

Identification of Lake Baikal Plankton Dinoflagellates from the Genera *Gyrodinium* and *Gymnodinium* Using Single-Cell PCR

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Abstract—Most of eukaryotes is represented by unicellular organisms (protists) which are still poorly investigated owing to a number of methodological problems. In this study, single-cell PCR was used for the analysis of two unarmoured dinoflagellate species from Lake Baikal. DNA fragments from living and fixed *Gymnodinium baicalense* and *Gyrodinium helveticum* cells were successfully sequenced, which indicates the usefulness of the method in the study of uncultivated dinoflagellates. However, this approach is too laborious to be introduced into traditional monitoring studies. The data obtained confirmed the earlier suggestion that the Baikal population of heterotrophic species *G. helveticum* was wrongly described as an endemic species of Lake Baikal, *Gymnodinium coeruleum*. The sequences of 28S rRNA gene fragment and ITS2 region were determined for the first time for *G. helveticum*. Phylogenetic trees inferred from the 28S rRNA and 18S rRNA genes confirm the assignment of *G. helveticum* to the genus *Gyrodinium*. Analysis of 18S rRNA gene fragments from the GenBank database revealed a large number of unknown eukaryotes from different water reservoirs similar to *G. helveticum*. This is could be because of the existence of cryptic diversity within morphospecies *G. helveticum*, as well as the existence of yet unknown species from the genus *Gyrodinium*. Our analysis stimulates more intensive study of *G. helveticum* phylogeography in particular and the diversity of *Gyrodinium* in general.

Keywords: phylogenetics, phylogeography, protists, dinoflagellates, dinophyta, *Gyrodinium*

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INTRODUCTION

Unicellular eukaryotes (protists) are a diverse group of organisms, which belong to almost all known eukaryotic kingdoms. In comparison with multicellular organisms, their genetic diversity is very high and they are grouped only by the fact that they are made up of a single cell. This stipulates a number of similar developmental routes (complex life cycle, the ability to spread over long distances). The mechanisms of their speciation, systematics, and biogeography are still poorly investigated in comparison with multicellular organisms and even bacteria. This is associated with their sizes, with the complexity of cultivation in the laboratory, and with poorly expressed morphological differences. Dinoflagellates is a large group of protists from the kingdom Alveolata, occupying almost all ecological niches as free-living autotrophs, heterotrophs and mixotrophs, symbionts, and parasites. They live in both marine and freshwater systems, but in the latter, they are less studied, which makes it impossible to assess their real diversity [1]. Photosynthetic dinoflagellates occupy the second place after diatom algae to provide our planet with oxygen [2].

The ecological role of heterotrophic dinoflagellates has been less studied, although it was demonstrated that marine dinoflagellates actively feed on bacteria and different plankton organisms, including larvae of multicellular animals [3, 4], and may inhibit the growth of toxic microalgae [5]. Obviously, the studies on the diversity of freshwater heterotrophic dinoflagellates using modern methods are necessary to better understand functioning of freshwater ecosystems.

Our understanding of the diversity of protists began to improve considerably when it became possible to study them by molecular biological methods [6]. Today, these methods should be routine in protistology. DNA analyzes of protists, that omitting the stage of their cultivation, are actively developed. In addition to enabling work with uncultivated organisms, these methods make it possible to investigate real genetic diversity, rather than the mutations accumulated during the culture growth for a number of years in the laboratory conditions. One such approach is associated with metagenomics, i.e., the analysis of total DNA isolated from a natural sample. The disadvantage of metagenomics is the inability to associate

sequenced DNA with morphological characteristics of organisms. Another approach, lacking such a shortcoming, is associated with the isolation of DNA or RNA from a single cell. At present, methods of whole genome and transcriptome sequencing from a single cell have begun to be developed [7]. However, for phylogeographic and monitoring studies, such a technique is redundant (genomes of protists are sometimes larger than the human one), and a faster and cheaper approach is needed. Many protists can be identified using several marker genes, like 18S and 28S rRNA genes, internal transcribed spacers (ITS1 and ITS2), and some mitochondrial genes. Amplification of such segments is possible not only from DNA extracted from a relatively large volume of biomass but also from DNA obtained from one or several cells (single-cell PCR, SC-PCR) [8]. Moreover, this method can be used to investigate genetic diversity of protists from already fixed samples (for example, [9]). Different variants of single-cell PCR techniques were tested on diatoms [10], ciliates [11], dinoflagellates [12], etc.

In this study, a detailed description of the single-cell PCR technique used in [13] is presented. With some modifications of the authors, the method was applied to planktonic dinoflagellates, both living and fixed in 70% ethanol and 1% Lugol's iodine solution. The objective of the study was to obtain new genetic data on unarmored dinoflagellates from Lake Baikal and to evaluate the applicability of the used approach in the monitoring of freshwater lakes. *Gyrodinium helveticum*, that is one of the few known heterotrophic freshwater dinoflagellates, was studied in more detail.

MATERIALS AND METHODS

Sample Collection

Plankton samples were collected during March–May 2012 in the surface water layer of Southern Baikal in the littoral zone near the villages Listvyanka and Bolshie Koty. Samples were collected using Apstein's net (10 μm mesh size) after drilling a hole in the ice. Part of the samples were immediately fixed in 70% ethanol and 1% Lugol's iodine solution. Identification of dinoflagellates in the samples was carried out using an Axiovert 200 optical inverted microscope (Zeiss).

Single-Cell DNA Amplification and Sequencing

About 20 μL of the sample containing the examined dinoflagellates were transferred to a droplet of deionized water on an autoclaved glass slide. An intact dinoflagellate cell was isolated with a glass microcapillary and photographed with a Pixera Penguin 600CL (DiRector™) camera at 400 \times magnification. Each cell was washed in 6–8 droplets of deionized MilliQ water using a sterile micropipette to prevent contamination with DNA from other organisms or their fragments. After washing, the cell was transferred to a droplet containing about 4 μL of water, destroyed with

a glass needle made of a glass micropipette. The whole droplet with the destroyed cell was immediately transferred to a PCR tube on ice. The last droplet of water from the washing stage was also transferred to a separate PCR tube to be used as a negative PCR control. All tubes were stored at -70°C for at least one hour (or overnight). Then, the tubes were thawed on ice and the PCR reagents were added directly to them. Alternatively, the following approaches were used after washing procedure. (1) The cell in 10 μL of water was incubated for 30 min at 55°C with 200 $\mu\text{g}/\text{mL}$ of proteinase K (Qiagen, United States) with subsequent inactivation for 10 min at 95°C and freezing, or (2) the cell isolated in 4 μL was not treated in any way, but several freeze/thaw cycles were performed.

PCR was performed in a volume of 20 μL , and the amplification parameters depended on the kit (*Taq* polymerase (Eurogen) or high-fidelity *Phusion* polymerase (Phusion, Finnzyme)) and primers used. 18S rRNA gene fragment (1179 bp) was amplified using dinoflagellate-specific primers L1: TTGGCCTACCGTGGCAATGAC, R1: TCCAATCTCTAGTCGCATGGT [14], and Dino1662R: TTATTCACCGGAWCACTCAATCGG [15]. Amplification of ITS2–28S rRNA fragment (1463 bp) was performed with primers 5L: GTGAATTGCAGAATTCCGTGAAC [14] and R28Lo: CTTACCAAAAATGGCCCACTTAGAG [16].

Multiplex PCR was carried out using two primer pairs (L1 and Dino1662R, 5L and R28Lo). Then, each pair of primers was used separately with the corresponding product of the first PCR, extracted from the agarose gel after electrophoresis, as a template. In theory, one DNA fragment, from primer L1 to R28Lo, could be amplified. However, in practice, such a long fragment could not be amplified from a single cell. Electrophoresis was carried out in a 1% low-melting agarose at $U = 80$ V for 30–40 min in $0.5 \times$ Tris–borate buffer. PCR was considered successful when a distinct band of appropriate size was seen on the gel. DNA fragments were sequenced using an ABI 3130XL automated sequencer (Applied Biosystems).

Phylogenetic Analysis

The obtained sequences were edited manually and assembled using BioEdit v. 7.1.3 [17]. Alignments of the DNA sequences were constructed using the MAFFT v. 6.952 software program [18]. Analysis of the genetic differences between DNA sequences was carried out using MEGA v. 6.0 [19].

Although three different DNA markers were sequenced for *Gyrodinium helveticum*, it was impossible to construct one phylogenetic tree on the basis of them because of considerable differences between the species from which they were sequenced. Phylogenetic analyses of the 18S rRNA and 28S rRNA genes were

performed using the maximum-likelihood (ML) method implemented in the RAxML program [20], robustness of the ML tree topology was assessed using bootstrap analysis with 1000 replicates. In addition, phylogenetic trees were constructed using the Bayesian inference (BI), as implemented in the MrBayes-3.1.2 [21]. Two hot and one cold Markov chain were run in two replicates for 2×10^6 generations, sampling every hundredth generated tree. Of these, the first 25% of trees were discarded. On the basis of other trees having stable estimates of the nucleotide substitution and likelihood model parameters, a phylogenetic tree and a posteriori probabilities of its branching pattern were obtained. The Akaike criterion implemented in jModelTest 2.1.1 [22] indicated that the General Time Reversible model of nucleotide substitution, with Gamma (G) distributed rates across sites and a proportion of invariable sites (I), was the most appropriate evolutionary model for the studied alignments. The model was used in all phylogenetic analyses. *Perkinsus marinus*, closely related to dinoflagellates, was used as outgroup [23]. New DNA sequences obtained in the study were deposited in the GenBank database under the accession numbers MG255302, MG255303, and MG493227.

RESULTS AND DISCUSSION

Dinoflagellate Identification

The fragments of the 18S rRNA gene (1179 bp) from unicellular organisms isolated from different samples of Baikal plankton were sequenced: two cells of *Gymnodinium baicalense*, three cells of *G. baicalense* var. *minor*, and two cells that were intermediate in size and shape between the species and its variety. All these fragments were identical to each other and coincided with the data obtained for this species earlier [24]. Moreover, species-specific ITS2 markers from Baikal water samples containing both *G. baicalense* and *G. baicalense* var. *minor* sequenced in our earlier study [25] demonstrated genetic homogeneity of dinoflagellates from these samples. A monoculture of *G. baicalense* grown in the laboratory from a single cell contained dinoflagellates, the sizes of which corresponded to both the main morphospecies and its variation (unpublished data). Moreover, the variation smaller in size dominated during exponential culture growth. It can be suggested that active division made it impossible for these cells to grow to typical sizes of *G. baicalense*. These facts, in combination with new data, do not provide a basis for isolation of the subspecies of *G. baicalense* var. *minor*.

The 18S rRNA gene fragment was also sequenced from a single dinoflagellate cell known as *Gymnodinium coeruleum* [26]. Earlier, from a Baikal water sample containing *G. baicalense*, *G. coeruleum*, and *Peridinium* sp., a fragment of the 18S rRNA gene belonging to *Gyrodinium helveticum* was sequenced. Having

discovered that morphological descriptions of this species and Baikal *G. coeruleum* were the same, we suggested that the latter was in fact the ubiquitous species *G. helveticum* [25]. Indeed, the sequence of the 18S rRNA gene fragment determined in the present study directly from the cell of *G. coeruleum* was identical to the corresponding sequence from the study of Annenkova et al. [25], as well as to the DNA sequence of *G. helveticum* from the classical description of this species [27]. Analysis of two other more variable markers from three cells of Baikal *G. coeruleum* (including the one from which the 18S rRNA gene fragment was sequenced) showed that they belonged to the same species (see below). In the literature, it was reported that, initially, *G. coeruleum* was described for marine waters and, then, it was detected only in salt water [28]. Thus, its existence in such a freshwater reservoir as Lake Baikal is extremely doubtful. All these data make it possible to exclude *Gymnodinium coeruleum* from the list of Baikal dinoflagellates and suggest assigning the corresponding morphotype to the species *Gyrodinium helveticum* (the following discussion will show the probability of the existence of cryptic genetic diversity within this species in Lake Baikal. To solve this problem, a separate population genetic study is needed.) Hereinafter, the analyzed dinoflagellate will be referred to as Baikal *G. helveticum*.

Phylogenetic analysis of the 18S rRNA gene fragment is shown in Fig. 1. *G. baicalense* clusters with verified representatives of the genus *Gymnodinium* with high statistical support, as in the earlier analysis [24]. Representatives of the genus *Gyrodinium* were grouped into one clade but with low statistical support (ML 44). This clade did not include only one *Gyrodinium* sp. (GenBank accession number AB001438), but it seems that this dinoflagellate has no close relationship with the genus *Gyrodinium* and was named so because of its similarity to *Karenia mikimotoi* (earlier known as *Gyrodinium nagasakiense*), with which it clusters with high statistical support. Within the *Gyrodinium* clade, one group is formed by a number of known species (*G. helveticum*, *G. rubrum*, *G. cf. gutrula*, *G. heterogrammum*, etc.) and unidentified eukaryotes (Clade I, Fig. 1, ML 72, BI 99), and another group is formed by *Gyrodinium fusiforme* (also known as *G. fusus* [29]) and *Gyrodinium spirale* (Clade II, Fig. 1, ML 75, BI 99).

The 28S rRNA gene fragment (1260 bp) and the ITS2 region (203 bp) were sequenced from the same *G. coeruleum* cell as the fragment of the 18S rRNA gene, as well as from two additional cells (Figs. 2a–2c). All three ITS2 variants were identical, and in one of the 28S rRNA gene fragments, there were two substitutions in compare with others. In the previous paragraph, it was discussed that Baikal *G. coeruleum* was actually *G. helveticum*. This means that, in the present study, the 28S rRNA gene fragment and ITS2 region of this morphospecies were sequenced for the first time. The ITS2 region is a promising marker for the analysis of closely related species and subspecies. The



Fig. 1. Phylogenetic analysis of the 18S rRNA gene fragments. The numbers in branches correspond to a posteriori probabilities (above, <90 not shown) and bootstrap support values (below, <50 not shown). In bold, species examined in the present study and *Gyrodinium rubrum*, known as the closest relative of *Gyrodinium helveticum*. Groups I and II correspond to the two clades with high statistical support into which DNA sequences of dinoflagellates similar to the genus *Gyrodinium* are divided. Group Ia corresponds to a separate clade, which of known species includes only *G. helveticum* from Lake Baikal and Lake Shikotsu, and also consists of unknown eukaryotes the closest to this species. * The complete list of sequences includes AY664972, KF129831, KF130000, KC488437, FJ914489, EU780608, and 31 sequences isolated from a water sample taken from the depth of 5 m in the San Pedro Channel, North Pacific (see Table 1).

knowledge of its structure will be useful in the future for phylogeographic analysis of *G. helveticum*. 28S rDNA can already be used to clarify the relationships of *G. helveticum* with other dinoflagellates. The phylogenetic tree based the 28S rRNA gene fragment (Fig. 3) reflects the evolutionary relationships between the known representatives of the genus *Gyrodinium* and the family Gymnodiniaceae. In general, the tree topology corresponds to earlier analyses of the family using different DNA markers (for example, [30]). As follows from Fig. 3, almost all species of the genus *Gyrodinium* (with the exception of *G. lebouriae* and *G. undulans*) statistically significantly fell into one large clade (ML 96, BI 100). This clade, as in the case of the 18S rRNA tree (Fig. 1), is formed by two groups. One group includes marine species and freshwater *G. helveticum* (group I, Fig. 3, ML 100, BI 100), another group includes marine members of *Gyrodinium* (*G. fusus*, *G. fusiforme* and three *Gyrodinium* representatives without exact species identification) (group II, Fig. 3, ML 98, BI 100). These groups do not coincide with the subgenera identified using a number of morphological characters [31].

On the basis of the constructed trees, it is possible to make the following general remarks about representatives of the genus *Gyrodinium*. A typical species of this genus is *Gyrodinium spirale*. The fragments of the 18S rRNA gene (Fig. 1) and 28S rRNA gene (Fig. 3) of this species obtained from different isolates [27, 32], as well as those from *G. cf. spirale* isolated from the same sample [30], were included in the analysis. The fragments of the 18S rRNA gene of *G. spirale* and *G. cf. spirale*, as well as the fragment of the 28S rRNA gene of *G. cf. spirale*, clustered with *G. fusiforme* (Figs. 1 and 3). At the same time, the fragment of the *G. spirale* 28S rRNA gene (AY571371) obtained from the cell culture, that was used for the complex analysis of this species [32], did not cluster with *G. cf. spirale* and *G. fusiforme* (group II, Fig. 3). It was the member of another group of the *Gyrodinium* representatives (group I, Fig. 3). In [30], it was reported that *G. cf. spirale* differed from *G. spirale* only in somewhat smaller size. However, genetic data indicate that *G. cf. spirale* from [30] and *G. spirale* from [27] are the same species, and *G. spirale* from [32] is a different species. The problem will probably be solved by more detailed study of their morphology and ultrastructure. However cryptic genetic diversity cannot be excluded. Concerning other *Gyro-*

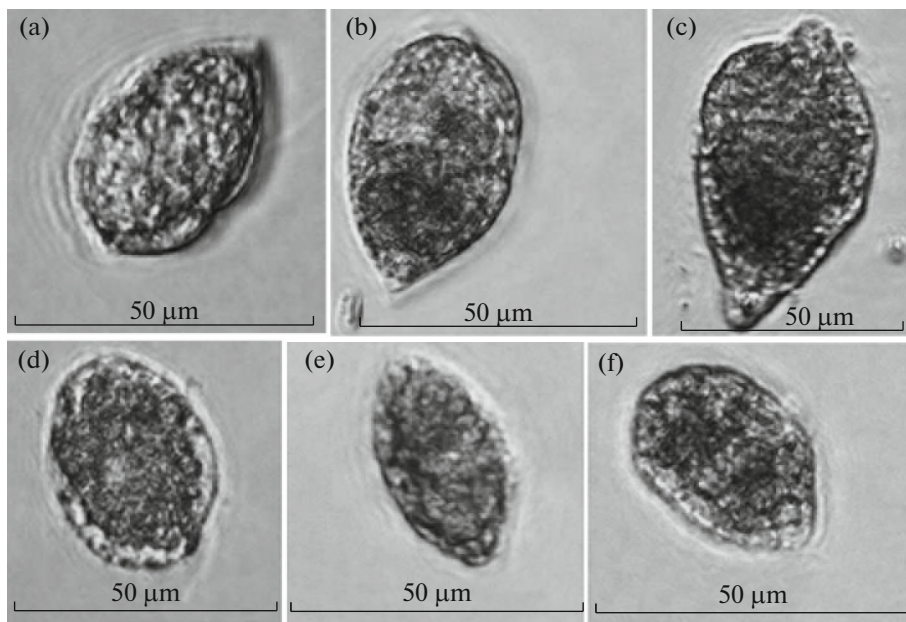


Fig. 2. Some of dinoflagellate cells used in single-cell PCR analysis: (a–c) *Gyrodinium helveticum* (= *Gymnodinium coeruleum* Antipina); (d–f) *Gymnodinium baicalense*. Optical microscopy.

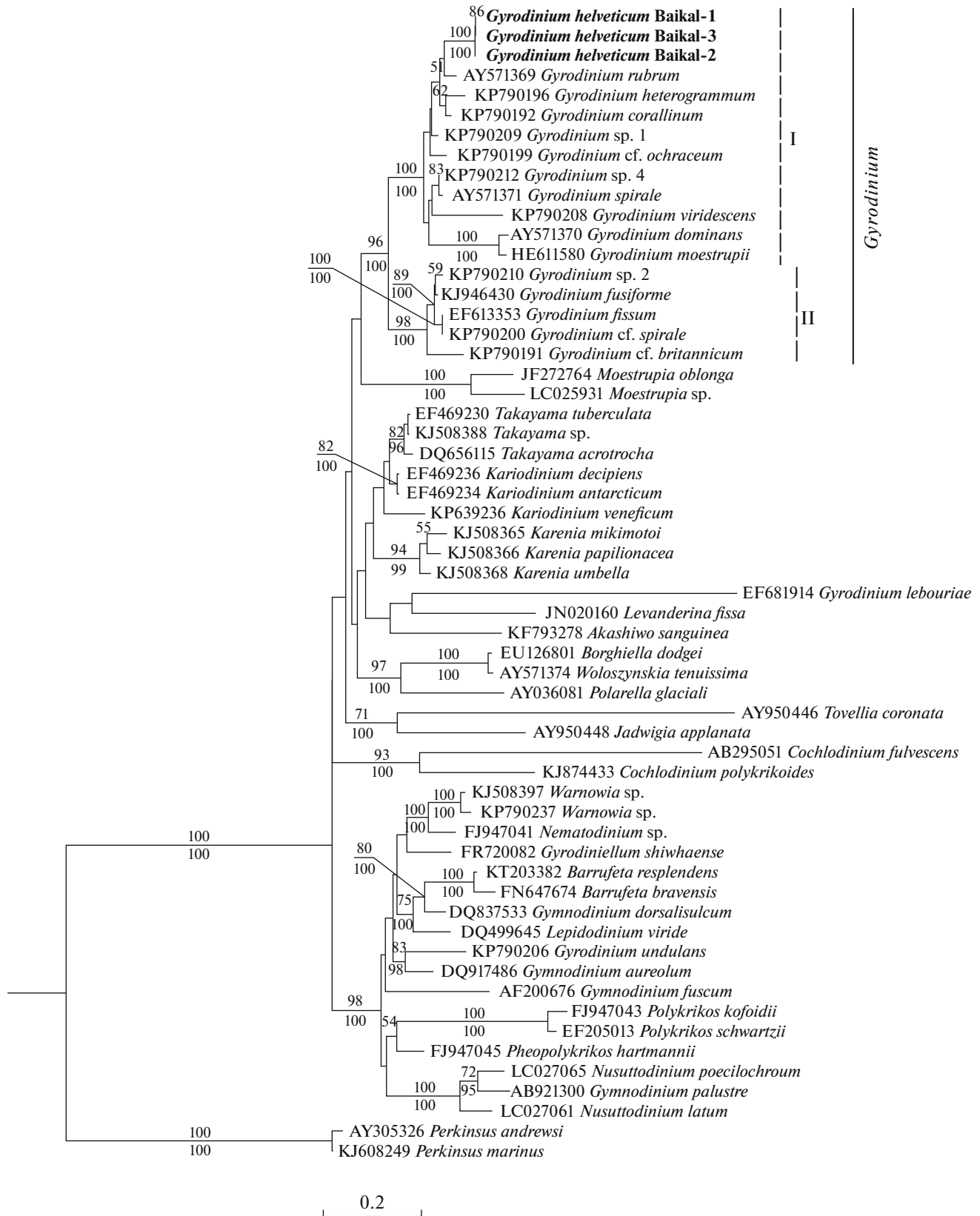


Fig. 3. Phylogenetic analysis of the 28S rRNA gene fragments. The numbers in branches correspond to a posteriori probabilities (above, <90 not shown) and bootstrap support values (below, <50 not shown). Groups I and II correspond to the two clades with high statistical support into which DNA sequences of dinoflagellates similar to the genus *Gyrodinium* are divided (correspond to the same groups I and II in Fig. 1). In bold, species examined in the present study.

dinium representatives, it should be noted that, in Fig. 3, *G. undulans* and *G. lebouriae* were not included in the main clade of this genus, which indicates the need to verify generic assignment of these species. Today, many species of the family Gymnodiniaceae are re-described using more detailed morphology and molecular phylogeny. For example, *G. fissum* has already been redetermined and isolated in a separate genus *Levanderina*, to which no other species belong yet [33]. According to Fig. 3 this genus is not close to the genus *Gyrodinium*.

According to the analyses of 18S and 28S rRNA gene fragments (Figs. 1 and 3), the most closely related species to *G. helveticum* among known species examined in this study is the marine species *Gyrodinium rubrum*. The finding corresponds to the results of an earlier phylogenetic analysis [27]. However, this closeness has low statistical support in the analysis of 28S rRNA gene fragments (Fig. 3). Although it is considered that the D1–D2 region of the 28S rRNA gene separates the dinoflagellate species quite well, in our case, the information was not enough. In addition, analysis of 18S rRNA (Fig. 1) points to the presence of unknown species (or of ones not sequenced at this marker) closely related to the species under study. Obviously, reliable determination of the closest relative of *G. helveticum* requires the use of additional markers and more representatives of the genus. In [34], the closeness of *G. helveticum* and *G. rubrum* was considered as one of the few cases of recent colonization of fresh waters by marine species. Indeed, all dinoflagellates included together with these species in clade I (Fig. 3) are also marine. However, according to the better studied 18S rRNA gene (Fig. 1), there can be freshwater dinoflagellates that are evolutionarily closer to *G. helveticum* than *G. rubrum* and other marine representatives of the genus (for example, according to this marker, eukaryotes AY919730 and AY919690 differ from *G. helveticum* by 0.6 and 1.4%, respectively, and *G. rubrum* differs from the latter by 1.5%). In particular, at the beginning of the last century, two more freshwater representatives of the genus *Gyrodinium* (*G. hyalinum* and *G. pusillum*) were described [31]. However, there are no molecular genetic data on them. If these or other freshwater *Gyrodiniums* are closer to *G. helveticum* than the marine ones, this means that either representatives of freshwater *Gyrodinium* separated from the marine representatives in the rather distant past (and during this time they managed to evolve into different species), or recent radiation took place among the freshwater *Gyrodinium* representatives. To verify these hypotheses, phylogeographic analysis of freshwater representatives of *Gyrodinium* is necessary.

Prevalence of the DNA Sequences Closely Related to the DNA of Gyrodinium helveticum

Gyrodinium helveticum is a widespread heterotrophic species. It was found in freshwater reservoirs of Europe [35], North America [36], and New Zealand [37]. It is included in the checklist of Baltic Sea phytoplankton species, but as an invasive freshwater species, not tolerant to the local salinity level [38]. Its presence in the Black Sea and the ability to tolerate these brackish waters was reported [39]. The latter statement requires additional verification. It is known that the salt barrier plays an important role in the evolution of dinoflagellates, and this barrier appears even in the very recent adaptation of the species to fresh water [15, 16]. It should be noted that many reports on *G. helveticum* are based on data of optical microscopy, which is not sufficient for reliable description of the organism at the species level. Despite wide distribution of the species, genetic analysis was carried out only for a specimen from Lake Shikotsu (Japan) and from Lake Baikal (Russia). Searching across the GenBank database reveals a large number of sequenced fragments of the 18S rRNA gene of unknown eukaryotes most similar to the corresponding sequence of *G. helveticum* DNA. Do all these sequences belong to different populations of *G. helveticum* or other evolutionarily close species? In what places do these species live?

To discuss the problem posed, all 18S rRNA gene fragments from unknown eukaryotes with 98–99% similarity (according to BLAST analysis) to the species under study were included in the phylogenetic analysis (Fig. 1). As a result, 96 sequences were included in one clade with the DNA sequences of *G. helveticum* from Lake Baikal and Lake Shikotsu (clade Ia, Fig. 1). The genetic distances between all these sequences (1219 nucleotides) average 1.3%. This clade has statistically significant a posteriori probability values (BI 99), but low bootstrap support. A similar effect was previously reported in the analysis of the 18S rRNA gene of another group of dinoflagellates [14]. This finding can be probably explained by weak phylogenetic signal (the DNA sequences from this clade contain 60 parsimony informative sites, while in the alignment used in this study, there are 131 such sites). The existence of a large number of close but not identical sequences of 18S rRNA gene can be explained by two reasons. First, these are artifacts of different nature: the stage of amplification and, in many cases, molecular cloning upon DNA sequencing from natural samples could contribute to the accumulation of artifact substitutions. In addition, the presence of intracellular variability of rRNA genes was proved for several dinoflagellate species. In particular, in *Alexandrium fundyense*, the differences between the 18S rDNA gene and pseudogene fragments sequenced from one strain reached 2.9% [40]. It cannot be excluded that a similar pattern can be observed in *Gyrodinium*. However, it is unlikely that such a large

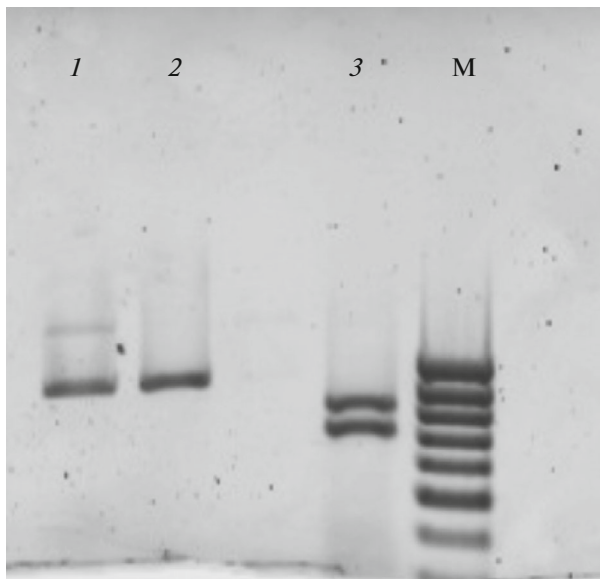


Fig. 4. Electropherogram of single-cell PCR products: (1) multiplex PCR, fragments of the 18S rRNA gene (1315 bp) and ITS2–28S rRNA gene region (1050 bp), (2) PCR with single primer pair, fragment of the ITS2–28S rRNA gene region; (3) multiplex PCR, fragments of 18S and 28S rRNA genes (698 and 781 bp, respectively). M, 1 kb DNA ladder (Thermo Fisher). As can be seen from lanes 1 and 3, the DNA fragments that are more similar in size are amplified approximately equally, while in the case of considerable size differences, the longer fragment is synthesized in smaller amounts.

number of DNA fragments from different habitats can be explained only by the above-mentioned artifacts. Moreover, in clade Ia (Fig. 1), they form several distinct groups, including dinoflagellates from different studies. The second, more probable, reason is that at least some of the DNA fragments that are similar to the *G. helveticum* 18S rRNA gene reflect real biodiversity.

The DNA sequences closely related to *G. helveticum* were found in different water reservoirs (Table 1). The 18S rRNA gene fragments, that were identical to Baikal *G. helveticum* DNA fragment, belong to *G. helveticum* from oligotrophic, relatively ancient (40000 years) Lake Shikotsu and to unknown eukaryote from oligotrophic Lake George (USA). Three sequences of unknown eukaryotes from Char Lake (the Arctic) are different from the Baikal species only in one or two substitutions in the analyzed 18S rRNA gene fragment. All these lakes, although far from each other, are ecologically rather similar to Lake Baikal (oligotrophy, low temperature, high transparency), which, apparently, determined the distribution of *G. helveticum* in them. Interestingly, two DNA fragments from Lake George are different from *G. helveticum* by 0.6–1.4%, which increases the probability of their belonging to a different species. Earlier, in Lake Baikal, we also found DNA fragments of unknown dinoflagellates which differed from the corresponding *G. helve-*

ticum DNA sequence by 2% and clustered with it in phylogenetic analysis [14] (for example, FJ823475 and FJ823476, Fig. 1). Preliminary analysis of 18S rDNA metabarcoding data from the Baikal plankton revealed an even greater diversity of 18S rRNA gene sequences clustering exclusively with *G. helveticum*, but differing from it at this marker by up to 4% (unpublished data). In the future, it is necessary to check whether closely related lineages/species of the genus *Gyrodinium* originated in the same lake (Lake Baikal, Lake George) owing to sympatric evolution. The remaining eukaryotes, the DNA sequences of which were included into clade Ia (Fig. 1), were found in marine waters (Table 1), mainly in cold seas and at different depths. It is impossible to say whether any of these sequences belong to the species like *G. corallinum* and *G. cf. ochraceum*, which, according to the analysis of the 28S rRNA gene, are in the same group as *G. helveticum* (Fig. 3), but for which the 18S rRNA gene is not sequenced. However, it can be suggested that some of these sequences belong to the yet undescribed (or insufficiently described) *Gyrodinium* representatives. For example, a ten-year study of the waters of Western Spitsbergen revealed that more than 30% of the *Gyrodinium* and *Gymnodinium* of these waters were not identified on the species level [41].

The taxonomy of the genus *Gyrodinium* and the family Gymnodiniaceae as a whole is paradoxical, since they include a great number of species; often, one species appears under several names and, consequently, the number of species in these taxa is obviously overestimated [1]. At the same time, a number of *Gyrodinium* species have not yet been described according to the current study. This situation is explained not only by the lack of data on a number of habitats (open ocean, distant lakes) but also by the complexity of the protist cultivation (especially of heterotrophic ones, like *G. helveticum*) and the fragility of unarmored cells. These methodological problems are overcome by using single-cell PCR.

Success of Single-Cell PCR in Dinoflagellates

The single-cell PCR approach described in the Materials and Methods section was used to study unarmored dinoflagellates both from unfixed samples and those preserved in 70% ethanol or 1% Lugol's iodine solution. Thirty cells of *Gymnodinium baicalense* from each sample variant and five cells of Baikal *Gyrodinium helveticum* from fixed samples were used (Fig. 2). Amplified DNA fragments from ten cells were sequenced to ensure that these were the desired sequences (fragments of the dinoflagellate 18S and 28S rRNA genes). Then, the success of the performed amplification was verified by obtaining a distinct electrophoretic band of the appropriate size (Fig. 4).

The efficiency of the method (successful amplification of the desired DNA fragment) with the use of living or ethanol-fixed cells was comparable, consti-

Table 1. List of unknown eukaryotes included together with *Gyrodinium helveticum* in clade Ia in Fig. 1

GenBank accession number	Origin
KJ: 762012, 762373, 762416*	Arctic Ocean, 35 m
EU371148	Arctic Ocean, 2 m
KJ: 757865, 758274, 758000	Ross Sea, 20 m
HM: 581744, 581708	Arctic Ocean, 20 m
HQ: 438164, 438151	North Pole, water from 170 m under sea ice
AY: 664930, 664955, 664956, 664972	Sargasso Sea
KC771213	Bering Sea
JN832834	386 m above the Nyegga cold methane seep, mid-Norwegian continental margin, 325 m
JN832725	Water at the coral reef, 365 m, mid-Norwegian continental margin
JQ226600	Saanich Inlet (northern part of the Pacific Ocean), 10 m
KJ: 762550, 762570, 762582, 762591, 762796	San Pedro Channel (northern part of the Pacific Ocean), 500 m
KJ: 762846, 762858, 762864, 762900, 762904, 762911, 762917, 762948, 762997, 762932, 763017, 763030, 763037, 763052, 763069, 763072, 763081, 763094, 763102, 763152, 763174, 763190, 763211, 763219, 763238, 763261, 763283, 763308, 763332, 763343, 763398, 763375, 763414, 763417, 763425	San Pedro Channel (northern part of the Pacific Ocean), 5 m
KF: 129831, 130000, KP404853	South China Sea (Pacific Ocean)
KJ: 763721, 763886	Gulf Stream (North Atlantic Ocean), 105 m
KC: 488402, 488437	The coast of Nova Scotia (northern part of the Atlantic Ocean), 30 m
FJ914489	The coast near the southeast of North Carolina (northern part of the Atlantic Ocean)
EU780608	Atlantic Ocean near North Africa
JQ692033, KX602140, KX602056	Surface coastal waters, Argentina (southern part of the Atlantic Ocean)
EU780635	South Atlantic Ocean
FJ832124	Ballast water from a ship from Singapore
KJ: 925398, 925460, 925439, 925394, 925468	Coastal zone of the Columbia River
AY: 919690, 919730, 919799	Lake George, Adirondack Park
JF: 730773, 730778, 730801	Lake Char (the Arctic)
FJ: 823475, 823476	Lake Baikal, tissues of Lubomirskiidae sponges

* The letter designations refer to each number.

tuting on average about 75% using Phusion DNA polymerase and about 30% with standard *Taq* polymerase. In the study about diatom algae [10], the single-cell PCR efficiency was comparable to that obtained in the present study and comprised from 30 to 70% of successful PCR. In the experiments with cells fixed in Lugol's iodine solution, the percentage of successful DNA fragment amplification was lower. With cells that were stored in Lugol's solution for more

than 8 months, PCR was unsuccessful. The desired DNA fragment was amplified after using different approaches for cell destruction (mechanical destruction, incubation with proteinase K, thawing/freezing), but the most effective was mechanical destruction. Earlier, the technique with the same success level was used in our study for a number of armored dinoflagellates, both living and fixed [16].

The success of single-cell PCR depends not only on the quality of the starting material and the DNA polymerase sensitivity but also on the nature of the amplified fragment. The shorter is the DNA fragment and the more its copies are in the genome, the more effective is its amplification. In particular, in our experiments, a 400-bp fragment containing the ITS-2 region was amplified in amounts sufficient for its sequencing in one amplification reaction, while amplification of a 1200-bp fragment containing a part of the 18S rRNA gene required repeated PCR, where the product of the first reaction served as the template (Fig. 4). Earlier, using the same approach, we obtained fragments of the mitochondrial cytochrome *b* gene [16]. Although this DNA fragment was small (588 nucleotides), it was rather difficult to amplify it from a single cell, since its copy number was lower than that of ribosomal genes. In addition, sequencing of this fragment faced the problem of multiple signals at one location. In such difficult cases, additional PCR, careful selection of specific primers and reaction conditions, cloning of the resulting DNA fragment may be required. At the same time, working with ribosomal genes, it was possible to use multiplex PCR, during which fragments of both the 18S and 28S rRNA genes and the ITS2 region were amplified from a single cell. Due to different lengths of these fragments, it was possible to separate them using electrophoresis. Then the amount of the material could be increased using additional PCR and sequenced. The experiments showed that, for the most efficient multiplex PCR, the amplified fragments should not be considerably different in size (otherwise the longer fragment would be synthesized in overly small amounts) and the primer pairs should not differ in their annealing temperature by more than 5°C.

In general, the technique presented in this study enables sequencing of traditional DNA marker fragments from single cells of different dinoflagellates, both living and fixed. This is especially important, since in many cases it is not possible to keep living samples collected in distant areas. In selecting a fixative for subsequent single-cell PCR, the following should be considered. Storing cells of unarmed dinoflagellates in ethanol can cause deformation of their cell wall, which will make it impossible to obtain adequate photomicrographs (in our study, we observed both normal and deformed cells, which allowed us to choose the former). During fixation in Lugol's iodine solution, the cells are colored brown, which can also interfere with the study of their morphology. It is possible to remove staining by adding a solution of sodium thiosulfate immediately before the experiment, and this will also increase the success of PCR with cells fixed in Lugol's iodine solution [9]. Choosing Lugol's iodine solution as a cell fixative, one should consider that, in time, the efficiency of DNA amplification will be substantially reduced.

The present study showed that, while in the first analyses of DNA from individual protist cells an opinion on wide opportunities of this approach was expressed, now it becomes clear that it has more limited applicability. Our experience with dinoflagellate cells shows that the efficiency of the method is not high enough to introduce it into the routine monitoring of water reservoirs, during which a large number of samples must be treated. For such monitoring, DNA metabarcoding is probably more suitable. However, amplification of traditional phylogenetic markers from individual cells using standard reagents should be used in the studies focused on specific organisms, including those not growing in laboratory conditions and rarely found in the samples. The method can be used to compare protist cells growing in the same culture. In this study, it was successfully applied to obtain new data on *G. baicalense* and *G. helveticum*, the analysis of which, in particular, revealed the need to study different populations of *G. helveticum* in Lake Baikal and outside of it, as well as to search for closely related species.

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