

Observations of the genus *Muelleria* (Bacillariophyceae) from the Republic of North Macedonia

Zlatko Levkov^{1,*}, Danijela Vidaković², Aleksandra Cvetkoska¹, Danijela Mitić-Kopanja¹,
Svetislav Krstić¹, Bart Van de Vijver^{3,4} & Paul B. Hamilton⁵

¹Institute of Biology, Faculty of Natural Sciences, Ss Cyril and Methodius University, Arhimedova 3, 1000 Skopje, Republic of North Macedonia

²University of Belgrade, Institute of Chemistry, Technology and Metallurgy, Njegoseva 12, 11000 Belgrade, Republic of Serbia

³Meise Botanic Garden, Research Department, Nieuwelaan 38, B-1860 Meise, Belgium

⁴University of Antwerp, Department of Biology, ECOBE, Universiteitsplein 1, B-2610 Wilrijk, Belgium

⁵Phycology Section, Research and Collections Division, Canadian Museum of Nature, P.O. Box 3443, Station D, Ottawa, Canada

*Author for correspondence: zlevkov@pmf.ukim.mk

Background and aims – An increased interest in the diatom flora from subaerial habitats in the Republic of North Macedonia has revealed the presence of a large number of interesting and often unknown, species. During a recent biodiversity survey, seven *Muelleria* species were recorded, several of which could not be identified based on the available literature.

Methods – Using both Light Microscopy and Scanning Electron Microscopy techniques, the morphology of several *Muelleria* species has been analysed. The morphology of all taxa is described, illustrated and compared with other possible similar *Muelleria* taxa, known worldwide.

Key results – Four already documented *Muelleria* species, *M. gibbula*, *M. islandica*, *M. terrestris* and *M. undulata* (= *Navicula gibbula* f. *undulata*) were identified, while two distinct species are described as new. These new species belong to the *M. gibbula* complex, and can be differentiated by valve size, stria density, shape of the proximal raphe ends and the shape of external areola foramina. The identity of one taxon remains uncertain due to a low number of observed specimens in the samples. As their ultrastructure is almost entirely unknown, they are briefly discussed.

Conclusions – The diversity of *Muelleria* in Europe and the North Hemisphere is most likely underestimated and some of the previous records of *M. gibbula* belong to other species. Observations of diatoms from “extreme” habitats such as intermittent ponds, wet mosses and soils can reveal the existence of interesting and new species.

Key words – Diatoms, *Muelleria*, intermittent ponds, new species, subaerial habitats.

INTRODUCTION

The genus *Muelleria* (Freng.) Freng., described in 1924 as a subgenus and later (1945) raised to genus level (Frenguelli 1924, 1945), is morphologically separated from *Navicula* Bory by unilaterally deflected proximal raphe ends, the presence of a longitudinal canal on each side of the raphe and a loculate valve wall. *Muelleria* is considered a medium-sized

genus with at present more than 40 species, mostly distributed in the Southern Hemisphere (principally South America, South Africa and Antarctica (Spaulding & Stoermer 1997, Spaulding et al. 1999, Van de Vijver et al. 2010, 2014, Zidarova et al. 2016, Kochman-Kędziora et al. 2017). This distribution, however, might be the result of limited collections from habitats typical for *Muelleria* species (Spaulding & Stoermer

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Table 1 – Samples used in this study that contained *Muelleria*, including their accession numbers in the Macedonian National Diatom Collection (MKNDC), Skopje, Republic of North Macedonia.

Accession no. MKNDC	Mountain	Locality	Collection date	Coordinates	Substrate
002488	Deshat	Lake North Gashevo	2 Aug. 2006	41.86694°N, 21.79889°E	surface sediment
003079	Shar Planina	intermittent pond on Radika	18 Jul. 1998	42.90833°N, 21.75833°E	surface sediment
003083	Shar Planina	intermittent pond below Džinibeg	20 Jul. 1995	42.36010°N, 20.7705°E	surface sediment
011389	Stogovo	sand below ice patch, Kenanica	12 Jun. 2017	41.49462°N, 20.65545°E	surface sand
011448	Shar Planina	intermittent pond, springs of river Pena	23 Jun. 2017	41.97397°N, 20.77032°E	surface sediment
011490	Shar Planina	intermittent pond below Džinibeg	12 Jun. 2017	42.3601°N, 20.7705°E	surface sediment
011861	Osogovo	wet mosses in intermittent spring near the road in village Sasa	31 Mar. 2018	42.035°N, 22.58331°E	wet mosses

1997). Recent observations from Europe and North America have resulted in both recording of already known species and description of several new species (e.g. Lange-Bertalot & Genkal, 1999, Reichardt 2004, Bahls 2014, Kociolek et al. 2014). Published diatom data indicate the historic presence of the genus *Muelleria* in Northern Europe, mostly at higher latitudes (Cleve 1894, Petersen 1915, Foged 1955, Hustedt 1961–1966). Two species, *M. gibbula* (Cleve) S.A.Spauld. & Stoermer and *M. terrestris* (J.B.Petersen) S.A.Spauld. & Stoermer are considered to be widely distributed (“cosmopolitan”) in subaerial habitats (Germain 1937, Krasske 1938, Allorge & Manguin 1941, Foged 1960, 1964, 1974, Dodd 1981, Reichardt 1997).

The genus *Muelleria* has been observed sporadically in the Republic of North Macedonia. So far, the literature only mentions two records of *M. gibbula* (Hustedt 1945, Levkov et al. 2005) and one record of *M. terrestris* (Levkov et al. 2007). However, the limited recorded distribution of *Muelleria* species in the country most likely results from underreporting due to the undersampling of the genus’ preferred microhabitats (mosses, moist soils and shallow ponds). Biogeographical and taxonomical phycological research in the Republic of North Macedonia over the last two decades was restricted to Lake Ohrid (e.g. Levkov et al. 2007, Levkov & Williams 2006, 2014, Pavlov et al. 2013). However, occasionally other habitats were analysed for the revision of particular genera such as *Amphora* Ehrenb. ex Kütz., *Gomphonema* Ehrenb. and *Luticola* D.G.Mann (Levkov 2009, Pavlov & Levkov 2013, Levkov et al. 2013, 2016). More recently, subaerial habitats have become the focus of detailed biogeographic observations (Tofilovska et al. 2014, Levkov et al. 2017), resulting in the description of a number of new species. Several populations of unknown *Muelleria* taxa were also observed during these studies, many of them recorded in subaerial habitats and intermittent alpine ponds. In the current research, morphological features and identity of these unknown *Muelleria* are studied based on detailed light microscope (LM) and scanning electron microscope (SEM) observations.

MATERIAL AND METHODS

Samples used for this study were collected during various sampling campaigns over the last 20 years. More than 6000 samples were observed, but only seven contained fairly large populations of *Muelleria* species (with more than 10 valves per slide) and were selected in this study for further analysis using detailed LM and SEM observations. The samples from this work are deposited in the Macedonian National Diatom Collection (MKNDC); see table 1 for details.

The first site where fairly large numbers of *Muelleria* cells were found is Lake North Gashevo, located on Mountain Deshat at an elevation of 1770 m a.s.l. The lake has a surface area of 631 m² and maximum depth of 2.2 m (average depth: 1.2 m) (Vasilevski 2008). The second site is a small, shallow, intermittent pond with a depth of 20 cm on Radika (part of Shar Planina mountain), at 2240 m a.s.l. The third and fourth site are two small intermittent ponds below Džinibeg at elevations of 2240 and 2230 m a.s.l. The ponds are shallow (20 cm) with a maximum length of c. 50 m and a width of c. 30 m with surface area around 1000 m². The fifth site is a pond near the River Pena springs; it is slightly smaller (length c. 40 m and width c. 20 m) with a surface area of c. 500 m² at an elevation of 1570 m a.s.l. The sixth sample was collected from a small intermittent spring in the village of Sasa, near the mountain Osogovo at an elevation of 1300 m a.s.l.

Organic material in the samples was removed by digestion with 2 ml of K₂MnO₄ and 4 ml of HCl added to a small (c. 2 ml) diatom subsample. The acid was removed from the processed sample through repeated dilutions and permanent slides were mounted with Naphrax®. Slide observations were performed using a Nikon E–80i light microscope, equipped with a Nikon Coolpix 600 digital camera. For SEM analyses, samples of cleaned material were dried onto aluminium stubs and coated with gold-palladium (Polaron SC7640 sputter coater [Quorum Technologies, Ashford, UK], or a Desk II sputter coater [Denton Vacuum, New Jersey, USA]). SEM

Table 2 – Morphometrical features of observed *Muelleria* species in the Republic of North Macedonia.

Species	shape	length (µm)	width (µm)	striae (in 10 µm)	valve margin	proximal raphe ends	areolae shape
<i>Muelleria gibbula</i>	linear-elliptic	22.0–53.5	8.0–11.0	15–19	slightly convex	extending to first row of areolae	circular
<i>Muelleria islandica</i>	linear to linear-elliptic	17.5–34.0	6.5–8.0	21–26	slightly convex	terminate within the central area to long reaching the first two rows of areolae	c-like to slit-like
<i>Muelleria sasaensis</i>	lanceolate	25.0–32.0	5.5–6.5	22–25	slightly convex	terminating before first row of areolae	circular
<i>Muelleria coxiana</i>	lanceolate to elliptic-lanceolate	16.0–28.5	6.0–7.5	20–24	slightly undulate	short and distantly from the first row	circular
<i>Muelleria terrestris</i>	linear	26.0–27.0	5.5–6.0	16–18	parallel	long reaching the first two rows of areolae	NA
<i>Muelleria undulata</i>	linear with undulate valve margins	20.0–24.0	4.5–5.0	26–28	undulated	extending to first row of areolae	NA
<i>Muelleria</i> sp. 1	linear-lanceolate	31.0	8.5	16–18	undulated	extending to first row of areolae	NA

micrographs were made with field emission microscopes, a Cambridge Instruments S4 Stereoscan using accelerating voltages of 5–10 kV and a FEI Apreo using accelerating voltages of 3–5 kV. Slides and samples were deposited in the Macedonian National Diatom Collection (MKNDK). Taxonomic identifications were based on Spaulding & Stoermer (1997), Spaulding et al. (1999), Esposito et al. (2008), Van de Vijver et al. (2010, 2014), Bahls (2014) and Kociolek et al. (2014). Terminology followed Van de Vijver et al. (2010), except the terminology relating to valve outline following Hendey (1964) and raphe morphology following Round et al. (1990).

RESULTS

Seven *Muelleria* taxa were observed in the samples from the Republic of North Macedonia (table 2). Four species were identified, *M. gibbula*, *M. islandica*, *M. terrestris* and *M. undulata* (Krasske) comb. & stat. nov. (= *N. gibbula* f. *undulata* Krasske). Two taxa could not be identified based on the literature and are described here as new species: *Muelleria sasaensis* sp. nov. and *Muelleria coxiana* sp. nov. Since for a third unknown taxon, an insufficient number of specimens could be found, it was impossible to characterize its population size and shape structure and the taxon is reported as *Muelleria* sp. 1.

1. *Muelleria gibbula* (Cleve) S.A. Spauld. & Stoermer

Figs 1, 2 & 10A–E

The taxonomy and nomenclatural history of *M. gibbula* was discussed by Spaulding & Stoermer (1997) and Spaulding et al. (1999). At present, our understanding of its phenotypic plasticity is limited. There appear to be, however, at least two morphological forms in the type slide. Four specimens were illustrated from the type slide (Spaulding et al. 1999: figs 63–66). The designated lectotype in fig. 63 in Spaulding et al. (1999) is narrow and slightly tumid in the middle. The specimen depicted in fig. 65 (in Spaulding et al. 1999) dif-

fers in its morphological features from the lectotype and two other isolectotype specimens (Spaulding et al. 1999: figs 64, 66; isolectotypes cited as “isotypes”) being linear-lanceolate, slightly tumid in the mid-valve and narrower (valve width 8 µm) versus linear-elliptic with broader valves (width 10–11 µm). Based on the morphological and numerical features, it is very unlikely that all valves are conspecific. Additionally, one specimen of *M. gibbula* was observed by SEM from syntype material (Spaulding & Stoermer 1997: figs 18–20, Spaulding et al. 1999: fig. 78). It is characterized by the presence of long proximal raphe ends extending to the first row of areolae and have circular areola foramina, similar to the specimen depicted in fig. 1A & C (this study). In general, the observed population from a small pond on Radika (part of Shar Planina Mountain) has morphological and numerical features (valve shape and size, stria density) matching the descriptions provided by Spaulding & Stoermer (1997) and Spaulding et al. (1999).

Morphology of *Muelleria gibbula* found in the Republic of North Macedonia

LM – Valves linear-elliptic with broadly rounded apices. Valve dimensions (n = 26): length 22.0–53.5 µm, valve width 8.0–11.0 µm. Axial area rather narrow, linear, slightly widening towards small, elliptical central area. Raphe filiform to slightly lateral, straight with clearly unilaterally bent long proximal raphe ends extending almost to first row of areolae. Terminal raphe fissures weakly deflected to same valve side as proximal raphe ends. Striae in central area (5–7 striae) more distantly spaced than other striae and clearly radiate whereas other striae slightly radiate, becoming almost parallel near apices. Central striae 15–19 in 10 µm, distal striae 21–26 in 10 µm. Areolae circular and clearly discernible in LM.

SEM – Frustules rectangular in girdle view. Valve face flat with gradual (fig. 2A) to abrupt (fig. 2B) valve face/mantle junction. Striae uniseriate, composed of single row of areolae. Areolae with external circular (fig. 2B & D) or slightly elongated foramina (fig. 2C, black arrows). All areola fo-

ramina are nearly same size, including areolae bordering axial area. Proximal raphe ends clearly unilaterally deflected. Proximal raphe ends long, bent and terminating within central area (fig. 2C & D) or at first row of areolae (fig. 2A). Terminal raphe fissures unilaterally deflected, covered by lacinia (= siliceous flap) giving impression of bifurcation (fig. 2A & B). Girdle bands open, bearing single row of small circular poroids (fig. 2C). Internally striae uniseriate composed of loculate areolae occluded by vela (fig. 2E & G). Areola with round to elliptical internal foramina. Longitudinal canal present on each side of raphe (fig. 2E), discontinuous in central nodule (fig. 2F) and terminating at helictoglossae (fig. 2E). Small areolae associated with longitudinal canal present on internal side of canal (black arrows in fig. 2F). Canal puncta not observed (fig. 2E). Raphe branches straight terminating proximally with rectelevatum, while distally with short helictoglossae (fig. 2E).

Remarks – Based on valve shape, *M. gibbula* is similar to *M. agnellus* Bahls (Bahls 2014: 75, figs 98–112), a species found in the Mission Mountains (Montana) and the Wind River Mountains (Wyoming), characterized by lanceolate

to linear-lanceolate valves, 26.9–59.6 μm long and 9.5–12.3 μm wide and proximal raphe ends terminating before the first row of areolae. *Muelleria agnellus* has complex areolae that vary in shape and the valves have a lower stria density (13–18 in 10 μm). Another species comparable to *M. gibbula* is *M. tetonensis* Bahls, recently described from a small alpine tarn in Grand Teton National Park (Wyoming, USA) (Bahls 2014: figs 134–160). *Muelleria tetonensis* can be differentiated by the cuneate apices, parallel valve margins and areolae with slit-shaped foramina that are oriented apically or transapically. *Muelleria spauldingiae* Bahls, which was also described from Wyoming (Bahls 2014: figs 127–142) has a different shape (elliptic to linear-elliptic) with wider valves (width 11.7–15.0 μm) and lower stria density (14–17 in 10 μm). The Maritime Antarctic species *Muelleria sabbei* Van de Vijver & S.A.Spauld. (in Van de Vijver et al. 2010: figs 77–85), has a comparable valve size (length 25–52 μm , width 8.0–12.0 μm) and stria density (16–17 in 10 μm at the valve centre), but the shape is different with broadly lanceolate to rhombic-lanceolate valves with strongly convex margins. Furthermore, the distal raphe fissures are not divergent

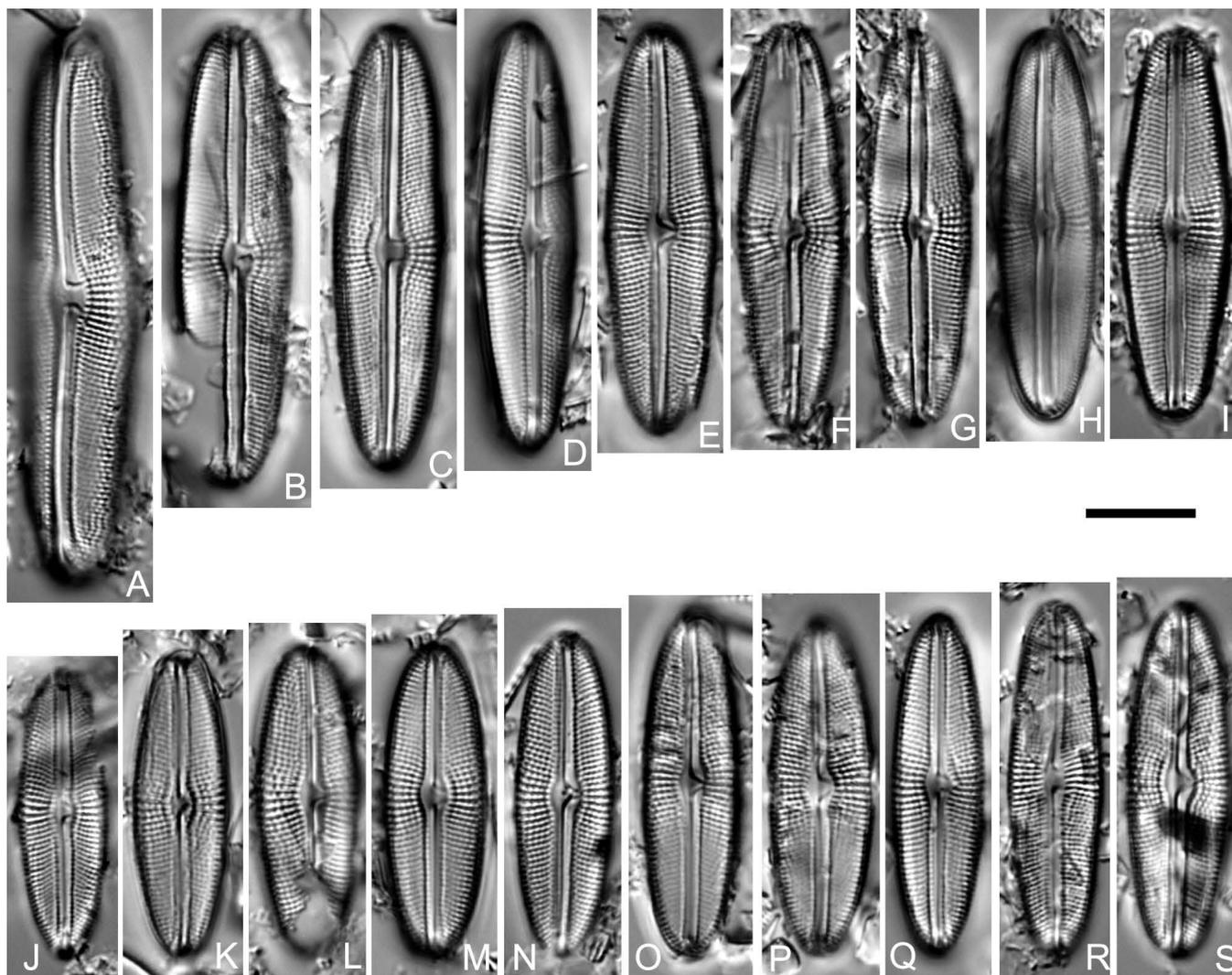


Figure 1 – LM micrographs of a population of *Muelleria gibbula* from an intermittent pond on Radika, Shar Planina showing the variability of the population (slide MKNDC 003709). Scale bar = 10 μm .

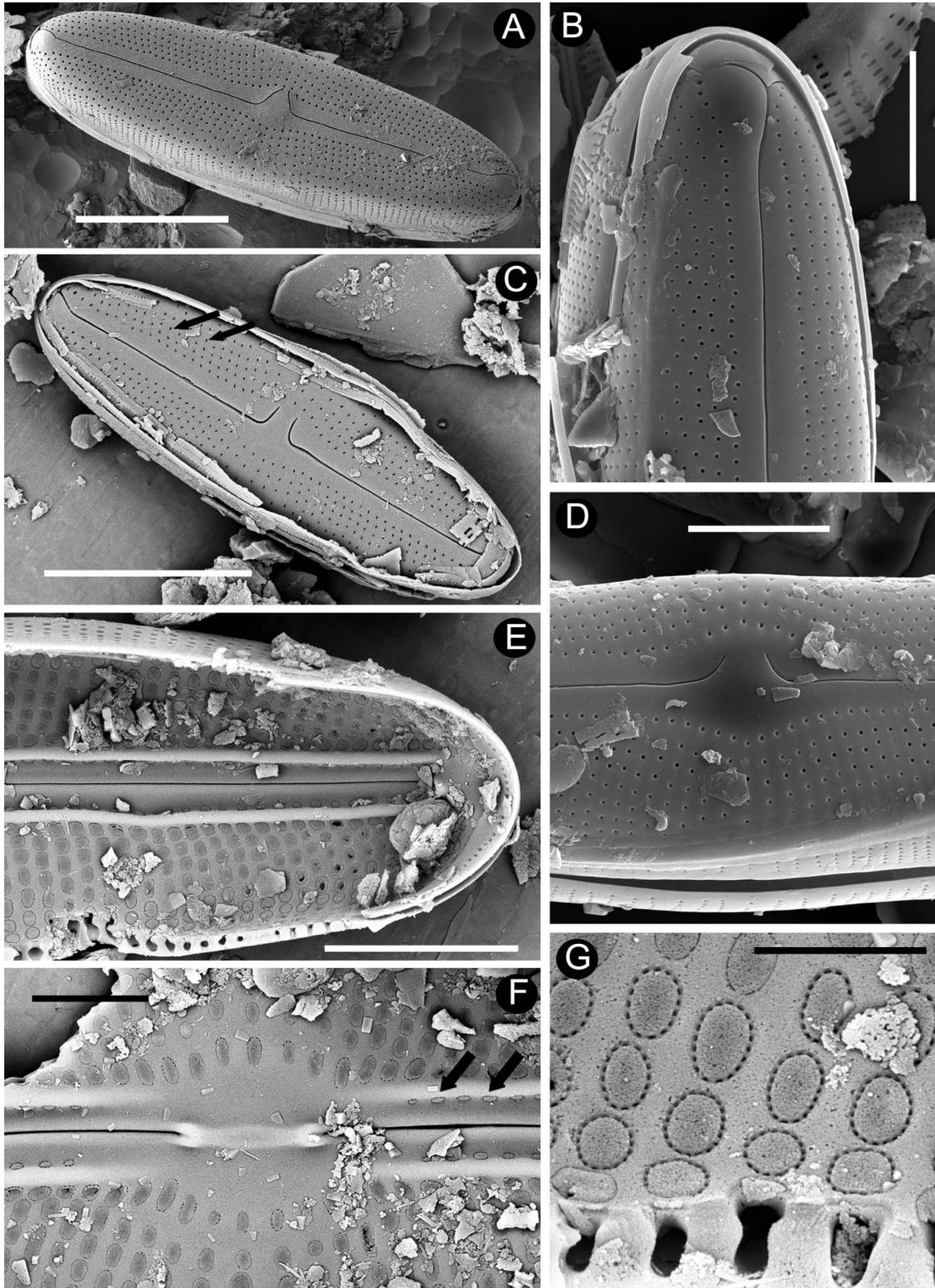


Figure 2 – SEM electron micrographs of *Muelleria gibbula* (sample MKNDC 003079): A, external view of an entire valve; B, detailed view of the valve apex showing the lacinia overlapping the terminal raphe fissures; C, external view of an entire valve, black arrows showing the external areolae openings; D, detailed view of the mid-valve showing the external areola foramina and proximal raphe ends; E, internal view of valve apex showing areolae occlusions and longitudinal ribs; F, internal detailed view of the mid-valve showing the areola occluded by hymenes and fine silica separations (vela) and rectelevatum; G, detailed internal view showing loculate valve wall and areolae occluded by velum. Scale bars: A & C = 10 µm; B, D & E = 5 µm; F = 2 µm; G = 1 µm.

and do not extend onto the valve mantle. Another Maritime Antarctic species, *Muelleria regigeorgiensis* Van de Vijver & S.A.Spauld. (in Van de Vijver et al. 2010: figs 60–71), has a similar valve size, but the shape is different (lanceolate) and the areolae are c-shaped to reniform and two large canal puncta are located near the terminal raphe fissures. For comparisons with *M. islandica* see the Remarks section of this species, as well as table 3.

2. *Muelleria islandica* Lange-Bert.

Figs 3, 4, 5 & 10F–J

Navicula islandica Østrup (Østrup 1918: 26, fig. 3: 36) is a later homonym of *N. islandica* Grunow (in Cleve & Möller 1879) and thus represents an invalid name. The holotype of *N. islandica* was reexamined by Lange-Bertalot & Genkal (1999: fig. 23: 15) and clearly belongs to the genus *Muelleria*. However, according to the ICN (Turland et al. 2018: art. 58.1) the final epithet in an illegitimate name may be reused in a different name and the resulting name should then be treated either as a replacement name with the same type as the illegitimate name, name or as the name of a new taxon with a different type. Since Lange-Bertalot (in Lange-Bertalot & Genkal 1999) intended *M. islandica* as a replacement name and did not designate a different type, the valid name of the species should be *Muelleria islandica* Lange-Bert. and not *M. islandica* (Østrup) Lange-Bert. Specimens observed on several locations in North Macedonia fit the description of *N. islandica* Østrup (1918: 26, fig. 3: 36) and are identified in this study as *M. islandica*.

Morphology of *Muelleria islandica* found in the Republic of North Macedonia

LM – Valves linear to linear-elliptic with broadly rounded ends. Valve dimensions (n = 38): length 17.5–34.0 µm (initial cell 37.5 µm), valve width 6.5–8.0 µm (initial cell 9.0 µm). Axial area narrow, linear, slightly widening near small, elliptical central area. Raphe filiform, straight with clearly unilaterally bent, long proximal raphe ends extending almost to first row of areolae or reaching first two rows of areolae. Terminal raphe fissures weakly deflected to same side of valve as proximal raphe ends. Central striae in central area (5–7 striae) more distantly spaced than other striae and clearly radiate whereas other striae slightly radiate, or becoming nearly parallel near apices. Central striae 21–26 in 10 µm, distal striae 26–30 in 10 µm. Areolae round and fine, but discernible in LM.

SEM – Valve face flat with rounded valve face/mantle junction (figs 4B, 5A, B & D). Striae uniseriate, composed of single row of areolae. External areola foramina with variable shape depending on the population, thus irregular to c-like (fig. 4B & D) foramina for the specimens found in the sample from Lake North Gashevo, or slit-shaped to round (fig. 5A–E) for those specimens found in the sample of the intermittent pond below Džinibeg. First row of areolae bordering axial area has foramina with both long and short slits, separated from rest of striae by narrow hyaline area (fig. 5B–D). Hyaline area gradually widening towards central area (fig. 5C & D). Areolae becoming smaller toward margins and receiving elliptical to circular foramina. Proximal

raphe ends clearly unilaterally deflected. Length and shape of proximal raphe ends variable, ranging from simple bent or doubly curved, terminating within central area for the specimens of Lake North Gashevo (fig. 4A–C) to long, curved, reaching first (two) row of areolae around central area for those of the intermittent pond below Džinibeg (fig. 5A–D). Terminal raphe fissures unilaterally deflected, overlapped with lacinia (figs 4D & 5E). Internally, striae uniseriate composed of loculate areolae occluded by hymenes (figs 4F, G & 5G). Some hymenes perforated along their edges by series of holes which appear as dark dots. Other hymenes (those closer to raphe) with smaller perforation. Areolae with elliptical internal foramina. Areola on valve face appearing larger than areolae on valve margin. Longitudinal canal present on each side of raphe (figs 4E & F, 5F) discontinuous in central nodule (figs 4E & 5F) and terminating at helictoglossae (fig. 4F). Longitudinal canals bounded by narrow ribs. Small areolae associated with longitudinal canal present on internal side of canal. Canal puncta not observed (fig. 4F). Raphe branches straight terminating proximally with rectelevatum (figs 4G & 5G), while distally with short helictoglossae (fig. 4F).

Remarks – Two populations of *M. islandica* were observed during this study. Both populations occurred in similar habitats, specifically small and shallow, glacial ponds/lakes in high mountains. Both populations have similar valve outlines (linear to linear-elliptic), size and stria density, and internal valve structure. However, differences between populations can be documented using SEM with respect to the external areola foramina and length of proximal raphe ends. The first population (fig. 4A–D) is characterized by the presence of irregular to c-like areolae and short, deflected raphe endings which terminate in the central area. The second population (figs 5A–D) has slit-like to round areolae and long, curved proximal raphe ends which extend to the first or second row of central areolae. Also in this population the areolae in the first row (adjacent to the axial area) are characterized by slit-like foramina which are longer than the rest of the areolae (figs 5C & D). In this study only two samples with *M. islandica* (samples 002488 & 011490) and one with *M. gibbula* (sample 003079) were present with higher numbers which allowed for LM and SEM observations of morphological and ultrastructural variability. These three populations showed differences with respect to the areolae shape and length of the proximal raphe ends. Though, the question about phenotypic stability of these features is still open, due to insufficient data to provide clear evidence for the range of morphological variability of the species.

Muelleria islandica is similar to *M. aequistriata* Van de Vijver & S.A.Spauld. (in Van de Vijver et al. 2010: figs 2–14) concerning the valve outline, but can be differentiated from *M. aequistriata* by several morphological features (table 3) such as valve outline (linear-lanceolate with almost parallel valve margins in *M. aequistriata*) and stria density (18–20 in 10 µm in *M. aequistriata*). *Muelleria islandica* also resembles *M. gibbula* with respect to ultrastructure. However, differences between these two species can be found in their valve width, stria density, the length of the proximal raphe ends and the shape of the external expression of the areolae. *Muelleria gibbula* is wider than *M. islandica* (8.0–11.0 µm vs. 6.5–8.0 µm), and has coarser striae with lower density (15–19 in 10

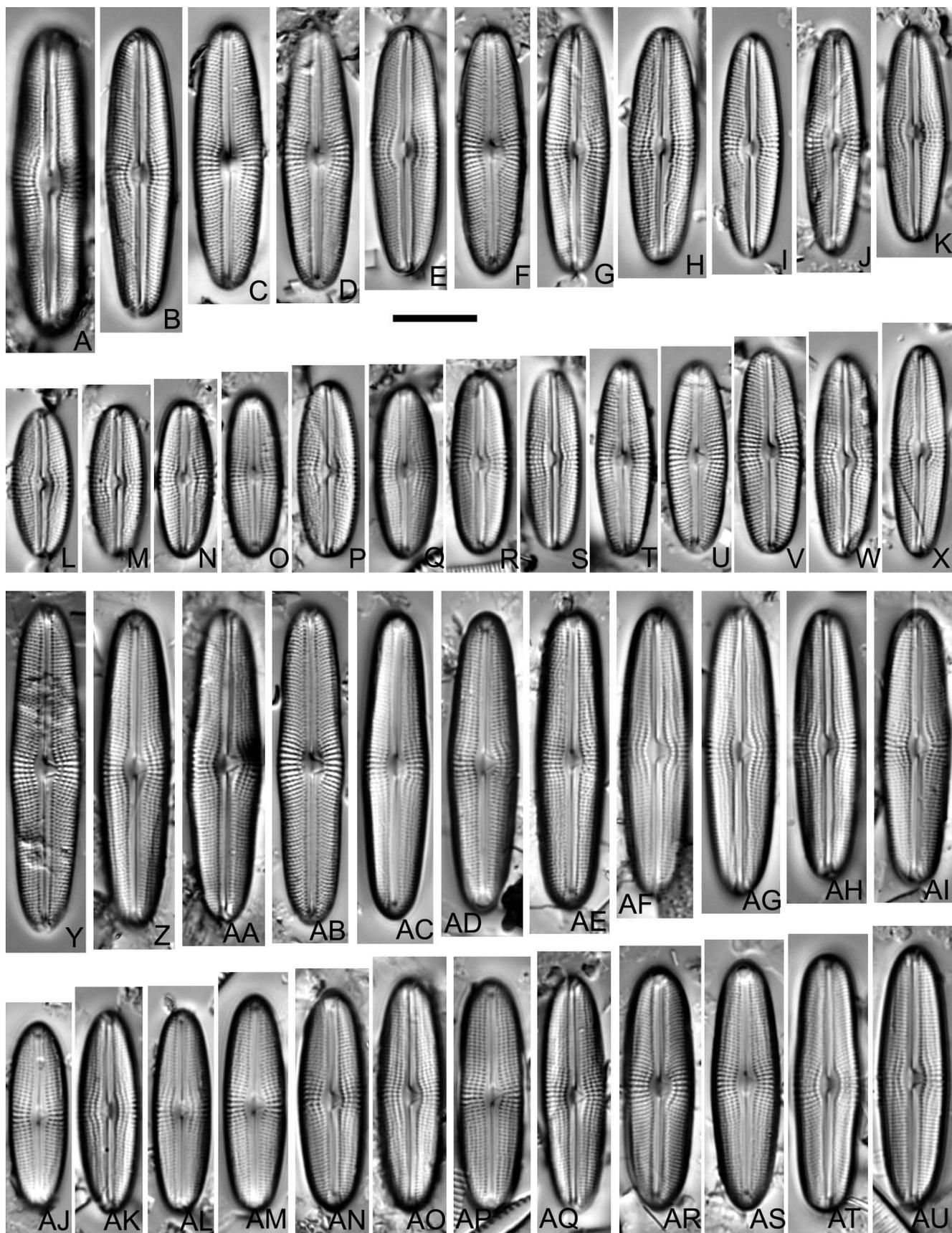


Figure 3 – LM micrographs of two populations of *M. islandica*: A–X, from Lake North Gashevo, mountain Deshat (slide MKNDC 002488); Y–AU, from intermittent pond below Džinibeg, Shar Planina (slide MKNDC 011490) showing the size reduction series. Scale bar = 10 µm.

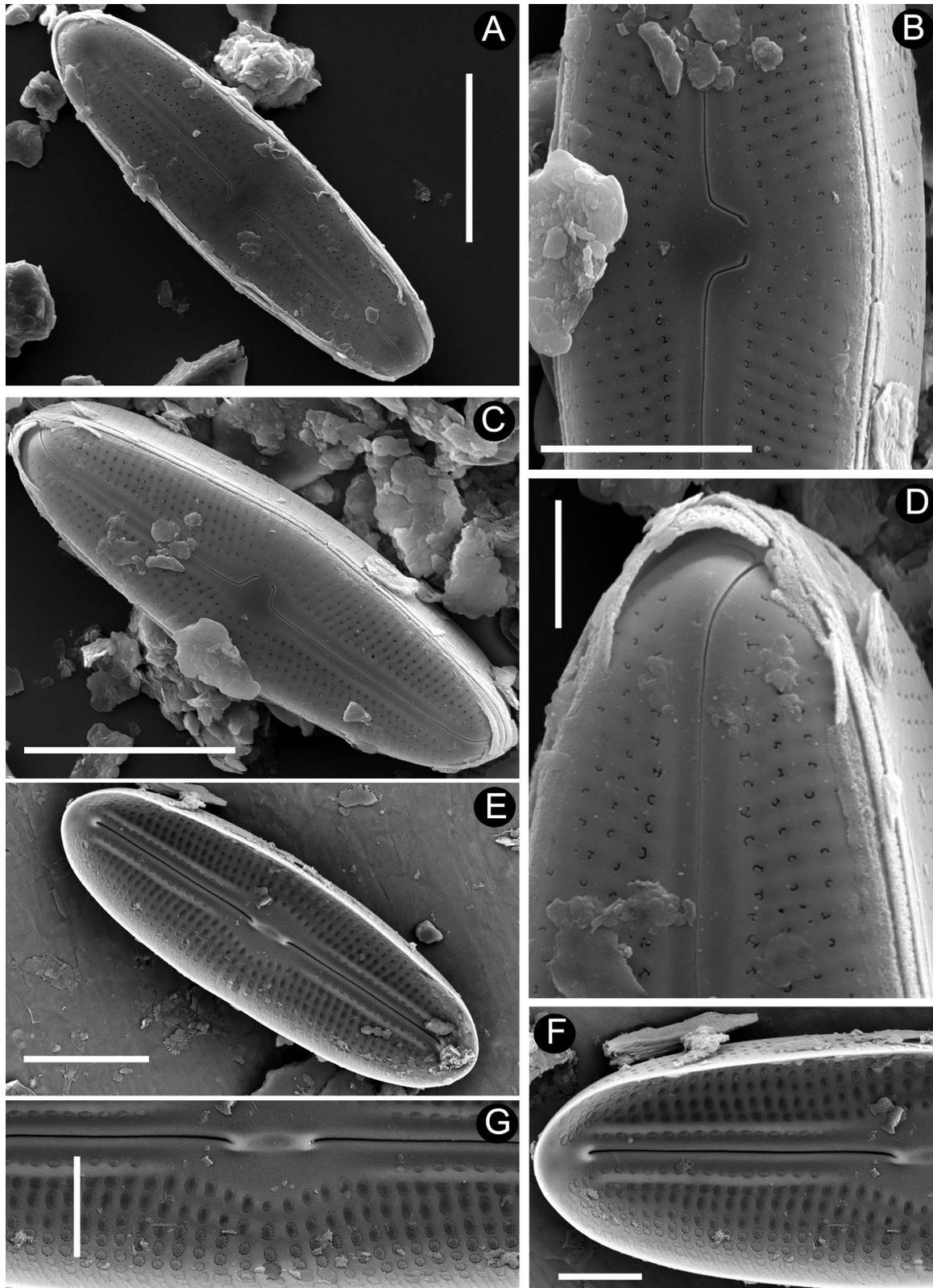
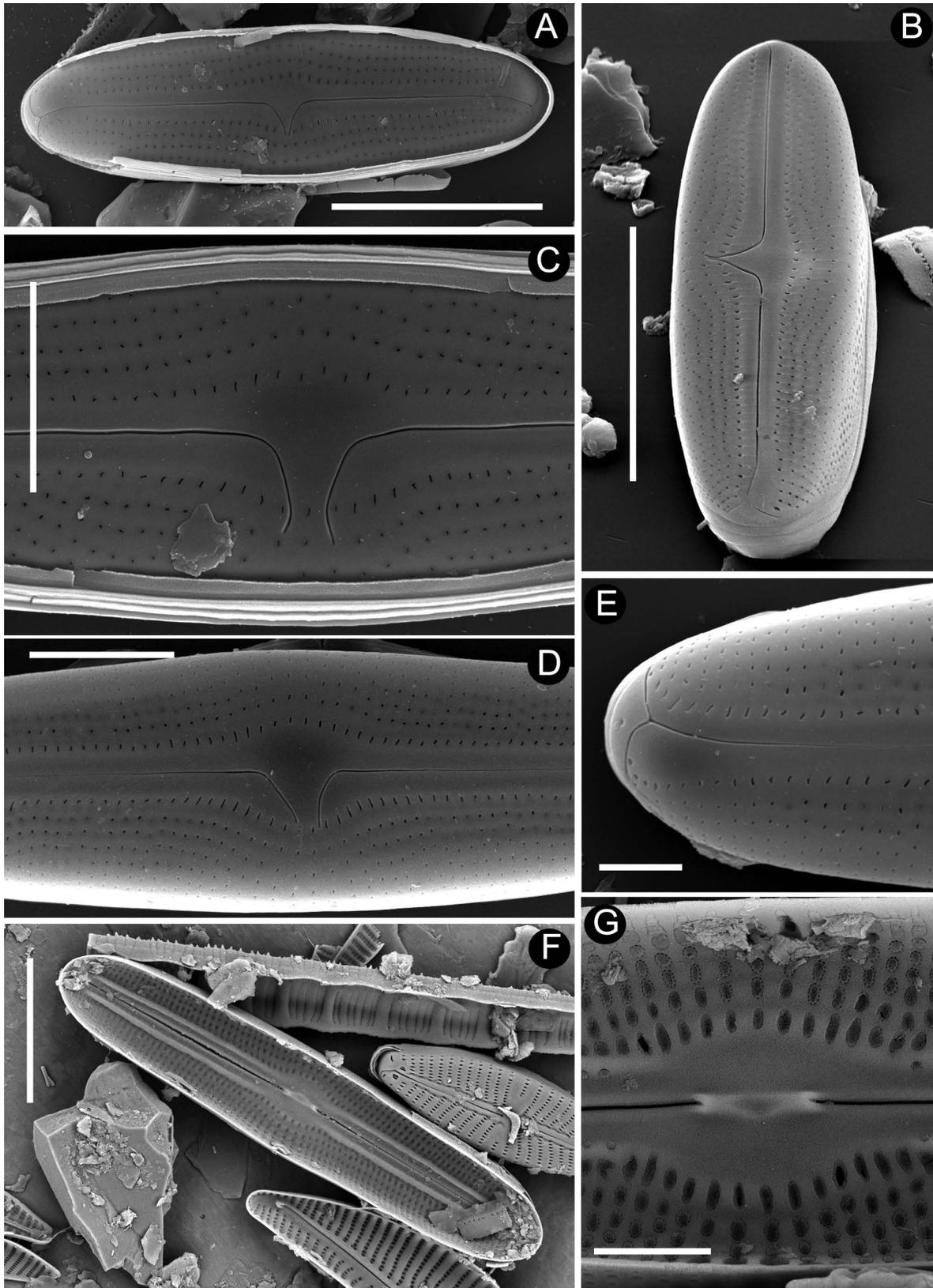


Figure 4 – SEM micrographs of *M. islandica* (sample MKNDC 002488): A, external view of an entire valve; B, detailed view of the mid-valve showing the external areola with c-like foramina and proximal raphe ends terminating within the central area; C, external view of an entire valve; D, detailed view of the valve apex showing the lacinia overlapping the terminal raphe fissures; E, internal view of whole valve; F, internal view of valve apex showing areolae occlusions, canal perforated with occluded pores and longitudinal ribs; G, internal detailed view of the mid-valve showing the areolae occluded by hymenes and fine silica separations (vela) and strongly thickened rectelevatum. Scale bars: A & C = 10 μm ; B & E = 5 μm ; D, G & F = 2 μm .



Figures 5 – SEM micrographs of *M. islandica* (sample MKNDC 011490): A & B, external view of an entire valve; C & D, detailed view of the mid-valve showing the external areola with slit-like foramina and proximal raphe ends extending into the first two rows of areolae; E, detailed view of the valve apex showing the lacinia overlapping the terminal raphe fissures; F, internal view of whole valve; G, internal detailed view of the mid-valve showing the areola occluded by hymenes and fine silica separations (vela) and strongly thickened rectelevatum. Scale bars: A, B & F = 10 μ m; C & D = 5 μ m; E & G = 2 μ m.

Table 3 – Comparison of the morphological and morphometric characteristics of *M. sasensis* and *M. coxiana* with some similar taxa.

* To complement the only published morphological data of *M. islandica* (Østrup 1910, Lange-Bertalot & Genkal 1999; fig. 23: 15), data from the two *M. islandica* populations found in this study is also provided here.

Species	Valve outline	Valve margin	Valve apices	Proximal raphe ends	Areolae shape	Valve length (µm)	Valve width (µm)	Stria density (in 10 µm)	Source
<i>M. sasensis</i>	lanceolate	slightly convex	narrowly rounded	terminate before or at the first row of areolae	circular	25.0–32.0	5.5–6.5	22–25	this study
<i>M. agnellus</i>	lanceolate to linear-lanceolate	convex and slightly gibbous	broadly rounded	terminate before or at the first row of areolae	complex and variable in shape	26.9–59.6	9.5–12.3	13–18	Bahls (2014)
<i>M. gibbula</i>	linear to elliptical	slightly convex	broadly rounded	extend into the first or second row	circular	22.0–46.0	6.0–10.0	14–19	Spaulding et al. (1999)
<i>M. islandica</i>	elliptical	convex	broadly rounded	terminate before or at the first row of areolae	NA	22.0	8.0	20	Østrup (1918)
<i>M. islandica</i>	linear to linear-elliptical	slightly convex	broadly rounded	terminate within the central area to long reaching the first two rows of areolae	c-like to slit-like	17.5–34.0	6.5–8.0	21–26	this study*
<i>M. linearis</i>	linear to elliptical	convex	broadly rounded	terminate past the second row of areolae	circular	25.0–32.0	6.0–8.0	15	Spaulding et al. (1999)
<i>M. relicta</i>	linear	parallel to slightly convex	broadly rounded	NA	NA	29.5	6.5	23	Reichardt (2004)
<i>M. terrestris</i>	linear-elliptical	parallel to slightly convex	broadly rounded	terminate in the first or second row of areolae	NA	24.0–37.0	5.0–7.0	17–22	Spaulding et al. (1999)
<i>M. coxiana</i>	lanceolate to elliptic-lanceolate	slightly undulate	slightly protracted	short, terminating distantly from first row of areolae	circular	16.0–28.5	6.0–7.5	20–24	this study
<i>M. aequistriata</i>	linear-lanceolate	slightly convex	broadly rounded	short, terminating before the first row of areolae	circular	25.0–48.5	5.8–9.0	18–20	Van de Vijver et al. (2010)
<i>M. kristinae</i>	linear-lanceolate to lanceolate	parallel to convex	acutely rounded	short, terminating before the first row of areolae	circular	11.0–25.0	5.0–6.7	18–21	Van de Vijver et al. (2010)
<i>M. olechiae</i>	linear-lanceolate to elliptical	convex	broadly rounded	short, terminating before the first row of areolae	circular	13.0–28.5	5.0–7.0	18–21	Kochman-Kędziora et al. (2017)
<i>M. undulatooides</i>	linear	undulate	weakly subcapitate	short, terminating before the first row of areolae	circular	23.0–29.0	6.0–7.5	23–26	Van de Vijver et al. (2014)

μm vs. 21–26 in 10 μm). Additionally, as mentioned above, there is a difference in the shape of areolae, whereas in *M. gibbula* areolae have round foramina, while in *M. islandica* areolae have c-like or slit-like foramina. *Muelleria agnellus* is also wider (9.5–12.5 μm) and the areolae are complex and externally occluded (table 3). *Muelleria tetonensis* has wider valves (8.6–11.7 μm), with slit-like external expression of the areolae that are orientated in various directions. In addition, the central area of *M. tetonensis* is narrower. *Muelleria regigeorgiensis* has a similar valve size (6.0–9.5 μm) and the proximal raphe ends also terminate within the central area. Differences, however, are found in valve shape and form of the areolae, which are c-shaped to reniform and possess two large canal puncta near the distal raphe ends.

3. *Muelleria sasaensis* Levkov, Vidaković, Cvetkoska, Mitić-Kopanja, Krstić, Van de Vijver & P.B.Hamilton, **sp. nov.**

Figs 6, 7 & 10K–O

Type material – Republic of North Macedonia, wet mosses in intermittent spring near the road in village Sasa, mountain Osogovo (42.035°N, 22.58331°E), 31 Mar. 2018, *Z. Levkov* s.n. (holo-: MKNDC, slide MKNDC 11861; iso-: BRM, slide BRM Zu11/23, CANA, slide CANA 128351).

Description: LM – Valves lanceolate with narrowly rounded ends. Valve dimensions (n = 21): length 25.0–32.0 μm , width

5.5–6.5 μm . Axial area narrow, linear, slightly widening near small, elliptical central area. Raphe filiform to slightly lateral, straight with clearly unilaterally bent, long proximal raphe ends extending nearly to first row of areolae. Terminal raphe fissures weakly deflected to same side of valve as proximal raphe ends. Striae in central area (4–6 striae) more distantly spaced than other striae and radiate whereas other striae only slightly radiate. Central striae 22–25 in 10 μm (measured in 5 μm), distal striae 26–30 in 10 μm . Areolae fine, circular and barely discernible in LM.

Description: SEM – Valve face flat with gradual valve face/mantle junction (fig. 7B & C). Striae are uniseriate, composed single type of areolae. Areolae have circular foramina (figs 7A–E). All areola foramina have same size, including areolae bordering axial area (fig. 7B & E). Proximal raphe ends deflected to same side, terminating before or at first row of areolae within central area (fig. 7B & E). Terminal raphe fissures unilaterally deflected and covered with lacinia (fig. 7C & D). Internally, striae uniseriate composed of loculate areolae occluded by perforated or solid hymenes (fig. 7F–H). Areolae with transversally elongated to elliptical internal foramina. Longitudinal canal present on each side of raphe (fig. 7G). Canal discontinuous in central nodule and terminating at helictoglossae (fig. 7G). Longitudinal canals bounded by narrow ribs. Small areolae associated with longitudinal canal present on internal side of canal (fig. 7G). Canal puncta not observed (fig. 7G). Raphe branches straight terminating

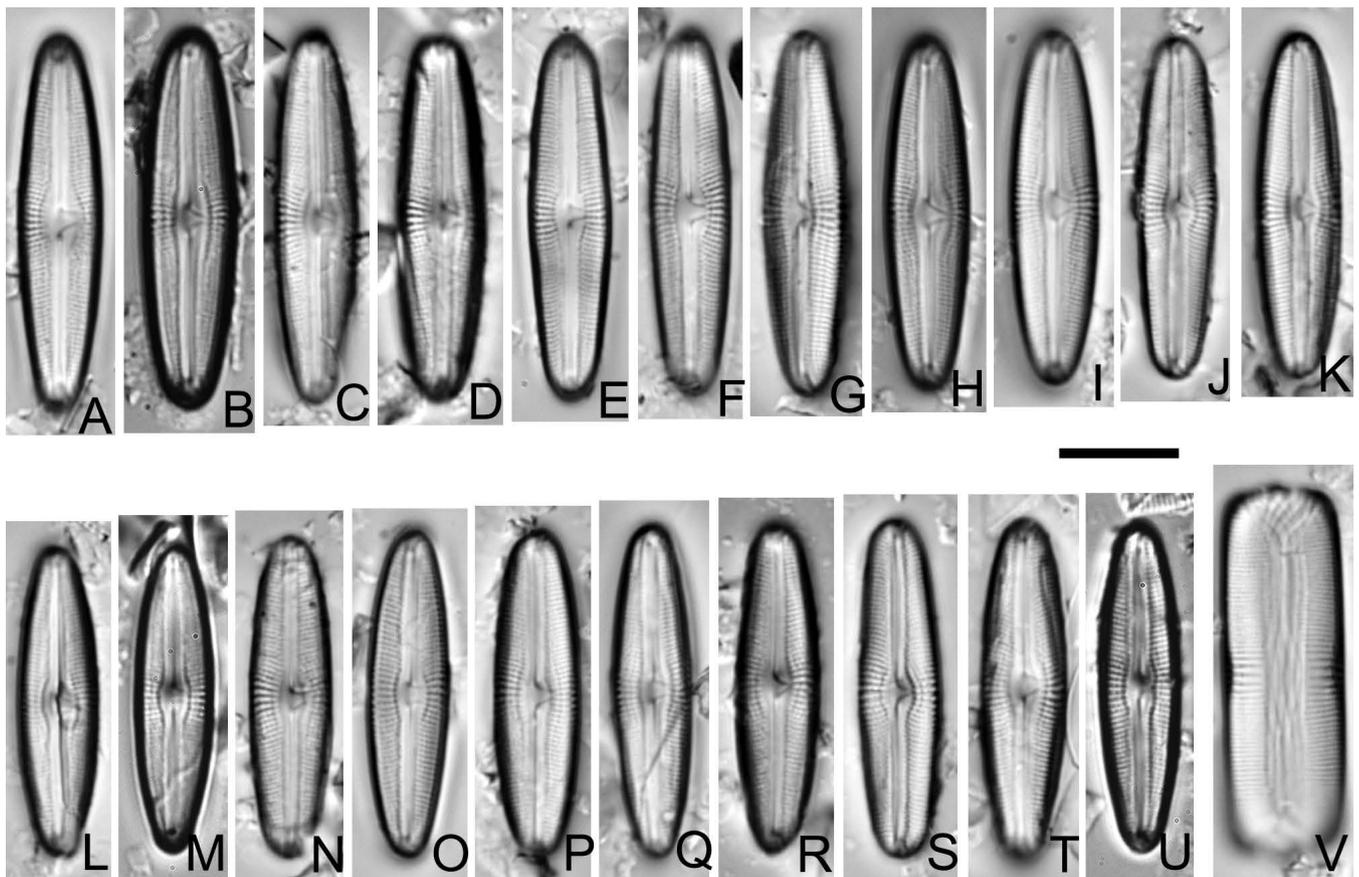


Figure 6 – LM micrographs of a type population of *M. sasaensis* from wet mosses near the road in village Sasa, Mountain Osogovo showing the variability of the population (slide MKNDC 011861). Scale bar = 10 μm .

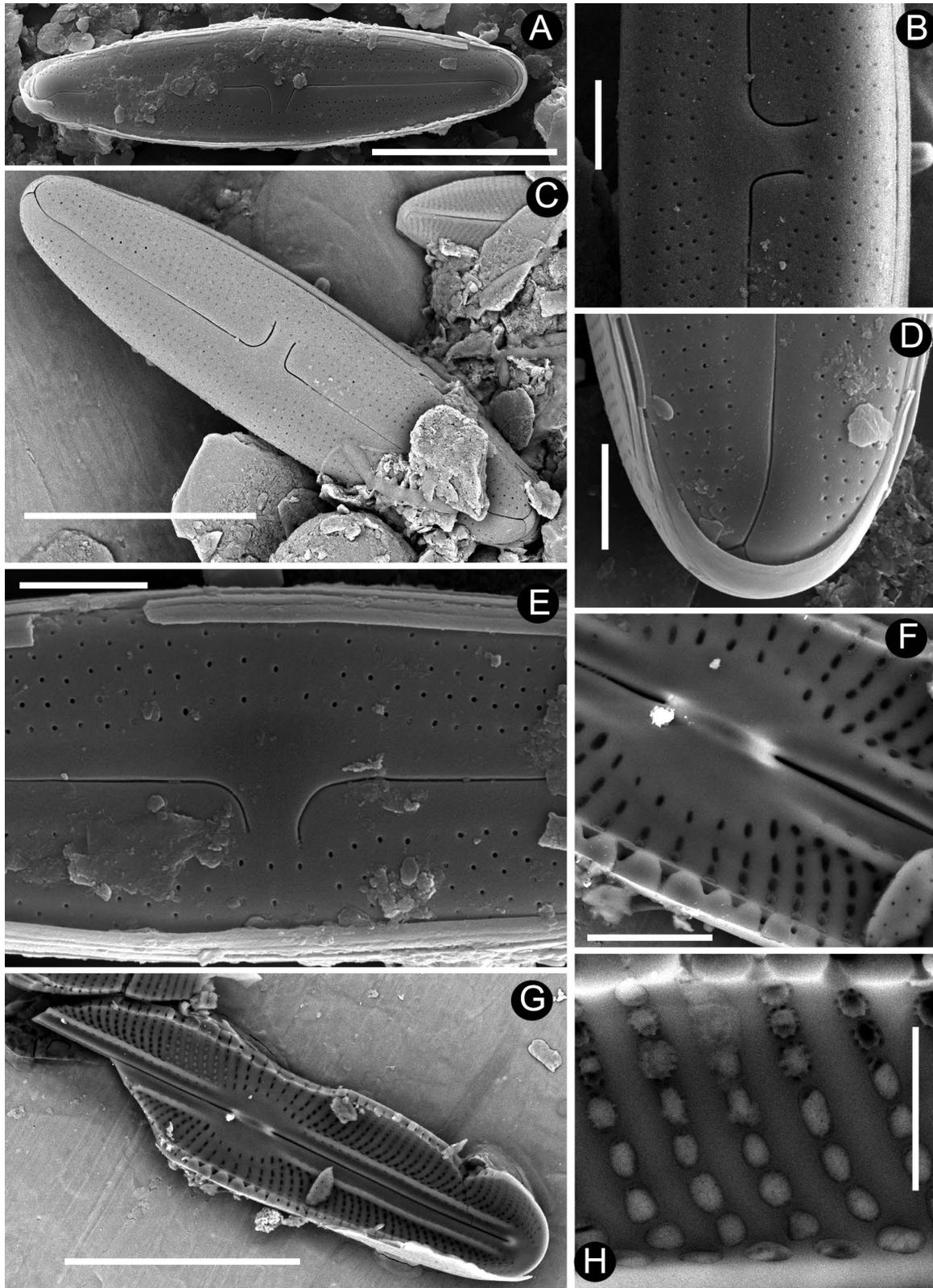


Figure 7 – SEM micrographs of *M. sasaensis* (sample MKNDC 011861): A & C, external view of an entire valve; B & E, detailed view of the mid-valve showing the areola with small round foramina and proximal raphe ends terminating within the central area near the first row of areolae; D, detailed view of the valve apex showing the lacinia overlapping the terminal raphe fissures; F, internal detailed view of the mid-valve showing the areola occluded by hymenes and fine silica separations (vela) and rectelevatum; G, internal valve view; H, internal detailed view of the mid-valve showing the areola occluded by hymenes and fine silica separations (vela). Scale bars: A, C & G = 10 μ m; B, D–F = 2 μ m; H = 1 μ m.

proximally with an interrupted rectelevatum (fig. 7F), while distally with short helictoglossae (fig. 7G).

Etymology – The specific epithet “*sasaensis*” refers to the village Sasa where this species was discovered.

Remarks – *Muelleria sasaensis* is another representative of the species complex around *M. gibbula*. However, both species can be differentiated by valve outline (lanceolate with narrowly rounded ends vs. linear-elliptic with broadly rounded apices), valve width (8.0–11.0 μm vs. 5.5–6.5 μm), and stria density (22–25 in 10 μm vs. 15–19 in 10 μm), and also by where the proximal raphe ends terminate (table 3). The new species can be differentiated from *M. agnellus* by the valve size (length 26.9–59.6 μm , width 9.5–12.3 μm in *M.*

agnellus) and a lack of a complex cribra occlusion over the external areolae (table 3). Other taxa like *Muelleria islandica* have wider and more linear valves with different external areola foramina (c-like or slit-like). The most similar species to *M. sasaensis* is *M. linearis* (O.Müller) Freng. (Spaulding & Stoermer 1997: figs 1, 2). Typification of *M. linearis* was performed by Spaulding et al. (1999: figs 1, 2) although the type slide had already been observed by Spaulding & Stoermer (1997). On the type slide only two specimens were observed, differing in valve shape and size. *Muelleria sasaensis* appears similar to the isolectotype specimen (Spaulding et al. 1999: fig. 2 = Spaulding & Stoermer 1997: fig. 1) with respect to valve size and shape (25 μm long and 6 μm wide).

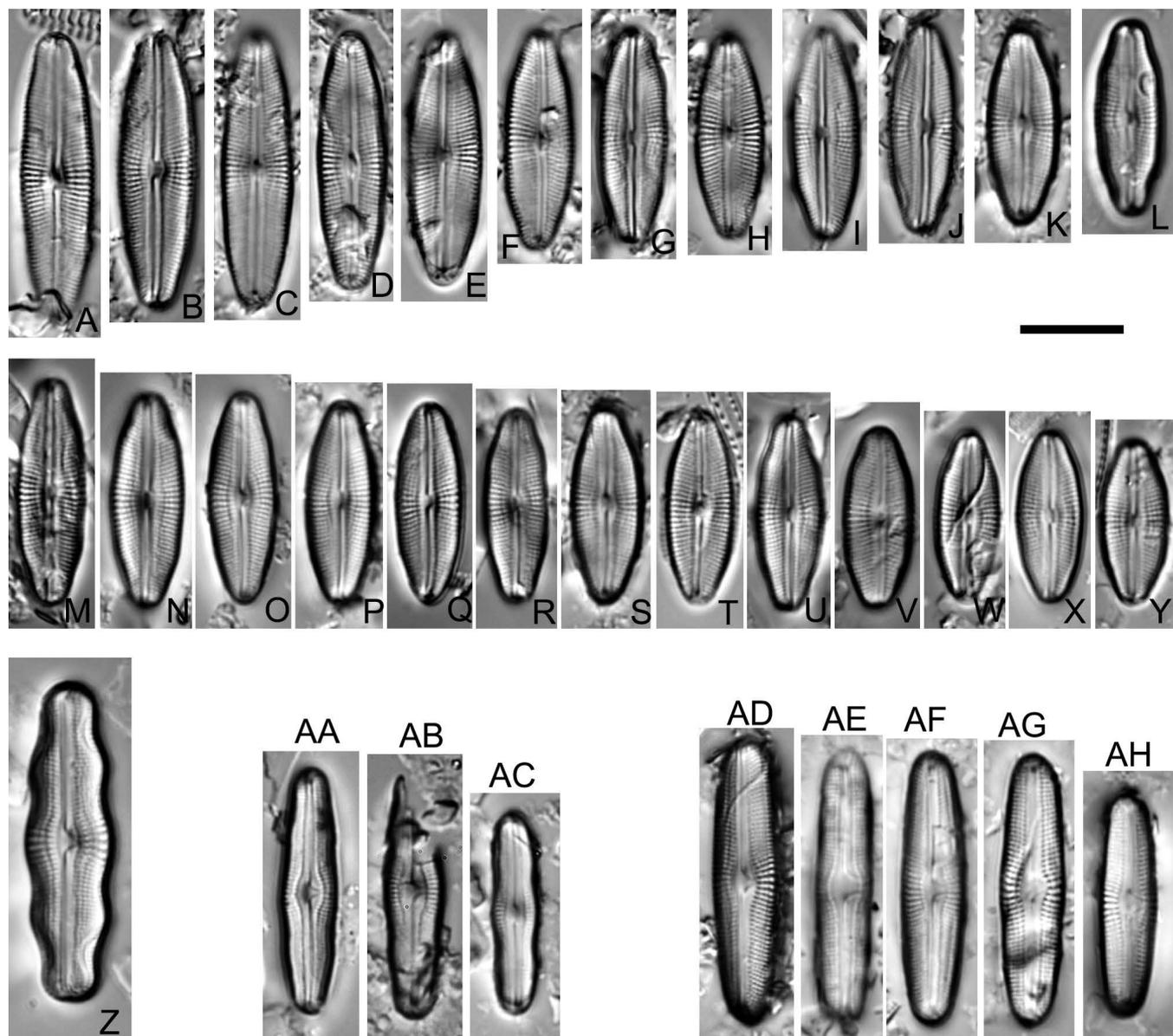


Figure 8 – LM micrographs of *Muelleria* species: A–L, *M. coxiana* population from intermittent pond on Radika, Shar Planina (slide MKNDC 003079); M–Y, *M. coxiana* population from intermittent pond, near the springs of river Pena, Shar Planina (slide MKNDC 011448); Z, *Muelleria* sp. 1. from intermittent pond below Džinibeg, Shar Planina (slide MKNDC 003083); AA–AC, *M. undulata* from sand below ice patch, Kenanica, mountain Stogovo (slide MKNDC 011389); AD–AH, *M. terrestris* from sand below ice patch, Kenanica, mountain Stogovo (slide MKNDC 11389). Scale bar = 10 μm .

However, it is questionable if this specimen belongs to *M. linearis*, since it differs from the protologue (Müller 1909, p. 13, fig. 15) in most of its morphological and numerical features. *Muelleria terrestris* was also typified by Spaulding et al. (1999, fig. 98). The latter is characterized by its linear valves with broadly rounded ends and a large elliptical central area contrary to the lanceolate valves with narrowly rounded ends and a small central area in *M. sasaensis*. *Muelleria relicta* (McCall) E.Reichardt as depicted by Reichardt (2004: fig. 4: 7) is characterized by linear valves, slightly gibbous in the mid-valve and a large central area. These features are in concordance with the holotype specimen of *M. terrestris* and in general no significant morphological difference can be noticed between the two species. Additional observations of other populations and SEM might reveal differences between these taxa.

4. *Muelleria coxiana* Levkov, Vidaković, Cvetkoska, Mitić-Kopanja, Krstić, Van de Vijver & P.B.Hamilton, **sp. nov.**

Figs 8A–Y & 9

Type material – Republic of North Macedonia, intermittent pond, springs of river Pena, Shar Planina, sediment (41.97397°N, 20.77032°E), 23 Jun. 2017, Z. Levkov s.n. (holo-: MKNDC, slide MKNDC 11448; iso-: BRM, slide BRM Zu11/24, CANA, slide CANA 128352).

Description: LM – Valves lanceolate to elliptic-lanceolate with slightly undulated margins and slightly protracted and rounded ends. Valve dimensions (n = 24): length 16.0–28.5 µm, width 6.0–7.5 µm. Axial area narrow, linear, slightly widening near central area. Central area narrow and weakly expressed, elliptical to slightly asymmetric. Raphe filiform to slightly lateral, straight with very short unilaterally bent proximal raphe ends terminating in central area, distantly from first row of areolae. Terminal raphe fissures weakly deflected to same side of valve as proximal raphe ends. Striae in central area (4–6 striae) more distantly spaced than other striae and clearly radiate whereas other striae only slightly radiate. Central striae 20–24 in 10 µm (measured in 5 µm), distal striae 26–30 in 10 µm. Areolae fine, circular and barely discernible in LM.

Description: SEM – Valve face flat with gradually curving valve face/mantle junction (fig. 9A) Striae uniseriate, composed of single type of areolae (fig. 9A–E). Areolae with circular foramina (fig. 9C–E). All areola foramina with same size, including areolae bordering axial area (fig. 9A & B). Proximal raphe ends short and slightly deflected to same side. Proximal raphe ends bent, terminating within central area usually distantly from first row of areolae (fig. 9C & D). Terminal raphe fissures unilaterally deflected, with lacinia (fig. 9E). Internally, striae uniseriate composed of elliptical areolae occluded by hymenes, occasionally perforated or imperforated (fig. 9F & G). Longitudinal canal present on each side of raphe (fig. 9F) and terminating at helictoglossae. Longitudinal canals bounded by narrow ribs. Small areolae associated with longitudinal canal present on internal side of canal (fig. 9G). Canal puncta not observed (fig. 9F). Raphe branches straight terminating proximally with rectelevatum (fig. 9G), while distally with short helictoglossae (fig. 9F).

Etymology – The species is named in honour of Dr. Eileen J. Cox for her extensive contributions to diatom research.

Remarks – *Muelleria coxiana* has a unique set of characters that justify its description as a new species. It shows morphological similarity only with *Muelleria kristinae* Van de Vijver (in Van de Vijver et al. 2010: figs 36–48). The latter species has a comparable valve outline (linear-lanceolate to lanceolate) and dimensions (length 11–25 µm, width 5.0–6.7 µm), but can be separated based on the shape of its valve apices (acutely rounded to even cuneate), the width of its axial area (moderately broad), the stria density and orientation (coarse and only weakly radiate) with its central stria not distantly spaced (table 3). *Muelleria olechiae* Kochman-Kędziora et al. (in Kochman-Kędziora et al. 2017: figs 1–27) is another small-celled species (length 13.0–28.5 µm, width 5.0–7.0 µm) but the latter has a different valve outline (linear-lanceolate with a clearly gibbous central part) and longer proximal raphe ends. *Muelleria terrestris* shares a similar valve size (length 24–37 µm, width 5.0–7.0 µm), but has a different valve outline (linear-elliptical with broadly rounded and not protracted apices), a broader central area (elliptical) and coarser striae (17–22 in 10 µm). *Muelleria relicta* has an outline similar to *M. gibbula* (linear with almost parallel valve margins), a large central elliptical central area and longer proximal raphe ends (Reichardt 2004: fig. 7). *Muelleria undulatoides* Van de Vijver, Zidarova & Kopalová (Van de Vijver et al. 2014: figs 52–61) has linear valves with clearly undulated margins and absent to weakly subcapitate apices, deflected (not bifurcated) terminal raphe fissures and weakly convergent striae near the apices. These features distinguish *M. undulatoides* and *M. coxiana* without difficulties. *Muelleria aequistriata* and *M. gibbula* have a different valve outline and longer proximal raphe ends, coarser striae and a larger central area.

5. *Muelleria* sp. 1.

Fig. 8Z

Description: LM – Valve linear-lanceolate with strongly undulated margin and protracted to subcapitate apices. Valve dimensions (n = 1): length 31.0 µm, width 8.5 µm. Axial area narrow, linear, slightly widening near small, elliptical to slightly asymmetrical central area. Raphe weakly lateral, straight with clearly unilaterally bent, long proximal raphe ends extending almost to first row of areolae. Terminal raphe fissures weakly deflected to same valve side as proximal raphe ends. Striae in central area (6–7 striae) more distantly spaced than other striae and clearly radiate whereas other striae only slightly radiate, becoming nearly parallel near apices. Central striae 16–18 in 10 µm (measured in 5 µm), and distal striae 26–28 in 10 µm. Areolae round and fine, not discernible in LM.

Remarks – Only one specimen of this distinct *Muelleria* species was observed after scanning eight slides from the intermittent pond below Džinibeg, Shar Planina. It is presented here as information for further research on this genus.

6. *Muelleria undulata* (Krasske) Levkov, P.B.Hamilton & Van de Vijver, **comb. & stat. nov.**

Fig. 8AA–AC & 10V–X

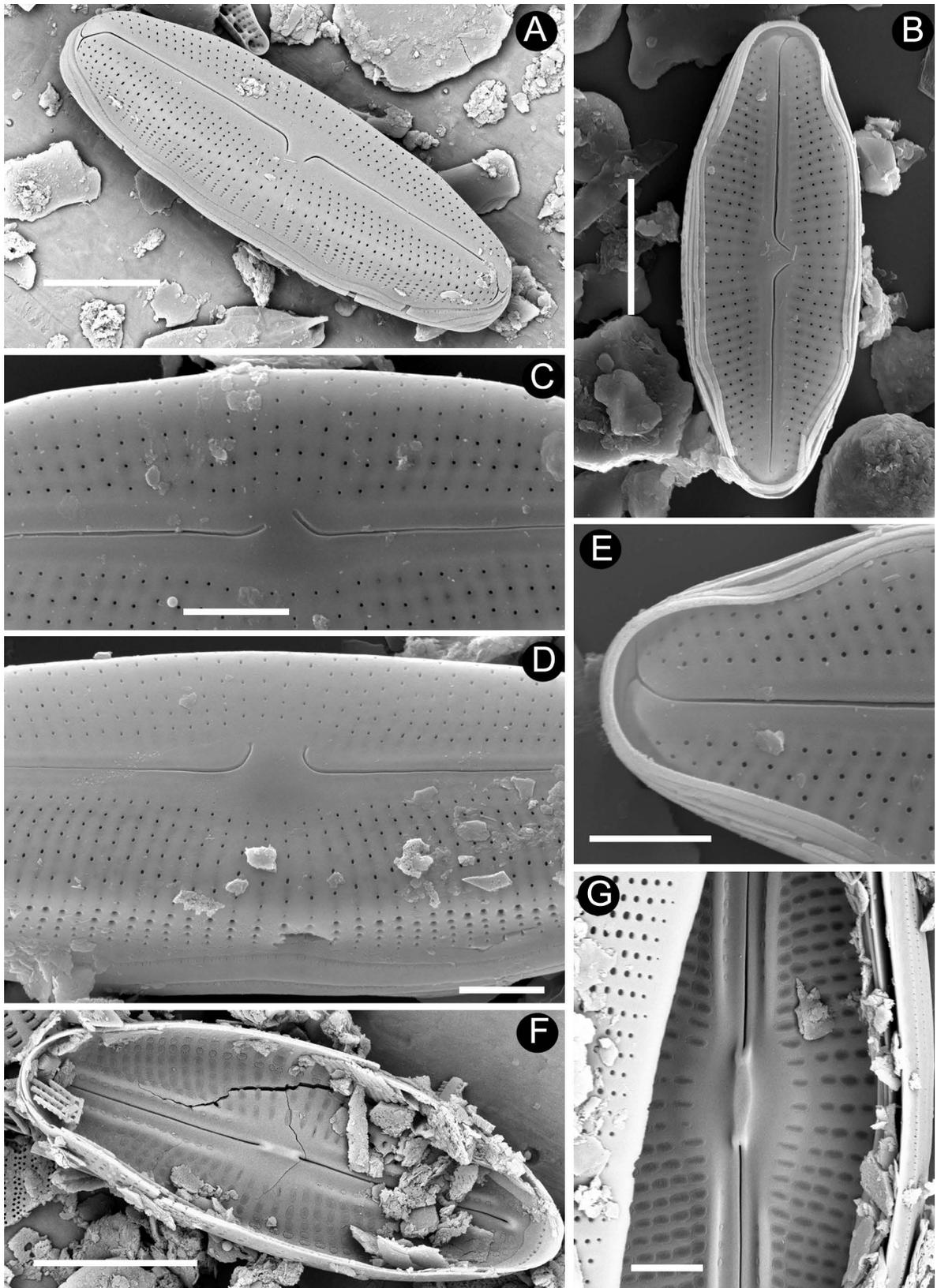


Figure 9 – SEM electron micrographs of *M. coxiana* (sample MKNDC 011448): A & B, external view of an entire valve; C & D, detailed view of the mid-valve showing the areola with small round foramina and short proximal raphe ends terminating within the central area distantly from the first row of areolae; E, detailed view of the valve apex showing the lacinia overlapping the terminal raphe fissures; F, internal valve view; G, internal detailed view of the mid-valve showing the areola occluded by hymenes and fine silica separations (vela) and rectelevatorum. Scale bars: A, B & F = 5 μ m, C–E & G = 2 μ m.

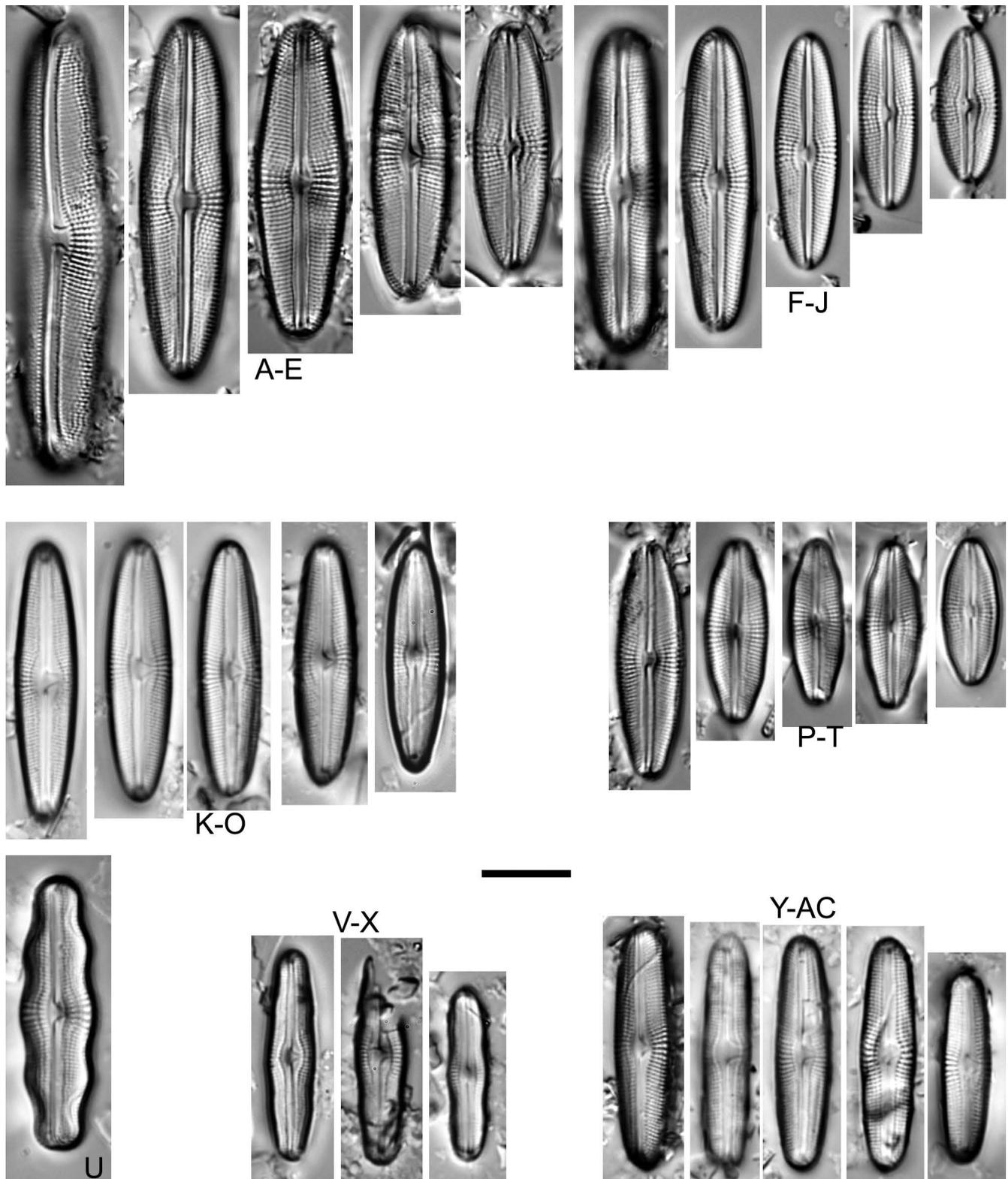


Figure 10 – LM micrographs of the seven *Muelleria* taxa observed in this study to facilitate inter species comparison under LM; A–E, *M. gibbula* from an intermittent pond on Radika, Shar Planina (slide MKNDC 003709); F–J, *M. islandica* from Lake North Gashevo, mountain Deshat (slide MKNDC 002488); K–O, *M. sasaensis* from wet mosses near the road in village Sasa, Mountain Osogovo (slide MKNDC 011861); P–T, *M. coxiana* from intermittent pond, near the springs of river Pena, Shar Planina (slide MKNDC 011448); U, *Muelleria* sp. 1. from an intermittent pond below Džinibeg, Shar Planina (slide MKNDC 003083); V–X, *M. undulata* from sand below an ice patch, Kenanica, mountain Stogovo (slide MKNDC 011389); Y–AC, *M. terrestris* from sand below an ice patch, Kenanica, mountain Stogovo (slide MKNDC 11389). Scale bar = 10 μ m.

Basionym – *Navicula gibbula* var. *undulata* Krasske, Archiv für Hydrobiologie 33: 513–514. 1938 (Krasske 1938). – Type: Iceland, Hverakot, sample C II 85 (lecto-: KASSEL).

Synonym – *Navicula gibbula* f. *undulata* Hust. (Hustedt 1942).

Morphology of *M. undulata* found in the Republic of North Macedonia

LM – Valve linear with undulated valve margins and broadly rounded apices. Valve dimensions ($n = 3$): length 19.5–24.0 μm , width 4.5–6.0 μm . Axial area narrow, linear, slightly widening near central area. Central area wide elliptical. Raphe weakly lateral, straight with clearly unilaterally bent, long proximal raphe ends extending almost to first row of areolae. Terminal raphe fissures deflected to same side of valve as proximal raphe ends. Striae in central area (7–8 striae) coarse, more distantly spaced than other striae and clearly radiate whereas other striae only slightly radiate, becoming nearly parallel near apices. Striae 22–24 in 10 μm (measured in 5 μm), and distal striae 26–28 in 10 μm . Areolae round and fine, not discernible in LM.

Remarks – During this study only three specimens were observed from sand below an ice patch, Kenanica, mountain Stogovo (sample 011389), with undulate valve margins and the specimens resembled *N. gibbula* var. *undulata* Krasske. The same taxon was later described by Hustedt (1942) as *N. gibbula* f. *undulata* Hust. Later Hustedt (1961–1966) synonymized his taxon with Krasske's variety. Lange-Bertalot et al. (1996) provided images of two specimens found in the lectotype slide from the Krasske collection (KASSEL, slide C II 85). This taxon considerably differs from *M. gibbula* with respect to the valve size and shape, and therefore should be treated as a separate species.

7. *Muelleria terrestris* (J.B.Petersen) S.A.Spauld. & Stoermer

Fig. 8AD–AH & 10Y–AC

Morphology of *M. terrestris* found in the Republic of North Macedonia

LM – Valves linear to linear-lanceolate with broadly rounded ends. Valve dimensions ($n = 5$): length 26.0–27.0 μm , width 5.5–6.0 μm . Axial area narrow, linear, slightly expanded near large, elliptical central area. Raphe filiform, straight with clearly unilaterally bent, long proximal raphe ends extending to first or second row of areolae. Terminal raphe fissures weakly deflected to same side of valve as proximal raphe ends. Striae in central area (7–8 striae) more distantly spaced than other striae and clearly radiate whereas other striae only slightly radiate, becoming almost entirely parallel near apices. Central striae 16–18 in 10 μm (measured in 5 μm), and distal striae 22–26 in 10 μm . Areolae round and fine, barely discernible in LM.

Remarks – Only five specimens of *M. terrestris* were observed during this study from wet mosses in an intermittent spring near the road in village Sasa, mountain Osogovo (sample 011389). Probably rare or the typical habitat for this species was not collected and analyzed during this study.

DISCUSSION

Previous taxonomical studies *Muelleria* (Spaulding & Stoermer, 1997, Van de Vijver et al. 2010, 2014, Kochman-Kędziora et al. 2017) have suggested that the highest species diversity of the genus is situated in the southern Hemisphere, with only a few species recorded elsewhere (Lange-Bertalot & Genkal, 1999, Reichardt 2004, Bahls 2014). There are two likely reasons for this hypothesis. The first is that typical habitats for *Muelleria* species, such as intermittent ponds, wet rocks, mosses, wet soil, are not frequently sampled and studied. Most of the prior research on diatoms in the northern Hemisphere targeted environmentally and economically important habitats such as rivers or lakes with the aim of establishing reference conditions and ecological status following the Water Framework Directive of the European Union. Also focused studies on phylogeny, taxonomy of widely distributed species complexes or new species from remote areas or biodiversity hotspots generally did not include extremely rare species. Meanwhile, studies of diatoms from terrestrial or subaerial microhabitats have been few (e.g. Antonelli et al. 2017, Pfister et al. 2017) and most have been limited to the sub-Antarctic region (e.g. Zidarova et al. 2014, Van de Vijver et al. 2014, Chattová et al. 2017), while ephemeral waters (such as intermittent alpine ponds or wet habitats) in Europe are seldom investigated (e.g. Petersen 1928, Krasske 1932, Bock 1963, Lange-Bertalot et al. 2003, Novais et al. 2014). The second reason for the observed low diversity of *Muelleria* in Europe maybe the general use of a broad species concept. Techniques such as SEM and molecular analyses have revealed the existence of a large number of unknown, sometimes cryptic diatom species (e.g. Evans et al. 2008, Vanormelingen et al. 2007, 2008, Lundholm et al. 2012). In many cases separate species sharing similar morphological features (characters such as raphe, stria, areolae morphology) were identified under a common name in the literature, although difference existed between those taxa (e.g. Krammer & Lange-Bertalot 1986). In a previous study of diatom distributions from the Republic of North Macedonia, specimens of *M. gibbula* have been mixed with other *Muelleria* taxa (Levkov et al. 2005).

However, the question of why a relatively high diversity of *Muelleria* taxa is observed in the Republic of North Macedonia still remains open. Only a few genetic studies have been performed on the diatom flora of the country, most of them specifically on Lake Ohrid diatoms (e.g. Ruck et al. 2016, Stelbrink et al. 2018). Speciation rates in diatoms from ancient lakes are relatively low. Most of the pennate species present in the contemporary flora of Lake Ohrid have been recorded in the fossil record at ages < 1.3 million years (Levkov et al., pers. obs.). Environmental conditions in the lake were relatively stable and thus climate changes did not force new adaptations of diatoms or create new habitats; the lake has high resilience and resistance to pulse and press events (Jovanovska et al 2016). However, subaerial habitats are highly variable in many environmental aspects causing various stresses (desiccation, temperature and light fluctuation, availability of nutrients) to organisms and thus considered as “extreme” habitats. Such habitats have a low number of species but these are highly specialized to micro-

environments. The species often have limited distributions and known from a single or limited number of localities. Restricted distributions are likely the result of habitat isolation and these ephemeral ponds have no direct connection to each other. They could be considered “aquatic islands” in a “sea of land”. Any neo-endemic that arises in one of these ponds (through mutation of *M. gibbula* or other ancestor species) is not likely to be transported to other ponds. Also, the limited number of studies on such habitats including genetic evaluations restricts our understanding of species distributions. Recent observations about the diatom flora of the Republic North Macedonia have shown that some species that were previously considered rare are quite frequent in specific habitats such as intermittent ponds or wet mosses. However, such habitats were rarely analysed in the past (Levkov et al. 2013, 2016).

Spaulding et al. (1999) provided a hypothesis for the high diversity of *Muelleria* in the Antarctic region. According to the authors, the most plausible hypothesis is that the species evolved in remote areas independently from marine or brackish water progenitors, thus the species would be more closely related to one or several different outgroup taxa, rather than to one another. However, the monophyly of *Muelleria* does not support a hypothesis of multiply colonisations. Most of the species observed in this study belong to a species complex around *M. gibbula* (except *Muelleria* sp. 1). All of them share similar valve morphology (linear-elliptic to linear-lanceolate) and have comparable valve lengths (overlapping to some extent). Additionally, with SEM all observed species share the same internal ultrastructural features, indicating that they belong to the same species complex and are related to *M. gibbula*. It is supposed that all these species, including *M. gibbula*, evolved from some even more remote ancestor. Probably, the ancestor was an aerophilic, cosmopolitan species that colonized freshwater habitats and evolved in several species. Such evolution can take place in isolated ponds anywhere on Earth.

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