC and phase microscopy at × 320. Epifluorescence microscopy was used on fresh samples at the time of collection to determine whether particular species were phototrophic or heterotrophic. Other sub-samples were analysed for pH, conductivity, temperature, inorganic nutrients (NH_4, PO_4, NO_3) dissolved organic carbon (DOC), and chlorophyll *a*. Aliquots (1 L) were filtered through ashed 47 mm GF/F filters and the filtrate was used for chemical analyses of nitrate (NO_3–N) and ammonium (NH_4–N) content according to the methods of Parsons et al. (1984) and soluble reactive phosphorus (PO_4–P) using the protocol of Eisenriech et al. (1975). DOC was measured using a Shimadzu TOC 5000 carbon analyser (GMI, Inc., Ramsey, USA). Chlorophyll *a* on the filters was extracted in methanol at –20 °C overnight and assayed spectrophotometrically according to the method of Talling (1974).

Photosynthetically active radiation (PAR) profiles, using a PAR sensor (LI-COR LI-192 SA, flat cosine collector (LI-COR, Lincoln, Nebraska, USA)), was measured on the ice surface and at 1 m depths intervals. Ice thickness and snowcover was measured on each sampling occasion.

Dinoflagellate Abundance. Dinoflagellate mean cell counts were calculated for the top 8 m of the lakes by making volume-weighted calculations. Volumes of 2 m depth layers (0–2, 2–4, 4–6, 6–8) were estimated using lake area, maximum depth, and mean depth. Since the morphometry of the lakes was unknown, a conical shape for the lake was assumed. The volume of each depth interval, was then calculated by assuming a circular surface area and using the formula V = mean depth × radius^2 × 1.45 (Wetzel 2001). The contribution (weight) of each 2 m layer in the lake was determined by setting the smallest (6–8 m) volume to 1. The cell counts for each 2 m layer were then averaged, taking the weight of each into account. In other words, surface layers with the largest volume usually accounted for 1.6 × that of the deepest layer. Integrated cell counts were of the same order as simple non-weighted averaging of the abundance at different depths, and differed by 5–25%. Physico-chemical parameter means were calculated by averaging the top 8 m.

Light climate. To compare the light climate in the three lakes, the extinction coefficient, Kd, was calculated for each lake and each sampling occasion using the equation (Kirk 1994):

$E_z = E_o \times e^{-Kdz}$. E_z is the light intensity at depth z, and E_0 at the depth above, using 2 m intervals. Kd was determined from loglinear regressions using Microsoft Excel.

Statistical analyses. In order to determine which environmental factors may have influenced the dinoflagellate abundance in each lake, a prin-

cipal component analysis (PCA) was performed. All the measured physico-chemical data and *Mesodinium* abundance (a dinoflagellate competitor) was included. All values were natural log transformed to achieve normality. Components with Eigen values >1 were retained and tested for statistical significance with regression analysis. The factors of significant components with an absolute loading over 0.5 were considered to be important, and were subsequently tested one by one by Spearman's correlation analysis against dinoflagellate abundance.

Key lake parameters (pH, conductivity, temperature, nutrients, DOC, dinoflagellate abundance) were compared between lakes using one-way-ANOVA. All statistical analyses were performed using SPSS v. 11 for Macintosh (SPSS Inc., Chicago, USA).

Establishment of single-cell cultures, DNA extraction, sequencing and genetic analyzes. In the Antarctic summer of 2004, dinoflagellates were isolated from different lakes in the Vestfold Hills area. Tow samples using 10 µm plankton sieves were taken from lakes included in this study, as well as some additional lakes. Single cells were isolated from tow samples using micro capillary pipettes. Cells were placed in f/2 medium based on GF/C-filtered lake water. The two species that survived in culture were later identified based on ribosomal DNA sequences and SEM analyses.

DNA was extracted from clonal culture strains (our isolates and the public available ones CCMP1383/2088) following a phenol-chloroform protocol (see Adachi et al. 1994). For this work, we used partial small subunit nuclear (SSU) ribosomal DNA (rDNA). SSU PCR amplifications were done using 25 ng of template genomic DNA, 0.125 mM of each nucleotide, 3.0 mM $MgCl_2$, 1X PCR buffer, 0.4 µM of each primer and 0.5 u of Taq DNA Polymerase (AmpliTaq, Applied Biosystems, Foster City, California, USA) in 25 µL total volume reactions. For the SSU PCR we used the combination of the universal primers 4616 (forward) 5′-AACCTGGTTGATCCTGCCAG-3′ and 4618 (reverse) 5′-TGATCCTTCTGCAGGTTCACCTAC-3′. The SSU PCR started with 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C, 2 min at 72 °C and ended with 7 min at 72 °C. The complete PCR product was around 1700 bp. For one strain, *Scrippsiella* aff. *hangoei*, the small subunit ribosomal size was 1232 bp since we were not able to sequence the entire fragment, although the complete PCR product was also around 1700 bp.

All PCR amplicons were cleaned using PCR-M™ Clean-Up System (Viogene, Taiwan). SSU amplicons were directly sequenced using the PCR primer 4616, plus the sequencing primers 516F 5′-CACATCTAAGGAAGGCAGCA-3′, 528F 5′-CGGTAATTCCAGCTCC-3′, 690F 5′-CAGAGGTGAAATTCT-3′ and 1055F 5′-GGTGGTGCATGGCCG-3′ (Edvardsen et al. 2003). The sequencing reaction was carried out using BigDye (v1.1, Applied Biosystems, Foster City, California, USA) chemistry and the products were precipitated according to the manufacturer's instructions and then loaded into an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, California, USA). The obtained sequences were edited and assembled by carefully analyzing the chromatograms using Bioedit (ver. 7.0.4.1; (Hall 1999)). Sequences were deposited in Genbank under the accession numbers: EF434275/6/7, EF417317, EF417318. The obtained SSU sequences were manually aligned and visually analyzed, and the sequences were aligned along with the most similar sequences obtained by a BLAST search in Genbank (*Peridinium aciculiferum* Lemmermann /*Scrippsiella hangoei* (Schiller) Larsen and *Polarella glacialis* Montresor, Procaccani & Stoecker) using ClustalX (v1.8;(Thompson et al. 1997)). The percentage of nucleotide differentiation between these species was calculated.

SEM analyses. Two different fixation protocols were used for SEM preparation. Protocol 1, used for the species identified as *Polarella glacialis* (Abraxas-strain) was a slightly modified version of Párducz's (1967) protocol: 300 µL culture was added to 300 µL fixative cocktail consisting of 200 µL saturated $HgCl_2$ and 100 µL OsO_4. Cells were fixed for 30 min while settling on a poly-L-lysine covered coverslip. Protocol 2 used for the species identified as *Scrippsiella* aff. *hangoei* (strain High-4): Cells were fixed in 5% glutaraldehyde and stored at 5 °C for nearly 10 months. Cells were then concentrated onto a 5 µm Isopore filter (Millipore, Billerica, USA) for further processing.

In both protocols the material was washed in DH_2O for one hour and dehydrated in an ethanol series: 30%, 50%, 70%, 96%, and 99.9 for 10 min in each change and finally twice in 100% ethanol for 30 min in each change, achieved by the addition of molecular sieves to 99.9% ethanol. Critical point drying was made using a BAL-TEC CPD-030 (Boeckeler Instruments, Inc., Tucson, Arizona, USA). Filters or coverslips were mounted on stubs and coated with Pt, and examined using a JEOL JSM-6335F (JEOL Ltd., Tokyo, Japan) field emission scanning electron microscope.

Results

Dinoflagellate species composition. The dinoflagellate communities in the saline lakes were typically dominated by only 3–4 dinoflagellate species including heterotrophic taxa. In the slightly brackish Highway Lake (salinity ~5), there were two dominant phototrophic species; one (or more) unidentified naked gymnodinoid species referred to as '*Gymnodinium*' spp. of 15–25 µm (Fig. 2a) and one thecate species. Using our single-cell isolated cultures, the thecate species could be identified by SEM and the partial SSU sequence. The plate pattern corresponded to that of brackish *Scrippsiella hangoei*. Some variations were noted in the shape of plate 1a, 2a and 4", similar to the variations observed by Larsen et al. (1995). Thus, in some cells plate 4" was almost quadrangular and separated from plate 1a (penta), which was 5-sided (Fig. 3c). In other cells, plate 1a was six-sided and shared a side (suture) with plate 1a. Penta and hexa configurations of plate 2a were also observed (not shown). When analyzed, the *Scrippsiella* aff. *hangoei* partial SSU sequences turned out to be very similar (0.2% difference in 1200 bp) yet not identical to those of the morphospecies *Scrippsiella hangoei* / *Peridinium aciculiferum* (data not shown). Since the partial SSU sequence was not identical, and other sequences (e.g. ITS , Logares et al. submitted) also had discrepancies,

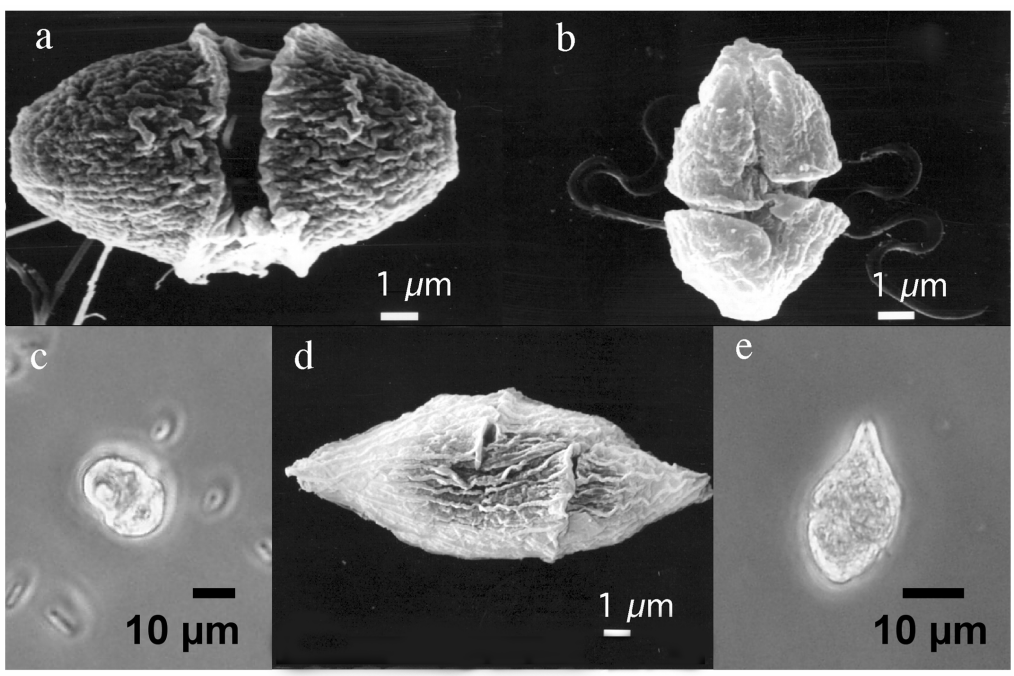

Figure 2. SEM and light microscope (LM) images of Lugol's preserved '*Gymnodinium*' spp. cells from Highway Lake (a–c) and SEM and LM images of *Gyrodinium glaciale* in natural samples from Ace Lake (d–e). SEMs courtesy of Fiona Scott, Antarctic Australian division, cells fixed in glutar-aldehyde and prepared as described in Scott and Marchant (2005).

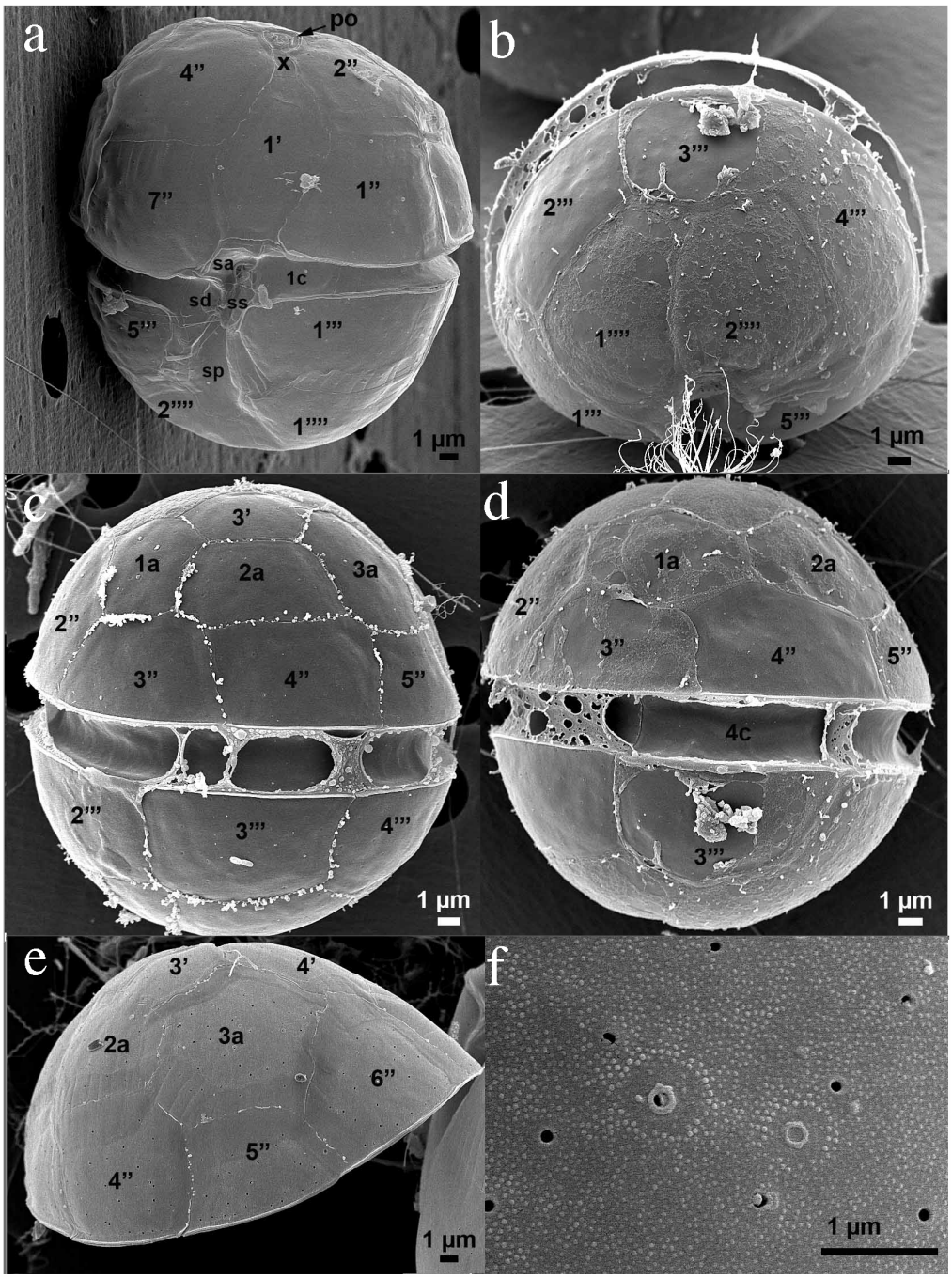

Figure 3. SEM of *Scripsiella* aff. *hangoei* culture established from Highway Lake. a) Ventral view. b) Antapical view. c & d) dorsal views showing variations in the shape of plates 1a, 2a and 4”. e) Right-dorsal view of a separated epitheca. f) High magnification of a thecal plate revealing its knobby surface and trichocyst pores.

we cannot with any certainty determine whether or not it is the same species as the Baltic *S. hangoei* cultures. Thus, we have decided to use the term affinis (aff.) as having affinity with but not identical with. The third dominant species was the marine heterotrophic species *Gyrodinium glaciale* Hada, which was identified by SEM-analyses (Fig. 2b) from plankton tows. In previous studies (Laybourn-Parry et al. 2002, Perriss and Laybourn-Parry 1997), this species has been referred to as *G. lachryma* (Meunier) Kofid & Swezy, but based on its small size and large girdle displacement we suggest that it is a *G. glaciale.* Balech (1976) observed small circular chloroplasts in this species (probably kleptoplasts). Although we did not observe these, their presence cannot be ruled out.

In Pendant Lake (salinity 16–20), there were four dominant dinoflagellate species. These included *G. glaciale*, the *S.* aff. *hangoei* species also found in Highway,'*Gymnodinium*' spp (Fig. 2), and the marine bipolar species *Polarella glacialis* (Montresor et al. 1999, 2003) (Fig. 4). *P. glacialis* was the dominant phototrophic dinoflagellate in Pendant Lake. The complete SSU rDNA sequences (from cultures isolated in Ekho and Abraxas lakes since no culture survived from Pendant Lake) were identical to the *P. glacialis* clones CCMP 1383 from Antarctica (McMurdo Sound) and CCMP 2088 from the Arctic (Baffin Bay, Canada). The two CCMP sequences were identical to each other when we sequenced them anew, contrary to the findings of Montresor et al. 2003. SEMs of both vegetative cells and cysts corresponded to the general morphology of *P. glacialis* (Fig. 4).

In saline, meromictic Ace Lake (17–34 psu), the dinoflagellate community was dominated by the heterotrophic or possibly mixotrophic *G. glaciale*, '*Gymnodinium*' spp., and low numbers of *Polarella glacialis*.

Seasonal dynamics. Highest mean and median abundances of dinoflagellates (Table 1) were found in Pendant Lake. Here, yearly mean abundances were over 7, 000 cells · L^{-1}, which is almost 2.5 times higher than in Highway Lake (ANOVA, p = 0.024). In Highway Lake, the community was 95% (by abundance) phototrophic (i.e. excluding *G. glaciale*) while in the other two lakes the heterotrophic-mixotrophic component was much higher with only ~ 45% phototrophs.

Highway Lake was characterized by very low dinoflagellate abundance in the winter (Fig. 5a)

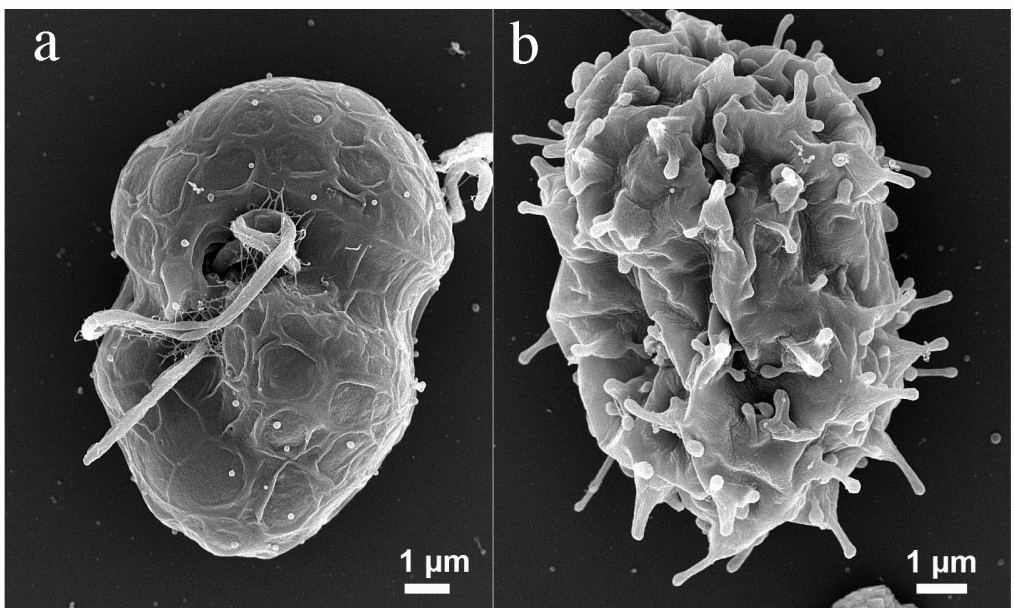

Figure 4. SEM images of *Polarella glacialis* culture established from Lake Abraxas (salinity similar to Pendant), morphology identical to cells from Pendant and Ace lankes. a) ventral view of a vegative cell. b) Spiny resting cyst.

Table 1. Year mean abundance of dinoflagellates and *Mesodinium*.

Lake	Chlorophyll a Mean ($\mu g \cdot L^{-1}$)	Dinoflagellate abundance Mean (cells $\cdot L^{-1}$)	Dinoflagellate abundance Mean (cells $\cdot L^{-1}$)	Phototrophic Dinoflagellate abundance (cells $\cdot L^{-1}$)	Proportion phototrophic dinoflagellates	*Mesodinium rubrum* abundance (cells $\cdot L^{-1}$)
Highway	2.2^{ab}	2645^{b}	224	2532	0.96	19075^{ab}
Pendant	4.4^{b}	7076^{b}	4402	3309	0.46	1173^{b}
Ace	4.2^{a}	4829	3682	2057	0.42	5845^{a}

[a] or [b] indicate significant difference (ANOVA, LSD post hoc, $p<0.001$) between pairs with the same letter. Photorophic dinoflagellates included all species except *G. glaciale*.

and distinct peaks in abundances during the summer. All three major species (including the *G. glaciale*) peaked in abundance in December of both years. From April – November there were few or no dinoflagellates present in the water samples. By mid-November dinoflagellate abundance started increasing again. In contrast to the other lakes, the abundance of *G. glaciale* was overall low, never exceeding 1,000 cells $\cdot L^{-1}$.

In Pendant Lake, the phototrophic *P. glacialis* dominated the community during the summer reaching up to a maximum of 8,000 cells $\cdot L^{-1}$ (Fig. 5b). However, total abundance of dinoflagellates was highest in the winter. All four species were present in the water from November until the end of June, when solar radiation was close to zero. At this time, a peak of *G. glaciale* up to 15,000 cells $\cdot L^{-1}$ formed, and then remained in the water throughout the winter. By the end of November the other species appeared in the water samples again.

Ace Lake also had a low abundance of phototrophic species (*P. glacialis* and '*Gymnodinium*' spp.) throughout the year, which did not increase substantially even during summer (Fig. 5c). The *P. glacialis* maximum was only about 1,000 cells $\cdot L^{-1}$. Instead, *G. glaciale* dominated the community as in Pendant Lake, reaching a maximum of 15,000 cells $\cdot L^{-1}$ in May.

In all lakes, the ciliate *Mesodinium rubrum* (=*Myronecta rubra*) was also enumerated, as this organism is functionally a phototrophic and mixotrophic species, similar in size to the dinoflagellates. Thus, *M. rubrum* is a potential dinoflagellate competitor. Moreover it dominated the ciliate communities of the lakes and can contribute significantly to phytoplankton photosynthesis, up to

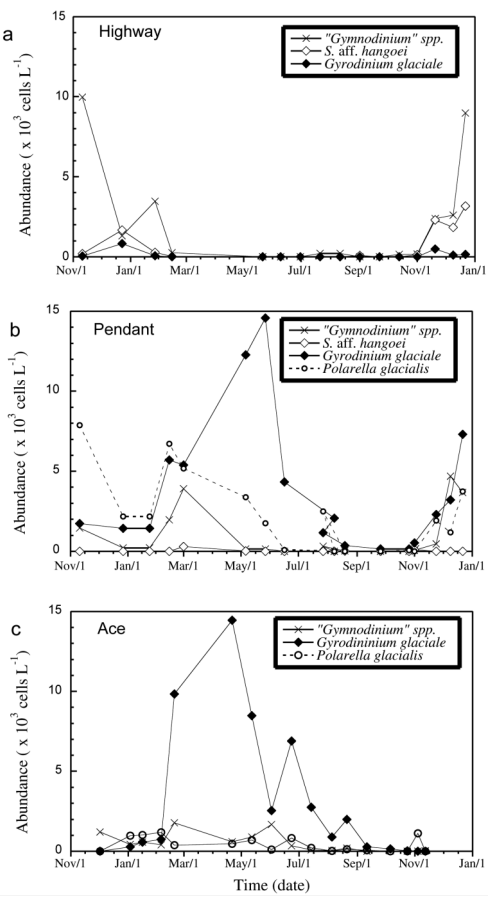

Figure 5. Dinoflagellate abundance (cells $\cdot L^{-1}$) from November 2002 to January 2004, volume weighted and averaged from samples from 2, 4, 6, and 8 m depth. a) Highway Lake, b) Pendant c) Ace Lake

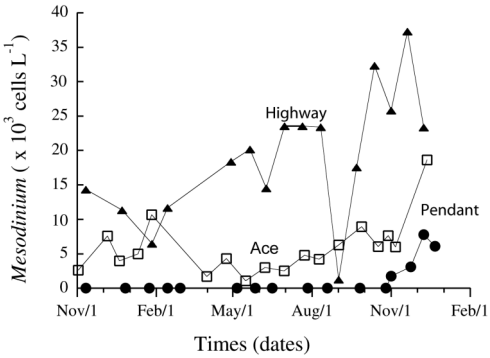

Figure 6. *Mesodinium rubrum* abundance (cells · L^{-1}) in all three lakes, volume integrated averages from 2 to 8 m, November 2002 to January 2004. Highway (filled triangles), Pendant (filled circle), and Ace Lake (open squares).

26% in Ace Lake and 40% in Highway Lake (Laybourn-Parry and Perriss 1995). The cell counts showed significantly higher mean abundance (19, 075 cells · L^{-1}) in Highway compared to Pendant and Ace (1,173 and 5,845 cells L^{-1}, respectively) (Table 1). In both Highway and Ace Lakes, *Mesodinium* was present in the water throughout the year (Fig. 6).

Physico-chemical parameters and dinoflagellate abundance. The PCA analyses were generally unable to reduce the number of parameters that may affect the variance in dinoflagellate abundance, except in Pendant Lake (Table 3). Dinoflagellate abundance in Highway had a significant ($p<0.05$) negative correlation with conductivity, nitrate, Kd and DOC, while there was a positive correlation with pH ($p<0.01$, $r = 0.68$) and temperature ($p<0.01$). In contrast, dinoflagellate abundance in Pendant Lake was negatively correlated to both orthophosphate and nitrate (Fig. 7) ($p<0.05$), and no other factors. In Ace Lake, there was a positive correlation ($p<0.05$) between light extinction (Kd) and dinoflagellate abundance (high numbers of *G. glaciale* in the winter when Kd was high). Orthophosphate, DOC and conductivity were negatively correlated with dinoflagellate cell concentration (Fig. 8). No parameter was significant in all three lakes.

The main difference between the lakes was salinity, in this study measured as conductivity (Table 2, Fig. 8). While Ace Lake had an annual mean conductivity (0–10 m) of 24.3 mS · cm^{-1},

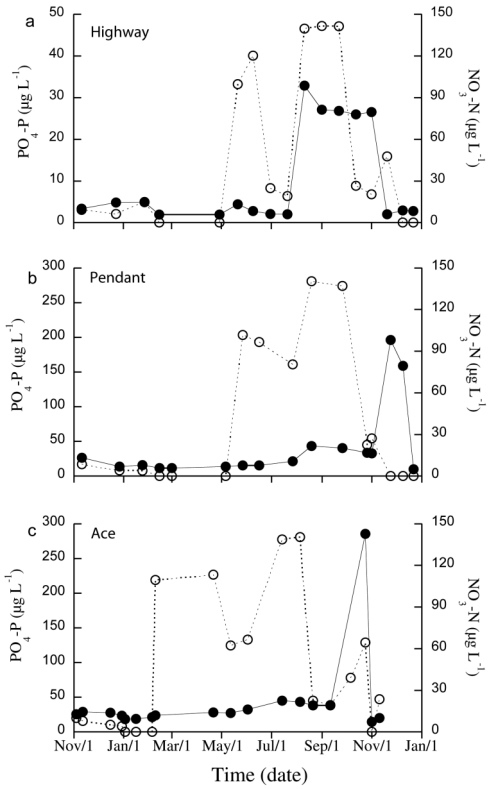

Figure 7. Concentrations of orthophosphate (filled circles) and nitrate (open circles), averaged from samples from four depths (2, 4, 6, 8 m) sampled from November 2002 to January 2004. a) Highway Lake, b) Pendant Lake, c) Ace Lake.

Pendant had 18.6 mS · cm^{-1} and Highway only 6.3 mS · cm^{-1} . Pendant and Ace lakes were both significantly different from Highway ($p<0.001$). In addition, both Pendant and Ace Lakes had higher salinities in the deeper layers of the water column. The seasonal variations were small in Highway, but varied between 15 and 25 in the upper mixolimnion of meromictic Ace Lake, presumably due to the melting of the ice cap (Fig. 9). Ace Lake had an average pH of 8.01, which was significantly higher than the pH of the other two lakes which were close to neutral (7.1–7.2) ($p<0.01$).

Another significant difference between the three lakes was their trophic status, as determined by orthophosphate concentrations. Orthophosphate in Pendant and Ace Lakes had an annual

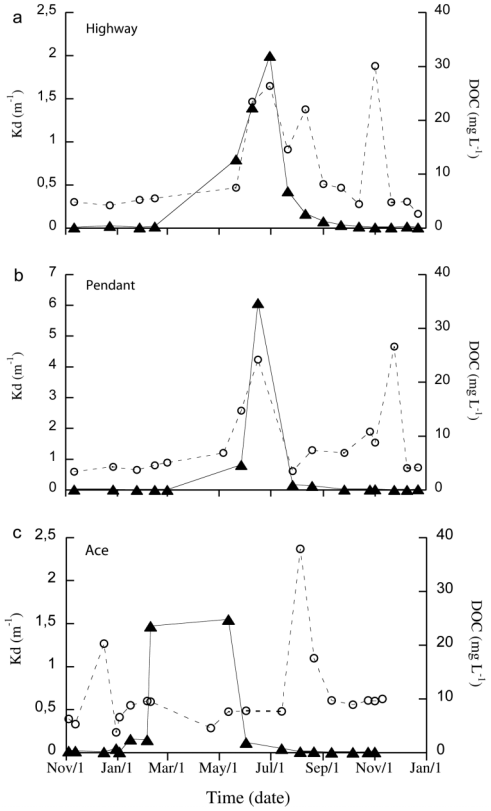

Figure 8. DOC (dissolved organic carbon) (open circles) averaged from 4 depths (2,4,6,8 m) and light extinction coefficient (calculated from surface to 8 m) Kd (filled triangles) from November 2002 to January 2004. a) Highway Lake, b) Pendant Lake, c) Ace Lake.

mean of 41.3 and 43.7 μg · L^{-1} respectively, and was thus higher ($p<0.05$) than in Highway Lake (mean10.6 μg · L^{-1}). Nitrate and ammonium concentrations were low in all three lakes and did not differ significantly. The seasonal dynamics of nitrate was very similar in all lakes, with very low levels (below 30 μg · L^{-1}) in the summer months and a maximum in late winter (September) reaching 150 μg · L^{-1} (Fig. 7). Orthosphate maxima were observed in spring /early summer in all lakes, and reached high levels (above 200 μg · L^{-1}) in Pendant and Ace lakes in November/December 2003.

Average annual temperature did not differ significantly between the lakes (Table 2), nevertheless there were differences in the dynamics. While

Table 2. Year median and mean values of the physico-chemical parameters.

Lake	Annual median/ mean	pH	Temp. (°C)	Conductivity (mS · cm^{-1})	PO_4–P (μg · L^{-1})	NH_4–N (μg · L^{-1})	NO_3–N (μg · L^{-1})	DOC (mg · L^{-1})	Kd (m^{-1})
Highway	median	7.2	1.0	6.3	3.9	16.2	21.9	6.5	0.03
	mean	7.2^{a}	0.9	6.3^{ab}	10.6^{ab}	18.3	46.3	11.0	0.32
Pendant	median	6.9	–0.3	18.8	18.6	13.3	6.3	6.0	0.02
	mean	7.1^{b}	–0.1	17.8^{a}	41.3^{a}	15.4	38.9	8.7	0.74
Ace	median	8.1	–0.8	24.3	27.7	10.9	20.9	8.9	0.02
	mean	8.01^{ab}	0.72	23.2^{b}	43.7^{b}	18.4	44.7	10.6	0.21

[a] or [b] indicates statistically significant differences (ANOVA, LSD post hoc, $p<0.01$ for conductivity and pH, and $p<0.05$ for phosphate) between pairs with the same letter.

Table 3. Component loadings of abiotic factors for the three lakes and percentage of variance explained by the retained principal component axis.

	Highway			Pendant	Ace		
Source	PC1	PC2	PC3	PC1	PC1	PC2	PC3
variance explained	42.5	22.1	15.7	49.1	36.8	20.9	16.4
eigenvalues	3.82	1.99	1.42	3.93	3.31	1.88	1.48
Temp.	–0.343	**–0.874**	0.108	**0.904**	–0.060	**0.825**	–0.301
Conductivity	**0.679**	0.421	0.146	–0.463	**0.954**	–0.214	–0.008
pH	**–0.831**	–0.322	0.115	**0.517**	**–0.618**	**0.559**	**–0.500**
Nitrate	**0.745**	0.436	–0.128	**–0.837**	**0.878**	–0.281	0.261
Phosphate	**0.865**	–0.250	0.090	0.060	**0.815**	0.385	–0.341
Ammonium	0.402	0.288	**–0.759**	**0.764**	0.050	0.106	0.147
Kd	–0.090	**0.939**	–0.107	–0.090	0.030	–0.030	**0.969**
DOC	0.321	**0.729**	0.308	–0.157	0.153	–0.157	–0.251
Mesodinium	–0.327	0.197	**0.830**	not det.	–0.185	**0.751**	0.376
p-values	0.01	<0.001	0.047	<0.001	0.004	0.027	0.015

Important (values > 0.5) loadings are shown in bold. P-values refer to the linear regression testing the difference in scores.

the winter temperature in all three lakes was close to –1°C, summer temperature varied more. In Ace the maximum temperature measured was 6.5°C, in Highway 4°C, while in Pendant the maximum was 1°C. Ice thickness data (not shown) revealed that Highway and Ace were ice-free in December and January, while Pendant retained most of its ice cover, accounting for the differences in temperature. There were no differences in light availability between the lakes, despite differences in ice thickness during summer. The calculated average and median Kd was low and similar in all lakes. DOC levels were also relatively low and similar for all lakes.

Discussion

In this study we investigated the species composition and seasonal dynamics of dinoflagellates in three Antarctic lakes, which differed in salinity. To our knowledge, this is the first study on dinoflagellate community dynamics in landlocked coastal saline lakes. In these Antarctic lakes, we found at least two species that have not previously been described in saline limnic habitats, one possibly a new species. All the dinoflagellate species that we identified to the species level in the lakes were representatives of the polar seawater dinoflagellate community, and not freshwater species. Although species diversity was much lower than in the polar seas, the total abundance was higher. Finally, we found that the dinoflagellates occur year-round despite extremely low PAR during winter, suggesting significant mixotrophic or heterotrophic activity.

The dinoflagellate communities in the saline Vestfold Hills lakes had small numbers of species, which is in sharp contrast to the species-rich polar waters. In a study of the Scotia Sea and Polar Front Zone, sixty-three armored dinoflagellates were identified and enumerated (Mckenzie and Cox 1991). Moreover, McMinn and Scott (2005) listed 70 common Antarctic dinoflagellates plus an additional twenty species which are rarely reported from Antarctic waters. Although we were not able to identify or enumerate a few rare species in our lakes, we concluded that the total dinoflagellate species richness is substantially reduced (10 to 20-fold) compared to the polar marine communities. These findings correspond to previous surveys of these lakes' summer plankton communities (Perriss and Laybourn-Parry 1997). Moreover, although species richness was lower in the lakes studied than the nearby Ellis Fjord, total dinoflagellate abundance was much higher. In the tidal Ellis Fjord, thirteen dinoflagellate species

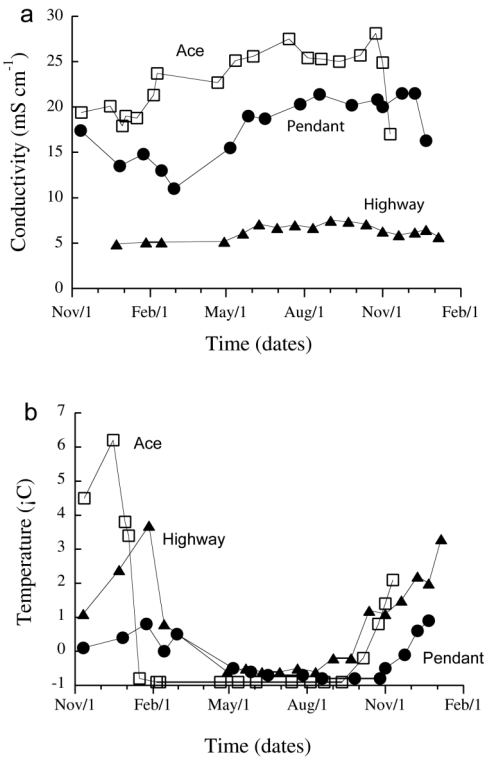

Figure 9. a) Conductivity (mS · cm^{-1}) as a proxy for salinity, averaged from 4 depths (2, 4, 6, 8 m) for all three lakes. b) Temperature (°C) averaged from 4 depths (2,4,6, 8 m) for all three lakes. Highway (filled triangles), Pendant (filled circle), and Ace Lake (open squares).

were identified and counted during winter and spring (May – October) (Grey et al. 1997). The total abundance never amounted to more than 100 cells · L^{-1}, which is substantially less than the thousands of dinoflagellates encountered during winter and spring in the studied Antarctic lakes. Likewise, a relationship between high diversity/low biomass has previously been observed in Antarctic deep sea plankton (López-García et al. 2001).

The low species richness of dinoflagellates in our lakes supports the predictions of island biogeography theory (Macarthur and Wilson 1967). This theory can be directly applied on the Antarctic saline lake system, where the lakes can be considered as separate islands, and the ocean as the mainland. Island theory explains species richness as a balance between immigration and extinction rate, and that there is a positive relationship between species richness and island (lake) area. Accordingly, small lakes would be expected to hold a lower number of species than the ocean. Smith et al. (2005) recently showed that phytoplankton adhere to this species-area relationship, and found that ecosystem size had a strong effect on local phytoplankton species richness. However, the effects of local filtering may have restricted the Antarctic lake species pool further. These lakes represent habitats that are quite different from the sea, with variable salinity, low nutrient levels (except Pendant), extreme light limitation, and different predators. Both nutrient levels and predation are known to have strong effects on diversity (Worm et al. 2002), as may island (lake) age (Borges and Brown 1999). Moreover, the fact that the lakes are young and have undergone physical evolution (salinity changes) could also contribute to the low species numbers, since changing conditions may have resulted in unusually high extinction rates or low colonization rates.

Another saline lake reported to contain marine dinoflagellates is Salton Sea in California. This large (975 km^2) shallow lake is located in the Californian desert and is inhabited raphidophytes and haptophytes in addition to marine dinoflagellates (Reifel et al. 2002). Twenty-two species of dinoflagellates have been observed in Salton Sea waters, as well as several unidentified dinoflagellates (Reifel et al. 2002 and K Steidinger, unpublished data). Just as in the Antarctic lakes, Salton Sea appears to contain fewer dinoflagellate species than marine ecosystems. However, Salton Sea has a larger number of species than the Antarctic lakes, which is consistent with the species area-relationship, as Salton Sea has a lake area that is almost 5,000 times larger than the Antarctic lakes. In contrast to the Vestfold Hill lakes, Salton Sea has never been connected to the sea, has a temperature range from 17–21°C, a salinity of about 43, and is very young (100 years) (Reifel et al. 2002).

Total dinoflagellate abundance was lowest in the brackish Highway Lake. This was also the lake with the highest proportion of phototrophic dinoflagellates, i.e. *S.* aff. *hangoei* and '*Gymnodinium*' spp. species. Highway is the lake that appears to have undergone the most significant change since its isolation from the ocean. The salinity has

decreased from >30 down to 5. Consequently, significant selection pressure for species tolerant to low salinities would be expected in this lake. Salinity differences impose an important physiological barrier to many species, and a recent study shows that only a few dinoflagellate taxa have made the evolutionary transitions between marine and freshwater environments (Logares et al. In press). Nevertheless, several dinoflagellates (e.g. *S. hangoei*) (Logares et al. 2007) can tolerate wide salinity ranges (Kremp et al. 2005). Thus, it is likely that a similar diversity of dinoflagellates to that found today in Ellis Fjord were trapped in Highway Lake. Following the break of the marine connection and as salinity declined due to freshwater input, most species became extinct.

The species we identified in the Vestfold Hills lakes were either identical to or similar to known marine species. In a previous study, (Perriss and Laybourn-Parry 1997), *Gonyaulax* cf *tamarensis* was reported as the most common phototrophic thecate taxa in the brackish lakes (including Highway and Pendant). Although *Gonyaulax tamarensis* Lebour has been transferred to *Alexandrium tamarense* (Lebour) Balech, we have no evidence that this was the species observed previously, nor that *A. tamarense* was present in the lakes. We suggest that "*Gonyaulax* cf *tamarensis"* was misidentified and is the *Scrippsiella* aff. *hangoei* species that we have described. The plate pattern of this species corresponded to that of *S. hangoei* as described by Larsen (1995). However, the partial SSU rDNA region differed in a few base pairs (~0.2%) from the *S. hangoei* isolated and sequenced from the Baltic Sea (Logares et al. 2007). Although the difference is small, the sequences are not identical, suggesting that these populations have not diverged very recently. As we have earlier observed identical SSU, LSU, and ITS sequences of *S. hangoei* and *P. aciculiferum*, despite different morphology, physiology, and habitat (Logares et al. 2007), we cannot with any certainty claim that the Baltic *S. hangoei* and the Antarctic *S*. aff. *hangoei* are the same species. The two populations may have undergone rapid allopatric speciation, but this issue will be further discussed elsewhere (Logares & Rengefors, unpublished data).

Most likely, *S.* aff. *hangoei* in the Vestfold lakes originate from a population in the polar ocean (rather than long-distance dispersal from the Baltic Sea). Although we observed cells that look like the *S.* aff. *hangoei* species in our samples from the sea, we were not successful in establishing these in culture and thereby unable to confirm their identity. These observations indicate that *S.* aff. *hangoei* can tolerate a salinity between 25 – 35. Despite this, *S.* aff. *hangoei* was not observed in Ace Lake nor any of the hypersaline lakes (Perriss and Laybourn-Parry 1997) suggesting low tolerance to high salinities. Similarily, the sibling species *S. hangoei* from the brackish Baltic Sea has been shown to have a salinity tolerance range from 0 to 30 (Logares et al. 2007), yet is not found in the adjacent saline North Sea. Possibly it is the salinity fluctuations, rather than just high salinity that prevent *S.* aff. *hangoei* from becoming common in Ace Lake, but further experimentation is necessary to elucidate this point.

In the more saline lakes (Pendant and Ace) we found that the bi-polar marine species *Polarella glacialis* was a common and abundant component of the dinoflagellate community. This species was recently described from Antarctic and Arctic seawater and ice (Montresor et al. 1999, 2003, Thomson et al. 2004). In contrast to *S.* aff. *hangoei*, the SSU sequences were identical for all the *P. glacialis* strains that we sequenced. Moreover, inspection of SEM micrographs of an unidentified gymnodinoid species and its cysts from McMurdo Sound, Antarctica in Stoecker (1992), suggests that this is also *P. glacialis*. The various cold-water habitats in which *P. glacialis* has been found indicate that it has a wide salinity tolerance. However, in the least saline lake (Highway), *P. glacialis* was not observed, suggesting that there could be a minimum salinity requirement for this species. However, other factors such as competition and predation cannot be ruled out. Indeed, in contrast to the other lakes, Highway is inhabited by the large freshwater cladoceran *Daphniopsis studeri*, a potential grazer.

In the saline lakes studied, many of the dinoflagellates were encountered year round despite the extremely low PAR during a large portion of the year. This implies that most species were either mixotrophic or heterotrophic. In an environment with a poor light climate, phototrophic dinoflagellates have two possible strategies. They can either form cysts and rest during the periods of low light and cold temperatures as is the case

for many marine and freshwater dinoflagellates (Dale 1983, Rengefors 1997). The other option is to adopt mixotrophic nutrition and acquire carbon through osmo- or phagotrophy (Caron et al. 1990, Hansen et al. 1994, Jacobson and Anderson 1996, Legrand and Carlsson 1998). *P. glacialis* cysts were observed in the water samples, and in July to October cell numbers were at or close to zero. The data suggest that this species overwinters in the benthos, but it is possible that some cells survive in the water as our cell counts indicate. Cysts (equivalent to those observed in the field) were also formed in old clonal cultures. The *S. hangoei*-like taxon was absent from the water samples most dates during May – October, suggesting that this species also forms overwintering cysts. Cysts were observed in clonal cultures of this species as well as has been found in the Baltic *S. hangoei* (Kremp and Parrow 2006). Nevertheless, it is possible that both species have the additional potential for mixotrophic nutrition, utilizing osmotrophy or phagotrophy to supplement photosynthesis.

Gyrodinium glaciale was the dominant heterotrophic dinoflagellate in all three lakes. We consider the species referred to as *Gyrodinium lachryma* by Laybourn-Parry et al. (2000, 2002, 1997) as *G. glaciale*, since the cells in our material were not larger than 25 µm, while *G. lachryma* have large cells ranging from 60 to 135 µm long (Balech 1976). In Pendant and Ace Lakes, this species forms a population peak in May–June, corresponding to early – mid-winter. The reason for the absence of this peak in Highway Lake is unknown, but it could be related to the lower salinity or higher abundance (over 20,000 cells · L^{-1} during winter) of the mixotrophic ciliate *M. rubrum*, a potential competitor, in this lake. The '*Gymnodinium*' spp. species in the three lakes did not survive in culture, its (their) identity therefore remains uncertain, and can only be resolved by a future study.

Contrary to what one might suppose, Antarctic lakes function throughout the year. The ice usually lacks snow cover allowing good transmission of light. Photosynthesis starts as soon as the light returns in late winter and bacterial production continues throughout the winter (Laybourn-Parry 2002, 2003). Consequently significant levels of dissolved organic carbon are present throughout the year as are bacteria and both heterotrophic and phototrophic flagellated protozoa. Mixotrophy is a common phenomenon among some of the major phototrophic components in Antarctic lakes involving the ingestion of bacteria and dissolved organic carbon, thereby allowing active populations to function over the winter period (Laybourn-Parry et al. 2005, Marshall and Laybourn-Parry 2002). Moreover, there is now evidence of high virus activity within Antarctic lakes suggesting that viral activity may play a significant role in carbon recycling (Madan et al. 2005).

Our PCA analyses between dinoflagellate dynamics and physico-chemical parameters revealed that all parameters measured could in part explain the variation in dinoflagellate abundance. However, only temperature, nitrate, and pH were important in all three lakes. Nevertheless, none of these factors were significantly correlated to dinoflagellate abundance on their own. These results are not surprising as light, temperature, and nutrients are all known to affect phytoplankton growth, while it is also known that phytoplankton can influence these parameters as well as DOC and pH. The only clear trend was that salinity (measured as conductivity) and phosphate appeared to have a substantial effect on both abundance and species composition in our between-lake comparison.

The saline lakes of the Vestfold Hills offer a remarkable natural laboratory in which to study how planktonic protists have adapted to a range of evolving physico-chemical conditions. The present study illustrates how the ancestral marine community has undergone radical simplification leaving a small number of well-adapted species. Over the relatively short time since isolation from the sea after the last major glaciations, distinct lacustrine populations have most probably evolved. Further work will show whether these populations are phenotypically and/or genetically distinct from each other, and from populations of the same species in the sea.

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V

Patterns of genetic diversity and differentiation among five protist (Dinophyceae) species

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Little is known on the genetic diversity within and among microbial lake-populations. Here, we have investigated the patterns of genetic variation in strains from five protist (dinoflagellate) species. These species are present in lacustrine and marine habitats in polar and temperate regions. A total of 68 dinoflagellate strains were investigated using Amplified Fragment Length Polymorphism (AFLP). To reduce potential biases introduced by cryptic species, all strains from the same species had homogeneous ITS nuclear ribosomal DNA. Overall, our results suggest that a multitude of dinoflagellate genotypes coexist within lake-populations, instead of a few genotypes dominating each lake. We have found a wide variability in the levels of intraspecific genetic diversity when the five species were compared (between 20–90 % of polymorphic loci; Nei's gene diversity between 0.08 – 0.37). We have also found evidence of genetic populations, which appeared not correlated with the lakes. Some of the genetic populations were sympatric within lakes, indicating that different genetically homogeneous groups could coexist despite the apparent presence of recombination.

Introduction

Little is known about the genetic diversity patterns within and among microbial populations inhabiting lakes and ponds. Marine environments do not have obvious geographical features which could be considered to act as barriers for microbial dispersal. Conversely, available data indicate that the exchange of aquatic microbes between lake-populations can be restricted (Hughes Martiny et al., 2006). Such dispersal restrictions can affect the genetic composition of microbial lake-populations.

For several marine microbial species, high amounts of intraspecific genetic diversity have been found on both spatial and temporal scales (e.g. Bolch et al., 1999a; Bolch et al., 1999b; Medlin et al., 2000; Rynearson and Armbrust, 2000; Shankle et al., 2004; Evans et al., 2005; Ig-

lesias-Rodriguez et al., 2006; Nagai et al., 2007). The few available studies also indicate high intraspecific genetic diversities within microbial lake and pond populations (e.g. Hayhome et al., 1987; Kusch et al., 2000; Coleman, 2001; De Bruin et al., 2004; Muller et al., 2005; Wilson et al., 2005; Zhang et al., 2006).

Several marine protist populations have recently been shown to be genetically differentiated in time and space (e.g. Medlin et al., 2000; Rynearson and Armbrust, 2004; Shankle et al., 2004; Nagai et al., 2007). For ponds and lakes, there is little data on the genetic differentiation among and within populations. In the cyanobacteria *Microcystis*, there is one study showing significant genetic differentiation among lake-populations (Wilson et al., 2005), while another study did not indicate such genetic structuring pattern (Janse et al., 2004). In lacustrine ciliates, high genetic diversity was found within populations, but little differentiation among them (Kusch et al., 2000; Zhang et al., 2006). It should be noted however, that the contradictory findings of some of the previous studies could be due to different species having been considered as different populations. This concern is based on recent observations indicating that several well-known microbial morphospecies actually consist of species complexes (e.g. Coleman, 2001; Casamatta et al., 2003; Montresor et al., 2003b; Bensch et al., 2004; Beszteri et al., 2007).

To date, the possibility that genetically differentiated and sexually reproducing microbial populations can coexist within the same lake has been little explored. A number of studies have found ecophysiological evidence suggesting that genetically differentiated populations of protists can coexist within one lake (see Weisse, 2002; Weisse and Rammer, 2006). In higher animals, where the structuring patterns of the genetic diversity have been studied much more extensively, there are a variety of examples of intra-lake genetically differentiated populations (e.g. Dynes et al., 1999; Wilson et al., 2004). Due to the high genetic diversity observed in some microbial populations, the presence of genetic structure within lakes is a likely scenario. However, the latter remains to be demonstrated.

Many eukaryotic microbes are characterized by extensive asexual reproduction and episodic sex. This reproductive strategy could affect the genetic diversity within microbial lake-populations (e.g. De Meester et al., 2006). In stable habitats, theoretical models predict that populations of species which reproduce asexually and sexually will become dominated by relatively few well-adapted genotypes (see Williams, 1975; Maynard Smith, 1978). In contrast, in environments where there are moderate levels of variation and disturbance, higher levels of genotypic diversity would be expected (Williams, 1975). More recent investigations indicate that other variables should be considered in order to understand the diversity found within lacustrine multi-clonal planktonic populations with episodic sex. For instance, evidence from zooplankton studies indicates that the length of the planktonic phase, the size of the waterbody, the egg population-size in the sediments, and the strength of selection, can affect the genetic diversity of multi-clonal planktonic populations (De Meester et al., 2006; Ortells et al., 2006; Vanoverbeke et al., 2007).

Although the factors that affect the genetic diversity have been investigated in planktonic populations of lacustrine metazooplankton (e.g. De Meester et al., 2006), little is known about planktonic protists with similar reproductive strategies, such as the dinoflagellates. Most lacustrine dinoflagellate species reproduce clonally by cell division during the planktonic phase, and sexually when the conditions for clonal growth are not favorable anymore (Pfiester and Anderson, 1987). In several dinoflagellate species, sexual reproduction is linked to the production of resting cysts which have environmental resistance and dispersal functions (see Pfiester and Anderson, 1987). In lakes and ponds, the germination of cysts may restore the multi-clonal planktonic populations when favorable environmental conditions return.

Dinoflagellates are ubiquitous unicellular eukaryotes with important roles in marine and freshwaters communities. They have a high diversity of life strategies, with symbionts, parasites, photosynthesizers, heterotrophs and mixotrophs (Hackett et al., 2004). Planktonic dinoflagellates are normally haploid (Von Stosch, 1973). Some species can produce potent toxins during algal blooms, thus representing an important concern for human and ecosystem health as well as local economies (Hallegraeff, 1993).

Here, we have investigated the genetic diversity patterns of variation among strains from five dinoflagellate species. These species occur in marine and lacustrine (freshwater-hypersaline) habitats of polar and temperate regions. Specifically we asked: (1) does one or a few dinoflagellate genotypes dominate the population of a lake, or are there several coexisting genotypes (2) is the genetic diversity within a lake structured into different genetic populations, and finally (3) are dinoflagellate lake-populations genetically differentiated among each other? To investigate these questions, we have used the Amplified Fragment Length Polymorphisms (AFLP) technique (Vos et al., 1995) on dinoflagellate strains from five species. AFLP has proved very useful to resolve and assign strains and clones of microbes (e.g. Duim et al., 2000). Within each species, all strains had homogeneous Internal Transcribed Spacer (ITS) sequences in the nuclear ribosomal DNA (nrDNA). The ITS nrDNA is a very rapidly evolving marker which is used to discriminate among species and cryptic species in dinoflagellates (see Litaker et al., 2007). The use of dinoflagellate strains with homogeneous ITS nrDNA was intended to minimize potential problems which could arise due to cryptic species. This is specially relevant since several cases of cryptic species have been reported for dinoflagellates (e.g. Montresor et al., 2003b; Kim et al., 2004; Lilly et al., 2005; Gribble and Anderson, 2007).

Materials and Methods

Collection of dinoflagellates and culturing

All dinoflagellate clonal cultures were obtained by isolating single cells from plankton samples, except when specified otherwise. The *Peridinium aciculiferum* Lemmermann isolates were obtained from the lakes Tovel (Italy), Stora Pildammen (Sweden), Brodammen (Sweden), Erken (Sweden) and Österträsk (Finland) [see details in Table 1]. *Peridinium cinctum* PCGY strains were obtained from the lake Gyllebosjön (Table 1). The remaining *Peridinium cinctum* strains were obtained from culture collections: SAG2017 (Sammlung von Algenkulturen der Universität Göttingen; SAG), CCAC 0102 (Culture Collection of Algae at the University of Cologne; CCAC). The strains of *Peridiniopsis borgei* were all isolated from Stora Kalkbrottsdammen (Table 1). *P. aciculiferum* was cultured in modified Woods Hole medium (Guillard and Lorenzen, 1972; MWC, 0 salinity) prepared with MilliQ water (Millipore Corp., Bedford, USA). *P. cinctum* and *Ps. borgei* were cultured in MWC medium based on sterile-filtered Gyllebosjön and Stora Kalkbrottsdammen water respectively.

In the Antarctic summer of 2004/5, dinoflagellates were isolated from different lakes in the Vestfold Hills area (68° S, 78° E), Eastern Antarctica. The Vestfold Hills is an ice-free coastal area in Princess Elizabeth Land, that was formed by isostatic uplift after the last glaciations ~6,000 year ago (Zwartz et al., 1998). The lakes sampled included Lake Abraxas (brackish; *Polarella glacialis*), Ekho Lake (hypersaline; *Pa. glacialis*), Highway Lake (brackish; *Scrippsiella* aff. *hangoei*), Vereteno Lake (brackish; *S.* aff. *hangoei*), Lake Hand (brackish; *S.* aff. *hangoei*), and McNeill Lake (brackish; *S.* aff. *hangoei*) [see details in Table 1]. These lakes are usually ice-free between 4–5 weeks each year. Plankton samples were collected through holes in the ice-cover using 10 µm plankton nets. Single cells of *Pa. glacialis* and *S.* aff. *hangoei* (identified by SEM and SSU rDNA; Rengefors et al., 2008) were isolated from the plankton samples using micro capillary pipettes. The cells were placed in f/2 medium (Guillard and Ryther, 1962) based on sterile-filtered lake water. Cells were subsequently slowly adapted to f/2 based on Øre Sound seawater and grown at either full strength (salinity ~30), half strength or quarter strength salinity, as an approximation of the original lake salinity. In addition to our own strains (Table 1), we used the publicly available marine *Pa. glacialis* strains. From the Provasoli-Guillard National Center for Culture of Marine Phytoplankton we obtained cultures isolated from the McMurdo Sound, Ross Sea, Antarctica (CCMP1383), and Baffin Bay, Arctic (CCMP2088) [Table 1]. Freeze-dried cultures from the strains MBIC10563/4/5 isolated from the Antarctic sea outside Showa Island (Antarctica) were obtained from the Japanese Marine Biotechnology Institute Culture Collection.

For morphological taxonomic identification, armor plate patterns were analyzed by light microscopy. *Peridinium cinctum* and *Peridiniopsis*

Table 1. Description of analyzed strains, collection sites, isolation dates and accession numbers.

Morphospecies	Isolate	Collection site	Coordinates	~Salinity	Elevation (~ m a.s.l.)	Isolation	Genbank Accession Numbers SSU	Genbank Accession Numbers ITS
Peridinium aciculiferum [A]	PAER-1	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	15	1995	AY970653	AY970649
Peridinium aciculiferum [A]	PAER-2	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	15	2004	EF417314	DQ022927
Peridinium aciculiferum [A]	PAER-3	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	15	2004	–	DQ022928
Peridinium aciculiferum [A]	PAER-8	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	15	2004	–	AY970650
Peridinium aciculiferum [A]	PAER-9	Lake Erken, Sweden	59° 51′ N, 18° 36′ E	0	15	2004	–	AY970651
Peridinium aciculiferum [A]	PASP-1	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417292
Peridinium aciculiferum [A]	PASP-2	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417293
Peridinium aciculiferum [A]	PASP-3	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417294
Peridinium aciculiferum [A]	PASP-4	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417295
Peridinium aciculiferum [A]	PASP-5	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417296
Peridinium aciculiferum [A]	PASP-6	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417297
Peridinium aciculiferum [A]	PASP-9	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417298
Peridinium aciculiferum [A]	PASP-10	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	–	EF417299
Peridinium aciculiferum [A]	PASP-11	St. Pildammen, Sweden	55° 35′ N, 12° 59′ E	0	20	2006	EF417315	EF417300
Peridinium aciculiferum [A]	PABR-1	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	20	2006	–	EF417287
Peridinium aciculiferum [A]	PABR-2	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	20	2006	–	EF417288
Peridinium aciculiferum [A]	PABR-3	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	20	2006	EF417313	EF417289
Peridinium aciculiferum [A]	PABR-4	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	20	2006	–	EF417290
Peridinium aciculiferum [A]	PABR-5	Brodammen, Sweden	55° 32′ N, 12° 58′ E	0	20	2006	–	EF417291
Peridinium aciculiferum [A]	PAFI-1	Lake Österträsk, Finland	60° 21′ N, 20° 00′ E	0	16	2006	–	EF417286
Peridinium aciculiferum [A]	PATO-1	Lake Tovel, Italy	46° 15′ N, 10° 49′ E	0	1178	2003	–	EF417285
Peridiniopsis borgei [A]	PBSK-A	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2005	EF058241	*
Peridiniopsis borgei [A]	PBSK-B	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2005	–	*
Peridiniopsis borgei [A]	PBSK-C	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2005	–	*
Peridiniopsis borgei [A]	PBSK-D	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2005	–	*
Peridiniopsis borgei [A]	PBSK-E	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2005	–	*
Peridiniopsis borgei [A]	PBSK-06-1	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-2	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-3	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-4	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-5	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-6	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-7	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*

Peridiniopsis borgei [A]	PBSK-06-8	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-9	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridiniopsis borgei [A]	PBSK-06-10	St. Kalkbrottsdammen	55° 31′ N, 12° 55′ E	0.6	4	2006	–	*
Peridinium cinctum [A]	PCGY-1	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-2	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-4	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	EF058245	*
Peridinium cinctum [A]	PCGY-10	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-11	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-12	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-13	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-14	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-17	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-18	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-21	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2003	–	*
Peridinium cinctum [A]	PCGY-06-9	Gyllebosjön	55° 36′ N, 14° 12′ E	0.1	70	2006	–	*
Peridinium cinctum	SAG2017	Marburg, Germany	–	–	–	1983	EF058243	*
Peridinium cinctum	CCAC 0102	Spiekeroog, Germany	–	–	–	1998	EF058244	*
Polarella glacialis [A]	CCMP1383	Ross Sea, Antarctica	77° 50′ S, 166° 30′ E	32	–	1991	EF417317	*
Polarella glacialis [A]	CCMP2088	Baffin Bay, Arctic	74° 29′ N, 78° 35′ W	35	–	1998	EF434275	*
Polarella glacialis [A]	MBIC10563	Showa Island, Antarctica	69° 00′ S, 39° 34′ E	35	–	1994	–	*
Polarella glacialis [A]	MBIC10564	Showa Island, Antarctica	69° 00′ S, 39° 34′ E	35	–	1994	–	*
Polarella glacialis [A]	MBIC10565	Showa Island, Antarctica	69° 00′ S, 39° 34′ E	35	–	1994	–	*
Polarella glacialis [A]	PGAB-A	Lake Abraxas	68° 29′ 20′′ S, 78° 17′ 13′′ E	18	13	2005	–	*
Polarella glacialis [A]	PGAB-C	Lake Abraxas	68° 29′ 20′′ S, 78° 17′ 13′′ E	18	13	2005	–	*
Polarella glacialis [A]	PGAB-E	Lake Abraxas	68° 29′ 20′′ S, 78° 17′ 13′′ E	18	13	2005	EF434276	*
Polarella glacialis [A]	PGEK-AH	Ekho Lake	68° 31′ 16′′ S, 78° 16′ 12′′ E	40	–2	2005	–	*
Polarella glacialis [A]	PGEK-BH	Ekho Lake	68° 31′ 16′′ S, 78° 16′ 12′′ E	40	–2	2005	EF434277	*
Polarella glacialis [A]	PGEK-EH	Ekho Lake	68° 31′ 16′′ S, 78° 16′ 12′′ E	40	–2	2005	–	*
Scrippsiella aff. *hangoei* [A]	SHHI-1	Highway Lake	68° 27′ 47′′ S, 78° 13′ 24′′ E	5	8	2005	–	EF417301
Scrippsiella aff. *hangoei* [A]	SHHI-4	Highway Lake	68° 27′ 47′′ S, 78° 13′ 24′′ E	5	8	2005	–	EF417302
Scrippsiella aff. *hangoei* [A]	SHMC-A	McNeill Lake	68° 31′ 40′′ S, 78° 21′ 44′′ E	5	26	2005	EF417318	*
Scrippsiella aff. *hangoei* [A]	SHMC-B	McNeill Lake	68° 31′ 40′′ S, 78° 21′ 44′′ E	5	26	2005	–	*
Scrippsiella aff. *hangoei* [A]	SHHA-1	Lake Hand	68° 33′ 03′′ S, 78° 18′ 50′′ E	5	9	2005	–	*
Scrippsiella aff. *hangoei* [A]	SHVE-B	Vereteno Lake	68° 33′ 03′′ S, 78° 18′ 50′′ E	5	9	2005	–	EF417306
Scrippsiella aff. *hangoei* [A]	SHVE-C	Vereteno Lake	68° 30′ 54′′ S, 78° 24′ 51′′ E	5	0	2005	–	EF417307
Scrippsiella aff. *hangoei* [A]	SHVE-B2	Vereteno Lake	68° 30′ 54′′ S, 78° 24′ 51′′ E	5	0	2005	–	*
Scrippsiella aff. *hangoei* [A]	SHVE-K2	Vereteno Lake	68° 30′ 54′′ S, 78° 24′ 51′′ E	5	0	2005	–	*

[A] Indicates cultures used for AFLP
* Awaiting for accession numbers

borgei cultures were fixed in 5 % formaldehyde. Dinoflagellate plate detachment between slide and cover slip was carried out with the aid of diluted sodium hypochlorite instillation. Squashed empty thecae and detached plates were observed under a Standard 14 Zeiss optical microscope with Nomarsky interference contrast illumination. The *P. aciculiferum* cultures PATO, PAFI, PASP, PABR have been previously identified in Logares et al. submitted. The cultures *P. aciculiferum* PAER had previously been analyzed by Scanning Electron Microscopy (SEM) and plate patterns are presented in Rengefors and Legrand (2001) and Logares *et al.* (2007a). The strains of *Scrippsiella* aff. *hangoei* and *Pa. glacialis* from Antarctic lakes have previously been identified by SEM and SSU rDNA in Rengefors et al. (2008).

DNA extraction, PCR and sequencing

DNA was extracted following Adachi *et al.* (1994) or using a GENERATION Capture Column Kit (Gentra Systems, Minneapolis). For this work, we amplified and sequenced the Internal Transcribed Spacer 1 and 2 (ITS1/2) and 5.8S (altogether ITS) of the nuclear ribosomal DNA (nrDNA).

PCR amplifications were done using 25 ng of template genomic DNA, 0.125 mM of each nucleotide, 1.5 (3.0 for SSU) mM of $MgCl_2$, 1X PCR buffer, 0.4 μM of each primer and 0.5 u of Taq DNA Polymerase (AmpliTaq, Applied Biosystems) in 25 μl total volume reactions. The primers ITS1 (forward) 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 (reverse) 5′-TCCTCCGCTTATTGATATGC-3′ were used. The ITS PCR temperature profile consisted of an initial denaturing step of 5 min at 95 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 45 °C, 1 min at 72 °C, and ended with 10 min at 72 °C. All PCR amplicons were cleaned using PCR-M™ Clean-Up System (Viogene, Taiwan). ITS amplicons were directly sequenced using the same PCR primers. The sequencing reaction was carried out using BigDye (v1.1, Applied Biosystems) chemistry and the products were precipitated following the manufacturer instructions and then loaded into an ABI Prism 3100 sequencer (Applied Biosystems). The obtained sequences were edited and assembled by analyzing carefully the chromatograms using BioEdit (v7.0.4.1; Hall, 1999). Sequences were deposited in GenBank (accession numbers shown in Table 1).

Amplified Fragment Length Polymorphism (AFLP)

AFLP was carried out in duplicates or triplicates (including one set of negatives) following a fluorescein protocol based on Vos et al. (1995). The set of 68 dinoflagellate strains used for AFLP are described in Table 1 (21 strains of *P. aciculiferum*, 15 of *Ps. borgei*, 12 of *P. cinctum*, 11 of *Pa. glacialis*, 9 of *S.* aff. *hangoei*). The DNA (250 ng total) from each clone was digested during 1 h at 37 °C using 2.5 u of EcoRI (Amersham Pharmacia), 2.5 u of TruI (Fermentas), 1 μg of BSA and 1X TA-buffer in each reaction (20 μl final volume). Ligation of adaptors was carried out for 3 h at 37 °C using 0.5 μM of E adaptor, 5 μM of M adaptor, 0.5 u of T4 ligase (USB®) and 1X Ligation-buffer in 5.0 μl reactions. The ligation product was diluted ten times and subsequently used as a template for the pre-amplification step. The pre-amplification reactions (20 μl final volume) consisted of 10 μl of the ligation product, 0.4 u of Taq DNA Polymerase (AmpliTaq, Applied Biosystems), 0.3 μM of E-primer (5′-GACTGCGTACCAATTCT-3′) and 0.3 μM of M-primer (5′-GATGAGTCCTGAGTAAC-3′), 0.2 mM of dNTPs, 1X PCR-buffer and 2.5 mM of $MgCl_2$. The preamplification thermal profile included an initial denaturing step of 2 min at 94 °C, 20 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C, followed by a final step of 10 min at 72 °C. The pre-amplification product was diluted ten times and used as a template for the selective amplification step.

The selective amplification step was carried out using four primer pairs with the strains of *P. aciculiferum*, *P. cinctum* and *Ps. borgei*: Mix 1: E_{TCG}-M_{CGG}; Mix 2: E_{TCT}-M_{CAC}; Mix 3: E_{TGA}-M_{CGC} ; Mix 4: E_{TAG}-M_{CCG}. With the strains from *Pa. glacialis* and *S.* aff. *hangoei* six primer pairs were used: Mix A: E_{TGA}-M_{CGA}; Mix B: E_{TGA}-M_{CAG}; Mix C: E_{TCG}-M_{CAG}; Mix D: E_{TCG}-M_{CGA} ; Mix E: E_{TAG}-M_{CAG} ; Mix F: E_{TCG}-M_{CAA}. The E-primers were labelled with fluorescein. Although different primers were used for the polar species the same AFLP protocol was used. Since several primer pair

combinations and hundreds of loci were used, the use of different primer combinations should not affect the results. Selective amplifications reactions (10 µl final volume) included 2.5 µl of preamplification product, 0.04 u of Taq DNA polymerase (AmpliTaq, Applied Biosystems), 0.2 mM of dNTP, 0.6 µM of each selective primer, 2.5 mM of $MgCl_2$ and 1X PCR buffer. The temperature profile consisted of an initial denaturing step of 2 min at 94 °C, 12 cycles of 30 s at 94 °C, 30 s at 65 °C –0.7 °C/cycle, 60 s at 72 °C, continued by 23 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C, followed by a final step of 10 min at 72 °C. After the incubation, 10 µl of formamide dye (100% formamide, 10mM EDTA, 0.1 % xylene cyanol, 0.1 % bromophenol blue) were added to the reactions and then products were stored overnight at 4 °C before further analysis. Subsequently, selective amplification products were denatured (3 min at 95 °C) and then 3.5 µl were loaded onto 6 % polyacrylamide gels. AFLP fragments were separated using 30 W during 90 min, and detected by the fluorescein labeled E-primers in a Typhoon imaging system 9200. (Amersham Biosciences). Reproducible bands in duplicates or triplicates were scored as 1 (presence) or 0 (absence) for the surveyed loci (see Table 2).

AFLP analyses

Analyses of AFLP polymorphism were carried out with AFLPsurv (V1.0; Vekemans, 2002) using the option for haploid data. AFLPsurv was also used to calculate the Nei's gene diversity index (*H*) [Nei, 1987]. The program TFPGA (v1.3; Miller, 1997) was used to calculate UPGMA dendrograms based on Nei's 1972 genetic distances (Nei, 1972). The support for the branching pattern was calculated through 1000 bootstrap pseudoreplicates. The TFPGA program was also used to calculate the percentage of polymorphic loci (PPL). F_{st} analyses were carried out among the *P. aciculiferum* lake-populations from Brodammen, St. Pildammen and Erken as well as between the *Ps. borgei* isolates PBSKA-E and PBSK06-1/10, which were collected in different years. Moreover, F_{st} analyses were carried out between marine and lacustrine *Pa. glacialis* strains.

Bayesian clustering analyses

We used the program STRUCTURE (v2.2; Pritchard et al., 2000) to estimate the number of *K* unknown genetic clusters (genetic populations) in which the multilocus AFLP data could be divided. This program uses a Bayesian algorithm to

Table 2. AFLP variation among the strains of the three analyzed species.

Species	Population	# Strains	# Loci	# poly_loci	PPL	H	Var (H)
P. aciculiferum	All	21	106	82	77.0	0.19	0.0003
P. aciculiferum	Brodammen	5	106	34	32.0	0.13	0.0004
P. aciculiferum	Pildammen	9	106	46	43.4	0.11	0.0002
P. aciculiferum	Erken	5	106	20	18.8	0.07	0.0002
Ps. borgei	St. Kalkbrot.	15	125	122	97.6	0.37	0.0001
P. cinctum	Gyllebosjön	12	118	56	47.4	0.17	0.0004
Pa. glacialis	All	11	175	45	25.7	0.08	0.0001
Pa. glacialis	Abraxas	3	175	3	1.7	0.01	0.0000
Pa. glacialis	Ekho	3	175	11	6.3	0.03	0.0001
Pa. glacialis	MBIC	3	175	11	6.3	0.03	0.0001
S. aff. *hangoei*	All	9	154	35	22.7	0.08	0.0001
S. aff. *hangoei*	Highway	2	154	11	7.1	0.05	0.0002
S. aff. *hangoei*	McNeill	2	154	8	5.2	0.04	0.0002
S. aff. *hangoei*	Vereteno	4	154	15	9.7	0.05	0.0002

Loci= total number of scored loci; # poly_loci = total number of polymorphic loci; PPL = percentage of polymorphic loci; H = Nei's gene diversity (Nei, 1987); Var (H) = variance of H.

estimate the most likely K and also to estimate the probability that each one of the analyzed strains belong to the inferred K clusters.

The program STRUCTURE was run initially with K values ranging from 1 to N_i (number of strains within each species) with a total of 20,000 generations and a burn-in of 10,000 generations. We identified the K values that gave the highest posterior probabilities for the data [Ln P(D)], and then we ran analyses with 200,000 generations (with burn-in =100,000) for the range of K values which have produced the highest Ln P(D). Increasing the number of generations can give more accurate Bayesian estimates.

The analyses were run under the "admixture model" (Pritchard et al., 2000), allowing individuals to have a mixed ancestry from the K populations. Models allowing for allele frequencies to be correlated (Falush et al., 2003) and independent (Pritchard et al., 2000) among genetic clusters were tested. For each selected value of K we estimated the average coefficients of membership (Q) of the sampled individuals to the inferred clusters. Strains were assigned to the inferred clusters using a threshold of $q_i > 0.80$.

Results

ITS nrDNA sequences

The ITS nrDNA sequences within the analyzed strains of *Peridinium aciculiferum*, *Peridiniopsis borgei* and *Peridinium cinctum* PCGY, *Polarella glacialis* and *Scrippsiella* aff. *hangoei* did not show any intraspecific variation (Accession numbers in Table 1). Among the *P. cinctum* strains obtained from culture collections (SAG2017, and CCAC0102) and the strains from Gyllebosjön (PCGY) there was an ITS nrDNA differentiation ranging between 3.7 – 5.9 % (based on a 552-character alignment). The strains from the culture collections were not analyzed with AFLP since they may be cryptic species.

AFLP

Our AFLP analyses have revealed a wide variability among the species in their levels of intraspecific genetic diversity (20 < PPL < 90; 0.08 < H < 0.37; PPL = Percentage of Polymorphic Loci; H = Nei's gene diversity; Fig. 1; Table 2). For all the strains of *P. aciculiferum*, *Ps. borgei*, *P. cinctum*, *Pa. glacialis* and *S.* aff. *hangoei* the number of scored loci ranged between 106 and 175 (see Table 2). More loci were scored in the polar species (*Pa. glacialis* and *S.* aff. *hangoei*) since the strains initially appeared to harbor comparatively low levels of diversity. However, the scoring of more loci confirmed the low genetic variation among the polar strains.

The correlation between the percentages of polymorphic loci (PPL) with the number of analyzed strains was tested since we used unequal number of strains within each species. In Figure 1, we show the increase of the PPLs with the number of scored strains. The samples sizes were too small to carry out curve fitting analyses in all species, except *P. aciculiferum.* In this species, the PPL appeared to increase logarithmically with the number of scored strains ($p < 0.05$; $R^2 \sim 0.94$). The general trend for all species seems to be a sharp increase in PPL for the first six samples analyzed, with a subsequent leveling off. We have also calculated the Nei's gene diversity index (hereafter H; Nei, 1987) within populations and species as an alternative measurement of genetic diversity.

Overall, the PPL (97.6) and H index (0.37) among the *Ps. borgei* strains were relatively higher than these values in the four other species (Fig. 1; Table 2). The second species with most variable PPL (77.0) among their strains was *P. aciculiferum* (Fig. 1). However, H was very similar between *P. aciculiferum* (H=0.19) and *P. cinctum* (H=0.17) strains (Table 2). The PPL among *P. cinctum* (47.4) strains was in between the PPLs of *Ps. borgei-P. aciculiferum* and the polar species [Fig. 1]. The strains from the polar species showed comparatively low PPLs (22.7–25.7) and H values (0.08) [Fig.1; Table 1].

The Nei's (1972) genetic distances measure the degree of genetic differentiation between strains. These distances ranged between 0.02–0.63 among *P. aciculiferum* strains, between 0.08–0.34 among *P. cinctum* and between 0.16–0.70 among *Ps. borgei* strains. For the polar species, the distances ranged between 0.01–0.15 among *Pa. glacialis* strains and between 0.01–0.14 among *S.* aff. *hangoei* strains. There were no strains with identical genotypes among our samples.

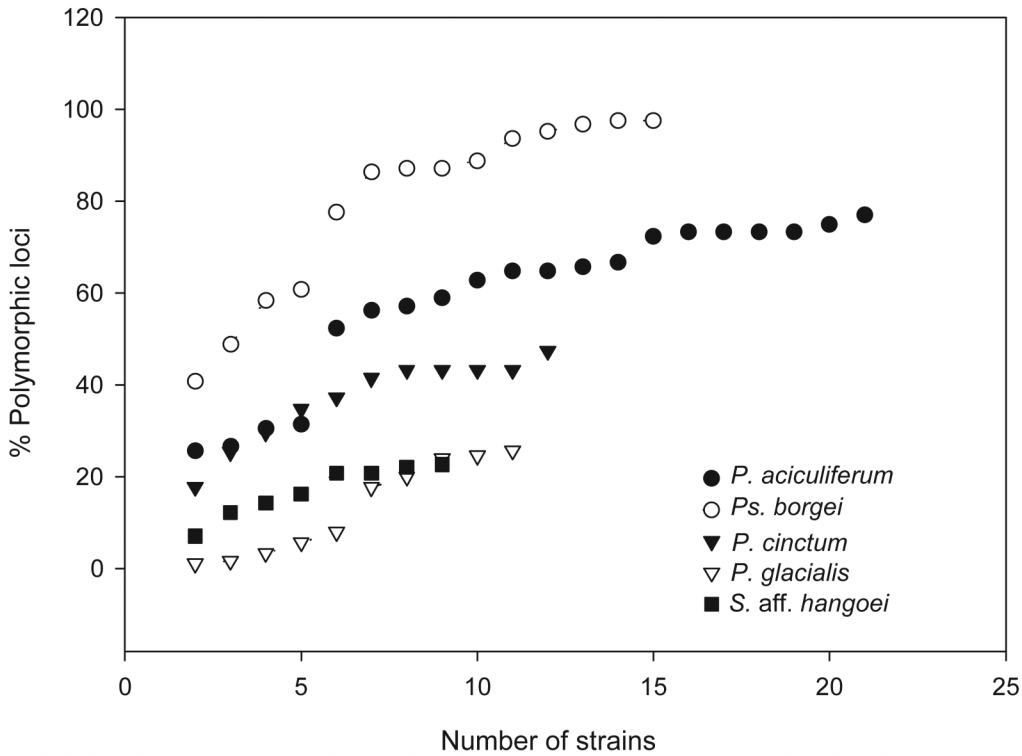


Figure 1. Percentage of polymorphic AFLP loci as a function of the number of strains (sample size).

In the UPGMA analyses of intraspecific genetic variation, some significantly supported (i.e. bootstrap support [BV] > 0.70) clusters of strains became apparent. Within *P. aciculiferum*, the strains clustered into two major groups, one composed of strains from Brodammen only, and another one comprising all the remaining strains (Fig. 2 A). Three major clusters were identified among the analyzed strains of *Ps. borgei* from the lake St. Kalkbrottsdammen (Fig. 2 B). Within *P. cinctum*, no bootstrap supported clusters were identified, although two strains (PCGY-06-9, PCGY-11) were considerably divergent from the others (Fig. 2 C). Within *Pa. glacialis*, three major clusters have been identified (Fig. 3 A). The first cluster groups Antarctic lacustrine strains from the lakes Ekho and Abraxas (Fig. 3 A). These lacustrine strains were significantly (BV > 0.70) separated from the marine strains. The second *Pa. glacialis* cluster groups marine Antarctic strains from the vicinity of Showa Island (Fig. 3 A; MBIC). Interestingly, the third cluster groups the Antarctic and Arctic strains (Fig. 3 A; CCMP). Among the lacustrine Antarctic *S.* aff. *hangoei* strains, no clear clustering patterns were found (Fig. 3 B). Only two strains from Lake Vereteno formed a divergent cluster with significant support (SHVE-B/B2) [Fig. 3 B].

Bayesian clustering analyses of AFLP diversity

The *P. aciculiferum* strains from the five sampled lakes across Sweden, Finland and Italy clustered into two genetic populations (i.e. two genetically homogeneous groups; K=2 had highest posterior probability) when considering the correlated allele frequencies model (Figs. 4 A, 5). Considering a $q_i > 0.80$ (q_i = coefficient of membership for the isolate i) threshold for assigning strains to the different clusters, three strains were assigned to cluster 1 (PABR-1/4/5) and 16 to cluster 2 (PABR-2/PASP-2/3/4/5/6/9/10/11, PAER-1/2/3/8/9, PATO-1, PAFI-1), out of a total of 21 strains (see Fig. 4 A). Only two strains were admixed (PABR-

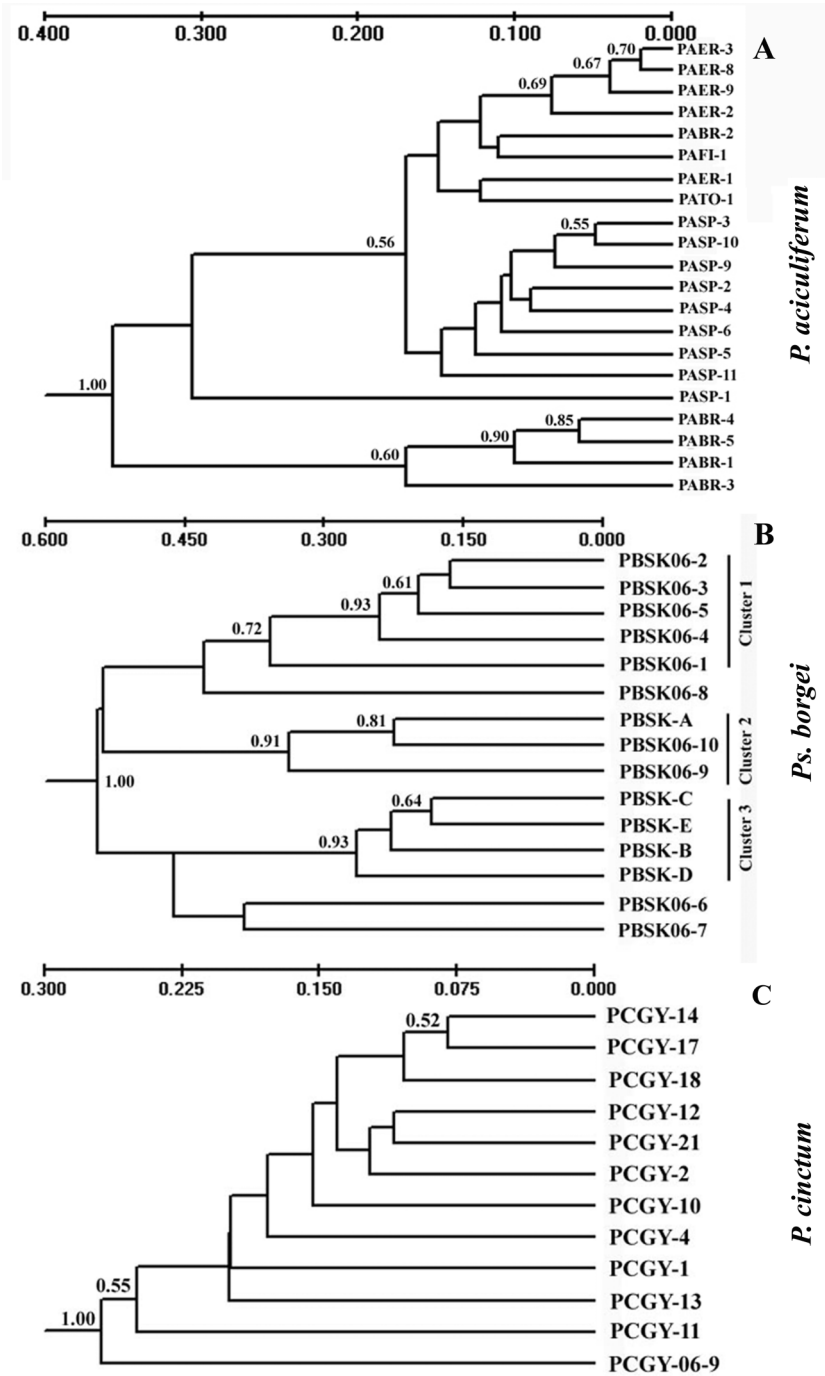


Figure 2. UPGMA trees based on Nei´s distances (1972). Support values were calculated over 1000 bootstrap pseudoreplicates (values > 0.50 are shown). A = *Peridinium aciculiferum*; B = *Peridiniopsis borgei*; C = *Peridinium cinctum*. The scalebar on the top shows approximate Nei's genetic distances.

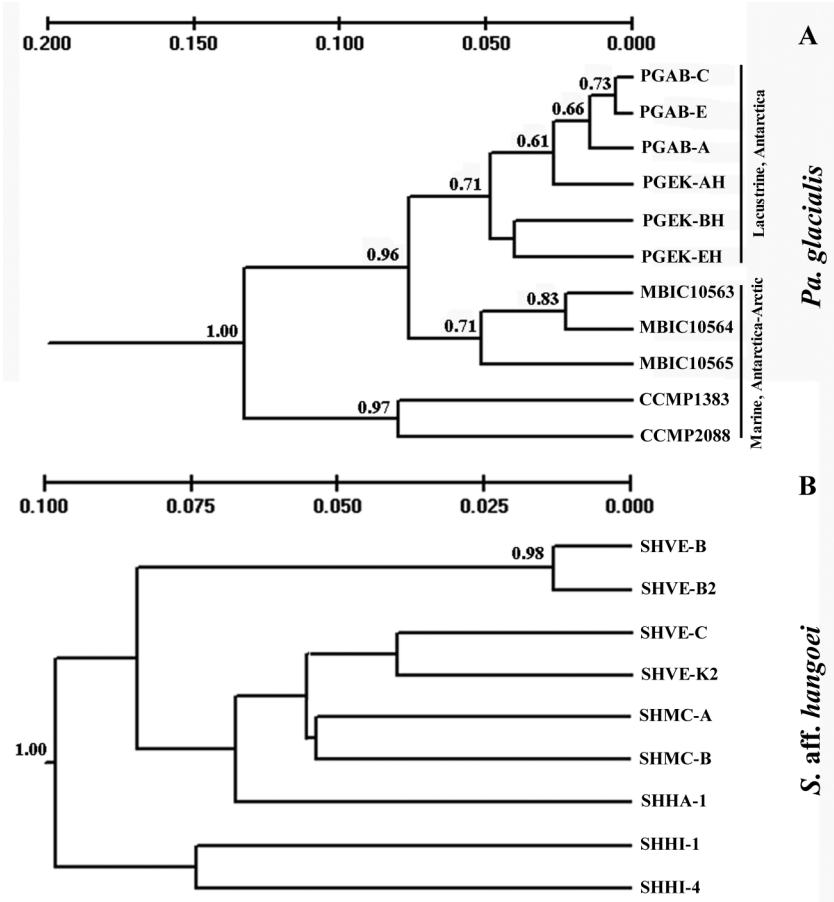
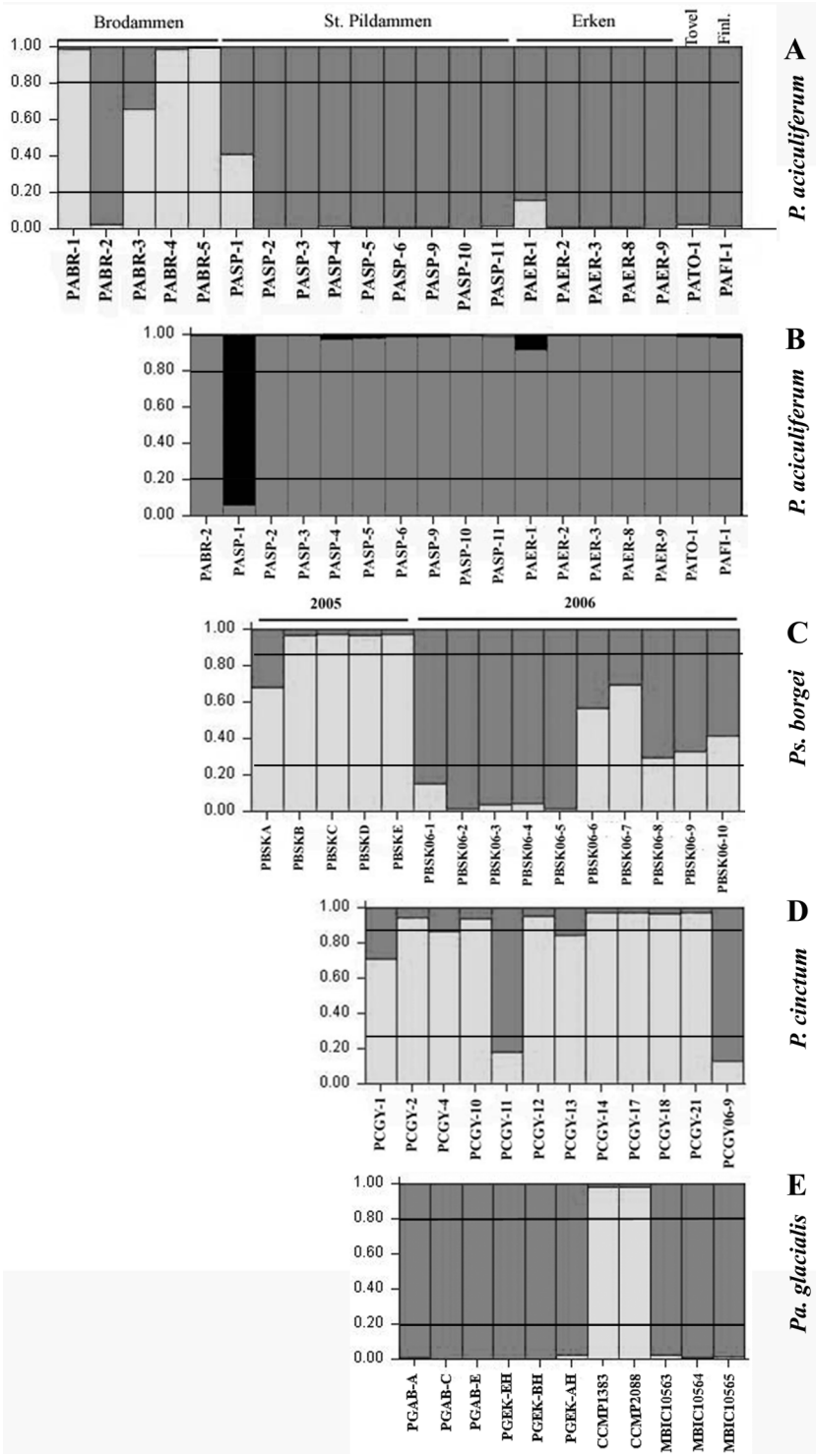


Figure 3. UPGMA trees based on Nei´s distances (1972). Support values were calculated over 1000 bootstrap pseudoreplicates (values > 0.50 are shown). A = *Polarella glacialis*; B = *Scrippsiella* aff. *hangoei*. The scalebar on the top shows approximate Nei's genetic distances.

3, PASP-1) [0.20 < q_i < 0.80; their genetic composition was a mixture of the *K* inferred clusters]. Under the independent allele frequencies model, three genetic clusters gave a better explanation of the data (Fig. 5). To test the putative subdivision of *P. aciculiferum* into three genetic clusters, we ran analyses excluding the strains PABR1/3/4/5, which were for the most part associated to cluster 1. These analyses supported the presence of a third genetic cluster, represented basically by one isolate, PASP1 (Fig. 4 B). The two inferred genetic populations within *P. aciculiferum* did not present any obvious correlation to the lake-populations (Fig. 4 A). However, the cluster 1 was predominantly represented in the small pond Brodammen, while the cluster two had representatives in all the studied lakes.

The strains of *Ps. borgei* from the lake St. Kalkbrottsdammen were structured into two genetic populations (Fig. 4 C). Using a criteria of q_i > 0.80, four strains were assigned to cluster 1 (PBSK-B/C/D/E) and five to cluster 2 (PBSK-06-1/2/3/4/5) out of a total of 15 strains (Fig. 4 C). The remaining strains were admixed (PBSK-A, PBSK-06-6/7/8/9/10). The independent allele frequencies model gave the highest posterior probabilities for two genetic populations (*K*=2; Fig. 5). All strains assigned to cluster 1 were obtained in 2005, while all strains assigned to cluster 2 were sampled in 2006.


Brodammen
St. Pildammen
Erken
Tovel
Fml.
A
P. aciculiferum
1.00
0.80
0.60
0.40
0.20
0.00
PABR-1
PABR-2
PABR-3
PABR-4
PABR-5
PASP-1
PASP-2
PASP-3
PASP-4
PASP-5
PASP-6
PASP-9
PASP-10
PASP-11
PAER-1
PAER-2
PAER-3
PAER-8
PAER-9
PATO-1
PAFI-1
B
P. aciculiferum
PABR-2
PASP-1
PASP-2
PASP-3
PASP-4
PASP-5
PASP-6
PASP-9
PASP-10
PASP-11
PAER-1
PAER-2
PAER-3
PAER-8
PAER-9
PATO-1
PAFI-1
2005
2006
C
Ps. borgei
PBSKA
PBSKB
PBSKC
PBSKD
PBSKE
PBSK06-1
PBSK06-2
PBSK06-3
PBSK06-4
PBSK06-5
PBSK06-6
PBSK06-7
PBSK06-8
PBSK06-9
PBSK06-10
D
P. cinctum
PCGY-1
PCGY-2
PCGY-4
PCGY-10
PCGY-11
PCGY-12
PCGY-13
PCGY-14
PCGY-17
PCGY-18
PCGY-21
PCGY06-9
E
Pa. glacialis
PGAB-A
PGAB-C
PGAB-E
PGEK-EH
PGEK-BH
PGEK-AH
CCMP1383
CCMP2088
MBIC10563
MBIC10564
MBIC10565

The *P. cinctum* strains from Gyllebosjön appear to belong to either one or two genetic populations. Under the correlated allele frequencies model, the posterior probabilities were very similar for *K*=1 and 2 (Ln P[D] = –379.1 and –377.2 respectively; Fig. 5) Under the independent allele frequencies model, the posterior probabilities for *K*=1 and 2 were also very similar (Ln P[D] = –383.9 and –383.1 respectively; Fig. 5). Overall, *K*=2 under the correlated allele frequencies model obtained the highest posterior probabilities. However, under this model all the strains were fairly admixed ($0.20 < q_i < 0.80$), which is an indication of no structure (data not shown) [see STRUCTURE v2.2 documentation]. Whereas, for *K*=2 under the independent allele frequencies model, eight strains have been assigned to cluster 1 (PCGY-2/4/10/12/14/17/18/21) and two strains to cluster 2 (PCGY-11 and PCGY06-9; Fig. 4 D). This differential assignment of strains to the *K* inferred clusters is an indicator of genetic structure [see STRUCTURE v2.2 documentation], suggesting that the independent allele frequencies model fits better to our data.

Two genetic populations were indicated for *Pa. glacialis* (Fig. 4 E). The independent allele frequencies models with a *K*=2 had the highest posterior probabilities (Fig. 6). The strains CCMP1383 (Antarctic marine) and 2088 (Arctic marine) were strongly assigned to one of the genetic clusters, while the rest of the strains were strongly assigned to the other cluster (Fig. 4 E).

For *S.* aff. *hangoei*, there was no evidence for the presence of different genetic populations among our strains (*K*=1 appears as the most likely scenario; Fig. 6).

F_{st} analyses

Population differentiation analyses between the three *P. aciculiferum* lake-populations that allowed for this tests (PABR, PASP, PAER), indicated a significant population differentiation (F_{st} = 0.46; $p < 0.05$; i.e. lake populations are more genetically differentiated than random assemblages of strains). The estimated F_{st} between PASP – PAER was F_{st} = 0.37, between PABR – PASP was F_{st} = 0.49, and between PABR–PAER was F_{st} = 0.51. The estimated F_{st} between the two groups of strains of *Ps. borgei* obtained in 2005 and 2006 from the same lake was F_{st} = 0.26 ($p < 0.05$). For the lacustrine Antarctic strains, the sample sizes for determining the genetic differentiation among lakes were too low. However, the sample sizes allowed for testing the genetic differentiation between lacustrine and marine *Pa. glacialis* populations, and this test indicated a significative genetic differentiation between them (F_{st} = 0.29; $p < 0.05$).

Relationships between UPGMA, Bayesian and F_{st} analyses

Among the *Ps. borgei* isolates, the UPGMA analyses identified three clusters (Fig. 2 B). The Bayesian analyses indicated the presence of two genetic populations of *Ps. borgei* within the lake St. Kalkbrottsdammen (Figs. 4 C, 5). The Bayesian and

Figure 4. Estimated membership of each of the sampled strains to the *K*-inferred genetic clusters for *P. aciculiferum* (A, B), *Ps. borgei* (C), *P. cinctum* (D) and *Pa. glacialis* (E) [*S.* aff. *hangoei* strains are not presented since all strains appeared to belong to the same cluster]. Each strain is represented as a vertical bar, partitioned into *K* segments, which are proportional to the estimated membership to the *K* inferred clusters. A = Membership of the *P. aciculiferum* strains to two inferred clusters (*K* = 2; cluster 1= light grey; cluster 2 = dark grey). The lake of origin of each strain is written above the bars. Note that genetic clusters do not necessarily coincide with lakes. B = Test of further population subdivision within *P. aciculiferum*. When strains predominantly belonging to cluster 1 (PABR-1/3/4/5) are removed, a third genetic population is suggested (cluster 3 = black). This cluster is represented by only one isolate (PASP-1). C = Membership of the *Ps. borgei* strains from the lake St. Kalkbrottsdammen to two inferred clusters. The year of origin of the different strains is indicated above the bars. D = Membership of the *P. cinctum* strains from Gyllebosjön to two inferred clusters. E = Membership of the *Pa. glacialis* strains to two inferred clusters. These results come from Bayesian runs of 200,000 generations, with a burn-in of 100,000. In these runs, the correlated allele frequencies model was considered in A, whereas the independent allele frequencies model was used in B–E. Codes below the bars indicate individual strains that are specified in Table 1.

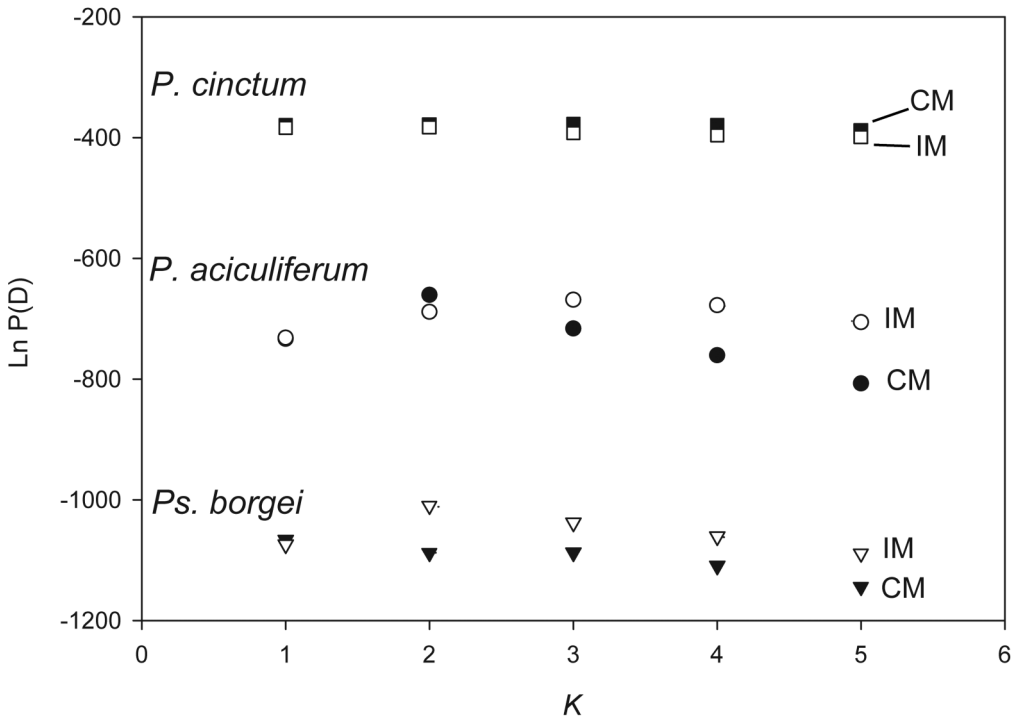


Figure 5. Posterior probability of the data Ln P(D) given the number of *K* (1–5) clusters. CM = correlated allele frequencies model; IM = independent allele frequencies model. The *K* values with highest Ln P(D) indicate the number of genetic populations with highest likelihood.

UPGMA results were congruent. The UPGMA cluster 1 (Fig. 2 B) was associated to strains which were strongly assigned to one of the genetic populations in the Bayesian analyses (Fig. 4 C). The UPGMA cluster 2 (Fig. 2 B) was linked to admixed individuals, and the UPGMA cluster 3 (Fig. 2 B) was associated to strains strongly assigned to another genetic population (Fig. 4 C). Two admixed strains (PBSK 06-6/7) were not included in any of the mentioned UPGMA clusters (Fig. 2 A). The segregation of strains collected during 2005 and 2006 indicated by the UPGMA and Bayesian analyses was supported by the F_{st} (F_{st} = 0.26; $p < 0.05$).

No bootstrap-supported clustering pattern was observed within the *P. cinctum* strains (Fig. 2 C) in the UPGMA analyses. Accordingly, the Bayesian analyses gave similar posterior probability values for *K*=1–3 under the independent and correlated allele frequencies models (Fig. 5). According to the recommendations of the STRUCTURE authors, the lowest *K* should be chosen when similar Ln P(D) are obtained (in this case, *K*=1) [see STRUCTURE v2.2 software documentation]. However, when using *K*=2 under the independent allele frequencies model, we observed that two strains (PCGY-11 and PCGY 06-9; Fig. 4 D) are assigned to one genetic population while most of the rest are assigned to the other genetic population. The same two strains are the most divergent ones in the UPGMA analyses (Fig. 2 C).

Among *P. aciculiferum* strains, our UPGMA analyses indicated a significant separation between some strains from the pond Brodammen (PABR-1/3/4/5) and the rest of the *P. aciculiferum* strains (Fig. 2 A). Accordingly, this divergent cluster was for the most part composing one of the genetic populations in the Bayesian analyses (Fig. 4 A). The isolate PABR-2, also from Bro-

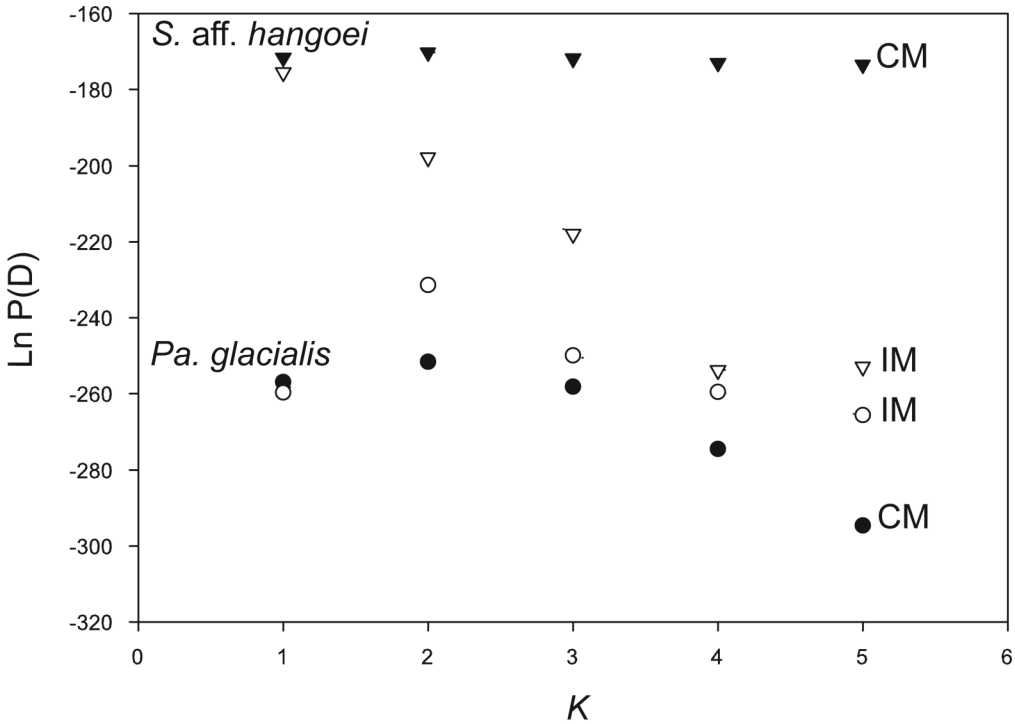


Figure 6. Posterior probability of the data Ln P(D) given the number of *K* (1–5) clusters. CM = correlated allele frequencies model; IM = independent allele frequencies model. Observe that *K*=2 obtained the highest probability for *Pa. glacialis* under the correlated and independent allele frequencies models. Whereas, *K*=1 obtained the highest probability for *S.* aff. *hangoei.*

dammen, clustered with strains from other lakes in the UPGMA analyses (Fig. 2 A). Correspondingly, PABR-2 was not assigned to the same genetic population as most strains from Brodammen (Fig. 4 A). In the UPGMA analyses, the isolate PASP-1 showed a significant degree of divergence, not clustering with any of the other strains (Fig. 2 A). Likewise, this isolate has been indicated to belong to a third genetic population in the Bayesian analyses (Fig. 4 B). Our F_{st} analyses supported the genetic differentiation of the lake populations from the waterbodies St. Pildammen, Erken and Brodammen. The separation between strains from St. Pildammen and Erken was obtained in our UPGMA analyses, although it was not supported by high bootstrap (Fig. 2 A).

In *Pa. glacialis*, the UPGMA analyses supported a clear segregation between lacustrine and marine strains, which was also supported by the F_{st} analyses (Fig. 3A). Our Bayesian results indicated that strains from two genetic populations were present among our samples of *Pa. glacialis* (Fig. 4 E). One *Pa. glacialis* genetic population was constituted by all the lacustrine strains from the Vestfold Hills as well as the marine strains from the neighborhood of the Showa Island (MBIC) [Fig. 4 E]. The Antarctic and Arctic strains CCMP1383-2088 respectively, were assigned to the second genetic population. This segregation was congruent also with the UPGMA analyses, in which the latter two strains were separated from all the other *Pa. glacialis* strains (Fig. 3 A). However, the lacustrine and marine MBIC strains were in the same genetic population.

No bootstrap-supported UPGMA clusters were observed among *S.* aff *hangoei* strain (Fig. 3 B). Accordingly, the Bayesian analyses indicated that only one genetic population was present (Fig. 6).

Discussion

Overall, our results suggest that a multitude of dinoflagellate genotypes coexist within lake-populations. A total of 68 dinoflagellate strains from five species were investigated by AFLP, and no identical genotypes were detected. These species are present in lacustrine and marine habitats of polar and temperate regions. The levels of intraspecific genetic diversity displayed a wide variability among the species. We found evidence that in four species, the genetic variability among strains was structured into genetic populations (i.e. genetically homogeneous clusters). Although it cannot be ruled out, we did not find conclusive evidence indicating that genetic populations are associated to different lakes.

The presence of cryptic species can represent a major problem for estimating indices of genetic diversity, especially among microbes, where the morphospecies concept is the basis for the present taxonomy. The source of this problem is that genetic differences among species can be considered as inter- or intrapopulational differences. In dinoflagellates, there is evidence of cryptic species in several taxa (e.g. Montresor et al., 2003b; Kim et al., 2004; Lilly et al., 2005; Gribble and Anderson, 2007). In this work, our criteria for avoiding the introduction of biases due to cryptic species was to use strains with homogeneous ITS nrDNA. Strains sharing the same ITS are likely to originate from the same species. The ITS is one of the most variable fragments in dinoflagellates, and its variation has been used to delineate different but morphologically identical/very similar species (see Litaker et al., 2007). According to Litaker et al. (2007), a threshold of 4 % in the ITS differentiation delineates most dinoflagellate species analyzed. Here, we found an ITS differentiation among *P. cinctum* strains from different lakes ranging between 3.7 – 5.9 %, suggesting that they might belong to different cryptic species. Therefore, only the strains from Gyllebosjön were considered for AFLP analyses. All the strains of *P. cinctum* (from Gyllebosjön), *Ps. borgei*, *P. aciculiferum*, *Pa. glacialis* and *S.* aff. *hangoei* had plate patterns corresponding to their species definitions as well as homogeneous ITS sequences at the intraspecific level. Therefore, we considered them to belong to the same species.

Genetic diversity among the five dinoflagellate species

Our AFLP analyses have revealed a wide variability among the species in their levels of intraspecific genetic diversity ($20 < PPL < 90$; $0.08 < H < 0.37$). In our study, the addition of more populations in the analyses could change the estimations of the intraspecific genetic diversities depending on the species. For example, adding other populations of *P. cinctum* and *P. aciculiferum* could increase considerably their intraspecific genetic diversities in the light of what had happened when a second population of *P. aciculiferum* was added to the initial Brodammen population (2 to 5; Fig. 1). A similar reasoning can be applied to *S.* aff. *hangoei*, especially if marine representatives are analyzed. In *Ps. borgei*, on the other hand, the observed levels of genetic diversity are already high (~ 95 %) within the single lake-population. Therefore, the addition of more populations will not change the conclusion that this species is genetically diverse. In contrast, the addition of more *Pa. glacialis* populations will most likely not change the conclusion that this species probably has a low genetic diversity across all its distributional range. In particular, since this study includes strains from both marine and lacustrine environments from the Arctic and Antarctic.

The relatively high levels of intraspecific genetic diversity found in the studied non-polar species (*P. aciculiferum*, *Ps. borgei*, *P. cinctum*) parallel similar findings in marine microbes from several taxa (Bolch et al., 1999a; Bolch et al., 1999b; Medlin et al., 2000; Rynearson and Armbrust, 2000; Shankle et al., 2004; Evans et al., 2005; Iglesias-Rodriguez et al., 2006) as well as freshwater (Hayhome et al., 1987; Kusch et al., 2000; De Bruin et al., 2004; Muller et al., 2005; Wilson et al., 2005; Zhang et al., 2006; Beszteri et al., 2007). However, the relatively low levels of diversity found among the isolates from the two polar species require a more detailed discussion. In *S.* aff. *hangoei*, this genetic homogeneity could reflect the relatively recent (in geological terms) colonization of the studied Antarctic lakes by a few marine ancestral strains (occurred no more than 6,500 years ago [Zwartz et al., 1998]). In *Pa. glacialis*, the relatively low intraspecific genetic diversity cannot be explained by a recent colonization,

since some of the strains were obtained from both marine and lacustrine habitats in the Antarctic and Arctic. Alternatively, the relatively low genetic diversity within *Pa. glacialis* can be due to the interplay of population expansion and extinction processes, where a few clones have distributed recently across a variety of habitats in distant geographical locations, while several others became extinct.

While in culture, it is possible that some mutations could have arisen among our strains, thus affecting the values of genetic diversity (see paper 7 in Figueroa, 2005). However, with the exception of PAER-1, all the cultures have been growing in the laboratory for less than three years, thus restricting the amount of possible mutations. In addition, the observed genetic structure in some species (e.g. bootstrap-supported genetic clusters and genetic populations) does not suggest the occurrence of extensive mutations during culturing. It is very unlikely that strains collected at different times and locations cluster together with high support as a result of random mutations occurring in less than three years of culturing. Nevertheless, mutations could have increased the genetic distances among some strains, but most likely they have not modified the clustering patterns. Comparative AFLP studies in green algae indicate that the effects on the AFLP results of mutations occurring during culturing are negligible (Muller et al., 2005). However, other studies in dinoflagellates suggest that higher amounts of cells with mutations could be present in cultures that have been maintained for a long time (see paper 7 in Figueroa, 2005).

Patterns of genetic differentiation

Our UPGMA results indicated that several relatively highly differentiated genotypes of *Ps. borgei* are present within the lake St. Kalkbrottsdammen. Such relatively high genetic differentiation among strains could be due to a rapid within-lake diversification following its colonization (Orr and Smith, 1998), the immigration of several genotypes, but not due to an old in-situ divergence, since St. Kalbrottsdammen is less than 60 years old (G. Cronberg, pers. com.). The UPGMA analyses identified three clusters (Fig. 2 B), which could correspond to three independent colonization lines, or several more colonizations from three main *Ps. borgei* lineages. The Bayesian analyses indicated the presence of two genetic populations of *Ps. borgei* within the lake St. Kalkbrottsdammen. Most of the individuals collected in 2005 were assigned to one genetic population while around half of the individuals collected in 2006 were assigned to the second genetic population. Thus, the genetic populations dominating the lake during 2005 and 2006 could have been different. The differential germination of cysts present in the sediments, harboring genotypes assembled in different years, could account for some of the observed genetic variability. It remains to be tested if the detected genetic populations are present every year, whether their presence is coupled to some environmental factor or if their occurrence was simply an outcome of random germination of cyst genotypes.

No clear clustering pattern was observed within the *P. cinctum* strains in the UPGMA analyses. Likewise, the Bayesian analyses were not conclusive if one or two genetic populations were present among the *P. cinctum* strains. However, when assuming two populations under the independent allele frequencies model, we observed that two strains (PCGY-11 and PCGY 06-9; Fig. 4 D) are assigned to one genetic population while the rest are assigned to the other genetic population. The same two strains are the most divergent ones in the UPGMA analyses. This correspondence suggests that strains from two genetic populations were present among our samples from Gyllebosjön.

The UPGMA analyses separated most *P. aciculiferum* strains from the pond Brodammen from the rest of the strains, as did the Bayesian analyses. This separation was also supported by phylogenetic studies using the mitochondrial gene cytochrome b (Logares, et al., submitted). Since the pond Brodammen is less than 15 years old, an ancient in situ diversification would not account for the observed differences. Interestingly, an atypical morphotype of *P. aciculiferum* was observed within Brodammen, which has only one clearly developed posterior spine (instead of four) and a slight posterior asymmetry on the antapical plate 2´´´. This asymmetry appears to increase with the growth of the cells (Boltovskoy. et al., unpublished data). Thus, both genetic and mor-

phological data suggest that a distinct population of *P. aciculiferum* is present in Brodammen.

In *Pa. glacialis* several clusters supported by bootstrap were evident in the UPGMA analyses. In contrast, no clear clusters were observed among *S.* aff *hangoei* strains. Particularly, the UPGMA analyses supported a clear segregation between lacustrine and marine *Pa. glacialis* strains, which was also supported by the F_{st} analyses. Our Bayesian results indicate that strains from two genetic populations were present among our samples of *Pa. glacialis*. No admixed strains were identified when assuming two genetic populations. One genetic population was constituted by all the lacustrine strains from the Vestfold Hills as well as the marine strains from the neighborhood of the Showa Island (MBIC). These two locations are separated by around 1,900 KM. The Antarctic and Arctic strains CCMP1383-2088 respectively, were assigned to the second genetic population, despite these strains are separated by around 20,000 KM. This segregation was congruent also with the UPGMA analyses, in which the latter two strains were separated from all the other *Pa. glacialis* strains.

Despite belonging to the same genetic population, the *Pa. glacialis* strains from lacustrine Antarctic habitats and the marine Antarctic strains from the vicinity of Showa Island were significantly differentiated according to the UPGMA results. If transitions between marine and lacustrine environments were frequent, then we would have expected a mixed pattern consisting of interspersed marine and lacustrine *P. glacialis* strains. However, no marine *Pa. glacialis* strain occurring in the neighborhood of the studied lakes could be included in this study, and therefore we can not ascertain that if included, a mixed pattern would emerge. Nevertheless, it is possible that the genetic differences between lacustrine and marine *Pa. glacialis* arose in the last 6,000 years, the age of the studied Antarctic lakes (Zwartz et al., 1998). The salinity of the lakes in question has undergone important changes from the ancestral marine conditions (salinity ~ 32). While the salinity of one of the two lakes with *Pa. glacialis* has decreased to brackish (Lake Abraxas; salinity ~ 18), the second lake has become hypersaline (Ekho Lake; salinity ~ 40). Salinity appears to be an important variable that affects microbial distributions (see Scheckenbach et al., 2006; Alverson et al., 2007; Logares et al., 2007b), most probably due to its effects on cell osmoregulation (see Lee and Bell, 1999). The different natural selection regimes due to salinity gradients that ancestrally marine *Pa. glacialis* populations encountered in the Antarctic lakes could have promoted a rapid evolutionary diversification. Despite that the studied Antarctic lakes are roughly only 6,000 years old, strong and disruptive natural selection regimes can potentially produce extremely rapid divergences (see Orr and Smith, 1998). If adaptation to different lake salinities has occurred, then the *Pa. glacialis* strains from Lake Abraxas and Ekho Lake should have segregated into separate genetic groups in the UPGMA analyses. This segregation was observed except for one isolate (Fig. 3 A). A higher sample size is needed to ascertain if Ekho and Abraxas harbor genetically differentiated populations of *Pa. glacialis.*

Similar to lakes Ekho and Abraxas, the lakes with *S.* aff. *hangoei* have also suffered major changes in their salinity composition over the last 6,000 years. Originally filled with marine waters (salinity 32), the salinity of the lakes Highway, Vereteno, Hand and McNeill have decreased considerably (salinity ~ 5 in the five lakes). The apparent lack of genetic differentiation among *S.* aff. *hangoei* strains from different lakes could be due to the reason that all strains were exposed to similar conditions of reduced salinity (i.e. natural selection acting on the same direction). However, a more extensive sampling is needed to determine if lake-populations have developed certain degrees of genetic differentiation.

What generates and maintains genetic diversity and differentiation?

Our results indicated variable amounts of intraspecific genetic diversity in the species studied. The sources of the observed diversity could be 1) new mutations, 2) immigrants bringing new alleles and genotypes and, 3) recombination, generating new genotypes. To our knowledge, there are no published data on the mutation and dispersal rates occurring in dinoflagellate populations. Nevertheless, due to the huge population sizes and high reproductive rates of dinoflagellates, it is likely that many new mutations emerge in their

populations per any given amount of time. The occurrence of elevated amounts of mutations could explain in part the relatively high diversity we have found within some lakes.

Sexuality among dinoflagellates has been documented in many species (Pfiester and Anderson, 1987) and there are reasons to believe that most dinoflagellates can reproduce sexually (Figueroa, 2005). Among the dinoflagellates we have studied, sexuality has been only confirmed in *P. cinctum* (Pfiester, 1975), and could possibly occur in the other studied species since resting cysts, which many times are associated to sex, have been observed in all of them (Rengefors and Anderson, 1998; Boltovskoy, 1999; Montresor et al., 2003a; Rengefors et al., 2008). However, there are also examples where resting cyst production is not necessarily associated to sex (Kremp and Parrow, 2006). The occurrence of recombination in the studied species could partially explain the relatively high diversity of genotypes we have found. However, the considerable amounts of differentiation among genotypes in some species suggest that recombination might not necessarily be high.

Part of the observed genetic diversity could also be explained by the characteristics of the dinoflagellate life cycle. Several dinoflagellate species produce sexual cysts at the end of the growing season that accumulate in the sediments and can remain dormant for several years before germinating (Pfiester and Anderson, 1987). Subsequent multi-clonal planktonic populations are regenerated every year from those cyst banks. Thus, each planktonic population would constitute a subset sample of the many genotypes present in the sediments. Previous studies have also proposed that the dinoflagellate life cycle can contribute to the high genetic diversity found within several marine dinoflagellate species (John et al., 2004).

Several strains were strongly assigned to a genetic population in most of the Bayesian analyses where genetic structure was indicated. This suggests the occurrence of few inter-population recombinant genotypes within the species we have studied. Moreover, in most of the species in which genetic populations were inferred, strains from different genetic populations were found coexisting within at least one lake. The subsequent question is how the different genetic clusters can coexist within a lake in the presence of recombination. This indicates that some mechanisms might prevent the genetic homogenization of strains from different genetic populations within lakes. For instance, mechanisms of gamete recognition (Starr et al., 1995), where cells from the same genetic population tend to recombine; temporal or spatial separation of strain from different genetic populations or selection against recombinant genotypes.

Concluding remarks

Lakes can be regarded as discrete patches with different natural selection regimes (in comparison to marine environments). It could be expected that microbial populations inhabiting lacustrine environments will become locally adapted and this adaptation would be reflected in their patterns of genetic variation. Despite that AFLP screens a considerable fraction of the genome (including neutral and selected regions) we did not find evidence that a few highly adapted genotypes dominate each dinoflagellate lake-population. In contrast, our data suggest that lakes and ponds are populated by a multitude of genotypes, paralleling similar findings in marine (Bolch et al., 1999a; Bolch et al., 1999b; Medlin et al., 2000; Rynearson and Armbrust, 2000; Shankle et al., 2004; Evans et al., 2005; Iglesias-Rodriguez et al., 2006) and other freshwater microbes (Kusch et al., 2000; De Bruin et al., 2004; Muller et al., 2005; Wilson et al., 2005; Zhang et al., 2006). Still, it remains to be investigated whether these relatively high levels of genetic diversity have an adaptive advantage or if they simply represent neutral background variation that accumulates due to the large population sizes. Finally, we have not found conclusive evidence indicating that lakes harbor genetically distinct microbial populations. Future studies using a larger number of strains should investigate the degree of adaptation of dinoflagellate populations to different lake conditions.

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Extensive dinoflagellate phylogenies indicate infrequent marine–freshwater transitions

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Abstract

We have constructed extensive 18S–28S rDNA dinoflagellate phylogenies (>200 sequences for each marker) using Maximum Likelihood and Bayesian Inference to study the evolutionary relationships among marine and freshwater species (43 new sequences). Our results indicated that (a) marine and freshwater species are usually not closely related, (b) several freshwater species cluster into monophyletic groups, (c) most marine–freshwater transitions do not seem to have occurred recently and, (d) only a small fraction of the marine lineages seem to have colonized fresh waters. Thus, it becomes apparent that the marine–freshwater boundary has acted as a barrier during the evolutionary diversification of dinoflagellates. Our results also shed light on the phylogenetic positions of several freshwater dinoflagellates which, to date, were uncertain.

Keywords: Dinoflagellates; Freshwater; Colonization; Phylogeny

1. Introduction

Most major microbial lineages originated in ancient oceans (e.g. Cavalier-Smith, 2006) and subsequently colonized fresh waters. From an evolutionary perspective, oceans can be considered as continents and lakes as islands for aquatic organisms. However, there is an important difference to take into account in this analogy. Marine and fresh waters are two environments which differ in their general physicochemical characteristics, and that has no parallel when continents and islands are compared. For most aquatic animals, the differences in osmotic pressure and ionic concentrations between marine and fresh waters represent a strong barrier that cannot be crossed by most species, which are normally adapted to one environment or the other, but not to both (Lee and Bell, 1999). It is unclear, however, to what extent the physicochemical differences affect the exchange of microbes between marine and fresh waters. In contrast to macroorganisms, most microbes have massive population sizes, high reproductive rates, high genetic diversity (e.g. Snoke et al., 2006) as well as the potential capability for long distance dispersal (Finlay, 2002). These characteristics suggest that frequent migration between marine and freshwater environments as well as rapid ecological diversification within species may be possible.

Traditionally, morphological classifications have suggested that several protist groups are segregated into predominantly marine and freshwater lineages (e.g. Taylor, 1987; Popovsky and Pfiester, 1990; Graham and Wilcox, 2000; Sims et al., 2006). However, the ambiguities of morphological phylogenies precluded further investigations on the phylogenetic relationships between marine and freshwater taxa. Today, molecular phylogenies open a new opportunity for studying the number and timing of marine–freshwater transitions during the evolutionary history

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of a microbial lineage. For instance, the presence of a few monophyletic clusters of freshwater species that are distantly related to all other marine species within an ancestral marine lineage, would indicate that freshwater colonizations are rare and probably not recent events. On the other hand, the presence of many closely related marine and freshwater species or strains would suggest that marine–freshwater transitions have occurred frequently. Recent molecular phylogenies comprising and array of prokaryote and microeukaryote taxa indicate that marine and freshwater species are normally not closely related (Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Saldarriaga et al., 2004; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; von der Heyden and Cavalier-Smith, 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Lefevre et al., 2007). Despite the insight these works have provided, they normally include relatively few freshwater species and are mostly focused in other questions than marine–freshwater transitions.

Dinoflagellates, an ancestral marine protist lineage which is present in both marine and fresh waters, constitute a well suited group for investigating marine–freshwater transitions in microbes. DNA sequences (especially 18S and 28S rDNA) from many dinoflagellate taxa are publicly available and there is also a large amount of data on dinoflagellate morphology, physiology, ecology, and fossil record. Dinoflagellates appear to have diverged from ciliates and apicomplexans around 900 million years ago [MYA] (Escalante and Ayala, 1995). At the beginning of the Mesozoic (~250 MYA), dinoflagellates showed a tremendous evolutionary radiation (Fensome et al., 1996, 1999). Dinoflagellates have an enormous diversity of life strategies, with symbionts, parasites, free living planktonic or benthic photosynthesizers, heterotrophs and mixotrophs (Hackett et al., 2004). Usually, dinoflagellates spend most of their life cycles as haploid cells that proliferate by mitotic division (Von Stosch, 1973). Sexuality can be induced by endo- and exogenous factors, and in many cases, results in a diploid resting cyst with environmental resistance and dispersal functions (Pfiester and Anderson, 1987). Dinoflagellates have key roles in the functioning of marine and freshwater ecosystems, and several species are well known toxin producers (e.g. Hallegraeff, 1993).

The phylogenetic relationships between marine and freshwater dinoflagellates have for long been considered unclear (e.g. see Boltovskoy, 1999). To date, most molecular phylogenies have only included a small number of freshwater dinoflagellates, thus contributing little to the clarification of their phylogenetic positions. As a consequence, the number and timing of marine–freshwater transitions in dinoflagellates have so far remained a matter of speculation.

The main objectives of this study are thus: (a) to get insight into the role of the marine–freshwater boundary throughout the diversification of dinoflagellates by analyzing molecular phylogenies, and (b), to shed light into the phylogenetic relationships between marine and freshwater species. Our approach consisted of constructing extensive 18S and 28S (D1/D2) rDNA phylogenies, including 43 new dinoflagellate sequences and publicly available ones. In total, the used sequence dataset represent a major fraction of the dinoflagellate diversity. Our phylogenetic results using Maximum Likelihood and Bayesian Inference suggest that marine–freshwater transitions have been infrequent events during the diversification of dinoflagellates and that in most cases have not occurred recently. In addition, our results suggest the phylogenetic positions of several freshwater dinoflagellate species which to date have been uncertain.

2. Materials and methods

2.1. Morphospecies and DNA extraction

Freshwater and brackish photosynthetic dinoflagellate morphospecies were either obtained from cultures established at our laboratory from plankton samples, or from one of the following sources: Culture Collection of Algae at the University of Cologne, Germany (CCAC); National Institute for Environmental Studies, Japan (NIES); Culture Collection of Algae at the University of Göttingen, Germany (SAG); Culture Collection of Algae and Protozoa, UK (CCAP); The Culture Collection of Algae at the University of Texas at Austin, US (UTEX); Carolina Biological Supplies; private collections from A. Kremp [*Woloszynskia halophila* (Biechler) Kremp et al. WHTV, *Scrippsiella hangoei* (Schiller) Larsen SHTV (brackish)] and E. Kim [*Peridinium limbatum* (Stokes) Lemmermann PLCB, *P. willei* Huitfeld-Kaas PWCL] (Table 1). DNA was extracted from clonal single-cell isolated cultures following Adachi et al (1994).

2.2. Morphospecies identification

The dinoflagellate morphospecies isolated and cultured from our own plankton samples were fixed in formaldehyde 5% for further taxonomic identification. A number of dinoflagellate morphospecies obtained from culture collections were fixed in the same manner for taxonomic corroboration. Dinoflagellate plate detachment between slide and cover slip was carried out with the aid of diluted sodium hypochlorite instillation. Squashed empty thecae and detached plates were analyzed under a Standard 14 Zeiss optical microscope with Nomarsky interference contrast illumination. The morphospecies that were identified or corroborated for this work are specified in Table 1.

2.3. PCR and sequencing

For this study we have chosen the small (18S) and large (28S) subunit ribosomal DNA (SSU and LSU rDNA, respectively) because they have been sequenced for many marine dinoflagellates representing most major taxa, as

Table 1
Freshwater dinoflagellates included in the analyses and the 43 sequences obtained for this work

Morphospecies name	Strain	Geographical origin	GenBank accession number	
			SSU	LSU (D1/D2)
Ceratium hirundinella	None	Lake in San Juan island, Washington, USA	AY443014	—
Ceratium hirundinella	HZ-2004	Mirror Lake, Storrs, CT, USA	AY460574	—
Ceratium sp.	HCB-2005	Old Woman Creek NER, USA	DQ487192	—
Cystodinium phaseoulus	ASW12002	Lake Lunzer, Obersee, Austria	EF058235	—
Dinophyceae	Clone L	Lake Tovel, Italy	—	AY827950
Esoptrodinium gemma	None	University of Aveiro Campus, Portugal	—	DQ289020
Glenodiniopsis steinii	NIES 463	Shizukuishi, Iwate, Japan	AF274257	EF058255
Kryptoperidinium foliaceum	SAG 38.80	Assateague Nat. Wildlife Refuge, USA	—	EF058256
Gloeodinium montanum	CCAC0066	Marburg, Nordeck, Germany	EF058238	EF058258
Gymnodinium impatiens	CCAC0025	Bradenburg; Neuglobsow, Germany	EF058239	EF058259
Gymnodinium palustre	AJC14-732	?	—	AF260382
Gymnodinium sp.	None	Lake Tovel, Northern Italy	AY840208	—
Gymnodinium sp.	LaTo2	Lake Tovel, Northern Italy	AY829527	—
Gymnodinium sp.	Clone M	Lake Tovel, Northern Italy	—	AY829529
Gymnodinium sp.	Clone N	Lake Tovel, Northern Italy	—	AY829530
Gyrodinium helveticum	None	Lake Shikotsu, Hokkaido, Japan	AB120004	—
Hemidinium nasutum	NIES 471	Tsuchiura, Ibaraki, Japan	AY443016	EF058260
Jadwigia applanata	CCAC0021	Biebergemund-Bieber, Lochmuhle, Germany	EF058240	AY950447
Jadwigia applanata	FW 145	?	—	AY950448
Peridiniopsis borgei[a]	PBSK-A	St. kalkbrottsdammen, Skåne, Sweden	EF058241	EF058261
Peridinium aciculiferum	PAER-1	Lake Erken, Sweden	AY970653	AY970652
Peridinium bipes	HY971028T	Korea	—	AY359682
Peridinium bipes f. globossum	NIES495	Lake Onogawa, Fukushima, Japan	EF058242	EF058262
Peridinium bipes f. occultatum	None	Japan	AF231805	—
Peridinium bipes f. occultatum	HYSS0312-04	Korea	—	AY733011
Peridinium centenniale[a]	CCAC0002	Cornwall, England	EF058236	EF058254
Peridinium cf. centenniale[a]	ASW12003	Jakobshaven, Greenland	EF058237	EF058257
Peridinium cinctum	AJC4	?	—	AF260385
Peridinium cinctum	CCAC0102	Spiekeroog, Germany	EF058244	EF058264
Peridinium cinctum	CCAP 1140/1	Plußsee, Germany	DQ166209	EF058263
Peridinium cinctum	None	Kiritappu Moor, eastern Hokkaido, Japan	AB185114	—
Peridinium cinctum[a]	PCGY-4	Lake Gyllebo, Skåne, Sweden	EF058245	EF058265
Peridinium cinctum[a]	SAG2017	Cappeler Weiher, Marburg, Germany	EF058243	EF058266
Peridinium gatunense	CCAP?	?	DQ166208	—
Peridinium gatunense[a]	PGDA-1	Lake Dagstorpsjön, Skåne, Sweden	EF058246	EF058267
Peridinium inconspicuum	CCAP1140/3	Kl. Ukleisee,Germany	EF058247	EF058268
Peridinium inconspicuum	UTEX LB2255	?	AF274271	—
Peridinium limbatum	PLCB-1	Crystal Bog, Oneida County, Wisconsin, USA	—	EF058269
Peridinium palatinum	AJC4cl-a	?	—	AF260394
Peridinium polonicum	NIES 500	Japan	AY443017	—
Peridinium pseudolaeve	AJC6-798	?	—	AF260395
Peridinium sp.	Carolina	USA	DQ166210	—
Peridinium sp.	None	?	AF022202	—
Peridinium sp.	HCB-2005	Old Woman Creek, Ohio, USA	DQ487197	—
Peridinium sp.	None	Lake Tovel, Northern Italy	AY827955	—
Peridinium volzii	NIES501	Pond, Tsuchiura, Ibaraki, Japan	EF058248	EF058270
Peridinium wierzejskii	NIES 502	Tsuchiura, Ibaraki, Japan	AY443018	—
Peridinium willei	PWCL1	Crystal Lake, Oneida County, Wisconsin, USA	EF058250	—
Peridinium willei	NIES366	Pond, Tsuchiura, Ibaraki, Japan	EF058249	EF058273
Peridinium willei	NIES 304	Tsukiyono, Gunma, Japan	AF274272	EF058271
Peridinium willei	NIES 365	Pond, Ajiro, Iwate, Japan	AF274280	EF058272
Peridinium willei	AJC2-675	?	—	AF260384
Peridinium willei	TK007	Kiritappu Moor, eastern Hokkaido, Japan	AB232669	AB232669
Peridinium willei	PWCA-1	Glenmore Reservoir, Calgary, Alberta, Canada	DQ166211	EF058274
Peridinium willei[a]	PWGY-B	Gyllebosjön, Skåne, Sweden	EF375879	—
Phytodinium sp.	ASW12001	Peat bog Neuhauser Moor, Mariazell, Austria	EF058251	—
Prorocentrum foveolatum	PFBL01	Tasmania, Australia	—	AY259172
Prorocentrum playfairii	PPWL01	Tasmania, Australia	—	AY259174
Scrippsiella-like	High-1-a	Highway lake, Vestfold Hills, Antarctica	—	EF058275
Tovellia coronata	Clone F1	Pond near Aneboda, Sweden	—	AY950446
Tovellia leopoliensis	NIES 619	Mitsukaido, Ibaraki, Japan	AY443025	—

(*continued on next page*)

Table 1 (*continued*)

Morphospecies name	Strain	Geographical origin	GenBank accession number	
			SSU	LSU (D1/D2)
Woloszynskia halophila	WHTV-S1	Baltic Sea, Finnish coast	EF058252	—
Woloszynskia pascheri	CCAC0075	Göttingen, Gemany	EF058253	EF058276
Woloszynskia pseudopalustris	AJC12cl-915	?	—	AF260402
Woloszynskia tenuissima	SCCAP K-0666	?	—	AY571374

The sequences obtained for this work start with "EF". Strain abbreviations are specified in Section 2.
[a] Indicates morphospecies identified for this work. The complete SSU/LSU sequence datasets are shown in Supplementary Materials.

well as a number of freshwater morphospecies. In addition, the SSU and LSU have slow or moderate evolutionary rates, which allows for the construction of phylogenies of distantly related taxa.

PCR amplifications were done using 25 ng of template genomic DNA, 0.125 mM of each nucleotide, 3.0 mM $MgCl_2$, 1× PCR buffer, 0.4 μM of each primer, and 0.5 U of Taq DNA Polymerase (AmpliTaq®, Applied Biosystems, Foster City, California) in 25 μl total volume reactions. For the SSU PCR we used the combination of the universal primers 4616 (forward) 5′-AACCTGGTTG ATCCTGCCAG-3′ and 4618 (reverse) 5′-TGATCC TTCTGCAGGTTCACCTAC-3′. The SSU PCR started with 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C, 2 min at 72 °C, ending with a final hold of 7 min at 72 °C. For the domains D1/D2 of the LSU rDNA we used the primers DinFi (forward) 5′-GCATATAAGTAMGYGGWGG-3′ and DinRi (reverse) 5′-CCGTGTTTCAAGACGGGTC-3′. The LSU PCR temperature profile was equivalent to the SSU except in that it consisted of 30 amplification cycles with a primer annealing temperature of 50 °C. All PCR amplicons were cleaned using PCR-M™ Clean-Up System (Viogene, Taiwan). LSU fragments were directly sequenced from both sides using the same PCR primers. SSU amplicons were directly sequenced using the PCR primer 4616 plus the sequencing primers 516F 5′-CACATCTAAGGAAGG CAGCA-3′, 528F 5′-CGGTAATTCCAGCTCC-3′, 690F 5′-CAGAGGTGAAATTCT-3′ and 1055F 5′-GGTGG TGCATGGCCG-3′ (Edvardsen et al., 2003). The sequencing reactions were carried out using BigDye (v1.1, Applied Biosystems) chemistry and the products were precipitated following the manufacturer instructions and then loaded into an ABI Prism 3100 sequencer (Applied Biosystems). The obtained SSU and LSU sequences were edited and assembled by analyzing carefully the chromatograms using Bioedit (v7.0.4.1; Hall, 1999). Sequences were deposited in GenBank under the Accession Nos. EF058235–EF058276/EF375879 (Table 1).

3. Phylogenetic analyses

3.1. Alignment construction

SSU and LSU sequences representing most dinoflagellate lineages were downloaded from GenBank (downloaded freshwater sequences are indicated in Table 1; the complete sequence datasets are indicated in Supplementary Materials). After the elimination of identical and apparently erroneous sequences, we created alignments using ClustalX (v1.8; Thompson et al., 1997). We constructed two initial alignments containing 238 sequences and 1850 characters for the SSU and 203 sequences and 619 characters for the LSU. Since in some cases the SSU and LSU were not available for the same species, SSU and LSU alignments differed in species composition.

To check for the potential influence of ambiguously aligned positions and divergent regions on the phylogenies, we constructed more stringent alignments (i.e. with more removed positions) using the program Gblocks (v0.91b, Castresana, 2000) as well as visual examination. The more stringent SSU and LSU alignments consisted of 237 sequences with 1592 characters and 200 sequences with 479 characters, respectively. The elimination of positions can also eliminate phylogenetic signal. For this reason, we ran phylogenetic analyses with alignments which differed in the number of removed positions (see Table 2).

We also constructed alignments with fewer sequences to check if this would have an effect on the phylogenetic reconstructions. The eliminated sequences belonged to significantly supported marine clades with no freshwater relatives. The number of excluded sequences was not high since there are studies indicating that more taxa for a given marker can improve tree reconstruction (see Pollock et al., 2002; Hedtke et al., 2006). The original 238-sequence SSU alignment was reduced to 168 sequences and 1828 characters. In the same manner, the original 203-sequence LSU alignment was reduced to 150 sequences and 619 characters. G blocks along with visual edition were also used on these sequence-reduced alignments. As a result, we generated a 168-sequence alignment with 1610 characters for the SSU and a 147-sequence alignment with 450 characters for the LSU. All these alignments were used for phylogenetic construction using models that do not consider the secondary structure of the rRNA (see Table 2). Alignments are available upon request.

3.2. Phylogenetic inference without considering rRNA secondary structure

Phylogenies were estimated using Maximum Likelihood (ML) and Bayesian Inference (BI) as implemented in

Table 2
Constructed phylogenies: datasets, evolutionary models, and resulting parameters

Tree #	Dataset			Method	Boot	Model	−lnL (±10)	α (±0.01)	Pinvar
	rDNA	Seq	Char						
1a	SSU	238	1850	BI	—	GTR+Γ+COV	−54950	0.39	—
1b	SSU	238	1850	BI	—	GTR+Γ+COV	−54954	0.39	—
2a	SSU	238	1850	BI	—	GTR+Γ+COV[a]	−55265	0.54	—
2b	SSU	238	1850	BI	—	GTR+Γ+COV[a]	−55260	0.55	—
3a	SSU	237	1592	BI	—	GTR+Γ+COV	−49423	0.38	—
3b	SSU	237	1592	BI	—	GTR+Γ+COV	−49431	0.38	—
4	SSU	238	1850	ML	1000	GTR+Γ+I	−50779	0.66	0.23
5	SSU	237	1592	ML	100	GTR+Γ+I	−46421	0.63	0.20
6a	SSU	168	1828	BI	—	GTR+Γ+COV	−43109	0.33	—
6b	SSU	168	1828	BI	—	GTR+Γ+COV	−43114	0.33	—
7a	SSU	168	1610	BI	—	GTR+Γ+COV	−39726	0.33	—
7b	SSU	168	1610	BI	—	GTR+Γ+COV	−39716	0.33	—
8	SSU	168	1828	ML	100	GTR+Γ+I	−40794	0.66	0.30
9	SSU	168	1610	ML	100	GTR+Γ+I	−36741	0.66	0.32
10a	LSU	203	619	BI	—	GTR+Γ+COV	−36987	0.71	—
10b	LSU	203	619	BI	—	GTR+Γ+COV	−36990	0.71	—
11a	LSU	200	479	BI	—	GTR+Γ+COV	−26825	0.71	—
11b	LSU	200	479	BI	—	GTR+Γ+COV	−26816	0.71	—
12	LSU	203	619	ML	1000	GTR+Γ+I	−33827	0.78	0.04
13	LSU	200	479	ML	1000	GTR+Γ+I	−24004	0.73	0.05
14a	LSU	150	619	BI	—	GTR+Γ+COV	−29792	0.65	—
14b	LSU	150	619	BI	—	GTR+Γ+COV	−29803	0.65	—
15a	LSU	147	450	BI	—	GTR+Γ+COV	−19036	0.66	—
15b	LSU	147	450	BI	—	GTR+Γ+COV	−19031	0.65	—
16	LSU	150	619	ML	1000	GTR+Γ+I	−26458	0.78	0.08
17	LSU	147	450	ML	1000	GTR+Γ+I	−16633	0.75	0.06

BI, Bayesian MCMC Inference; ML, Maximum Likelihood; Seq, number of sequences in the alignment; Char, number of characters in the alignment; Boot, number of bootstrap pseudoreplicates (only ML); GTR, General Time Reversible model; Γ, gamma distributed rate of variation across sites; COV, covarion model; I, proportion of invariable sites model; −lnL, log-likelihood: in ML, highest log-likelihood tree within the bootstrap pseudoreplicates; in BI, harmonic mean of the log-likelihood of the cold chain after the burn-in phase; α, shape parameter of the gamma distribution: in ML, alpha corresponds to the highest log-likelihood tree; in BI: alpha corresponds to the mean value after the burn-in phase; pinvar, proportion of invariable sites (only for ML, corresponds to the highest log-likelihood tree).
All Bayesian MCMC analyses were run for 5×10^6 generations with seven Markov chains and a burn-in of 2×10^6 generations.
a and b indicate replicates of each Bayesian run.
[a] Doublet and 4by4 models for the stem and loop areas of the SSU correspondently.

GARLI (serial version, v0.942; Zwickl, 2006) and MrBayes (v3.1.2 parallel version [MPI]; Metropolis-coupled Markov Chain Monte Carlo model [MCMC] approach for approximation of Bayesian posterior probabilities [PPs]; Huelsenbeck and Ronquist, 2001; Altekar et al., 2004). The hierarchical likelihood ratio test (Huelsenbeck and Crandall, 1997) and the Akaike information criterion (Akaike, 1974), as implemented in ModelTest (v3.7; Posada and Crandall, 1998) indicated that the General Time Reversible (GTR) model of nucleotide substitution, with a Gamma (Γ) distributed rate of variation across sites and a proportion of invariable sites (I) was the most appropriate evolutionary model for our SSU and LSU datasets. In ML and BI analyses, the shape parameter (α) of the Gamma (Γ) distribution and the proportion of invariable sites (I) were estimated from the datasets using default options in the programs GARLI and MrBayes.

All Bayesian MCMC analyses were run with seven Markov chains (six heated chains, one cold) for 5×10^6 generations and the trees were sampled every 100 generations, which resulted in 5×10^4 sampled trees. Each analysis used default (flat) priors and was repeated at least twice from random starting trees. The evolutionary model used in Bayesian analyses was the GTR+Γ+COV. The Covarion Model (COV) allows substitution rates to change across positions through time (Miyamoto and Fitch, 1995; Huelsenbeck, 2002). The COV model can be regarded as a general case of the proportion of invariable sites model (Huelsenbeck, 2002) permitting sites to change between invariable and variable states in an "on–off" fashion independently from each other (as implemented in MrBayes). The covarion model was used since previous phylogenetic analyses with dinoflagellate rDNA (Shalchian-Tabrizi et al., 2006b), along with studies in other taxa, indicate that this model gives a better explanation of rDNA data (Galtier, 2001; Huelsenbeck, 2002; Shalchian-Tabrizi et al., 2006a). The obtained posterior probability (PP) values for the branching patterns as well as marginal likelihoods for the tree reconstructions were compared to ensure convergence. Consensus trees and PPs were calculated using the 3×10^4 trees after the log-likelihood stabilization (burn-in phase).

ML analyses in GARLI were run with 100 or 1000 bootstrap (Felsenstein, 1985) pseudoreplicates. All parameters were used in default options, except for the number of generations that the program should run with no significant improvements in the scoring of the topology, which was set to 5000. All analyses in GARLI were run under the GTR+Γ+I model, since the covarion model is not implemented. Consensus trees from the bootstrap output were generated using MrBayes.

Phylogenetic analyses with MrBayes and GARLI were run at the University of Oslo Bioportal (http://www.bioportal.uio.no/). The trees generated with MrBayes and GARLI were visualized in TreeView (v1.6.6; Page, 1996) and further edited in MEGA (v 3.1; Kumar et al., 2004).

3.3. Phylogenetic inference considering rRNA secondary structure

The rRNA molecule has a complex secondary structure that comprises stems (paired nucleotides) and loops (unpaired nucleotides) (Hillis and Dixon, 1991). To maintain this secondary structure, mutations in stem nucleotides are usually compensated by other stem mutations, therefore nucleotide variation in stem areas can be correlated, violating an assumption made by most models of nucleotide substitution. The effect of differential variation in loop and stem areas for phylogenetic construction has been investigated (e.g. Dixon and Hillis, 1993) and models that take into account this covariation have been proposed (e.g. Schoniger and von Haeseler, 1994; Muse, 1995). Some researchers have reported considerable improvements in phylogenetic reconstructions after implementing models which consider nucleotide covariation (e.g. Murray et al., 2005; Telford et al., 2005). In order to investigate the effects of nucleotide covariation in our study, we have constructed SSU phylogenies considering the secondary structure. The SSU rRNA secondary structure of the dinoflagellate *Peridinium aciculiferum* was estimated with MFOLD (Zuker et al., 1999) and used as a general model for estimating the covariating sites in the SSU alignment consisting of 238 sequences and 1850 characters. This secondary structure was used only for estimating the covariating SSU sites and not for trying to improve the alignments. Partitioned Bayesian MCMC phylogenetic analysis were carried out using the Doublet model (based on the SH model of Schoniger and von Haeseler, 1994) for the covariating stem areas and the 4by4 for the loop areas. For stem and loop areas, the GTR+Γ+COV model was used. Bayesian MCMC analyses were run with seven Markov chains (one cold and six heated) for 5×10^6 generations, and the trees were sampled every 100 generations, which resulted in 5×10^4 sampled trees. Consensus trees and PPs were calculated from the 3×10^4 trees after the log-likelihood stabilization. The generated trees were visualized and edited in the same way as indicated above. The comparison between the model considering the secondary structure (H_1 = doublet+4by4) and the one not considering it (H_0 = 4by4) was done using a Bayes factor (B_{10}), which is equal to the ratio of the posterior probabilities of H_1 and H_0 given that the prior probabilities for H_1 and H_0 are equal. The marginal likelihood for each model was estimated using the harmonic mean of the likelihood values obtained during the stationary phase of the MCMCs, as suggested by Newton et al. (1994). Bayes factors were interpreted as suggested by Kass and Raftery (1995). We did not carry out secondary structure analysis with the LSU since the results with the SSU did not indicate that major changes occur by using this model.

4. Results

4.1. Phylogenetic results

All the data presented here represent a summary of the results obtained from a total of 26 constructed phylogenies (Table 2). Except when specified, we do not refer to a clade seen in a single consensus tree, but to clades which were shared among all or most of the different phylogenies. The trees in Figs. 1 and 2 correspond to trees 1a and 10a in Table 2, respectively. These trees are intended to represent the entire set of phylogenetic results instead of a single phylogenetic reconstruction. Posterior Probabilities (PPs) and Bootstrap support values (BVs) for some clades recurrently obtained across the phylogenies are presented in Table 3.

Around 70% of the analyzed freshwater SSU (19 out of 27) and LSU (24 out of 30) sequences clustered with other freshwater sequences (Figs. 1 and 2; sequences from the same morphospecies were counted only once in the calculation of the percentage). Only two freshwater sequences (*Peridinium aciculiferum* and *Woloszynskia pseudopalustris*) affiliated very closely with marine species (Figs. 1 and 2). Some freshwater clades and single species did not show any consistent affiliation with any other lineage (e.g. the clades PESS, PBOR, TOVE [see Table 3 for definitions], *Hemidinium nasutum*), while other freshwater clades or species affiliated to marine groups (e.g. WOLO, *Gyrodinium helveticum*, *Gymnodinium palustre*) (Figs. 1 and 2). Several freshwater sequences or clades displayed long branches in the phylogenies (e.g. TOVE, PESS, WOLO, *Hemidinium nasutum*; Figs. 1 and 2).

Most SSU and LSU phylogenies had weakly defined backbone topologies but several well supported internal clades (Figs. 1 and 2, Table 3). In most SSU and LSU trees, several clades were repeatedly obtained using different datasets, tree-reconstruction methods and evolutionary models (examples of recurring clades in Table 3). There were some cases where moderately or well supported clades by one tree reconstruction technique or dataset were poorly supported, or not present at all, using different methods or datasets (e.g. clades 9 and 14 in Table 3).

The implementation of the model not considering the SSU secondary structure [4by4] (H_0) was ~310 lnL units

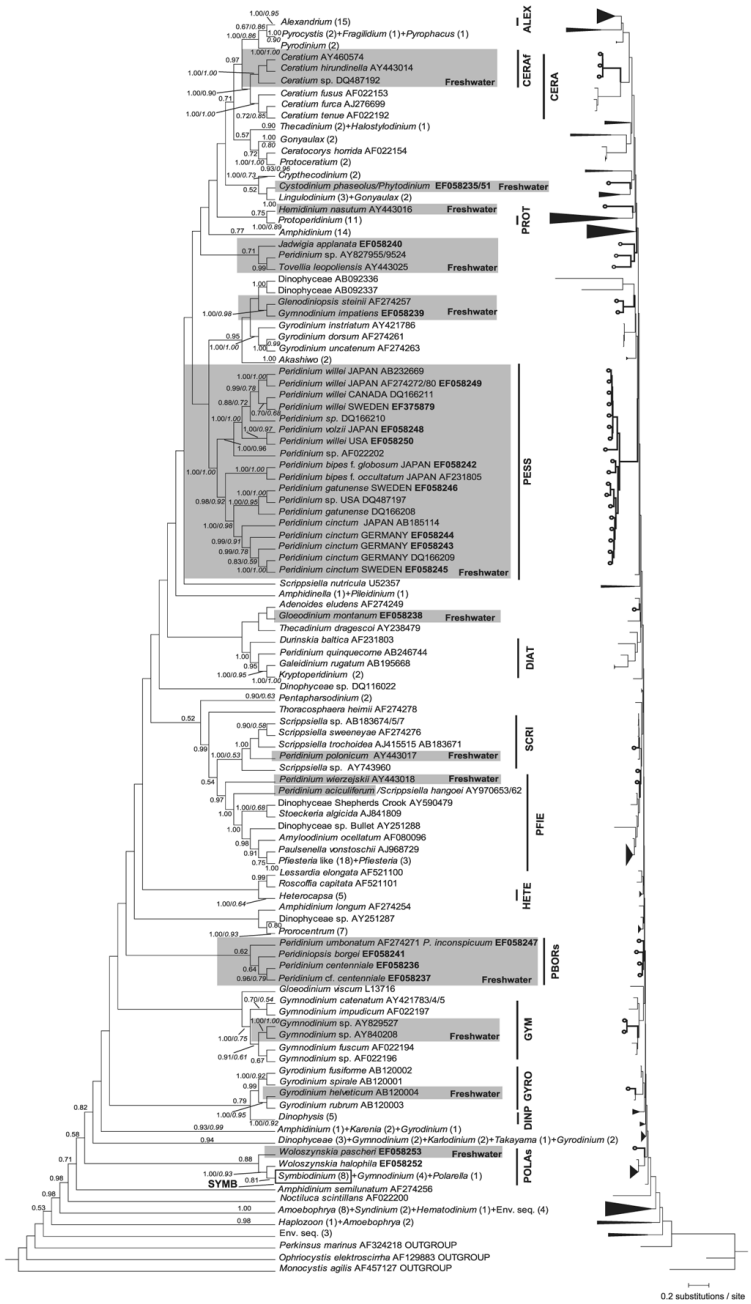
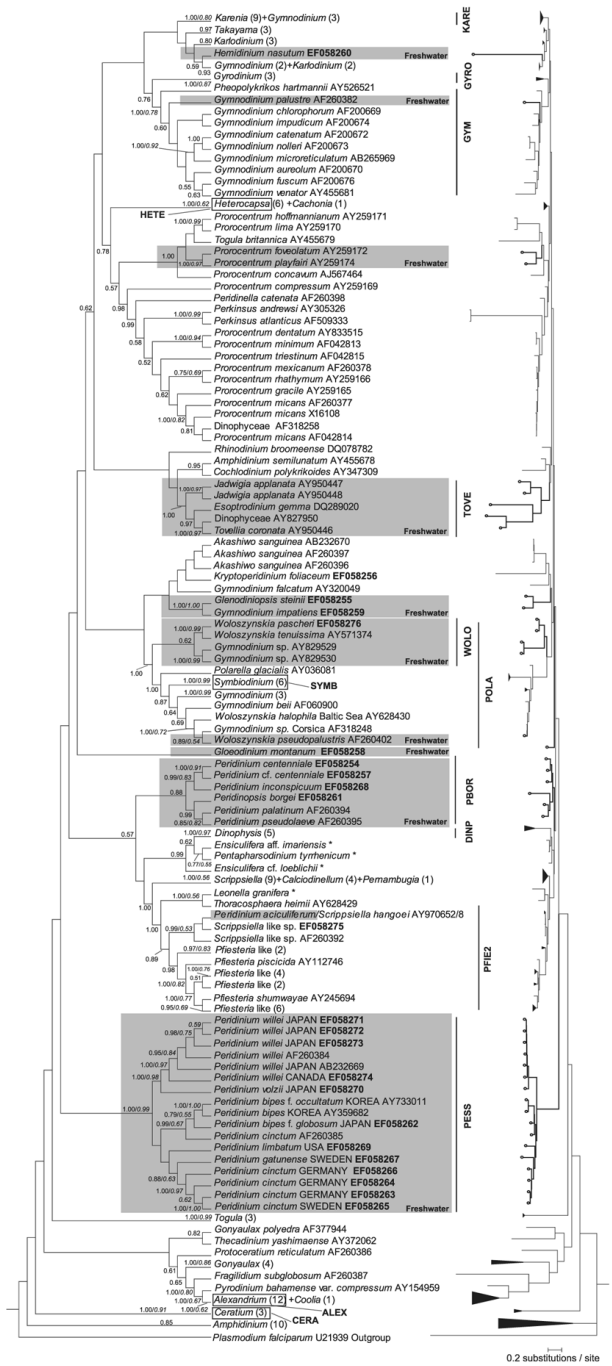


Fig. 1. Consensus SSU rDNA phylogeny constructed from an alignment with 238 sequences and 1850 characters under the GTR+Γ+COV (Bayesian Inference) and GTR+Γ+I (Maximum Likelihood) models. This consensus phylogeny corresponds to the trees 1a and 4 (Table 2) and intends to represent the whole set of constructed SSU trees (Table 2). The tree on the left shows the topology and posterior probabilities (normal print)/bootstrap support (italics) values >0.50. The tree on the right shows the evolutionary distances obtained by Bayesian MCMC Inference. Freshwater taxa and clusters are shown within frames in the cladogram (left) and indicated with a small terminal circle in the phylogram (right). The triangles in the phylogram correspond to marine clades that were collapsed. The number of collapsed sequences is indicated within parenthesis. The clusters indicated with the vertical lines or rectangles are described in Table 3 or Section 4.

better than the Doubles+4by4 model (H_1), thus producing a Bayesian factor which indicates that there is no phylogenetic improvement considering the rDNA secondary structure (Table 2).



In most cases, ML and BI phylogenies using SSU and LSU supported (BVs and PPs > 0.80) the monophyly of some major dinoflagellate genera (Table 3, Figs. 1 and 2). In general, the PP values were higher than the bootstrap values (Table 3). This is most probably related to the fact that BVs are normally more conservative than PPs (see Cummings et al., 2003; Simmons et al., 2004). In several cases, there was a slight decrease in the PPs and BVs of clades in trees constructed with reduced character datasets (Table 3).

4.2. Phylogenetic relationships between marine and freshwater dinoflagellates

In the SSU and LSU phylogenies using ML and BI, there was always a high support (BVs and PPs > 0.97) for a clade comprising freshwater morphospecies of the *Peridinium* sensu stricto (=PESS) group (i.e. *P. willei* Huitfeld-Kaas, *P. cinctum* Ehrenberg, *P. gatunense* Nygaard, *P. bipes* Stein, *P. limbatum* (Stokes) Lemmermann, *P. volzii* Lemmermann) [group proposed by Boltovskoy (1979, 1999)]. The PESS clade was separated by a relatively longer branch from other marine and freshwater groups (Figs. 1 and 2). The internal topology of PESS was well defined in BI and ML analyses for both the SSU and LSU (Figs. 1 and 2).

Another cluster, mostly freshwater, that was suggested by the SSU and LSU phylogenies is PBOR [composed of: *Peridinium umbonatum* Stein, *Peridinium inconspicuum* Lemmermann, *Peridiniopsis borgei* Lemmermann, *Peridinium centenniale* (Playfair) Lefèvre, *Peridinium* cf. *centenniale*, *Peridinium palatinum* Lauterborn and *Peridinium pseudolaeve* Lefèvre (Figs. 1 and 2; Table 3)]. Within this group all species are fully freshwater, except for *Ps. borgei*, which is normally found in brackish limnic habitats, but not marine brackish environments. The PBOR clade did not show consistent affiliation to any other marine or freshwater clade.

Another clade indicated by the LSU and SSU phylogenies is POLA (Table 3; Figs. 1 and 2). Within POLA, the freshwater LSU sequences *Woloszynskia pascheri*, *W. tenuissima*, and the gymnodinioids AY829529–AY829530 formed the subclade WOLO (Fig. 2). In the ML and BI LSU phylogenies, the freshwater *W. pseudopalustris* grouped with significant support with a *Gymnodinium* from the Mediterranean (Corsica) and *W. halophila* from the Baltic Sea (PPs > 0.90, BVs > 0.70; Fig. 2). *W. pseudopalustris* was very closely related (very short branch lengths) to the Mediterranean *Gymnodinium* sp. and *W. halophila*. This cluster was distantly related to the WOLO subclade (Fig. 2).

SSU and LSU phylogenies suggested the clusters PFIE and PFIE2 (see Figs. 1 and 2) which included the freshwater morphospecies *Peridinium wierzejskii* Woloszynska and *P. aciculiferum* Lemmermann. The cluster PFIE and PFIE2 received significant PPs (>0.80), but was poorly supported by ML phylogenies (see Table 3). In the SSU phylogenies, *P. wierzejskii* and the pair *P. aciculiferum*/*Scrippsiella hangoei* (these species share identical rDNA; Gottschling et al., 2005; Logares et al., 2007) appeared in basal positions (Fig. 1). In the LSU BI and ML trees (which did not include *P. wierzejskii*) the freshwater *P. aciculiferum* (along with the brackish *S. hangoei*) clustered with marine-brackish *Scrippsiella*-like (EF058275 and AB260392) morphospecies (not available for SSU phylogenies) with a relatively high PP support (>0.90) but low BVs [~0.50] (Fig. 2).

SSU phylogenies also suggested the clade SCRI (Fig. 1), which included the freshwater *Peridinium polonicum* (Woloszysnka) Bourrelly. SCRI received high PPs (>0.90) but low BVs (~0.39) (Table 3).

All SSU and LSU phylogenies strongly supported a clade composed of sequences from *Ceratium* morphospecies (PPs and BVs > 0.90). In all analyses, the three freshwater SSU *Ceratium* sequences formed a highly supported subclade (=CERAf) distinctively separated from the marine cluster (PPs and BVs > 0.90) (Fig. 1).

The ML and BI LSU phylogenies suggested a freshwater cluster (=TOVE) composed by *Jadwigia applanata* Moestrup et al., *Esoptrodinium gemma* Javornicky, *Tovellia coronata* (Woloszynska) Moestrup et al., and the dinoflagellate sequence AY827950 (Fig. 2). TOVE received variable support (ranging from high to very low) in the LSU phylogenies (Clade 18, Table 3). In the SSU phylogenies, which included less sequences within TOVE than the LSU, the freshwater *J. applanata* sometimes affiliated to the freshwater pair *Tovellia leopoliensis* (Woloszynska) Moestrup et al. and *Peridinium* AY827955 (Fig. 1). The clustering of *T. leopoliensis* and *Peridinium* AY827955 was highly supported across BI phylogenies (PP > 0.90).

In the phylogenies, the freshwater *Gymnodinium palustre* Schilling (LSU; Fig. 2) and the freshwater sequences *Gymnodinium* sp. AY829527–AY840208 (SSU; Fig. 1) clustered with marine *Gymnodinium* species (Figs. 1 and 2; GYM clade) with significant support (PPs and BVs > 0.70) [In LSU phylogenies, *Pheopolykrikos hartmannii* (Zimmer-

Fig. 2. Consensus LSU rDNA phylogeny constructed from an alignment with 203 sequences and 619 characters under the GTR+Γ+COV (Bayesian Inference) and GTR+Γ+I (Maximum Likelihood) models. This consensus phylogeny corresponds to the trees 10a and 12 (Table 2) and intends to represent the whole set of constructed LSU trees (Table 2). The tree on the left shows the topology and posterior probabilities (normal print)/bootstrap support (italics) values >0.50. The tree on the right shows the evolutionary distances obtained by Bayesian MCMC Inference. Freshwater taxa and clusters are shown within frames in the cladogram (left) and indicated with a small terminal circle in the phylogram (right). The triangles in the phylogram correspond to marine clades that were collapsed. The number of collapsed sequences is indicated within parenthesis. The clusters indicated with the vertical lines or rectangles are described in Table 3 or Section 4. *Subsequent to our analyses, the GenBank access to the sequences indicated with * was precluded by the sequence authors due to a delayed publication. Nevertheless, the accession numbers for these sequences are shown in Supplementary Materials with a proper disclaimer.

Table 3
PP and BV support for 18 selected clades which were normally found across most LSU and SSU phylogenies

	Clade #																		**PP_m BV_m**	**SD**
	Tree #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
SSU	1a	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.97	0.62	1.00	0.88	0.99	1.00		—	—	—	**0.96**	**0.11**
	2b	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.67	0.61	1.00	1.00	0.79	0.92	—	—	—	—	**0.92**	**0.14**
	3a	1.00	0.88	1.00	1.00	1.00	1.00	—	1.00	0.97	0	1.00	0.97	0.94	1.00	—	—	—	—	**0.90**	**0.27**
	4	*0.89*	*0.95*	*0.92*	*0.97*	*0.90*	*1.00*	—	*0.64*	*0.10*	*0.24*	*0.45*	*0.23*	*0.40*	*0.53*	—	—	—	—	***0.63***	***0.33***
	5	*0.90*	*0.62*	*0.94*	*0.98*	*0.88*	*1.00*	—	*0.58*	*0.11*	*0.10*	*0.46*	*0.25*	*0.38*	*0.37*	—	—	—	—	***0.58***	***0.33***
	6a	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.56	0.92	1.00	0.99	0.99	1.00	—	—	—	—	**0.95**	**0.12**
	7b	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.95	0	1.00	0.90	0.96	1.00	—	—	—	—	**0.91**	**0.27**
	8	*0.95*	*1.00*	*0.96*	*0.98*	*0.89*	*1.00*	—	*0.85*	*0.15*	*0.24*	*0.46*	*0.23*	*0.52*	*0.13*	—	—	—	—	***0.64***	***0.36***
	9	*0.89*	*1.00*	*0.91*	*1.00*	*0.91*	*1.00*	—	*0.80*	*0.09*	*0*	*0.35*	*0.24*	*0.44*	*0.54*	—	—	—	—	***0.63***	***0.37***
LSU	10a	—	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	—	—	1.00	—	0.88	1.00	0.99	1.00	**0.96**	**0.09**
	11b	—	1.00	1.00	1.00	1.00	1.00	0.71	0.82	—	—	—	—	1.00	—	0.66	1.00	1.00	0	**0.85**	**0.30**
	12	—	*0.62*	*0.97*	*0.99*	*0.91*	*0.94*	*0.80*	*0.62*	—	—	—	—	*0.87*	—	*0.27*	*0.56*	*0.23*	*0.18*	***0.66***	***0.30***
	13	—	*0.59*	*0.96*	*0.98*	*0.94*	*0.86*	*0.76*	*0.44*	—	—	—	—	*0.87*	—	*0.26*	*0.56*	*0.35*	*0.12*	***0.64***	***0.30***
	14a	—	1.00	1.00	1.00	1.00	1.00	1.00	0.67	—	—	—	—	1.00	—	0.92	1.00	0.89	0.58	**0.92**	**0.14**
	15b	—	1.00	0	1.00	1.00	1.00	0.98	0.98	—	—	—	—	1.00	—	0.41	1.00	0.61	0.79	**0.81**	**0.32**
	16	—	*1.00*	*1.00*	*1.00*	*0.99*	*0.97*	*0.80*	*0.61*	—	—	—	—	*0.96*	—	*0.24*	*0.51*	*0.23*	*0.22*	***0.71***	***0.33***
	17	—	*0.99*	*0*	*1.00*	*1.00*	*0.98*	*0.59*	*0.42*	—	—	—	—	*0.90*	—	*0.20*	*0.44*	*0.12*	*0.16*	***0.57***	***0.39***
	%	100	100	88	100	100	100	100	100	100	66.7	100	100	100	100	100	100	100	100		
	PP_m	**1.00**	**0.99**	**0.89**	**1.00**	**1.00**	**1.00**	**0.92**	**0.94**	**0.82**	**0.43**	**1.00**	**0.95**	**0.97**	**0.98**	**0.71**	**1.00**	**0.87**	**0.59**		
	SD	0	0.04	0.33	0	0	0	0.14	0.12	0.19	0.41	0	0.05	0.07	0.03	0.23	0	0.18	0.43		
	BV_m	***0.91***	***0.85***	***0.83***	***0.99***	***0.93***	***0.97***	***0.73***	***0.62***	***0.11***	***0.15***	***0.43***	***0.24***	***0.67***	***0.39***	***0.24***	***0.52***	***0.23***	***0.17***		
	SD	*0.03*	*0.2*	*0.34*	*0.01*	*0.05*	*0.05*	*0.10*	*0.15*	*0.03*	*0.12*	*0.05*	*0.01*	*0.25*	*0.19*	0.03	0.06	0.09	0.04		

Tree #, corresponds to trees in Table 2. In BI analyses, only the replicate with the highest −lnL harmonic mean was considered. Clades 1–8 (clades include all analyzed species within each genus and nothing else): 1, PROT (*Protoperidinium* spp.); 2, ALEX (*Alexandrium* spp.); 3, DINP (*Dinophysis* spp.); 4, SYMB (*Symbiodinium* spp.); 5, CERA (*Ceratium* spp.); 6, PESS (*Peridinium* sensu stricto ssp., *P. willei*, *P. limbatum, P. gatunense*, *P. cinctum*, *P. bipes*, *P. volzii*); 7, KARE (*Karenia* spp., includes some sequences misnamed *Gymnodinium*); 8, HETE (*Heterocapsa* spp.). Clades 9–18 (clades include at least all mentioned species/sequences): 9, PFIE (*Peridinium wierzejskii, P. aciculiferum-Scrippsiella hangoei*, *Pfiesteria*-like, *Pfiesteria* spp.); 10, PBORs (*Peridnium umbonatum, P. inconspicuum*, [6] *Peridiniopsis borgei*, *Peridinium* cf. *centenniale,*, *Peridinium centenniale*); 11, DIAT (*Durinskia baltica*, *Peridinium quinquecorne*, *Galeidinium rugatum*, *Kryptoperidinium foliaceum*); 12, POLAs (*Woloszynskia pascheri*, *W. halophila*, *Symbiodinium* spp., *Gymnodinium* spp., *Polarella glacialis*); 13, GYRO (*G. helveticum*, *G. fusiforme, G. spirale, G. rubrum, G. dominans*); 14, SCRI (*Peridinium polonicum*, *Scrippsiella* sp. AB183674, *S. sweeneyae*, *S. trochoidea*, *Scrippsiella* sp. HZ2005); 15, PBOR (clade 10 + *Peridinium palatinum*, *P. pseudolaeve*); 16, POLA (clade 12 + *Woloszynskia tenuissima*, *W. pseudopalustris, Gymnodinium* sp. AY829529, AY829530); 17, PFIE2 (*Peridinium aciculiferum- Scrippsiella hangoei*, *Scrippsiella*-like EF058275, *Scrippsiella* sp. AF260392, *Pfiesteria* sp., *Pfiesteria*-like spp.); 18, TOVE (*Jadwigia applanata*, *Esoptrodinium gemma*, *Tovellia coronata*, Dinophyceae AY827950). 0, clade broken (no support); —, clade not applicable for the specified tree; BVs, bootstrap values (in italics); PPs, posterior probability values (in regular fonts); %, presence of a given clade across the phylogenies in percentage; PP_m and BV_m, median PPs or BVs for a clade across the phylogenies (columns) or for all the selected clades along one phylogeny (files); SD, standard deviation.

mann) Matsuoka & Fukuyo is included in GYM; Fig. 2]. In both cases, the freshwater *Gymnodinium* species were not closely related to the marine *Gymnodinium* species (Figs. 1 and 2).

In the SSU analyses the freshwater *Gyrodinium helveticum* (Penard) Takano & Horiguchi clustered with significant PP support (>0.75) within a clade (=GYRO) composed by other marine *Gyrodinium* species (Fig. 1). Within GYRO, *G. helveticum* had a noticeable degree of sequence divergence from the marine species (Fig. 1).

In the LSU phylogenies, the freshwater *Prorocentrum playfairii* Croome & Tyler and *P. foveolatum* Croome & Tyler clustered with high PP (>0.90) support to other marine *Prorocentrum* sequences as well as the marine *Togula britannica* (Herdman) Flø Jørgensen et al. (=PRO1 clade) (Fig. 2). Within PRO1, *P. playfairii* and *P. foveolatum* always clustered together with high PP and BV support (>0.90).

The freshwater *Glenodiniopsis steinii* (Lemmermann) Woloszynska and *Gymnodinium impatiens* Skuja were strongly associated (PPs and BVs > 0.90) throughout all the SSU and LSU phylogenies (Figs. 1 and 2). In SSU and LSU phylogenies, the pair did not show consistent affiliation to any other clade.

A number of solitary freshwater sequences appeared in very different positions depending on the marker or the method used for tree reconstruction. In several cases, those solitary sequences displayed long branches. The morphospecies associated to these sequences are *Cystodinium phaseolus* Pascher ASW12002, *Hemidinium nasutum* Stein NIES 471 and *Gloeodinium montanum* Klebs CCAC0066 (Figs. 1 and 2).

In the LSU phylogenies *Perkinsus* sp. appeared in different positions depending on the method for phylogenetic reconstruction and alignment dataset. This is almost certainly an artifact (*Perkinsus* is considered basal to dinoflagellates, as the SSU phylogenies indicate) and will not be further considered.

5. Discussion

Here, we have investigated the role of the marine–freshwater boundary on the historical diversification of dinoflagellates. Our results indicated that (a) marine and freshwater dinoflagellates are usually not closely related, (b) several freshwater species cluster into monophyletic groups, (c) only a small fraction of the marine lineages seem to have colonized fresh waters, and (d) most marine–freshwater transitions do not seem to have occurred recently. Our results also gave insight on the phylogenetic positions of several freshwater dinoflagellate species, which so far have been unclear.

5.1. Marine and freshwater taxa were normally not closely related

In the SSU and LSU phylogenies, ~70% of the investigated freshwater species appeared more closely related to other freshwater species than to marine species. In some cases, freshwater sequences could be related to marine clades (the opposite was not observed). However, a number of freshwater sequences did not affiliate to any other of the analyzed marine and freshwater dinoflagellates (*Cystodinium phaseolus* Pascher ASW12002, *Hemidinium nasutum* Stein NIES 471 and *Gloeodinium montanum* Klebs CCAC0066). In several cases, the freshwater species/clades displayed a significant degree of sequence divergence (long branches) in comparison to the marine species/clades. Altogether, this indicates the segregation of marine and freshwater dinoflagellates, and also that freshwater invasions may have occurred long time ago or that the rate of molecular divergence has increased in invading lineages. Morphological data also suggest the separation between marine and freshwater lineages (see Taylor, 1987; Popovsky and Pfiester, 1990), although this separation can be ambiguous due to the uncertainties of morphology-based phylogenies. A historical segregation of marine and freshwater dinoflagellates is also suggested by the fossil record. Several fossil freshwater dinoflagellates are not identifiable with any known marine fossil genera or species (Batten, 1989). The segregation of marine and freshwater taxa can also be observed in other microbial phylogenetic studies (e.g. Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Lefevre et al., 2007). However, these works for the most part include few freshwater species and investigate questions different than marine–freshwater transitions. Interestingly, long branches separating freshwater species/clades from marine taxa are also observed in other microbial groups (e.g. Hoef-Emden et al., 2002; Von der Heyden et al., 2004). These long branches could be product of ancient divergences, an increase of the evolutionary rates during marine–freshwater transitions, or a mix of both.

5.2. Several freshwater species clustered into monophyletic groups

A central result of this study is that several freshwater dinoflagellate species clustered into monophyletic groups. In particular, our results strongly supported the monophyly of the freshwater *Peridinium* sensu stricto group [=PESS], which was proposed by Boltovskoy (1979) on a morphological basis (e.g. number of cingular plates, sulcal structure, ecdysial openings, capsulate cysts), but which monophyly was still under debate (Figs. 1 and 2). The relatively long branch separating PESS from the rest of the dinoflagellates suggests an ancient divergence of this clade from the other taxa (Figs. 1 and 2), or an increase of the evolutionary rates during the marine–freshwater transition. Interestingly, there are two fossil dinoflagellate morphospecies, *Palaeoperidinium pyrophorum* and *Pa. cretaceum*,

from the early Cretaceous (145-135 MYA) which are considered to be members of the ancestral group from which the modern PESS have derived (see Boltovskoy, 1979; Bujak and Davies, 1983; Harding, 1990). *Pa. pyrophorum* was found in marine sediments while *Pa. cretaceum* was obtained from brackish paleoenvironments. These data suggest that *Pa. cretaceum* could have been an intermediate species during the marine–freshwater transition that eventually led to PESS. In contrast to many dinoflagellate groups, the internal topology of the PESS clade was highly resolved, with subgroups that correspond to morphospecies definitions (Figs. 1 and 2) [morphologically, the six species within PESS are distinguished by their outline body shapes; degree of dorso-ventral compression; bilateral symmetry in the plate disposition; relative position, shape and size of plates; and presence or absence of the apical pore complex and of apical and antapical horns and lists (Evitt and Wall, 1968; Boltovskoy, 1973, 1975, 1976; Imamura and Fukuyo, 1990a, b)]. In addition, both the SSU and LSU indicated a significant degree of sequence divergence within most of these morphospecies (cryptic diversity). This suggests that each morphospecies could actually constitute a species complex, as was reported for other dinoflagellates (e.g. Scholin et al., 1994; Montresor et al., 2003; Kim et al., 2004; Lilly et al., 2005). Our results also show that a number of freshwater and marine morphospecies currently assigned to *Peridinium* are evolutionary unrelated to PESS (*Peridinium* sensu stricto). These include *Peridinium polonicum*, *P. pseudolaeve*, *P. palatinum*, *P. inconspicuum*, *P. umbonatum*, *P. aciculiferum*, *P. wierzejskii*, *P. centenniale*, and *P. quinquecorne*.

Other monophyletic freshwater groups which are indicated by our analyses are PBOR (except for *Peridiniopsis borgei* that is present in limnic-brackish habitats), TOVE, CERAf, and WOLO (Figs. 1 and 2). The clade PBOR does not seem to have been reported before, however, results from other authors have already suggested the existence of the TOVE clade (Lindberg et al., 2005; Calado et al., 2006; Moestrup et al., 2006; Hansen et al., 2007). In the phylogenies, the marine and freshwater *Ceratium* appeared clearly separated (Fig. 1), suggesting that this lineage has not invaded fresh waters recently. Some morphological and biological differences between the marine and freshwater *Ceratium* also suggest that these lineages did not diverge recently: the freshwater species of *Ceratium* have six plates composing the cingulum in contrast to marine species which have five; freshwater *Ceratium* species produce resting cysts, whereas the far more numerous marine species do not encyst (Wall and Evitt, 1975). An early freshwater colonization for the CERAf lineage is suggested also by fossil data, which indicate the presence of shared morphological characteristics between living freshwater *Ceratium* and Cretaceous *Ceratium*-like marine fossils (as the presence of pendant horns instead of the upswept horns observed in many modern marine *Ceratium*, the development of a fourth horn, and the presence of the sixth cingular plate) (Wall and Evitt, 1975). The present study is probably one of the first to report the freshwater WOLO subclade (within the POLA clade) (Fig. 2). The clear separation between the WOLO clade and other marine species within POLA suggests an early freshwater invasion by the WOLO lineage (Fig. 2).

5.3. An indication of infrequent freshwater colonizations by a few ancestrally marine lineages

The clustering of several freshwater sequences into monophyletic groups suggests that freshwater colonizations were not common during the diversification of dinoflagellates and that the diversification of several freshwater taxa occurred after the invasion of fresh waters. If freshwater colonizations would have been more common, a relatively higher number of evolutionary distantly related freshwater taxa would have been expected (i.e. several freshwater colonization lines from a spectrum of marine lineages). In addition, in our phylogenies, several marine groups did not include any freshwater relative, suggesting that relatively few of the living marine dinoflagellate lineages have colonized fresh waters. Morphological studies also suggest that only a handful dinoflagellate lineages have colonized fresh waters. It is estimated that out of 14 dinoflagellate orders, only five have freshwater representatives that are distributed within about 12 families. Four of those families comprise more than 90 % of the freshwater morphospecies (estimated from Taylor (1987) and Popovsky and Pfiester (1990)). In our study, we have covered ~70% of the freshwater genera, with two or more species, that are recognized in Popovsky and Pfiester (1990). Despite that a more extensive taxon sampling will most probably reveal new freshwater dinoflagellate lineages, their total number will most likely continue to be much lower than the total number of marine lineages. A similar pattern to ours was observed in diatoms, where apparently only a fraction of the marine lineages have been successful in colonizing fresh waters (Mann, 1999).

5.4. Most freshwater colonizations do not seem to have occurred recently

Except for two cases (*Peridinium aciculiferum* and *Woloszynskia pseudopalustris*), our results indicate that most of the studied freshwater dinoflagellates belong to lineages that have colonized fresh waters a long time ago. This is suggested by (a) the very small number of closely related marine and freshwater species, and (b) by the presence of relatively long branches separating several freshwater species/clades from other marine groups (Figs. 1 and 2). Nevertheless, the observed long branches could also be partially explained by a putative increase in the evolutionary rates during freshwater colonizations.

An increased taxon sampling will most probably identify other freshwater species which are a product of recent freshwater colonizations. Yet, the number of species

belonging to lineages that have colonized fresh waters long time ago will most likely be much higher than the number of species that have colonized freshwater recently. Early freshwater colonizations are also indicated by the fossil record. The freshwater dinoflagellate fossil record extends back until the Mesozoic (~140 MYA), with several freshwater fossil morphospecies that are not identifiable with marine fossils (Batten, 1989). This indicates the occurrence of ancient freshwater invasions by marine dinoflagellates and the posterior evolution of truly freshwater species. The changes in the sea levels that occurred during the Mesozoic and Cenozoic flooded large continental areas (Haq et al., 1987). This could have promoted the early invasion of continental waters by some dinoflagellate lineages. Sims et al. (2006) proposed a similar scenario for the invasion of continental waters by some marine diatom lineages.

5.5. Other freshwater colonization lines

Apart from the discussed freshwater clades, we have identified several other freshwater colonization lines that were represented in most cases by only one or two species. For instance, the freshwater *Peridinium polonicum* has probably diverged from marine *Scrippsiella* species (Fig. 1). *P. aciculiferum* has probably colonized fresh waters very recently, since it is very closely related to the marine-brackish *Scrippsiella hangoei*, and both morphospecies cluster with marine-brackish species (see Logares et al., 2007) (Figs. 1 and 2). According to the SSU phylogenies, the freshwater *P. wierzejskii* is related to a group of mostly marine-brackish dinoflagellates which includes *Pfiesteria* and associated species (Fig. 1), although this relationship is not supported by morphology (different plate patterns). The freshwater species *Gymnodinium* AY829527, *Gymnodinium* AY840208 and *G. palustre* most probably represent the freshwater colonization of a marine *Gymnodinium* clade (GYM clade; Figs. 1 and 2). Likewise, the freshwater *Gyrodinium helveticum* represents a freshwater colonization line of the marine *Gyrodinium* clade (altogether forming the clade GYRO; Fig. 1). Similarly, the freshwater *Prorocentrum playfairii* and *P. foveolatum* represent a freshwater colonization line of a marine *Prorocentrum* clade (Fig. 2). Within the clade POLA, the freshwater *Woloszynskia pseudopalustris* clustered with a marine *Gymnodinium* and the brackish *W. halophila* (Fig. 2). In this case, the freshwater colonization seems to have occurred recently, since the three morphospecies were very closely related. The freshwater *Glenodiniopsis stenii* and *Gymnodinium impatiens* did not show any clear affiliation to any other lineage across the phylogenies (the fact that this and other *Gymnodinium* species did not cluster together in the phylogenies clearly indicates that this genus is polyphyletic). There were also other solitary freshwater sequences which drifted across the phylogenies not affiliating with any other group or species (i.e. *Cystodinium phaseolus, Hemidinium nasutum*, *Gloeodinium montanum*).

Altogether, each one of these freshwater colonization lines could represent undersampled freshwater lineages, which colonized fresh waters a long time ago. If the marine–freshwater transitions leading to these species had happened more recently, then those species would most probably have affiliated with marine relative groups. However, despite our extensive taxon sampling of marine lineages, it is also possible that the marine relatives of these solitary freshwater sequences have not been sequenced yet.

5.6. Methodological comments on phylogeny reconstruction

Several clades were significantly supported by the SSU and LSU phylogenies (e.g. PESS, DINP, SYMB, ALEX, CERA; see Table 3). In general, at the genus level, our molecular phylogenies agreed with morphological classifications (the agreement between molecular phylogenies and morphological classifications in dinoflagellates has been addressed in other studies [e.g. Saldarriaga et al., 2004; Taylor, 2004] and will not be discussed here). On the other hand, we have obtained a low support for the backbone of most phylogenies. Both patterns agree with other studies (e.g. Saldarriaga et al., 2004; Zhang et al., 2005; Shalchian-Tabrizi et al., 2006b). The low resolution of the treeś backbones does not represent a problem for this work, which does not intend to resolve deep phylogenetic relationships.

Despite that other studies have reported an improvement in the phylogenetic reconstructions when considering the SSU secondary structure (e.g. Murray et al., 2005; Telford et al., 2005), the results of our analyses did not differ significantly whether or not the secondary structure was considered. This could be the consequence of a non-optimal modeling of the SSU secondary structure or that the Doublets model was not the most appropriate for the analyses. Another possibility is that the use of an extensive taxon sampling or the covarion model improved the quality of the trees that did not consider the secondary structure (see Galtier, 2001; Huelsenbeck, 2002; Pollock et al., 2002; Hedtke et al., 2006; Shalchian-Tabrizi et al., 2006b), which then showed similar results to the ones that considered the secondary structure.

A fraction of the clades present in most of the phylogenies received significant BVs and PPs (e.g. ALEX, DINP, SYMB, CERA, PESS; Table 3), whereas other clades received high PPs and low BVs (e.g. PFIE, PFIE2, POLA, SCRI). This contradiction is puzzling. A number of studies indicate that Bayesian Inference (BI) overestimates and bootstrap underestimates support values (e.g. Cummings et al., 2003; Simmons et al., 2004). In our results, it seems that the differences are too large and consistent to be only a product of over- or underestimation. Another possibility is that these clades represent attractive topologies for Bayesian analyses and were visited more often in the MCMC explorations of the parameter space, giving them a higher support (see Cummings et al., 2003). However, some clades

that obtained low-moderate BVs and PPs with the SSU obtained higher BVs and PPs in the LSU phylogenies, which happened to included more sequences within them (compare POLAs and POLA, PBORs, and PBOR; Table 3). This suggests that the low support values could have been the product of reduced phylogenetic signal caused by the presence of fewer sequences.

The diversity of dinoflagellates is still being uncovered, and new species and lineages are being discovered regularly (e.g. Burkholder et al., 1992; Montresor et al., 1999; Lopez-Garcia et al., 2001; de Salas et al., 2003; Jeong et al., 2005; Lindberg et al., 2005; Lin et al., 2006). We have considered if future analyses including newly discovered freshwater and marine dinoflagellates could lead to different patterns (e.g. closely related freshwater and marine species in several lineages), and concluded that this possibility is unlikely. In this study, we have included 88 SSU and LSU sequences associated to around 40 freshwater dinoflagellate morphospecies (out of ~220 recognized). This covers ~70% of the freshwater genera [as recognized in Popovsky and Pfiester (1990)] which contain more than two species. The SSU and LSU have been sequenced for numerous marine dinoflagellates and sequences representing most lineages have been included in our analyses. If closely related marine and freshwater dinoflagellates were common in nature, then we should have detected a higher number of them (more than only two cases) due to our extensive taxon sampling.

The existence of an enormous hidden diversity of marine and freshwater dinoflagellates that if analyzed could lead to radically different conclusions is also an unlikely scenario. Conspicuous (>10 μm) marine and freshwater dinoflagellates have been widely studied by taxonomists, and there is no indication that the total number of recognized species will increase by orders of magnitude after new taxonomic descriptions. In contrast, a potentially high diversity could be hidden in small (<10 μm) and poorly studied dinoflagellates. Environmental DNA surveys of marine and freshwater small (<10 μm) microeukaryotes found new dinoflagellate lineages and species. However, these studies do not indicate that the unknown diversity of small dinoflagellates is enormous (e.g. Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001; Richards et al., 2005; Lin et al., 2006; Lefevre et al., 2007). In particular, not many freshwater picodinoflagellates (i.e. pico-sized) were found in environmental DNA surveys of two freshwater lakes in Europe and North America, suggesting that the unknown amount of picodinoflagellate diversity might not be very high (Richards et al., 2005; Lefevre et al., 2007). Interestingly, the freshwater picodinoflagellates detected by Richards et al. (2005) and Lefevre et al. (2007) clustered into two clades, and are apparently not evolutionary closely related to marine picodinoflagellates. Thus, as it seems to be the case for several picoeukaryote lineages (see Richards et al., 2005), freshwater and marine picodinoflagellates might also be separated into marine and freshwater lineages.

5.7. Evolutionary remarks

In summary, our molecular results, along with fossil and morphological data, indicated that during the evolution of dinoflagellates, the marine–freshwater margin has acted as a barrier that relatively few lineages have been able to cross. Molecular data from other microbial lineages also support the contention that marine–freshwater transitions have not been frequent in the evolution of microbes (Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Alverson et al., 2007; Lefevre et al., 2007). Altogether, these data indicate that habitat differences prevent the free interchange of microbes between marine and fresh waters in a similar manner as it does with metazoans (Lee and Bell, 1999). Likewise, several studies on multicellular organisms also indicate that only a few marine lineages have been successful in colonizing fresh waters (e.g. Lee and Bell, 1999; Miller and Labandeira, 2002; Vermeij and Wesselingh, 2002; Lovejoy et al., 2006). Despite that the development of complex osmoregulatory mechanisms is probably one of the main barriers for the colonization of freshwater by metazoans, it is unclear what constitutes the barrier that prevents marine microbes from the constant recolonization of fresh waters and the reverse. Contrary to metazoans, microbes normally have massive population sizes, high reproductive rates, enormous genetic variability (e.g. Snoke et al., 2006) and capabilities for long distance dispersal (Finlay, 2002). Due to these characteristics, it would be expected that microbial strains from several lineages would acquire the necessary mutations to make the environmental transition. Future studies will need to investigate if the marine–freshwater barrier is generated only by salinity gradients or there are other factors, like competitive exclusion by adapted residents (De Meester et al., 2002) or high extinction rates in small limnic habitats (island biogeography theory), playing a role.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.08.005.

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VII

Diversification of unicellular eukaryotes: Cryptomonad colonizations of marine and fresh waters inferred from revised 18S rRNA phylogeny

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The cryptomonads is a well-defined lineage of unicellular eukaryotes, composed of several marine and freshwater groups. However, the evolutionary relationships among marine and freshwater groups are unclear due to conflicting inferences between phenotypic traits and molecular phylogenies. Here, we have inferred the interrelationships among marine and freshwater species in order to better understand the importance of the marine/freshwater boundary on the evolutionary diversification of cryptomonads. We have constructed molecular phylogenies of cryptomonads by taking into account both rate-variation across sites and sequences (covarion substitutions), and by analyzing the vast majority of publicly available cryptomonad sequences, including environmental phylotypes. The resulting phylogenies included 58 freshwater sequences, and revealed two novel freshwater cryptomonad clades (CRY1 and CRY2) and a large hidden diversity of cryptomonads. CRY1 was placed deeply within the cryptomonad phylogeny together with all the major freshwater lineages (i.e. *Goniomonas* and *Cryptomonas)*, while CRY2 was placed within a lineage of marine species identified as *Plagioselmis*-like with the aid of a new sequence generated from a cultured species (N 750301, Klaveness 1981). The inferred phylogenies suggest only few independent colonisations of freshwater. Most of the cryptomonad transitions seem to be unidirectional, from marine to freshwater, but re-colonizations of marine habitats have also taken place. This implies that the differences in the biogeophysical conditions between marine and fresh waters constitute a substantial barrier for the transitions of cryptomonads between these two habitats.

Introduction

For most multicellular organisms, the marine-freshwater boundary represents a barrier for the exchange of individuals between the two types of environments (Lee and Bell, 1999). For microbes, it is still unclear whether the marine-freshwater boundary represents a barrier for the exchange of individuals, although some recent studies indicate it does (see Alverson *et al.,* 2007; Logares *et al.,* 2007 [Paper 6, this thesis]). Usually, eukaryotic micro-organisms have immensely larger population sizes than multicellular organisms, (e.g. Fenchel & Finlay 2004, Green and Bohannan, 2006; Snoke *et al.,* 2006) and they have the potential capability of long-distance dispersal (see Finlay, 2002; Hughes Martiny *et al.,* 2006), two characteristics which could enable them to cross successfully and repeatedly the boundary between marine and fresh waters. Simply due to the enormous number of individuals, the chances of a successful marine-freshwater transition would be much higher in microbes than in multicellular organisms. In addition, the relatively high genetic diversity found in several microbial populations (e.g. Medlin *et al.,* 2000) may also increase the probability for successful crossings of the marine-freshwater boundary and establishment of viable populations in the newly colonized environment.

The cryptomonads is an ideal group for investigating the significance of the marine/freshwater boundary among unicellular eukaryotes, because they have a cosmopolitan distribution and are found in both oceans and in most types of freshwater habitats (Klaveness 1988, 1989). In addition, the cryptomonads is a relatively small and easily recognized group of protists with about 100–200 (or more) species – depending upon the acceptance of the original description (e.g. the descriptions of 42 new species of cryptomonads in Schiller 1957; see also Hoef-Emden *et al.,* 2002). Recently, the cryptomonads have been suggested as the sistergroup of haptophytes, being closely related to katablepharids and possibly *Telonemia* (Okamoto and Inouye, 2005; Shalchian-Tabrizi *et al.,* 2006a).

Traditionally, the species diversity of cryptomonads has been mainly described on the basis of morphological (e.g. cell shape and size, organelle location and morphology, cf. Pringsheim 1968, Klaveness 1985, 1991a,b) and biochemical characters (colour, phycobiliprotein pigments, cf. Butcher 1967; Gantt 1979; Hill and Rowan 1989; Rowan 1989). However, some of these characters have shown to be unreliable for species demarcations and higher order classifications (Deane et al., 2002; Hoef-Emden *et al.,* 2002; Hoef-Emden and Melkonian, 2003). The omnipresence of cryptomonads is widely recognized (e.g. Klaveness 1988), but due to the difficulties with morphological species identification, the actual number of cryptomonad species is probably underestimated (Hoef-Emden and Melkonian, 2003). In other protist groups, molecular studies have recently uncovered a significant amount of hidden diversity (Lopez-Garcia *et al.,* 2001; Not *et al.,* 2007; Shalchian-Tabrizi *et al.,* 2007), and a similar situation may be expected in cryptomonads.

Recent revisions of the cryptomonad classification and phylogeny based on inferences from rRNA genes suggest two separate orders, Goniomonadales and Cryptomonadales and about a total of 20 genera (Hoef-Emden *et al.,* 2002; von der Heyden *et al.,* 2004). However, the phylogenetic relationships between the main cryptomonad lineages remain unclear due to low statistical support for the basal branching order in molecular phylogenies (Deane *et al.,* 2002; Hoef-Emden *et al.,* 2002; von der Heyden *et al.,* 2004). The lack of resolution in the deepest branches may be due to highly different evolutionary rates in freshwater and marine lineages (Hoef-Emden *et al.,* 2002; von der Heyden *et al.,* 2004). If the occurrence of highly heterogeneous evolutionary rates is not taken into account in the applied phylogenetic methods, they may generate artificial phylogenetic relationships and statistical support (Lockhart *et al.,* 2006; Shalchian-Tabrizi *et al.,* 2006b).

Molecular phylogenies suggest that the majority of the known freshwater cryptomonads are comprised in the genus *Cryptomonas* as well as in a subgroup within the heterotrophic genus *Goniomonas* (Deane *et al.,* 2002; von der Heyden *et al.,* 2004). In addition, morphological studies have reported some freshwater species for the genera *Rhodomonas, Plagioselmis, Campylomonas, Storeatula, Hemiselmis and Komma* (e.g Hill 1991 a,b, Kugrens & Clay 2003), but the paucity of good

diacritical characters have left some of them in a long-lasting state of taxonomic transition (e.g. strain N 750301, cf. Klaveness 1981, Novarino *et al.* 1994, Javornicky 2003).

Over the last few years, several surveys of environmental 18S rRNA sequence diversity from different marine and freshwater habitats have resulted in thousands of publicly available sequences (Moon-van der Staay *et al.* 2001; Lefranc *et al.,* 2005; Richards et al., 2005; von der Heyden *et al.* 2005; Lefevre *et al.,* 2007). However, these sequences have not yet been extensively used for inferring the phylogenetic relationships between marine and freshwater microbes. Here, we have generated improved phylogenetic reconstructions in cryptomonads in order to infer the evolutionary relationships between freshwater and marine species. For this purpose, we have used publicly available 18S sequences as well as a new sequence from a cultured freshwater *Plagioselmis* species (strain N 750301; Klaveness 1981). The aim was to find out if the different biogeophysical conditions occurring in marine and freshwater habitats represent a substantial barrier for the permanent migration of cryptomonads between these two environments. Uneven evolutionary rates as seen in earlier cryptomonad trees were accommodated by taking into account both rate variation across sites (i.e. gamma distributed rate of variation) and across sequences (i.e. covarion model), which have been suggested appropriate for rRNA analysis of eukaryotes (Galtier, 2001; Shalchian-Tabrizi *et al.,* 2006a).

Experimental Procedures

Cultures, DNA isolation and PCR

One cryptomonad freshwater strain that was identified as *Rhodomonas* (Klaveness 1981) and now defined as *Plagioselmis nannoplanctic* (Novarino *et al.* 1994), was grown at 17 °C and 14/10 h light/dark cycles in the medium of Guillard and Lorenzen (1972, without organic buffer). The culture was harvested by centrifugation and used for DNA extraction. DNA was extracted using Direct kit (Invitrogen, Carlsbad, CA) according to the manufacturers instructions.

The *P. nannoplanctic* 18S rRNA gene was amplified by PCR using universal 18S rRNA primers as previously described (Medlin *et al.,* 1988; Klaveness *et al.,* 2005) and cloned using a TOPO-TA kit (Invitrogen, Carlsbad, CA). Sequences from two clones were identified as nuclear cryptomonad (and not from plastid nucleomorph) by BLAST searches against the NCBInr database.

Data mining and phylogenetic analyses

Publicly available sequences from freshwater cryptomonad species were collected from GenBank by either keyword searches in Entrez or BLAST searches using query sequences from all main cryptomonad groups (i.e. query from the *Goniomonas, Cryptomonas, Chroomonas, Rhodomonas* and *Teleaulax/Plagioselmis* groups; Table 1). These searches were finalized August 15th 2007. Whenever possible, the correct freshwater or marine origin of the sequences was confirmed with the corresponding publications.

All retrieved freshwater sequences of potential cryptomonad origin were manually added to a global eukaryote 18S alignment containing 229 sequences and 1239 characters from the majority of eukaryote groups (Alignment 1 = AL1). Chimearic sequences were omitted. After deletion of ambiguously aligned sites, the sequence matrix was used for phylogenetic analyses. Based on the resulting tree topology, all sequences with clear cryptomonad origin were selected for a second analysis together with sequences from katablepharids and glaucophytes (the two latter were chosen as outgroups). By deleting unrelated eukaryote groups in the AL1 inference, more unambiguously aligned characters could be included for better resolution of the cryptomonad phylogeny in Alignment2 (AL2; 134 taxa and 1582 characters). A third alignment (AL3) was generated by removal of terminal characters with a lot of missing data from the environmental sequences, in order to investigate the impact of missing data on the inferred tree topology. This caused inadequate sequence lengths for some of the taxa and the sequences were subsequently removed from the data (i.e. in total 112 taxa and 1013 characters).

The largest alignment (AL1) was analyzed with the Maximum Likelihood (ML) criterion as implemented in the GARLI program (Zwickl 2006). Ten heuristic tree searches were done using

Table 1. Environmental freshwater cryptomonad-like sequences used in the phylogenetic analyses.

Sample locality	Accession number	Sequence length	Clone number
Chevreuse, France	AY821952	1605	CH1_2B_6
Chevreuse, France	AY821950	1592	CH1_2A_33
Chevreuse, France	AY821953	1584	CH1_5A_2
Chevreuse, France	AY821955	1604	CH1_S1_20
Lake Aydat, France	AY642740	1293	A54
Lake George, USA	AY919723	1697	LG11-10
Lake George, USA	AY919805	1697	LG35-01
Lake George, USA	AY919781	1698	LG27-07
Lake George, USA	AY919779	1699	LG27-01
Lake George, USA	AY919733	1698	LG14-10
Lake George, USA	AY919695	1697	LG05-02
Lake George, USA	AY919739	1698	LG17-07
Lake George, USA	AY919764	1701	LG23-04
Lake George, USA	AY919727	1698	LG13-03
Lake George, USA	AY919729	1699	LG14-01
Lake George, USA	AY919784	1697	LG29-02
Lake George, USA	AY919686	1697	LG03-02
Lake George, USA	AY919746	1710	LG19-05
Lake George, USA	AY919707	1711	LG08-05
Lake La Godivelle lac d'en haut, France	AY642724	1328	G5.11
Lake La Godivelle lac d'en haut, France	AY642733	1330	PG5.34
Lake Pavin, France	AY642699	1770	P1.25
Lake Pavin, France	DQ244012	1770	PFF1AU2004
Lake Pavin, France	AY642716	1712	P1.31
Lake Pavin, France	AY642712	1371	P34.3
Lake Pavin, France	AY642715	1696	P1.30
Lake Pavin, France	AY642713	1388	P1.27
The Alps, France	EF196794	963	B400
The Alps, France	EF196752	963	BI103
The Alps, France	EF196706	824	BA373
The Alps, France	EF196685	809	BA25
The Alps, France	EF196694	823	BA123
The Alps, France	EF196687	808	BA39
The Alps, France	EF196691	853	BA91
Villerest reservoir, Roanne, France	DQ409122	974	VP18

the General Time Reversible (GTR) model, with a gamma distributed rate of variation across sites and a proportion of invariable sites (GTR+G+I model). Likelihood scores estimated from the resulting 10 trees were compared and the tree with highest likelihood score was chosen. The bootstrap analysis with GARLI was done with 100 pseudoreplicates using the GTR+G+I model. Both the other alignments (AL2 and AL3) were analyzed with ML and Bayesian methods. The RaxML program (Stamatakis et al., 2005) was used with the CAT evolutionary model and random starting trees. The tree robustness was estimated by 100 bootstrap replicates. Bayesian phylogenetic analyses were done with MrBayes (Ronquist and Huelsenbeck, 2003), applying the GTR+G+I and GTR+G+I+COV (COV=covarion) models; the latter included two covarion parameters (sites being on > off and off > on). Each Bayesian inference was run from a tree reconstructed by GARLI using GTR+G+I model. The MCMC chains included three heated and one cold chain that lasted for 4 – 20,000,000 generations. The posterior probability values and harmonic mean likeli-

hood values were calculated from the sampled trees after the burn-in phase, which was assessed from the marginal likelihood scores of the sampled trees. Both the harmonic mean and posterior probability values for the internal branching pattern were almost identical in independent runs, suggesting convergence of the MCMC chains. All phylogenetic analyses were performed on the Bioportal at University of Oslo (http://www.bioportal.uio.no).

Results and Discussion

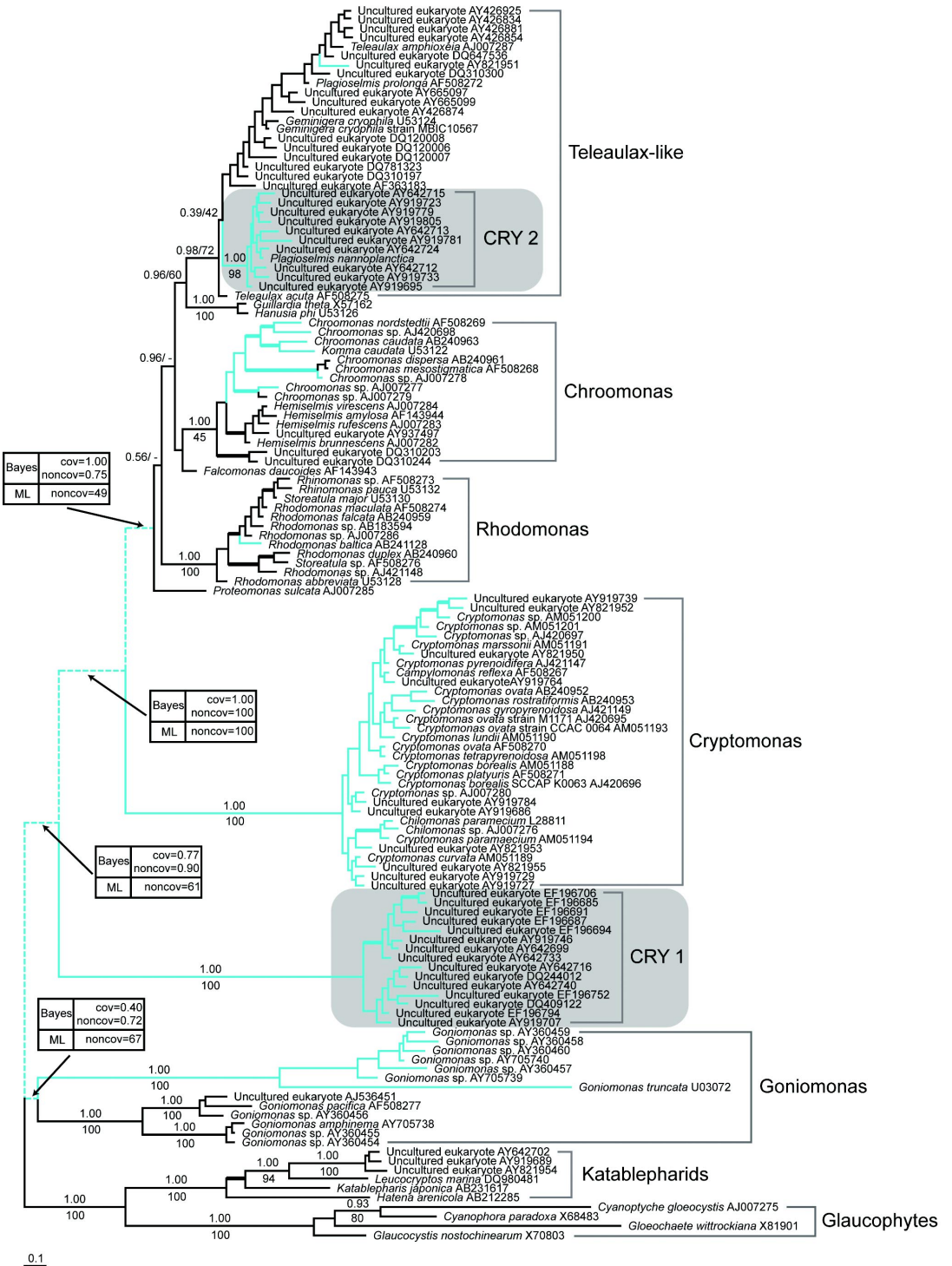
Identification of cryptomonad freshwater phylotypes

Our extensive search for 18S rRNA sequences in GenBank uncovered several potential cryptomonad sequences generated from previous surveys of environmental clone libraries. The identified sequences were imported into an alignment where all main eukaryote lineages were represented. The constructed maximum likelihood (ML) phylogeny uncovered in total 58 sequences, from freshwaters, that clustered within the cryptomonads (results not shown). In addition, we sequenced the 18S rRNA sequence from the cultured freshwater species *Plagioselmis nannoplanctic.*

The covarion model improves cryptomonad phylogeny

By using a small sample of eukaryotes belonging to katablepharids, glaucophytes (both defined as outgroups) and a wider sample of cryptomonads, we increased the number of usable aligned characters (AL2). Model comparisons between the GTR+G+I and the GTR+G+I+COV Bayesian trees, showed a 216.26 (or Bayes factor = 432.52) difference in the marginal likelihood of the trees (i.e. harmonic mean of the sampled trees: covarion= –10414.78, noncovarion = –10631.04) in favour of the covarion model. Interpretation of the likelihood differences in a Bayesian framework in accordance with Kass and Raftery (1995) and Newton and Raftery (1994) suggests that the covarion model is vastly fitter to the data than the best model without covarion parameters. Nevertheless, the tree topologies produced with these two models showed overall similarity (Figs. 1, 2). At the base of the cryptomonad tree, the heterotrophic *Goniomonas* has been considered to constitute a distinct branch that is divided into two freshwater and marine subgroups (Deane *et al.,* 2002; von der Heyden *et al.,* 2004). This pattern was also found in our analyses of the AL2 dataset, albeit weakly supported in the Bayesian covarion analysis (Fig. 1). Moreover, the *Goniomonas* was excluded from the other groups with variable support values. The phylogeny of the other cryptomonads that we have obtained was congruent with recently published 18S rRNA trees, showing high support for separate clusters of *Cryptomonas* (including *Chilomonas* and *Campylomonas*), *Rhodomonas* (including *Storeatula* and *Rhinomonas*) and *Teleaulax* (including *Geminigera* and *Plagioselmis* genera lineages (> 70% bootstrap support and 0.95 posterior probability; hereafter only pp; Deane et al., 2002; Hoef-Emden *et al.,* 2002). The genus *Chroomonas* (including *Hemiselmis* and *Komma)* was highly supported only in the Bayesian inference (1.00pp). Four other species with unclear affiliation in previous studies (Deane *et al.,* 2002; Hoef-Emden *et al.,* 2002), *Falcomonas, Proteomonas*, *Guillardia* and *Hanusia,* are robustly placed within the Cryptomonadales but excluded from any of the groups within the order. The covarion-based tree showed higher posterior probability than the non-covarion tree for placing the freshwater *Cryptomonas* as sister to all other Cryptomonadales (i.e. excluded from the other Cryptomonadales with covarion=1.00pp vs. noncovarion=0.75pp).

Phylogenetic reconstruction of AL2 (Fig. 2) showed large congruence with Fig. 1, and importantly all the main cryptomonad groups identified in Fig. 1 were also recovered with similar support (>79% and 0.95pp values). However, in analyses of AL2 *Cryptomonas* was less supported as the sister taxa of the other Cryptomonadales groups. In Fig. 2. the genus *Goniomonas* was split into two independent branches, but the changes were not significantly supported. Taken together, the variable support for the clustering of *Goniomonas* in Fig. 1 and the split of this genus in Fig. 2 (i.e. using fewerer characters) show that the phylogeny within *Goniomonas* should be interpreted with caution. Interestingly, the four species with unclear affiliation in the analysis of the AL1 dataset, *F. daucoides*, *P. sulcata*, *G. theta* and *H.*


Teleaulax-like
CRY 2
Chroomonas
Rhodomonas
Cryptomonas
CRY 1
Goniomonas
Katablepharids
Glaucophytes
Bayes cov=1.00 noncov=0.75
ML noncov=49
Bayes cov=1.00 noncov=100
ML noncov=100
Bayes cov=0.77 noncov=0.90
ML noncov=61
Bayes cov=0.40 noncov=0.72
ML noncov=67
0.1

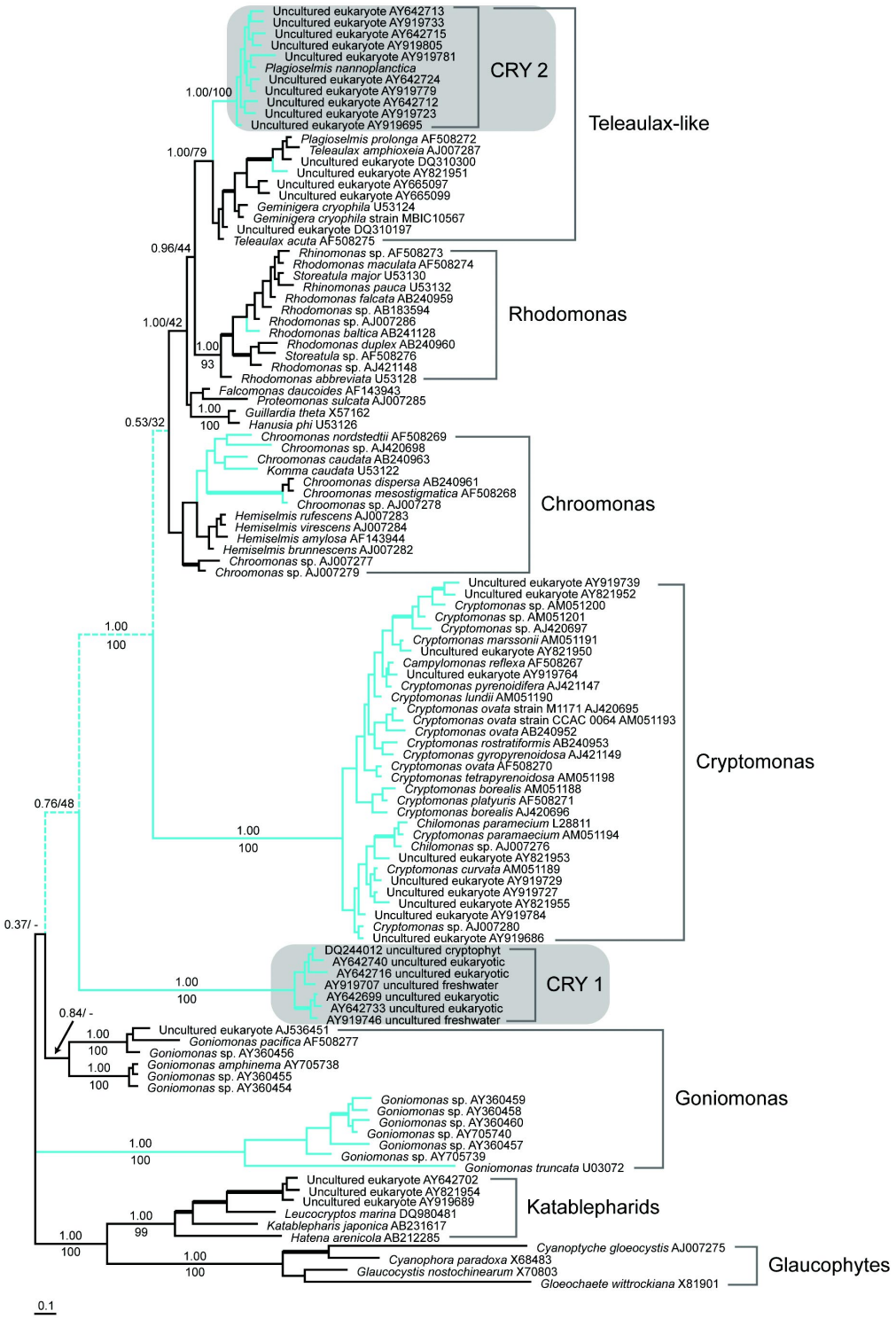
phi, were weakly clustered together in AL2. Thus, compared to the Fig.1, the removal of terminal missing characters caused no substantial changes in the overall ML and Bayesian tree topology (Fig. 2), but reduced the statistical support at some deep nodes. Importantly, the removal of sites did not change significantly the phylogenetic relationships between the marine and freshwater cryptomonads.

New freshwater cryptomonad lineages

A major finding of this work is the identification of two novel cryptomonad lineages that are entirely composed of freshwater phylotypes (both supported by 100% bootstrap and 1.0pp [pp=posterior probabilities]). One of these lineages, here named Cryptomonad Group 1 (CRY1), is composed of 15 sequences, and together with the *Goniomonas*, it is excluded robustly from the order Cryptomonadales (100% and 1.00pp), suggesting a ancient origin of this lineage within the cryptomonads. CRY1 and the *Goniomonas* group were mutually excluded with moderate support in Fig. 1, but with less support in the Fig. 2. Within the CRY1, no major subgroups were found. A striking feature of CRY1, which is shared with almost all freshwater cryptomonads, is the long basal branch separating CRY1 from all other cryptomonads (see also Hoef-Emden *et al.,* 2002). Since no species with proper description was placed within the CRY1, the identity of the species composing this lineage remains unclear. Nevertheless, the members of CRY1 are likely to be small (i.e. pico or nano size) as all of them were reported from picoplankton surveys (Lefranc *et al.,* 2005; Slapeta *et al.,* 2005; Richards *et al.,* 2005; Lefevre *et al.,* 2007; Lepere *et al.,* 2007). It is unclear if they contain plastids or are heterotrophic as *Goniomonas*, but the deep and distinctness of the group suggest that it constitutes either the most divergent plastid-containing cryptomonad clade or a new heterotrophic group at the base of cryptomonad tree. Despite these uncertainties, the clear distinctness of the group supports either a third order within cryptomonads, or a new genus belonging to the order Goniomonadales.

In contrast to the unknown identity of the-CRY1 members, the second identified freshwater lineage, CRY2, includes the nano-sized *Plagioselmis nannoplanctica* (Skuja) Novarino, Lucas et Morrall 1994. The group CRY2 clustered to the *Teleaulax*-like group, suggesting that all the affiliated phylotypes may have a *Plagioselmis*-like morphology and cell size. However, the other included *Plagioselmis* (*P. prolonga* Butcher) is placed together with the marine genera *Teleaulax* and *Geminigera*. Clearly, some of the *Plagioselmis* have been misclassified. All the remaining sequences belonging to CRY2 have been obtained from pico-size filtered freshwater, supporting the scenario that both CRY1 and CRY2 are composed of pico/nano-sized cells. The shorter basal and internal branches in the CRY2 and *Teleaulax*-like groups, in comparison with the other freshwater groups, suggest either slower evolutionary rates or a more recent diversification. Moreover, the short internal branches within the CRY2 clade indicate that the group is more homogeneous than CRY1 and that the different phylotypes may represent different strains of the same species. In addition to CRY1 and CRY2, a single freshwater sequence was placed within the *Teleaulax* group (i.e. accession number AY821951). This could represent another freshwater colonization line within *Teleaulax*. Interestingly, we could not identify any novel cryptomonad clade from marine samples, despite the numerous surveys of marine environments around the world. This indicates that the largest unknown diversity of cryptomonads is likely found in freshwater.

Figure 1. Phylogeny of cryptomonads inferred from Bayesian inferences using the GTR+G+I+Covarion model of evolution and the AL2 18S rRNA alignment. New freshwater groups CRY1 and CRY2 identified from GenBank are marked with grey boxes. Freshwater and marine cryptomonad lineages are marked with blue and black lines, respectively. Lines with unclear marine or freshwater origin are indicated with dashed lines. Groups with posterior probability > 0.90 and bootstrap support > 75 % are marked with thick branches due to limited space. Values at internal nodes represent posterior probability values and maximum likelihood bootstrap analysis. Thick lines indicate posterior probability > 0.95% and bootstrap > 85%.


Uncultured eukaryote AY642713
Uncultured eukaryote AY919733
Uncultured eukaryote AY642715
Uncultured eukaryote AY919805
Uncultured eukaryote AY919781
Plagioselmis nannoplanctica
Uncultured eukaryote AY642724
Uncultured eukaryote AY919779
Uncultured eukaryote AY642712
Uncultured eukaryote AY919723
Uncultured eukaryote AY919695
CRY 2
1.00/100
Teleaulax-like
Plagioselmis prolonga AF508272
Teleaulax amphioxeia AJ007287
Uncultured eukaryote DQ310300
Uncultured eukaryote AY821951
Uncultured eukaryote AY665097
Uncultured eukaryote AY665099
Geminigera cryophila U53124
Geminigera cryophila strain MBIC10567
Uncultured eukaryote DQ310197
Teleaulax acuta AF508275
1.00/79
0.96/44
Rhinomonas sp. AF508273
Rhodomonas maculata AF508274
Storeatula major U53130
Rhinomonas pauca U53132
Rhodomonas falcata AB240959
Rhodomonas sp. AB183594
Rhodomonas sp. AJ007286
Rhodomonas baltica AB241128
Rhodomonas duplex AB240960
Storeatula sp. AF508276
Rhodomonas sp. AJ421148
Rhodomonas abbreviata U53128
Rhodomonas
1.00/42
1.00
93
Falcomonas daucoides AF143943
Proteomonas sulcata AJ007285
1.00
100
Guillardia theta X57162
Hanusia phi U53126
0.53/32
Chroomonas nordstedtii AF508269
Chroomonas sp. AJ420698
Chroomonas caudata AB240963
Komma caudata U53122
Chroomonas dispersa AB240961
Chroomonas mesostigmatica AF508268
Chroomonas sp. AJ007278
Hemiselmis rufescens AJ007283
Hemiselmis virescens AJ007284
Hemiselmis amylosa AF143944
Hemiselmis brunnescens AJ007282
Chroomonas sp. AJ007277
Chroomonas sp. AJ007279
Chroomonas
1.00
100
Uncultured eukaryote AY919739
Uncultured eukaryote AY821952
Cryptomonas sp. AM051200
Cryptomonas sp. AM051201
Cryptomonas sp. AJ420697
Cryptomonas marssonii AM051191
Uncultured eukaryote AY821950
Campylomonas reflexa AF508267
Uncultured eukaryote AY919764
Cryptomonas pyrenoidifera AJ421147
Cryptomonas lundii AM051190
Cryptomonas ovata strain M1171 AJ420695
Cryptomonas ovata strain CCAC 0064 AM051193
Cryptomonas ovata AB240952
Cryptomonas rostratiformis AB240953
Cryptomonas gyropyrenoidosa AJ421149
Cryptomonas ovata AF508270
Cryptomonas tetrapyrenoidosa AM051198
Cryptomonas borealis AM051188
Cryptomonas platyuris AF508271
Cryptomonas borealis AJ420696
Chilomonas paramecium L28811
Cryptomonas paramaecium AM051194
Chilomonas sp. AJ007276
Uncultured eukaryote AY821953
Cryptomonas curvata AM051189
Uncultured eukaryote AY919729
Uncultured eukaryote AY919727
Uncultured eukaryote AY821955
Uncultured eukaryote AY919784
Cryptomonas sp. AJ007280
Uncultured eukaryote AY919686
Cryptomonas
0.76/48
1.00
100
0.37/ -
DQ244012 uncultured cryptophyt
AY642740 uncultured eukaryotic
AY642716 uncultured eukaryotic
AY919707 uncultured freshwater
AY642699 uncultured eukaryotic
AY642733 uncultured eukaryotic
AY919746 uncultured freshwater
CRY 1
1.00
100
0.84/ -
1.00
100
Uncultured eukaryote AJ536451
Goniomonas pacifica AF508277
Goniomonas sp. AY360456
1.00
100
Goniomonas amphinema AY705738
Goniomonas sp. AY360455
Goniomonas sp. AY360454
Goniomonas sp. AY360459
Goniomonas sp. AY360458
Goniomonas sp. AY360460
Goniomonas sp. AY705740
Goniomonas sp. AY360457
Goniomonas sp. AY705739
Goniomonas truncata U03072
Goniomonas
1.00
100
Uncultured eukaryote AY642702
Uncultured eukaryote AY821954
Uncultured eukaryote AY919689
Leucocryptos marina DQ980481
Katablepharis japonica AB231617
Hatena arenicola AB212285
Katablepharids
1.00
99
1.00
100
Cyanoptyche gloeocystis AJ007275
1.00
100
Cyanophora paradoxa X68483
Glaucocystis nostochinearum X70803
Gloeochaete wittrockiana X81901
Glaucophytes
0.1

Cryptomonad phylogeny indicates few marine-freshwater transitions

The inclusion of a broad taxon sampling as well as sequences from environmental surveys in the reconstructed phylogenies has allowed a rigorous examination of the relationships between freshwater and marine cryptomonads. The results uncovered that in most cases, marine and freshwater species were not closely related, but separated into distinct clades. This indicates that marine-freshwater transitions have not been common during the evolutionary diversification of cryptomonads. Otherwise, many more closely related marine and freshwater species should have been present. The clustering pattern of the freshwater species suggests that most of the colonizations took place relatively early in the evolution of cryptomonads. In addition, most freshwater species seem to have diversified subsequent to the colonization events. On the other hand, the less diversified freshwater clades and the solitary freshwater sequences (e.g. AY821951, AJ007286, AF143944) are most probably either the product of more recent freshwater colonizations or could represent larger freshwater clades that were undersampled. Our analyses also suggest that most transition events have been unidirectional, from marine to fresh waters, but because three *Chroomonas* species (i.e. *C. mesostigmatica, C. dispersa* and *Chroomonas sp.)* are placed within a freshwater clade, at least two marine re-colonization events seem to have taken place.

Although some of the basal branching patterns receive higher statistical support by the covarion analysis, the relationships between several of the cryptomonad groups remain unclear. The exact number of freshwater colonizations is therefore difficult to evaluate on the basis of only the 18S rRNA gene phylogeny. Improved resolution of the branching order between the major clades of cryptomonads, and thus a more precise estimation of the number of colonization events, should be further investigated by employing a larger diversity of genes. Nevertheless, the current work clearly shows that marine-freshwater transitions have taken place only at relatively few occasions during the evolution of cryptomonads, suggesting that differences in freshwater and marine biogeophysical conditions constitute a considerable barrier for the transitions of cryptomonads between these two environments. Similar evidence has recently been found in studies of other protist groups, such as diatoms (Alverson *et al.* 2007), dinoflagellates (Logares *et al.* 2007 [Paper 6, this thesis]) and *Ancyromonas* (Schekenbach *et al.* 2006). Altogether, ours and others' studies suggest that the differences between marine and freshwater environments constitute a general barrier for the exchange of protists between these two habitats. Probably, this barrier has a considerable impact on the diversification patterns of unicellular eukaryotes.

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Figure 2. Phylogeny of cryptomonads. The tree is reconstructed with Bayesian inferences using the GTR+G+I+Covarion model of evolution and the AL3 dataset. Numbers on the internal nodes represents Bayesian posterior probability and bootstrap support values. Thick lines denote posterior probability > 0.95% and bootstrap > 85%. New freshwater groups CRY1 and CRY2 are marked with grey boxes. Freshwater and marine cryptomonad lineages are marked with blue and black lines, respectively. Lines with unclear marine or freshwater origin are indicated with dashed lines.

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