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# Recent evolutionary diversification of a protist lineage

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## Summary

**Here, we have identified a protist (dinoflagellate) lineage that has diversified recently in evolutionary terms. The species members of this lineage inhabit cold-water marine and lacustrine habitats, which are distributed along a broad range of salinities (0–32) and geographic distances (0–18 000 km). Moreover, the species present different degrees of morphological and sometimes physiological variability. Altogether, we analysed 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 analysed 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 versus lacustrine communities) and salinity. Probably, this evolutionary diversification was promoted by the variety of natural selection regimes encountered in the different environments.**

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## 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. local adaptation, geographic isolation, etc.; see, e.g. Futuyma, 1998; Coyne and Orr, 2004). The same mechanisms are probably promoting 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 apparent 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 gene flow which would restrain the opportunities for microbial diversification. Furthermore, the relative importance of natural selection and genetic drift in the diversification of microbes needs to be investigated. For example, large microbial populations most probably have a greater tolerance to the random fixation of mutations (i.e. genetic drift) than relatively smaller populations of multicellular counterparts. Thus, it is likely that the relative importance of natural selection and genetic drift in promoting the diversification and formation of spatial-distribution patterns in microbes and in multicellular organisms is not equivalent.

During the last 15 years, molecular studies have revealed new data and patterns, which have improved our understanding on microbial diversity and biogeogra-

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phy. For example, several molecular studies indicate that the microbial diversity is much higher than previously estimated (e.g. Huber *et al*., 2007), 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. Our previous work suggested the existence of a group of closely related dinoflagellate (protists) species composed by *Scrippsiella hangoei*, *Peridinium aciculiferum* and *Scrippsiella* aff. *hangoei* (Logares *et al*., 2007a; Rengefors *et al*., 2008). However, the number of strains and markers were not sufficient for achieving conclusive results. Here, after including several new strains and more genetic markers we have confirmed that the analysed dinoflagellate lineage represents a case of recent evolutionary diversification. Moreover, we have investigated the evolutionary relationships among the strains/species within this dinoflagellate lineage, 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 analysed the patterns of ribosomal and mitochondrial DNA (mtDNA) 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 versus 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.

## Results

### *Morphological analyses*

The optical microscopy analyses confirmed the morphospecies identity of all the *P. aciculiferum* isolates. However, the isolates from Stora 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 and colleagues (1995) (Fig. 1 and Fig. S1; see also Rengefors *et al*., 2008). 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 *P. aciculiferum*, the general morphology between them was different (Fig. 1 and Logares *et al*., 2007a).

### *Nuclear ribosomal DNA homogeneity versus cytochrome b mtDNA differentiation*

The analysed cold-water dinoflagellate strains showed three different nuclear ribosomal DNA (nrDNA) ribotypes (Figs 2 and 3). All the *P. aciculiferum* isolates from lakes in Sweden, Finland and Italy, as well as the *S. hangoei* isolates from the Baltic Sea had identical nrDNA sequences (Figs 2 and 3). The second ribotype was found among the *S.* aff. *hangoei* isolates from the Antarctic Vereteno and Highway lakes (Figs 2 and 3). The third ribotype was found in the Arctic *S.* aff. *hangoei* (Figs 2 and 3). Between the pair *P. aciculiferum*–*S. hangoei* and the bipolar *S.* aff. *hangoei* isolates, there was a very low nrDNA differentiation. The sequences comprising the Internal Transcribed Spacer 1 and 2 (= ITS) (ITS1/2 are the most variable areas within nrDNA) differed less than 1.43% among the 30 analysed strains (Figs 2 and 3). The ITS differentiation between the bipolar *S.* aff. *hangoei* was ~0.90% (Figs 2 and 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% (Figs 2 and 3). A total of 558 ITS nucleotides were analysed to obtain these percentages. The D1/D2 Large Subunit (LSU) sequence from the Arctic *S.* aff. *hangoei* differed by ~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 Small Subunit (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.

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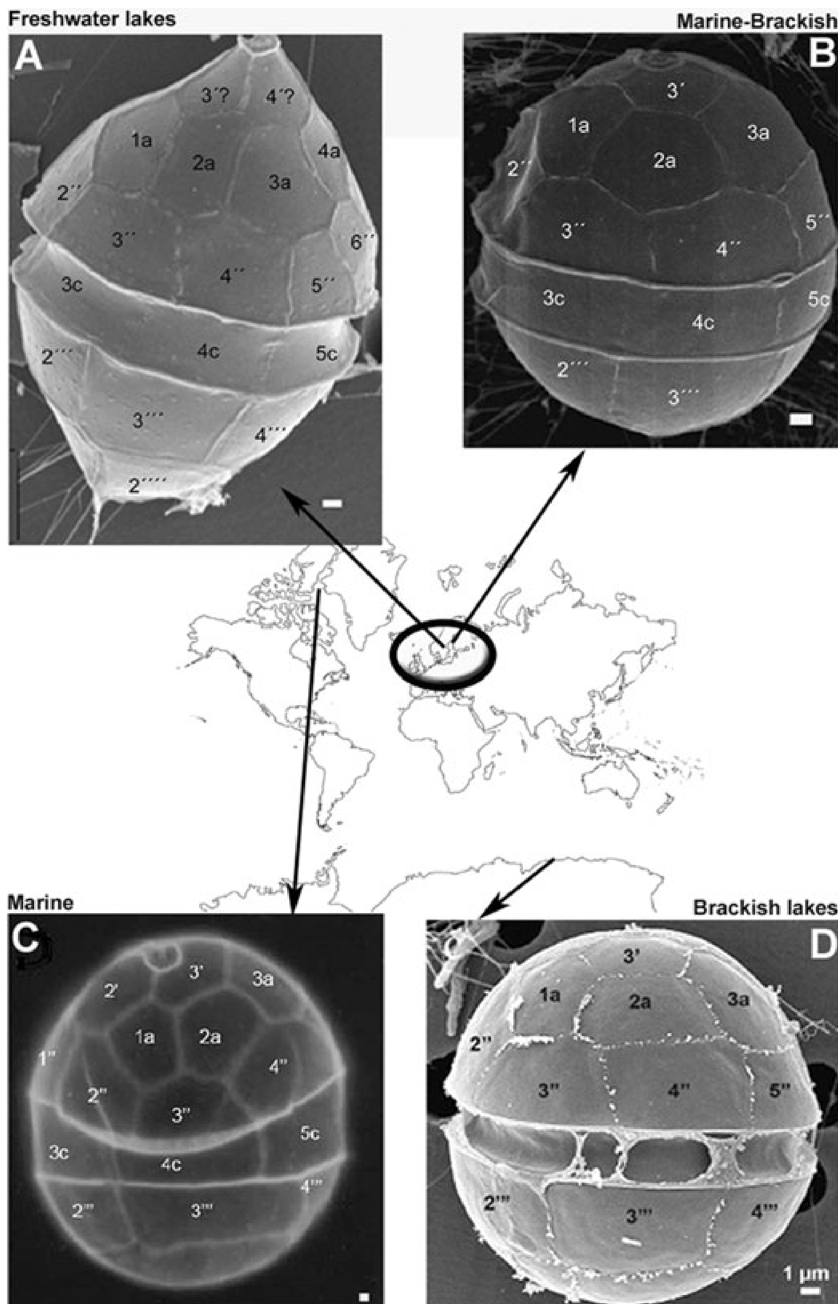


**Fig. 1.** Morphology and geographic source of the investigated dinoflagellate strains/species.
A. *Peridinium aciculiferum*: freshwater lakes in Northern-Central Europe (picture from Logares *et al*., 2007a).
B. *Scrippsiella hangoei*: Marine-brackish, Baltic Sea (picture from Logares *et al*., 2007a).
C. *Scrippsiella* aff. *hangoei*: Marine, Arctic (picture from this study; detailed morphological description in Fig. S1).
D. *Scrippsiella* aff. *hangoei*, brackish Antarctic lakes (picture from Rengefors *et al*., 2008).
The scale bar = 1 μm. The numbers over the cell armour correspond to the dinoflagellate Kofoidian tabulation. Note the difference in general morphology between *Peridinium* (A) and *Scrippsiella* (B–D).

A total of 13 cytochrome b (COB) mtDNA haplotypes, with a genetic differentiation ranging between 0.12% and 2.40%, were identified among the 30 analysed strains (Fig. 3). Within the 21 *P. aciculiferum* isolates from five lakes, eight haplotypes with a differentiation ranging between 0.12% and 1.32% were detected (Fig. 3). The three haplotypes that were detected within the four *S. hangoei* isolates from the Baltic had a differentiation ranging between 0.12% and 0.30% (Fig. 3). Only one haplotype was detected within the four *S.* aff. *hangoei* isolates from the two Antarctic lakes. The haplotype of the Arctic *S.* aff. *hangoei* differed by only 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 a lack of mtDNA heteroplasmy.

A total of 23 variable COB sites were identified, out of 836 analysed, among the 13 recognized haplotypes. These 23 variable sites accounted for 24 mutations, of

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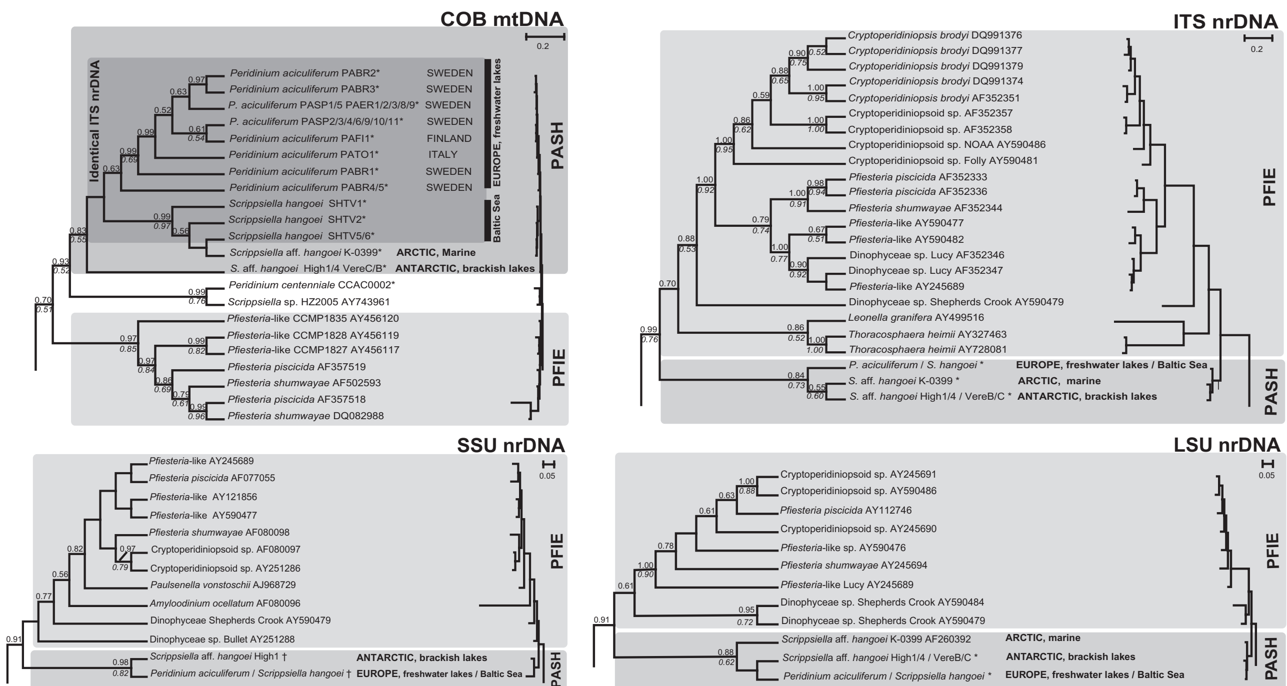


**Fig. 2.** Bayesian Inference (BI) and Maximum Likelihood (ML) consensus phylogenies constructed with the COB mtDNA and ITS, SSU, LSU nrDNA. The BI and ML phylogenies were constructed under the GTR+*G*+I (COB) or the GTR+*G*+COV (nrDNA) models. The trees on the left show the topologies and Posterior Probabilities (PPs; normal print above the nodes)/Bootstrap support values (BVs; Italics below the nodes) $> 0.5$. The trees on the right show the evolutionary distances obtained by Bayesian MCMC inference (the top-right scale bars indicate substitutions per site). Posterior Probabilities were calculated from the $3 \times 10^4$ trees obtained after the log-likelihood stabilization, while BVs were calculated from 1000 bootstrap pseudoreplicates. The frames indicate the PASH (*P. aciculiferum*, *S. hangoei*, *S.* aff. *hangoei* Arctic-Antarctic) and PFIE (*Pfiesteria* and *Pfiesteria*-like species) clusters. Note that several species that were not related to the clades of interest were removed from these trees. Full trees 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). 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.

which only six generate amino acid replacements, resulting in a total of eight different amino-acid haplotypes (Table S2).

### *Phylogenetic relationships*

Across the 16 constructed nrDNA and COB mtDNA phylogenies using Bayesian Inference (BI) and Maximum Likelihood (ML), *P. aciculiferum* from European freshwater lakes, *S. hangoei* from the Baltic Sea, *S.* 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) [PPs = Posterior Probabilities; BVs = Bootstrap Values]. In the nrDNA and COB mtDNA phylogenies, PASH clustered with *Pfiesteria* and *Pfiesteria*-like species (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). 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).

## 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 versus lacustrine communities) and salinities, implying in some cases the dispersal across great geographic distances. The presence of the studied lineage in such variety of

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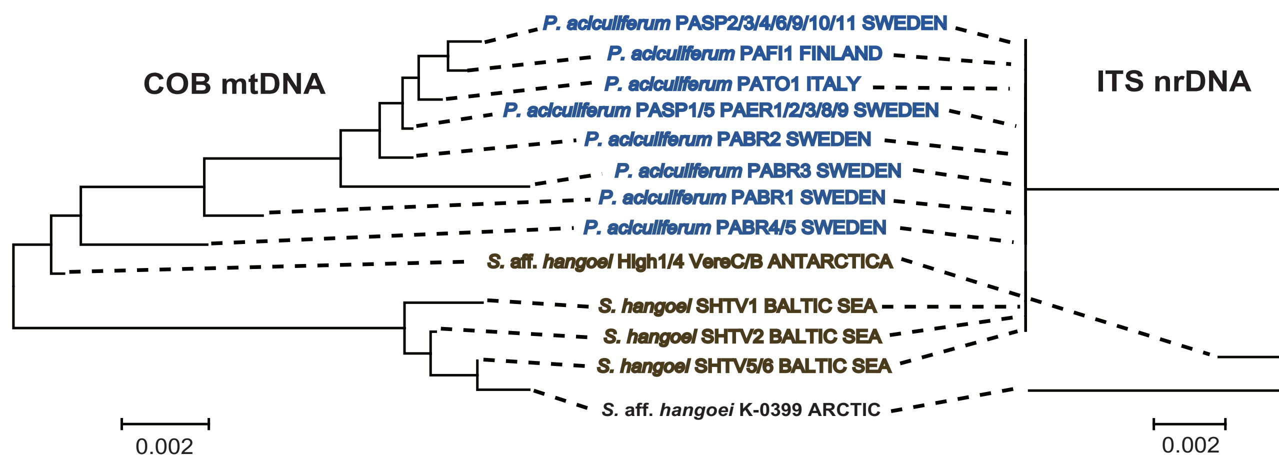


**Fig. 3.** Cytochrome b mtDNA variation versus ITS nrDNA homogeneity. Contrasting Neighbour-Joining phylograms based on uncorrected genetics distances (*P*) between COB haplotypes (left) and the corresponding ITS sequences (right). Freshwater lacustrine strains/species appear in light blue, brackish 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 scale bar indicates *P*.

environments suggests that natural selection could have had a major role in promoting diversification.

### *Recent evolutionary diversification*

The low nrDNA differentiation between *P. aciculiferum*, *S. hangoei* and the bipolar *S.* aff. *hangoei* indicates a recent divergence between these strains/species. In particular, the ITS (= ITS1/2–5.8 s), which harbours two rapidly evolving introns (ITS1/2) normally used for investigating the 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 neighbouring lakes, had a much higher level 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 among all 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.,* 2007a; Rengefors *et al.*, 2008), which do not appear to be product of phenotypic plasticity (see Logares *et al.*, 2007a; Rengefors *et al.*, 2008). Thus, the studied cold-water dinoflagellates do not appear to constitute one global interbreeding population despite their close evolutionary relationships. Overall, our results contrast with several other studies showing considerable genetic variation among morphologically identical or very similar microbial strains (e.g. Potter *et al.*, 1997; Montresor *et al.*, 2003; Kim *et al.*, 2004; Boenigk *et al.*, 2005; Lilly *et al.*, 2005).

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 ($P < 0.02$) would place them into the same 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 species (see Logares *et al.*, 2007a; Rengefors *et al.*, 2008).

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 (see Logares *et al.*, 2007a). Such differentiation was indicated by Amplified Fragment Length Polymorphism, a fingerprinting technique that screens the whole genome (Bensch and Akesson, 2005). Other organisms which are known to have diversified recently

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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). Even though the nrDNA similarity between the studied dinoflagellates does not appear to be due to slow evolutionary rates, we cannot 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; 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.*, 2007a)].

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 and 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* indicate that their divergence could have occurred between four and two million years ago (MYA). On the other hand, the divergence between the bipolar *S.* aff. *hangoei* could have occurred between 1.5 and 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, because differential natural selection regimes can be a strong motor of divergence (e.g. Orr and Smith, 1998). Such diversification appears to have occurred in parallel with transitions between environments with different salinity (freshwater, brackish, marine) and ecologies (marine versus 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. Resting cysts have been identified in the strains/species *S. hangoei, S.* aff. *hangoei* and *P. aciculiferum* (Rengefors *et al.*, 1998; 2008; Kremp and Parrow, 2006). 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 was 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; 2008; Kremp and Parrow, 2006). 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 and colleagues (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 6000 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.*, 2008). Most likely, the divergence between *P. aciculiferum* and *S. hangoei* (and the colonization of fresh waters by *P. aciculiferum*) occurred very recently, because these morphospecies still share identical ITS nrDNA, despite presenting genetic differentiation at the genome level (Logares *et al.*, 2007a). *Scrippsiella 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.*, 2007a). 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

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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 analysed strains. The ML BVs and BI PPs for the PASH clade using the SSU, LSU, ITS and COB ranged from moderate (~0.60) to high (> 0.90) (see Table S1). 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 armour 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); see also Rengefors *et al*., 2008].

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). *Pfiesteria* and *Pfiesteria*-like dinoflagellates are marine-brackish and in several cases toxin producers (e.g. Steidinger *et al*., 1996; 2001; Marshall *et al*., 2000; 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). As 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 versus 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) 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, because 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.

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*Concluding remarks*

The presence of the studied dinoflagellate 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*., 2007b). The role of genetic drift during this diversification is unclear, although the huge population sizes that dinoflagellates normally have indicated 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.

## Experimental procedures

*Dinoflagellate cultures and morphospecies identification*

All dinoflagellate clonal cultures were obtained by isolating single cells from plankton samples, except when specified otherwise. *Peridinium aciculiferum* Lemmermann isolate PATO was obtained from Lake Tovel, which is located at 1178 m above sea level (a.s.l.) in the Italian Alps and was formed after the last glaciations (~15 000 years ago) (Kulbe *et al*., 2005; Table 1). *Peridinium aciculiferum* isolates PASP were obtained during March 2006 from Stora Pildammen, an approximately 100-year-old artificial pond located in Southern Sweden (Table 1). *Peridinium aciculiferum* isolates PABR were obtained during March 2006 from Brodammen, a small artificial pond ~10 years old located in Southern Sweden (Table 1). Both Stora Pildammen and Brodammen are located ~20 m a.s.l. and remain ice-covered from December to March approximately. *Peridinium aciculiferum* isolates PAER were obtained from Lake Erken, Eastern-Central Sweden (Table 1), which is 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. *Peridinium aciculiferum* PAFI was isolated during March 2006 from Lake Österträsk, Åland, Finland (Table 1). 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 (Table 1) during 2002. The Baltic Sea is a semi-enclosed brackish Sea with a salinity gradient ranging from 1 to 25 (see Voipio, 1981). The post-glacial history of the Baltic Sea started about 8500 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 months per year. The *S.* aff. *hangoei* isolates High and Vere were obtained from Highway Lake (8 m a.s.l.) and Vereteno Lake (sea level), respectively, during the summer of 2004/2005 (Table 1). 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 ~6000 years ago (Zwartz *et al*., 1998). Highway and Vereteno lakes are brackish and are usually ice-free for around 4 weeks each year. Plankton samples were collected through holes in the ice-cover. The Arctic isolate of *S.* 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 (Table 1) 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). *Peridinium 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 for 6 years in seawater-based Throndsen medium (Throndsen, 1978; salinity 30). 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, armour plate patterns were analysed by light microscopy. The *P. aciculiferum* cultures (PATO, PAFI, PASP, PABR) were fixed in 5% formaldehyde. Dinoflagellate plate detachment between slide and coverslip 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 *S.* 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 and colleagues (2007a) and Rengefors and colleagues (2008) (see also Fig. 1).

*DNA extraction, PCR and sequencing*

DNA was extracted following Adachi and colleagues (1994). For the Arctic *S.* aff. *hangoei* K-0399, DNA was extracted from a 200 μl frozen pellet, obtained from ~10 ml culture, using a GENERATION Capture Column Kit (Gentra Systems, Minneapolis).

For this work, we used different areas of the nrDNA molecule as well as the mitochondrial (mt) gene COB. The amplified and sequenced nrDNA fragments were: ITS1/2, 5.8S, SSU and the D1/D2 domains of the 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

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

| Morphospecies | Isolate | Collection site | Coordinates | ~Salinity | Isolation | GenBank accession numbers | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 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**[a] | 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**[a] | **EF417314**[a] | 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 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417292**[a] | **EF417324**[a] |
| *Peridinium aciculiferum* | PASP-2 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417293**[a] | **EF417325**[a] |
| *Peridinium aciculiferum* | PASP-3 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417294**[a] | **EF417326**[a] |
| *Peridinium aciculiferum* | PASP-4 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417295**[a] | **EF417327**[a] |
| *Peridinium aciculiferum* | PASP-5 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417296**[a] | **EF417328**[a] |
| *Peridinium aciculiferum* | PASP-6 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417297**[a] | **EF417329**[a] |
| *Peridinium aciculiferum* | PASP-9 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | – | **EF417298**[a] | **EF417330**[a] |
| *Peridinium aciculiferum* | PASP-10 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | **EF417311**[a] | – | **EF417299**[a] | **EF417331**[a] |
| *Peridinium aciculiferum* | PASP-11 | Stora Pildammen, Sweden | 55°35′N, 12°59′E | 0 | 2006 | – | **EF417315**[a] | **EF417300**[a] | **EF417332**[a] |
| *Peridinium aciculiferum* | PABR-1 | Brodammen, Sweden | 55°32′N, 12°58′E | 0 | 2006 | – | – | **EF417287**[a] | **EF417319**[a] |
| *Peridinium aciculiferum* | PABR-2 | Brodammen, Sweden | 55°32′N, 12°58′E | 0 | 2006 | – | – | **EF417288**[a] | **EF417320**[a] |
| *Peridinium aciculiferum* | PABR-3 | Brodammen, Sweden | 55°32′N, 12°58′E | 0 | 2006 | **EF417309**[a] | **EF417313**[a] | **EF417289**[a] | **EF417321**[a] |
| *Peridinium aciculiferum* | PABR-4 | Brodammen, Sweden | 55°32′N, 12°58′E | 0 | 2006 | – | – | **EF417290**[a] | **EF417322**[a] |
| *Peridinium aciculiferum* | PABR-5 | Brodammen, Sweden | 55°32′N, 12°58′E | 0 | 2006 | – | – | **EF417291**[a] | **EF417323**[a] |
| *Peridinium aciculiferum* | PAFI-1 | Lake Östersträsk, Finland | 60°16′N, 20°06′E | 0 | 2006 | **EF417310**[a] | – | **EF417286**[a] | **EF417333**[a] |
| *Peridinium aciculiferum* | PATO-1 | Lake Tovel, Italy | 46°15′N, 10°49′E | 0 | 2003 | **EF417312**[a] | – | **EF417285**[a] | **EF417334**[a] |
| *Scrippsiella* aff. *hangoei* | K-0399 | Foxe Basin, Arctic | 69°23′N, 81°45′W | 0–32 | 1989 | AF260392 | – | **EF506568**[a] | **EF506569**[a] |
| *Scrippsiella* aff. *hangoei* | High-1 | Highway Lake, Antarctica | 68°14′S, 78°28′E | 5 | 2005 | EF058275 | EF417318 | **EF417301**[a] | **EF417335**[a] |
| *Scrippsiella* aff. *hangoei* | High-4 | Highway Lake, Antarctica | 68°14′S, 78°28′E | 5 | 2005 | – | – | **EF417302**[a] | **EF417336**[a] |
| *Scrippsiella* aff. *hangoei* | Vere-B | Vereteno Lake, Antarctica | 68°30′S, 78°24′E | 4 | 2005 | – | – | **EF417306**[a] | **EF417337**[a] |
| *Scrippsiella* aff. *hangoei* | Vere-C | Vereteno Lake, Antarctica | 68°30′S, 78°24′E | 4 | 2005 | – | – | **EF417307**[a] | **EF417338**[a] |
| *Peridiniopsis borgei* | PBSK-A | St Kalkbrottsdammen | 55°31′N, 12°55′E | 0.6 | 2005 | EF058261 | **EF058241**[a] | – | **EF417339**[a] |
| *Peridinium centenniale* | CCAC0002 | Cornwall, England | – | 0 | – | EF058254 | EF058236 | – | **EF417340**[a] |
| *Polarella glacialis* | CCMP1383 | Antarctica | – | Marine | 1991 | – | EF417317 | – | **EF417341**[a] |

**a.** (Bold) sequences obtained for this work.

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DNA, 0.125 mM of each nucleotide, 1.5 (3.0 for SSU) mM of $MgCl_2$, 1× 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′ (White *et al*., 1990) 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′-TGATCCTTCTGCAGGTTCACCTAC-3′ (Medlin *et al*., 1988). 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′-CCGTGTTTCAAGACGGGTC-3′ (Logares *et al*., 2007a). 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′-ATGAAATCTCATTTACAWWCATATCCTTGTCC-3′, and Dinocob1R (reverse), 5′-TCTCTTGAGGKAATTGWKMACC TATCCA-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′-CGG TAATTCCAGCTCC-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 analysing 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 *S.* 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 *S. hangoei* and *P. aciculiferum* in Logares and colleagues (2007a). 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 (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 ML and 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 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 ($\alpha$) 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 $5 \times 10^6$ generations and the trees were sampled every 100 generations, which resulted in $5 \times 10^4$ 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 as 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 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 $3 \times 10^4$ trees after the log-likelihood stabilization.

Maximum Likelihood 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, as 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).

### *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 analyse the genetic polymorphism of the mtDNA sequences. The COB reading frame was obtained by analysing the sequences at the ExPASy Proteomics Server (http://www.expasy.org/). The protozoan mitochondrial genetic code was used to translate the COB sequences.

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## Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Light micrographs and schematic diagrams of *S.* aff. *hangoei* from the Canadian Arctic, isolate K-0399.

A and B. Differential interference contrast.

C–E. Fluorescence microscopy of CalcoFlour White stained cells.

F–I. Schematic diagram of the thecal plates deduced from fluorescence 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 labelled according to the Kofoidian plate formula in C–I. The plate tabulation for the Arctic *S.* aff. *hangoei* is po, x, 4′, 3a, 7″, 6c (t+5c), 6 s, 5‴, $2 \times 0$.

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

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

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