

Phylogenetic relations of the dinoflagellate *Gymnodinium baicalense* from Lake Baikal

Communication

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Abstract: Freshwater dinoflagellates still remain poorly studied by modern biological methods. This lack of knowledge prevents us from understanding the evolution and colonization patterns of these ecologically important protists. *Gymnodinium baicalense* is the most abundant, and possibly endemic, planktonic dinoflagellate from the ancient Lake Baikal. This dinoflagellate species blooms in the spring under the ice. This study analyzed the origin of this Baikalian dinoflagellate using three markers (two ribosomal and one mitochondrial DNA). It was found that this species is a true member of the order *Gymnodiniales* and has close relatives in the glacial melt waters of the Arctic Ocean. It seems that *G. baicalense* has diversified relatively recently from the arctic marine gymnodinioids. These results shed light on dinoflagellate biogeography and their colonizations in Lake Baikal biodiversity hotspot.

Keywords: Protists • Baikal • Biogeography • Evolution • 18S rRNA gene • ITS-2 • COI

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1. Introduction

Dinoflagellates are widespread aquatic protists. They occupy various ecological niches as photosynthetic, heterotrophic, or mixotrophic organisms. Some dinoflagellates are symbionts or parasites of other organisms. Many planktonic dinoflagellate species form seasonal blooms, and some marine species are toxic for humans [1]. Freshwater dinoflagellates are less studied than marine dinoflagellates; there are more than 1700 described species of free-living marine dinoflagellates and only about 220 described freshwater species [2]. Moreover, many of the known freshwater dinoflagellates have been described before electron microscopy and molecular genetic methods became available. Thus, identifying and comparing freshwater dinoflagellates from different groups and geographic locations is challenging. This lack of knowledge precludes an understanding of the evolution and colonization patterns of these protists.

Today there are two opposing opinions about the distribution of free-living microorganisms, including dinoflagellates. Some scientists claim that protists live in all environments that they can exploit [3], while others suggest the existence of endemic microorganisms is due to natural selection and geographical barriers (the moderate endemism hypothesis) [4]. In order to reject either of these two hypotheses, there is a need for a more comprehensive description of the taxonomy and distribution of these protists. Thus, data about poorly described or unknown species are needed. Also, prior cases of dinoflagellate endemism should be revisited using modern methods. For example, Gómez [5] argued that many endemic dinoflagellates species from the Mediterranean Sea were poorly described and that they were actually cosmopolitan species. On the contrary, endemic species may not be detected due to the lack of resolution of the morphospecies concept (based on light microscopy data) and the use of taxonomic literature based on European species. Molecular genetic

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information about such dinoflagellates is therefore highly desirable, since unicellular organisms are often difficult to distinguish solely by morphological methods [6].

Lake Baikal, the deepest (1637 meters) and oldest (about 27 million years) lake in the world [7], is a unique, low-mineralized freshwater reservoir. This is one of the world's biodiversity hotspots with more than 2500 described animal species, approximately 1000 plant species, and many species still unidentified. The main planktonic dinoflagellate bloom in the lake occurs between late February and May. These dinoflagellates may attain similar biomass levels as diatoms, which usually dominate the spring Baikalian plankton [8]. Nevertheless, Baikalian dinoflagellates remain poorly studied, despite a relatively high species/genetic diversity [9].

The most abundant planktonic Baikalian dinoflagellate species belongs to an unarmored, photosynthetic species. This species blooms under the ice from late February and disappears immediately after ice-out in May. In high-crop years, the number of cells per liter may reach 3×10^6 in the 0-50 meter layer [8]. In 1955, Antipova described this species as an endemic dinoflagellate, *Gymnodinium baicalense* [10]. Since this publication (available only in Russian), the taxonomic position of *G. baicalense* has not been discussed. At present, the affiliation of this dinoflagellate within the *Gymnodiniales* order is questionable because this group of gymnodinioids has been shown to be polyphyletic [11]. Also, endemism among protists is rare, so more evidence is needed to verify that *G. baicalense* is endemic to Lake Baikal.

The aim of the present study was to characterize the phylogenetic position of *Gymnodinium baicalense* among other known dinoflagellates. Three DNA markers were analyzed to address the position of this species.

2. Experimental Procedures

2.1 Sample collection

Gymnodinium baicalense is the first species that appears in the spring season, thus, it can easily be distinguished from other Baikalian dinoflagellates.

A sample of Baikalian plankton was collected with a plankton net (mesh size, 20 μ m) at the surface water layer under the ice in the region of Listvyanka village (south part of Lake Baikal) on March 9, 2008. According to microscopic observations, the sample contained many *G. baicalense* cells (identified using the Antipova description [10]) and just a few cells of another dinoflagellate species (*Gyrodinium helveticum*). A preliminary molecular genetic analysis of the sample revealed eight identical 710 bp 18S rDNA fragments of a *Gymnodinium*-like dinoflagellate and one 18S rDNA fragment of *Gyrodinium helveticum* [12].

2.2 DNA extraction, PCR amplification and sequencing

Total DNA was extracted using the DNA sorb B kit for DNA extraction from the plankton sample (InterLab Service, Russia). The PCR amplifications were performed with dinoflagellate specific oligonucleotide primers (Table 1) and reagents from Amplisens (Russia) on a MJ Research Peltier Thermal Cycler 220. PCR products from three different DNA regions (18S, ITS-2, COI) were analyzed by electrophoresis and were cloned into InstAclone PCR Cloning Kit (Fermentas, EU). Inserts were amplified using forward and reverse primers and sequenced on an automated CEQ 8800 sequencer (Beckman Coulter, USA). For the 18S rDNA sequence, two internal primers (5'-GGGCATCACAGACCTG-3' and 5'-TGATCCTTCTGCAGGTT-3') were also used. Sequences obtained in the present study were deposited in GenBank (Accession Numbers HQ270472 – HQ270474, FJ024302 – FJ024304).

2.3 Phylogenetic analyses

Sequences were manually edited using BioEdit 7.0.5.2 [13]. The analyzed 18S rDNA and COI fragments were aligned using BioEdit 7.0.5.2 [13] and Gblock [14]. The hypervariable fragments containing partial 5.8S rDNA, 28S rDNA and complete second internal transcribed spacer (ITS2) were aligned with Mafft 6.240 [15] using model L-INS-I and then manually verified.

Phylogenetic trees were constructed using Bayesian (BI) and Maximum-Likelihood (ML) analyses. The

Origin	Forward, 5' to 3'	Reverse, 5' to 3'	T _a , °C
18S RNA gene	TTGGCCTACCGTGGCAATGAC	GTTACGGAGTTCTGCAATTCAC	60
ITS2-5.8S-28S RNA gene	GTGAATTGCAGAACTCCGTGAAC	ATCCCTGTTCAATCGCCATTACT	52
COI mitochondrial gene	GAAAACCAGAACTTCTATAATG	CGGTAAGGATATCAAGATTG	51

Table 1. Dinoflagellate specific primers.

Bayesian information criterion (BIC) implemented in jModeltest 0.1 [16] was used to select the substitution models for the analyses. The General Time Reversible (GTR) model of nucleotide substitution, with Gamma (G) distributed rates across sites and a proportion of invariable sites (I) (GTR+G+I) was chosen for the 18S rDNA datasets, while the GTR+G model was used for CO1 dataset. The Hasegawa–Kishino–Yano (HKY) model with Gamma distributed rates across sites and a proportion of invariable sites (HKY+G+I) was chosen for the 5.8S-ITS2-28S rDNA datasets. In addition, the Covarion Model was used for BI analyses performed with MrBayes-3.1.2 [17]. The BI MCMC analysis for 18S rDNA was run with seven Markov chains (six heated chains, one cold) for 7×10^6 generations with 2 independent runs for each analysis. Trees were sampled every 100th generation with the first 30,000 samples discarded as “burn-in”. The BI MCMC analyses for 5.8S-ITS2-28S rDNA fragments and COI fragments were run with five Markov chains (four heated chains, one cold) for 4×10^6 generations with 2 independent runs for each analysis. Trees were sampled every 100th generation with the first 10,000 samples discarded as “burn-in”. The average standard deviation of split frequencies and convergence diagnostics for the posterior probabilities of bipartitions were used in all cases to check for convergence. The ML analyses were conducted first in the Phyml and then in the morePhyML online programs to obtain accurate phylogenetic trees (<http://mobyle.pasteur.fr/cgi-bin/portal.py> [18]). The reliability of internal branches was assessed using the non-parametric bootstrap method with 1000 replicates. The trees were visualized in FigTree (<http://tree.bio.ed.ac.uk/>).

3. Results

3.1 Sequences

Five clones with an insert of the 18S rDNA sequences (1553 nucleotides) were sequenced. These sequences were identical to each other with the exception of one substitution in the V8 domain of one sequence. Two clones were sequenced and were found to carry identical copies of the mitochondrial cytochrome oxidase I gene (COI, 759 nucleotides). Nine clones for the third marker (second internal transcribed spacer (ITS-2, 259 nucleotides), part of 5.8S (87 nucleotides) and 28S rDNA (31 nucleotides)) were sequenced, and three of them had a single substitution. All distinct DNA sequences were used in the following analyses.

A BLAST search was conducted on the new sequences to find related sequences in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>).

In addition, known sequences that belong to different gymnodinioids were included into the analyses. The final 18S rDNA alignment contained 48 sequences and 1398 positions, the COI alignment contained 40 sequences and 486 positions, and the ITS2 alignment contained 21 sequences and 273 positions. Both the ML and BI methods resulted in similar topologies of phylogenetic trees based on each of the three markers (Figures 1, 2, 3).

3.2 Phylogenetic relations based on partial 18S rDNA data

The most informative data on phylogenetic relations of *G. baicalense* were provided by the 18S rDNA marker, because the 18S rDNA gene has been sequenced for many dinoflagellate species and for various environmental samples. The Baikalian 18S rDNA sequences formed one clade with members of the *Gymnodiniales* order (BI 1; ML 93, Figure 1) including the following families: *Gymnodiniaceae*, *Polykrikaceae* and *Warnowiaceae*. Within this clade *G. baicalense* sequences were similar to the 18S rDNA fragment from the *Gymnodiniaceae* family (*G. aureolum*), but this had low statistical support (Figure 1). Members of the *Gymnodiniaceae* did not form one group within the *Gymnodiniales* clade but were split into three groups. This pattern may be explained by a lack of 18S rDNA sequences of freshwater gymnodinioids, thus, the *Gymnodiniaceae* cluster is unstable. For example, two sequences of *Gymnodinium* sp. from Lake Tovel grouped with *G. aureolum* and the Baikalian cluster on the BI tree, but were situated separately on the ML tree (Figure 1). The closest relative to *G. baicalense* on the 18S rDNA tree is a eukaryote from the Austre Brøggerbreen ice, Arctic ocean, Norway (BI 1; ML 93, Figure 1). The DNA sequence for this arctic dinoflagellate had only two substitutions when compared with *G. baicalense*. Thus, the *p*-distance between these two DNA sequences was only 0.1% (SD 0.002), though this current analysis found that a *p*-distance of up to 3.7% (SD 0.005) is typical for other dinoflagellate species from the family *Gymnodiniaceae*.

3.3 Phylogenetic relations based on ITS-2 data

The ITS-2 region has been extensively studied in dinoflagellates and has successfully been used as a molecular marker for dinoflagellate phylogenetics at the species level. It has been shown that a 4% difference in the ITS is usually sufficient to delineate species of free-living dinoflagellates [19]. The Baikalian ITS-2 sequences were clearly distinct from corresponding sequences of known dinoflagellates: the *p*-distances between the Baikalian samples and other dinoflagellates were greater

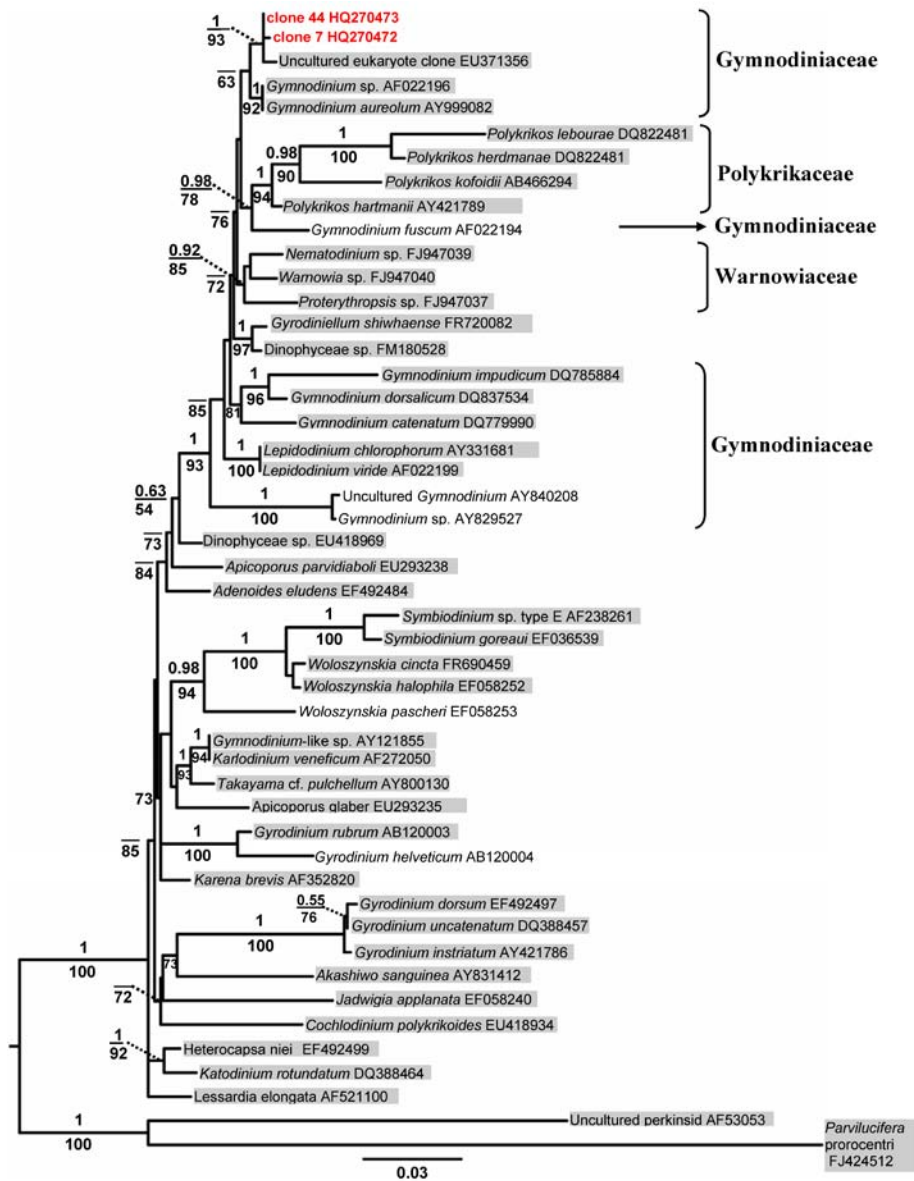


Figure 1. Phylogenetic analysis of the dinoflagellate 18S rDNA sequences. The tree shown is the ML tree. Values above vertical lines are BI posterior probabilities, values below vertical lines are bootstrap support for the ML analyses, (<50 are not shown). Rooted at midpoint. Sequences of marine and brackish water species are on grey background, Baikalian sequences are red colored.

than 13%, with two exceptions. Sequences of the marine *G. aureolum* and *Gymnodinium* sp. differ from those of *G. baicalense* by 4.4% (SD 0.012) and 5.5% (SD 0.016), respectively. *Gymnodinium baicalense* forms one clade with marine members of the genera *Gymnodinium* and *Lepidodinium* from the *Gymnodiniaceae* family on the ITS-2 tree with high statistical support (BI 1; ML 100, Figure 2). Similar to the 18S rDNA sequences, the ITS-2 sequence of the marine *G. aureolum* was the most similar to the Baikalian sequences (BI 98; ML 86, Figure 2).

3.4 Phylogenetic relations based on partial COI gene data

The phylogenetic analysis of the COI gene placed the Baikalian sequence, with sufficient statistical support (BI 1; ML 78, Figure 3), within the clade including members of the *Gymnodiniaceae* family: the marine species *Gymnodinium catenatum*, *G. impudicum* and *Lepidodinium chlorophorum*. The latter species is known to be phylogenetically close to the *Gymnodinium* genus [20]. The phylogenetic analysis of the COI amino acid sequences showed the same tree topology. To date, only

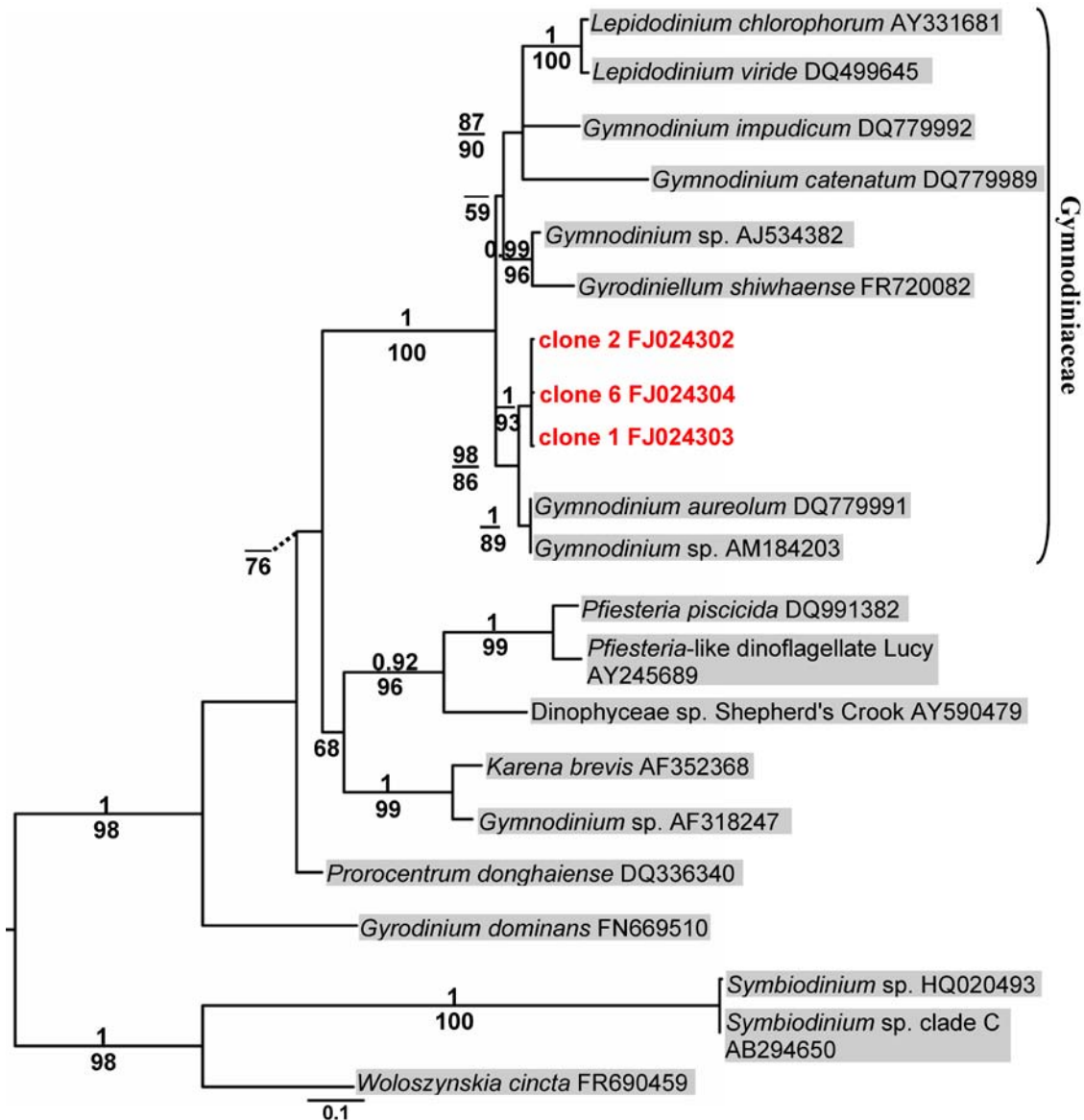


Figure 2. Phylogenetic analysis of the dinoflagellate ITS-2 sequences. The tree shown is the ML tree. Values above vertical lines are BI posterior probabilities, values below vertical lines are bootstrap support for the ML analyses. (<50 are not shown). Rooted at midpoint. Sequences of marine and brackish water species are on grey background, Baikalian sequences are red colored.

a few dinoflagellate COI genes have been sequenced, so it is still impossible to identify close relatives of the Baikalian species using this marker. Currently this gene is being actively studied for various dinoflagellates due to its use for barcoding [21], and in the future will become more useful for the determination of phylogenetic relationships.

4. Discussion

According to the Antipova description [10], atecal cells of *Gymnodinium baicalense* and its variation *G. baicalense*

var *minor* are oval, 28-90 µm long and 16-32 µm width, with a slightly compressed ventral side. The nucleus is in the middle of the cell, the chloroplasts are golden-brown, and the eyespot is absent. Dinoflagellates from the studied sample had the same features, though there were no cells longer than 60 µm.

The results obtained from this study allow us to make two main conclusions about this species. First, all molecular phylogenetic analyses showed that *Gymnodinium baicalense* clustered with true members of the *Gymnodiniales*, thus, it belongs to this taxonomical group. Historically, *Gymnodiniales* is a polyphyletic order,

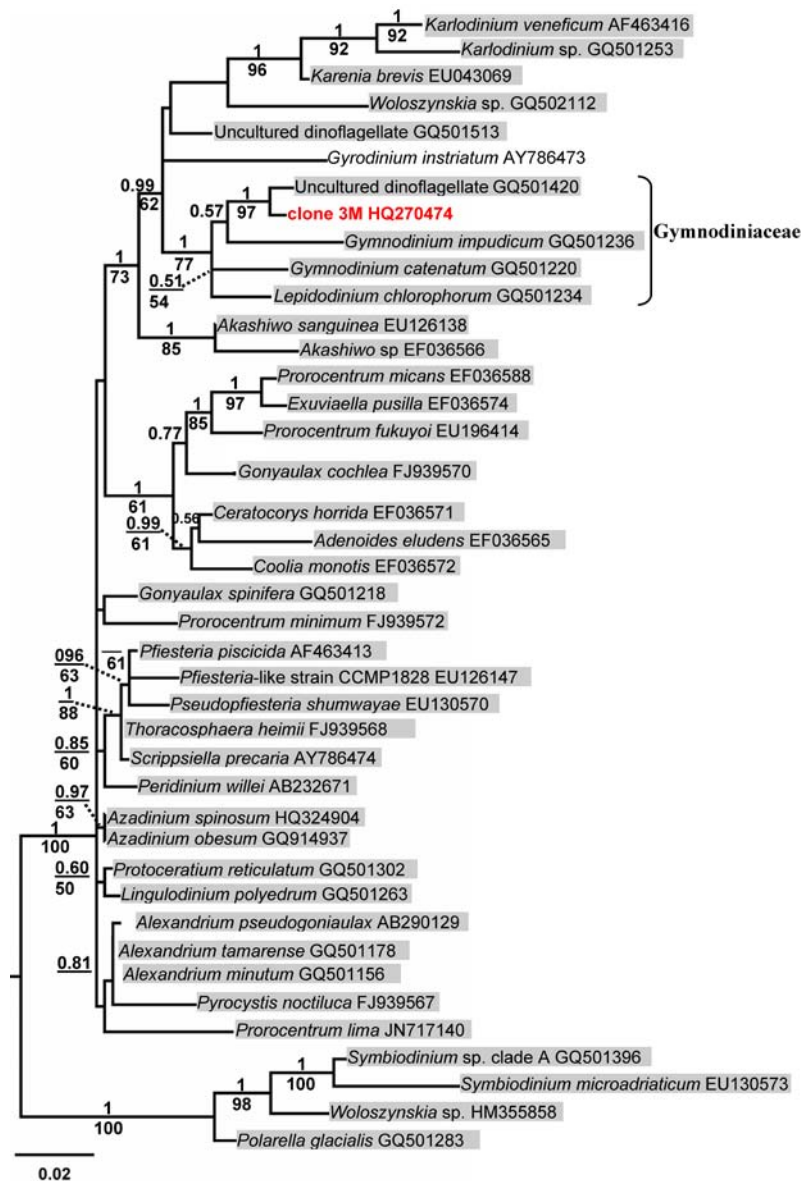


Figure 3. Phylogenetic analysis of the dinoflagellate partial CO1 gene sequences. The tree shown is the ML tree. Values above vertical lines are BI posterior probabilities, values below vertical lines are bootstrap support for the ML analyses, (<50 are not shown). Rooted at midpoint. Sequences of marine and brackish water species are on grey background, Baikalian sequence are red colored.

and many organisms were placed in it by mistake [11]. The reason for those erroneous placements was the problematic identification of unarmored dinoflagellates by light microscopy. In many cases, taxonomic differences among species can be seen only when electron microscopy in combination with molecular genetic data are used. Daugbjerg *et al.* [22] reduced the genus *Gymnodinium* to include only the following species: the freshwater *G. fuscum* and *G. palustre*, the marine *G. aureolum*, *G. catenatum*, *G. impudicum*, *G. nolleri*, *G. cf. placidum* and *G. chlorophorum* (because of a few morphological features the latter is now known as *Lepidodinium*

chlorophorum [20]). Nevertheless, a large number of species are still informally known as members of the *Gymnodinium* genus. This is especially true for many freshwater dinoflagellates because their classification is questionable [23]. *Gymnodinium baicalense* may be a true member of the genus *Gymnodinium* (or belong to a closely related genus), because *G. baicalense* clustered with the confirmed *Gymnodinium* species *G. aureolum* [23] (Figures 1, 2). *Gymnodinium aureolum* is an unarmoured, autotrophic dinoflagellate with brown chloroplasts. It was previously thought that *Karenia mikimotoi* may be synonymous to this species, but this

is not true according to the recent studies [24]. A detailed study of the *G. baicalense* ultrastructure is necessary to determine whether it truly belongs to the *Gymnodinium* genus.

The second conclusion from this study concerns the potential colonization patterns of Baikalian dinoflagellate species. The closest relative of the Baikalian species is an unknown arctic dinoflagellate based on 18S rDNA sequences (Figure 1). Because only two substitutions are between *Gymnodinium baicalense* and the arctic dinoflagellate sequences, it's unlikely to find a closer relative, even if more freshwater gymnodinioids are included in the analysis. This arctic dinoflagellate was sequenced from the glacial melt water sample from Austre Brøggerbreen, Arctic ocean, [25] which means that it can survive in water with low salinity.

Similarly, both the Arctic and Baikalian dinoflagellates grow in water that is ice-covered. *Gymnodinium baicalense* also grows well in interstitial ice water during the spring [26]. These facts allow us to suggest that *G. baicalense* (or its freshwater ancestor) diversified relatively recently from the northern marine gymnodinioids. The Pliocene–Pleistocene glaciations (about 3 million years ago) may have facilitated the colonization from arctic waters to Baikal. For dinoflagellates, the long-distance dispersal is possible because they have resistance resting stages (cysts) in their life cycle, which may persist for up to 100 years [27]. Baikal is a cold water lake that is ice-covered at least 5 months a year and is a good environment for psychrophilic dinoflagellates. Thus, dispersion from the Arctic ocean to the center of Eurasia is possible despite the large distance. Interestingly, *G. baicalense* is more closely related to the marine bloom-forming *G. aureolum* (or an unknown marine dinoflagellate on the COI tree) than to *G. fuscum* from oligotrophic freshwaters. *Gymnodinium aureolum* is also common in temperate and even sub-arctic marine waters [28].

One can speculate that *G. baicalense* is not an endemic organism that was formed in Lake Baikal, rather, it may be a relict organism that was preserved in Lake Baikal when the climate became warmer. This hypothesis is supported by findings of *G. baicalense*-

like morphospecies in several mountain lakes in the Baikalian region, including Lake Oron, also known as the “northern brother of Baikal” [26]. Furthermore, there are other relict organisms in Lake Baikal, *i.e.* the diatom *Aulacoseira baicalensis*, which also lives under the Baikalian ice [29]. An alternative scenario for *G. baicalense* is that an unknown ancestor migrated to Baikal and evolved some species-specific feature while in the lake. To test these two hypotheses, *Gymnodinium*-like dinoflagellates from additional lakes in the Baikalian region and the whole of Eurasia must be studied using molecular genetic methods.

This study presents a molecular genetic description of a dinoflagellate species that plays a key role in the spring phytoplankton community in Lake Baikal. Phylogenetic analyses based on three DNA markers revealed that this Baikalian dinoflagellate belongs to the *Gymnodiniales* order and has close relatives in the Arctic Ocean. This work is part of a growing body of research aimed at clarifying the phylogenetic positions of freshwater protists in relation to each other and to marine species. The results of this study warrant further investigations on freshwater dinoflagellate biogeography and phylogeny. To fully understand the biogeography and possible endemic nature of *G. baicalense*, other freshwaters in Eurasia must be examined for the presence of this dinoflagellate. It is also important to compare *G. baicalense* with its Arctic relatives and to try to find any local adaptations of the Baikalian species. Additionally, deeper investigation of *G. baicalense* in Baikal is needed to understand its genetic diversity, life cycle, and ecology.

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