

## **High Mitochondrial Diversity in a New Water Bear Species (Tardigrada: Eutardigrada) from Mountain Glaciers in Central Asia, with the Erection of a New Genus *Cryoconicus***

Author(s): Krzysztof Zawierucha, Daniel Stec, Dorota Lachowska-Cierlik, Nozomu Takeuchi, Zhongqin Li and Łukasz Michalczyk

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# HIGH MITOCHONDRIAL DIVERSITY IN A NEW WATER BEAR SPECIES (TARDIGRADA: EUTARDIGRADA) FROM MOUNTAIN GLACIERS IN CENTRAL ASIA, WITH THE ERECTION OF A NEW GENUS *CRYOCONICUS*

KRZYSZTOF ZAWIERUCHA<sup>1,2,6\*</sup>, DANIEL STEC<sup>3\*</sup>,  
DOROTA LACHOWSKA-CIERLIK<sup>3</sup>, NOZOMU TAKEUCHI<sup>4</sup>,  
ZHONGQIN LI<sup>5</sup> and ŁUKASZ MICHALCZYK<sup>3</sup>

<sup>1</sup>*Department of Animal Taxonomy and Ecology, Adam Mickiewicz University, Umultowska 89, 61-614, Poznań, Poland*

<sup>2</sup>*Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburska 89, 277-21 Libečov, Czech Republic*

<sup>3</sup>*Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland*

<sup>4</sup>*Department of Earth Sciences, Graduate School of Science, Chiba University, Chiba, Japan*

<sup>5</sup>*State Key Laboratory of Cryospheric Sciences and Tien Shan Glaciological Station, Chinese Academy of Sciences, 320 Donggang West Road, Lanzhou 730000, China*

<sup>6</sup>*Corresponding author: k.p.zawierucha@gmail.com*

*\*equal contributions*

**Abstract.**— Glaciers and ice sheets are considered a biome with unique organism assemblages. Tardigrada (water bears) are micrometazoans that play the function of apex consumers on glaciers. Cryoconite samples with the dark-pigmented tardigrade *Cryoconicus* **gen. nov.** *kaczmareki* **sp. nov.** were collected from four locations on glaciers in China and Kyrgyzstan. The erection of the new genus is based on a unique combination of morphological traits as well as on phylogenetic analyses. The analysis of COI sequences in the new species revealed high genetic differentiation with 9 haplotypes shared among 13 sequenced individuals from three sequenced populations. There was no apparent geographic structure in COI haplotype diversity, which might indicate effective dispersal abilities of the new species. A recovery of numerous live individuals from a sample that was frozen for 11 years suggests high survival rates in the natural environment. The ability to withstand low temperatures, combined with dark pigmentation that is hypothesised to protect from intense UV radiation, could explain how the new taxon is able to dwell in an extreme glacial habitat. We also found that a rare mountain tardigrade *Ramazottius cataphractus* (Maucci, 1974) is morphologically similar to the new species, therefore we propose to transfer it to the new genus. Our study indicates that glacier invertebrate fauna is still poorly known and requires intense research.



**Key words.**— animals, biodiversity, cryoconite, dark pigmentation, dispersion, extremophiles, fauna, glaciers, endangered habitats, supraglacial zone

## INTRODUCTION

Glaciers and ice sheets constitute *ca.* 10% of the land surface and are the reservoirs of *ca.* 70% of fresh-water on Earth (*e.g.* Anesio and Laybourn-Parry 2012). Despite low temperatures, high doses of UV radiation, intense melting and water drainage from glacier surface, they are habitats for a number of organisms, including microinvertebrates (Mueller *et al.* 2001, Takeuchi *et al.* 2001, Zawierucha *et al.* 2015, 2016, 2018). Glaciers and ice sheets are currently considered biomes with distinct biogeographical structuring and unique organism assemblages adapted to extreme conditions (Anesio and Laybourn-Parry 2012, Boetius *et al.* 2015). The surface of glaciers is typically covered with mineral and organic dust which, together with microorganisms, form glaciers granules (*e.g.* McIntyre 1984, Hodson *et al.* 2008, Cook *et al.* 2015) termed the 'cryoconite'. These granules are highly biologically active consortia of microorganisms that reduce glacier albedo and influence ice melting and formation of water reservoirs, 'cryoconite holes' (Wharton *et al.* 1985, Takeuchi *et al.* 2001, Hodson *et al.* 2010, Cook *et al.* 2015). The ecology and biology of cryo-sphere has been the subject of intensive studies in recent years (*e.g.* Boetius *et al.* 2015, Cook *et al.* 2015, Hotaling *et al.* 2017). The majority of previous works focused on microbe diversity (*e.g.* Grzesiak *et al.* 2015, Franzetti *et al.* 2016), organic matter and carbon budget (Stibal *et al.* 2010, 2012, 2015), biocryomor-phology (*e.g.* Cook *et al.* 2016), physiology and meta-bolism of cryoconite microorganisms (*e.g.* Gawor *et al.* 2016), bioalbedo (*e.g.* Yallop *et al.* 2012, Di Mauro *et al.* 2017), and contamination of supraglacial ecosystems (*e.g.* Ferrario *et al.* 2017). However, despite the first studies on organisms inhabiting glacial ecosystems took place as long ago as at the end of the 19<sup>th</sup> century (*e.g.* Drygalski 1897), our knowledge of the diversity and ecology of glacier microinvertebrates, which often are apex consumers in these extreme ecosystems, is far from satisfactory (Zawierucha *et al.* 2015, 2018).

One of such poorly studied metazoan groups found on glaciers are tardigrades. Tardigrada, also known as water bears, are a cosmopolitan phylum of microinvertebrates (*ca.* 0.5 mm in length) that live in a wide variety of aquatic and terrestrial environments, from ocean depths to mountain tops, including harsh ecosystems such as polar deserts and glaciers (*e.g.* Nelson *et al.* 2015, Zawierucha *et al.* 2015). Although all tardigrades require a film of liquid water to be active, many lim-noterrestrial species can withstand unfavourable environmental conditions thanks to the ability to enter cryptobiosis, a latent state under which tardigrade metabolism is undetectable (*e.g.* Wright 2001, Jönsson and Bertolani 2001, Nelson *et al.* 2015). For example, thanks to anhydro- and cryobiosis, water bears can

survive periodic drying and freezing, respectively. In fact, during the cryptobiotic state, tardigrades are even able to withstand the open space conditions and hence they are considered to be among the toughest animals on Earth (Guidetti *et al.* 2012). Up to now, more than 1200 tardigrade species have been described throughout the globe (Guidetti and Bertolani 2005, Degma and Guidetti 2007, Degma *et al.* 2009–2016). However, only several of them have been reported exclusively from glaciers, specifically from cryoconite holes: *Hypsibius klebelsbergi* Mihelčič, 1959 from the Alps, *H. jane-tscheki* Ramazzotti, 1968 and *H. thaleri* Dastych, 2004 from the Himalayas, *Hypsibius* sp., *Isohypsibius* sp. and one species of Ramazzottiidae from Svalbard archipelago (Dastych *et al.* 2003, Dastych 2004, Zawierucha *et al.* 2015, 2016).

The family Ramazzottiidae (Eutardigrada: Parachela) was erected by Sands *et al.* (2008) and currently comprises four genera with 37 species, with the most species rich being the genus *Ramazzottius* Binda and Pilato 1986. The family is characterised by asymmetrical double claws elongated external and posterior primary branches, apophyses for the insertion of stylet muscles in the form of 'blunt hooks', and ornamented eggs. Whereas the basal claw and both branches are unified into a single element in internal and anterior claws, the external claws have the primary branch joined to the secondary branch and the basal part by a flexible, sometimes transparent, junction (Marley *et al.* 2011). Currently, molecular phylogenetic analyses within limnoterrestrial Tardigrada are limited to only a few studies (Sands *et al.* 2008, Guil and Giribet, 2012, Guil *et al.* 2013, Bertolani *et al.* 2014), all with fragmentary data on Ramazzottiidae. Moreover, molecular data on glacier dwelling tardigrades are restricted only to one species, *Hypsibius klebelsbergi* Mihelčič, 1959, inhabiting Alpine glaciers (Kiehl *et al.* 2007, D'Haese *et al.* 2011, Dabert *et al.* 2015). Jørgensen *et al.* (2007) showed high mitochondrial diversity in a species of a terrestrial heterotardigrade. They found 11 COI haplotypes in 14 populations of *Echiniscus testudo* (Doyère, 1840) collected from three continents, and they interpreted the wide geographic range of the species as evidence for high dispersal potential of *E. testudo* (see also Jørgensen *et al.* 2013 and Gašiorrek *et al.* 2017). Moreover, mitochondrial DNA diversity within glacier invertebrate species has been reported by Hartzell *et al.* (2005) and Dial *et al.* (2012) in oligochaetes endemic to North America, *Mesenchytraeus* sp. (also known as ice worms), and by Shain *et al.* (2016) in Icelandic rotifers. Hartzell *et al.* (2005), using COI, revealed the presence of two geographically distinct clades of oligochaetes in North America (northern and southern). Dial *et al.* (2012) found similar results and, by sequencing COI and 18S rRNA, showed restricted active gene flow between glaciers

throughout evolutionary history of the species (Dial *et al.* 2012). A study conducted on Iceland showed that rotifers collected from three glaciers exhibit high mitochondrial diversity that suggesting the persistence of rotifer lineages during glacial/interglacial cycles (Shain *et al.* 2016). The handful of available examples show that despite an increasing interest in glacier invertebrates, data on their morphological as well as molecular diversity are still extremely scarce (Zawierucha *et al.* 2015, 2016).

With *ca.* 174000 square kilometres of ice cover, Asia is the fourth most glaciated region in the world, surpassed only by the Antarctic, Arctic and South America (World Glacier Monitoring Service, Narama *et al.* 2010, Li *et al.* 2011, Farinotti *et al.* 2015). The glacier-covered area in Central Asia has shrunk significantly within last fifty years, with the estimated decrease of *ca.* 9–35% (Bolch 2007, Narama *et al.* 2010, Li *et al.* 2011, Farinotti *et al.* 2015). Despite the fact that glaciers are among the most endangered habitats on the planet (Hodson *et al.* 2008, Anesio and Laybourn-Parry 2012, Vaughan *et al.* 2013), the number of studies on animals of Asian glaciers is limited to a few faunistic surveys (Zawierucha *et al.* 2015). Due to rapid climate change, studies on the organisms constituting the glacier biome are an urgent task (Anesio and Laybourn-Parry 2012, Zawierucha *et al.* 2015).

In our study, we have analysed cryoconite samples collected at 3934–4830 m a.s.l. from glaciers in Tien Shan (China and Kyrgyzstan) and Qilian Shan (China). In these samples, we found a new tardigrade genus that we describe here by means of integrative taxonomy, *i.e.* by combining light and scanning electron microscopy imaging with sequencing of nuclear and mitochondrial DNA fragments, two conservative (18S rRNA, 28S rRNA) and two variable (ITS-2, COI). Moreover, we investigated the variability of the mitochondrial COI sequences within and between the populations of the new taxon, separated by distances from *ca.* 525 to *ca.* 1700 km.

## MATERIAL AND METHODS

### Samples and specimens

Cryoconite samples were collected from four mountain glaciers between 2003 and 2007 (see Table 1 and Fig. 1 for sampling details and localities, more information on the study area can be found in Takeuchi *et al.* 2005, 2008, Takeuchi and Li 2008). As described in the previous studies (Takeuchi *et al.* 2005, 2008, Takeuchi and Li 2008), the ablation ice surfaces on all of the four glaciers were densely covered with cryoconite

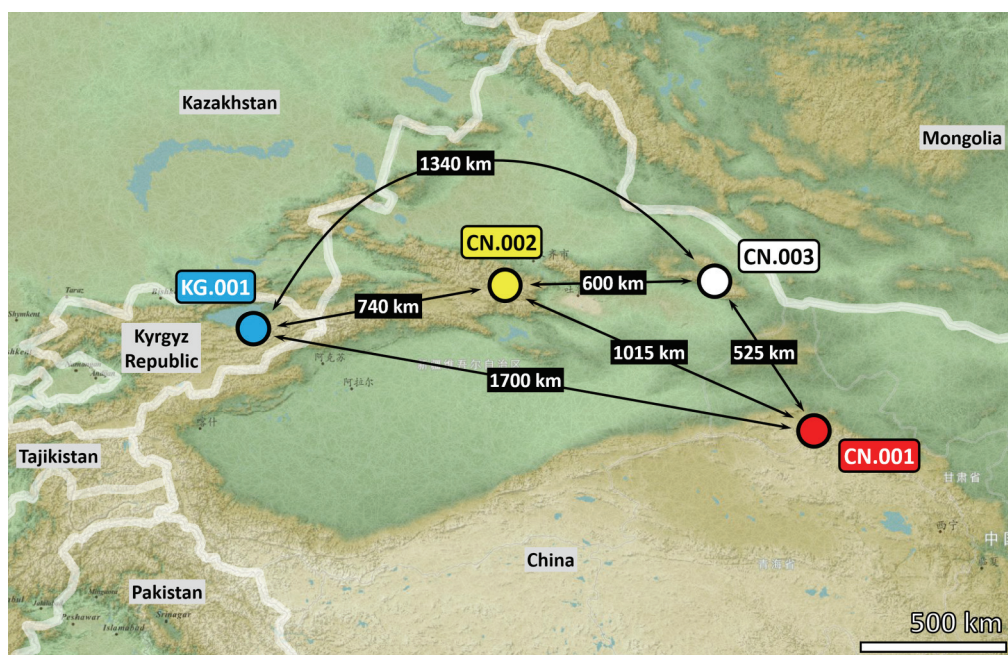


Figure 1. Study area with sampled populations of *Cryoconicus* gen. nov. *kaczmareki* sp. nov. (see Table 1 for details) and geographic distances between them. Colour codes for the four sampled populations: red = CN.001 (Qilian Shan, Qiya Glacier), yellow = CN.002 (Tien Shan, Ürtümqi No.1 Glacier), white = CN.003 (Tien Shan, Miaoergou Glacier), blue = KG.001 (Tien Shan, Grigoriev Ice Cap). Population colours correspond to colours in the COI haplotype network (Fig. 11). Colours of the map correspond with topographical features: green = flatlands, brown = mountain ranges. Map from Apple iPhoto Map.



Table 1. The list of the cryoconite samples, all collected by Nozomu Takeuchi, containing *Cryoconiticus* gen. nov. *kaczmareki* sp. nov. investigated in this study, with collection, analysis and type designation details. Abbreviations: E – ethanol, F – formaldehyde, I – water ice, QY, UM, MG are slide codes, PCM – phase contrast light microscopy, SEM – scanning electron microscopy, DNA – DNA sequencing. More details on the study area are available in Takeuchi *et al.* (2005), Takeuchi and Li (2008), and Takeuchi *et al.* (2008).

Sample code	Coordinates and altitude	Country	Mountain range	Glacier	Collection date	Preservation method	Type designations (slide codes)
CN.001	39°14'30"N 97°45'17"E 4830 m a.s.l.	China	Quilian Shan	Qiyi	2005.08	E, F	10 paratypes (QY)
CN.002	43°06'40"N 86°48'30"E 3934 m a.s.l.	China	Tien Shan	Ürümqi No.1	2006.08	E, F, I	holotype (UM 10/3) and 28 paratypes (UM and CN.002)
CN.003	43°08'11"N 94°11'44"E 4330 m a.s.l.	China	Tien Shan	Miaoergou	2003.08	E, F	7 paratypes (MG)
KG.001	41°58'18"N 77°54'43"E 4300 m a.s.l.	Kyrgyzstan	Tien Shan	Grigoriev Ice Cap	2007.08	E	I

ranging from *ca.* 30 to *ca.* 1100 g m<sup>-2</sup> (Fig. 2(A–B)). The samples, each approximately of 15–45 cm<sup>3</sup> in volume (15 cm<sup>2</sup> in area and 1–3 cm in depth, including *ca.* 36 cm<sup>3</sup> of cryoconite granules), were collected with a stainless-steel scoop. The samples were then preserved with 1 ml of 70% ethanol, or 1 ml of 36% formaldehyde, or frozen (see Table 1 for details). Tardigrades were isolated from the samples in May–June 2014 and processed for various analyses, depending on preservation method. Specifically, all specimens from formaldehyde and some from ethanol were mounted on permanent slides for imaging and morphometry in phase contrast light microscopy (PCM) or fixed for imaging in scanning electron microscopy (SEM). The remaining ethanol-preserved individuals were used for DNA sequencing. Some of animals from the defrosted samples were alive (35 individuals) and were placed on culture dishes in an attempt to obtain eggs (of which none were isolated from the original samples) whereas dead tardigrades were mounted on microscope slides. Live animals were cultured according to Stec

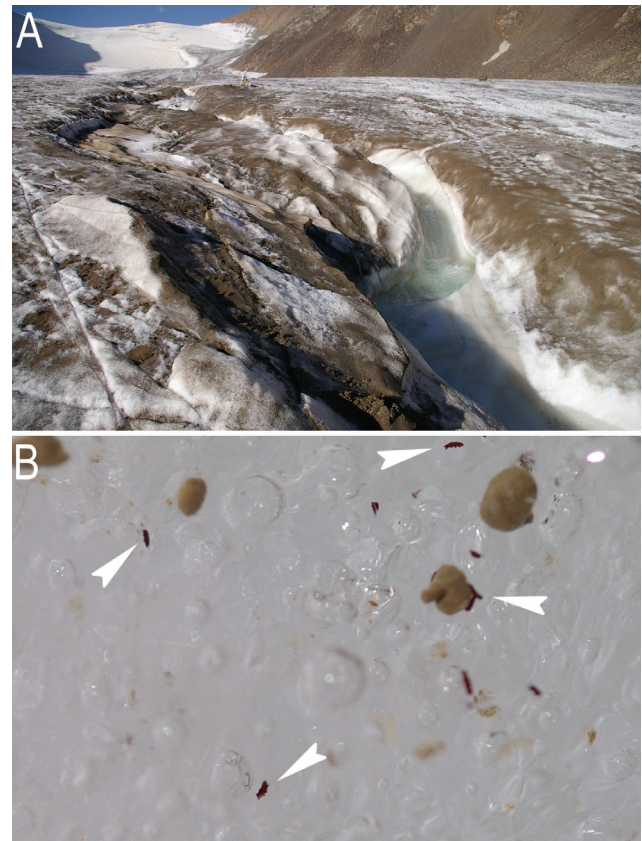


Figure 2. The Urumqi No.1 Glacier (site CN.002–003): A – a typical habitat of *Cryoconiticus* gen. nov. *kaczmareki* sp. nov., B – A close-up of the glacier surface with water film, cryoconite granules and individuals of *Cryoconiticus* gen. nov. *kaczmareki* sp. nov. (several are indicated by arrowheads).

*et al.* (2015). Additionally, several individuals of five different *Ramazottius* species from various locations (including a strain of *Ramazottius varieornatus* Bertolani and Kinchin, 1993 kindly provided to us by Daiki Horikawa and Kazuharu Arakawa), were used to obtain ITS-2, 28S rRNA and COI sequences for comparisons with the new genus (details in Table 2).

## Microscopy and imaging

For PCM, specimens were mounted in Hoyer's medium prepared according to Morek *et al.* (2016) and examined under a *Nikon Eclipse 50i* associated with a *Nikon Digital Sight DS-L2* digital camera. Tardigrades for SEM were processed according to Stec *et al.* (2015), i.e. subjected to a water/ethanol and an ethanol/acetone series, dried in CO<sub>2</sub> critical point, and sputter-coated with an ultrathin layer of gold. Bucco-pharyngeal apparatuses were extracted following a sodium hypochlorite (NaClO) protocol by Eibye-Jacobsen (2001) with modifications described thoroughly in Gąsiorek *et al.* (2016). Specimens and buccal apparatuses were examined under high vacuum in a *Versa 3D DualBeam* SEM at the ATOMIN facility of the Jagiellonian University. All figures were assembled in *Corel Photo-Paint X6*, ver. 16.4.1.1281. For deep structures that could not be fully focused in a single photograph, a series of 2–5 images were taken every *ca.* 0.2  $\mu$ m and then assembled manually into a single deep-focus image.

## Morphometrics and nomenclature

Sample size for morphometrics was chosen following recommendations by Stec *et al.* (2016). All measurements are given in micrometres ( $\mu$ m) and were performed under PCM with the *Nikon Digital Sight DS-L2* software. Structures were measured only if their orientations were suitable. Body length was measured from the anterior to the posterior end of the body, excluding the hind legs. The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube, expressed as a percentage (Pilato 1981). The morphology of the apophyses for the insertion of the stylet muscles were compared with Pilato (2013). Macroplacoid length sequence is given according to Kaczmarek *et al.* (2014a). Terminology for the structures within the bucco-pharyngeal apparatus and for the claws follows that of Pilato and Binda (2010) and Gąsiorek *et al.* (2017). Claws were measured following Beasley *et al.* (2008). Morphometric data were handled using the "Parachela" ver. 1.2 template available from the Tardigrada Register (Michalczyk and Kaczmarek 2013). Tardigrade taxonomy follows Bertolani *et al.* (2014).

Table 2. The list of *Ramazottius* taxa, with their collection details, genotyped for the phylogenetic and genetic distances analysis.

Species	Sample code	Coordinates, altitude	Country	Locality	Sample type	Collection date	Collector
<i>Ramazottius</i> sp.	AT.002	47°54'56"N, 16°41'42"E, 130 m a.s.l.	Austria	Purbach	moss on stone	2014.05	Aneta Rumler
<i>Ramazottius</i> sp.	DE.002	52°16'53"N, 13°38'23"E, 37 m a.s.l.	Germany	Königs Wusterhausen	moss on roof	2014.04	Łukasz Michalczyk
<i>Ramazottius</i> cf. <i>rupeus</i>	NO.001	78°59'44"N, 12°24'22"E, 49 m a.s.l.	Norway	Svalbard, Spitsbergen	moss on stone	2013.08	Krzysztof Zawierucha
<i>Ramazottius subanomalous</i>	PL.019	52°24'11"N, 16°53'19"E, 88 m a.s.l.	Poland	Poznań	moss on stone	2014.05	Marta Ostrowska
<i>Ramazottius varieornatus</i>	n/a	43°03'48"N, 141°22'24"E, 13 m a.s.l.	Japan	Sapporo, Japan	moss on concrete	2004	Daiki Horikawa

## Genotyping

In total, 17 specimens from three populations were genotyped (population CN.001: 5 individuals, CN.002: 8 ind. and KG.001: 4 ind.). A *Chelex*<sup>®</sup> based DNA extraction from individual animals, amplification and sequencing of the chosen markers followed protocols described in detail in Stec *et al.* (2015). Four genomic fragments were amplified using universal and specific primers (Table 3): **18S rRNA** (nuclear small ribosome subunit, nDNA), **28S rRNA** (nuclear large ribosome subunit, nDNA), **ITS-2** (internal transcribed spacer, nDNA), and **COI** (mitochondrial cytochrome *c* oxidase subunit I, mtDNA). Universal primers for COI used widely in tardigrade barcoding, LCO1490 and HCO2198 or HCOoutout (Folmer *et al.* 1994, Prendini *et al.* 2005), produced no amplifications for the new genus, thus we designed a specific forward primer, COI\_Ram.var\_F, homologous with LCO1490, based on a fragment of the mtDNA genome of *R. varieornatus* kindly provided to us by Daiki Horikawa and Kazuharu Arakawa (Hashimoto *et al.* 2016). We also designed a specific reverse primer, COI\_Ram.sub\_R, homologous with HCO2198, based on a *Ramazzottius subanomalous* (Biserov, 1985) COI sequence obtained with universal primers. Primer properties were checked with an online calculator by Kibbe (2007). The primer pairs of COI\_Ram.var\_F and HCO2198 or COI\_Ram.sub\_R gave satisfactory results and were used to amplify the COI fragment for all individuals of the new genus. COI sequences for *Ramazzottius* species listed in Table 2 were obtained with universal primers. Reads were obtained with the *ABI 3130xl* sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of the Jagiellonian University, Kraków, Poland. Sequences were processed in *BioEdit ver. 7.2.5* (Hall 1999), checked using Basic Local Alignment Search Tool (BLAST, Altschul *et al.* 1990) and submitted to GenBank.

## Phylogenetic analyses

To find the phylogenetic position of the new genus within the superfamily Hypsibioidea, we constructed a phylogram based on available 18S rRNA sequences (references to the used sequences are given below) for the representatives of the Hypsibioidea families. Then, to strengthen our inference on the new genus hypothesis, we also conducted a phylogenetic analysis based on concatenated sequences of three molecular markers 28S rRNA, ITS-2 and COI. In addition to the sequences of *Ramazzottius* spp. obtained by us (Table 2, GenBank accession numbers in Table S1), we also utilised published sequences for the superfamily Hypsibioidea as well as sequences for the outgroups deposited in GenBank (i.e. Jørgensen and Kristensen 2004, Faurby *et al.* 2008, Sands *et al.* 2008, Welnicz *et al.* 2011, Guil and Giribet 2012, Michalczyk *et al.* 2012, Bertolani *et al.* 2014, Gašiorek *et al.* 2016, Morek *et al.* 2016, Stec *et al.* 2016, 2017, Gašiorek *et al.* 2017; all GenBank accession numbers and relevant references are presented in Table S1). We aligned all sequences with the ClustalW Multiple Alignment tool (Thompson *et al.* 1994) implemented in *BioEdit* and trimmed the aligned fragments to the size of the shortest available alignment, i.e. 785, 711, 415 and 567 bp for 18S rRNA, 28S rRNA, ITS-2 and COI, respectively. The concatenated sequences comprised 28S rRNA, ITS-2 and COI alignments (734, 451, and 468 bp long, respectively). For phylogeny reconstruction, we used both Bayesian inference (BI) and Maximum likelihood (ML). We used the Akaike Information Criterion (AIC) in MrModeltest 2.3 (Nylander *et al.* 2004) and in conjunction with PAUP\* (Swofford 2002) to determine the best-fitting nucleotide substitution model. In the result, we chose the GTR+I+G model for 18S rRNA (proportion of invariable sites (I) = 0.4865, gamma distribution shape parameter (G) = 0.6762), GTR+I+G model for concatenated data set (proportion of invariable sites

Table 3. Primers used for sequencing of the four DNA fragments (three nuclear and one mitochondrial) of *Cryoconicus* gen. nov. *kaczmareki* sp. nov.

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Source
18S rRNA	SSU01_F	forward	AACCTGGTTGATCCTGCCAGT	Sands <i>et al.</i> (2008)
	SSU82_R	reverse	TGATCCTTCTGCAGGTTACCTAC	
28S rRNA	28SF0001	forward	ACCCVCYNAATTTAAGCATAT	Mironov <i>et al.</i> (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	
ITS-2	ITS3	forward	GCATCGATGAAGAACGCAGC	White <i>et al.</i> (1990)
	ITS4	reverse	TCCTCCGCTTATTGATATGC	
COI	COI_Ram.var_F	forward	TTTCAACAAACCATAAAGATATCGG	<b>this study</b>
	COI_Ram.sub_R	reverse	TAAACTTCTGGGTGGCCAAAAAATCA	
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	



(I) = 0.1322, gamma distribution shape parameter (G) = 0.5138). We ran BI using MrBayes 3.1 (Huelsenbeck and Ronquist 2001, Huelsenbeck *et al.* 2001) with 1 cold and 3 heated Markov chains for 10 million generations, and trees were sampled every 1000<sup>th</sup> generation (we ran each simulation twice). We estimated the convergence of Bayesian analyses using Tracer v. 1.5.0 (Rambaut and Drummond 2009). We discarded the 'burnt in' trees, and used the remainder to reconstruct a 50% majority rule consensus tree. Maximum likelihood (ML) analyses for the concatenated data set were performed using RAxMLv. 8.1.11 (Stamatakis 2014) applying a rapid bootstrapping analysis with 1000 bootstrap pseudoreplicates and a general-time-reversible nucleotide substitution model. Branch support was assessed using 1000 bootstrap replicates. All trees were visualised with TreeView 1.6.6 (Page 1996). Additionally, uncorrected pairwise distances between the new genus and *Ramazzottius* spp. for all available DNA fragments were calculated using MEGA version 6 (Tamura *et al.* 2013) and are provided in Table S2. The median-joining networks (Bandelt *et al.* 1999) from COI sequences was generated using PopART ver. 1.7 (<http://popart.otago.ac.nz>) for each specimen from all three populations in order to study the relationships between haplotypes and their geographic distribution.

## Data deposition

Raw data underlying the description of the new taxon are deposited in the *Tardigrada Register* (Michalczyk and Kaczmarek 2013) under [www.tardigrada.net/register/0047.htm](http://www.tardigrada.net/register/0047.htm). All new DNA sequences were submitted to GenBank ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)). Files with GenBank accession numbers (S1), list of haplotypes with calculated genetic distances (S2) as well as raw sequences of the new species (S3) are additionally provided in the Supplementary Materials deposited under <http://dx.doi.org/10.7910/DVN/FL5T20>.

## RESULTS

### Taxonomic account

Phylum: **Tardigrada** (Doyère, 1840)

Class: **Eutardigrada** Richters, 1926

Order: **Parachela** Schuster, Nelson, Grigarick and Christenberry, 1980

Superfamily: **Hypsibioidea** Sands, McInnes, Marley, Goodall-Copestake, Convey and Linse, 2008

Family: **Ramazzottiidae** Marley, McInnes and Sands, 2011

Genus: ***Cryoconicus*** gen. nov.  
(Figs 3–8, 10, Table 4)

**Etymology.** The name *Cryoconicus* refers to cryoconite granules, among which the new genus was discovered.

**Diagnosis.** Juveniles light-brown, adults intensely dark-brown (Fig. 3A). Peribuccal lamellae and papulae absent. Buccal apparatus of the *Ramazzottius* type, with asymmetrical apophyses for the insertion of the stylet muscles and two macroplacoids; microplacoid and septulum absent (Fig. 5A–F). Claws of the *Ramazzottius* type, but without accessory points (Fig. 6A and 7A–F). The posterior primary branch almost uniform in diameter from the base to the curving (Fig. 7A–D and 8A–B). Wide, semi-transparent cuticular bars under claws I–III (Fig. 6A and 8C, arrowheads).

### Genus composition (two species)

***Cryoconicus cataphractus*** (Maucci, 1974) comb. nov.

*Hypsibius cataphractus*: *Locus typicus*: Austria, vicinity of Grossglockner (Maucci 1974), Spitsbergen (Dastych 1985), Greenland (Maucci 1991).

*Ramazzottius cataphractus*: Canada (Jørgensen and Kristensen 1991), Greenland (Maucci 1991, Maucci 1996).

**Material studied.** photomicrographs of paratypes (courtesy of Roberto Bertolani and the Civic Museum of Natural History of Verona), and drawings from the original description of the species by Maucci (1974).

**Notes.** Given that claw morphology, body pigmentation, and cuticular sculpturing of *R. cataphractus* match those of the new genus, we transfer the species to *Cryoconicus* gen. nov. It is also worth noting that *C. cataphractus* comb. nov., by inhabiting polar and montane environments, exhibits thermal preferences similar to those of the new species. Nevertheless, despite the similarities, the new species can be readily differentiated from *C. cataphractus* comb. nov. by the size of cuticular granulation (*ca.* 1  $\mu$ m in diameter and difficult to observe in PCM in *C.* gen. nov. *kaczmareki* sp. nov. *vs ca.* 2  $\mu$ m in diameter and easily identifiable in PCM in *C. cataphractus* comb. nov.) and by the length of the posterior primary branch (up to 35  $\mu$ m in *C.* gen. nov. *kaczmareki* sp. nov. *vs* up to 45  $\mu$ m in *C. cataphractus* comb. nov.).

***Cryoconicus kaczmareki*** sp. nov.  
(Figs 3–7, Table 4)

**Type depositories.** holotype (slide number: UM 10/3) and 39 paratypes (slides: UMS1/2–UMS1/6, QY29/1–QY29/6, MG53/1–MG53/5, UM10/1–UM10/5) are deposited in the Department of Animal Taxonomy and



Ecology at the Adam Mickiewicz University, Poznań, Poland, further 6 paratypes (slides: CN.002/01–05) are preserved at the Institute of Zoology and Biomedical Research of the Jagiellonian University, Kraków, Poland.

**Etymology.** We take great pleasure in dedicating the new species to our friend and a prolific Polish

tardigradologist, dr. Łukasz Kaczmarek of the Adam Mickiewicz University, Poznań, Poland.

**Description.** Juveniles light brown, adults brown to dark brown (Fig. 3A). Eyes absent in all live specimens (Fig. 3A). Elliptical organs placed dorso-laterally on the head (Fig. 3C–E). Legs IV with a single small gibbosity, not always well visible (Fig. 6B–D). A flat

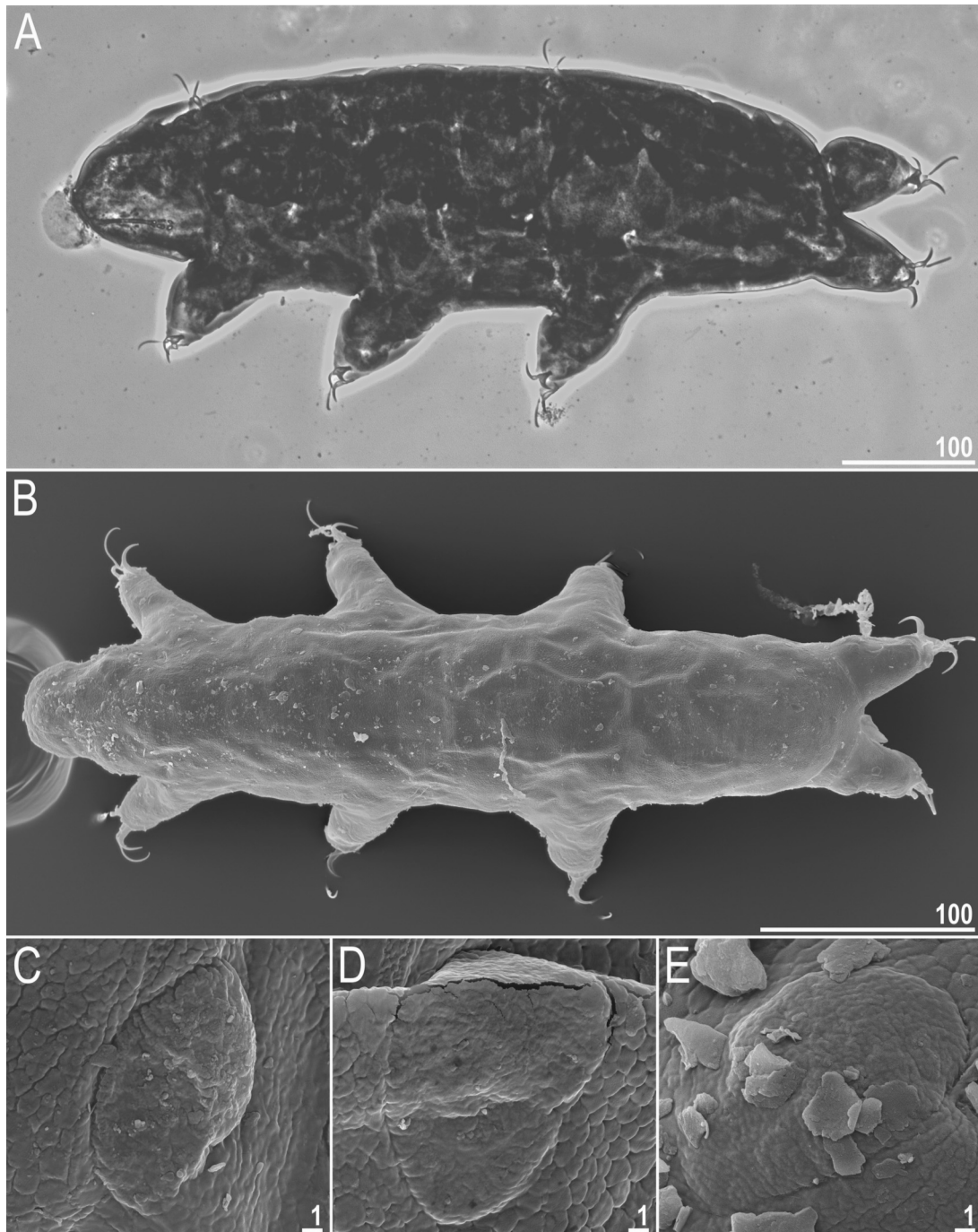


Figure 3. *Cryoconicus* gen. nov. *kaczmareki* sp. nov.: A – Dorsal view, habitus (holotype, PCM), B – Dorsal view (paratype, SEM), C–E – Cephalic elliptical organs (paratypes, SEM). Scale bars in  $\mu\text{m}$ .

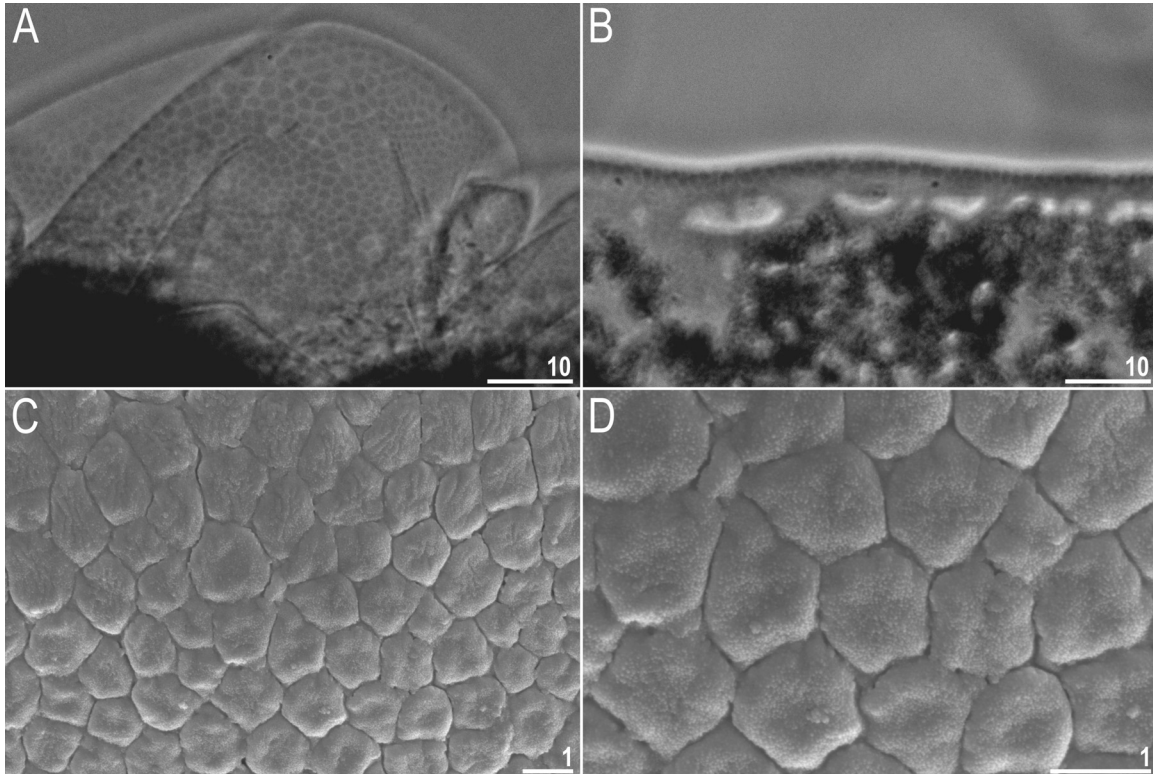


Figure 4. *Cryoconicus* gen. nov. *kaczmareki* sp. nov.: A – Lateral cuticle separated from internal tissues with clearly visible sculpturing (paratypes, PCM), B – Sculpture on the lateral cuticle (paratype, PCM), C–D – Cuticular sculpture on the dorsum (paratype, SEM). Scale bars in  $\mu\text{m}$ .

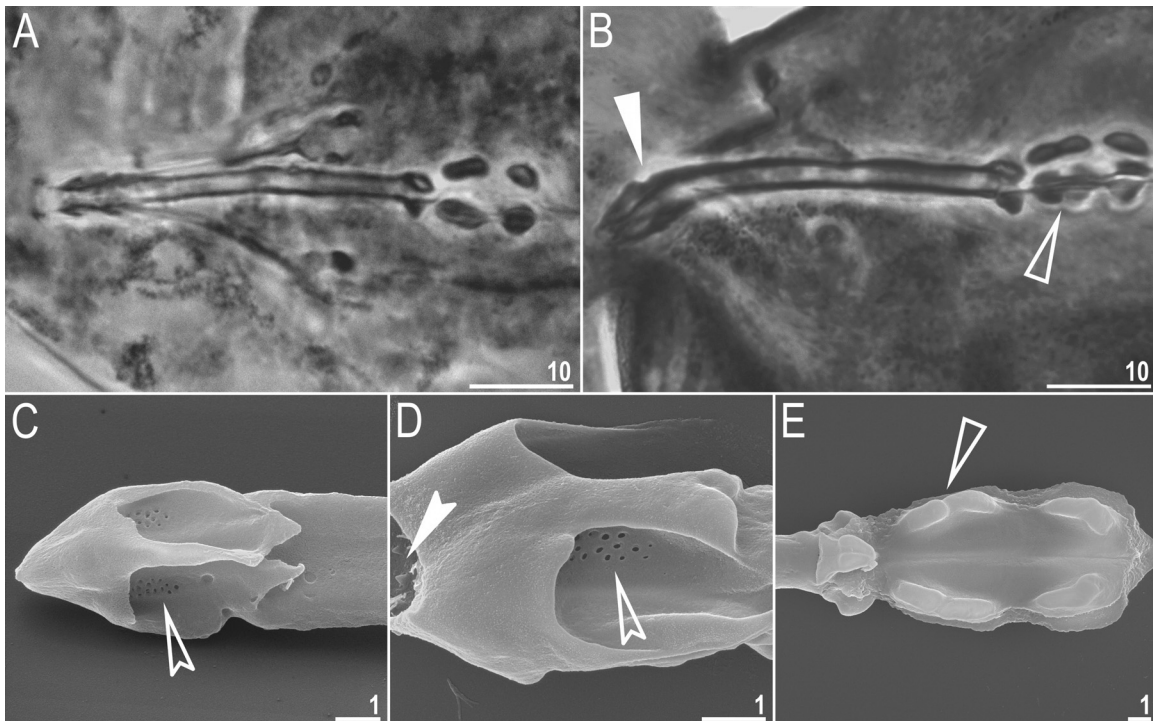


Figure 5. *Cryoconicus* gen. nov. *kaczmareki* sp. nov.: A – Buccal apparatus, dorso-ventral projection (paratype, PCM), B – Buccal apparatus, lateral projection (paratype, PCM), C, D – Buccal crown (paratype, SEM), E – Macroplacoids (paratype, SEM). The flat filled arrowhead indicates the dorsal AISM, the flat empty arrowhead indicates the constriction in first microplacoid, the indented filled arrowhead indicates teeth in the oral cavity and indented empty arrowheads indicate porous areas on the lateral sides of the crown. Scale bars in  $\mu\text{m}$ .



papilla-like cuticular fold, visible in SEM only, present on the lateral sides of legs IV (Fig. 6E–F). Dorso-lateral cuticle covered with densely arranged small (0.8–1.6  $\mu\text{m}$ ) and flat polygon-shaped tubercles (visible clearly in SEM but poorly in PCM, Fig. 4A–D). Mouth antero-ventral. Peribuccal lamellae and papulae absent. The

oral cavity armature composed of a band of medium-size conical teeth visible only in SEM (Fig. 5D). Two distinct porous areas on the lateral sides of the buccal crown (visible in SEM only, Fig. 5C–D). The apophyses for the insertion of the stylet muscles asymmetrical, of the *Ramazzottius* type (Fig. 5B). Stylet support

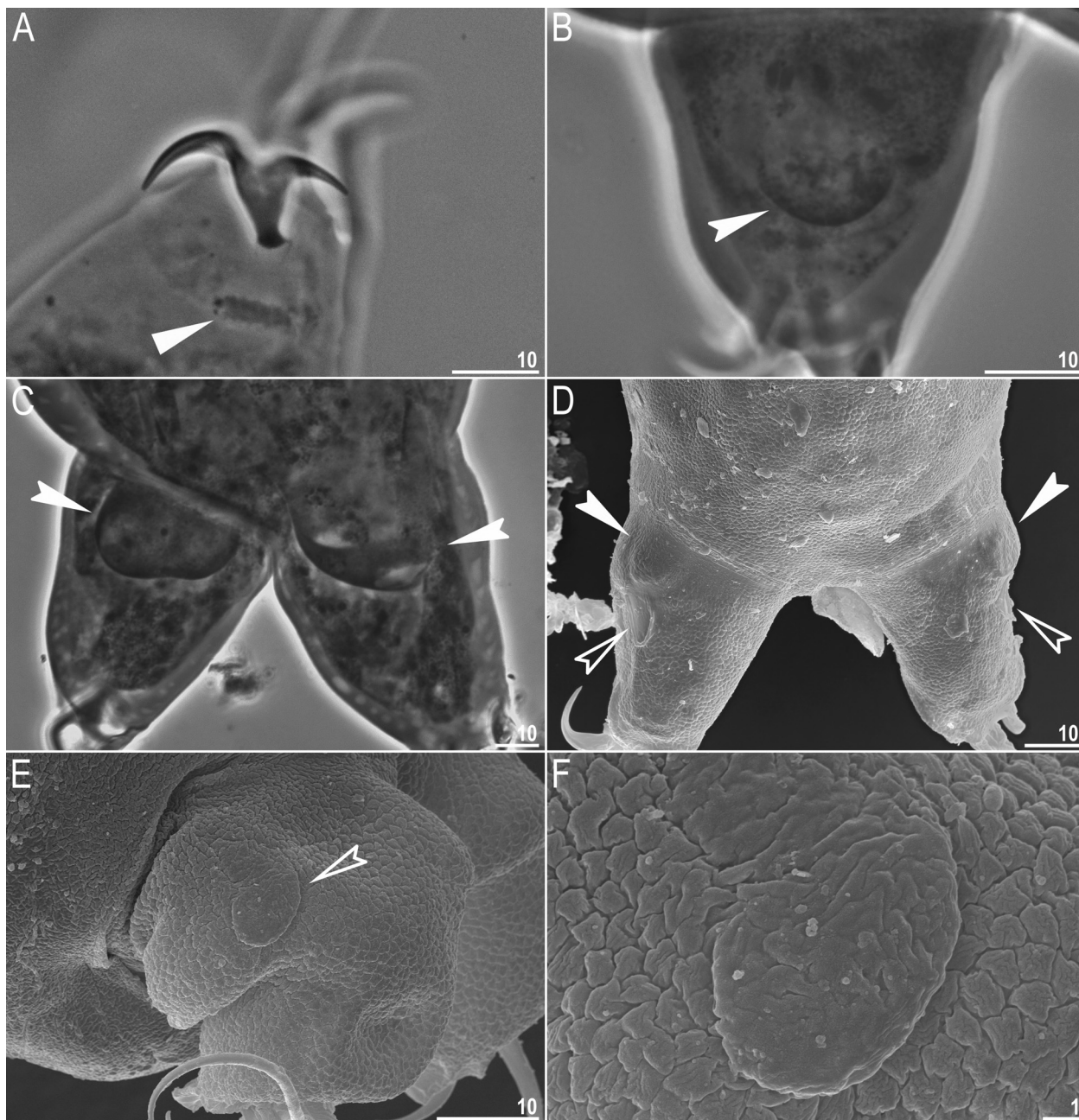


Figure 6. *Cryoconicus* gen. nov. *kaczmareki* sp. nov.: A – Internal claw II with the wide and semi-transparent cuticular bar below (paratype, PCM), B – A gibbosity on a hind leg (paratype, PCM), C – Gibbosities on hind legs (paratype, PCM), D – Gibbosities on hind legs (paratype, SEM), E – Hind leg with the papilla-like cuticular fold (paratype, SEM), F – The papilla-like cuticular fold on a hind leg (paratype, SEM). The flat filled arrowhead indicates the cuticular bar, indented filled arrowheads show hind leg gibbosities, and the indented empty arrowhead indicates the papilla-like cuticular fold. Scale bars in  $\mu\text{m}$ .



insertion point in posterior position. Buccal tube narrow, with a distinctly thickened wall posterior to the stylet support insertion point. Bulbus with apophyses and two macroplacoids, microplacoid and septulum absent (Fig. 5A–B and E). The first macroplacoid with a central constriction, second without constrictions.

Macroplacoid length sequence  $2 < 1$ . Apophyses and the first macroplacoid as well as the first and the second macroplacoid clearly separated (Fig. 5A–B and E). Claws slender, similar to the *Ramazzottius* type claws (Fig. 7A–D and 8A–B). The primary branches of external and posterior claws very thin, almost filament-like

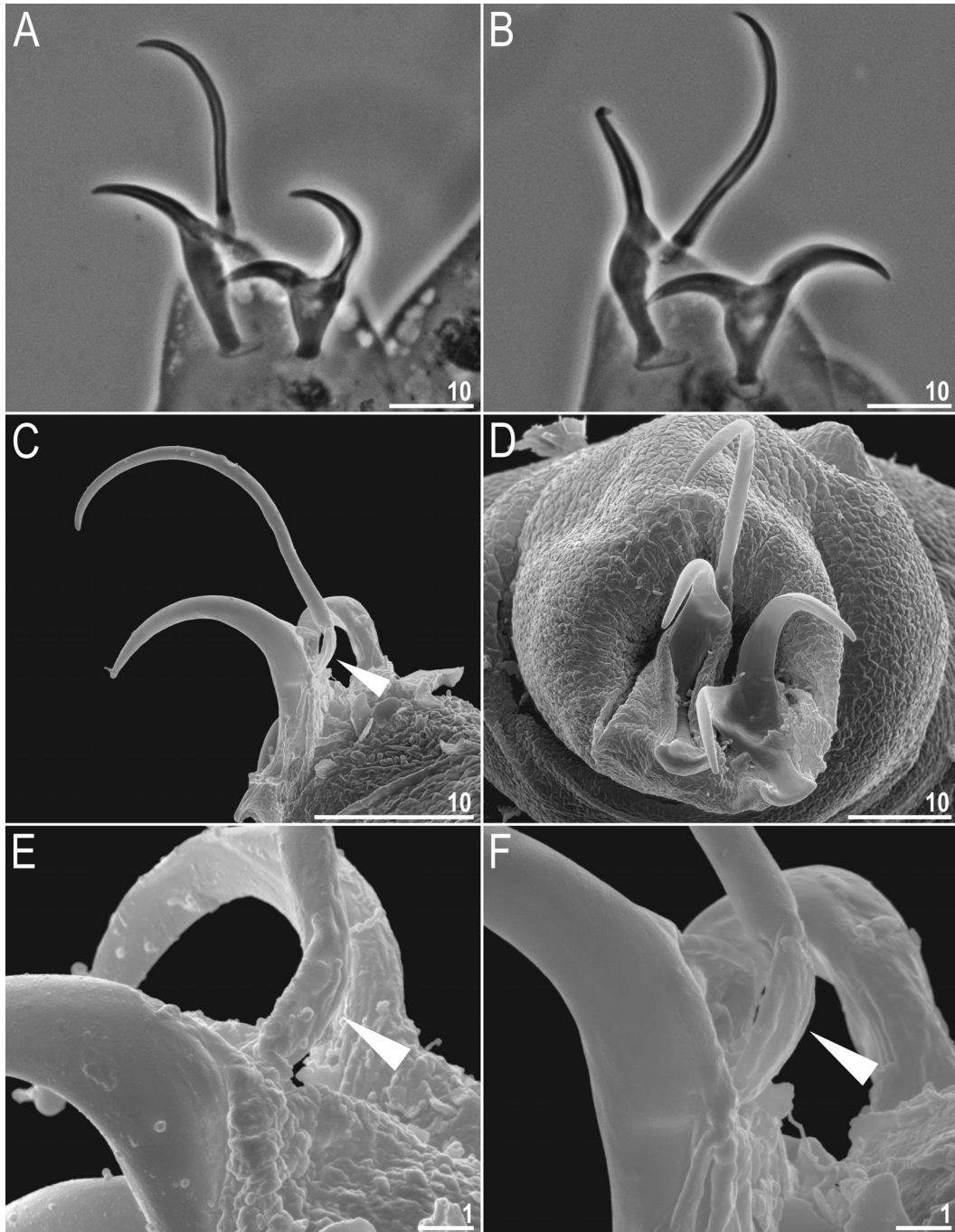


Figure 7. *Cryoconicus* gen. nov. *kaczmareki* sp. nov.: A – Claws III (paratype, PCM), B – Claws IV (paratype, PCM), C – External claw III (paratype, SEM), D – Claws IV (paratype, SEM), E – The non-sclerotized flexible junction between the secondary and the primary branch of the external claw II (paratype, SEM), F – The non-sclerotized flexible connector between the secondary and the primary branch of the external claw III (paratype, SEM). Arrowheads indicate the non-sclerotized flexible connector between the secondary and the primary branches. Scale bars in  $\mu\text{m}$ .

Table 4. Measurements [in  $\mu\text{m}$ ] of selected morphological structures of individuals of *Cryoconicus* gen. nov. *kaczmareki* sp. nov. from samples CN.001–3. Specimens mounted in Hoyer's medium (N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens, SD – standard deviation).

Character	N	Range		Mean		SD		Holotype	
		$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>	$\mu\text{m}$	<i>pt</i>
Body length	24	292–652	981–1470	467	1236	93	150	568	1397
Buccopharyngeal tube									
Buccal tube length	24	28.7–40.7	–	35.8	–	4.2	–	40.7	–
Stylet support insertion point	25	18.1–26.0	60.5–65.1	22.8	62.4	2.1	1.1	25.6	63.0
Buccal tube external width	23	2.1–3.5	7.2–9.5	2.9	8.1	0.5	0.7	3.2	7.9
Buccal tube internal width	23	1.0–2.1	3.1–6.0	1.6	4.4	0.4	0.7	1.7	4.1
Placoid lengths									
Macroplacoid 1	26	3.8–6.4	12.9–16.5	5.1	14.2	0.7	1.1	6.2	15.3
Macroplacoid 2	26	2.4–4.6	7.5–12.5	3.7	10.1	1.3	1.2	4.2	10.2
Macroplacoid row	26	7.8–13.3	25.2–34.6	10.5	28.9	3.1	2.4	12.0	29.5
Claw 1 lengths									
External base	22	9.2–17.1	28.0–41.9	13.4	35.8	2.7	4.5	15.5	38.0
External primary branch	23	14.2–25.8	45.2–65.9	19.9	54.5	3.3	5.8	22.4	55.0
External secondary branch	20	8.6–21.0	31.1–53.6	14.0	40.4	3.3	7.3	14.6	35.8
Internal base	17	5.8–13.6	17.9–30.4	9.2	23.0	2.3	4.0	9.8	24.2
Internal primary branch	11	7.2–15.5	24.8–38.1	11.6	30.5	5.5	4.0	15.5	38.1
Internal secondary branch	15	6.4–12.9	19.4–35.4	9.9	26.7	2.3	6.1	11.0	27.0
Claw 2 lengths									
External base	20	9.6–19.7	31.7–47.1	14.1	38.8	3.0	5.1	15.4	37.8
External primary branch	28	14.2–28.8	51.4–72.5	22.1	61.6	4.6	6.6	24.9	61.1
External secondary branch	22	8.0–21.6	32.3–61.4	14.7	43.5	4.2	10.2	14.4	35.4
Internal base	21	5.6–11.6	19.7–31.2	9.2	25.3	2.6	3.1	10.2	25.0
Internal primary branch	12	10.6–17.9	28.7–45.8	13.1	35.6	4.8	5.8	13.5	33.1
Internal secondary branch	19	6.4–13.1	19.7–34.1	10.0	27.2	2.4	3.9	11.8	29.1
Claw 3 lengths									
External base	23	8.1–19.2	34.2–49.0	14.8	40.7	3.0	4.7	16.0	39.2
External primary branch	26	16.0–30.4	55.4–77.4	23.0	63.6	4.3	7.5	23.6	58.0
External secondary branch	24	6.3–22.1	30.6–66.9	15.1	43.8	4.5	10.2	14.3	35.1
Internal base	22	5.0–11.6	18.1–31.5	8.8	25.0	2.2	4.1	11.3	27.8
Internal primary branch	10	7.6–15.4	28.8–40.6	12.1	34.8	2.5	4.6	12.3	30.3
Internal secondary branch	22	4.8–16.9	18.3–41.5	10.2	27.7	2.9	5.8	16.9	41.5
Claw 4 lengths									
Anterior base	19	7.2–13.2	21.3–35.5	9.5	26.1	6.5	3.9	11.3	27.8
Anterior primary branch	12	10.1–18.2	31.6–45.5	14.3	39.1	2.6	4.7	18.2	44.8
Anterior secondary branch	19	6.8–14.8	21.0–39.9	11.0	30.1	2.5	5.3	12.6	31.0
Posterior base	14	8.6–20.2	31.6–45.7	13.4	37.5	3.0	3.7	16.3	40.0
Posterior primary branch	20	17.3–35.3	58.3–80.5	24.7	67.0	4.9	7.0	26.1	64.2
Posterior secondary branch	13	10.0–18.9	32.1–53.6	14.7	42.1	3.2	7.7	16.1	39.6

and attached to the secondary branch by a not sclerotized, flexible and light-refracting connector (Fig. 7A–F and 8A–B). The posterior primary branch without the basal swelling, *i.e.* almost uniform in diameter from the base to the curving. All primary branches without accessory points (Fig. 6A, 7A–D and 8A–B). Claw

lengths increasing slightly from legs I to IV (Table 4). Pseudolunules present on all claws (Fig. 7A–B) sometimes hardly visible. Wide, semi-transparent cuticular bars under claws I–III present, but visible only in lightly pigmented/thoroughly fixed specimens (Fig. 6A). Eggs unknown.

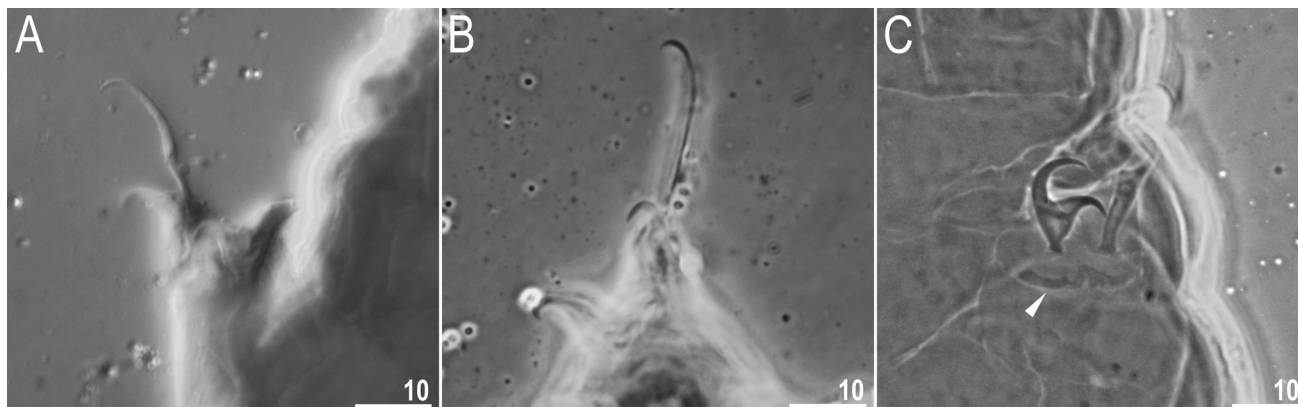


Figure 8. *Cryoconicus* gen. nov. *cataphractus* comb. nov. (Maucci, 1974): A – External claw I (paratype, DIC), B – External claw IV (paratype, PCM), C – Wide semi-transparent cuticular bar under claw II (holotype, PCM). Scale bars in  $\mu\text{m}$ . Photomicrographs courtesy of Roberto Bertolani and the Civic Museum of Natural History of Verona.

**Remarks.** The morphology of the buccal apparatus of the new species is typical for a herbivorous, non-predatory tardigrade (Guidetti *et al.* 2012, Guil and Sanchez-Moreno 2013), but the undigested matrix of the gut suggests that the new genus feeds on cyanobacteria or fungi and could be a microbivore (Fig. 10A–B). Thus, more data are needed to establish the dietary preferences of the new species. Although eggs were not found, we should probably expect them to be ornamented and laid freely as the new genus is phylogenetically embedded among taxa that lay such eggs (see below

for details). Moreover, no exuvia with eggs were found and these are easier to observe than single eggs that could be obscured by cryoconite particles and overlooked.

**DNA sequences.** We found single haplotypes in the conservative nuclear regions, 18S rRNA and 28S rRNA (accession numbers MG432796 and MG432797, respectively), across 17 sequenced individuals from the three examined populations. In contrast, the two more variable markers, ITS-2 and COI, exhibited multiple haplotypes. The ITS-2 was represented by three haplotypes

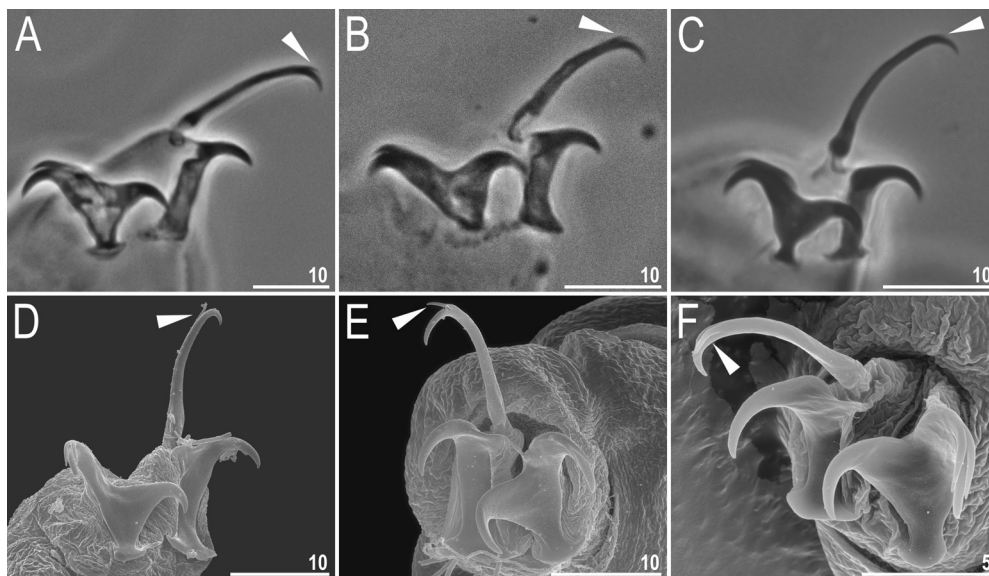


Figure 9. Claws of *Ramazzottius* species: A – *Ramazzottius subanomalous* (Biserov, 1985) – claws IV (PCM), B – *Ramazzottius* sp. (PL.028) claws IV (PCM), C – *Ramazzottius* cf. *rupeus* (Biserov, 1999) claws IV (PCM), D – *Ramazzottius subanomalous* claws I (SEM), E – *Ramazzottius subanomalous* claws IV (SEM), F – *Ramazzottius bunikowskiae* Kaczmarek *et al.* 2006 claws II (SEM). Arrowheads indicate accessory points on external primary branches. Scale bar in  $\mu\text{m}$ .



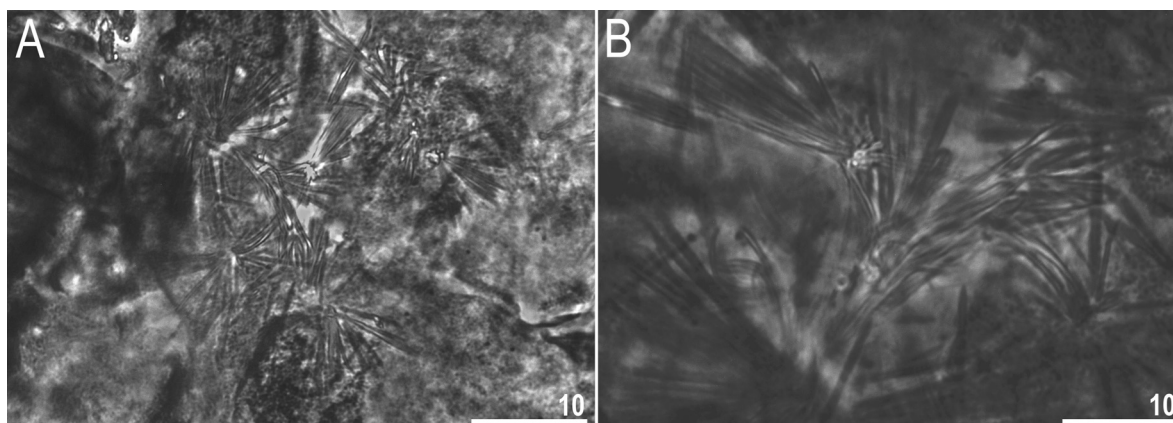


Fig. 10. *Cryoconicus* gen. nov. *kaczmareki* sp. nov.: intestine contents (paratypes, PCM). Scale bar in  $\mu\text{m}$ .

that differed by two single nucleotide polymorphisms (SNPs, Table 5, MG432798–800) whereas nine haplotypes with eighteen variable sites were recovered for COI (Table 6, MG432801–9). The uncorrected p-distances between the three ITS-2 haplotypes varied from 0.3% to 0.6% (0.4% on average) and from 0.2% to 2.1% (1.1% on average) between the nine COI haplotypes (see also Supplementary Materials S2 deposited under <http://dx.doi.org/10.7910/DVN/FL5T20>). The most abundant ITS-2 haplotype was H1 (15 of 17 specimens, present in all three sequenced populations), whereas haplotypes H2 and H3 were represented only by single individuals in populations CN.002 and CN.001, respectively. There was no clear geographic structuring of the COI haplotypes (Table 6, Fig. 11): seven haplotypes (H1–3 and H6–9) were found only in single populations and two haplotypes were shared between two populations (H5 in samples CN.002 and KG.001, *ca.* 740 km apart, H4 in samples CN.001 and CN.002, *ca.* 1015 km apart). The p-distances between haplotypes within and between the sampled populations were of a similar magnitude: 0.2–1.6% and 0.0–2.1%, respectively. The COI haplotypes formed four haplogroups which were connected by hypothetical intermediate haplotypes (Fig. 11). The first haplogroup consisted of haplotypes

H1–4, the second one of only haplotype H5, the third one of haplotypes H6 and H7, and the fourth one comprised haplotypes H8 and H9 (Fig. 11). The sequences of all haplotypes for all sequenced DNA markers are given in the Supplementary Materials (S3 deposited under <http://dx.doi.org/10.7910/DVN/FL5T20>).

### Differential diagnosis and the phylogenetic position of the new genus

The new genus belongs to the family Ramazzottidae Marley *et al.* 2011, which is defined by the presence of body pigmentation, two elliptical sensory organs on head, asymmetrical AISM in the shape of ‘blunt hooks’ (the dorsal being different from the ventral, resulting in asymmetry with respect to the frontal plane), the presence of a thin and flexible connector between the basal claw and the primary branch in the posterior claws, and by freely laid ornamented eggs (Pilato and Binda 1990, Marley *et al.* 2011). Until recently, the family comprised two genera, *Ramazzottius* Binda and Pilato, 1986 and *Hebesuncus* Pilato, 1987, but Bertolani *et al.* (2014) provisionally broadened the definition by

Table 5. The list of ITS-2 haplotypes and their variable sites detected in the three analysed populations of *Cryoconicus* gen. nov. *kaczmareki* sp. nov. The haplotype 1 is treated as reference and different nucleotide in each loci are bolded. GenBank accession numbers are given in brackets. See Tab. 2 for collection data.

Haplotype (accession no.)	Population(s)	Single Nucleotide Polymorphisms ( <i>loci</i> )	
		169	366
1 (MG432798)	CN.001–2, KG.001	G	T
2 (MG432799)	CN.002	C	T
3 (MG432800)	CN.001	G	C

Table 6. The list of COI haplotypes and their variable sites detected in the three analysed populations of *Cryoconicus* gen. nov. *kaczmareki* sp. nov. The haplotype 1 is treated as reference and different nucleotide in each loci are bolded. GenBank accession numbers are given in brackets. See Table 1 for collection data.

Haplotype (accession no.)	Population(s)	Single Nucleotide Polymorphisms (loci)																			
		29	98	98	122	137	140	173	185	221	266	272	320	332	344	371	404	416	458	566	
1 (MG432801)	KG.001	A	T	T	G	C	A	G	A	A	A	T	A	C	A	A	T	C	A	C	
2 (MG432802)	CN.002	A	T	<b>A</b>	<b>A</b>	C	A	G	A	A	<b>G</b>	A	C	C	A	A	T	C	A	C	
3 (MG432803)	CN.001	A	T	<b>A</b>	<b>A</b>	C	A	G	A	A	T	A	C	C	A	A	T	C	A	C	
4 (MG432804)	CN.001–2	A	T	<b>A</b>	<b>A</b>	C	A	G	A	A	T	A	C	C	A	A	T	C	A	C	
5 (MG432805)	KG.001, CN.002	A	T	<b>A</b>	<b>A</b>	C	A	G	A	A	T	<b>G</b>	<b>T</b>	<b>T</b>	<b>G</b>	<b>C</b>	<b>T</b>	<b>T</b>	<b>G</b>	<b>C</b>	
6 (MG432806)	KG.001	<b>G</b>	T	<b>A</b>	<b>A</b>	C	A	<b>A</b>	A	<b>G</b>	T	<b>G</b>	<b>T</b>	<b>T</b>	A	C	C	A	C		
7 (MG432807)	CN.002	<b>G</b>	T	<b>A</b>	<b>A</b>	<b>T</b>	A	<b>A</b>	<b>G</b>	<b>A</b>	T	<b>G</b>	<b>T</b>	<b>T</b>	A	T	C	A	C		
8 (MG432808)	CN.002	A	<b>C</b>	<b>C</b>	<b>A</b>	C	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	T	<b>G</b>	<b>T</b>	<b>T</b>	<b>G</b>	T	C	A	<b>T</b>		
9 (MG432809)	CN.002	A	<b>C</b>	<b>A</b>	<b>A</b>	C	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>G</b>	<b>T</b>	<b>T</b>	<b>G</b>	T	C	A	<b>T</b>		

restricting the diagnostic criteria to claw morphology. This resulted in a tentative inclusion of two further genera, *Ramajendas* Pilato and Binda, 1990, and *Thalerius* Dastych, 2009, in the family. However, the systematic position of these two taxa is not certain. Whereas *Ramazzottius* and *Hebesuncus* share a number morphological traits, the similarities with *Ramajendas* and *Thalerius* are limited to claw morphology as other traits are different or not known. Specifically, *Ramajendas* lacks body pigmentation and the cephalic sensory organs, it has the AISM similar to the *Isohypsibius* type and deposits smooth eggs in shed exuviae (*e.g.* Pilato and Binda 1990, Kaczmarek *et al.* 2014b) whereas *Thalerius* lacks body colouration and the cephalic sensory organs, but the AISM shape and egg type are not known (Dastych 2009). However, the lack of body pigmentation, general appearance of the buccal apparatus and the presence of non-transparent (opaque) cuticular bars under claws suggests affinity with the family Isohypsibiidae. Moreover, the close phylogenetic relationship between *Ramazzottius* and *Hebesuncus* has been confirmed by molecular studies (*e.g.* Bertolani *et al.* 2014) whereas DNA sequences for *Ramajendas* and *Thalerius*, which would allow an independent verification of the systematic status of these genera, are not currently available. It is, therefore, possible that the claw morphology in *Ramajendas* and *Thalerius* evolved independently and is only superficially similar to that of *Ramazzottius* and *Hebesuncus*. Thus, taking all this into consideration, in our opinion, *Ramajendas* and *Thalerius* should be provisionally placed in the family Isohypsibiidae.

In contrast to *Ramajendas* and *Thalerius*, the new genus exhibits all key animal traits that allow to confidently place it in the family Ramazzottiidae, *i.e.* cephalic sensory organs as well as the *Ramazzottius* type claw and AISM morphology. Moreover, the systematic position of the new genus inferred from morphology is congruent with that based on DNA sequences (see below for details). Given the controversies regarding the composition of the Ramazzottiidae, we differentiate the new genus only from *Ramazzottius* and *Hebesuncus*, the genera that undoubtedly belong to the family.

*Cryoconicus* gen. nov., differs from *Ramazzottius* and *Hebesuncus* by a number of traits, specifically by body pigmentation (dark brown in *Cryoconicus* gen. nov. *vs* colouring from pink, violet, orange to red and light brown in the two other genera), the shape of the posterior primary branch (almost uniform in diameter from the base to the curving in *Cryoconicus* gen. nov. *vs* clearly thicker at base, forming a basal club-like swelling and thinner towards the curving in the two other genera, Fig. 9A–F), the lack of the accessory points (accessory points are present on all primary

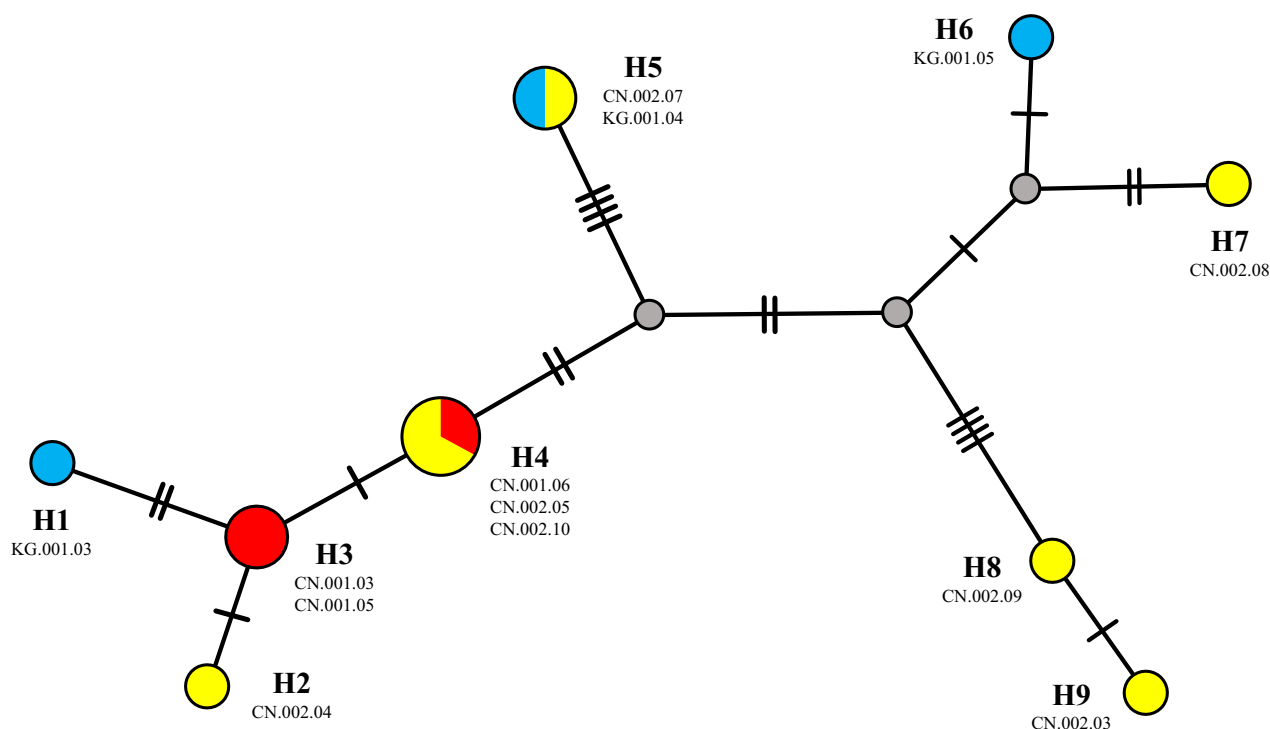


Figure 11. Haplotype Median Joining network for the mitochondrial DNA haplotypes of *Cryoconicus* gen. nov. *kaczmareki* sp. nov. Haplotypes are represented by coloured circles, the surface area of which is proportional to the number of individuals exhibiting a particular haplotype (population and specimen codes are listed next to the circles, colours correspond with those in Fig. 1, i.e. red – CN.001, yellow – CN.002, blue – KG.001). Grey circles indicate hypothetical intermediate haplotypes that were not found, but are necessary to link the observed haplotypes. Hatch marks in the network represent single mutations.

branches in the two other genera (for *Ramazzottius* see Fig. 9A–F), and by the presence of wide semi-transparent cuticular bars under claws I–III (although these are visible only in mounted individuals in which the pigment has faded). In contrast to *Hebesuncus*, in which external claws are similar to the *Hypsibius* claw type, with only the posterior claws being of the *Ramazzottius* type, *Cryoconicus* gen. nov. and *Ramazzottius* share a similar morphology of both external and posterior claws but, as mentioned above, the two genera differ in the shape of the primary branches and in the presence/absence of the accessory points.

The uncorrected p-distances between *Cryoconicus* gen. nov. and sequenced *Ramazzottius* species were as follows (full distance matrices are provided in supplementary materials S3 deposited under <http://dx.doi.org/10.7910/DVN/FL5T20>):

- **18S rRNA:** from 1.1% (*Ramazzottius oberhaeuseri* (Doyère, 1840) from Spain, FJ435728) to 2.1% (*Ramazzottius* sp. from moss/lichen sample, Disko Island, West Greenland (S. McInnes pers. comm.), EU266959), with the average p-distance of 1.4%;
- **28S rRNA:** from 4.7% (*Ramazzottius subanomalous* (Biserov, 1985) from Poland, MF001998) to 7.0% (*Ramazzottius varieornatus* Bertolani

and Kinchin, 1993 from Japan, MG432818), with the average p-distance of 5.9%;

- **ITS-2:** from 10.6% (*R. varieornatus* from Japan, MG432816) to 13.6% (*R. subanomalous* from Poland, MG432819), with the average p-distance of 11.8%;
- **COI:** from 17.8% (*Ramazzottius oberhaeuseri* from Spain, FJ43579) to 21.2% (*R. varieornatus* from Japan, MG432813), with the average p-distance of 19.6%.

Thus, all sequences for the new genus are clearly different from sequences deposited in the GenBank. All phylogenetic analyses, i.e. both trees based on the conservative marker (18S rRNA) and on the concatenated sequences (28S rRNA+ITS-2+COI) as well as both trees generated by Bayesian Interference (BI) and Maximum Likelihood (ML), supported the erection of the new genus (Fig. 12). All but one analysis returned the phylograms with highly supported nodes and *Cryoconicus* gen. nov. as the sister group to other *Ramazzottius* taxa (Fig. 12). Only in the case, in the ML analysis of the 18S rRNA fragment (Fig. 12B), a single *Ramazzottius* sp. from Greenland (EU266959) was an outgroup to the *Cryoconicus* gen. nov. + *Ramazzottius* clade. Importantly, however, this identification



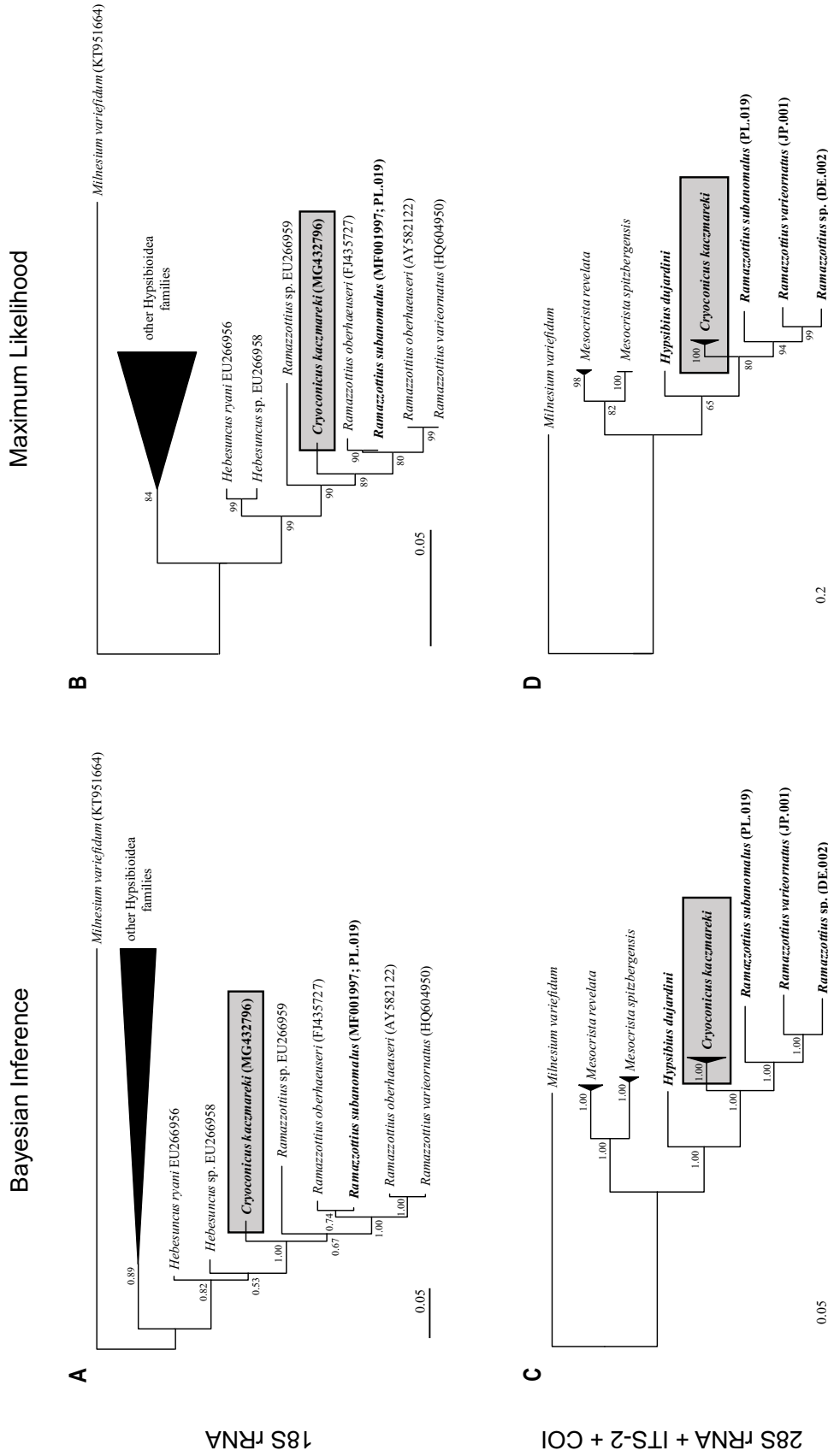


Figure 12. The phylogenetic position of *Cryoconicus* gen. nov. *kaczmareki* sp. nov. (grey rectangle) within the Hypsibioidae tree constructed from available Hypsibioidae 18S rRNA nucleotide sequences (see S1). B - Maximum Likelihood (based on the Kimura 2-parameter model) Hypsibioidae tree constructed from available Hypsibioidae 18S rRNA nucleotide sequences (see S1). C - Bayesian Hypsibioidae tree constructed from concatenated nucleotide sequences of the three molecular markers: COI, ITS-2 and 28S rRNA (see S1). D - Maximum Likelihood (based on the General Time Reversible model) Hypsibioidae tree constructed from concatenated nucleotide sequences of the three molecular markers: COI, ITS-2 and 28S rRNA (see S1). Numbers at nodes indicate Bayesian posterior probabilities or bootstrap support values, respectively. Scale bars represent substitutions per position.

(Sands *et al.* 2008) is uncertain and the exceptionally long branch for this taxon suggests this sequence could represent another new ramazzottiid genus rather than a *Ramazzottius* species. It needs to be noted, however, that the number of published ramazzottiid sequences is low and effort should be made to improve sampling in the future, including *Hebesuncus*, for which ITS-2 and COI sequences are currently unavailable.

To conclude, both morphological and molecular traits congruently support the erection of *Cryoconicus* gen. nov. and suggest *Ramazzottius* as its closest affinity. Although *C. cataphractus* comb. nov. was described earlier than *C. kaczmareki* sp. nov., the latter taxon is described in much more detail, thus we designate it as the nominal species for the genus.

## DISCUSSION

In this study, we showed high diversity of a mitochondrial DNA marker in a species representing a new tardigrade genus discovered in mountain glaciers in central Asia. The identification of as many as nine COI haplotypes from only 13 individuals suggests that the genetic diversity within the species might be considerable. Moreover, there seem to be no differences in the intra- and inter-population variability in COI sequences, regardless of the geographic distance dividing the populations. Also, identical haplotypes were found in populations separated by up to *ca.* 1000 km and, at the same time, within a single population haplotypes separated by a considerable p-distance of 1.6% were identified. Although the sample size of 17 sequenced individuals is relatively low, the observed patterns may allow some tentative inference on the biology of the new species. Specifically, high COI diversity suggests that (1) the investigated populations have been inhabiting the glaciers for a considerable time. This, in turn, implies (2) successful adaptations to the harsh conditions of mountain glaciers that include low temperatures and extensive UV exposition. Moreover, haplotypes shared between distant populations may be interpreted as evidence for (3) good dispersal abilities.

Although glaciers are considered to be one of the harshest environments on Earth, they harbour their own persistent ecosystems (*e.g.*, Hodson *et al.* 2008, Cook *et al.* 2015). For example, a recent study on rotifers inhabiting Icelandic glaciers showed that the animals endured in the environment over glacial/interglacial cycles throughout the Pleistocene (Shain *et al.* 2016). Such persistence is also likely in tardigrades since they can survive under cryptobiosis through unfavourable conditions such as high and low temperatures, drought as well as high doses of irradiation (*e.g.* Guidetti *et al.* 2012). An example of an Antarctic tardigrade, *Acutuncus antarcticus* (Richters, 1904),

shows that tardigrades can recover and reproduce after being frozen for thirty years (Tsujimoto *et al.* 2015). Similarly, in the present study numerous individuals of *Cryoconicus* gen. nov. were recovered after eleven years of being frozen, which suggests high survival rates in the natural environment of the new species.

Another factor that may limit survival on high altitude glaciers is the ultraviolet radiation (*e.g.* Sommaruga 2001), the intensity of which increases by *ca.* 8% and up to *ca.* 24% per 1000 meters with solar exposition of 60° and 20°, respectively (Blumthaler *et al.* 1997). Interestingly, numerous high mountains animals, such as collembolans, enchytraeids or tardigrades, contain dark pigment granules in the epidermis. Such an apparent convergence strongly suggests a high adaptive advantage of dark pigmentation, which may both protect from UV radiation (*e.g.* Shain *et al.* 2000, Potapow 2001, Greven *et al.* 2005, Makowska *et al.* 2016), and possibly also help to absorb heat from sunlight. Apart from *C. kaczmareki* sp. nov., the only three tardigrades found so far in high mountain cryoconite holes, *Hypsibius janetscheki* Ramazzotti, 1968 and *Hypsibius thaleri* Dastych, 2004 from the Himalayas and *Hypsibius klebelsbergi* Mihelčič, 1959 from Alps (Mihelčič 1959, Ramazzotti 1968, Dastych 2004), are all dark-pigmented. Tardigrades in high alpine glaciers were previously found at *ca.* 2660 m asl (Dabert *et al.* 2015), thus, in places with at least twice as the amount of irradiation as in lowlands (Blumthaler *et al.* 1997).

There is not much data on tardigrade dispersal, but three available molecular studies suggest that tardigrades can disperse across vast areas. Specifically, sampling in Europe, north Africa and south Greenland revealed multiple populations of a single species, *Echiniscus testudo* Doyère, 1840 (Jørgensen *et al.* 2007, see also Gašiorek *et al.* 2017). In a more recent study Dabert *et al.* (2015) showed that two *H. klebelsbergi* populations found in cryoconite holes in glaciers in the Ötztal Alps, separated by a geographic barrier (a mountain ridge) shared identical 18S rRNA, 28S rRNA and COI haplotypes. Moreover, Cesari *et al.* (2016) demonstrated that multiple populations of common Antarctic tardigrade *Acutuncus antarcticus* (Richters, 1904) share identical 18S rRNA, but exhibit various COI haplotypes. Our study is, therefore, in accordance with previous findings and it adds to the poor knowledge on tardigrade zoogeographic distributions.

Although previously tardigrades were found in the Arctic, Antarctic, Alpine and Himalayan cryoconite holes (Zawierucha *et al.* 2015, 2016, 2018), they have never been reported in cryoconite granules on glacier surfaces. The description of a new genus from glaciers may not be surprising as with over 1200 tardigrade species known worldwide, only 20 tardigrade taxa have

been reported from glaciers and as few as 12 of them were identified to the species level (Zawierucha *et al.* 2015, 2016). Taking into consideration that glaciers constitute *ca.* 10% of the Earth's surface, they are a grossly underexplored environment in terms of studies on tardigrade ecology and diversity.

## SUMMARY AND CONCLUSION

- By the implementation of the integrative taxonomy, we were able to erect the new genus and species *Cryoconicus* gen. nov. *kaczmareki* sp. nov. from Asian glaciers;
- We detected a high intraspecific variation in the COI sequence, which suggests that the investigated populations have been inhabiting the glaciers for a considerable time;
- *Cryoconicus* gen. nov. *kaczmareki* sp. nov. recovered after eleven years of being frozen, which suggests high survival rates in the natural environment of the new species;
- An ability to withstand low temperatures, combined with dark pigmentation that is hypothesised to protect from intense UV radiation, could explain how the new taxon is able to dwell in high mountain glaciers;
- Taking in to consideration that glaciers constitute *ca.* 10% of the Earth's surface, they are a grossly underexplored environment for studies on tardigrade ecology and diversity.

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