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ABSTRACT
Diatoms from the extreme cold habitats in northern areas of East Siberia are still poorly studied. Ecological significance of the lakes of the Pole of Cold of the Northern Hemisphere consists in their pristine environment characterized by special environmental factors, such as the long ice cover period, low temperatures, short vegetative period, low photosynthetically active radiation, deficiency of nutrients. Such water systems can thus be used as sensitive indicators of environmental changes. The diatom *Ulnaria ulna* (Nitzsch) Compère from the genus *Ulnaria* (Kützing) Compère is widespread in fresh and brackish waters of the world, both in plankton and in benthos, which raises questions about the actual distribution of this species and the presence of cryptic species hidden under this name. In this study, monoclonal cultures of *Ulnaria* were isolated from epilithic microphytobenthos collected in Lake Labynkyr, located in the northern Pole of Cold region in the Sakha Republic (Yakutia), and were investigated in detail by light and scanning electron microscopy and phylogenetic analysis of the *rbc*L gene fragments. Cells in culture were able to attach to the substrate with mucilage strands secreted through the pore fields of both valves at one end of the cell. The results of the morphometric and phylogenetic analyses enabled to identify the isolated clones as *Ulnaria pilum* Kulikovskiy & Lange-Bertalot and showed that species morhoplogical variability is larger than originally described.

KEY WORDS
Lake Labynkyr, diatoms, *Ulnaria*, *rbc*L, morphology, phylogeny, Alcian blue staining.

RÉSUMÉ

Description morphologique et phylogénie moléculaire de deux clones de diatomées du genre Ulnaria *(Kützing) Compère isolés d'un lac ultraoligotrophe de l'une des zones les plus froides de l'hémisphère nord, République de Sakha (Yakoutie), Russie.*

Les diatomées des habitats de froid extrême dans les régions du nord de la Sibérie orientale sont encore peu étudiées. L'importance écologique des lacs de l'une des zones les plus froides de l'hémisphère nord réside dans leur environnement vierge caractérisé par des facteurs environnementaux particuliers, tels que la longue période de couverture par la glace, les basses températures, la courte période végétative, le faible rayonnement photosynthétique actif, la carence en nutriments. Ces systèmes d'eau peuvent donc être utilisés comme indicateurs sensibles des changements environnementaux. La diatomée *Ulnaria ulna* (Nitzsch) Compère du genre *Ulnaria* (Kützing) Compère est très répandue dans les eaux douces et saumâtres du monde, tant dans le plancton que dans le benthos, ce qui soulève des questions sur la répartition réelle de cette espèce et la présence d'espèces cryptiques cachées sous ce nom. Dans cette étude, des cultures monoclonales d'*Ulnaria* ont été isolées à partir de microphytobenthos épilithiques collectées dans le lac Labynkyr, situé dans la région de l'une des zones les plus froides en République de Sakha (Yakoutie), et ont été étudiées en détail par microscopie optique et électronique à balayage ainsi que par analyse phylogénétique des fragments du gène *rbc*L. Les cellules en culture ont été capables de se fixer au substrat avec des brins de mucilage sécrétés par les champs de pores des deux valves à une extrémité de la cellule. Les résultats des analyses morphométriques et phylogénétiques ont permis d'identifier les clones isolés comme étant *Ulnaria pilum* Kulikovskiy & Lange-Bertalot et ont montré que la variabilité morphologique des espèces est plus importante que ce qui avait été décrit à l'origine.

MOTS CLÉS
Lac Labynkyr, diatomées, *Ulnaria*, *rbc*L, morphologie, phylogénie, coloration bleue d'Alcian.

INTRODUCTION

Lake Labynkyr is located in the one of the coldest places in the world, in the northern Pole of Cold in the Oymyakon region of the Sakha Republic (Yakutia), Russia. The mean annual air temperature is –16°C, while the average January temperatures range from –40 to –65°C. The lake is covered with ice for eight months a year. The lake of tectonic origin is situated in east of the republic at the junction of the Suntar-Khayat range and the Oymyakon highland at an elevation of 1020 m above sea level. The water of the lake is ultra-fresh and contains a high concentration of dissolved oxygen (not lower than 8.7 mg/L at the bottom) (Tomberg *et al.* 2017). The area of Labynkyr Lake belongs to the territories of Yakutia, which are little-studied and almost uninhabited due to its remoteness and inaccessibility by any transport. There is a little information about the diatom taxa from plankton and benthos in the lake (Komarenko & Vasilyeva 1975; Kopyrina 2012). Using light microscopy, 116 taxa of benthic and planktonic diatoms mainly represented by the genera *Cyclotella* Kützing, *Gomphonema* Ehrenberg, *Eunotia* Ehrenberg, *Navicula* Bory, *Cymbella* Agardh, *Pinnularia* Ehrenberg, and *Fragilaria* Lyngbye have been described. The species of the genus *Ulnaria* (Kützing) Compère has not been previously detected in the lake.

The freshwater genus *Ulnaria* is mostly composed of species previously placed in *Synedra* Ehrenberg (Williams 1986, 2011). *Ulnaria* was typified by *Ulnaria ulna* (Nitzsch) Compère (synonym of *Synedra ulna* (Nitzsch) Ehrenberg) (Compère 2001) and, in the absence of appropriate type material, an epitype was proposed (Lange-Bertalot & Ulrich 2014). A number of new species have recently been described (Morales *et al*. 2014; Kulikovskiy *et al*. 2016; Van De Vijver & Cocquyt 2009; Van De Vijver *et al.* 2017; Liu *et al*. 2017, 2019; Cantonati *et al.* 2018). *Ulnaria ulna* is said to be a widespread species, found in fresh and brackish waters in Europe, Asia, North and South America, Australia, New Zealand (Podunai *et al*. 2017) and Africa (Triest *et al*. 2012; Smith *et al*. 2015). Studies on this species are cited worldwide (Guiry & Guiry 2019), which raises the question concerning its actual distribution and the number of species possibly hidden under this name (Williams 2011). Recently, Kulikovskiy *et al.* (2016) isolated two strains from Lake Baikal which are resembling *U. ulna*. Based on the combined molecular and morphological approach, the authors described *U. pilum* Kulikovskiy & Lange-Bertalot as a new species. In our study, we obtained monoclonal cultures within the genus *Ulnaria* from Lake Labynkyr and studied it in detail with light microscopy (LM), scanning electron microscopy (SEM), and a phylogenetic analysis of the *rbc*L gene fragment.

MATERIAL AND METHODS

The material for the study was taken from epilithic samples from Lake Labynkyr. Stones with fouling were sampled in early June 2017 by divers from a depth of 6 m in the Northern part of the lake (62°33'33"N; 143°36'27"E) at the source of the Labynkyr River (Fig. 1). Microphytobenthos was brushed off and placed in sterile plastic flasks with Diatom Medium (DM) (Thomson *et al.* 1988); enrichment cultures were maintained at a temperature of +10-15°C under natural light.

FIG. 1. — Study site (62°33'33"N; 143°36'27"E), Lake Labynkyr, Sakha Republic (Yakutia), Russia.

Monoclonal diatom cultures were obtained by the selection of individual cells with a micropipette under an inverted light microscope (Axiovert 200; Carl Zeiss, Jena, Germany). Cells were grown in a micro-incubator at + 10°C and illuminated with 16 µEinstein m^{-2} s^{-1}, with a 12:12 h day:night cycle in a 96-well plate with DM. Two monoclonal cultures, *U. pilum* LAB55 and *U. pilum* LAB59, were transferred to a 100 mL Erlenmeyer flasks for further growth.

The clones were studied using a light microscope (Axiostar Plus, Carl Zeiss, Jena, Germany) and a scanning electron microscope (Quanta 200; FEI, Hillsboro, United States). To remove organic matter diatom cells were treated using a standard procedure with 10% HCl and 30% peroxide. Cleaned diatom material was air-dried on coverslips and mounted onto glass slides with Naphrax® for LM. For SEM, samples were sputtered with gold by a vacuum evaporation device (SCD 004; Balzers, Liechtenstein). At least 50 valves of each clone were measured to obtain their morphometric characteristics. To investigate the mucilage excretions using SEM, diatom cells were grown on a cover glass in a Petri dish with DM medium for five days. The cells were then fixed with 2.5% glutaraldehyde solution (Sigma, USA) for 40 min. The fixed cells were washed with phosphate buffer solution (0.1 M, pH 7.4) and dehydrated in an ethanol series with ascending concentrations (30, 50, 70, and 100%) for 5 min in each solution. Then, the cover glass was removed from the Petri dish, filled carefully with a drop of 100% ethanol, and dried in a Critical point dryer (CPD 030, BAL-TEC AG, Balzers, Liechtenstein).

To stain the polysaccharides on the cell surface, the suspension of diatoms was fixed with 2.5% glutaraldehyde and stained with a 0.1% solution of Alcian blue (pH 2.5 and 1.0) for 10 min in a drop on the glass. Then the sample was washed and analysed under an inverted light microscope (Axiovert 200, Carl Zeiss, Jena, Germany).

DNA was isolated from the cell biomass as described previously (Marchenkov *et al.* 2018). The primers for amplification of the gene fragment of the large subunit of ribulose-bisphosphate carboxylase (*rbc*L) (*rbc*L-F: ATGTCTCAATCTGTATCAGAACGG; *rbc*L-R: CAACCTTGTGTAAGTCTCACTATTC) were chosen based on of the multiple alignment of *rbc*L genes and their environment to 13 chloroplast genomes of diatoms: KC509519.1 *Asterionella formosa* Hassall, KC509520.1 *Asterionellopsis glacialis* (Castracane) Round, KJ958484.1 *Cerataulina daemon* (Greville) Hasle, NC_024082.1 *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C.Lewin, AP011960.1 *Fistulifera solaris* S.Mayama, M.Matsumoto, K.Nemoto & T.Tanaka, GU591328.1 *Kryptoperidinium foliaceum* (F.Stein) Lindemann, EF067920.1 *Phaeodactylum tricornutum* Bohlin, KR709240.1 *Pseudo-nitzschia multiserie* (Hasle) Hasle, EF067921.1 *Thalassiosira pseudonana* Hasle & Heimdal, KJ958485.1 *Thalassiosira weissflogii* (Grunow) G.A.Fryxell & Hasle, Z67753.1 *Odontella sinensis* (Greville) Grunow, JQ088178.1 *Synedra acus* Kützing, and *Fragillariopsis cylindrus* (Grunow ex Cleve) Helmcke & Krieger (https://genome.jgi.doe.gov/Fracy1/Fracy1.home.html).

The *rbc*L gene fragment (approximately 1500 bp) was amplified with *Taq* DNA-polymerase (Evrogen, Moscow Russia). The reaction mixture contained 1 × reaction buffer, 1.25 units of *Taq* DNA-polymerase, 0.4 mM mixture of dNTP, 2.8 mM Mg^{2+}, 0.2 µM primers, and 10 ng of gDNA template. The reaction temperature profile was as follows: 95°C for 3 min, 30 cycles (95°C for 20 s, 56.5 °C for 20 s, and 72°C for 1.5 min), and 72°C for 5 min. The reaction products were analysed in a 1% agarose gel, then cleaned from the reaction mixture components by sorption onto magnetic particles AMPure XP (Agencourt, United States) and sequenced directly using the Sanger sequencing method on an automated gene analyzer 3130XL (Applied Biosystems, United States) in the Genomics Core Facility, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia.

The nucleotide sequences were analysed using Vector NTI 11 Academic software (Invitrogen, United States). Alignment of the nucleotide sequences was performed using the CLUSTALW algorithm using the BioEdit 7.2.5 (Hall 1999). For the phylogenetic analysis, a data set from previously published work was used (Kulikovskiy *et al.* 2016). The phylogenetic relationship of *rbc*L gene fragments was reconstructed using

MEGA 6.06 software (Tamura *et al*. 2013) by means of the maximum likelihood method chosen during the search for the best models with a bootstrap support equal to 1000 replicas (Zuckerkand & Pauling 1965). The evolutionary distances were estimated using the General Time Reversible (GTR) method (Lanave *et al*. 1984) with gamma distributed + I equal four. The sequences obtained in this study were deposited in GenBank under the following Accession Numbers: *U. pilum* LAB55 (MK673271) and *U. pilum* LAB59 (MK492923).

RESULTS

Sampling Site

At the time of the study, the lake was completely covered with ice and snow. The thickness of the ice was 1.1 m, the thickness of the snow crust varied from 0.5 to 1 cm, and the temperature of the bottom water varied from 1.3°C to 2°C. Stones and sediments were abundantly colonized by the charophyte *Nitella flexilis* (Linnaeus) C.Agardh (Romanov & Kopyrina 2014), and the diatoms *Hannaea arcus* (Ehrenberg) R.M.Patrick and *Didimosphenia geminata* (Lyngbye) M.Schmidt.

Description, light, scanning electron microscopy and expanded diagnosis of the species

Ulnaria pilum valves linear-lanceolate with almost parallel margins, tapering gradually from the centre to the poles, the distal part of the poles slightly expanded (Fig. 2A). Frustules rectangular in girdle view (Fig. 3A, C), hypovalve with valvocopula and two copulae, as for epivalve (Fig. 3C). Sternum narrow and linear, central area more or less rectangular (Fig. 2B) to oval (Fig. 2C), composed of small striae, sometimes rare, even absent (Fig. 2A-C). Valve length 218-264 µm, breadth 4.4-6.2 µm at centre, 1.7-2.9 µm towards poles, length/breadth ratio 42-53. Striae located transversely, mostly opposite, sometimes not, with 9-13 striae in 10 µm (Fig. 2B, C), consisting of 6-8 areola, the more distal striae with 3-4 areola, areola density 31-50 in 10 µm. Pore fields of the ocellulimbus type, situated at the valve apices consisting of sets of small pores arranged in regular, closely spaced rows parallel to the central valve axis, situated on the pole, set away from the mantle (Fig. 2F). Two apical spines of conical shape parallel to one another overhang valve face margin (Fig. 2F). Rimoportulae located at both poles, at oblique angle relative to sternum (Fig. 2D, E).

The description of the morphological variability of the species *U. pilum* is expanded according to the following features: the breadth of the valve proximally 4.4-6.2 µm and subapically 1.7-2.9 µm, the number of striae 9-13 and areoles 31-50 in 10 µm (Table 1).

Exopolymer production and Alcian blue staining

Alcian blue is used to stains mucous secretions of living organisms (Steedman 1950). This dye forms complexes with sulphated (pH 1.0) and carboxylated (pH 2.5) groups of acidic polysaccharides and stains them blue, and is used to identify transparent exopolymer particles formed from polysaccharides secreted by many species of phytoplankton (Passow & Alldredge 1995). The staining showed that an exopolysaccharide layer is well expressed as a capsule around cells of *U. pilum* and as two mucilage strands at one end of the cell in the area of the apical spines. Due to mucilage strands, the diatom cells attach to the substrate either as a single cell (Fig. 3A) or several cells (15-20) joint to each other to form micro-colonies (Fig. 3B). SEM showed that mucilage strands are present only at one end of the cell (Fig. 3C) and are excreted through the marginal pore field of both valves (Fig. 3A, D).

Phylogenetic analysis data

All the *rbc*L gene fragments obtained in this study from clones *U. pilum* LAB55 and *U. pilum* LAB59 were 99.8% identical to each other. Both clones were resolved within a lineage encompassing other members of the genera *Ulnaria* and *Fragilaria* species with high statistical support (Fig. 4). Within this lineage, they are divided into two branches: *U. pilum* LAB55 and *U. pilum* LAB59 form one highly supported node with *U. pilum*, *U. ulna*, *U. ferefusiformis* Kulikovskiy & Lange-Bertalot, and *U. acus* (Kützing) Aboal; and *Fragilaria*-like species are grouped into the second clade. A similar clade formation with such a distribution of species into two sister groups has been previously shown (Kulikovskiy *et al*. 2016).

DISCUSSION

The two clones isolated from benthic samples of Lake Labynkyr were assigned to the species *U. pilum* by combining the morphometric and sequence data. At the same time the combination of characteristics of these clones is resembling *U. ulna*. Specimens of the epitype of *U. ulna* are needle-shaped, length 230-320 µm, breadth 6.3-7.7 µm in the central part, 3-4 µm at the ends, stria density 9.2-10.4 in 10 µm, areola density 28-33 in 10 µm (Lange-Bertalot & Ulrich 2014). In light of these data *U. ulna* is distinguished from *U. pilum* LAB55 and *U. pilum* LAB59 by having wider valves breadth proximally and subapically, less stria – and areola densities. A greater range of striae and areolae densities relative to the epitype of *U. ulna* was previously detected in specimens from populations in Lake Mull in Scotland (Williams 1986), as well as in clones isolated from Lake Hubsugul (Mongolia), the Biya River, Ob River, Aduy River (Russia), and in natural populations in Belgium, France, and the United Kingdom (Podunai *et al*. 2017).

Ulnaria pilum was originally described based on two strains isolated from the plankton samples of Lake Baikal (Kulikovskiy *et al.* 2016). The described species is resembling the taxon already known from Lake Baikal: *Ulnaria danica* (Kützing) Compère & Bukhtiyarova (synnym of *S. ulna* subsp. *danica* (Kützing) Skabitschewsky) in terms of valve breadth, but it differs in having a fewer striae in 10 µm. Kulikovskiy *et al.* (2016) gave measurements of *U. pilum* in the range of 218-295 µm at length, 5.6-6.3 µm at width proximally, 2.0-2.2 µm at width subapically, and 10.0-11.5 striae and 35-40 areolae in 10 µm. Our data resemble the description of *U. pilum*,

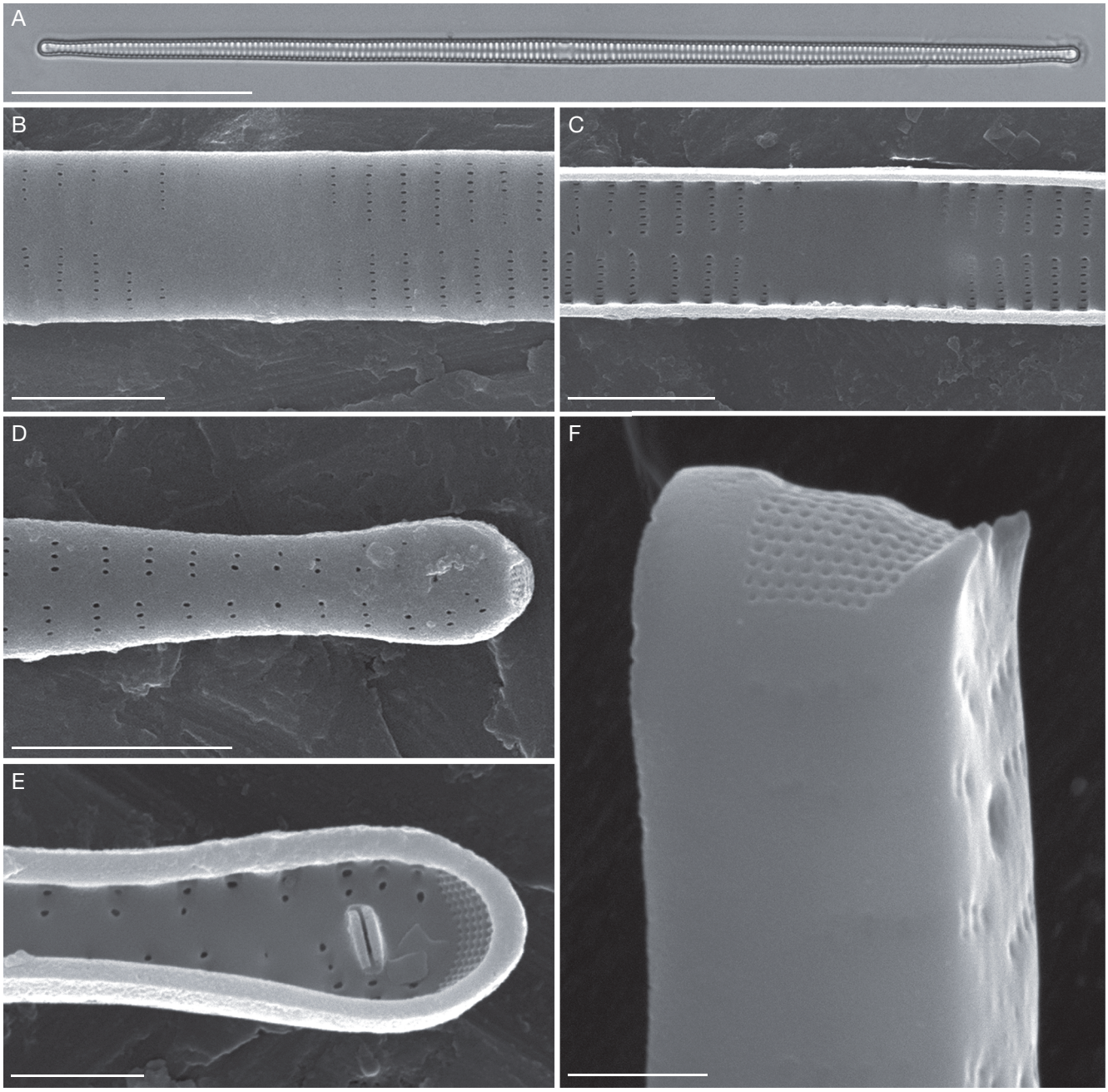

Fig. 2. — *Ulnaria pilum* Kulikovskiy & Lange-Bertalot LAB59: **A**, LM; **B**, SEM, external view and central area; **C**, internal view and central area; **D**, SEM, external view and the valve apex; **E**, internal view and the valve apex, rimoportula is located at an oblique angle relative to the central sternum; **F**, SEM, external view, two small spines at the apex. Scale bars: A, 50 µm; B-E, 5 µm; F, 1 µm.

but differ in a wider range of proximal and distal widths, and the number of striae and areolae in 10 µm (Table 1). Using molecular data from a number of species from *Ulnaria* and *Fragilaria* species, results show them to be sister to one another (Fig. 4), found in the same lineage but independent of one another (Kulikovskiy *et al*. 2016). The *rbc*L gene fragments of *U. pilum* LAB55 and *U. pilum* LAB59 have the highest similarity (99.8 %) to each other and to *U. pilum* B160 KR336760 strain. They also have high similarities to and form a clade with *U. ulna* (Theriot *et al*. 2010; Kermarrec *et al*. 2013), *U. acus* (Galachyants *et al*. 2012) and *U. ferefusiformis* (Kulikovskiy *et al*. 2016).

It is known that, depending on the mobility of algae and environmental conditions, benthic and planctonic diatoms produce various amounts of extracellular polymeric substances, mainly polysaccharides, which enable the diatoms to develop a variety of colonial life forms and actively grow over various surfaces (Molino & Wetherbee 2008; Aumeier & Menzel 2012; Bosak *et al.* 2012). In microphytobenthos, diatoms are represented by a wide variety of forms associated with the substrate; they move along the substrate or attach to it by means of mucilage strands, tubes, or pads. The isolated clones of *U. pilum* LAB55 and *U. pilum* LAB59 are epilithic forms. The cells of the species investigated are able to actively

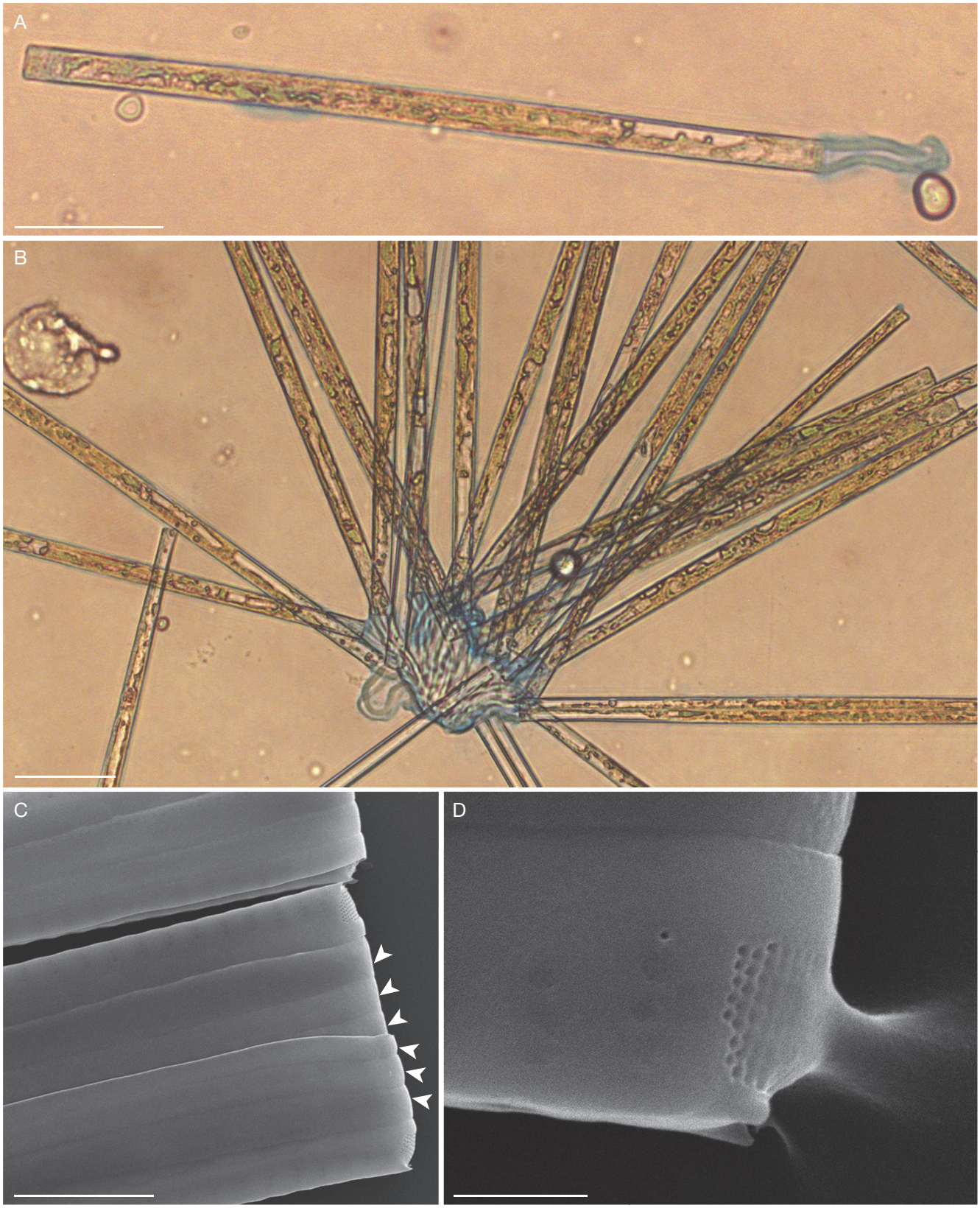

Fig. 3. — Cells in the culture *Ulnaria pilum* Kulikovskiy & Lange-Bertalot LAB55 attached to substrate: **A**, LM, mucilage strands are stained by Alcian blue in single cell; **B**, LM, cell aggregates; **C**, SEM, there are no traces of mucilage on the unattached ends of cells, tree closed girdle bands of each valve are pointed by **arrowheads**; **D**, SEM, attachment occurs with mucilage excreted through the pore field. Scale bars: A, B, 50 µm; C, 5 µm; D, 1 µm.

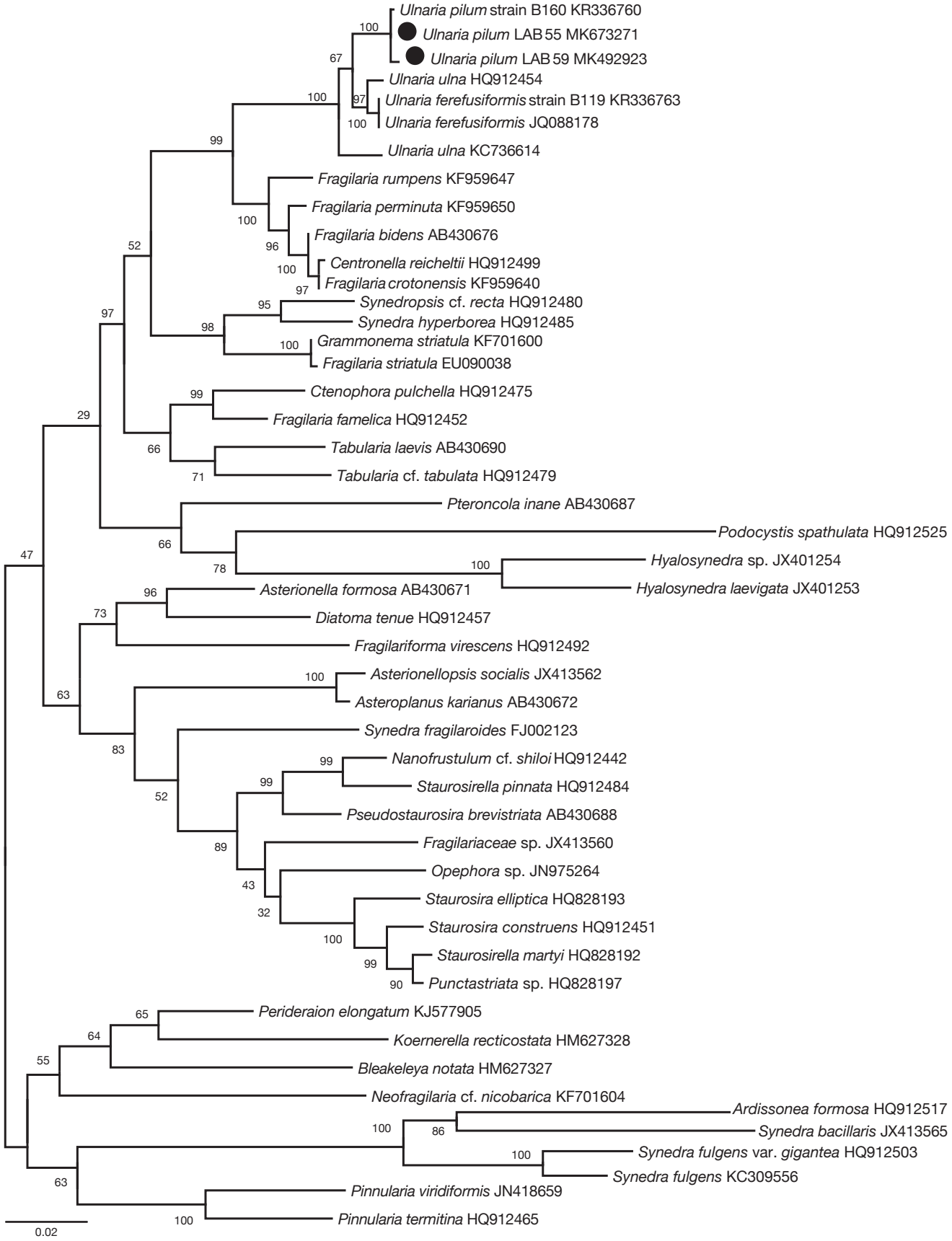

FIG. 4. — Phylogenetic analysis of *rbc*L gene fragments, carried out by the maximum likelihood method. In the tree nodes there are bootstrap values obtained for n = 1000 replicas. Evolution distances are determined by the GTR method with Gamma distributed equal to four. • Marked sequences obtained in this work.

Table 1. — The range of morphological features measurement of the studied taxa (expanding of morphometric parameters).

Taxons	Length (µm)	Breadth proximally (µm)	Breadth subapically (µm)	Striae in 10 µm	Areolae in 10 µm	Resources
U. ulna (Nitzsch) Compère	230-320	6.3-7.7	3.0-4.0	9.2-10.4	28-33	Lange-Bertalot & Ulrich 2014
U. pilum M.Kulikovskiy & H.Lange-Bertalot	218-295	5.6-6.3	2.0-2.2	10-11.5	35-40	Kulikovskiy *et al*. 2016
U. pilum LAB55	258-264	4.4-6.2	1.9-2.9	9.0-11.0	36-50	Present study
U. pilum LAB59	218-241	5.2-5.8	1.7-2.4	10.0-13.0	31-48	Present study
Expanded diagnosis of the species *U. pilum*	218-295	4.4-6.3	1.7-2.9	9.0-13.0	31-50	

overgrow the surface, due to attachment to the substrate with the help of two mucilage strands, which are excreted from the pore field on the apical valve ends. Colonies can be formed in the same way. For diatoms of the genus *Ulnaria*, the ability to live an attached lifestyle using mucilage secretions of various shapes is known (Morales *et al*. 2014; Liu *et al*. 2017). In addition, it has previously added reference that "in the natural populations, *Ulnaria ulna* grew either as single cells or in clusters of cells being attached to the substratum by common mucilage pad. In cultures, if conditions were good, rapidly growing clones formed loose colonies on the bottom of Petri dishes" (Podunay *et al*. 2014). However the original description of *U. pilum* (Kulikovskiy *et al.* 2016) did not include the ability to form colonies and attach to the substrate using mucous cords in isolated strains.

Thus, the study extends the boundaries of the genus *Ulnaria* distribution and contributes to our understanding of its habitat. The isolated clones from oligotrophic Lake Labynkyr are epilithic forms of the genus *Ulnaria* growing on a rocky substrate. Their ability to attach to the surface of the substrate and to form colonies using two mucilage strands excreted from the pore field at the valve's apical end was demonstrated. According to the results of the morphometric and phylogenetic analyses, the clones have been assigned to the species *U. pilum.* Our data allow an expansion of the range of morphological variability of the species with respect to the valve proximally and subapical width and the striae and areoles number in 10 µm (Table 1).

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