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Morphological and molecular evidence support description of two new diatom species from the genus *Ulnaria* in Lake Baikal

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Abstract: Light and scanning electron microscopical observations are made on two non–raphid pennate species from Lake Baikal, Russia. Based on their morphologies, they are assigned to the genus *Ulnaria*. Molecular data from *rbc*L sequences were also generated from cultures of these two taxa. Combined morphological and molecular data suggest one of the species is the same as a species commonly referred to as *Synedra acus*, a species commonly reported from Lake Baikal and used as a model species for studies on valve silicification and nanotechnology applications. The second species is similar to *Ulnaria ulna*. Both taxa are important players in the ecology of Lake Baikal. Based on the combined molecular and morphological approach, and comparison of the species with recently designated types for both *U. ulna* and *U. acus*, we conclude that the two species are not synonymous with either *U. acus* or *U. ulna*, or other known species, and present full descriptions of them as species new to science.

Key words: ancient lakes, diatoms, Lake Baikal, morphology, new species, phylogeny, *Ulnaria*

Introduction

Lake Baikal is a hotspot of endemic diatoms (Kulikovskiy et al. 2012b, 2015b; Kulikovskiy & Kociolek 2014; Vishnyakov et al. 2014). Comprehensive study of diatoms from this lake reveals remarkable taxonomic diversity, and a few hundred new species were described (Kociolek et al. 2013; Kulikovskiy et al. 2011, 2012a,c, 2013, 2014a,b, 2015a). These investigations relate to our understanding of species diversity in the benthic diatom community. Planktonic diatoms were studied previously and data about their diversity were summarized in Popovskaya et al. (2002, 2011). These authors described planktonic diatom communities in Lake Baikal, including centric diatoms (*Aulacoseira* Thwaites, *Cyclotella* (Kützing) Brébisson and *Stephanodiscus* Ehrenberg), non–raphid pennate taxa from the genera *Fragilaria* Lyngbye, *Synedra* Ehrenberg, *Asterionella* Hassall, *Hannaea* Patrick, *Diatoma* Bory, *Meridion* Agardh, *Tabellaria* Ehrenberg, and *Belonastrum* (Lemmermann) Round et Maidana, and the raphid genera *Nitzschia* Hassall and *Cymatopleura* Smith (Popovskaya et al. 2002, 2011).

Recently there has been a revision of the planktonic species in the genera *Fragilaria* and *Ulnaria* (Kützing) Compère, including the typification of several species (Lange–Bertalot & Ulrich 2014). Species of the non–raphid pennate genus *Ulnaria* play important roles in phytoplankton communities of Lake Baikal (Popovskaya et al. 2002, 2011). They dominate in some years generating so–called "*Synedra* years" versus centric diatom–dominated assemblages ("*Aulacoseira* years") (Popovskaya & Genkal 1998). Previously all *Ulnaria* species were included to the large genus *Synedra* (Compère 2001). Skabitschewsky (1960) was the first to prepare a revision of planktonic diatoms from Lake Baikal. Popovskaya et al. (2002, 2011) fully accepted his opinion about taxonomic diversity for this group of diatoms in the lake. These authors reported four taxa, and in terms of currently accepted taxonomic position, these four taxa would be assigned to the genus *Ulnaria*, but commonly they are referred to as:

Synedra acus Kützing, *S. acus* var. *radians* (Kützing) Hustedt, *S. ulna* (Nitzsch) Ehrenberg, and *S. ulna* var. *danica* (Kützing) Grunow (Skabitschewsky 1960; Popovskaya et al. 2011). A word here about what could be confusion regarding the nomenclature of some of these taxa. Skabitchewsky (1960) commented that he preferred to use the designation "subspecies" versus variety. Skabitchewsky did not intend to create new names with his designation of subsp., so we use the older trinomial for our designations of *S. acus* var. *radians* and *S. ulna* var. *danica* (var. instead of subsp.).

Members of the genus *Ulnaria* (under the name *Synedra*) from Lake Baikal have, for a long time, been used as model freshwater organisms for various studies due to their relatively easy cultivation. In particular, there are numerous studies about molecular biosilification mechanisms in vivo (Grachev et al. 2005; Safonova et al. 2007; Basharina et al. 2012; Annenkov et al. 2013), design of new experimental approaches to study biosilification (Annenkov et al. 2010, 2013), and the role of the cytoskeleton in the morphogenesis of siliceous frustule (Kharitonenko et al. 2015). The investigations help us in understanding chemical principles of silification and how such process occur in living cells. This knowledge contributes to various nanotechnological applications (Losic et al. 2009). Moreover *S. acus* is the first freshwater diatom with completely documented mitochondrial and chloroplast genomes (Ravin et al. 2010; Galachyants et al. 2012). Its interaction with bacterial community is also under investigation (Zakharova et al. 2010). Finally, given the importance of the non–raphid pennate planktonic diatoms to the ecosystem structure and function in Lake Baikal (Hampton et al. 2014), including their responses to climate and other anthropogenic environmental changes (Mackay et al. 1998; Moore et al. 2009), it becomes clear how important it is to identify the taxa correctly and communicate efficiently and effectively via biological names (de Queiroz & Gauthier 1994).

The aim of this work is to produce the first revision of planktonic diatoms from the genus *Ulnaria,* including combined molecular and morphological data, to understand the species occurring in Lake Baikal.

Material and Methods

Samples were collected from Lake Baikal by M.S. Kulikovskiy in July, 2012, and strains were isolated by E.S. Gusev. Strains of *Ulnaria ferefusiformis* sp. nov. were isolated from the following samples: B114, B118 – plankton near Bolshoi Ushkaniy Island, 28.07.2012, 53°51'129"N, 108°35'795"E; B119 – plankton from Solnechnaya Bay, 29.07.2012, 54°17'327"N, 108°29'106"E. Strains of *Ulnaria pilum* sp. nov. were isolated from the following samples: B155 – plankton from Ludarskaya Bay, 30.07.2012, 55°22'429"N, 109°12'652"E; B160 – plankton from Sludianskaya Bay, 30.07.2012, 55°28'078"N, 109°09'828"E. Water temperature was about 17 °C and conductivity 111 $\mu S.cm^{-1}$ in studied places. Water mineralization and temperature measurements were performed using the Hanna Combo (HI 98129) device, Hanna Instruments, Inc., USA.

A subsample of each collection was added to WC liquid medium (Guillard & Lorenzen 1972). Monoclonal strains were established by micropipetting single cells under an inverted microscope. Nonaxenic unialgal cultures were maintained in WC liquid medium at 10 °C in a growth chamber with a 12:12 h light/dark photoperiod.

Strains for LM and SEM investigations have been processed by means of a standard procedure involving, treatment with 10% HCl and concentrated hydrogen peroxide. After treatment the sample was washed with deionized water. Permanent diatom preparations have been mounted in Naphrax®. Light microscopic (LM) observations have been performed by means of a Zeiss Axiovert microscope equipped with oil immersion objective (×100/n.a.1.4, DIC). Valve ultrastructure was examined with a JSM–6510LV field emission scanning electron microscope (Borok, Russia).

Total DNA of monoclonal cultures was extracted using a NucleoSpin® Plant II Mini kit (Macherey–Nagel, Germany) according to the manufacturer's protocol. Fragments of partial *rbc*L plastid genes (912 bp) were amplified using primers from Abarca et al. (2014) for *rbc*L fragments

For amplification of *rbc*L we used reaction volumes of 25 µl, with 10 ng of gDNA, 0.8 mM of forward primer, 0.8 mM of internal reverse primer, 3 mM $MgCl_2$, 0.5 M Betaine monohydrate, 250 µM dNTP, 10 mM Tris–HCl, 50 mM KCl, 2.5 nl Tween 20 and 0.75 units of PeqLab Hot Taq DNA Polymerase. The conditions of amplification were: an initial denaturation of 2 min at 94 °C, followed by 40 cycles at 94 °C for denaturation (1 min), 52.8 °C for annealing (45 s) and 72 ºC for extension (1.5 min), and a final extension of 10 min at 72 °C. (Abarca et al. 2014).

The obtained sequences were edited manually and assembled using BioEdit v7.1.3 (Hall 1999). Analysis of the genetic differences between the sequences was performed with MEGA v.6.1 (Tamura et al. 2011). Newly determined sequences and DNA fragments from 50 other members of the group "clade II non–raphid pennate pennate diatoms, which were downloaded from GenBank (taxa and Accession Numbers are given in the tree, Fig. 31), were included in the alignment. Two raphid diatoms (Pinnularias) were chosen as outgroup. The alignment was constructed using Mafft v6.952 based on the G–INS–I model with default parameters (Katoh & Toh 2010). Bayesian information criterion (BIC) implemented in jModelTest 2.1.1 (Darriba et al. 2012) indicated, that the General Time Reversible (GTR) 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 *rbc*L DNA alignment. Phylogenies of these sequences were constructed based on this model using Bayesian inference (BI) and Maximum Likelihood (ML) analysis.

BI analysis was conducted with MrBayes–3.1.2 (Ronquist & Huelsenbeck 2003) and was run with 5 Markov chains (four heated chains, one cold) for 2·106 generations and 2 independent runs in each analysis. Trees were sampled every 100th generation, the first 25% samples were discarded as ''burn–in''. ML analysis was conducted using GARLI 2.0 on–line program (Bazinet & Cummings 2008). The tree was constructed with collapsing the branch into a polytomy. It is important when bootstrapping, since no support should be given to a branch that doesn't really exist. Subtree–pruning–regrafting (SPR) first tree swapping algorithm and non–para-

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metric bootstrap analysis with 100 replicates were used. The statistical support values were drawn on the ML tree visualized using iTOL (http://itol.embl.de/).

Sequences from *Ulnaria* obtained in this study were deposited to GenBank under following Accession Numbers *Ulnaria pilum* strain B155 (KR336759), *Ulnaria pilum* strain B160 (KR336760), *Ulnaria ferefusiformis* strain B114 (KR336761), *Ulnaria ferefusiformis* strain B118 (KR336762), *Ulnaria ferefusiformis* strain B119 (KR336763).

Results and Discussion

Formal descriptions of new species

***Ulnaria ferefusiformis* Kulikovskiy, Lange–Bertalot sp. nov. (Figs 1–13)**

Description

Light microscopy (Figs 1–8): Valves approximately fusiform, i.e. weakly spindle–shaped, with parallel or slightly concave margins proximally slightly ca. halfway to the ends, then narrowing more strongly and finally tapering towards subcapitate ends. Length 76–152 µm, breadth proximally 4.0–4.8 µm, distally below the apices 0.7–1.2 µm, at apices 1.4–1.6 µm. Axial area very narrow distally, becoming wider proximally to ca. 0.3 µm. Central area somewhat indistinctly defined due to a more or less clear, but weak, ghost striae. Other striae in alternate or opposite position, 12–14, rarely up to 15 in 10 µm, becoming slightly more densely–spaced near the ends. Areolae not discernible.

Scanning electron microscopy (Figs 9–13): Areolae about 50 in 10 µm, lacking or reduced in number in the central area; distally strongly reduced to two, finally a single one on either side of the valve face and an additional one on valve mantles. Poles are covered by seriate pore fields (ocellulimbi); each pole with a large rimoportula internally. External opening of the rimoportula is a simple circular poroid. Marginal spines lacking.

Etymology: The Latin epithet means approximately spindle–shaped.

Type locality: Russia, Lake Baikal, sample B114 (53°51'129"N, 108°35'795"E), leg. M. Kulikovskiy, coll. date 28/07/2012.

Holotype (designated here): slide B114m (IBIW, Russia).

Iconotype: Fig. 3.

***Ulnaria pilum* Kulikovskiy, Lange–Bertalot sp. nov. (Figs 14–30)**

Description

Light microscopy (Figs 14–24): Valves needle–shaped, tapering from the central part with almost parallel margins towards the ends, however not continually, since distal parts are drawn out, i.e. more strongly narrowed below the subcapitate apices. Length 218–295 µm, breadth proximally 5.6–6.3 µm, subapically 2.0–2.2 µm. Length–to–breadth ratio is ca. 40–50. Axial area very narrow, linear. Central area distinct, rectangular, variable in length, ghost striae scarce or missing. Striae 10.0–11.5 in 10 µm; position predominantly opposite proximally, predominantly alternate distally. Areolae difficult to discern.

Scanning electron microscopy (Figs 25–30): Large ocellimbi are present on the mantle as are, two apical spines. Rimoportula are present at both poles, similar to most other taxa of the *Ulnaria ulna* cluster. Marginal spines lacking. Areolae 35–40 in 10 µm. Proximal striae of the valve face consist of 6–9 areolae, however distal striae have at most three or two areolae or a single areola on either side. Other features as described for *U. baicalensis*.

Etymology: The Latin *pilum*, in English javelin, was

Table 1. Measurements of main quantitative features of studied taxa.

Taxon	Length (µm)	Breadth (µm)	Striae in 10 µm	Resources
Ulnaria ferefusiformis sp. nov.	76–152	4–4.8	12–14	This study
Synedra acus Kützing	90–300	4.5–5	12–14	Skabitschewsky 1960
Synedra acus Kützing	90–250	4–5	12–14	Popovskaya et al. 2002
Ulnaria pilum sp. nov.	218–295	5.6–6.3	10–11.5	This study
Synedra ulna var. *danica* (Kützing) Grunow	89–480	4.5–6.4	8–11	Skabitschewsky 1960
Synedra ulna var. *danica* (Kützing) Grunow	235–442	8–10	8–10	Popovskaya et al. 2002
Synedra ulna var. *danica* (Kützing) Grunow*	–	5.4	8	Popovskaya et al. 2002, 60: 2, 3
Synedra acus var. *radians* (Kützing) Hustedt	200–500	2–5	12–18	Skabitschewsky 1960
Synedra acus var. *radians* (Kützing) Hustedt	142–288	2.2–5	11–22	Popovskaya et al. 2002
Synedra acus var. *radians* (Kützing Hustedt	142–288	2.8–5	11–18	Popovskaya & Genkal 1998

* counted by us from pictures given in publications

an important weapon of the infantry of the Roman Empire.

Type locality: Russia, Lake Baikal, sample B155 (55°22'429"N, 109°12'652"E), leg. M. Kulikovskiy, coll. date 30/07/2012.

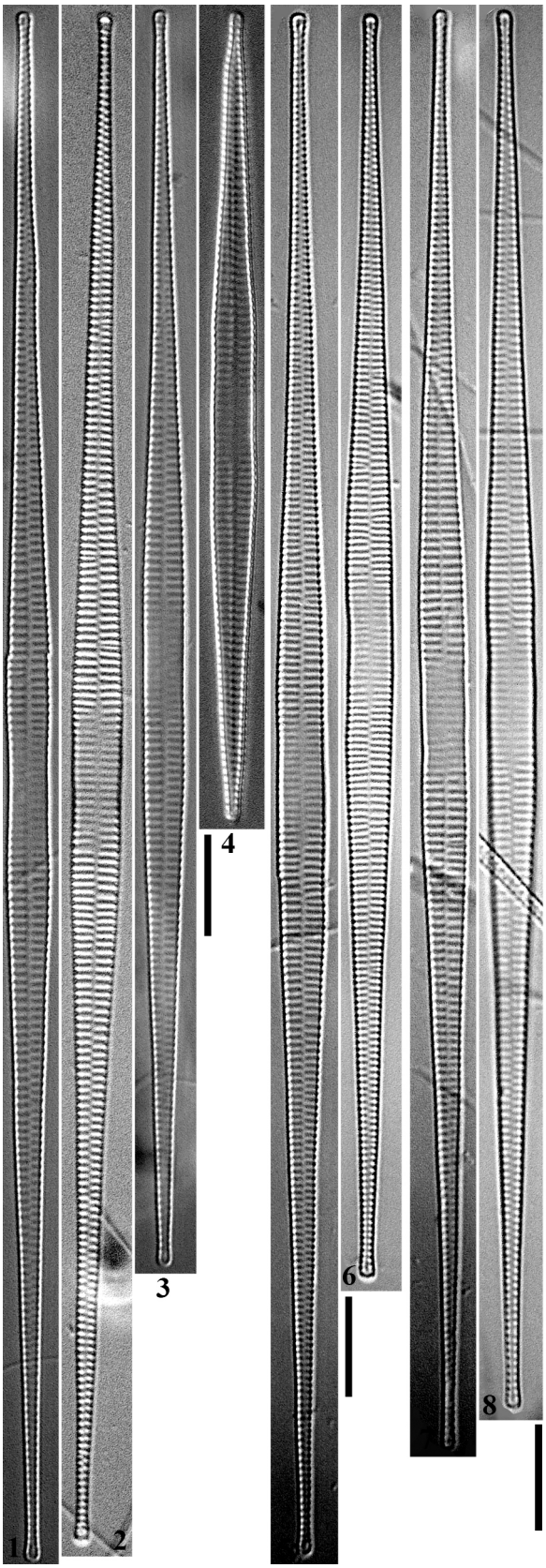
Holotype (designated here): slide B155m (IBIW, Russia).

Iconotype: Fig. 14.

Phylogenetic analysis data

All the *rbc*L DNA fragments of the studied *Ulnaria ferefusiformis* strains were identical to each other and to the diatom named "*Synedra acus"*, which was collected previously from Lake Baikal (Galachyants et al. 2012) and had the same morphology (see below) as *U. ferefusiformis* sp. nov. Thus, with this correspondence between morphology and molecular sequence data it seems they are the same species. DNA fragment of *Ulnaria ulna* differs from them by 0.7% (nine substitutions). The two strains of *Ulnaria pilum* sp. nov. investigated here have identical *rbc*L gene fragments, which clearly differ from partial *rbc*L gene of *U. ferefusiformis* sp. nov. Both studied species were included in clade I (Fig. 31). Within that clade *U. pilum* sp. nov. forms one highly supported clade with two strains of *U. ulna*, *U. ferefusiformis* sp. nov. strains and *"S. acus"* from Baikal (Fig. 31, clade Ia). This *Ulnaria*–like clade forms one group with *Fragilaria*–like species with high statistical support (ML 74, BI 100, clade I). These species clearly differ from any known *Synedra* species according to the phylogenetic tree. It should be mentioned that the two strains of *U. ulna*, which were included in the analysis, did not cluster together. This can be explained because of biases (artefacts during sequencing, in particular Next Generation Sequencing of the strain TCC 520; Kermarrec et al. 2013), wrong identification of one or both strains based on morphology, and / or the possibility of cryptic diversity within *U. ulna*.

The phylogenetic tree based on partial *rbc*L gene includes members of non–raphid pennate diatoms and outgroup from the raphid diatoms (*Pinnularia termitina* (Ehrenberg) Fricke and *Pinnulatia viridiformis* Krammer). Within non–raphid diatoms four species from the genus *Synedra* formed a distinct, separate clade with high statistical support (100 ML; 100 BI, Figure 31, VI group). Other non–raphid diatoms belonged to one clade but with moderate support (ML 64, BI 97, Figure 31). Within this clade there are five relatively big and highly supported groups (I – V groups, Figure 31) and certain species outside them. The phylogenetic relations between I – V groups and other non–raphid diatoms from this study stay unresolved. Similar results were obtained in the previous studies (e.g., Theriot et al. 2010). Investigations of both much more genetic data and diatom samples are needed for the identification of evolutionary relations between these non–raphid diatom genera. Anyway our



Figs 1–8. *Ulnaria ferefusiformis* sp. nov. LM: (1–4) valves from strain B114, (5–6) valves from strain B118, (7–8) valves from strain B119. Scale bars 10 μm.

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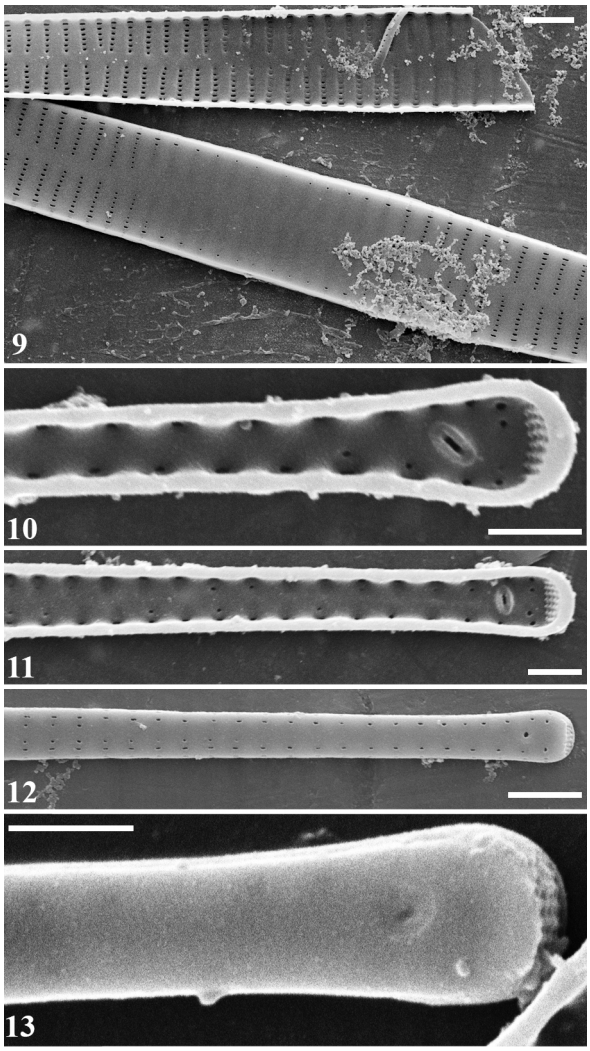
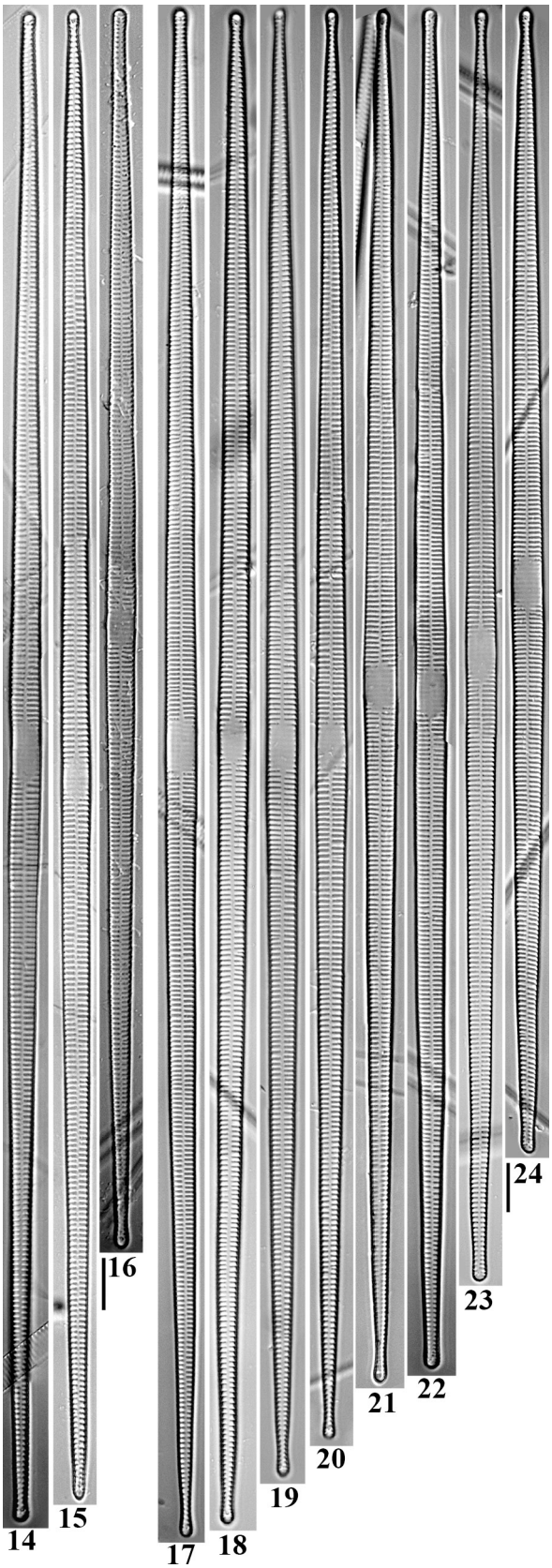


Figs 9–13. *Ulnaria ferefusiformis* sp. nov. SEM: (9, 12, 13) are external views, (10, 11) are internal views; (9) central are with "ghost striae" evident in the middle of the valve. Striae are uniseriate and alternate on either side of the evident sternum. (10, 11) valve apices with distinct rimoportulae and ocellulimbi present. Round areolae are scattered across the valve. (12, 13) apices, with round areolae and round opening of rimoportula present. Ocellulimbus is restricted to the valve mantle. Scale bar 2 µm (9, 12), 1 µm (10, 11, 13).

molecular data with using more species shown that *Ulnaria* is a close related but independent branch from *Fragilaria* sensu stricto, that supported data given by Medlin et al. (2012).

Taxonomy of new species

The species that most resembles *Ulnaria ferefusiformis* sp. nov. is *U. acus* (Kützing) Aboal 2003 according to various "second hand" concepts of other authors, except that of Kützing (1844), original describer of this taxon. The basionym *Synedra acus* Kützing 1844 has been neotypified recently by Lange–Bertalot & Ulrich (2014). Since the holotype material from "Hamburger Moor", the only location mentioned in Kützing's protologue. is not available the only other Herbarium number authorized by Kützing containing

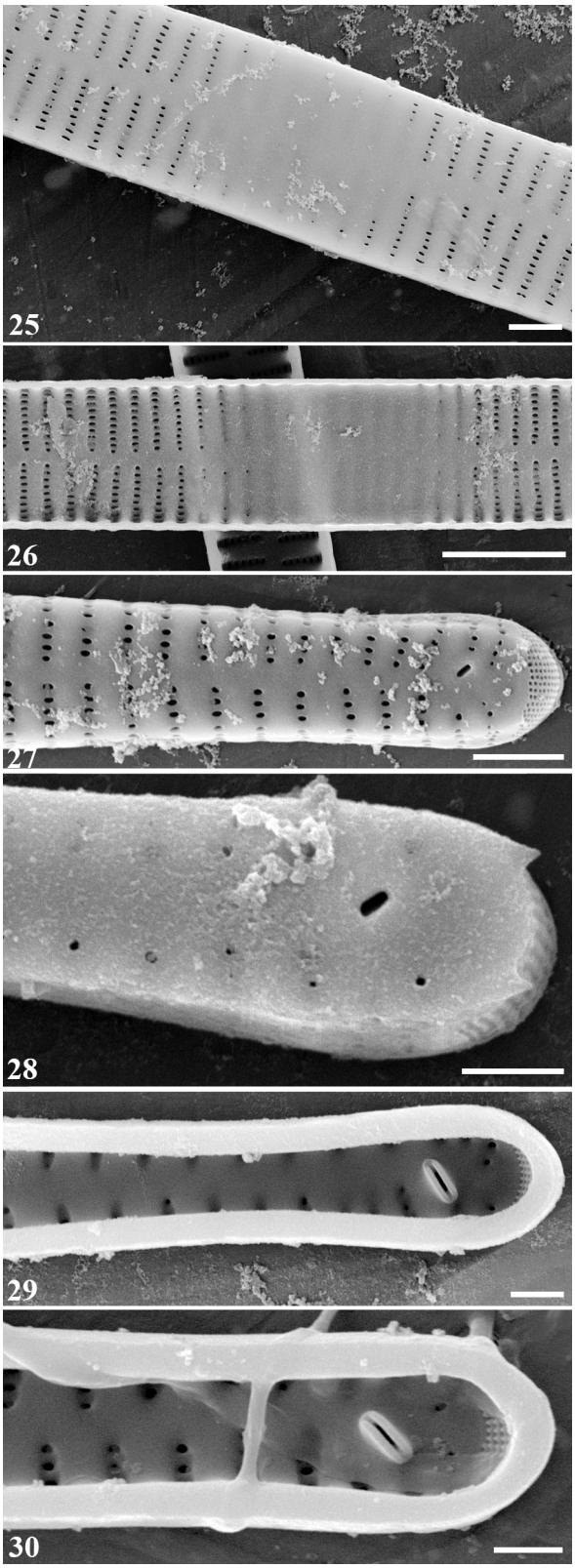


Figs 14–24. *Ulnaria pilum* sp. nov. LM: (14–16) valves from strain B155, (17–24) valves from strain B160. Scale bar 10 µm.

Synedra acus is no. 401 from Falaise, France, collected and sent to Kützing by De Brébisson. It is prepared in slide B.M. no. 18305 with several specimens conforming to Kützing's protologue. However, although identified by Kützing as *S. acus* it cannot be lectotypified but just neotypified, since this material was not listed in the original description by him. These specimens are 95–102 µm long, 4.6–5.0 µm broad proximally, resulting in a length–to–breadth ratio of about 20. Stria density is 11.5–13.0 in 10 µm proximally, becoming 13–15 near the ends. *Ulnaria acus* differs from *U. ferefusiformis* sp. nov. mainly by valve outlines which are not fusiform. Outline more similar to our new species can be found in *Ulnaria grunowii* Lange–Bertalot & Ulrich (2014), but these are narrower on average (2.7–4.5 µm; Lange–Bertalot & Ulrich 2014). The valves taper gradually from the center to the ends without a parallel or concave central part. The stria density of this species is similar near the cener of the valve, being 12–13 in 10 µm, but becomes more dense towards the apices, 14–16 / 10 µm.

Ulnaria ferefusiformis sp. nov. is similar to Skabtichewsky's (1960) and Popovskaya et al.'s (2002) concept of *Synedra acus* from Lake Baikal. Popovskaya et al. (2002) gave the measurements for *Synedra acus* with a range for length of 90–250 µm and of 4–5 µm for breadth. He recorded 12–14 striae in 10 µm. These data agree with measurements as given by Skabitshewsky (1960) with the same length and number of striae and breadth 4.5–5 µm. Our data about *U. ferefusiformis* sp. nov. conforms with data cited above, except it is shorter, with length measured at 76–152 µm (see Table 1). Drawing given by Skabitschewsky (1960: 86) and TEM pictures from Popovskaya et al. (2002: 56, 1–3) conform to the valve morphology studied by us on the basis valve morphology and quantitative characters (see Table 1).

U. pilum sp. nov. is characterized by very narrow and longer valves. On the basis of these features, our new described species is similar to two taxa known from Lake Baikal, these are *Synedra ulna* var. *danica* (Kützing) Grunow (=*S. ulna* subsp. *danica* (Kützing) Skabitschewsky) and *S. acus* var. *radians* (Kützing) Hustedt (=*S. acus* subsp. *radians* (Kützing) Skabitschewsky). These two infraspecific taxa share the same longer valves with *U. pilum* sp. nov. However, published data about *S. ulna* var. *danica* and *S. acus* var. *radians* do not allow to us associate these taxa with *U. pilum* sp. nov. as they differ with respect to valve breadth and striae number (see Table 1). *Synedra ulna* var. *danica* is similar to *U. pilum* sp. nov. in terms of valve breadth, but our species differs by having a fewer striae per 10 µm. Report of wider valves of *S. ulna* var. *danica* given in Popovskaya et al. (2002) as compared to other references (see Table 1) is possibly a mistake, and this contention is supported by our measurement of valves from published resources (see Table 1). *Synedra acus* var. *radians* differs from *U. pilum* by wider valves



Figs 25–30. *Ulnaria pilum* sp. nov. SEM: (25, 27, 28) external views. (26, 29, 30) internal views. (25) central are showing "ghost straie" and rectangular area. Uniseriate striae are opposite across the distinct central sternum. (26) central area. Unornamented central are is distinct. Areolae are elliptical in shape. (27, 28) apices elongated opening of rimoportulae angled relative to the central sternum. Pore field extends barely from the mantle onto the valve face and has rounded porelli. Two small spines are evident at the apex in Fig. 28. (29, 30) apices, with thickened, raised rimoportulae positioned at an oblique angle relative to the central sternum. A pseudoseptum appears to be present. Porelli are located on the mantle of the valve. Scale bar 2 µm (25, 27), 5 µm (26), 1 µm (28, 29, 30).

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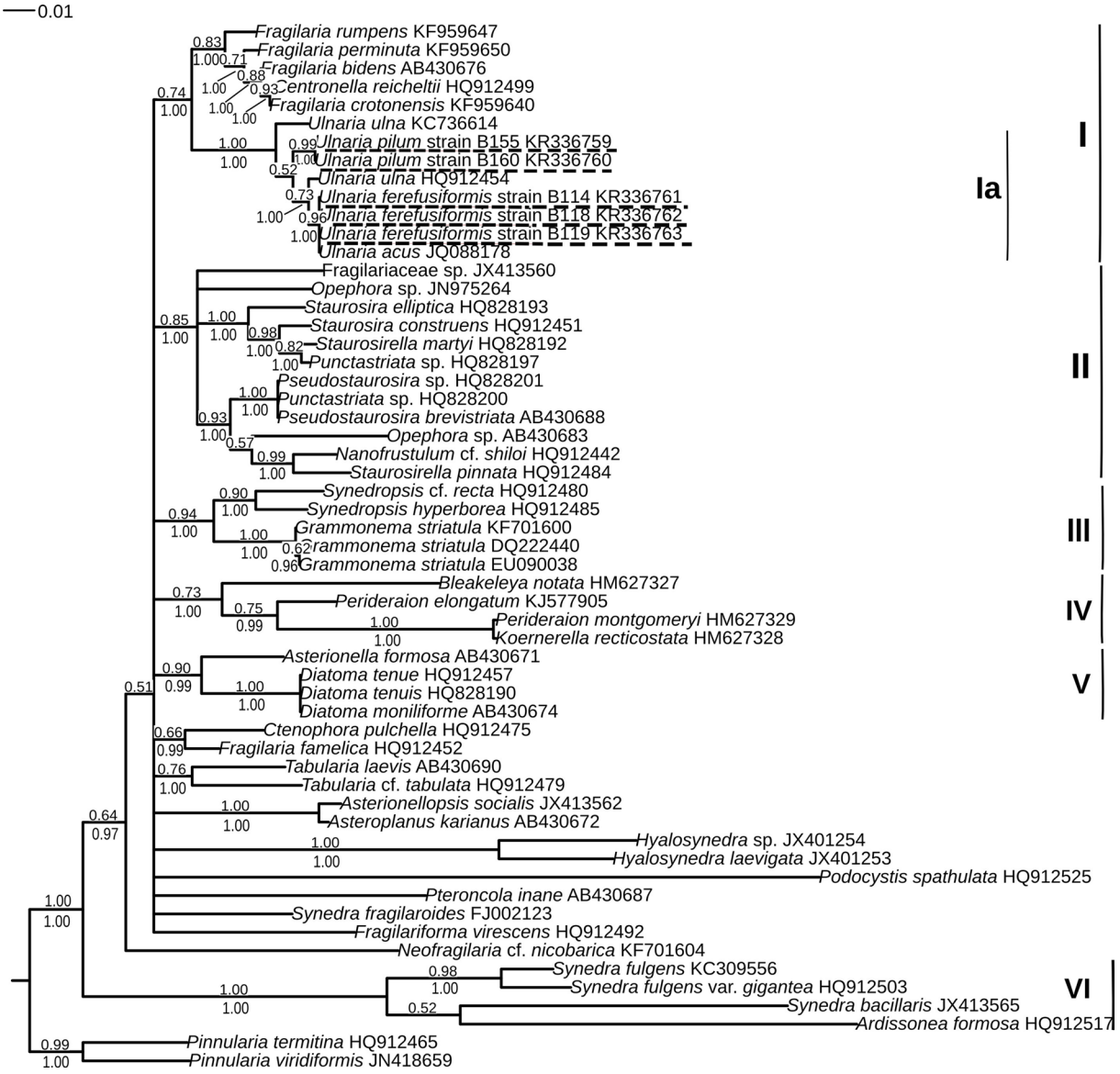

Fig. 31. Phylogenetic analysis based on partial *rbc*L gene. The tree shown is the ML tree. Values above vertical lines are bootstrap support for the ML analyses (<0.5 are not shown), Values below vertical lines are BI posterior probabilities (<0.9 are not shown). Species analyzed in this study are underlined.

and large number of striae in 10 µm (see Table 1). It is not excluded that diversity of planktonic *Ulnaria* in Lake Baikal is higher that observed previously and that different, closely–related taxa can occur as sympatric cryptic or pseudocryptic populations. Understanding of this phenomenon needs in comprehensive molecular investigation of planktonic *Ulnaria* from all parts of Lake Baikal. Presence of sympatric populations in diatoms was shown by Amato et al. (2007).

Another species closely–related to *U. pilum* sp. nov. is *Ulnaria ulna*. Recently an epitype of *Bacillaria ulna* Nitzsch 1817 (syn. *Synedra ulna* (Nitzsch) Ehrenberg syn. *Fragilaria ulna* (Nitzsch) Lange–Bertalot) has been selected, based on a particular line drawing in the protologue of that taxon chosen as lectotype by Lange–Bertalot & Ulrich (2014). Specimens of the epitype population are likewise needle–shaped, 230–320 µm long, 5.2–6.5 µm broad proximally, 3–4 µm distally below the weakly subcapitate apices; length–to–breadth ratio is 17–50; stria density 8.5–9.5 / 10 µm, areola density 28–33 in 10 µm; a central area is not developed (Lange–Bertalot & Ulrich 2014). By these data *U. ulna* and *U. pilum* sp. nov. are similar but not the same. Different are stria– and areola densities and, mainly, the outlines of the distal parts of valves, which are conspicuously less narrowed subapically in *U. ulna*. The slightly inflated subcapitate apices are 3.50–3.75 µm broad (vs. 2.2–2.6). *Ulnaria delicatissima* (W. Smith) Aboal et Silva (syn. *Fragilaria delicatissima* (W. Smith) Lange–Bertalot) is distinguished from *U. pilum* sp. nov. by having narrower valves proximally, about 4 µm as far as lectotypified specimens are concerned.

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