Microbial Ecology

Genetically Distinct Populations of the Dinoflagellate *Peridinium limbatum* in Neighboring Northern Wisconsin Lakes

E. Kim, L. Wilcox, L. Graham and J. Graham

Department of Botany, University of Wisconsin—Madison, 132 Birge Hall, 430 Lincoln Dr., Madison, WI 53706, USA

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Abstract

The extent to which free-living microorganisms exist in geographically isolated, genetically distinct populations is a subject of continuing debate. Some authorities contend that many microorganisms have cosmopolitan distributions, while others provide evidence that more limited geographical distribution of genetically distinct populations can occur. We report the occurrence of two morphologically similar, but genetically distinct, populations of the microbial eukaryote *Peridinium limbatum* (Stokes) Lemmermann from neighboring Northern Wisconsin freshwater bodies. Five strains of *P. limbatum* were cultured by single-cell isolation from both Crystal Lake and Crystal Bog (Oneida Co., WI). Genetic variation between the two populations encompassed 8.9% (mean of 35.4 of 397 nucleotides) of the nuclear ribosomal DNA internal transcribed spacer (ITS1 and ITS2) region. In contrast, 0.5% (mean of 2.25 of 397 nucleotides) variation was observed within the Crystal Lake population and 0.3% (mean of 1.21 of 397 nucleotides), within the Crystal Bog population. This difference between the two populations was highly statistically significant (p-value $<< 0.001$). The extent of genetic variation between the two *P. limbatum* populations was greater than that reported in the literature for some morphologically distinguishable microalgal species, suggesting the occurrence of cryptic sister species. On the other hand, hybrid sequences obtained from one of the Crystal Lake strains suggest that the two populations may still be members of a single sexually compatible biological species. Our data suggest that the two neighboring *P. limbatum* populations may be diverging genetically under conditions of limited gene flow, suggesting a mechanism for the origin of geographically isolated, genetically distinct populations of microbial eukaryotes.

Correspondence to: E. Kim; E-mail: eunsookim@wisc.edu

Introduction

Geographical distribution of free-living microorganisms has been the subject of recent controversy. The hypothesis that most free-living microorganisms have cosmopolitan distributions is widely accepted [2] and supported by several studies. For example, all of the 86 freshwater ciliates identified from one lake in Australia were described earlier from Northern Europe [4]. In addition, studies of free-living heterotrophic flagellates from various geographical locations (mainly marine environments), which were performed with the morphological species concept and at the generic level, also suggested cosmopolitanism [14]. High dispersal ability and abundance of free-living microorganisms have been suggested as major factors preventing geographical isolation of microbial populations [3]. The cosmopolitan hypothesis suggests that differences in microbial community composition among habitats can be explained by ecological factors. On the other hand, the occurrence of novel microorganisms at one site, but not other locations, suggests the possibility that at least some microorganisms are more locally distributed. Recent molecular population studies of thermophilic archaea and cyanobacteria from hot springs provided convincing evidence that prokaryotes could exhibit restricted geographical distributions [13, 19]. Molecular genetic data also suggest that some haptophyte microalgal species are more geographically restricted than was earlier suspected on the basis of morphological markers alone [10]. The occurrence of genetically distinct geographic types has been documented in the widespread neritic dinoflagellate *Scrippsiella trochoidea* [11].

In this study, we present evidence for the existence of genetically distinct populations of the dinoflagellate *Peridinium limbatum* (Stokes) Lemmermann in two neighboring Northern Wisconsin lakes. This study originated with the observation that initial isolates of *P.*

limbatum from the two lakes, which were indistinguishable at the light microscopic level, had different growth medium requirements, suggesting genetic divergence. In order to estimate genetic variation between the two *P. limbatum* populations, we isolated and cultured multiple strains from each freshwater body and sequenced nuclear ribosomal DNA internal transcribed spacer (ITS) and adjacent regions. We also conducted morphological comparisons at the light and scanning electron microscope levels.

Materials and Methods

Collection Sites. Crystal Bog (46.00.455 N 89.36.371 W) and Crystal Lake (46.00.165 N 89.36.734 W) are located in Oneida County, in the northern part of Wisconsin, USA. These two freshwater bodies, completely separated by ~400 m, are among the study sites for the Northern Temperate Lakes Microbial Observatory (NTLMO), which is affiliated with the Long Term Ecological Research (LTER) Program. These water bodies have been monitored by the NTLMO since 2000; water samples analyzed for the present study were collected during the summer of 2002. These two water bodies belong to the same watershed. Crystal Lake's elevation is ~2 m higher than that of Crystal Bog. Crystal Bog is smaller (0.5 ha) and shallower (mean depth: 1.7 m) than Crystal Lake (36.7 ha; 10.4 m). In addition, the two water bodies differ in water chemistry and fish abundance. Compared to Crystal Lake, Crystal Bog is slightly more acidic (pH 5.1 vs pH 6.0), is darker (Secchi depth 1.6 m vs 7.3 m), has more total P (19.2 μg/L vs 8.6 μg/L), and contains fewer fish species (1 vs 23) [12].

Isolation of Peridinium limbatum and Culture Media. Individual cells of *P. limbatum* from the two freshwater bodies were isolated with a finely drawn glass pipette [15] and cultured. At least eight culture strains were obtained from each lake population. Initial isolates of *P. limbatum* from Crystal Bog did not grow in DYIII medium [8], but did grow in a 1:1 mixture of DYIII and 0.45 μm-filtered Crystal Bog water. Isolates of *P. limbatum* from Crystal Lake were maintained in DYIII medium. All isolates were grown in a culture room at 15°C with 16-h daylength. Several isolates of *P. willei* Huitfeld-Kaas, used in phylogenetic analyses as outgroup taxa, were obtained from Crystal Lake and grown in DYIII medium.

DNA Preparation. Genomic DNA was isolated from one *P. willei* and 10 *P. limbatum* cultures (five strains of Crystal Bog *P. limbatum* and five strains of Crystal Lake *P. limbatum*), using DNeasy Plant Mini Kit (QIAGEN, Catalog No. 69104). Microcentrifuge tubes with cells were frozen in liquid nitrogen, thawed, and cells disrupted with small plastic pestles. DNA was extracted according to the manufacturer's suggested protocol.

PCR Amplification and Cloning. PCR primers were based on sequences of various dinoflagellates and designed to amplify partial 18S and full ITS1, 5.8S, and ITS2 regions. The forward primer was 5′-GTGGTGCATGGCCGTTCTTAGTT-3′ and the reverse primer was 5′-TCCTCCGCTTATTGATATGC-3′. The 30 μl reaction mixture contained 2.5 units of Takara Ex Taq (Takara Bio) 10× Ex Taq buffer, 0.2 mM of each dNTP, and 5% glycerol. PCR cyclic reactions consisted of a denaturation step at 95°C for 3 min; 30 cycles of 1 min at 95°C, 1 min at 45°C, and 1 min at 72°C; and a final 15 min at 72°C. The amplified fragments were cloned into pCR 4-TOPO vector for sequencing (Invitrogen, Carlsbad, CA).

Sequencing. Multiple bacterial clones were obtained from each *Peridinium limbatum* strain for sequencing. Cloned plasmids were isolated using the QIAquick Miniprep Kit (QIAGEN, Catalog No. 27104). Manufacturer's suggested sequencing primers (T3, T7, M13F, or M13R) were used to sequence both strands. Sequencing reactions were performed with 20 μL of reaction mixture, which included 2 μL Bigdye ver. 3.1 and 6 μL Bigdye buffer ver. 3.1. Cyclic sequencing reactions consisted of a denaturation step at 95°C for 3 min; 50 cycles of 10 s at 96°C and 4 min at 58°C; and a final 7 min at 72°C. The reaction mixtures were cleaned and sequences determined at the University of Wisconsin Biotechnology Center.

Detection of Putative Hybrid Sequences. To clarify the existence of the two putative hybrid sequences from Crystal Lake *P. limbatum* clone 9, the following experiment was performed. DNA was reextracted from a fresh culture of Crystal Lake *P. limbatum* clone 9 in case the initial DNA was contaminated. In addition to the previous primer pair, a new primer pair with a new forward primer specific for Crystal Bog type DNA (5′-CAATGTGGTGGTCAATTCTG-3′) was designed. PCR reactions were modified to have fewer cycles and longer extension time for minimizing PCR hybrid artifacts [18]. The reactions consisted of a denaturation step at 95°C for 3 min; 25 cycles of 10 s at 98°C, 30 s at 45°C, and 1.5 min at 72°C; and a final 10 min extension at 72°C. Subsequent cloning and sequencing were as above.

Genetic Variation Estimates and Phylogenetic Analysis. Sequences were visually aligned using MacClade [9]. The aligned sequences were compared using distance matrices generated from PAUP* [16]. The informative variable sites distinguishing sequences from Crystal Lake

Table 1. Strains and number of sequences obtained for the study

	Strain number	*No. of bacterial clones (full/short fragments)*
Crystal Lake *P. limbatum* strains	PLCL 1	2
	PLCL 3	5/2
	PLCL 8	3/1
	PLCL 9	3/1
	PLCL 14	2/2
	Total	15/6
Crystal Bog *P. limbatum* strains	PLCB 3	7
	PLCB 4	4
	PLCB 5	2
	PLCB 7	3
	PLCB 8	1
	Total	17
Crystal Lake *P. willei*	PWCL 3	4

and Crystal Bog were manually counted. A maximum parsimony tree was constructed from 100 independent rearrangements from random addition starting trees using the TBR branch-swapping algorithm in PAUP* [16]. Bootstrap values were calculated from fast stepwise addition with 1000 bootstrap replicates.

Light Microscopy. Cells from three clonal cultures from Crystal Lake (CL-1, CL-4, CL-9) and three clonal cultures from Crystal Bog (CB-3, CB-4, CB-7) were immobilized with 5% aqueous $NiSO_4$. More than 10 cells from each of the six samples were photographed with an Olympus digital camera and Zeiss Axioplan microscope at 400×. Mean cell lengths, widths, and length/width ratios were determined from the images and statistically tested for significant differences.

SEM. Cells of *P. limbatum* from each of the two contrasting water bodies were fixed in 2% osmium tetroxide and 4% glutaraldehyde for 30 min. Fixed cells were rinsed in phosphate buffer (pH 6.8) and dehydrated through a graded ethanol series. The cells were collected on 8 μm polycarbonate membranes (Whatman), critical point dried, mounted on specimen stubs with colloidal graphite, sputter coated with gold–palladium alloy, and imaged on a scanning electron microscope (Hitachi S-570 LaB_6 SEM).

Results

Genetic Variation between the Two P. limbatum Populations Was Detected. Table 1 shows the number of DNA sequences obtained from the Crystal Lake and Crystal Bog *P. limbatum* strains. In each of the five Crystal Lake *P. limbatum* isolates, additional shorter DNA fragments were amplified, whereas no such DNA bands were obtained from the Crystal Bog *P. limbatum* isolates. Compared to the full-length fragments, the shorter bands turned out to be missing 265 bp in the 3′ portion of 18S, the entire ITS1, and the 5′ part of 5.8S regions. However, PCR amplification with a new primer pair—the forward primer 5′-CTGARCGCTGAGCGA-ACTG-3′ (which bridges the missing region) and the same reverse primer—designed to detect the shorter fragments revealed that genomic DNA from the Crystal Bog *P. limbatum* also contained the shorter sequences.

Table 2 shows the genetic distances among sequences obtained in this study. While sequence differences in the ITS1 and ITS2 regions within each *P. limbatum* population were 0.5% (Crystal Lake) and 0.3% (Crystal Bog), the Crystal Lake and Crystal Bog sequences differed by 8.9% in the same ITS regions. This difference was significant, with p-value $<< 0.001$. Among a total of 1084 sites, 42 sites were informative in distinguishing the two *P. limbatum* populations. Though only 1.31% of the partial 18S sequences and 1.30% of the 5.8S sequences were informative sites, 8.3% of the combined ITS1 and

Table 2. Sequence variation within and between the two *P. limbatum* strains and between *P. limbatum* and *P. willei* strains

	18S (partial)	*ITS1*	*5.8S*	*ITS2*
Pairwise distances among the Crystal Lake *P. limbatum* sequences	0–7/533 (0–1.35%)	0–2/211 (0–0.95%)	0–3/154 (0–1.95%)	0–5/186 (0–2.76%)
Pairwise distances among the Crystal Bog *P. limbatum* sequences	0–7/533 (0–1.31%)	0–3/211 (0–1.42%)	0–2/154 (0–1.30%)	0–3/186 (0–1.64%)
Pairwise distances between the Crystal Lake and Crystal Bog *P. limbatum* populations	8–17/533 (1.50–3.20%)	18–21/211 (8.53–9.95%)	2–5/154 (1.30–3.25%)	16–21/186 (8.74–11.09%)
Pairwise distances between the *P. limbatum* and *P. willei* sequences	20–28/533 (3.78–5.25%)	116–119/211 (55.15–56.61%)	21–26/154 (13.64–16.88%)	81–87/189 (43.17–46.35%)
Variable sites distinguishing the Crystal Lake and Crystal Bog *P. limbatum* sequences	7/533 (1.31%)	18/211 (8.53%)	2/154 (1.30%)	15/186 (8.06%)
Variable sites distinguishing the *P. limbatum* and *P. willei* sequences	15/533 (2.81%)	112/217 (51.61%)	21/155 (13.55%)	74/189 (39.15%)

Uncorrected ("p") substitution model was used to estimate genetic distances. There was a highly significant difference (p-value $<< 0.001$) in the ITS regions distinguishing the typical Crystal Bog and Crystal Lake sequences.

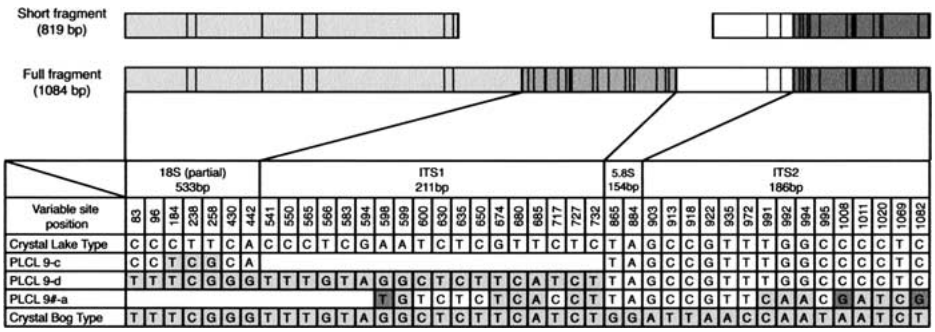

Figure 1. Variable sites distinguishing the two *P. limbatum* strains. The relative positions of the informative sites, 24 for short fragments and 42 for full fragments, are shown. Putative hybrid sequences PLCL 9-c, 9-d, and 9#-a had Crystal Bog-type sites as well as Crystal Lake-type sites. While PLCL 9-d had Crystal Bog-type sites in 18S and ITS1 regions, PLCL 9-c had only three Crystal Bog-type sites among the seven informative sites in the 18S region. PLCL 9#-a showed a more complex pattern of hybridization between Crystal Lake and Crystal Bog sequences. Unshaded positions indicate Crystal Lake-type and lightly shaded positions, Crystal Bog-type. Three sites in PLCL 9#-a (darkly shaded) differed from those in typical Crystal Lake or Crystal Bog-type sequences.

ITS2 sequences was able to distinguish the two populations (Table 2 and Fig. 1), reflecting the fact that ITS1 and ITS2 regions evolve much faster than 18S and 5.8S genes. Sequence variation between the *P. limbatum* and the *P. wiliei* sequences was over 55% in ITS1 and 43% in ITS2 (Table 2).

A strict consensus maximum parsimony tree from 42 sequences is shown in Fig. 2. Putative hybrid sequences (PLCL 9-c and 9-d) were excluded from this analysis. Sequences from the Crystal Lake *P. limbatum* strains were clearly distinguished from the Crystal Bog *P. limbatum* strain sequences with ≥ 90% bootstrap support. Sequences from the two populations were also separated using the 18S partial sequences alone, with more than 85% bootstrap support (not shown here).

Putative Hybrid Sequences from One of the Crystal Lake P. limbatum Strains. Four sequences were initially obtained from the Crystal Lake *P. limbatum* strains no. 9. Among them, two sequences were similar to sequences from the four other Crystal Lake *P. limbatum* strains, but the other two appeared to be hybrids between the Crystal Lake–type and Crystal Bog–type sequences (Fig 1). The 42 informative sites distinguishing the two *P. limbatum* populations were used to determine whether particular sequences were Crystal Lake–type or Crystal Bog–type. One of the two hybrid sequences (PLCL 9-d) had Crystal Bog–type 18S and ITS1 sequences with Crystal Lake–type 5.8S and ITS2 sequences. The other hybrid sequence (PLCL 9-c) was a short sequence missing 265 bp and had Crystal Lake-type 5.8S and ITS2 sequences. In the 18S sequences of the short hybrid, three informative sites were Crystal Bog–type while four informative sites were Crystal Lake–type.

A subsequent experiment was performed using a new primer set to amplify a part of the hybrid sequence PLCL 9-d. The amplified segment was ~600 bp, spanning the

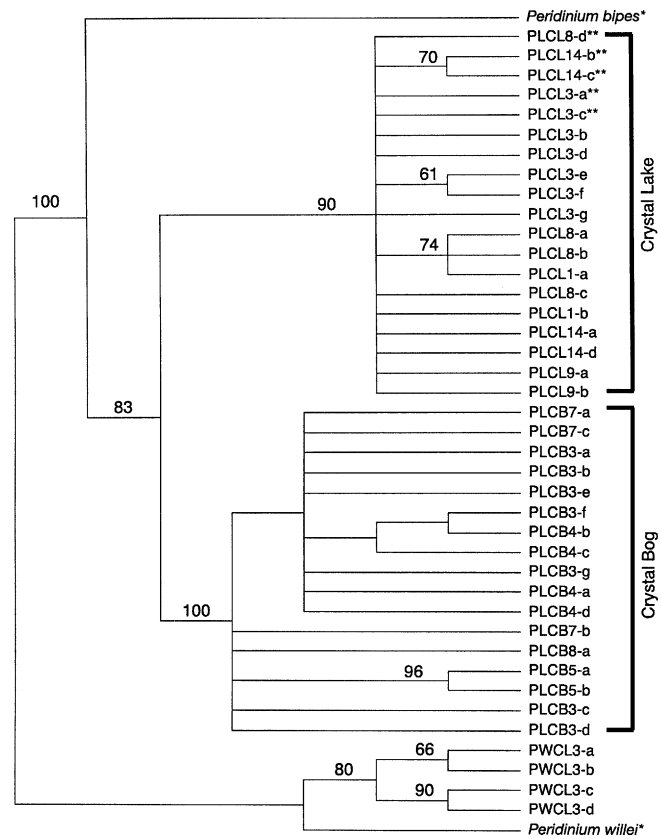

Figure 2. A maximum parsimony consensus tree was constructed from ribosomal DNA sequences. All characters were equally weighted. Among 1094 characters, 768 characters were constant and 277 characters were informative. Bootstrap values > 50 are shown on branches. Crystal Lake and Crystal Bog sequences were separated with ≥90% bootstrap values. Sequences with asterisks were obtained from GenBank (*P. bipes*—AF231805, *P. willei*—AF274272). Short sequences are indicated with double asterisks. These sequences contain only the 18S genes, not ITS1, 5.8S, or ITS2 genes. PL: *Peridinium limbatum*; PW: *Peridinium willei*; CL: Crystal Lake; CB: Crystal Bog.

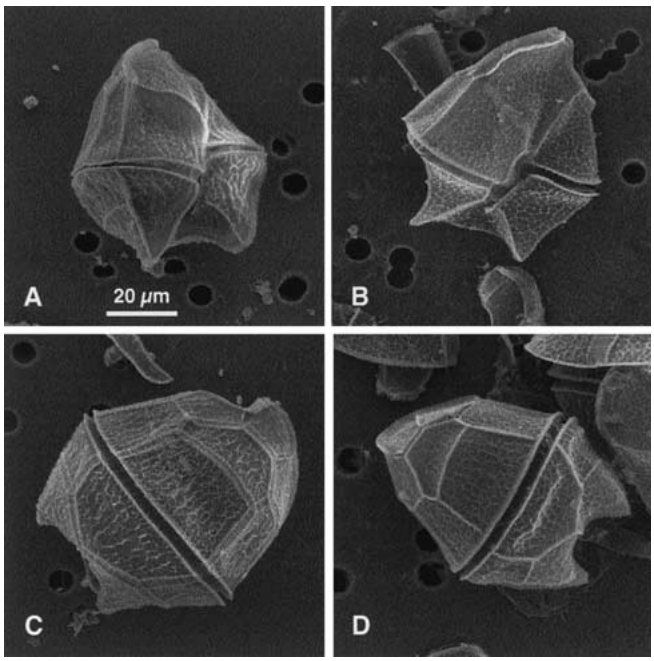

Figure 3. SEM of *P. limbatum*. (A) Ventral and (C) dorsal views of Crystal Lake *P. limbatum*. (B) Ventral and (D) dorsal views of Crystal Bog *P. limbatum*. Cells of both populations of *P. limbatum* had the same thecal plate pattern. Consistent morphological differences between the populations were not detected.

ITS1, 5.8S, and ITS2 regions. Of the eight sequences obtained, five were Crystal Lake–type despite two mismatches at the 3′ end of the forward primer. Of the three remaining sequences, one was Crystal Bog–type and the other two were identical to each other and appeared to be a Crystal Bog/Crystal Lake hybrid (PLCL 9#-a). This result suggests that in Crystal Lake *P. limbatum* clone 9, there are at least some copies of the Crystal Bog-type rDNA sequence in the genome. Eight new sequences were also obtained from reextracted PLCL 9 DNA using the initial primer set. All were Crystal Lake–type, suggesting that Crystal Lake–type sequences in the amplified region are more abundant than either hybrids or Crystal Bog–type sequences.

Consistent Morphological Differences Were Not Observed between the Two P. limbatum Populations in the LM and SEM Studies. SEM revealed that thecal plate patterns of *P. limbatum*, an important character in dinoflagellate systematics [6, 7], were the same for Crystal Bog and Crystal Lake strains (Fig. 3). Although slight differences in mean length and width occurred among clones, when the data for the three lake clones were pooled and compared with pooled data for the three bog clones, no significant differences were detected (Table 3). However, there was a significant difference (p-value < 0.001) in length to width ratios (Table 3). Light microscopic images did not reveal consistent differences in cell morphologies (Fig. 4).

Discussion

The two morphologically similar populations of *P. limbatum* that we cultured from neighboring water bodies were genetically unique in their fast evolving ITS regions. Similarly, some morphologically similar strains of *Scrippsiella trochoidea* also had distinct ITS sequences [11]. Our study originated with the observation that initial isolates of *P. limbatum* from Crystal Bog had different growth medium requirements than initial isolates from Crystal Lake, suggesting that there might be physiological differences between the two *P. limbatum* populations. Our isolates from Crystal Bog were initially unable to grow in DYIII medium, whereas new Crystal Lake isolates were able to grow in DYIII medium. Since isolates from Crystal Bog grew when we added filtered Crystal Bog water to DYIII medium, we speculate that certain trace elements present in the bog water (but not in lake water) were required for the growth of new isolates from Crystal Bog, but not those from Crystal Lake. Seasonal dynamics of *P. limbatum* also differed between Crystal Lake and Crystal Bog *P. limbatum*. A summer bloom of *P. limbatum* consistently occurred in Crystal Bog, strongly correlated with an extreme drop in bacterial diversity [5]. In contrast, *P. limbatum* in Crystal Lake did not form conspicuous blooms during summer. While this difference in population dynamics may have been

Table 3. Light microscopic measurements

	Mean length (μm)	*Mean width* (μm)	*Mean L/W*
PLCL clone 1	68.4	64.2	1.066
PLCL clone 4	70	64.2	1.094
PLCL clone 9	71.8	66.3	1.081
PLCL summary	70.1 (s = 4.65)	64.9 (s = 4.23)	1.081 (s = 0.0468)
PLCB clone 3	63.5	60.2	1.056
PLCBclone 4	69.3	67.3	1.033
PLCB clone 7	70.3	67.6	1.040
PLCB summary	67.7 (s = 5.42)	65.0 (s = 6.03)	1.043 (s = 0.042)

There were no significant differences in mean lengths and widths between the Crystal Bog and Crystal Lake clones ($p > 0.05$). However, the L/W ratios were different (p-value < 0.01).

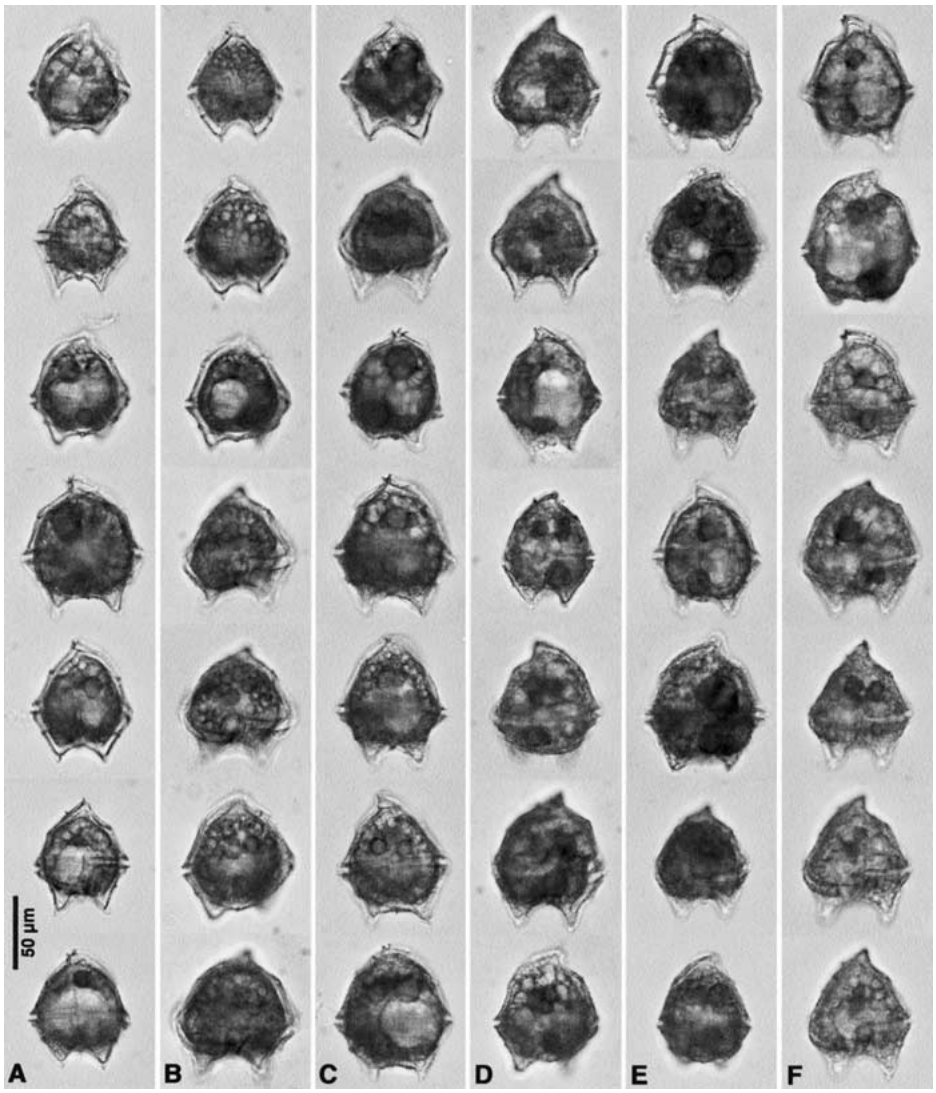

Figure 4. Light microscopic images of cultured *P. limbatum* cells. (A) PLCB 3, (B) PLCB 4, (C) PLCB 7, (D) PLCL 1, (E) PLCL 4, and (F) PLCL 9. Consistent morphological differences distinguishing Crystal Lake and Crystal Bog *P. limbatum* are not obvious.

influenced by differing aspects of lake versus bog environment, it also suggested the possibility of genetic divergence.

Genetic comparison between the two *P. limbatum* populations—molecular sequencing of ribosomal DNA sequences—did indeed reveal differences. First, there were differences in PCR amplification between the two populations. Additional DNA fragments, 256 bp smaller than the expected size, were consistently amplified from the Crystal Lake *P. limbatum* strains. Though shorter fragments occurred in the Crystal Bog *P. limbatum* strains, these were not visibly amplified. One possible explanation is that the ratio of shorter fragments to full fragments is lower in the Crystal Bog strains compared to the Crystal Lake strains, leading to a preferential amplification of full fragments. Second, sequence comparisons showed that there were 42 informative sites among a total of 1084 sites that distinguished the two populations. 33 informative sites were dispersed in ITS1 and ITS2 regions, accounting for 8.3% of the total sequence. Pairwise distances between sequences from the two populations in ITS1 and ITS2 regions ranged between 8.53% and 11.09%. This is greater than the genetic variation between two closely related species of the green alga *Scenedesmus*—about 4% variation in the ITS2 region—according to a previous study by Van Hannen et al. [17].

Two putative hybrid sequences were initially obtained from Crystal Lake clone no. 9. Our concern was a possible DNA contamination during PCR mixture preparation, which could have led to the formation of PCR hybrids. To eliminate this possibility, we reextracted DNA from a fresh culture and this fresh DNA was used for the subsequent molecular work. Crystal Bog–type and Crystal Bog/Lake hybrid sequences were obtained confirming that Crystal Lake clone 9 contains Crystal Bog-type sequences. One might suspect cross contamination between cultures, but each culture has been very carefully handled and we believe that cross contamination is unlikely. Even

if contamination had occurred, it is still hard to explain the putative hybrid sequence PLCL 9#-a. Figure 1 is a graphical representation of the three putative hybrid sequences. If PCR hybridization alone led to the formation of these sequences, there should be at least eight incidents of DNA template recombination for PLCL 9#-a, two for PLCL 9-c, and one for PLCL 9-d. In addition, in PLCL 9#-a, PCR hybridization would require DNA extension from a mismatched 3′-end, between position 599 and 600 and between 991 and 992. This evidence suggests that Crystal Lake *P. limbatum* clone 9 contains at least fragments of Crystal Bog–type rDNA sequences.

The extent of genetic variation between the two *P. limbatum* populations revealed in our study suggests possible emergence of sibling species, two reproductively isolated "biological species." However, the presence of hybrid sequences together with similar morphology would instead suggest that the two populations may be still reproductively compatible. The hybrid sequences may have originated from sexual recombination between the two populations and rapid biased concerted evolution of ribosomal DNA sequences toward the Crystal Lake-type, similar to rapid biased concerted evolution of ITS sequences in artificial hybrids of *Armeria* [1]. Sexual exchange has also been invoked to explain the occurrence of multiple ITS types in single cells of the dinoflagellate *Scrippsiella trochoidea* [11].

We conclude that these two *P. limbatum* are genetically distinct populations of the same morphological/biological species, which is diverging. We speculate that a single population, having become separated geographically, may now be experiencing limited genetic flow. Each physically separated population is now able to accumulate distinctive mutations. Bouts of hybridization occurring during early stages of speciation have also been proposed to explain ITS patterns in populations of *Scrippsiella* [11]. Studies of *P. limbatum* from additional geographical regions would shed more light on this issue. Our study adds to a growing body of data suggesting that previously recognized morphospecies may actually have geographically patchy distributions when studied at the molecular level.

Acknowledgments

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