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

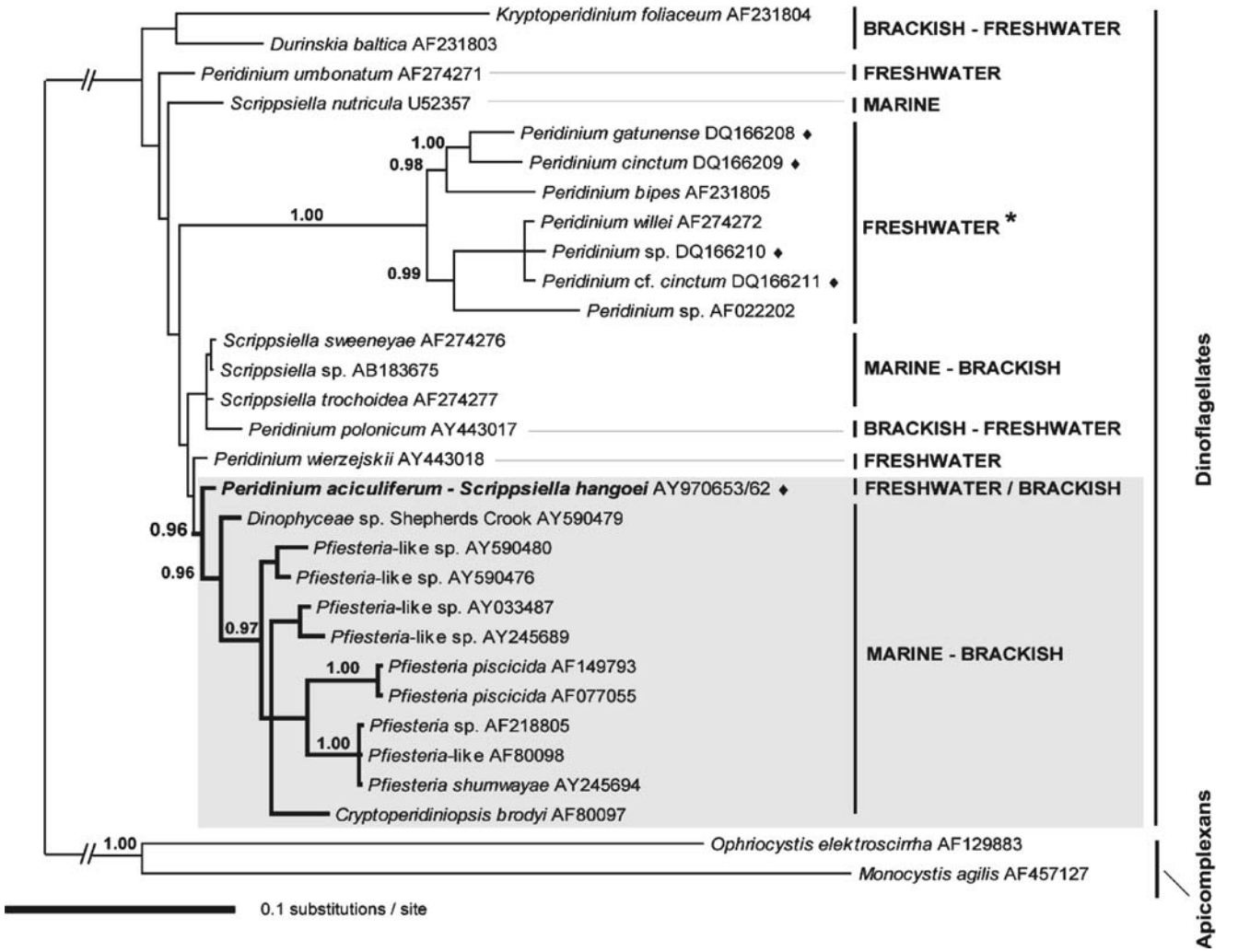

Figure 1. Bayesian SSU rDNA phylogram of 30 sequences based on 1749 nucleotides (GTR + G model; ran for 4×10^6 generations). The gray box indicates the relationship of *Peridinium aciculiferum* and *Scrippsiella hangoei* with other marine/estuarine species. Bayesian posterior probability branch support 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′-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 we used the combination of the 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, 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

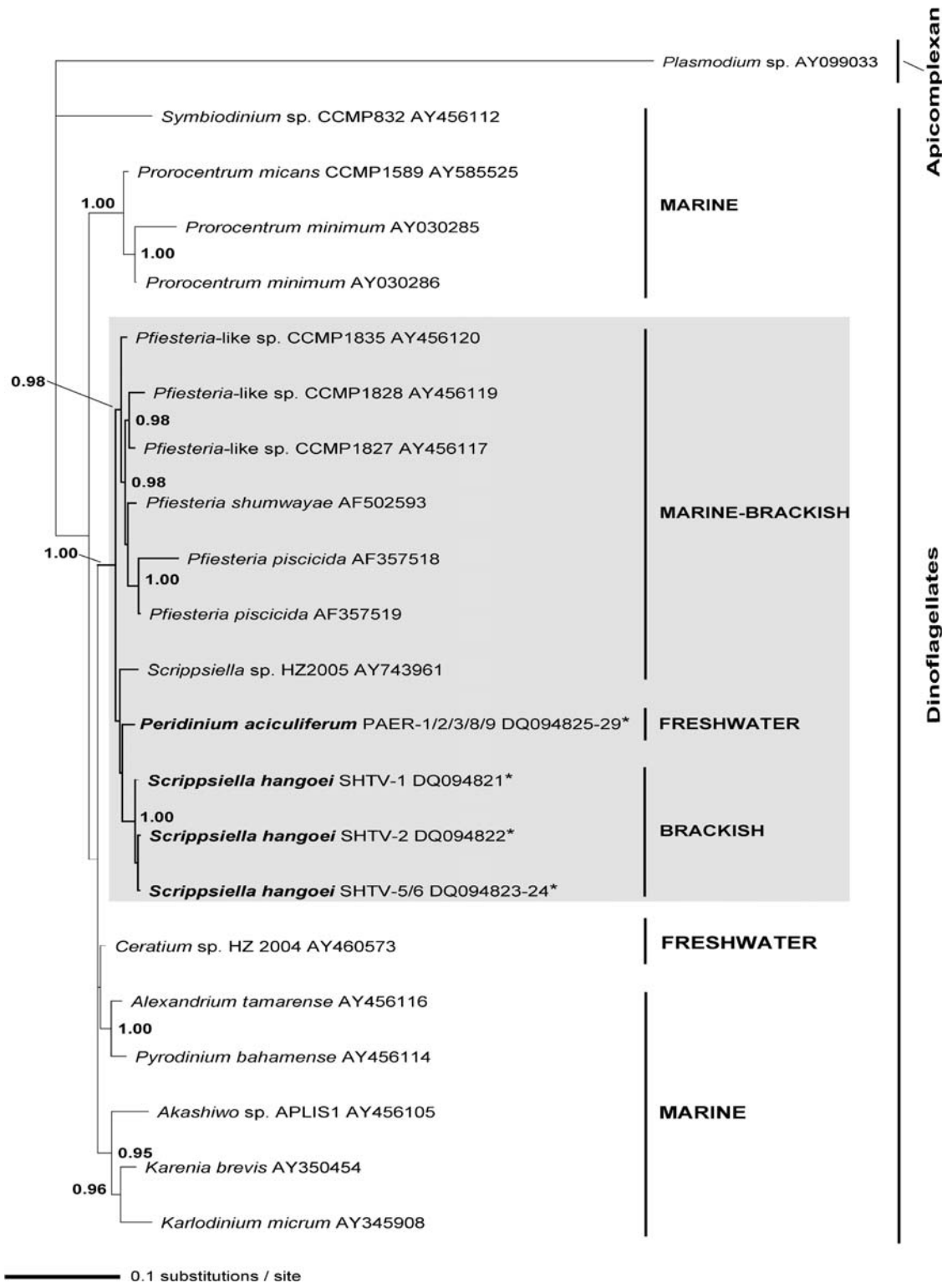

Figure 2. Bayesian *cob* mtDNA phylogram of 22 sequences using 826 nucleotides (GTR + G model; ran for 4×10^6 generations). The gray box indicates the relationship of *P. aciculiferum* and *S. hangoei* with other marine/estuarine species. Bayesian posterior probability branch support values ≥ 0.95 are 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.

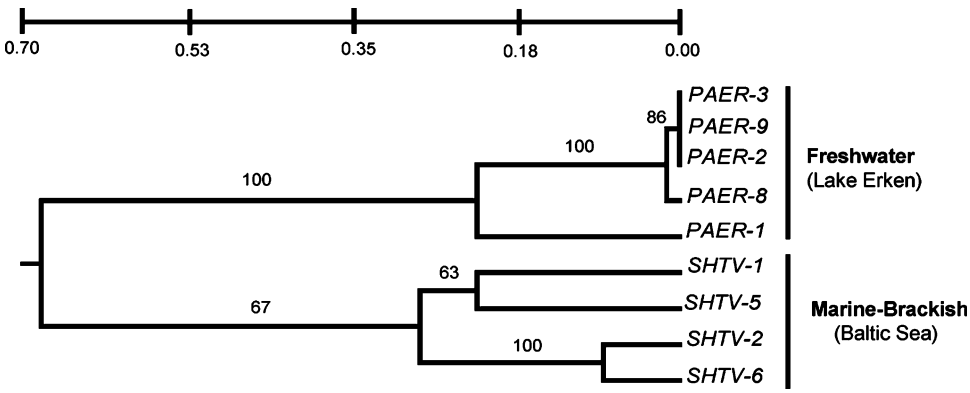

Figure 3. UPGMA phylogram based on Nei's minimum distances for the 156-screened AFLP loci. Five clonal cultures were used for *P. aciculiferum* and four for *S. hangoei*. PAER: *P. aciculiferum*; SHTV: *S. hangoei*. Values over branches indicate bootstrap support (1000 pseudoreplicates).

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.

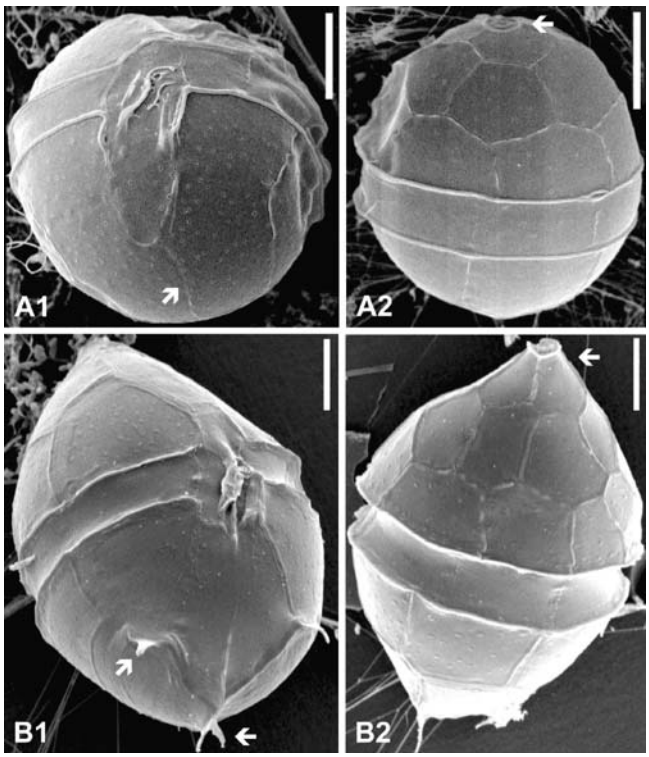

Figure 4. SEM micrographs showing the morphological differences between egg-shaped *S. hangoei* (A1, 2; Baltic Sea) and subspherical *P. aciculiferum* (B1, 2; Lake Erken). The presence of spines in *P. aciculiferum* and its absence in *S. hangoei* is shown in A1 and B1 (*arrows*). Differences in the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

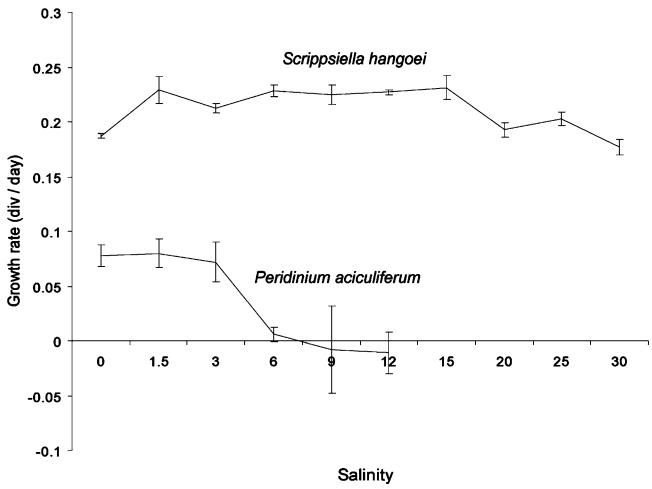

Figure 5. Growth of *S. hangoei* and *P. aciculiferum* in different salinity media. Data points refer to treatment means ± 1 SE. Note that *S. hangoei* was grown at 150 μmol photons m^{-2} s^{-1} and 14:10 h light–dark, whereas *P. aciculiferum* was grown at 20 μmol photons m^{-2} s^{-1} and 12:12 h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