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Genotypic diversity of *Ulnaria acus* (Kützing) Aboal from Eurasia

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ABSTRACT. *Ulnaria acus* is a cosmopolitan freshwater diatom. As a rule, diatom populations represent a combination of different genotypes. In early study, using a fragment of the *rbc*L gene of *U. acus* strains isolated from geographically distant regions, no significant genetic differences were found in the populations of this species. In this paper, we analyzed 41 strains isolated from different regions of Eurasia. Based on the analysis of the 18S rRNA gene fragment, it was shown that all strains form a single clade consisting of *U. acus*. Phylogenetic analysis of *cox*1 genes revealed that Eurasian strains of *U. acus* form three clades. Strains of *U. acus* from Lake Ritsa (Abkhazia) and Lake Matano (Indonesia) are not heterogeneous. Strains of *U. acus* isolated from Lake Baikal (Russia) and the r. Erdre (France) contained two clades and we can suggest that the genetic structure of the populations of this species is heterogeneous. All studied populations are conspecific taking into account their sexual compatibility of at least two generations.

Keywords: diatom, genetic diversity, genotype, *cox*1, 18S rRNA, sexual compatibility

1. Introduction

Diatoms are a widespread group of organisms that inhabit very diverse ecosystems: seas, oceans, freshwater rivers, lakes; they can inhabit the ice surface and be endosymbionts in dinoflagellates (Bondarenko et al., 2006; Nikulina and Kociolek, 2011; Gagat et al., 2014; Horner, 2018; Wolf et al., 2019). At present, the number of diatom species is debatable; according to some authors, taking into account cryptic species the total number of diatom species is estimated at 100,000 to 200,000 (Mann and Vanormelingen, 2013). The AlgaeBase database contains information on approximately 18 thousands extant and fossil diatom species (Guiry and Guiry, 2022).

Numerous studies have shown that diatoms selected at one point are genetically heterogeneous (Rynearson and Armbrust, 2004; Casteleyn et al., 2009; Evans et al., 2009; Härnström et al., 2011; MacGillivary and Kaczmarska, 2011; Kaczmarska et al., 2014; Tesson et al., 2014; Chen and Rynearson, 2016; Wolf et al., 2021). Genetic heterogeneity is maintained through the sexual process and there are suggestions that it is necessary for more efficient adaptation to changes in the environment (Rynearson and Armbrust, 2004; Tesson et al., 2014).

Previously, it was shown that the V3–V4 region of 18S rRNA can be used to identify diatoms, since the divergence in this fragment is sufficient to separate several hundred species (Zimmermann et al., 2011; Luddington et al., 2012). The exceptions are representatives of the genus *Stephanodiscus* Ehrenberg, and therefore, for their identification, it was proposed to use additionally a fragment containing internal transcribed spacers (ITS) and the 5,8S rRNA gene (Zimmermann et al., 2011). Thus, the use of the V3-V4 region of the 18S rRNA gene is sufficient to identify species. However, comparison of the level of 18S rRNA divergence with other marker genes (ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit – *rbc*L, cytochrome C oxidase subunit 1 – *cox*1, ITS, universal plastid amplicon – UPA) in diatoms showed that the rate of mutation accumulation in the gene *cox*1 is higher (Evans et al., 2007; Guo et al., 2015). Previously, it was found that phylogenetic analysis of the *cox*1 gene fragment in diatoms enables to differ genotypes of the same species isolated from geographically distant points (Ehara et al., 2000; Evans et al., 2007; Hamsher et al., 2011). Thus, using a fragment of the *cox*1 gene, perhaps, to establish the genotypes of species of the genera *Ulnaria* (Kützing)

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Compère and *Fragilaria* Lyngbye selected from different habitats in Lake Baikal.

The species *Ulnaria acus* (Kützing) Aboal was chosen as the object of study, which is a cosmopolitan species living in freshwater bodies of Eurasia (Aboal et al., 2003; Medlin et al., 2008; Kulikovskiy et al., 2016; Kahlert et al., 2019; Liu et al., 2019; Podunay et al., 2021). *U. acus* was found in water bodies in North America (Eberle, 2008; Smith, 2010; Bergey et al., 2017), South America (Vouilloud, 2003; Crossetti and Bicudo, 2008), as well as in other regions (Sherwood, 2004; Bostock and Holland, 2010; Marazzi, 2014; Khairy et al., 2017). The species is also one of the dominant species in the phytoplankton composition of Lake Baikal (Popovskaya and Genkal, 1998; Bondarenko et al., 2019). At present, there are no data on the diversity of genotypes of this species in Lake Baikal. The aim of this work was to identify the diversity of genotypes of *U. acus* strains isolated from different freshwater ecosystems of Eurasia based on the analysis of the gene fragment *cox*1. According to the concept of biological species, we examined sexual compatibility of the investigated populations.

2. Materials and methods

2.1. Sampling and culturing

To isolate monoclones, phytoplankton samples were placed in sterile plastic flasks with Diatom Medium (DM) (Culture collection..., 1988) and transported to the laboratory. A total of 31 strains were isolated from the samples collected at different parts of Lake Baikal (Supplementary Table S1) and in addition, 10 strains of *U. acus* were taken from the World Ocean Diatoms Collection (WODC) of the Karadag Scientific Station (Russia) (r. Sarthe, France; r. Erdre, France; Lake Ritsa, Abkhazia; Lake Matano, Indonesia and Lake Khuvsgul, Mongolia) (Supplementary Table S1). Isolated strains were grown in 250 ml Erlenmeyer flasks with DM at 8-18°C and illuminated with 16 μEinstein m–2 s–1 under a 12:12-h light:dark photoperiod (Zakharova et al., 2020).

2.2. DNA extraction, PCR and marker sequencing

DNA was extracted from the biomass of diatoms, as described earlier (Marchenkov et al., 2018). 18S rRNA fragment (~1100 bp) was amplified using primer pair 18S_F 5'–AACCTGGTTGATCCTGCCAGT-3' (Katana et al., 2001) and 18S_R 5'– GTTTCAGHCTTGCGACCATACTCC-3' (Guo et al., 2015). Amplification was performed with *Taq* DNA polymerase (Evrogen, Russia). PCR mixture consisted of 1x *Taq* Turbo buffer, 1.25 units/μ of *Taq* DNA polymerase, 0.2 mM dNTP mixture, 2.5 mM total Mg2+, 0.2 μM of each primer, and 50-100 ng DNA. PCR temperature profile was as follows: 3 min initial denaturation at 95°C, then 35 cycles of (30 sec at 95°C, 30 sec at 56.5°C, 70 sec at 72°C), and 3 min hold at 72°C.

Fragment gene of *cox*1 (~700 bp) was amplified using primer pair *cox*1_1F 5'-ATGAAGTTTGCTAATCGATGGT-3' and *cox*1_714R 5'-AAAAAGGTGTTGGAACAGTACAG-3', which were selected on the base of the chloroplast genome *U. acus* GenBank JQ088178.1 (Ravin et al., 2010). Amplification was performed with *Taq* DNA polymerase (Evrogen, Russia). PCR temperature profile was as follows: 3 min initial denaturation at 95°C, then 35 cycles of (30 sec at 95°C, 30 sec at 56.5°C, 45 sec at 72°C), and 3 min hold at 72°C.

PCR products were analyzed by electrophoresis in 1.5% agarose gel and purified with AMPure XP (Agencourt, USA) or Monarch® DNA Gel Extraction Kit (NEB, USA). They have been sequenced by use of BigDye Terminator v.3.1 (Applied Biosystems, USA) and analyzed on 3130XL or 3500XL genetic analyzer (Applied Biosystems, USA) in SB RAS Genomics Core Facility (Novosibirsk, Russia).

2.3. Phylogenetic analysis

The dataset *cox*1 fragment for phylogenetic reconstruction contains 40 monoclonal strains (32 strains from Lake Baikal, 8 strains from WODC) sequenced in this study and 46 sequences from dataset NCBI nr database were selected from different groups of diatoms (Supplementary Table S1).

The dataset 18S rRNA fragment for phylogenetic reconstruction contains 41 monoclonal strains (31 strains from Lake Baikal, 10 strains from WODC) sequenced in this study and 80 sequences from dataset NCBI nr database were selected from different groups of diatoms (Supplementary Table S1).

Best model of nucleotide substitutions was found using MEGA 6.0 (Tamura et al., 2013). Reconstruction of phylogenetic relationships for the 18S rRNA and the *cox*1 gene fragments was performed using MEGA 6.0 (Tamura et al., 2013). Maximum Likelihood (ML) trees constructed using the General Time Reversible (GTR) model (Lanave et al., 1984) with gamma-distributed rate variation across sites. Confidence of branching was calculated using 1000 bootstrap support replicas (Zuckerkandl and Pauling, 1965).

Estimates of Evolutionary Divergence between Sequences (*p*-distance) was conducted using MEGA 6.0 (Tamura et al., 2013).

2.4. Mating experiments

To check mating compatibility, clones were maintained in exponential growth phase and inoculated in pairwise combinations into fresh culture medium (10 ml) in glass Petri dishes (5 cm diameter). Culture conditions were the same as for vegetative growth. The mixtures were checked daily under the inverted microscope at the magnification 200x for the presence of sexual (gametes, zygotes, auxospores), initial and post-initial cells. An appearance of the latter suggests the sexual compatibility of parental clones. Several post-initial cells of the first generation were isolated in order to test their fertility. After reaching a suitable cell size, the descendant clones were mated with parental clones (backcrossing) or with clones known to

reproduce sexually. We concluded that the populations were conspecific, provided the second generation was viable (Supplementary Table S2).

3. Results and discussion

As a result of the determination of nucleotide sequences, 41 fragments of the V3-V4 region of the 18S rRNA and 40 fragments of the *cox*1 gene were obtained for monoclonal cultures of diatoms from Lake Baikal (Russia), r. Sarthe (France), r. Erdre (France), Lake Ritsa (Abkhazia), Lake Matano (Indonesia), and Lake Khuvsgul (Mongolia) (Fig. 1 and Supplementary Table S1. It should be noted that the amplicons for the strains from the Lake Khuvsgul (Mongolia) were not obtained with the selected primers. We assume that this is due to the presence of substitutions in the regions of the *cox*1 gene, with which the primers hybridize.

Phylogenetic analysis of the 18S rRNA gene fragment containing the V3-V4 region showed that all sequences formed one clade with a high degree of support (100%) that belongs to the species *U. acus* (Fig. 2). As shown earlier, the 18S rRNA gene fragment containing the V3-V4 region is sufficient to establish the species identity of diatoms (Zimmermann et al., 2011; MacGillivary and Kaczmarska, 2011; Luddington et al., 2012; Kaczmarska et al., 2014).

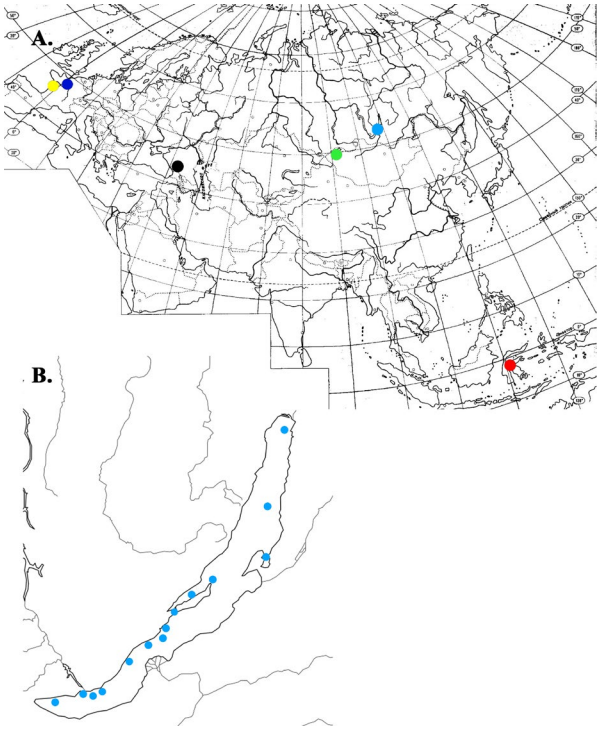

Fig.1. Scheme map of sampling sites. A – Eurasia; B – Russia, Lake Baikal. The places, from which the strains were isolated are marked with circles of different color: blue – Russia, Lake Baikal; black – Abkhazia, Lake Ritsa; dark blue – France, Le Mans, r. Sarthe; yellow – France, Nantes, r. Erdre; green – Mongolia, the Lake Khuvsgul , red – Indonesia, Lake Matano.

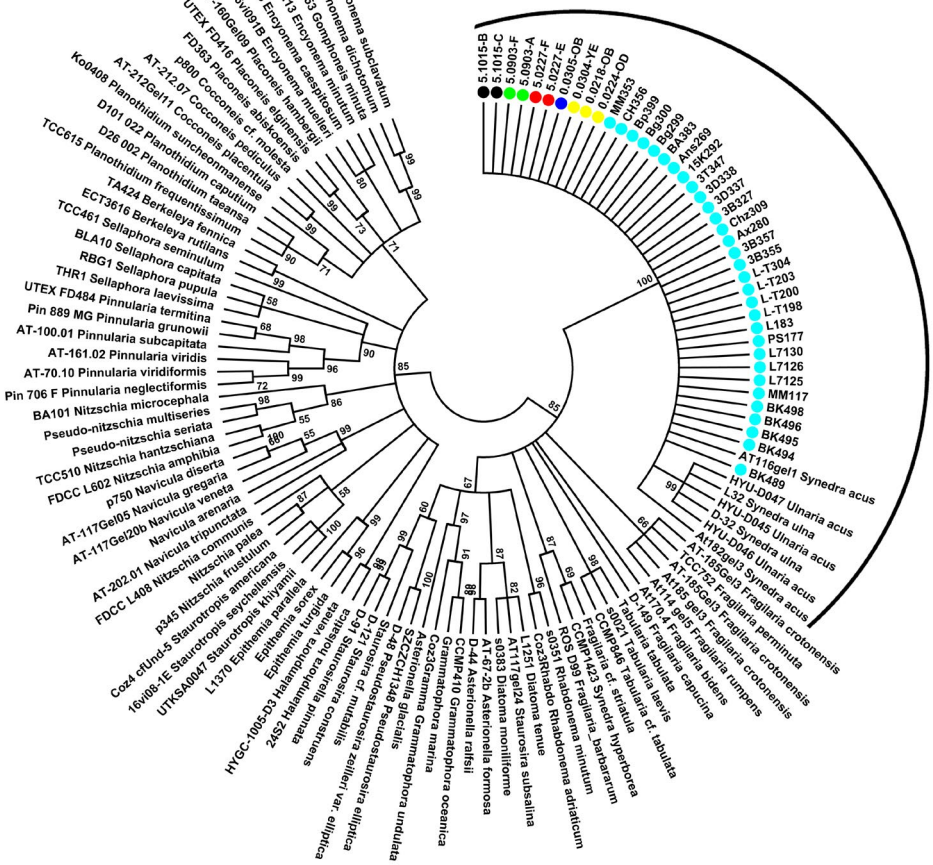

Fig.2. Maximum likelihood phylogenetic tree of gene 18S rRNA using model GTR with gamma-distributed rate variation across sites (MEGA6.0). The sequences obtained in this work are marked with circles of different color: blue – Russia, Lake Baikal; black – Abkhazia, Lake Ritsa; dark blue – France, Le Mans, r. Sarthe; yellow – France, Nantes, r. Erdre; green – Mongolia, the Lake Khuvsgul , red – Indonesia, Lake Matano.

Phylogenetic analysis of *cox*1 gene fragment showed bootstrap support for clade 1 (78 %), clade 2 (89 %) and clade 3 (91 %) (Fig. 3). Clade 1 contains sequences of 26 strains isolated from three basins of Lake Baikal, 2 strains from r. Erdre (France), 1 strain from r. Sarthe (France) and 2 strains from Lake Ritsa (Abkhazia) (Fig. 3). Clade 2 is formed by the sequences of 5 strains isolated from three basins of Lake Baikal as well as 1 strain from r. Erdre (France). In clade 3, it is formed by the *cox*1 sequences of two strains from Lake Matano (Indonesia) (Fig. 3). Clade 1 is the most numerous and contains sequences from different geographic localities.

The separation of Indonesian strains into an individual genotype may be caused by the fact that the strains were isolated from Lake Matana, which is located in the south-east of the central part of the island of Sulawesi. The island is located in the Wallacea region, which in biogeographic terms is a transitional zone between the Sundaland (in Prehistoric times it was connected with continental Southeast Asia) and the Sahul (in the past it formed a single continent with Australia) regions (New, 2002). The reason for the divergence may also be related to the fact that the isolated and relatively small island population without connection with the continent had more chances to accumulate differences in the *cox*1 gene.

The *p*-distance between clade 1 and clade 2 is 0.011-0.018, and between clade 1 and 3 is 0.056-0.064. Between clade 2 and clade 3 *p*-distance is 0.06. Thus, the number of substitutions between clade 1 and clade 2 for the *cox*1 gene is more than three times lower than with clade 3. This may indicate that the level of divergence of strains selected in Lake Matano (Indonesia) is higher than in the strains selected from other places. It should be noted that the *p*-distance *cox*1 between species of *Pseudo-nitzschia fukuyoi* and *Pseudo-nitzschia plurisecta* is 0.06 (Lim et al., 2018) that is comparable to our data for clade 3. However, according to results of mating experiments, all the investigated populations of *U. acus* turned to be sexually compatible (Supplementary Table S2).

Phylogenetic analysis of the fragments gene *cox*1 of strains *U. acus* isolated from a sample in Lake Baikal, 3 km from Baikalskoe settlement (Russia) showed that strains 3B357, 3B355 belong to clade 1, while strain 3B327 belongs to clade 2 (Fig. 3 and Supplementary Table S1). We observe a similar result for strains isolated from r. Erdre, Nantes, (France), where strains 0.0224-OD and 0.0304-YE are in clade 1, and strain 0.0218-OB is in clade 2 (Fig. 3 and Supplementary Table S1). Thus, we mark that different genotypes can be present simultaneously in one population of *U. acus*. Data on the heterogeneity of the *U. acus* population

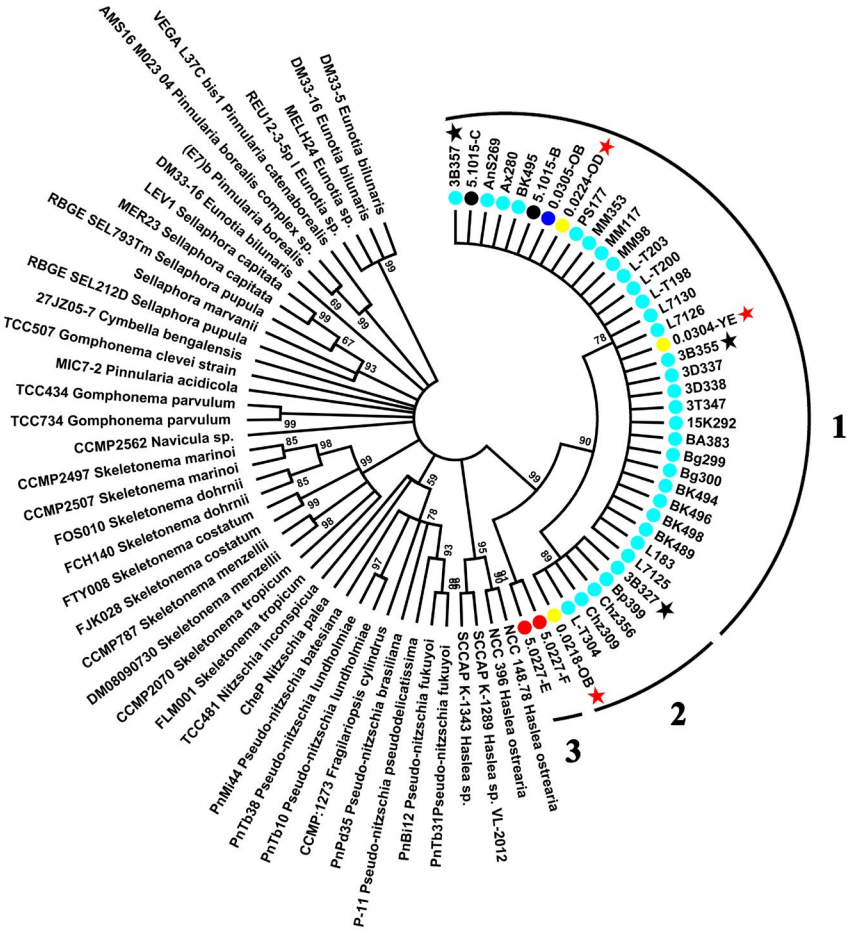

Fig.3. Maximum likelihood phylogenetic tree of *cox*1 using model GTR with gamma-distributed rate variation across sites (MEGA6.0). The sequences obtained in this work are marked with circles of different color: blue – Russia, Lake Baikal; black – Abkhazia, Lake Ritsa; dark blue – France, Le Mans, r. Sarthe; yellow– France, Nantes, r. Erdre; red – Indonesia, Lake Matano. Red stars mark clones belonging to two different clades isolated from the same sample France, Nantes, r. Erdre. Black stars mark strains belonging to two different clades isolated from the same sample in Russia, Lake Baikal.

are consistent with previously received resulting for other diatom species using *cox*1, ITS1-5,8S-ITS2 and microsatellites (Rynearson and Armbrust, 2004; Casteleyn et al., 2009; Evans et al., 2009; Härnström et al., 2011; MacGillivary and Kaczmarska, 2011; Kaczmarska et al., 2014; Tesson et al., 2014; Chen and Rynearson, 2016; Godhe and Rynearson, 2017; Wolf et al., 2021). Previously noted that using metabarcoding of the fragment gene *rbc*L in samples taken from Europe water bodies did not show genetic diversity in species of the genus *Ulnaria* and *Fragillaria* depending on the geographical location (Kahlert et al., 2022). The *cox*1 gene fragment was used to indicate that *U. acus* populations are genetically heterogeneous, and the *rbc*L gene fragment contains an insufficient number of informative sites. At the same time, we must emphasize that these genetic differences do not necessarily suggest reproductive isolation. On the contrarily, following biological species concept (Amato, 2010), it should be recognized that all the investigated populations, widespread on the Eurasian continent, belong to the same species *U. acus*.

In 2008, during the blooming of *Thalassiosira gravida* in the North Atlantic, 165 monoclonal cultures were isolated. Using microsatellite markers, 160 of them were found to be of different genotypes. A high level of genetic diversity was observed throughout the bloom and all taken samples coexisted simultaneously in the same location (Chen and Rynearson, 2016). In the study, the authors using allele-specific quantitative PCR on a mixed culture of *Thalassiosira hyalina* consisting of six different genotypes showed that when temperature and pCO2 change, the ratio of these genotypes also changes. Thus, the authors conclude that the genetic composition of a population may change as a result of intraspecific selection during adaptation to environmental changes (Wolf et al., 2019).

4. Conclusions

The intraspecific variability of diatoms plays an important role in the response of species to environmental changes (light, salinity, temperature, nutrients). At present, the intraspecific variability of *U. acus* has not been sufficiently studied. Our study has shown that analysis of the *cox*1 marker gene can separate *U. acus* into individual genotypes. It has been observed that the strains isolated from one point can belong to two separate clades, and this indicates the heterogeneity of the *U. acus* population in one habitat. Probably, the further use of a more variable genetic marker, such as microsatellites, or an increase in the sample size, will reveal more genotypes in the *U. acus* population. Further studies to identify the correlation between various *U. acus* genotypes and environmental parameters will allow us to approach the answer to question of how the abundance of this species changes during its blooming period.

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Conflict of interest

The authors declare no conflict of interest.

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