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## First records of limnoterrestrial tardigrades (Tardigrada) from Haida Gwaii, British Columbia, Canada

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### Abstract

Moss samples were collected from trees and rocks in Haida Gwaii, British Columbia, Canada, and examined for the presence of tardigrades. Specimens from 24 taxa were found in 17 out of the 22 examined samples. New species records for British Columbia are provided and undescribed *Grevenius* and *Crenubiotus* species were found; a division in four morphogroups of *Grevenius*, based on number and presence of placoids, is provided to aid in the future identification. In addition, three specimens of a new species belonging to a potential new undescribed *Diploechiniscus* species were identified. The finding of *Macrobiotus occidentalis occidentalis* also provides the occasion to transfer the latter one to the genus *Diaforobiotus*, for which a new dichotomous key for the identification of its species is given and to redefine the family Richtersiidae. The DNA sequences of selected taxa are also provided. The high number of tardigrade species collected from a relatively low number of samples highlight how still unexplored is tardigrade diversity, particularly in still-largely insular island systems like Haida Gwaii.

**Keywords:** *Integrative taxonomy, insular microfauna, faunistic, biogeography, Hecate Strait*

### Introduction

Tardigrades have been recorded from the Canadian Pacific coast, a region of the province of British Columbia, since the beginning of 20th century (Richters 1908; Murray 1910), when two species were firstly found and described from the harbor of the city of Victoria (*Echiniscus canadensis* Murray, 1910 and *Macrobiotus occidentalis occidentalis* Murray, 1910). An 80-year hiatus on the tardigrades research in British Columbia then ensued until R. D. Kathman (Kathman 1989, 1990a, 1990b; Kathman & Nelson 1989; Kathman & Dastych 1990; Kathman & Cross 1991) described three new species from the region (*Insuetifurca arrowsmithi* (Kathman & Nelson, 1989), *Ursulinus woodsae*

(Kathman, 1990), and *Platicrista cheleusis* Kathman, 1990) and provided an ecological characterization of the moss-dwelling tardigrades of this region (Kathman & Cross 1991). Following these discoveries, another 30-year hiatus ensued until two new species (*Sisubiotus hakaensis* Vecchi, Choong, & Calhim, 2022 and *Crenubiotus salishani* Vecchi, Choong, & Calhim, 2022) were identified and described from Calvert Island (Queen Charlotte Sound, Central Coast, BC) and Salt Spring Island (Gulf Islands, Strait of Georgia, BC), respectively (Vecchi et al. 2022b). These most recent results showed that in island systems, even those within relatively well-traversed regions, tardigrade diversity remains largely unexplored.

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At the same time, the Canadian Pacific coast region includes essentially isolated island systems like the Haida Gwaii archipelago, about 80 km offshore of northwestern British Columbia, which comprises several hundred islands and islets over an area of 1 million hectares. It is likely that Haida Gwaii flora and fauna had been isolated from the mainland since the inundation of the area of the present-day Hecate Strait by about 10,700 years ago (Fedje et al. 2021). Despite extensive development in many areas, significant protected areas, including Naikoon Provincial Park and associated reserves, still support high numbers of endemic, disjunct, and rare species (Golumbia 2000).

In this paper, we provide the first account on tardigrades from Haida Gwaii, with (when possible) DNA sequences to corroborate some of the identification and to allow future comparisons with the recovered taxa. In addition, we provide a division of the genus *Grevenius* in four morphogroups, based on number and presence of placoids, to aid in the future identification of species in this genus, an amended diagnosis for the family Richtersiidae, and an updated dichotomous key for the genus *Diaforobiotus* amending the one given by Stec et al. (2022).

## Materials and methods

### *Sampling and tardigrades extraction*

Moss samples (22 in total) were collected by H. Choong in several locations in Haida Gwaii as part of a biodiversity survey in collaboration with the BC Parks (i.e., an agency of the British Columbia Ministry of Environment and Climate Change Strategy) and colleagues from the Haida Nation (Table I; Figure 1; map prepared with the R package “ggOceanMaps”; Vihtakari 2022) from 1st to the 5th of June 2022. Moss samples were air dried and preserved in paper bags. To extract tardigrades from their substrates, fragments of all samples (1–3 g of dry sample) were placed in distilled water for ca. 30 minutes. After soaking, samples were sieved (sieves meshes: 500 and 32 µm) to separate tardigrades and eggs from the substrate; animals and eggs were then isolated using a needle and a glass pipette under a stereomicroscope.

The remaining fragments of the samples are vouchered in the Invertebrate Zoology Collections, Royal BC Museum, Victoria, BC, Canada (RBCM) and coded following Table I.

### *Microscopy and imaging*

Specimens were mounted at Department of Biological and Environmental Sciences, University

of Jyväskylä, Jyväskylä, Finland (JYU) on slides in Hoyer’s medium, dried at 60°C for 1 week and then sealed with transparent nail polish. Observations with Light Microscope (LM) and measurements were carried out under both phase contrast (PhC) and differential interference contrast (DIC) up to the maximum magnification (100× oil objective) with a Leica DM RB microscope equipped with a AmScope MU1803 digital camera, at the Department of Life Sciences, University of Modena and Reggio Emilia (Unimore). For structures that could not be satisfactorily focused on a single light microscope photograph, stacks of images were taken and assembled into a single deep-focus image with the AmScope Camera software. To ensure the presence or absence of minute structures of selected specimens, stacks of images were acquired taking advantage of the autofluorescence (light length excitation 488 nm and emission signal collected 509–614 nm) with the Confocal Laser Scanning Microscope [CLSM] Leica SP8 equipped with white laser available at “Centro Interdipartimentale Grandi Strumenti” at the Unimore and 3D reconstructions were performed with the software Leica LAS X (Guidetti et al. 2019; Massa et al. 2021, 2023). Photographs were assembled into figures with FigureJ (Mutterer & Zinck 2013).

Specimens are deposited in the Bertolani Collection (BC) at the Department of Life Sciences, University of Modena and Reggio Emilia, Italy, and in the Invertebrate Zoology Collections of the Royal BC Museum (RBCM). Microscope slides are reported in the taxonomic account with the sample code (Table I) and slide number both given at JYU (S#\_SL#) and BC (C#s#) or RBCM (RBCM#).

### *Genotyping*

The DNA was extracted at JYU from individual animals following a Chelex® 100 resin (BioRad) extraction method by Casquet et al. (2012) with modifications described in detail in Stec et al. (2020a). When possible, carcasses were recovered and mounted on slides as voucher specimens. Voucher specimens are deposited in BC. Slides with mounted voucher specimens are reported in the taxonomic account with the sample code and slide number both given at JYU (S#.Name.V#) and BC (C#v#). Amplification and sequencing of fragments of the 18S ribosomal RNA gene and of the COI mitochondrial gene were amplified and sequencing according to Stec et al. (2020a). Sequencing products were read with the ABI 3130x1 sequencer at the Department of Biological and Environmental Science of JYU. Sequences

Table I. List of moss samples used in this study with relative codes, localities, and coordinates expressed in decimal degrees (WGS84), and substrate on which moss samples were collected. Beached trees are western hemlock (*Tsuga heterophylla*). The other tree species mentioned are western redcedar (*Thuja plicata*) and Sitka spruce (*Picea sitchensis*). SC-JYU: sample code at University of Jyväskylä (Finland; S#); SC-RBCM: sample code at Royal BC Museum (Canada; HG1-#); SC-MO: sample code at University of Modena and Reggio Emilia (Italy; C#). \*: samples for which there is not remaining fragments.

SC-JYU	SC-RBCM	SC-MO	Locality	Coordinates	Substrate
S1995	HG1-2	C5069	Naikoon Provincial Park, Tlell Beach	53.579808–131.930108	Dead tree
S1996	HG1-3	C5070	Naikoon Provincial Park, Tlell Beach	53.579808–131.930108	Ground
S1997	HG1-4	C5071	Naikoon Provincial Park, Tlell Beach	53.579549–131.930463	Tree
S1998	HG1-5	C5072	Port Clements, Sunset Park	53.669442–132.200119	Beached tree
S1999	HG1-6	C5073	Port Clements, Sunset Park	53.669125–132.200164	Beached tree
S2000	HG1-7	C5074	Port Clements, Sunset Park	53.668708–132.199342	Tree base
S2001	HG1-8	C5075	Port Clements, Sunset Park	53.668708–132.199342	Tree stump
S2002	HG1-9	C5076	Morseby Island, Pallant Creek	53.050833–132.028611	Beached tree
S2003	HG1-10	C5077	Morseby Island, Pallant Creek	53.050833–132.028611	Beached tree
S2004	HG1-11	C5078	Morseby Island, Pallant Creek	53.051944–132.024722	Rock
S2005	HG1-12	C5079	Morseby Island, Mosquito Lake	53.067778–132.066111	Fallen tree
S2006	HG1-13	C5080	Morseby Island, Mosquito Lake	53.066944–132.065833	Rock
S2007	HG1-14	C5081	Naikoon Provincial Park, Rose Spit	54.161381–131.661864	Ground
S2008	HG1-15	C5082	Naikoon Provincial Park, Rose Spit	54.161806–131.662536	Fallen tree
S2009	HG1-25	C5083	Naikoon Provincial Park, Rose Spit	54.132797–131.665344	Tree
S2010	HG1-26	C5084	Graham Island, Gregory Beach, Duu Guusd Conservancy	53.403044–132.520417	Beached tree
S2011	HG1-27	C5085	Graham Island, Gregory Beach, Duu Guusd Conservancy	53.403044–132.520417	Beached tree
S2012	HG1-28	C5086	Graham Island, Gregory Beach, Duu Guusd Conservancy	53.403011–132.521103	Tree stump
S2013	HG1-32	C5087	Graham Island, Yakoun Lake, Yaaguun Suu Heritage Conservancy	53.353194–132.281478	Fallen tree
S2014	HG1-33	C5088	Graham Island Yakoun Lake, Yaaguun Suu Heritage Conservancy	53.353267–132.281525	Tree
S2015	HG1-34	C5089	Graham Island, Yakoun Lake, Yaaguun Suu Heritage Conservancy	53.353172–132.281189	Rock
S2016*	HG1-35	C5090	Graham Island, Yakoun Lake, Yaaguun Suu Heritage Conservancy	53.353172–132.281189	Rock

were processed in MEGA7 (Kumar et al. 2016) and submitted to NCBI GenBank (Accession numbers provided in Table II). To aid in the taxa identification to species level, the produced sequences were searched against all the NCBI nr database assigned to tardigrades (NCBI: txid42241) with the blastn algorithm (Madden 2002). Matches of taxa from this study and sequences in Blast are discussed in the respective taxonomic accounts. The blastn results are provided in SM.01.

## Results

Tardigrades and eggs (149 and 85 respectively) belonging to 24 taxa were found in 17 over 22 samples. The taxa found in each sample are presented in Table III.

### Taxonomic account

**Phylum: Tardigrada Doyère, 1840**

**Class: Heterotardigrada Marcus, 1927**

**Order: Echiniscoidea Richters, 1926**

**Family: Echiniscidae Thulin, 1928**

***Claxtonia mauccii* (Ramazzotti, 1965)**

- 1 individual (Slide S2011\_SL1 – C5885s1)
- 2 individuals sequenced (Voucher slides S2011. Clax.V01-2 – C5085v2-3)

By the presence of only cirri *A*, and two pair of lateral projections, a dorsal plates sculpture composed by large multangular granules with massive striae without intracuticular layer of pillars (Gąsiorek et al. 2023), the male individual can be confidently attributed to *C. mauccii*.

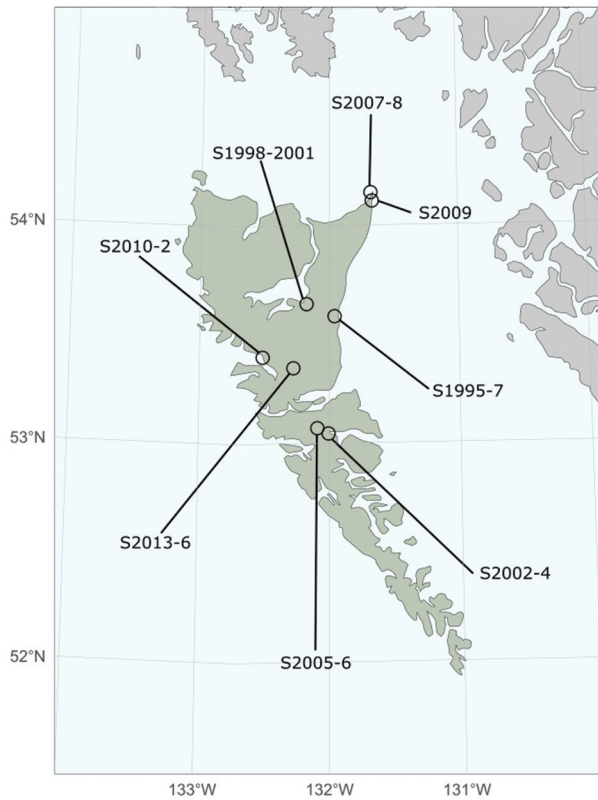


Figure 1. Map of Haida Gwaii (British Columbia, Canada) showing the sampling localities.

### *Claxtonia aff. wendti*

- 1 individual (Slide S2016\_SL1 – RBCM 023-00007-002)

One individual with a characteristic plate's ornamentation of the *Claxtonia wendti* morphotype (Gąsiorek et al.

2023) (large epicuticular granules, which at a high magnification show intracuticular layer composed of numerous pillars). Due to the unsuitable orientation of the individual, and the presence of multiple cryptic species in *Claxtonia* (Gąsiorek et al. 2023), it is not possible to confirm the identification of this species.

### *Diploechiniscus sp.*

- 3 individuals (Slide S2013\_SL1 – C5087s1)

A possible new species of *Diploechiniscus* (by having dorsolateral spicules). The description of this species will be the subject of future investigations.

### *Echiniscus cf. robertsi*

- 4 individuals (Slide S2010\_SL1 – C5084s1)

- 2 individuals sequenced (Voucher slides S2010. Ech.V03-6 – C5084v1-2)

By having only appendages in positions *A* and *D* lateral (Figure 2(a)) and a polygonal sculpture of the dorsal plate (Figure 2(b)), the individuals resemble *Echiniscus robertsi* Schuster & Grigarick, 1965 (a rare species recorded only twice by Schuster & Grigarick 1965 and Biserov 1998). Schuster and Grigarick (1965) described the presence of spines in position *D* lateral, while in the animals analyzed in this study, longer filaments were present in *D* lateral.

### *Echiniscus merokensis merokensis* Richters, 1904

- 1 individual (Slide 2015\_SL1 – RBCM 023-00006-004)

Table II. Sequences produced in this study. For each sequenced individual the specific name, the sample code as listed at University of Jyväskylä (Finland; JYU), the individual code as listed at JYU, the slide containing the voucher individual coded as listed at JYU (S#) and as deposited at Bertolani collection (BC) of the University of Modena and Reggio Emilia (Italy; C#v#), and accession number of GenBank for the relative sequences are reported. \*: carcass not recovered.

Species	Sample	Individual	Voucher code		18S SSU	COI
			JYU	BC		
<i>Adorybiotus granulatus</i>	S2011	Ado.2	S2011.Ado.V02	C5085v1	OQ029308	OQ029488
<i>Claxtonia maucii</i>	S2011	Clax.1	S2011.Clax.V01	C5085v2		OQ029484
		Clax.2	S2011.Clax.V02	C5085v3		OQ029485
		Cre.1*				
<i>Crenubiotus sp.</i>	S2009	Cre.1*				OQ029487
<i>Macrobiotus occidentalis occidentalis</i>	S2011	Dia.1	S2011.Dia.V01	C5085v4	OQ029309	OQ029491
		Dia.2	S2011.Dia.V02	C5085v5	OQ029310	OQ029492
<i>Echiniscus cf. robertsi</i>	S2010	Ech.3	S2010.Ech.V03	C5084v1	OQ029307	
		Ech.6	S2010.Ech.V06	C5084v2	OQ029306	OQ029482
<i>Macrobiotus hufelandi</i>	S2001	Mac.2*				OQ029489
		Mac.3*				OQ029490
<i>Paramurrayon cf. stellatus</i>	S2006	Mur.5*			OQ029313	OQ029486
		Mur.6	S2006.Mur.V06	C5080v1	OQ029312	
<i>Testechiniscus laterculus</i>	S2016	Tes.3	S2016.Tes.V03	C5090v1	OQ029311	OQ029483



By having lateral cirri in *A*, *C*, *D*, *E*, a spine in *Cd* and plates, ornamentation composed by irregular-shaped granules randomly distributed, the specimens can be confidently assigned to *E. merokensis merokensis*.

***Hypechiniscus daedalus* Gąsiorek, Oczkowski, Bartels, Nelson, Kristensen & Michalczyk, 2021**

- 3 individuals (Slide S2016\_SL1 – RBCM 023-00,007-001)
- 1 individuals (Slide S2016\_SL3 – C5090s2)

The morphology of the individuals perfectly fits the original description.

***Pseudechiniscus (Meridioniscus) dastychi* Roszkowska, Grobys, Bartylak & Kaczmarek, 2020**

- 2 individuals (Slide 2010\_SL1 – C5084s1)

Despite this species (Figure 2(c–d)) being described from the maritime Antarctic (Roszkowska et al. 2020) and the absence of DNA data on the newly found population, we confirm this record due to the striking morphological match with the original description.

***Testechiniscus laterculus* (Schuster et al. 1980)**

- 1 individual (Slide S2016\_SL1 – RBCM 023-00007-003)
- 1 individual sequenced (Voucher slide S2016.Tes.V03 – C5090v1)

The individuals found match the original description by Schuster et al. (1980) and the amended diagnosis by Gąsiorek et al. (2018). These are characterized by a peculiar ornamentation of dorsal plates (Figure 2(e)), the presence of evident spines on legs I (Figure 2(f)), and the presence of small upward spurs on the external claws of legs IV (Figure 2(g)). This species has been already recorded from British Columbia by Kathman and Dastych (1990).

**Class: Eutardigrada Richters, 1926**

**Order: Apochela Schuster et al., 1980**

**Family: Milnesiidae Ramazzotti, 1962**

***Milnesium granulatum* Ramazzotti, 1962**

- 1 individual (Slide S2000\_SL1 – C5074s1)

The morphology of the individuals fits the original description and the amended diagnosis by Michalczyk et al. (2012).

**Order: Parachela Schuster et al., 1980**

**Superfamily: Isohypsibioidea Sands et al., 2008**

**Family: Doryphoribiidae Gąsiorek, Stec, Morek & Michalczyk, 2019**

***Grevenius* sp.**

- 2 individuals + 4 eggs in one exuvia (Slide S2008\_SL1 – C5082s1)

An undescribed *Grevenius* species fitting the genus differential diagnosis by Gąsiorek et al. (2019) by the presence of Isohypsibioidea-type buccopharyngeal apparatus with peribuccal laminae, ridge-like symmetrical AISM, Isohypsibioidea-type claws with branches similar in length and pseudolunulae.

Due to the very few animals found, we refrain to describe this species; however, we present a detailed morphological account and a differential diagnosis compared to the other species in the genus.

In the buccal apparatus, lamellae absent and AISM present. Ventral lamina absent. Three grain-like macroplacoids and a microplacoid present (Figure 3(a)). The first and the second macroplacoid are almost fused and the third macroplacoid is spaced from the first two with a distance similar to the size of the microplacoid in length. Claws of the same leg are similar in size (Figure 3(b–c)). Pseudolunulae under each claw present (Figure 3(b)), larger under the external (or posterior in the hind legs) claws. Two cuticular bars present on each leg I–III: one intern to the internal claws and the other extern to the external claws. The cuticular bars are well visible only in one of the two specimens. Cuticular bar present claws IV. Cuticle sculptured from the II claws to the posterior end of the body, with small tubercles forming a faint reticulum (Figure 3(d)).

The species of the genus *Grevenius* can be divided into four morphological homogeneous groups based on the number of placoids reported in the original descriptions, therefore we propose four informal morphogroups to aid in the future identification of species in this genus:

- ***annulatus* morphogroup:** species with two macroplacoids and without the microplacoid. Composition: *Grevenius annulatus annulatus* (Murray, 1905); *Grevenius annulatus minor* (Ramazzotti, 1945); *Grevenius fuscus*



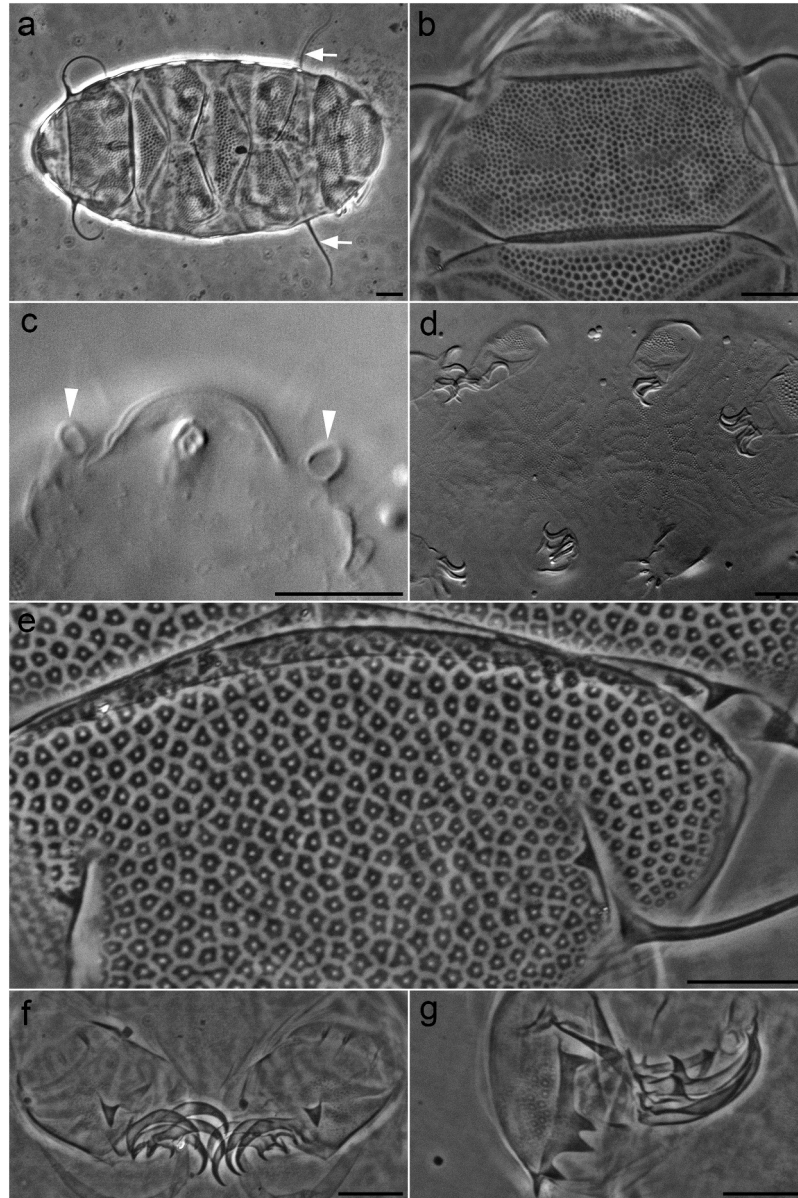


Figure 2. Echiniscidae. (a-b) *Echiniscus* cf. *robertsi*: in toto, (a) sculpture of the dorsal plate, (b) c-d. *Pseudechiniscus* (*Meridioniscus*) *dastychi*: cephalic papillae, (c) ventral sculpture of the cuticle (D). e-g. *Testechiniscus laterculus*: sculpture of the dorsal plate (e), first pair legs (f), fourth pair leg (g). Arrows: filaments in D lateral; arrowheads: cephalic papillae. a-b, e-g. DIC; c-d. PhC. a-g. z-stack. Scale bars: 10  $\mu$ m.

(Mihelčič, 1971/72); *Grevenius longiunguis* (Pilato, 1974); *Grevenius nipponicus* (Sudzuki, 1975); *Grevenius rusticus* (Pilato et al., 2015); *Grevenius sismicus* (Maucci, 1978); *Grevenius verae* (Pilato & Catanzaro, 1989); *Grevenius zappalai* (Pilato et al., 2015);

- **pulcher morphogroup** species with two macroplacoids and with the microplacoid. Composition: *Grevenius pulcher* (Mihelčič, 1971/72);
- **asper morphogroup**: species with three macroplacoids and without the microplacoid. Composition: *Grevenius asper* (Murray, 1906); *Grevenius baicalensis* (Ramazzotti, 1966);

*Grevenius baldii* (Ramazzotti, 1945); *Grevenius baldioides* (Tumanov, 2003); *Grevenius deconincki* (Pilato, 1971); *Grevenius deflexus* (Mihelčič, 1960); *Grevenius granulifer* (Thulin, 1928); *Grevenius kotovae* (Tumanov, 2003); *Grevenius myrops* (du Bois-Reymond Marcus, 1944); *Grevenius ladogensis* (Tumanov, 2003); *Grevenius pushkini* (Tumanov, 2003); *Grevenius brevitubulatus* (Rho, Chang & Kim, 1997); *Grevenius cryophilus* Zawierucha, Buda, Novotna Jaromerska & Gąsiorek, 2020; *Grevenius granditintinus* (Chang & Rho, 1996); *Grevenius hydrogogianus* (Ito & Tagami, 1993); *Grevenius karenae* (Zawierucha,

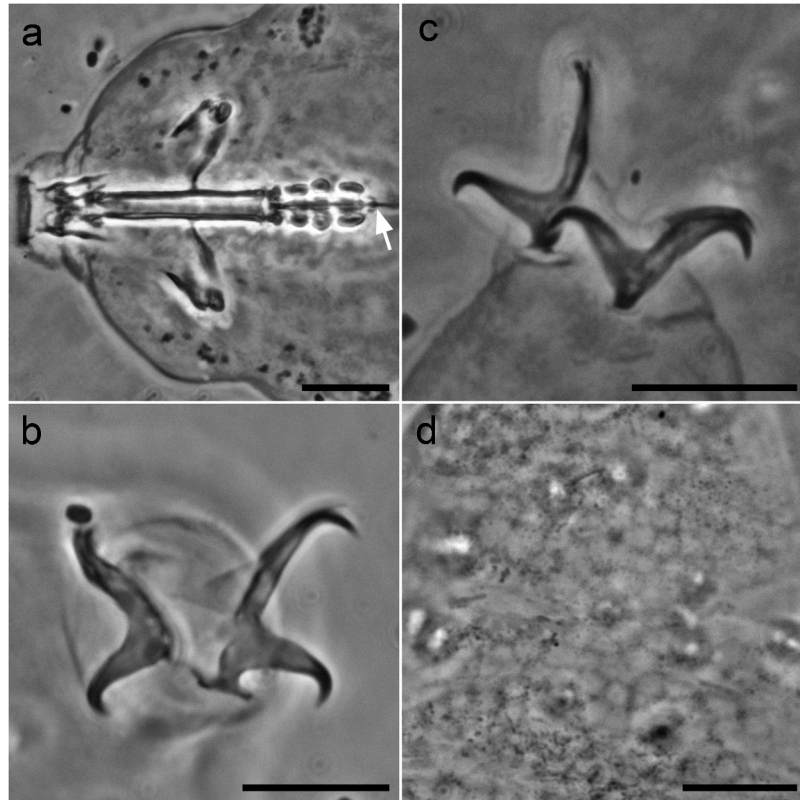


Figure 3. *Grevenius* sp.: buccopharyngeal apparatus (a), claws I (b), claws IV (c), dorsal sculpture of the cuticle (d). Arrow: microplacoid. a–d. DIC. a–c. z-stack; (d). single plane. Scale bars 10  $\mu$ m.

2013); *Grevenius kenodontis* (Kendall-Fite & Nelson, 1996); *Grevenius koreanensis* (Iharos, 1971); *Grevenius kristenseni* (Pilato et al., 1989); *Grevenius laevis* (McInnes, 1995); *Grevenius malawiensis* (Jørgensen, 2001); *Grevenius marii* (Bertolani, 1982); *Grevenius monoicus* (Bertolani, 1982); *Grevenius tubericulatus* (Pilato & Catanzaro, 1989); *Grevenius rugosus* (Guidi & Grabowski, 1996);

- ***lineatus* morphogroup**: species with three macroplacoids and the microplacoid. Composition: *Grevenius lineatus* (Mihelčič, 1969).

The individuals found belongs to an undescribed species in the *lineatus* morphogroup that differs from *G. lineatus* by the sculptured cuticle in form of tubercles creating a reticulum (sculpture in form of lines in *G. lineatus*).

**Superfamily: Hypsibioidea Pilato, 1969 in Marley et al. 2011**

**Family: Hypsibiidae Pilato, 1969**

***Diphascon* cf. *claxtonae***

- 1 individual (Slide S2010\_SL1 – C5084s1)

The species identification follows the key by Fontoura and Pilato (2007) for the species of the *D. pingue* group, to which *Diphascon claxtonae* Pilato & Binda, 1998 belongs, and it is characterized by having: smooth cuticle; pharyngeal bulb with apophyses, three macroplacoids, microplacoid, and septulum; lunules and other cuticular thickenings on the legs absent; claw bases without indentations (Fontoura et al. 2010). However, being the species of the *D. pingue* group differing only for few morphometric characters and our sample composed by one individual only, we prefer to be prudent and name this taxon *D. cf. claxtonae*.

***Hypsibius* cf. *convergens***

- 3 individuals (Slide S2002\_SL1 – C5076s1)

The morphology of the individuals fits the original description of *Hypsibius convergens* (Urbanowicz, 1925). The individuals are characterised by the presence of faint septula in the pharynx, together with II macroplacoid long two-thirds of the I macroplacoid which present a median constriction, and robust claws with short common tract. *Hypsibius convergens* was previously reported as cosmopolitan

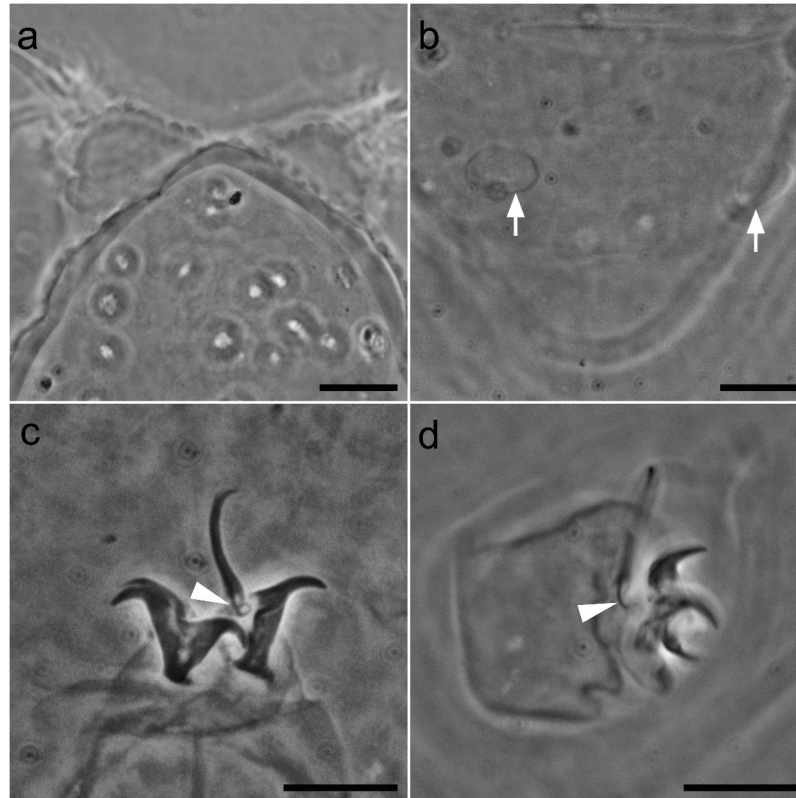


Figure 4. *Ramazzottius* spp. a–c. *Ramazzottius* sp. 1: dorsal posterior sculpture of the cuticle (a), head sensory areas (b), claw I (c). (d) *Ramazzottius* sp. 2: claws II (d). Arrows: sensory areas; arrowheads: filament within the LRU. a–d. DIC. a–d. z-stack. Scale bars 10  $\mu$ m.

(McInnes 1994). However, being the original description of the species very synthetic and our sample composed by three individual only, we prefer to be prudent and name this taxon *Hypsibius* cf. *convergens*.

#### ***Platicrista angustata* (Murray, 1905)**

- 1 individual (Slide S2013\_SL1 – C5087s1)

*Platicrista* without sculptured cuticle, no cuticular bars visible between or at the base of the claws and lunulae IV smooth. Following the identification table from Miller and Miller (2021), the individual is attributed to *P. angustata*.

#### **Family: Ramazzottiidae Sands et al., 2008**

##### ***Ramazzottius* sp. 1**

- 1 individual (Slide S1995\_SL1 – C5069s1)

*Ramazzottius* in early *simplex* stage (i.e., buccopharyngeal apparatus, reforming claws, and new cuticle absent) with sculptured cuticle (Figure 4(a)) on the dorsal and lateral sides of the body starting from the

first pair of legs. Hemispherical sensory areas are well visible on the head (Figure 4(b)). The sculpture is formed by tubercles becoming flat to hemispherical from the anterior to the posterior side. Leg sculpturing is present only on the IV pair of legs. Claws of the *Ramazzottius* type *oberhaeuseri* variant (Guidetti et al., 2019) (Figure 4(c)) with well-visible lunulae under the external (or posterior in the hind legs) claws and faint lunulae under the internal (or anterior in the hind legs) claws. On the main branches of each claw accessory points are present, those are thick on internal (or anterior in the hind legs) and faint on external (or posterior in the hind legs). Eggs unknown.

##### ***Ramazzottius* sp. 2**

- 2 individuals (Slide S2008\_SL1 – C5082s1)

*Ramazzottius* with smooth cuticle. Hemispherical sensory areas are not well visible on the head. Claws of the *Ramazzottius* type *oberhaeuseri* variant (Guidetti et al. 2019) with clear filaments of the LRU that never touch the secondary branch (Figure 4(d)), possibly because of mounting stress on the claws. Pale or

absent lunulae under each claw. Primary branch of the external (or posterior in the hind legs) claws slender. Internal (or anterior in the hind legs) claws stumpy. On the main branches of each claw accessory points are present, those are thick on internal (or anterior in the hind legs) and faint on external (or posterior in the hind legs). Eggs unknown.

***Ramazzottius* gr. *oberhaeuseri***

- 1 egg (Slide S1996\_SL1 – C5070s1)

Egg of the *Ramazzottius oberhaeuseri* complex (Stec et al. 2018) without evident cap-like structures on the hemispherical process and with wrinkled surface over the egg chorion and over the processes. The processes are surrounded by dots. The wall of each process is formed by an internal and an external wall. The internal is hemispherical and slightly flattened on the apex, the width is slightly higher than the height. The external presents in few processes an elongated tip with variable height. The space between the two walls has the same optical density of the walls. This egg could possibly belong to *Ramazzottius* sp. 1. considering its proximity to the two samples; however, due to the absence of an embryo, it is not possible to confidently assign it.

**Superfamily: Macrobitoidea Thulin, 1928 in Marley et al. 2011**

**Family: Adorybiotidae Stec et al., 2020 in Stec et al. 2020d**

***Adorybiotus granulatus* (Richters, 1903)**

- 6 individuals + 3 eggs (Slide S2011\_SL1 – C5085s1)
- 5 individuals + 2 eggs (Slide S2013\_SL1 – C5087s1)
- 1 egg (Slide S2013\_SL2 – C5087s1)
- 3 individuals + 1 egg (Slide S2014\_SL1 – RBCM 023-00005-001)

The morphology is consistent with the redescription and the neotypic material by Ramazzotti and Maucci (1983). A very evident thickening of the buccal tube wall is present after the stylet supports insertions (Figure 5(a)) Gonochoric population. Egg with embryo confirms the relation between the species and egg (Figure 5(b)).

***Crenubiotus* sp.**

- 11 individuals (Slide 2009\_SL1 – C5083s1)
- 7 individuals + 2 eggs (Slide 2009\_SL2 – RBCM 023-00004-001)
- 2 individuals + 9 eggs (Slide 2012\_SL1 – C5086s1)

- 1 egg (Slide 2013\_SL3 – C5087s3)
- 1 individual (Slide 2015\_SL1 – RBCM 023-00006-002)
- 1 individual sequenced (Sample S2009, no voucher slide available)

This undescribed species is similar to *C. crenulatus* s. s. but differs by the medioventral tooth of the transversal ridge. Tooth in addition to those present in *C. crenulatus* s.s. visible in some specimens (Figure 5(c)). Subterminal constriction on the second placoid absent (present in *C. crenulatus* s. s.) (Figure 5(d–e)). Elongated tips of the egg processes with no trabecular layer (Figure 5(f)). Egg with embryo confirms the relation between the species and egg (Figure 5(f)). The COI sequences have 98.3% identity with sequences found in GenBank (SM.01) and attributed to an undescribed *Crenubiotus* species (OM151284–5; *Crenubiotus* sp. GL.001) found in Greenland by Stec et al. (2020).

**Family: Macrobiotidae Thulin, 1928**

***Macrobiotus hufelandi* C.A.S. Schultz, 1834**

- 2 individuals + 10 eggs (Slide S1996\_SL1 – C5070s1)
- 8 individuals + 2 eggs (Slide S1997\_SL1 – C5071s1)
- 2 individuals (Slide S2000\_SL1 – C5074s1)
- 12 individuals + 9 eggs (Slide S2001\_SL1 – RBCM 023-00003-001)
- 1 individual (Slide S2005\_SL1 – C5079s1)
- 6 individuals + 5 eggs (Slide S2008\_SL1 – C5082s1)
- 1 individual (Slide S2010\_SL1 – C5084s1)
- 2 individuals (Slide S2011\_SL1 – C5085s1)
- 1 individual (Slide S2012\_SL1 – C5086s1)
- 1 individual (Slide S2016\_SL1 – RBCM 023-00007-004)
- 2 individuals sequenced (Sample S2001, no voucher slides available)

The individuals and eggs fit with the species redescription by Bertolani & Rebecchi (1993). The identification was also confirmed by the DNA sequencing (Table II). The COI sequences have >99% identity with sequences found in GenBank (SM.01) from the neotype population (HQ876584-7) of *M. hufelandi*, confirming the morphological identification.

***Macrobiotus occidentalis occidentalis* Murray, 1910**

- 4 individuals (Slide 2011\_SL1 – C5085s1)

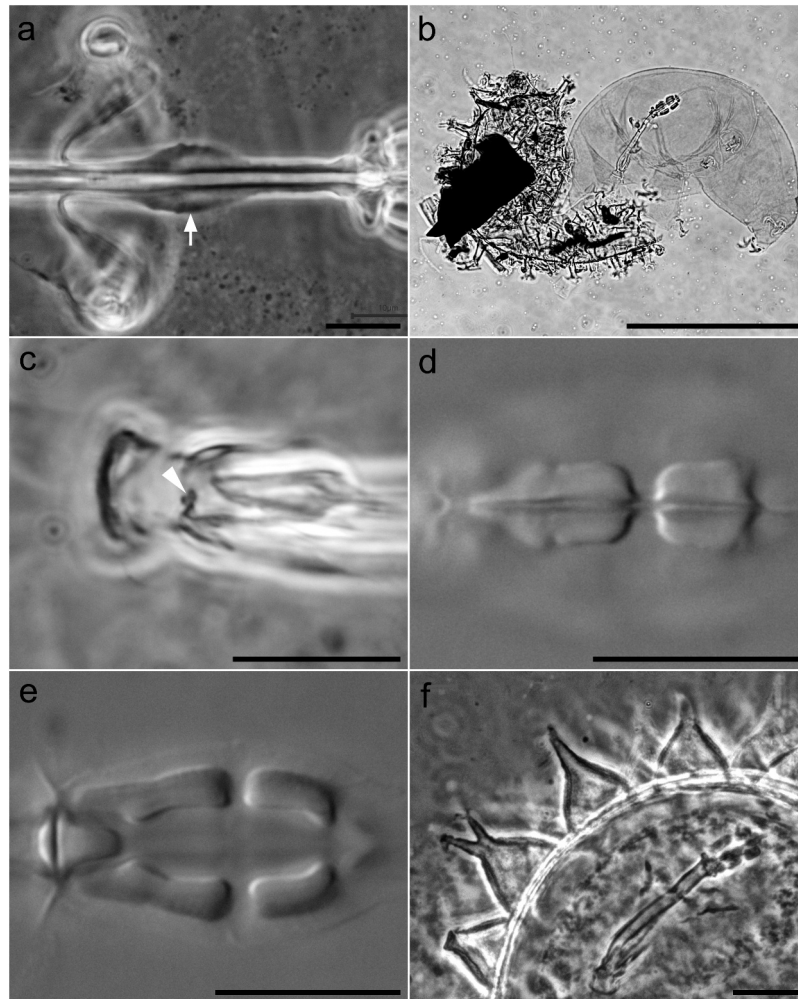


Figure 5. Adorybiotidae. a–b. *Adorybiotus granulatus*: buccopharyngeal apparatus (a), egg with embryo (b). c–f. *Crenubiotus* sp.: buccal armature of the ventral side (c), macroplacoids in dorsal view (d), cuticular annexes and cage of the pharynx (e), egg with embryo (f). Arrow: thickening of the buccal tube wall; arrowhead: additional tooth in the oral cavity armature. a–c, f. DIC; d–e. PhC. a, f. single plane. b–e. z-stack. Scale bars a, c–f. 10  $\mu$ m, b. 100  $\mu$ m.

- 5 individuals + 3 eggs (Slide 2015\_SL1 – RBCM 023-00006-003)
- 13 eggs (Slide 2016\_SL2 – C5090s1)
- 9 individuals (Slide 2016\_SL3 – C5090s2)
- 2 individuals sequenced (Voucher slides S2011. Dia.V01-2 – C5085v4-5)

Orange body ([https://youtu.be/Wsic3w\\_j0AY](https://youtu.be/Wsic3w_j0AY)). Oral cavity armature with an anterior band of teeth at the base of the buccal lamellae absent or not visible with LM; a posterior band of teeth covering the internal side of the buccal ring composed of irregular rows of granular teeth, increasing in size from the front to the back (well visible with LM; Figure 6(a–b)); a band of teeth present at beginning of the buccal tube is divided into the dorsal and the ventral portion. The ventral portion is composed of small faint teeth (in LM faintly visible as granular; Figure 6(a)). The dorsal portion is

composed of three evident granular teeth (Figure 6(b)). An additional tooth caudal and median to the dorsal portion (at the level of the buccal crown) in shape of a mucrone is also present (Figure 6(b)). Two macroplacoids without evident constrictions are present (Figure 6(c)), and a rugose-margined microplacoid is present, although not well visible in all the specimens (Figure 6(c), SM.02). Claws of the Richtersiidae type (Figure 6(d)), as described by Lisi et al. (2020). Lunulae under claws I–III smooth (Figure 6(d)), whereas lunulae under claws IV weakly dentate (Figure 6(e)). The eggs are light orange, spherical with slender conical processes, and a dotted surface (Figure 6(f)).

Measurements of animals and eggs are provided in SM.03.

In the alive individuals, a space between the cuticle and the epidermis is present. This space is filled

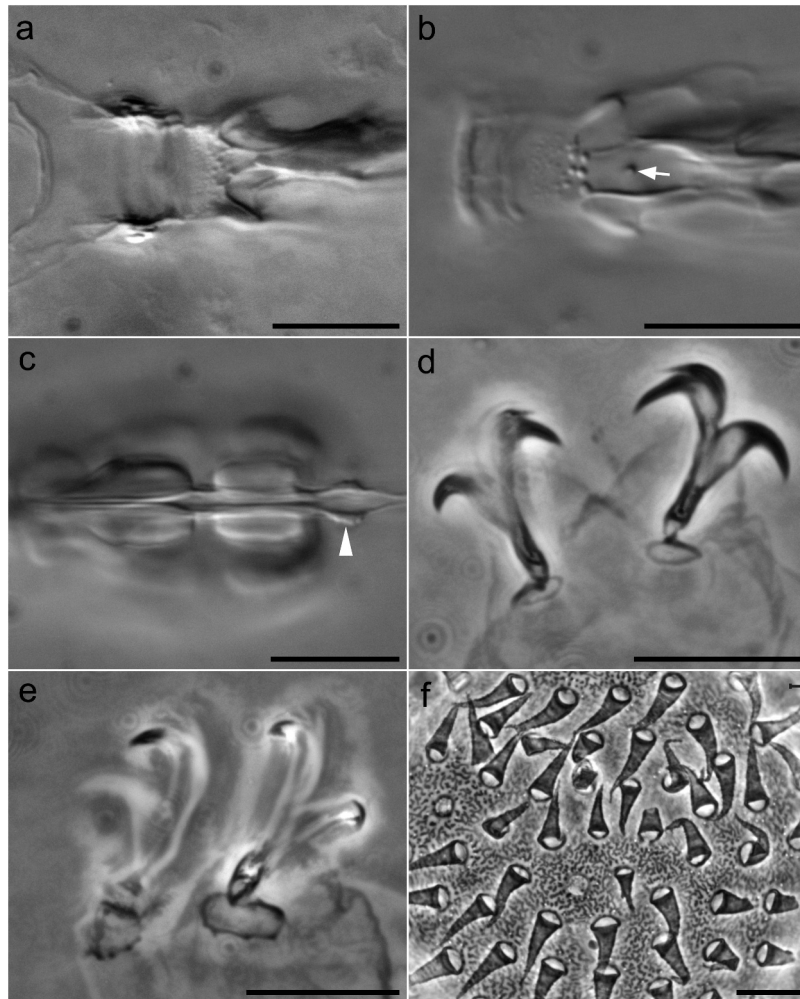


Figure 6. *Macrobotus occidentalis occidentalis*: ventral oral cavity armature (a), dorsal oral cavity armature (b), placoids in dorsal view (c), claws II (d), lunulae under the claws IV (e), eggs surface (f). Arrow: additional tooth in the oral cavity armature; arrowhead: rugose-margined microplacoid. a–c. PhC; d–f. DIC. a–f. z-stack. Scale bars 10  $\mu$ m.

with liquid in which crystal-like structures (refractive at PhC) float ([https://youtu.be/Wsic3w\\_j0AY](https://youtu.be/Wsic3w_j0AY)).

The specimens resemble *Macrobotus occidentalis occidentalis* Murray, 1910 described from Victoria Harbour (Vancouver Island, British Columbia), including the very peculiar floating crystal-like structures between the cuticle and the epidermis.

#### ***Mesobiotus* sp.**

- 3 individuals (Slide S2000\_SL1 – C5074s1)
- 2 individuals (Slide S2011\_SL1 – C5085s1)
- 5 individuals (Slide S2012\_SL1 – C5086s1)
- 1 individual (Slide S2015\_SL1 – RBCM 023-00006-001)
- 1 individual (Slide S2016\_SL2 – C5090s1)

*Mesobiotus* with oral cavity armature with anterior row of teeth not visible or very faint, posterior row composed by two line of large teeth, and transversal ridges divided into a ventral line composed by two ventral bars and a median mucrone and a dorsal line composed by three large bar-like transversal teeth. Faint granulation on IV legs not exceeding between legs. Lunule IV finely dentate. Accessory points lifted from the primary branch of each claw. Eggs unknown.

#### ***Paramacrobotus klymenki* Pilato et al., 2012**

- 3 individuals + 6 eggs (Slide S2010\_SL1 – C5084s1)

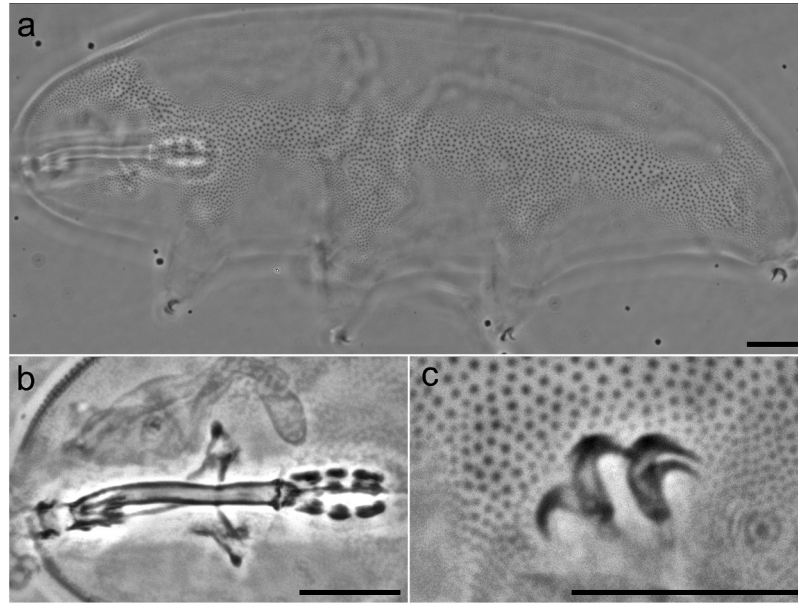


Figure 7. *Paramurrayon* cf. *stellatus*: *in toto* sculpture of the dorsal cuticle (a), buccopharyngeal apparatus (b), claws III (c). a–c. DIC. a–c. z-stack. Scale bars: 10  $\mu$ m.

The morphology of the individuals and eggs fits the original description.

#### ***Sisubiotus hakaiensis* Vecchi et al., 2022**

- 5 individuals + 12 eggs (S2013\_SL1 – C5087s1)
- 2 individuals + 1 egg (S2014\_SL1 – RBCM 023-00005-002)

The morphology of the individuals and eggs perfectly fits the original description.

#### **Family: Murrayidae Guidetti et al., 2000**

##### ***Paramurrayon* cf. *stellatus***

- 3 individuals (Slide S2006\_SL1 – C5080s1)
- 2 individuals sequenced (Voucher slide S2006. Mur.V06 – c5080v2; + 1 individual, no voucher slide available)

By having star-shaped cuticular pillars distributed in evident dorsolateral bands, the morphology of the individuals fits the original description of *Paramurrayon stellatus* (Guidetti, 1998) (Figure 7(a)). Buccal apparatus and claws morphology also conform to the genus diagnosis (Figure 7(b–c)). However, as eggs were not found, we prefer to be prudent and name this taxon *Paramurrayon* cf. *stellatus*.

#### **Discussion**

Despite its relatively sparse research history with few, limited, discontinuous sampling efforts, British Columbia has yielded an astonishing tardigrade species diversity, indicating that an incredible hidden biodiversity that only awaits to be unveiled. As shown in other tardigrade studies, an increase in sampling effort increases the number of species found (Nelson & Bartels 2013; Vuori et al. 2020; Massa et al. 2021), and it is similarly expected that additional efforts in characterizing the tardigrade fauna of Haida Gwaii will provide a large increase in the number of identified species. At the same time, due to climate change, anthropogenic effects, and resulting loss of habitats, species are becoming extinct even before we have the chance to find and describe them (Lees & Pimm 2015), and microscopic invertebrate like tardigrades are no exception to this trend (Vicente 2010; Zawierucha et al. 2018). Our results underscore the importance of continuing to sample and to document tardigrade diversity in this region, particularly in islands off the British Columbia coast such as Haida Gwaii and Vancouver Island. We provided the first account on tardigrades diversity from Haida Gwaii, showing tardigrade diversity and reporting of two *Macrobotus* species (*M. hufelandi* and *M. occidentalis occidentalis*) which provides the occasion to briefly discuss biogeography and taxonomic issues.

Historically, *Macrobotus hufelandi* was the first formally described tardigrade species (C.A.S. Schultze 1834) and was initially thought to be cosmopolitan. Subsequent studies involving fine morphology and DNA barcoding suggested instead the presence of multiple species with similar morphology (Bertolani & Rebecchi 1993; Cesari et al. 2009; Bertolani et al. 2011; Kaczmarek & Michalczyk 2017). Based on this, the cosmopolitan nature of *M. hufelandi* was questioned. In this study, we showed unequivocally (through DNA sequencing) that other than its type of locality in Germany, this species is present also in Canada. In addition, molecular data supporting the presence of this species in USA (Colorado; Vecchi et al. 2022) and Russia (Tumanov et al. 2022) expand the distribution of this species to most of the Boreal hemisphere and assigning this species to the Holarctic bioregion. Future integrative studies focusing on *M. hufelandi* specimens in the Austral hemisphere are needed to clarify whether the species is truly cosmopolitan or not and will be an important contribution in the discussion on the cosmopolitan *vs.* localized distribution of tardigrade species (see for example Jørgensen et al. 2007; Gąsiorek et al. 2019; Kaczmarek et al. 2020; Morek et al. 2021; Kayastha et al. 2023).

*Macrobotus occidentalis occidentalis* is an enigmatic species of which many worldwide records have been published (McInnes 1994). In its original description, based on different characters, Murray (1910) pointed out an affinity between *M. o. occidentalis* and many species now belonging to different genera (*Richtersiis coronifer* (Richters, 1903), *Adorybiotus granulatus* (Richters, 1903), *Crenubiotus crenulatus* (Richters, 1904), *Mesobiotus harmsworthi* (Murray, 1907), and *Diaforobiotus islandicus* (Richters, 1904)) but was recognized to be most similar to *D. islandicus*. Despite this ambiguity in the morphological affiliation of *M. o. occidentalis*, its taxonomic position has never been challenged *in extenso*, and it is still nowadays included in the genus *Macrobotus* (Guidetti & Bertolani, 2005; Degma & Guidetti, 2007, 2019–2023) despite the revolutions in tardigrades taxonomy that happened in the last decades after the introduction of the molecular phylogenesis in tardigrade taxonomy (e.g., Cesari et al. 2009; Guidetti et al. 2009; Bertolani et al. 2014). Numerous reports of *M. o. occidentalis* started accumulating quickly after its description from different localities, for example, Sweden (Thulin 1911) and France (Cuénot 1932). By the time of the publication of Marcus' monograph on tardigrades (Marcus 1936), *M. o. occidentalis* had also been recorded

from Ireland, Scotland, Peru, and Brasil. When McInnes (1994) published a list of tardigrade records of terrestrial and freshwater habitats in various countries, *M. o. occidentalis* was reported from all continents except Antarctica. One subspecies was described from North Korea (*Macrobotus occidentalis striata* Dastych, 1974). It should be noted that, in Murray (1910), the description of *M. o. occidentalis* from Australia (p. 139, indicated as “*Macrobotus occidentalis*, Murray?”) and Hawaii (p. 155–156, indicated as “*Macrobotus occidentalis*(?)”) comes before than the proper description of the species from Canadian material (p. 169–175, indicated with “*Macrobotus occidentalis* sp. n.”). This may be the reason that led Dastych (1988) to erroneously indicate its *locus typicus* in Australia instead of in Victoria, Vancouver Island, Canada.

Given the morphological characters reported for the individuals to *Macrobotus o. occidentalis* found in our survey (including the 18S similarity with *D. islandicus* SM.01) and the speculations on its affinities with *Diaforobiotus islandicus* already presented by Murray (1910), we propose *Diaforobiotus occidentalis* (Murray, 1910) comb. nov. stat. nov. The presence of a very small thickening within the pharynx that we found to be a microplacoid, confirming it with both LM and CLSM (SM. 02), is not in contrast with the original Murray (1910) description as due to the small size of this structure, that could have been easily overlooked at the time. A similar structure has also been reported for *Paramacrobotus areolatus* (Stec et al., 2020c) and in Stec et al. (2020b) Figure 4(e) a similar structure can be glimpsed for *Richtersiis coronifer*.

The genus *Diaforobiotus* was first described by Guidetti et al. (2016) as belonging to the family Richtersiidae, considered invalid name in Guidetti et al. (2021) that thus proposed the name Richtersiidae. Since its proposal by Guidetti et al. (2016) based on molecular and morphological data, the family Richtersiidae has undergone a first amendment by Lisi et al. (2020), followed by two redefinitions by Guidetti et al. (2021) and Stec et al. (2020d). Stec et al. (2020d), among the other features, added in the amended diagnoses the absence of the microplacoid as diagnostic, but the presence of a microplacoid within the pharynx in the *D. occidentalis* comb. nov. stat. nov. and its possible presence in *R. coronifer* imposes a further diagnostic amendment of the family Richtersiidae.

According to Stec (2022), which showed hidden cryptic diversity within the genus *Diaforobiotus*, and the morphological characters more affine to *Macrobotus* described by Dastych (1974) with



details for *Macrobiotus occidentalis striatus* Dastych 1974, we propose its elevation to the species level. Since *Macrobiotus striatus* Mihelčič, 1949 is already a valid name (even though considered *nomen dubium* by Dastych 2015 and *nomen inquirendum* by Stec et al. 2021), to avoid synonymies, we propose *Macrobiotus occidentalis* stat. nov. Dastych, 1974.

Only little of Murray's material survived (Morgan 1977), and among the remaining material conserved in the Royal Scottish Museum, there is no trace of *D. occidentalis* comb. nov. stat. nov. type specimens (Morgan 1977). As the type material of *D. occidentalis* comb. nov. stat. nov. can be considered lost, an integrative redescription of this species and a neotype assignation are needed. However, given the distance of the population found in this study to the type locality of *D. occidentalis* comb. nov. stat. nov. (700 km), we temporarily refrain from assigning a neotype and from providing an amended diagnosis of the species. However, an extensive descriptive account of *D. occidentalis* comb. nov. stat. nov. is given in the results.

Moss samples for tardigrade extraction in this study were collected at or about sea-level (Table I). Most of the moss samples were collected from "standard" terrestrial habitats (rocks, living trees, and tree stumps). Previous studies in British Columbia (e.g., Kathman & Nelson 1989) have focused on tardigrade diversity from elevations as high as 760 m. However, *Hypsibius* cf. *convergens* (S2002), *Echiniscus* cf. *robertsi*, *Pseudechiniscus* (*Meridioniscus*) *dastychi*, *Diphascion* cf. *claxtonae* (S2010), and *Claxtonia maucci* (S2011) (Table III) came from moss collected from beached trees in several localities in the intertidal zone (Figure 8). These beached trees, which are primarily

western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), are large and remain relatively stationary, having been in their approximate positions for at least 30 years (Chris Ashurst pers. comm). While partially submerged during high tide, the upper-most level of the beached trees remains exposed and have accumulated enough sediment to support a permanent assemblage of terrestrial vegetation, including mosses. The diversity and peculiarity of tardigrade taxa found in these "non-standard" habitats shows how keeping exploring new habitat types is important in obtaining a complete view and understanding of tardigrade diversity. Interestingly, there is no overlap between the taxa found at all beached tree locations. Past studies remain divided as to the extent to which ecological factors affect tardigrade distribution in the Americas (Meyer 2013), but the results indicate that further data are needed to determine whether a consistent pattern can be elucidated.

The exploration of Haida Gwaii conducted in this study also increases the knowledge on American tardigrades fauna adding seven taxa (Table I) to the 274 taxa already known for North America based on the last available checklist for this area (Kaczmarek et al. 2016) and nine taxa (Table I) to the 129 found in Canada (Kaczmarek et al. 2016; Kayastha et al. 2021; Vecchi et al. 2022b).

#### Family Richtersiidae amended diagnosis

Double claws Y-shaped, with the two branches forming an evident common tract of a variable length with system of internal septa. Teeth present or absent on lunulae I-III and always present on lunulae IV. Buccal tube with ventral lamina



Figure 8. Picture of beached tree (western hemlock - *Tsuga heterophylla*) in the intertidal zone, showing growth of moss and other terrestrial plants, on which sample of mosses used for this study were collected (see sample code in Table III).

exhibiting ventral thickening in its anterior portion (sometimes hardly visible under light microscope) and a cuticular thickening on the anterior, dorsal wall of the buccal tube (which can form a large apophysis). Transverse crests in the buccal armature absent. Two macroplacoids in the pharynx. Microplacoid present or absent (in most taxa). Cuticular pores (at least in a phase of the life cycle). Eggs laid freely with conical (usually spiky) processes and without areolation on their surface. Body and leg granulation absent in all currently recognized species.

### Dichotomous key for the genus *Diaforobiotus*

The key does not include *Diaforobiotus islandicus nicaraguensis* (Séméria, 1985) designated as nomen inquirendum by Stec (2022).

1. - Teeth present on lunulae of legs I-III (2)
  - Teeth absent on lunulae of legs I-III (3)
2. - The claw common tract longer than the half of the entire claw height, egg surface between processes with evenly distributed dark dots (seen in PCM) – *Diaforobiotus islandicus* (Richters, 1904)
  - The claw common track constitutes one-third of the entire claw length, egg surface between processes without dark dots (seen in PCM) – *Diaforobiotus caelicola* (Kathman, 1990)
3. - The claw common tract shorter than the half of the entire claw length, the dorsal portion of the third band of teeth in the oral cavity armature (OCA) – where the transversal ridge is generally present – comprises only one tooth – *Diaforobiotus hyperonyx* (Maucci, 1982)
  - The claw common tract longer than the half of the entire claw height, dorsal portion of the third band of teeth in the OCA – where the transversal ridge is generally present – comprises three teeth (4)
4. - Microplacoid absent, chorion surface with evenly distributed, minute, faintly visible light refracting dots in the egg surface between processes (visible in PCM), egg process base/height ratio between 56% and 83% – *Diaforobiotus svalbardicus* Stec, 2022
  - Microplacoid present (even if small and not always clearly visible), chorion surface with irregularly distributed dark dots in the egg surface between processes (visible in PCM), egg process base/height ratio between 23% and 50% – *Diaforobiotus occidentalis* (Murray, 1910)

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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### Data availability statement

The video showing *Diaforobiotus occidentalis* comb. nov. stat. nov. individuals (sample S2016) and the crystal-like structures floating in the space between epidermis and cuticle in the alive individual is available on YouTube at the link: [https://youtu.be/Wsic3w\\_j0AY](https://youtu.be/Wsic3w_j0AY).

### Supplementary material

Supplemental data for this article can be accessed online at <https://doi.org/10.1080/24750263.2023.2288824>

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