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Genetic Diversity Patterns in Five Protist Species Occurring in Lakes

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Little is known about the extent of the genetic diversity and its structuring patterns in protist species living in lakes. Here, we have investigated the genetic diversity patterns within five dinoflagellate species (*Peridinium aciculiferum*, *Peridinium cinctum*, *Peridiniopsis borgei*, *Polarella glacialis*, *Scrippsiella* aff. *hangoei*) that are present in lakes and sometimes, in marine habitats located in polar and temperate regions. A total of 68 clonal strains were investigated using Amplified Fragment Length Polymorphism (AFLP), a sensitive genetic fingerprinting technique. All used strains within each species had identical ITS nuclear ribosomal DNA sequences, a characteristic that indicates that they likely belong to the same species. We found a wide variability in the genetic diversity among species (between 20% and 90% of polymorphic loci; Nei's gene diversity between 0.08 and 0.37). In some cases, our analyses suggested the presence of different genetically homogeneous subgroups (genetic populations) within the same water body. Thus, it appears that different genetic populations can coexist within the same lake despite the likely occurrence of recombination that tends to homogenize the gene pool. Overall, our results indicated that a large number of dinoflagellate genotypes are present in lake populations, instead of a few dominating ones. In addition, our study shows that protists with identical ITS sequences can harbor considerable amounts of genetic diversity.

Key words: dinoflagellates; lakes; genetic variation; AFLP; ITS.

Introduction

The extent of the genetic diversity within microbial species occurring in lakes is still largely unknown. Moreover, there is little knowledge about the relationships between habitat type and the amount of genetic diversity and its structuring patterns. For instance, marine environments do not have obvious geographical features which could act as barriers for microbial dispersal and promote the emergence of structure in their genetic diversity. Therefore, the genetic diversity

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in marine microbial species would be expected to present less structure than in species inhabiting, for example, lakes, ponds or other discrete habitats where dispersal can be restricted (see e.g. Gibson et al. 2006; Hughes Martiny et al. 2006; Telford et al. 2006).

Despite predictions, several marine microbial species have shown a high intraspecific genetic diversity on both spatial and temporal scales (e.g. Bolch et al. 1999; Evans et al. 2005; Iglesias-Rodriguez et al. 2006; Nagai et al. 2007; Rynearson and Armbrust 2000; Shankle et al. 2004). The few available studies also indicate high intraspecific genetic diversity within microbial populations inhabiting lakes (e.g. Coleman 2001; De Bruin et al. 2004; Hayhome et al. 1987; Kusch et al. 2000; Müller et al. 2005; Wilson et al. 2005; Zhang et al. 2006). In particular, several marine protist populations have recently been shown to be genetically differentiated in time and space (e.g. Medlin et al. 2000; Nagai et al. 2007; Rynearson and Armbrust 2004; Shankle et al. 2004). However, there are little comparable data about the genetic differentiation among and within populations found in lakes. For the cyanobacterium *Microcystis*, Wilson et al. (2005) showed significant genetic differentiation among lake populations, while another study did not indicate such genetic structuring pattern (Janse et al. 2004). Beszteri et al. (2007) showed two genetically distinct populations of the diatom *Cyclotella meneghiniana*, which were separated by only 2 km. In lacustrine ciliates, high genetic diversity was found within populations, but little differentiation among them (Kusch et al. 2000; Zhang et al. 2006). It should be noted, however, that the contradictory findings of some of the previous studies could be due to different species having been considered as different populations. This concern is based on recent observations indicating that several well-known microbial morphospecies (i.e. species defined on a morphological basis) actually consist of species complexes (e.g. Bensch et al. 2004; Beszteri et al. 2007; Casamatta et al. 2003; Coleman 2001; Montresor et al. 2003a).

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

To date, the possibility that genetically distinct populations of sexually reproducing microbes can coexist within the same lake has been little explored. Overall, in the absence of barriers for sexual reproduction, the emergence of genetically differentiated populations would be prevented. In higher animals, where the structuring patterns of the genetic diversity have been studied extensively, there are a variety of examples of genetically differentiated populations coexisting within the same lake (e.g. Dynes et al. 1999; Wilson et al. 2004). In microbes, a number of studies have found ecophysiological evidence suggesting that genetically differentiated populations can coexist within one lake (see Weisse 2002; Weisse and Rammer 2006). Due to the high genetic diversity observed in some microbial populations, the presence of genetic structure within lakes is a likely scenario.

Although the intraspecific genetic diversity and its structuring patterns have been investigated in planktonic populations of lacustrine metazooplankton (e.g. De Meester et al. 2006), little is known about planktonic protists with similar reproductive strategies. Free-living protists generally alternate between asexual and sexual reproduction, but data on the frequency of sexual reproduction in the field is very rare and contradictory (reviewed by Weisse 2008). An important group of aquatic protists are the dinoflagellates, which often have a significant role in marine and freshwater communities. Some species can produce potent toxins during algal blooms, thus representing an important concern for human and ecosystem health as well as local economies (Hallegraeff 1993). Dinoflagellates have a high diversity of life strategies, including symbionts, parasites, photosynthesizers, heterotrophs, and mixotrophs (review in Hackett et al. 2004). Most lacustrine dinoflagellate species reproduce clonally by cell division during the planktonic phase, and sexually when the conditions for clonal growth are not favorable anymore (Pfiester and Anderson 1987). Normally, they are haploid during vegetative growth (Von Stosch 1973). In several dinoflagellate species, sexual reproduction is linked to the production of resting cysts which have environmental resistance and dispersal functions (see Pfiester and Anderson 1987). In lakes and ponds, the germination of cysts may restore the multi-clonal planktonic populations when favorable environmental conditions return. Nevertheless, recent evidence from laboratory studies shows that there are alternate pathways in dinoflagellate life cycles, allowing for sexual reproduction circumventing cyst production and proceeding with planktonic growth (Figueroa and Bravo 2005a, b). Hence, the frequency of sexual events in planktonic populations could be much more frequent than what has previously been assumed. To date, however, it has not been possible to measure sexual events in the field.

Here, we have investigated the amount of the genetic diversity and its structuring patterns within five dinoflagellate species (*Peridinium aciculiferum*, *Peridinium cinctum*, *Peridiniopsis borgei*, *Polarella glacialis*, *Scrippsiella* aff. *hangoei*). The studied species are present in freshwater lakes (*P. aciculiferum*, *P.cinctum*, *Ps. borgei*), saline lakes (*P. glacialis*, *S.* aff. *hangoei*), and sometimes also in marine habitats (*P. glacialis*) distributed across polar and temperate regions. Specifically we asked: (1) Do few dinoflagellate genotypes dominate the population of a lake, or are there several coexisting genotypes? (2) Do different dinoflagellate species harbor similar amounts of genetic diversity? And finally (3) does the genetic diversity within the studied species present any degree of structure? To investigate these questions, we have used the Amplified Fragment Length Polymorphisms (AFLP) technique (Vos et al. 1995) on dinoflagellate strains from five species. AFLP is a genetic fingerprinting technique that screens the genetic variation across the whole genome. This technique has proved very useful to resolve and assign strains and clones of microbes (e.g. Duim et al. 2000) and to investigate the genetic variation among very closely related species (e.g. Albertson et al. 1999; Logares et al. 2007).

In our study, all strains within each species had identical Internal Transcribed Spacer (ITS) sequences in the nuclear ribosomal DNA (nrDNA). The ITS nrDNA is a very rapidly evolving marker which is used to discriminate among species and cryptic species in dinoflagellates (see Litaker et al. 2007). The use of dinoflagellate strains with identical ITS nrDNA was intended to minimize potential biases due to cryptic species, since strains sharing the same ITS have considerable chances to belong to the same species (although there are exceptions, e.g. Logares et al. 2007). This is specially relevant since several cases of cryptic species have been reported for dinoflagellates (e.g. Gribble and Anderson 2007; Kim et al. 2004; Lilly et al. 2005; Montresor et al. 2003a). However, using only strains with identical ITS sequences restricted us in the number of samples that we could use. In addition, the distribution of some species made inter-lake comparisons unfeasible. For example, *Ps. borgei* occurs only in a few geographically-scattered lakes and we therefore focused on investigating only one lake. Conversely, we could sample species like *P. aciculiferum* and *Pa. glacialis* in a number of lakes and different environments (i.e. marine *Polarella*). Despite having variable numbers of samples for the different species, we included all of them in the analyses since this approach provides broader insight on the genetic diversity across species.

Results

AFLP

Our AFLP analyses indicated a wide heterogeneity among the studied species in their levels of intraspecific genetic diversity ($20 < \text{PPL} < 90$; $0.08 < H < 0.37$; PPL = Percentage of Polymorphic Loci; H = Nei's gene diversity; Fig. 1; Tables 1 and 2).

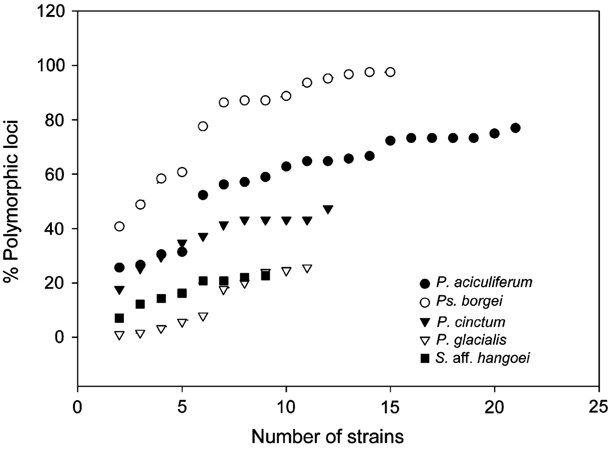


Figure 1. Percentage of polymorphic AFLP loci as a function of the number of strains (sample size).

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

Morphospecies	Isolate	Collection site	Coordinates	Elevation[a]			Genbank accession numbers	
				Salinity	(~m a.s.l.)	Isolation	SSU	ITS
Peridinium aciculiferum	PAER1 = SCCAP K0985	Lake Erken, Sweden	59°51′N, 18°36′E	0	15	1995	AY970653	AY970649
Peridinium aciculiferum	PAER2 = SCCAP K0986	Lake Erken, Sweden	59°51′N, 18°36′E	0	15	2004	EF417314	DQ022927
Peridinium aciculiferum	PAER3	Lake Erken, Sweden	59°51′N, 18°36′E	0	15	2004	—	DQ022928
Peridinium aciculiferum	PAER8	Lake Erken, Sweden	59°51′N, 18°36′E	0	15	2004	—	AY970650
Peridinium aciculiferum	PAER9	Lake Erken, Sweden	59°51′N, 18°36′E	0	15	2004	—	AY970651
Peridinium aciculiferum	PASP1	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417292
Peridinium aciculiferum	PASP2	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417293
Peridinium aciculiferum	PASP3	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417294
Peridinium aciculiferum	PASP4	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417295
Peridinium aciculiferum	PASP5	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417296
Peridinium aciculiferum	PASP6	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417297
Peridinium aciculiferum	PASP9	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417298
Peridinium aciculiferum	PASP10	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	—	EF417299
Peridinium aciculiferum	PASP11	St. Pildammen, Sweden	55°35′N, 12°59′E	0	20	2006	EF417315	EF417300
Peridinium aciculiferum	PABR1	Brodammen, Sweden	55°32′N, 12°58′E	0	20	2006	—	EF417287
Peridinium aciculiferum	PABR2 = SCCAP K0987	Brodammen, Sweden	55°32′N, 12°58′E	0	20	2006	—	EF417288
Peridinium aciculiferum	PABR3	Brodammen, Sweden	55°32′N, 12°58′E	0	20	2006	EF417313	EF417289
Peridinium aciculiferum	PABR4	Brodammen, Sweden	55°32′N, 12°58′E	0	20	2006	—	EF417290
Peridinium aciculiferum	PABR5	Brodammen, Sweden	55°32′N, 12°58′E	0	20	2006	—	EF417291
Peridinium aciculiferum	PAFI1	Lake Österträsk, Finland	60°21′N, 20°00′E	0	16	2006	—	EF417286
Peridinium aciculiferum	PATO1	Lake Tovel, Italy	46°15′N, 10°49′E	0	1178	2003	—	EF417285

Peridiniopsis borgei	PBSK-A	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2005	EF058241	EU445295[b]
Peridiniopsis borgei	PBSK-B	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2005	—	EU445296[b]
Peridiniopsis borgei	PBSK-C	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2005	—	EU445297[b]
Peridiniopsis borgei	PBSK-D	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2005	—	EU445298[b]
Peridiniopsis borgei	PBSK-E	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2005	—	EU445299[b]
Peridiniopsis borgei	PBSK06-1	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445300[b]
Peridiniopsis borgei	PBSK06-2	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445301[b]
Peridiniopsis borgei	PBSK06-3	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445302[b]
Peridiniopsis borgei	PBSK06-4	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445303[b]
Peridiniopsis borgei	PBSK06-5	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445304[b]
Peridiniopsis borgei	PBSK06-6	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445305[b]
Peridiniopsis borgei	PBSK06-7	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445306[b]
Peridiniopsis borgei	PBSK06-8	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445307[b]
Peridiniopsis borgei	PBSK06-9	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445308[b]
Peridiniopsis borgei	PBSK06-10	St. Kalkbrottsdammen, Sweden	55°31′N, 12°55′E	0.6	4	2006	—	EU445309[b]
Peridinium cinctum	PCGY1	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445310[b]
Peridinium cinctum	PCGY2	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445311[b]
Peridinium cinctum	PCGY4	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	EF058245	EU445312[b]
Peridinium cinctum	PCGY10	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445313[b]
Peridinium cinctum	PCGY11	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445314[b]
Peridinium cinctum	PCGY12	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445315[b]
Peridinium cinctum	PCGY13	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445316[b]
Peridinium cinctum	PCGY14	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445317[b]
Peridinium cinctum	PCGY17	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445318[b]
Peridinium cinctum	PCGY18	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445319[b]
Peridinium cinctum	PCGY21	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2003	—	EU445320[b]
Peridinium cinctum	PCGY06-9	Gyllebosjön, Sweden	55°36′N, 14°12′E	0.1	70	2006	—	EU445321[b]
Polarella glacialis	CCMP1383	Ross Sea, Antarctica	77°50′S, 166°30′E	32	—	1991	EF417317	EU445333[b]
Polarella glacialis	CCMP2088	Baffin Bay, Arctic	74°29′N, 78°35′W	35	—	1998	EF434275	EU445332[b]
Polarella glacialis	MBIC10563	Near Showa Island, Antarctica	69°00′S, 39°34′E	35	—	1994	—	EU445329[b]
Polarella glacialis	MBIC10564	Near Showa Island, Antarctica	69°00′S, 39°34′E	35	—	1994	—	EU445330[b]

Table 1. (*continued*)

Morphospecies	Isolate	Collection site	Coordinates	Elevation[a]			Genbank accession numbers	
				Salinity	(~m a.s.l.)	Isolation	SSU	ITS
Polarella glacialis	MBIC10565	Near Showa Island, Antarctica	69°00′S, 39°34′E	35	—	1994	—	EU445331 [b]
Polarella glacialis	PGAB-A = SCCAP K0981	Lake Abraxas, Antarctica	68°29′20″S, 78°17′13″E	18	13	2005	—	EU445323 [b]
Polarella glacialis	PGAB-C	Lake Abraxas, Antarctica	68°29′20″S, 78°17′13″E	18	13	2005	—	EU445324 [b]
Polarella glacialis	PGAB-E	Lake Abraxas, Antarctica	68°29′20″S, 78°17′13″E	18	13	2005	EF434276	EU445325 [b]
Polarella glacialis	PGEK-AH	Ekho Lake, Antarctica	68°31′16″S, 78°16′12″E	40	−2	2005	—	EU445326 [b]
Polarella glacialis	PGEK-BH	Ekho Lake, Antarctica	68°31′16″S, 78°16′12″E	40	−2	2005	EF434277	EU445327 [b]
Polarella glacialis	PGEK-EH	Ekho Lake, Antarctica	68°31′16″S, 78°16′12″E	40	−2	2005	—	EU445328 [b]
Scrippsiella aff. *hangoei*	SHHI-1	Highway Lake, Antarctica	68°27′47″S, 78°13′24″E	5	8	2005	—	EF417301
Scrippsiella aff. *hangoei*	SHHI-4 = SCCAP K0980	Highway Lake, Antarctica	68°27′47″S, 78°13′24″E	5	8	2005	—	EF417302
Scrippsiella aff. *hangoei*	SHMC-A	McNeill Lake, Antarctica	68°31′40″S, 78°21′44″E	5	26	2005	EF417318	EU445335 [b]
Scrippsiella aff. *hangoei*	SHMC-B	McNeill Lake, Antarctica	68°31′40″S, 78°21′44″E	5	26	2005	—	EU445336 [b]
Scrippsiella aff. *hangoei*	SHHA-1	Lake Hand, Antarctica	68°33′03″S, 78°18′50″E	5	9	2005	—	EU445334 [b]
Scrippsiella aff. *hangoei*	SHVE-B	Vereteno Lake, Antarctica	68°33′03″S, 78°18′50″E	5	9	2005	—	EF417306
Scrippsiella aff. *hangoei*	SHVE-C	Vereteno Lake, Antarctica	68°30′54″S, 78°24′51″E	5	0	2005	—	EF417307
Scrippsiella aff. *hangoei*	SHVE-B2	Vereteno Lake, Antarctica	68°30′54″S, 78°24′51″E	5	0	2005	—	EU445337 [b]
Scrippsiella aff. *hangoei*	SHVE-K2	Vereteno Lake, Antarctica	68°30′54″S, 78°24′51″E	5	0	2005	—	EU445338 [b]

[a] Elevation is indicated in meters above the sea level (m a.s.l.).

[b] Sequences obtained for this work.

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

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

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

For all the strains of *Peridinium aciculiferum* (freshwater lakes), *Peridiniopsis borgei* (freshwater lakes), *Peridinium cinctum* (freshwater lakes), *Polarella glacialis* (saline lakes and marine) and *Scrippsiella* aff. *hangoei* (saline lakes) the number of scored loci ranged between 106 and 175 (see Table 2). More loci were scored in the polar species (*Pa. glacialis* and *S.* aff. *hangoei*) since they initially appeared to harbor comparatively low levels of diversity. However, the scoring of more loci in these species did not modify the previous results.

Due to the high difficulty and variable success in establishing new dinoflagellate clonal-cultures with identical ITS sequences from environmental samples, we used unequal numbers of strains within each species. Therefore, the correlation between the percentages of polymorphic loci (PPL, as a proxy of genetic diversity) with the number of analyzed strains was tested. In Figure 1, the PPLs appear to increase with the number of scored strains. We have also calculated the Nei's gene diversity index (hereafter *H*; Nei 1987) within populations and species as an alternative measurement of genetic diversity. Overall, the PPL (97.6) and *H* index (0.37) within *Ps. borgei* were the highest among the analyzed species (Fig. 1; Table 2). The second species with highest PPL (77.0) was *P. aciculiferum* (Fig. 1). However, *H* was very similar in *P. aciculiferum* (*H* = 0.19) and *P. cinctum* (*H* = 0.17) (Table 2). The PPL in *P. cinctum* (47.4) was in-between the PPLs of *Ps. borgei*—*P. aciculiferum* and the polar species (Fig. 1). The polar species had comparatively low PPLs (22.7—25.7) and *H* values (0.08) (Fig. 1; Table 1).

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

In the UPGMA tree analyses of intraspecific genetic variation, some significantly supported (i.e. bootstrap values [BV] > 0.70) clusters of strains were observed. In *P. aciculiferum*, the strains clustered into two major groups, one composed of strains from Brodammen only, and another one comprising all the remaining strains (Fig. 2A). Three major clusters were identified among the analyzed strains of *Ps. borgei* from the lake St. Kalkbrottsdammen (Fig. 2B), with cluster three including strains from 2005 only. Within *P. cinctum*, no bootstrap supported clusters were identified, although two strains (PCGY06-9, PCGY11) were considerably divergent from the others (Fig. 2C). Within *Pa. glacialis*, three major clusters were identified (Fig. 3A). The first cluster groups Antarctic lacustrine strains from the lakes Ekho and Abraxas (Fig. 3A). These lacustrine strains were significantly (BV > 0.70) separated

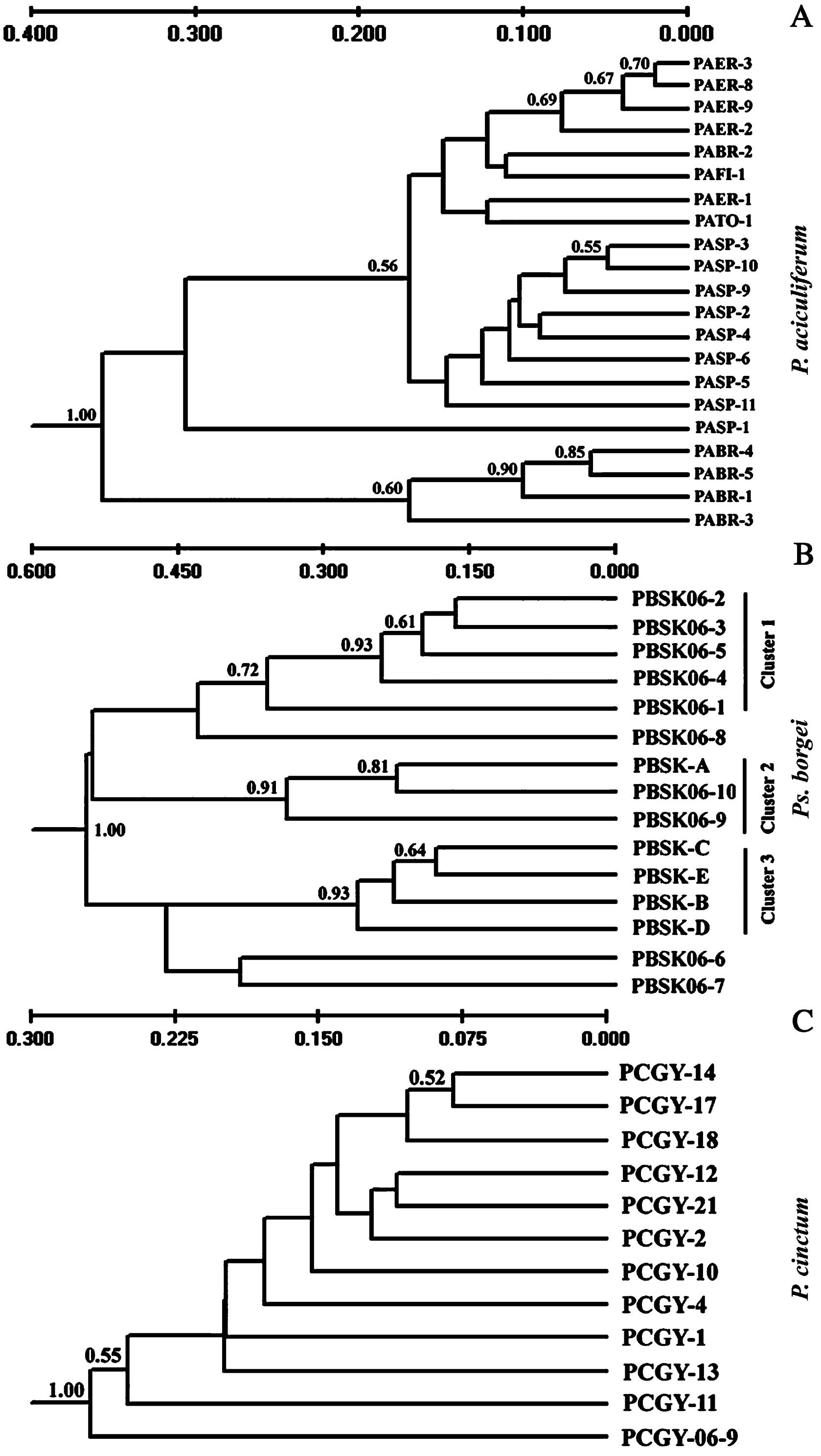


Figure 2. AFLP UPGMA trees based on Nei's distances (Nei 1972). Support values were calculated over 1000 bootstrap pseudoreplicates (values >0.50 are shown). **A** = *Peridinium aciculiferum*; **B** = *Peridiniopsis borgei*; **C** = *Peridinium cinctum*. The scale bar on the top shows approximate Nei's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

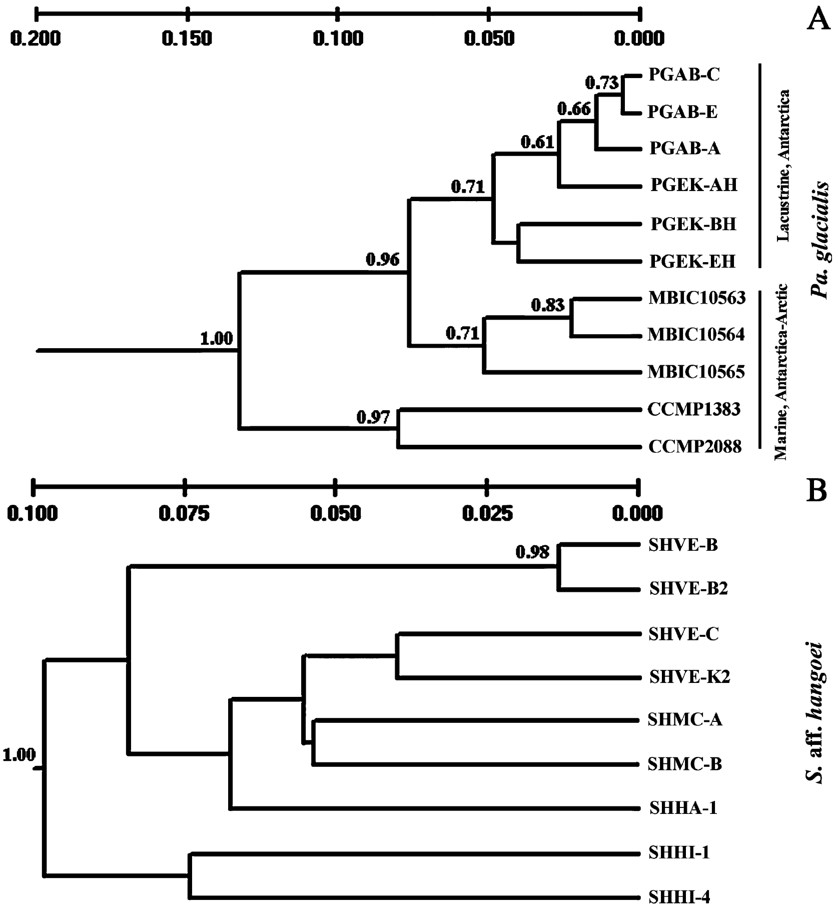
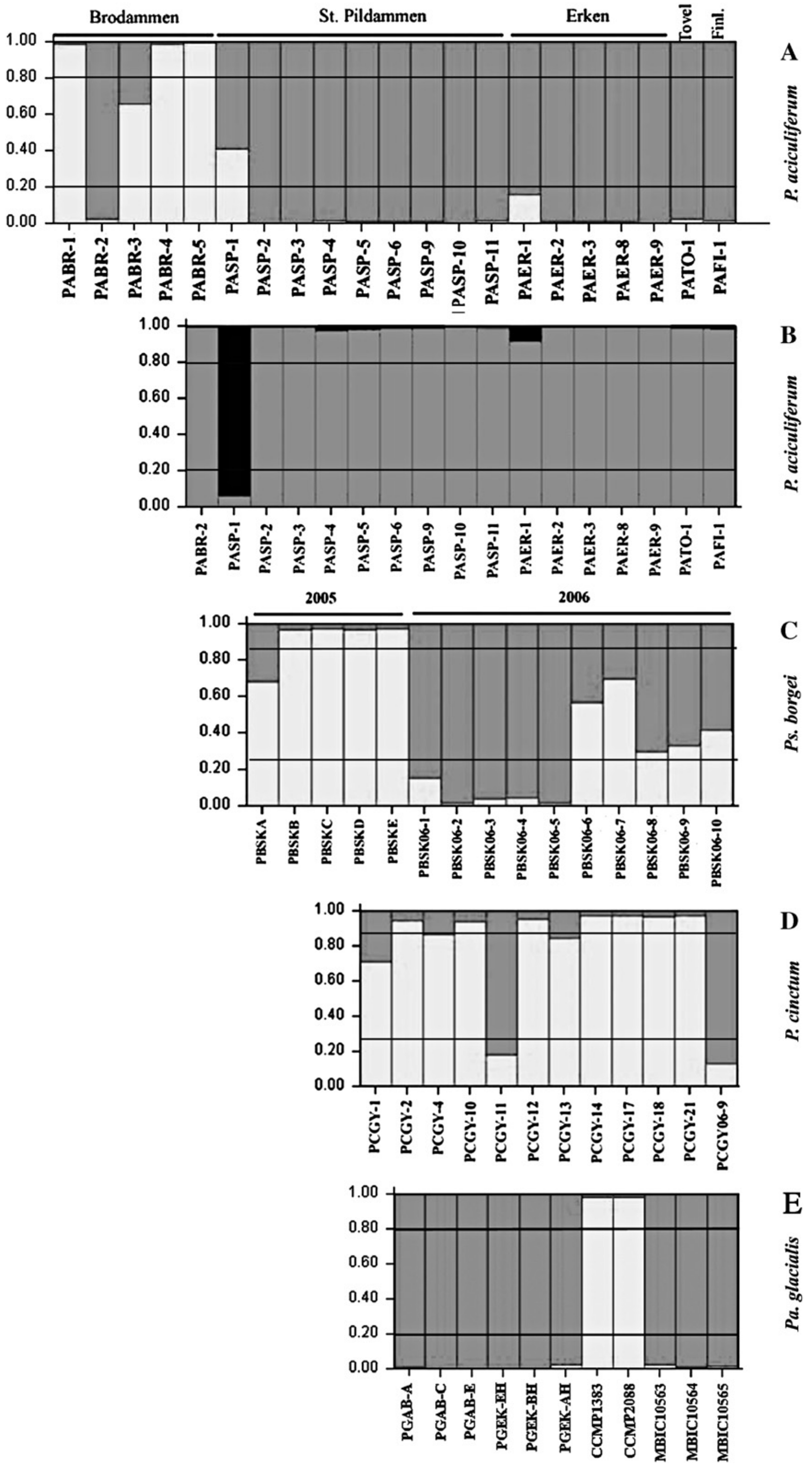


Figure 3. AFLP UPGMA trees based on Nei's distances (1972). Support values were calculated over 1000 bootstrap pseudoreplicates (values >0.50 are shown). **A** = *Polarella glacialis*; **B** = *Scrippsiella* aff. *hangoei*. The scale bar on the top shows approximate Nei's genetic distances.

from the marine strains. The second *Pa. glacialis* cluster groups marine Antarctic strains from the vicinity of Showa Island (Fig. 3A; MBIC). Interestingly, the third cluster groups the Antarctic and Arctic strains (Fig. 3A; CCMP strains). Among the lacustrine Antarctic *S.* aff. *hangoei* strains, no clear clustering patterns were found (Fig. 3B). Only two strains from Lake Vereteno formed a divergent cluster with significant support (SHVEB/B2) (Fig. 3B).

Bayesian Clustering Analyses of AFLP Diversity

The *P. aciculiferum* strains from the five sampled lakes in Europe clustered into two genetic populations (i.e. two genetically homogeneous groups independent of the geographic distribution of the strains; $K = 2$ had highest posterior probability) when considering the correlated allele frequencies model (Fig. 4A, Supplementary Fig. S1). Considering a $q_i > 0.80$ (q_i = coefficient of membership for the isolate *i*) threshold for assigning strains to the different clusters, three strains were assigned to cluster 1 (PABR1/4/5) and 16 to cluster 2 (PABR2/ PASP2/3/4/5/6/9/10/11, PAER1/2/3/8/9, PATO1, PAFI1), out of a total of 21 strains (see Fig. 4A). Only two strains were admixed (PABR3, PASP1) [$0.20 < q_i < 0.80$; their genetic composition was a mixture of the *K* inferred clusters]. Under the independent allele frequencies model, three genetic clusters gave a better explanation of the data (Supplementary Fig. S1). To test the putative subdivision of *P. aciculiferum* into three genetic clusters, we ran analyses excluding the strains PABR1/3/4/5, which were for the most part associated to cluster 1. These analyses supported the presence of a third genetic cluster, represented basically by one isolate, PASP1 (Fig. 4B). Overall, the inferred genetic populations within *P. aciculiferum* did not present any obvious correlation to the lakes (Fig. 4A, B). However, cluster one was predominantly represented by strains from the small pond Brodammen, while cluster two had



representatives in all the lakes where *P. aciculiferum* was studied (Fig. 4A).

The strains of *Ps. borgei* from Lake St. Kalkbrottsdammen were apparently structured into two genetic populations (Fig. 4C). Using a criteria of $q_i > 0.80$, four strains were assigned to cluster 1 (PBSK-B/C/D/E) and five to cluster 2 (PBSK06-1/2/3/4/5) out of a total of 15 strains (Fig. 4C). The remaining strains were admixed (PBSK-A, PBSK06-6/7/8/9/10). The independent allele frequencies model gave the highest posterior probabilities for two genetic populations ($K = 2$; Supplementary Fig. S1). All strains assigned to cluster 1 were obtained in 2005, while all strains assigned to cluster 2 were sampled in 2006.

The *P. cinctum* strains from Lake Gyllebosjön appear to belong to either the same or two genetic populations. Under the correlated allele frequencies model, the posterior probabilities were very similar for $K = 1$ and 2 ($\text{Ln}\,P[D] = -379.1$ and -377.2 respectively; Supplementary Fig. S1). Under the independent allele frequencies model, the posterior probabilities for $K = 1$ and 2 were also very similar ($\text{Ln}\,P[D] = -383.9$ and -383.1 respectively; Supplementary Fig. S1). Overall, $K = 2$ under the correlated allele frequencies model obtained the highest posterior probabilities.

Two genetic populations were indicated for *Pa. glacialis* (Fig. 4E). The independent allele frequencies models with a $K = 2$ had the highest posterior probabilities (Supplementary Fig. S2). The strains CCMP1383 (Antarctic marine) and CCMP2088 (Arctic marine) were strongly assigned to one of the genetic clusters, while the rest of the strains were strongly assigned to the other (limnic) cluster (Fig. 4E).

For *S.* aff. *hangoei*, there was no evidence for the presence of different genetic populations among our strains ($K = 1$ appears as the most likely scenario; Supplementary Fig. S2).

ITS nrDNA Sequences

The ITS nrDNA sequences from the analyzed strains of *Peridinium aciculiferum*, *Peridiniopsis borgei*, *Peridinium cinctum*, *Polarella glacialis* and *Scrippsiella* aff. *hangoei* were identical within each species (accession numbers in Table 1).

Discussion

Overall, our results suggest that a large number of dinoflagellate genotypes coexist within lake populations. A total of 68 dinoflagellate strains belonging to five species (*Peridinium aciculiferum*, *Peridinium cinctum*, *Peridiniopsis borgei*, *Polarella glacialis*, *Scrippsiella* aff. *hangoei*) were investigated by AFLP, and no identical genotypes were detected. Despite that identical genotypes will potentially be detected in larger sample sizes, our results are sufficient to contradict the contention that very few genotypes per species are present in each lake. Our study also shows that considerable amounts of genetic diversity may not be detected when using rapidly evolving neutral markers such as the ITS nrDNA.

Genetic Diversity among the Five Dinoflagellate Species

Our AFLP analyses indicated a wide variation among the studied species in their levels of intraspecific genetic diversity ($20 < \text{PPL} < 90$; $0.08 < H < 0.37$). However, the analysis of more lake populations and strains could modify these

Figure 4. Estimated membership of each of the sampled strains to the *K*-inferred genetic clusters for *P. aciculiferum* (**A**, **B**), *Ps. borgei* (**C**), *P. cinctum* (**D**) and *Pa. glacialis* (**E**) (*S.* aff. *hangoei* strains are not presented since all strains appeared to belong to the same cluster). Each strain is represented as a vertical bar, partitioned into *K* segments, which are proportional to the estimated membership to the *K* inferred clusters. **A** = Membership of the *P. aciculiferum* strains to two inferred clusters ($K = 2$; cluster 1 = light grey; cluster 2 = dark grey). The lake of origin of each strain is written above the bars. Note that genetic clusters do not necessarily coincide with lakes. **B** = Test of further population subdivision within *P. aciculiferum*. When strains predominantly belonging to cluster 1 (PABR1/3/4/5) are removed, a third genetic population is suggested (cluster 3 = black). This cluster is represented by only one isolate (PASP1). **C** = Membership of the *Ps. borgei* strains from the lake St. Kalkbrottsdammen to two inferred clusters. The year of origin of the different strains is indicated above the bars. **D** = Membership of the *P. cinctum* strains from Gyllebosjön to two inferred clusters. **E** = Membership of the *Pa. glacialis* strains to two inferred clusters. These results come from Bayesian runs of 200,000 generations, with a burn-in of 100,000. In these runs, the correlated allele frequencies model was considered in **A**, whereas the independent allele frequencies model was used in **B—E**. Codes below the bars indicate individual strains that are specified in Table 1.

estimations depending on the species. For example, analyzing more populations of *P. cinctum*, *S.* aff. *hangoei*, and *P. aciculiferum* could increase the estimations of their intraspecific genetic diversity. In *Ps. borgei*, on the other hand, the observed levels of genetic diversity were already high (~95%) within the single lake population that was studied. Therefore, the analysis of more populations will not change the conclusion that *Ps. borgei* harbors a relatively high genetic diversity as measured in percentage polymorphic loci, even if the total genetic diversity would increase with increased sampling effort. In contrast, the analysis of more *Pa. glacialis* populations will probably not change the conclusion that this species harbors a relatively low genetic diversity across its entire distributional range.

The moderate-to-high levels of intraspecific genetic diversity found in *P. aciculiferum*, *Ps. borgei* and *P. cinctum* parallel similar findings in marine microbes from several taxa (Bolch et al. 1999; Evans et al. 2005; Iglesias-Rodriguez et al. 2006; Rynearson and Armbrust 2000; Shankle et al. 2004) as well as a few analyzed freshwater species (Beszteri et al. 2007; De Bruin et al. 2004; Hayhome et al. 1987; Kusch et al. 2000; Müller et al. 2005; Wilson et al. 2005; Zhang et al. 2006). Contrastingly, the relatively low levels of genetic diversity found in the two polar species are more difficult to explain and could be due to the interplay of a variety of processes, like population expansion and extinctions. More data is needed to ascertain why they were genetically less diverse.

While in culture, it is possible that some mutations could have arisen among our strains, thus affecting the obtained values of genetic diversity (see paper 7 in Figueroa 2005). However, with the exception of PAER1, all the clonal cultures that we set-up have been growing in the laboratory for less than 3 years, thus restricting the amount of possible mutations. In addition, comparative AFLP studies in green algae indicate that the effects on the AFLP results of mutations occurring during culturing are negligible (Müller et al. 2005).

Patterns of Genetic Differentiation

Our analyses indicated that several relatively highly differentiated genotypes of *Ps. borgei* are present within the Lake St. Kalkbrottsdammen. Such relatively high differentiation among strains could be due to a rapid within-lake diversification or the immigration of several genotypes, but not due to an old in-situ divergence, since this lake is less than 60 years old (G. Cronberg, pers. com.). Our analyses also indicated the presence of different genetic populations dominating the water column during 2005 and 2006. The differential germination of cysts present in the sediments, harboring genotypes assembled in different years, could be the reason behind the occurrence of this pattern.

Both genetic and morphological data suggest that a distinct population of *P. aciculiferum* is present in the pond Brodammen. Our genetic analyses indicated a separation between most *P. aciculiferum* strains from Brodammen and the rest of the strains. This separation was also supported by phylogenetic studies using the mitochondrial gene cytochrome b (Logares et al. 2008). Interestingly, an atypical morphotype of *P. aciculiferum* was observed within Brodammen, which has only one clearly developed posterior spine (instead of four) and a slight posterior asymmetry on the antapical plate 2‴. This asymmetry appears to increase with the growth of the cells (Boltovskoy et al., unpublished data). Since the pond Brodammen is less than 15 years old, an ancient in situ diversification would not account for the observed differentiation.

No clear clustering patterns were observed in *P. cinctum* and *S.* aff *hangoei*, suggesting that all analyzed strains belonged to the same population. In contrast, several statistically supported clusters were evident in *Pa. glacialis*. Particularly, our analyses supported a clear segregation between lacustrine and marine *Pa. glacialis* strains. Our results also indicated that strains from two genetic populations were present among our samples of *Pa. glacialis*. One genetic population was constituted by all the lacustrine strains from the Vestfold Hills area as well as the marine strains from the neighborhood of the Showa Island (MBIC). These two Antarctic locations are separated by around 1900 km. The Antarctic and Arctic strains CCMP1383-2088 respectively, were assigned to the second genetic population, despite that these strains are separated by around 20,000 km. It is possible that the genetic differences between lacustrine and marine populations of *Pa. glacialis* arose in the last 6000 years, the age of the studied Antarctic lakes (Zwartz et al. 1998). The new natural selection regimes that the colonizing *Pa. glacialis* populations encountered in these lakes (especially salinity differences) could have promoted a relatively rapid genetic divergence from their marine ancestors.

What Generates the Observed Genetic Diversity Patterns?

Our results indicated variable and sometimes high levels of intraspecific genetic diversity among the studied species. Due to the large population sizes and high reproductive rates (sexual and asexual) of dinoflagellates, it is likely that a much higher number of mutations will emerge and persist in their populations at any given amount of time than in populations of multicellular organisms (see Mes 2008). The occurrence of recombination in the studied species could also partially explain the relatively high diversity of genotypes we have found. However, the considerable divergence among genotypes in some species suggests that recombination might be rare in some cases. Sexuality among dinoflagellates has been documented in many species (Pfiester and Anderson 1987) and there are reasons to believe that most dinoflagellates can reproduce sexually (Figueroa 2005). Among the dinoflagellates we have studied, sexuality has only been confirmed in *P. cinctum* (Pfiester 1975), and could possibly occur in the other studied species since resting cysts, which many times are associated to sex, have been observed in all of them (Boltovskoy 1999; Montresor et al. 2003b; Rengefors and Anderson 1998; Rengefors et al. 2008). However, there are also examples where resting cyst production is not necessarily associated to sex (Kremp and Parrow 2006).

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

In some cases (*Ps. borgei*, *P. cinctum*), strains belonging to different genetic populations were found to coexist within the same lake. The subsequent question is how the different genetic groups can coexist within one lake in the presence of recombination. Probably, some mechanisms might be preventing the homogenization of the gene pool. For instance, mechanisms of gamete recognition (Starr et al. 1995), where cells from the same genetic population tend to recombine; temporal or spatial separation of strains from different genetic populations, or selection against recombinant genotypes.

Concluding Remarks

Many unicellular eukaryotes reproduce sexually and asexually, and under stable environmental conditions, it has been predicted that populations will become dominated by a relatively few well-adapted genotypes (see Maynard Smith 1978; Williams 1975). Despite that stable environmental conditions could be potentially found in some lakes, our study indicates that they are populated by a large number of genotypes. Still, it remains to be investigated which mechanisms are involved in the maintenance and structuring of this diversity.

Methods

Collection of dinoflagellates and culturing: All dinoflagellate clonal cultures were obtained by isolating single cells from plankton samples, except when specified otherwise. The *Peridinium aciculiferum* isolates were obtained from the lakes Tovel (Italy), Stora Pildammen (Sweden), Brodammen (Sweden), Erken (Sweden) and Österträsk (Finland) (see details in Table 1) during winter/early spring (i.e. middle to end of the bloom). The *Peridinium cinctum* strains were obtained from the lake Gyllebosjön (Table 1). The strains of *Peridiniopsis borgei* were all isolated from Stora Kalkbrottsdammen (Table 1). All the mentioned lakes have been formed after the last glaciation (~15,000 years ago) and there is no boat traffic among them. However, Stora Pildammen, Brodammen and Stora Kalbrottsdammen are man-made and much more recent (i.e. less than 200 years). *P. aciculiferum* was cultured in modified Woods Hole medium (Guillard and Lorenzen 1972; MWC, 0 salinity) prepared with MilliQ water (Millipore Corp., Bedford, USA). *P. cinctum* and *Ps. borgei* were cultured in MWC medium based on sterile-filtered Gyllebosjön and Stora Kalkbrottsdammen water respectively.

In the Antarctic summer of 2004/5, dinoflagellates were isolated from different lakes in the Vestfold Hills area (68°S, 78°E), Eastern Antarctica. The Vestfold Hills is an ice-free coastal area in Princess Elizabeth Land, that was formed by isostatic uplift after the last glaciation ~6000 year ago (Zwartz et al. 1998). The lakes sampled included Lake Abraxas (brackish; *Polarella glacialis*), Ekho Lake (hypersaline; *Pa. glacialis*), Highway Lake (brackish; *Scrippsiella* aff. *hangoei*), Vereteno Lake (brackish; *S.* aff. *hangoei*), Lake Hand (brackish; *S.* aff. *hangoei*), and McNeill Lake (brackish; *S.* aff. *hangoei*) (see details in Table 1). These lakes are usually ice-free between 4 and 5 weeks each year. Plankton samples were collected through holes in the ice-cover using 10 µm plankton nets. Single cells of *Pa. glacialis* and *S.* aff. *hangoei* (identified by SEM and SSU rDNA; Rengefors et al. 2008) were isolated from the plankton samples using microcapillary pipettes. The cells were placed in f/2 medium (Guillard and Ryther 1962) based on sterile-filtered lake water. Cells were subsequently slowly adapted to f/2 based on Øre Sound seawater and grown at either full-strength (salinity ~30), half-strength or quarter-strength salinity, as an approximation of the original lake salinity. In addition to our own strains (Table 1), we used the publicly available marine *Pa. glacialis* strains. From the Provasoli-Guillard National Center for Culture of Marine Phytoplankton we obtained cultures isolated from the McMurdo Sound, Ross Sea, Antarctica (CCMP1383), and Baffin Bay, Arctic (CCMP2088) (Table 1). Freeze-dried *Pa. glacialis* cultures from the strains MBIC10563/4/5 isolated from the Antarctic sea outside Showa Island (Antarctica) were obtained from the Japanese Marine Biotechnology Institute Culture Collection. The cultures that we have isolated for this work are available on request or through the Scandinavian Culture Collection of Algae & Protozoa (SCCAP; Copenhagen, Denmark).

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

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

PCR amplifications were done using 25 ng of template genomic DNA, 0.125 mM of each nucleotide, 1.5 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. 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. All PCR amplicons were cleaned using PCR-M^{TM} Clean-Up System (Viogene, Taiwan). ITS amplicons were directly sequenced using the same PCR primers. The sequencing reaction was carried out using BigDye (v1.1, Applied Biosystems) chemistry and the products were precipitated following the manufacturer instructions and then loaded into an ABI Prism 3100 sequencer (Applied Biosystems). The obtained sequences were edited and assembled by analyzing carefully the chromatograms using BioEdit (v7.0.4.1; Hall 1999). The sequences were deposited in GenBank (accession numbers shown in Table 1).

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

The selective amplification step was carried out using four primer pairs with the strains of *P. aciculiferum*, *P. cinctum* and *Ps. borgei*: Mix 1: E_{TCG}—M_{CGG}; Mix 2: E_{TCT}—M_{CAC}; Mix 3: E_{TGA}—M_{CGC}; Mix 4: E_{TAG}—M_{CCG}. With the strains from *Pa. glacialis* and *S.* aff. *hangoei* six primer pairs were used: Mix A: E_{TGA}—M_{CGA}; Mix B: E_{TGA}—M_{CAG}; Mix C: E_{TCG}—M_{CAG}; Mix D: E_{TCG}—M_{CGA}; Mix E: E_{TAG}—M_{CAG} ; Mix F: E_{TCG}—M_{CAA}. The E-primers were labelled with fluorescein. Although different primer combinations were used for the polar species, the same AFLP protocol was used for all species. The use of different primer combinations should not affect the results, since several primer pair combinations and hundreds of loci were considered. Selective amplification reactions (10 µl final volume) included 2.5 µl of pre-amplification product, 0.04 u of Taq DNA polymerase (AmpliTaq, Applied Biosystems), 0.2 mM of dNTP, 0.6 µM of each selective primer, 2.5 mM of $MgCl_2$ and 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 of formamide dye (100% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) were added to the reactions and then products were stored overnight at 4 °C before further analysis. Subsequently, selective amplification products were denatured (3 min at 95 °C) and then 3.5 µl were loaded onto 6% polyacrylamide gels. AFLP fragments were separated using 30 W during 90 min, and detected by the fluorescein labeled E-primers in a Typhoon imaging system 9200 (Amersham Biosciences). Reproducible bands in duplicates or triplicates were scored as 1 (presence) or 0 (absence) for the surveyed loci (see Table 2). We have carefully considered the reproducibility of the AFLP banding patterns in our work. All AFLP digestions, ligations and amplifications were carried out in duplicates or triplicates from the same DNA extraction but in independent tubes from the beginning. In most cases (above 95%) the banding patterns from the replicates were congruent. In the few cases where there was ambiguity, that particular locus was either not scored or scored in comparison to the closest relatives (i.e. if all the closest relatives presented a band, and one strain lacked a band in one replicate, then the lack of that band was considered to be a protocol problem).

AFLP analyses: Analyses of AFLP polymorphism were carried out with AFLPsurv (V1.0; Vekemans 2002) using the option for haploid data. AFLPsurv was also used to calculate the Nei's gene diversity index (*H*) (Nei 1987). The program TFPGA (v1.3; Miller 1997) was used to calculate UPGMA dendrograms based on Nei's 1972 genetic distances (Nei 1972). The support for the branching pattern was calculated through 1000 bootstrap pseudoreplicates. The TFPGA program was also used to calculate the percentage of polymorphic loci (PPL).

Bayesian clustering analyses: We used the program STRUCTURE (v2.2; Pritchard et al. 2000) to estimate the number of K unknown genetic clusters (genetic populations which are characterized by a set of alleles frequencies at each locus) in which the multilocus AFLP data could be divided. STRUCTURE uses a Bayesian algorithm to estimate the most likely K and also to estimate the probability that each one of the analyzed strains belong to the inferred K clusters. One of the main assumptions when using STRUCTURE is that the markers are unlinked. We consider that our AFLP data fulfill this assumption in most cases, since the markers originate from several different regions of the genome.

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

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

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.protis.2008.10.004.

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