Some have drops and some do not, but can we rely on that? Re-investigation of *Diphascon tenue* (Tardigrada: Eutardigrada) with discussion of the phylogeny and taxonomy of the superfamily Hypsibioidea

Одни с каплями, другие без, но можно ли на это положиться? Переисследование *Diphascon tenue* (Tardigrada: Eutardigrada) с обсуждением филогении и таксономии надсемейства Hypsibioidea

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Abstract. An integrative description of a newly discovered Spitsbergen population of *Diphascon tenue* Thulin, 1928 was undertaken using high quality light microscopy, scanning electron microscopy and methods of molecular taxonomy. Phylogenetic analyses indicated the relationships of this species within the subfamily Itaquasconinae Bartoš in Rudescu, 1964 (the family Hypsibiidae Pilato, 1969). *Arctodiphascon* **gen. nov.** is erected with two species, *A. tenue*, **comb. nov.** and *A. wuyingensis* (Sun et al., 2020), **comb. nov.** Following the results of the phylogenetic molecular and morphological analysis, *Adropion greveni* (Dastych, 1984) and *A. mauccii* (Dastych et McInnes, 1996) are transferred to the genus *Diphascon* Plate, 1888 as *D. greveni* Dastych, 1984, **stat. resurr.** and *D. mauccii* Dastych et McInnes, 1996, **stat. resurr.** Following the results of the phylogenetic molecular and morphological analyses, the generic name *Meplitumen* Lisi et al., 2019, **syn. nov.** is recognised as synonymous to *Platicrista* Pilato, 1987. An emended diagnosis for the genus *Astatumen* Pilato, 1997 is given. The distribution of morphological characters within the superfamily Hypsibioidea is analysed and their taxonomic significance is discussed. A new phylogenetic tree of Hypsibioidea based on molecular data is presented. The subfamilies Itaquasconinae and Pilatobiinae Bertolani et al., 2014 are raised to family rank, as Itaquasconidae and Pilatobiidae **(status promotus)**. The subfamily Diphasconinae Dastych, 1992 is transferred to the Hypsibiidae, as a sister group to the subfamily Hypsibiinae Pilato, 1969.

Резюме. Приведено интегративное описание обнаруженной на Шпицбергене популяции вида *Diphascon tenue* Thulin, 1928, выполненное методами высокоразрешающей световой и сканирующей электронной микроскопии и молекулярной таксономии. Филогенетический анализ выявил положение этого вида в пределах подсемейства Itaquasconinae Bartoš in Rudescu, 1964 (семейство Hypsibiidae Pilato, 1969). Установлен новый род *Arctodiphascon* **gen. nov.**, включающий два вида: *A. tenue*, **comb. nov.** и *A. wuyingensis* (Sun et al., 2020), **comb. nov.** В соответствии с результатами филогенетического молекулярного и морфологического анализа виды *Adropion greveni* (Dastych, 1984) и *A. mauccii* (Dastych et McInnes, 1996) перенесены в род *Diphascon* Plate, 1888 как *D. greveni*

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Dastych, 1984, **stat. resurr.** и *D. mauccii* Dastych et McInnes, 1996, **stat. resurr.** В результате проведенного филогенетического молекулярного и морфологического анализа установлено, что родовое название *Meplitumen* Lisi et al., 2019, **syn. nov.** является синонимом названия *Platicrista* Pilato, 1987. Приведен уточненный диагноз рода *Astatumen* Pilato, 1997. Проанализировано распределение морфологических признаков в пределах надсемейства Hypsibioidea и их таксономическая значимость. Представлено новое молекулярно-филогенетическое дерево Hypsibioidea. Подсемейства Itaquasconinae и Pilatobiinae Bertolani et al., 2014 повышены в ранге до семейств (как Itaquasconidae и Pilatobiidae, **status promotus**). Подсемейство Diphasconinae Dastych, 1992 перенесено в семейство Hypsibiidae, являясь сестринской группой для подсемейства Hypsibiinae Pilato, 1969.

Key words: phylogeny, taxonomy, morphological evolution, molecular characters, integrative description, Tardigrada, Hypsibioidea, Hypsibiidae, Itaquasconinae, *Diphascon*, *Meplitumen*, *Astatumen*, new synonymy, new combination, new genus

Ключевые слова: филогения, таксономия, морфологическая эволюция, молекулярные признаки, интегративное описание, Tardigrada, Hypsibioidea, Hypsibiidae, Itaquasconinae, *Diphascon*, *Meplitumen*, *Astatumen*, новая синонимия, новые комбинации, новый род

Introduction

Tardigrades are a group of microscopic multicellular animals widely distributed in nature (Nelson et al., 2018). Extant tardigrades are water-dependent animals inhabiting all types of habitats with at least a thin layer of liquid water, from oceans to ephemeral continental water bodies. The highest species diversity of tardigrades is found within terrestrial hygroscopic substrates, mostly moss and lichen cushions. One of the two large tardigrade clades that successfully invaded these semiterrestrial environments is the class Eutardigrada Richters, 1926. This group has undergone significant simplification of its morphological structure during its evolutionary history, leaving only a limited set of morphological characters available for the phylogenetic analysis and identification (Jørgensen et al., 2018; Gąsiorek & Michalczyk, 2020). The introduction of molecular methods in the analysis of the animal phylogeny has significantly changed our understanding of tardigrade evolution and, as a result, affected the taxonomy of this group (e.g. Marley et al., 2011; Bertolani et al., 2014; Gąsiorek et al., 2019a; Tumanov, 2022). However, morphological analysis still plays a substantial role in tardigrade taxonomy, not least because molecular data are missing for many rare taxa of these animals.

The genus *Diphascon* Plate, 1888 is one of the oldest known genera within the Eutardigrada,

initially established based on the peculiar structure of the bucco-pharyngeal apparatus. For a long time after the description of the genus *Diphascon*, all Hypsibiidae species with the buccal tube divided into a rigid anterior and a flexible posterior part were attributed to this genus or subgenus, depending on the rank accepted by certain authors. Pilato (1987) in his detailed morphological analysis of the genus *Diphascon* introduced additional characters such as the shape of the apophyses for the insertion of the stylet muscles (AISM) and their caudal processes, the shape of the stylet furcae and the presence of a drop-like cuticular thickening between the buccal and pharyngeal tubes. Using these characters, he divided the genus *Diphascon* into four genera: (1) *Diphascon*, which incorporates the forms with AISM in the shape of a "semilunar hook", symmetrical with respect to the frontal plane, with the caudal processes pointing posteriorly and laterally and the furcae with the posterolateral processes thickened at their apices; (2) *Mesocrista* Pilato, 1987, which incorporates the forms with AISM in the shape of "wide and flat ridges", symmetrical with respect to the frontal plane, with the caudal processes well-developed and pointing posteriorly and laterally, and the furcae with the posterolateral processes thickened at their apices; (3) *Platicrista* Pilato, 1987, which incorporates the forms with AISM in the shape of "very wide and flat ridges",

symmetrical with respect to the frontal plane, with the caudal processes poorly developed and pointing laterally, and the furcae with the posterolateral processes spoon-like and tapering at their apices; (4) *Hebesuncus* Pilato, 1987, which incorporates the forms with AISM in the shape of "blunt hooks" where the dorsal hook is different in shape and size from the ventral one with consequent asymmetry with respect to the frontal plane. He also recognised two subgenera within the genus *Diphascon*: the subgenus *Diphascon* incorporated the species with a drop-like structure in the buccal tube and a new subgenus *Adropion* Pilato, 1987 incorporated the species without such a structure. Later phylogenetic analyses based on molecular data (Sands et al., 2008; Guil & Giribet, 2012; Bertolani et al., 2014) supported the validity of these species groups but also revealed the polyphyly of the genus *Diphascon*. In the study by Bertolani et al. (2014), species representing the subgenus *Adropion* were proved to be more closely related to the genera of the subfamily Itaquasconinae Bartoš in Rudescu, 1964 (*Astatumen* Pilato, 1997, *Itaquascon* de Barros, 1939 and *Platicrista*) than to the species of the subgenus *Diphascon*, resulted in rising *Adropion* to the genus level. Moreover, in the same work a separate clade was recovered that included some taxa of *Diphascon*, which resulted in the establishment of a new genus *Pilatobius* Bertolani et al., 2014 and a new subfamily Pilatobiinae Bertolani et al., 2014 within the family Hypsibiidae Pilato, 1969.

Pilato (1997) introduced two more characters of the bucco-pharyngeal apparatus, namely, the presence or absence of the stylet supports and the position of the transition point between the rigid and flexible parts of the bucco-pharyngeal tube. Both of these characters were initially used for the separation of the genera *Itaquascon* and *Astatumen* (Pilato, 1997) and later the position of the transition point between the rigid and flexible parts of the bucco-pharyngeal tube was used for the separation of a new genus *Meplitumen* Lisi et al., 2019 from *Platicrista* (Lisi et al., 2019).

The genus *Bindius* Pilato, 2009 was established for a single species *Bindius triquetrus* Pilato, 2009 based on the presence of evident asymmetry of the apophyses for the insertion of stylet muscles (AISM) (Pilato, 2009).

The work of Gąsiorek & Michalczyk (2020) devoted to the morphological and molecular phylogenetic analyses of the subfamily Itaquasconinae provided new important insights into the phylogenetic tree of this subfamily. The validity of the genus *Mesocrista* was confirmed and its position relatively distant from the genus *Platicrista* was revealed. A polyphyly of the genus *Adropion* was shown, which led to the establishment of a new genus *Guidettion* Gąsiorek et Michalczyk, 2020, and the presence of another unnamed clade of the genus-rank was demonstrated. In addition, the complex genetic structure of the group of species currently attributed to the genera *Astatumen* and *Itaquascon* was revealed. Two new genera, *Raribius* Gąsiorek et Michalczyk, 2020 and *Insulobius* Gąsiorek et Michalczyk, 2020 belonging to the *Astatumen-Itaquascon* morphotype but distant from the main *Astatumen* clade, were established using the presence/absence of stylet supports as a discriminating character (Gąsiorek & Michalczyk, 2020).

In a recent publication by Zawierucha et al. (2023), two new genera were described, *Kararehius* Zawierucha et al., 2023 and *Kopakaius* Zawierucha et al., 2023, both belonging to the subfamily Diphasconinae Dastych, 1992. Despite their phylogenetic position deep within the paraphyletic genus *Diphascon*, the morphology of both these genera shows significant similarity with Itaquasconinae. The genus *Kararehius* strongly resembles the genus *Adropion* in the absence of the drop-like structure in the buccal tube, while the genus *Kopakaius* is most similar to the genera *Itaquascon* and *Raribius* in the absence of distinct placoids in the pharynx, the widened bucco-pharyngeal tube, and in the absence of the drop-like structure in the buccal tube.

Currently, the genus *Diphascon* has no formal diagnosis (see discussion in Tumanov, 2018a) and includes species "with buccal tube followed by an annulated pharyngeal tube, with a cuticular thickening between them (often dropshaped, sometimes barely evident); pharyngeal bulb is an elongated oval, containing always 3 macroplacoids in a line (and sometimes with a microplacoid and/or septulum)" (cited from the emended diagnosis of the subfamily Diphasconinae Dastych, 1992, as given in Bertolani et al., 2014).

This genus now contains 38 species that fit this diagnosis [four of them are *nomina dubia* according to Dastych (2015)] and three are inadequately described species with two macroplacoids [two of them are *nomina dubia* according to Dastych (2015)].

Diphascon tenue Thulin, 1928 is a rare arctic species described from the Faroe Islands (Thulin, 1928) and later recorded from Scotland, Sweden, Norway, the Carpathians, Spitsbergen, Canada, and the Khibiny Mountains (McInnes, 1994; Tumanov, unpublished data). It is the third species described in the genus *Diphascon* in its current composition, while the two oldest species, *D. chilenense* Plate, 1888 (type species of *Diphascon*) and *D. alpinum* Murray, 1906, are considered as *nomina dubia* (Dastych, 2015).

During our investigation of the tardigrade fauna of Spitsbergen, abundant individuals identified as *D. tenue* were found in a moss sample. We used this material to perform an integrative investigation of this species using methods of light microscopy (LM) and scanning electron microscopy (SEM), and to set the phylogenetic position of this and some related taxa using molecular phylogenetic analysis.

Material and methods

Material examined

Diphascon tenue Thulin, 1928

The material is listed below, in the description of this species.

Adropion greveni (Dastych, 1984)

95 specimens mounted on slides in Hoyer's medium (five of these specimens used for DNA extraction); 7 specimens mounted on stubs for SEM investigation. **Antarctica**, *South Shetland Is.*, King George I., Fildes Peninsula, 62°09′09.5″S 58°56′17.3″W, 39 m a.s.l., mosses and algae on soil in shallow permanent freshwater stream, 18 Feb. 2020, A. Przhiboro leg., slides SPbU 263(005–053), SEM stubs SPbU Tar_19, SPbU Tar_35.

Astatumen sp.

One specimen mounted on slide in Hoyer's medium (and used for DNA extraction). **Russia**, *Murmansk Prov.*, 68°13′33.5″N 33°19′50.3″E, moss on stone, 10 Aug. 2021, D. Tumanov leg., slide SPbU 288(001).

Astatumen(?) sp. "Spitsbergen"

One specimen mounted on slide in Hoyer's medium (and used for DNA extraction). **Norway**, *West Spitsbergen*, 78°11′43.8″N 15°33′46.8″E, soil with grass roots, 13 Aug. 2019, N. Shunatova leg., slide SPbU 290(001).

Calohypsibius ornatus (Richters, 1900)

One specimen mounted on slide in Hoyer's medium (and used for DNA extraction). **Norway**, *West Spitsbergen*, 79°15′37″N 11°31′41.3″E, moss on soil, 17 Aug. 2019, N. Shunatova leg., slide SPbU 259(114).

Diphascon sanae Dastych, Ryan et Watkins, 1990

Two specimens mounted on slides in Hoyer's medium. **Antarctica**, *Larsemann Hills*, Progress Station, moss on rock, 12 March 1991, A. Ostrovskij leg., slide SPbU 75(001).

Diphascon cf. pingue (Marcus, 1936)

Three specimens mounted on slides in Hoyer's medium (and used for DNA extraction). **Russia**, *Leningrad Prov.*, 60°24′58″N 30°20′33.6″E, moss and lichens on soil, 20 Sept. 2020, E. Androsova leg., slides SPbU 275(107, 112, 113).

Guidettion sp.

Two specimens mounted on slides in Hoyer's medium (and used for DNA extraction). **Russia**, *Leningrad Prov.*, 59°43′30.9″N 30°23′32″E, moss on stone, 3 July 2020, D. Tumanov leg., slide SPbU 264(045).

Meplitumen sp.

Four specimens mounted on slides in Hoyer's medium (one of them used for DNA extraction). **Russia**, *Murmansk Prov.*, 68°13′33.5″N 33°19′50.3″E, moss on stone, 10 Aug. 2021, D. Tumanov leg., slides SPbU 288(001–004).

Microhypsibius sp. 1

One specimen used for DNA extraction (not mounted). **Norway**, *West Spitsbergen*, 79°15′37.7″N 11°31′40.1″E, moss on soil, 17 Aug. 2019, N. Shunatova leg.

Microhypsibius sp. 2

One specimen mounted on slide in Hoyer's medium (and used for DNA extraction). **Norway**, *West Spitsbergen*, 79°15′37″N 11°31′41.3″E, moss on soil, 17 Aug. 2019, N. Shunatova leg., slide SPbU 259(63).

Platicrista aff. angustata (Murray, 1905)

One specimen mounted on slide in Hoyer's medium. **Norway**, *West Spitsbergen*, Nordvest-Spitsbergen National Park, 79°15′37.0″N 11°31′41.3″E, moss

on soil, 17 Aug. 2019, N. Shunatova leg., slide SPbU 259(009).

Isolation of specimens

Tardigrades were extracted from fresh or frozen samples by washing them through two sieves (Tumanov, 2018b). The content of the fine sieve was examined under a Leica M205C stereo microscope.

Microscopy and imaging

Tardigrades found were fixed with acetic acid or relaxed by incubating live individuals at 60 °C for 30 min (Morek et al., 2016) and mounted on slides in Hoyer's medium. Permanent slides were examined under a Leica DM2500 microscope equipped with phase contrast (PhC) and differential interference contrast (DIC). Photographs were taken using a Nikon DS-Fi3 digital camera with NIS software.

For scanning electron microscopy (SEM) specimens were thermally relaxed at 60 °C (Morek et al., 2016), dehydrated in an ascending ethyl alcohol series (10%, 20%, 30%, 50%, 70%, 96%) and acetone, critical-point dried in CO_2, mounted on stubs and coated with gold. A Tescan MIRA3 LMU Scanning Electron Microscope was used for observations (Centre for Molecular and Cell Technologies, St Petersburg State University).

Morphometrics and terminology

All measurements are given in micrometres (μm). Structures were measured only if their orientation was suitable. Body length was measured from the anterior end of the body to the posterior end, excluding the hind legs. The bucco-pharyngeal tube was measured from the anterior margin of the stylet sheaths to the caudal end of the buccal tube, not including the buccal apophyses. Length of the buccal tube was measured excluding the drop-like structure. Terminology for the structures within the bucco-pharyngeal apparatus and for the claws follows Pilato & Binda (2010). AISM is used as an abbreviation for the apophyses for insertion of stylet muscles. Elements of the buccal apparatus were measured according to Kaczmarek & Michalczyk (2017). Claws were measured following Beasley et al. (2008), but the total length of the claws was also measured (according to Pilato et al., 2002) to ensure compatibility with older publications. The *pt* index used is the percentage ratio between the length of a structure and the length of the buccal tube (Pilato, 1981) and is presented here in italics. Morphometric data were handled using ver. 1.6 of the "Parachela" template, which is available from the Tardigrada Register (Michalczyk & Kaczmarek, 2013), with addition of the total length of the claws.

Genotyping

DNA was extracted from individual specimens using QuickExtract™ DNA Extraction Solution (Lucigen Corporation, USA; see description of complete protocol in Tumanov, 2020). Preserved exoskeletons were recovered, mounted on a microscope slide in Hoyer's medium and retained as the hologenophore (Pleijel et al. 2008).

Four genes were sequenced: a small ribosome subunit (18S rRNA) gene, a large ribosome subunit (28S rRNA) gene, internal transcribed spacer (ITS-2), and the cytochrome oxidase subunit I (COI) gene. PCR reactions included 5 μl template DNA, 1 μl of each primer, 1 μl DNTP, 5 μl Taq Buffer (10×) (−Mg), 4 μl 25 mM $MgCl_2$ and 0.2 μl Taq DNA Polymerase (Thermo Scientific™) in a final volume of 50 μl. The primers and PCR programs used are listed in Electronic supplementary material 1 (see Addenda). The PCR products were visualised in 1.5% agarose gel stained with ethidium bromide. All amplicons were sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the help of an ABI Prism 310 Genetic Analyzer in the Core Facilities Center "Centre for Molecular and Cell Technologies" of St Petersburg State University. Sequences were edited and assembled using ChromasPro software (Technelysium, USA). The COI sequences were translated to amino acids using the invertebrate mitochondrial code, MEGA11 (Tamura et al., 2021), in order to check for the presence of stop codons and therefore of pseudogenes.

Phylogenetic analyses

Sequences of 18S and 28S genes representing all genera of Hypsibioidea and available in GenBank at the time of the analysis were downloaded. Sequences of appropriate length that

were homologous to the sequences obtained and originated from publications with a reliable attribution of the investigated taxa were selected, with addition of the newly obtained sequences (see Addenda: Electronic supplementary material 2). Both 18S rRNA and 28S rRNA are nuclear markers used in phylogenetic analyses to investigate high taxonomic levels (Jørgensen et al., 2010, 2011; Guil & Giribet, 2012; Bertolani et al., 2014; Guil et al., 2019; Gąsiorek et al., 2019a, 2019b, 2019c). The COI and ITS-2 markers were not included into the analysis because the inclusion of fast-evolving genes may negatively affect the resolution of deep phylogeny (Betancur-R. et al., 2014; Chen et al., 2015; Klopfstein et al., 2017). Two sequences of Macrobiotoidea [*Macrobiotus shonaicus* Stec, Arakawa et Michalczyk, 2018 of Macrobiotidae and *Richtersius coronifer* (Richters, 1903) of Richtersiusidae Guidetti, Schill, Giovannini, Massa, Goldoni, Ebel, Förschler, Rebecchi et Cesari, 2021] were used as an outgroup.

Sequences were automatically aligned with the MAFFT algorithm (Katoh et al., 2002) with the software AliView version 1.27 (Larsson, 2014); the alignments were cropped to a length of 1673 bp for 18S and 823 bp for 28S. Sequences of both genes were concatenated using SeaView 4.0 (Gouy et al., 2010) (final alignment presented in Electronic supplementary material 3; see Addenda). The best substitution model and partitioning scheme for posterior phylogenetic analysis was chosen under the Akaike Information Criterion (AICc), using IQ-TREE multicore version 1.6.12 (Kalyaanamoorthy et al., 2017; Minh et al., 2020). IQ-TREE suggested retaining two predefined partitions separately, and GTR+F+I+G4 model was recognised as the most suitable for both datasets. Maximum-likelihood (ML) topologies were constructed using IQ-TREE software (Minh et al., 2020) with GTR+F+I+G4 model. Bayesian analysis of the same datasets was performed using MrBayes ver. 3.2.6, GTR model with gamma correction for intersite rate variation (8 categories) and the covariation model (Ronquist & Huelsenbeck, 2003). Analyses were run as two separate chains (default heating parameters) for 20 million generations, by which time they had ceased converging (final average standard deviation of the split frequencies was less than 0.01).

The quality of chains was estimated using built-in MrBayes tools. MrBayes program was run at the CIPRES ver. 3.3 website (Miller et al., 2010). Bayesian analysis quality was verified using the program Tracer v1.7.1 (Rambaut et al., 2018). Uncorrected pairwise distances were calculated using MEGA11 (Tamura et al., 2021) with gaps/missing data treatment set to "pairwise deletion".

Institutional acronyms

The specimens examined are kept at the following institutions and collections (the curator is given in parentheses): SpbU – Department of Invertebrate Zoology, Faculty of Biology, St Petersburg University, Russia (Denis Tumanov); ZMUC – Zoological Museum, University of Copenhagen, Denmark (Martin Vinther Sørensen).

Results and discussion

Phylum **Tardigrada** Doyère, 1840

Class **Eutardigrada** Richters, 1926

Order **Parachela** Schuster, Nelson, Grigarick et Christenberry, 1980

Superfamily **Hypsibioidea** Guil, Jørgensen et Kristensen, 2019

Family **Hypsibiidae** Pilato, 1969

Genus *Arctodiphascon* **gen. nov.**

Type species: *Diphascon tenue* Thulin, 1928

Diagnosis. Peribuccal structures consisted of six peribuccal lobes. AISM in shape of "semilunar hooks" asymmetrical with respect to frontal plane with dorsal apophyses being distinctly higher, shorter and thicker than ventral ones, caudal processes of both apophyses pointing posteriorly and laterally. Buccal tube followed by a flexible annulated pharyngeal tube, with drop-like apodeme, bearing annulation between them. Pharynx oval with three bar-like, elongate macroplacoids. Microplacoids or septula absent. Claws of *Hypsibius*-type. Lunules and cuticular bars on limbs absent. Eggs laid within exuviae, with numerous minute pillars-like processes on chorion surface.

Comparison. Arctodiphascon **gen. nov.** is most similar to *Bindius* (Pilato, 2009) in having an

asymmetrical AISM, a long thin bucco-pharyngeal tube and three rod-like macroplacoids, but differs clearly from the latter in having the drop-like apodeme of the bucco-pharyngeal tube, which is completely absent in *Bindius*. *Arctodiphascon* **gen. nov.** is also similar to *Guidettion* in having a long thin bucco-pharyngeal tube and three rod-like macroplacoids without microplacoids or septula, but differs clearly from it in having a drop-like apodeme of the bucco-pharyngeal tube and an asymmetrical AISM. *Arctodiphascon* **gen. nov.** is similar to *Diphascon* in having a drop-like apodeme of the bucco-pharyngeal tube and three macroplacoids, but clearly differs from it in having no microplacoid or septula. The only species lacking both microplacoids and septula currently assigned to *Diphascon* is *D. speciosum* (Mihelčič, 1971) (*nomen dubium* according to Dastych, 2015), but this species has a granulated body cuticle, while the body surface in *Arctodiphascon* **gen. nov.** lacks granulation.

Note. The establishment of *Arctodiphascon* **gen. nov.** is substantiated by the morphological and molecular phylogenetic analysis presented below.

Etymology. The name refers to the morphological similarity of the new genus to the genus *Diphascon*, and to the predominantly arctic distribution of its type species *Arctodiphascon tenue*, **comb. nov.**

Genus composition (two species): *Arctodiphascon tenue* (Thulin, 1928), **comb. nov.** and *A. wuyingensis* (Sun, Zhang, Wang, Zhao et Luo, 2020), **comb. nov.** (placed tentatively; for the position of *A. wuyingensis*, see discussion below).

Key to the species of the genus *Arctodiphascon* gen. nov.

1. Pharyngeal tube longer (buccal/pharyngeal tube length ratio 33–46%), pharyngeal apophyses extremely poorly developed, usually indiscernible with LM, second macroplacoid shorter relative to first macroplacoid (second/first macroplacoid ratio 0.87–1.30) . . . . ***A. tenue***
– Pharyngeal tube shorter (buccal/pharyngeal tube length ratio 84–98%), pharyngeal apophyses normally developed, discernible with LM, second macroplacoid longer relative to first macroplacoid (second/first macroplacoid ratio 1.37–1.59) . . . . ***A. wuyingensis***

Arctodiphascon tenue (Thulin, 1928), **comb. nov.**
(Figs 1–3)

Diphascon tenue Thulin, 1928: 255–256, fig. 26.

New material examined. 172 specimens and one exuviae with eggs mounted on slides in Hoyer's medium (six of these specimens used for DNA extraction); 15 specimens mounted on stubs for SEM investigation. **Norway**, *West Spitsbergen*, Nordvest-Spitsbergen National Park, 79°15′37.0″N 11°31′41.3″E, moss on soil, 17 Aug. 2019, N. Shunatova leg., slides SPbU 259(001–107), SEM stub SPbU Tar_38.

Type material examined. Syntype. **Denmark**, *Faroe Is.*, Trangesvaag, 250 m a.s.l., moss, slide ZMUC-TAR-774 (NHMD) [Thulin's collection, labelled as "type"].

Morphological description (based on new material). Body elongate, relatively thin, slightly widened on its caudal end, with evidently narrowed head region (Fig. 1; Table 1). Body transparent or whitish, without eyespots. Cuticle smooth in LM, with poorly developed rugosity in SEM, more evident dorsally (Fig. 1B–D). No cephalic sensory structures visible (Fig. 1E), except for two elongate porous areas laterally to mouth opening (visible in SEM only; Fig. 1F, G, white arrowheads), possibly being sensory structures or muscle attachment zones. Mouth opening anteroventral, surrounded by six peribuccal lobes (visible in SEM only; Fig. 1F, G).

Bucco-pharyngeal apparatus of *Diphascon*-type sensu Pilato & Binda (2010), with very thin and long bucco-pharyngeal tube (Fig. 2A–C). Oral cavity armature not visible in LM. Dorsal and ventral apophyses for insertion of stylet muscles (AISM) in shape of "semilunar hooks", asymmetrical with respect to frontal plane, with dorsal apophyses being distinctly higher, shorter and thicker than ventral ones (Fig. 2E–G), caudal processes of both apophyses pointed posteriorly and laterally, better developed dorsally (Fig. 2F, G). Stylet furcae typically developed, with swollen apices (Fig. 2D). Stylet supports well-developed (Fig. 2A–C). Pharyngeal tube with fine indistinct annulation (Fig. 2H). Most caudal zone of pharyngeal tube inside pharynx with slightly thickened walls and less developed annulation; annulation in this zone often not visible (Fig. 2K, black arrow). A well-developed drop-like dorsal apodeme present between buccal tube and pharyngeal tube
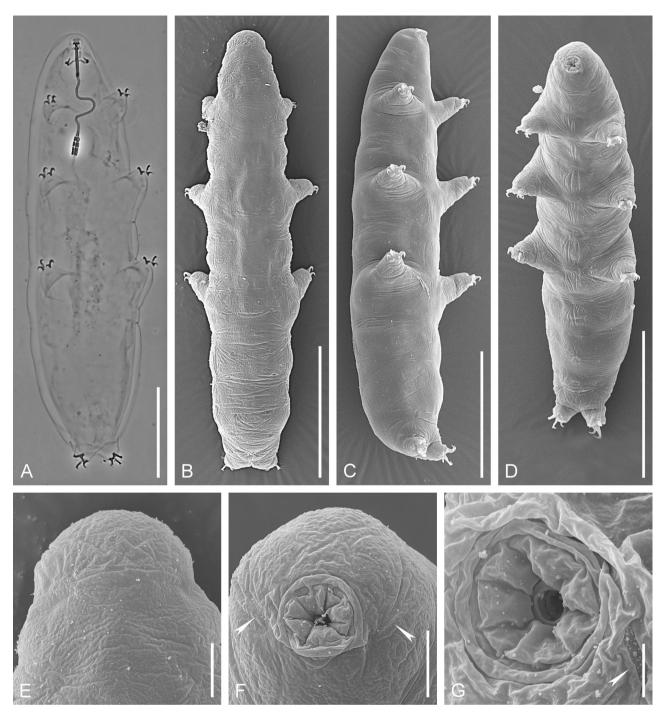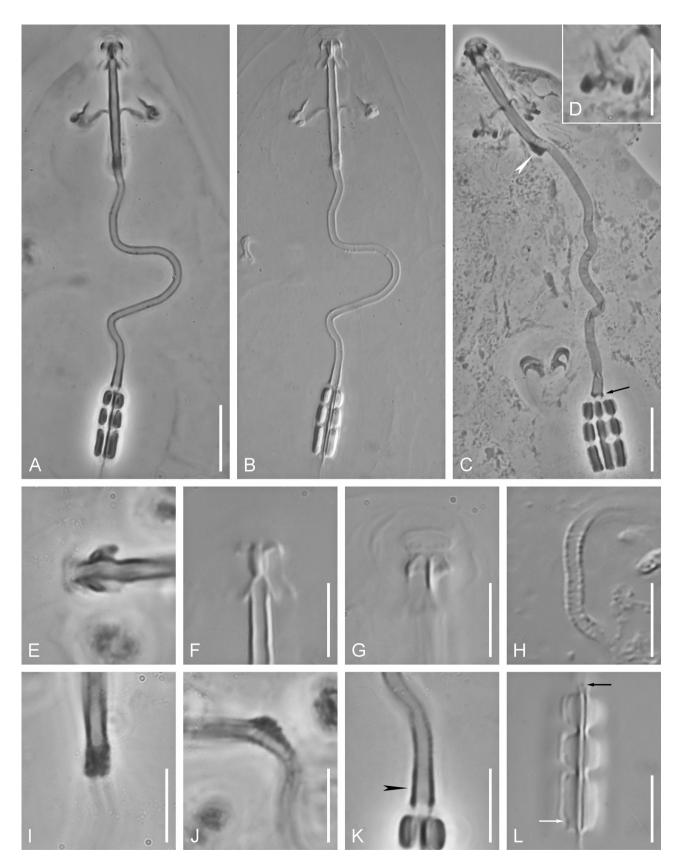
Fig. 1. *Arctodiphascon tenue* (Thulin, 1928), **comb. nov.**, total view and head. **A**, total view (PhC); **B**, dorsal view (SEM); **C**, lateral view (SEM); **D**, ventral view (SEM); **E**, dorsal view of head region (SEM); **F**, ventral view of head region (SEM); **G**, mouth opening (SEM). White arrowheads indicate porous areas. Scale bars: A–D – 50 µm; E–F – 5 µm; G – 2 µm.

(Fig. 2A–C, white arrowhead); surface of apodeme with annulation looking as continuation of pharyngeal tube annulation; annulation also present on pharyngeal tube wall below apodeme

(Fig. 2I, J). Extremely small pharyngeal apophyses rarely discernible with LM (Fig. 2L, black arrow) present; small thickenings of pharyngeal tube walls usually visible only caudally (Fig. 2C,

black arrow). Pharyngeal bulb subspherical, slightly elongate, with three rod-like macroplacoids, first and second being almost equal, third the longest (Fig. 2A–C, L). Third macroplacoid often with poorly developed pre-terminal incision (Fig. 2L, white arrow). No microplacoid, septulum or pseudoseptulum.

All legs with small claws of *Hypsibius*-type sensu Pilato & Binda (2010), slightly increasing in size from legs I to legs IV, with developed accessory points (Fig. 3A–E). No lunules (pseudolunules) at claw bases, but claw bases widened (visible in SEM only) (Fig. 3D, E). No cuticular bars below claws, but muscle attachment zone often forming a deep fold on ventral surface of legs I–III, which sometimes looking as a poorly developed bar-like structure in LM (Fig. 3A, B, D, white arrowhead).

Eggs laid in exuviae. Egg chorion with numerous short pillars, forming a granulated pattern on egg surface (Fig. 3F–H).

Comparison of the new material with the original description and the syntype of Diphascon tenue. Thulin (1928) described *D. tenue* from four syntypes. The original description given by Thulin (1928) is rather brief and does not meet modern standards for tardigrade species descriptions. However, it is accurate enough to provide essential details for the species recognition.

Our material corresponds perfectly to Thulin's description (1928) in all details, including some morphometric indices introduced by Thulin. These were *ms* (length of the structure in relation to body length) and *claw length index* (ratio between posterior and anterior claws lengths for legs IV). We did not use Thulin's *cph* index (length of the structure in relation to pharynx length), because the soft tissues can be strongly affected by the slide preparation process and mounting medium. Measurements provided by

Thulin were the following (measurements of our material are in parentheses): body length 231 μm (131–234 μm); pharynx length 21.6 μm (23.3 μm in a single measured specimen); length of posterior claw of legs IV 6.7 μm (5.3–8.2 μm); *ms* for posterior claws of legs IV 2.8% (2.9–3.7%); *claw length index* for legs IV 1.33 (1.09–1.42).

The presence of a drop-like apodeme of the bucco-pharyngeal tube was not mentioned in the original description, but it is visible in the Thulin's drawing (1928: 255, fig. 26). Moreover, this structure is well-recognisable in the syntype examined (Fig. 4). Unfortunately, specimens from the Thulin's collection are in poor state now because of the mounting medium degradation.

DNA sequences. The sequences of good quality for 18S rRNA and 28S rRNA gene fragments were obtained from six individuals [voucher slides SPBU 259(57–61)], those for COI gene fragment from five specimens [voucher slides SPBU 259(57–61)], and for ITS-2 marker from three specimens [voucher slides SPBU 259(57–59)]. No genetic polymorphism was revealed for all genes fragments: all sequences were identical for each of four genes. All obtained sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: OQ351311–OQ351316 (18S rRNA); OQ357540–OQ357545 (28S rRNA); OQ352269–OQ352273 (COI); OQ357877–OQ357879 (ITS-2).

Phylogenetic analysis of the superfamily Hypsibioidea

Both Bayesian and ML analyses of the 18S + 28S DNA sequences resulted in the identical phylogenetic tree (see the combined tree in Figs 5 and 6). The superfamily Hypsibioidea Pilato, 1969 was fully supported and divided into two fully

Fig. 2. *Arctodiphascon tenue* (Thulin, 1928), **comb. nov.**, bucco-pharyngeal apparatus. **A**, total view of bucco-pharyngeal apparatus (PhC); **B**, total view of bucco-pharyngeal apparatus (DIC); **C**, total view of bucco-pharyngeal apparatus, white arrowhead indicates dorsal apodeme in lateral position, black arrow indicates thickening of pharyngeal tube caudal end (PhC); **D**, stylet furca (PhC); **E**, lateral view of AISM (PhC); **F**, dorsal AISM (DIC); **G**, ventral AISM (DIC); **H**, fragment of pharyngeal tube with annulation (DIC); **I**, dorsal view of apodeme of bucco-pharyngeal tube with annulation on lateral sides (PhC); **J**, lateral view of apodeme of bucco-pharyngeal tube with annulation visible on apodeme and pharyngeal tube below (PhC); **K**, caudal part of pharyngeal tube, black arrowhead indicates thickened zone with reduced annulation (PhC); **L**, ventral row of macroplacoids, white arrow indicates pre-terminal incision, black arrow indicates minute pharyngeal apophyses (DIC). Scale bars: A–C – 10 μm; D–L – 5 μm.

supported clades: clade I, embracing the family Ramazzottiidae Sands et al., 2008, and clade II, comprising all remaining taxa currently attributed to Hypsibioidea. Within clade II, the family Calohypsibiidae Pilato, 1969 (currently represented by a single genus *Calohypsibius* Thulin, 1928) has an isolated position, while all other taxa form a monophyletic clade fully supported in Bayesian analysis (1.00) and moderately supported in ML analysis (83%). Within this clade, six monophyletic subclades with unresolved relations were revealed. Five clades were well supported: the family Microhypsibiidae Pilato, 1998 (currently represented by a single genus *Microhypsibius* Thulin, 1928), the family Acutuncidae Vecchi et al., 2023 [currently represented by a single genus *Acutuncus* Pilato et Binda, 1997 (Vecchi et al., 2023)], the genus *Mixibius* Pilato, 1992, the subfamily Pilatobiinae, and the clade embracing the subfamilies Hypsibiinae Pilato, 1969 and Diphasconinae. The subfamily Itaquasconinae clade was poorly supported in Bayesian analysis (0.78) and moderately supported in ML analysis (83%) (Figs 5 and 6).

Within the Itaquasconinae clade, three subclades with poorly resolved relations were revealed: the first comprising the genus *Guidettion* and a recently described species *Diphascon wuyingensis*, the second consisting of two undescribed *Adropion*-like species (*Adropion* "sp n 1_ PL 276" and *Adropion* "sp n 2_NO 018"; Gąsiorek & Michalczyk, 2020) and *D. tenue*, and the third comprising all remaining species of Itaquasconinae, currently attributed to the genera *Astatumen*, *Platicrista*, *Meplitumen*, *Insulobius*, *Raribius*, *Mesocrista*, *Itaquascon* and *Adropion* (Fig. 6).

In our analysis, *Adropion greveni* clearly falls within Diphasconinae (Fig. 5). *Meplitumen* sp. falls within the genus *Platicrista*. *Astatumen* sp. and *Astatumen*(?) sp. both fall within the genus *Astatumen*, forming a fully supported monophyletic clade with *Astatumen bartosi* (Węglarska, 1959) (Fig. 6).

Analyses of the similarity of gene sequences in *Arctodiphascon tenue*, comb. nov.

18S rRNA

Homology comparison of the obtained sequence of *A. tenue*, **comb. nov.** with the GenBank records (available on 2 October 2022) indicated high conformity to the phylum Tardigrada. The most closely related sequence, that of *Adropion* "sp n 1_PL 276" (MT126748; Gąsiorek & Michalczyk, 2020) from the family Hypsibiidae, subfamily Itaquasconinae was identical by 96.32% (query coverage was 93%, E value 0.0).

The ranges of uncorrected genetic *p*-distances between the studied specimens of *A. tenue*, **comb. nov.** and other Hypsibioidea species (see Addenda: Electronic supplementary material 4) were 3.52–14.24% (mean 6.78%), with *Astatumen trinacriae* (Arcidiacono, 1962) being the most similar (LR997449, unpublished) and *Kopakaius* sp. being the least similar (OP191643; Zawierucha et al., 2023).

28S rRNA

Homology comparison of the obtained sequence of *A. tenue*, **comb. nov.** with the GenBank records (available on 2 October 2022) indicated high conformity to the phylum Tardigrada. The most closely related sequence, that of *Adropion scoticum* (Murray, 1905) (OP035794; Tumanov et al., 2022) from the family Hypsibiidae, subfamily Itaquasconinae, was identical by 93.01% (query coverage was 99%, E value 0.0).

The ranges of uncorrected genetic *p*-distances between the studied specimens of *A. tenue*, **comb. nov.** and other Hypsibioidea species (see Addenda: Electronic supplementary material 4) were 10.04–27.24% (mean 18.29%), with *Pilatobius recamieri* (Richters, 1911) being the most similar (KX347527; Gąsiorek et al., 2017) and *Ramazzottius oberhaeuseri* (Doyère, 1840) being the least similar (MG573242; Stec et al., 2018).

ITS-2

Homology comparison of the obtained sequence of *A. tenue*, **comb. nov.** with the GenBank records (available on 2 October 2022) indicated high conformity to the phylum Tardigrada. The most closely related sequence, that of *Macrobiotus wandae* Kayastha et al., 2020 (MN435120; Kayastha et al., 2020) from the family Macrobiotidae was identical by 98.86% (query coverage was 18%, E value 1e-32).

The ranges of uncorrected genetic *p*-distances between the studied specimens of *A. tenue*,

Table 1. Summary of morphometric data for *Arctodiphascon tenue* (Thulin, 1928), **comb. nov.**

Character	n	Range		Mean		SD	
		µm	*pt*	µm	*pt*	µm	*pt*
Body length	30	131–234	*943–1431*	194	*1222*	23	*118*
Buccopharyngeal tube							
Buccal tube length	30	13.6–17.6	–	15.9	–	0.8	–
Pharyngeal tube length	30	30.5–48.7	*215.9–301.5*	40.5	*255.1*	4.4	*23.8*
Buccal/pharyngeal tube length ratio	30	33%–46%	–	40%	–	4%	–
Stylet support insertion point	30	8.6–11.0	*57.3–65.1*	9.9	*62.3*	0.6	*1.5*
Buccal tube external width	30	1.2–1.5	*7.6–9.8*	1.4	*8.7*	0.1	*0.5*
Buccal tube internal width	30	0.7–1.0	*4.6–6.4*	0.9	*5.5*	0.1	*0.5*
Placoid lengths							
Macroplacoid 1	30	1.6–2.4	*10.7–15.2*	2.0	*12.9*	0.2	*1.1*
Macroplacoid 2	30	1.4–2.8	*10.0–17.0*	2.2	*13.9*	0.3	*1.7*
Macroplacoid 3	30	2.2–4.1	*15.6–27.2*	3.5	*21.9*	0.5	*3.0*
Macroplacoid row	30	6.5–10.3	*46.3–65.6*	9.2	*57.7*	1.0	*5.3*
Second/first macroplacoid ratio	30	0.87–1.30	–	1.08	–	0.11	–
Claw 1 lengths							
External base	21	1.3–1.9	*8.2–12.6*	1.6	*10.0*	0.2	*1.3*
External primary branch	21	2.1–3.5	*13.8–20.6*	2.7	*17.3*	0.4	*2.0*
External secondary branch	21	1.5–2.8	*10.5–16.9*	2.2	*14.2*	0.3	*1.6*
External total	12	3.6–4.9	*24.5–31.7*	4.1	*26.3*	0.3	*2.1*
Internal base	15	1.4–1.8	*9.1–10.7*	1.6	*9.9*	0.1	*0.5*
Internal primary branch	15	1.8–2.6	*12.6–16.0*	2.3	*14.5*	0.2	*1.0*
Internal secondary branch	15	1.6–2.0	*10.2–12.7*	1.8	*11.2*	0.1	*0.8*
Internal total	14	3.1–3.9	*20.2–25.9*	3.6	*22.3*	0.3	*1.4*
Claw 2 lengths							
External base	21	1.3–2.2	*8.2–13.6*	1.7	*10.7*	0.2	*1.5*
External primary branch	21	2.4–4.1	*16.9–24.1*	3.2	*20.3*	0.4	*2.1*
External secondary branch	21	1.6–3.0	*10.6–18.9*	2.6	*16.3*	0.3	*1.9*
External total	15	3.9–5.2	*24.5–33.8*	4.7	*29.8*	0.5	*2.5*
Internal base	16	1.6–2.0	*10.2–12.0*	1.8	*11.2*	0.1	*0.5*
Internal primary branch	16	2.0–3.0	*13.7–17.9*	2.5	*15.7*	0.2	*1.2*
Internal secondary branch	16	1.8–2.6	*11.6–14.9*	2.1	*13.3*	0.2	*1.2*
Internal total	16	3.2–4.5	*22.3–26.3*	3.9	*24.5*	0.3	*1.2*
Claw 3 lengths							
External base	25	1.3–2.4	*9.1–15.3*	1.8	*11.3*	0.3	*1.7*
External primary branch	25	2.1–4.1	*15.2–24.3*	3.2	*20.2*	0.4	*2.1*
External secondary branch	25	1.6–3.2	*11.1–19.8*	2.5	*15.9*	0.4	*2.0*
External total	14	3.4–5.3	*25.1–35.1*	4.6	*29.8*	0.5	*2.7*
Internal base	17	1.5–2.0	*9.4–12.7*	1.8	*11.1*	0.1	*0.9*
Internal primary branch	17	2.1–3.2	*13.0–20.4*	2.6	*16.2*	0.3	*2.0*
Internal secondary branch	17	1.6–2.6	*9.8–15.4*	2.0	*12.5*	0.3	*1.5*
Internal total	15	3.2–4.4	*21.9–29.3*	4.0	*24.9*	0.3	*1.7*
Claw 4 lengths							
Anterior base	20	1.5–2.5	*10.2–16.2*	2.1	*13.3*	0.3	*1.5*
Anterior primary branch	20	2.3–3.9	*15.7–23.5*	3.3	*20.5*	0.4	*2.0*
Anterior secondary branch	20	1.8–3.2	*11.9–20.5*	2.7	*16.9*	0.4	*2.0*
Anterior total	20	3.8–5.8	*25.0–36.4*	5.0	*31.5*	0.5	*2.7*
Posterior base	18	1.8–3.0	*12.0–19.9*	2.5	*15.6*	0.3	*2.1*
Posterior primary branch	18	3.7–5.8	*24.6–33.9*	4.5	*28.2*	0.5	*2.4*
Posterior secondary branch	18	2.8–3.8	*17.6–23.6*	3.3	*20.2*	0.3	*1.7*
Posterior total	18	5.3–8.2	*34.7–47.9*	6.6	*41.1*	0.6	*3.4*
Posterior total to body length	18	2.9%–3.7%	–	3.3%	–	0.2%	–
Claw length index	15	1.09–1.42	–	1.31	–	0.09	–

Notes. Measurements are given in µm, *pt* values in % (*pt* index is a percentage ratio between the length of a structure and the length of the buccal tube). SD – standard deviation.
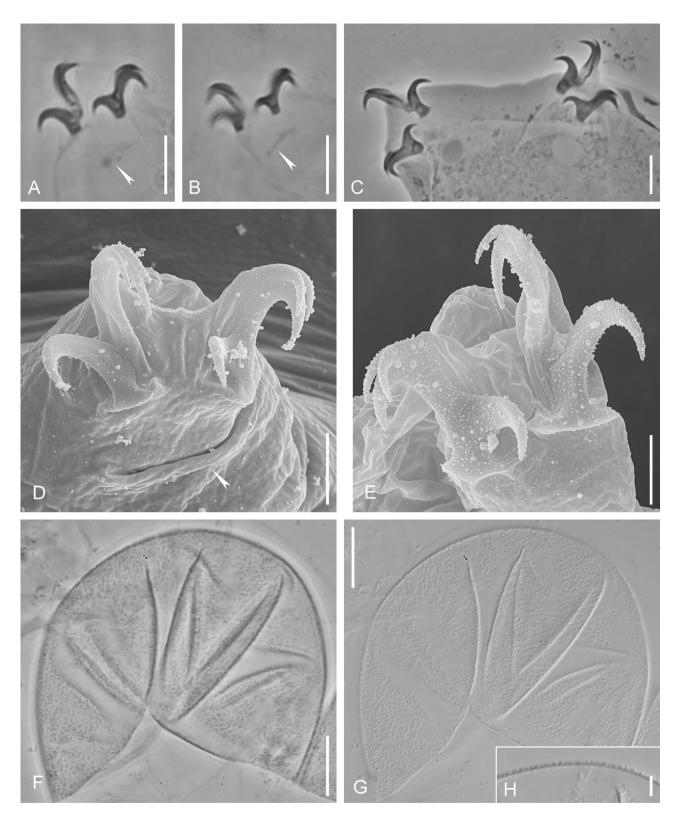
Fig. 3. *Arctodiphascon tenue* (Thulin, 1928), **comb. nov.**, claws and eggs. **A**, claws of leg III (PhC); **B**, claws of leg II, focused on the cuticular folding under the claw bases (PhC); **C**, claws of legs IV (PhC); **D**, claws of leg II (SEM); **E**, claws of legs IV (SEM); **F**, egg shell (PhC); **G**, egg shell (DIC), **H**, fragment of the optical section of egg shell with pillars visible (DIC). White arrowheads indicate cuticular folding under the claw bases. Scale bars: A–C – 50 µm; D–E, H – 2 µm; F, G – 10 µm.

comb. nov. and other Hypsibioidea species (see Addenda: Electronic supplementary material 4) were 38.90–116.80% (mean 71.97%), with *Mesocrista revelata* Gąsiorek et al., 2016 haplotype 3 being the most similar (KU528633; Gąsiorek et al., 2016) and *Ramazzottius subanomalus* (Biserov, 1985) haplotype 1 being the least similar (KU900019; Stec et al., 2016).

COI

Homology comparison of the obtained sequence of *A. tenue*, **comb. nov.** with the GenBank records (available on 2 October 2022) indicated high conformity to the phylum Tardigrada. The most closely related sequence, that of *Murrayon dianeae* (Kristensen, 1982) (FJ435801; Guil & Giribet, 2012) from the family Murrayidae, was identical by 79.53% (query coverage was 96%, E value 5e-166).

The ranges of uncorrected genetic *p*-distances between the studied specimens of *A. tenue*, **comb. nov.** and other Hypsibioidea species (see Addenda: Electronic supplementary material 4) were 25.44–40.28% (mean 31.13%), with *Ramazzottius* aff. *oberhaeuseri* 4 haplotype 3 being the most similar (MG573252; Stec et al., 2018) and *Astatumen* sp. n. 2 being the least similar (MT107471; Gąsiorek & Michalczyk, 2020).

Position of *Arctodiphascon tenue*, comb. nov. within Hypsibiidae

Our phylogenetic analyses revealed that despite the obvious morphological similarity with the genus *Diphascon* (presence of a drop-shaped dorsal apodeme between the rigid and the flexible part of the bucco-pharyngeal tube), the species *A. tenue*, **comb. nov.** clearly belongs to the phylogenetic clade currently designated as the subfamily Itaquasconinae (Fig. 6). Within this clade, *A. tenue* forms a monophyletic clade with two *Adropion*-like species that have not been formally described (Gąsiorek & Michalczyk, 2020). In our opinion, *A. tenue* should be excluded from the genus *Diphascon* due to its phylogenetic distance from this clade. However, taking into account its morphology, which is unique within the Itaquasconinae clade – the presence of

the drop-shaped dorsal apodeme of bucco-pharyngeal tube – this species cannot be included in any of the currently existing genera of the Itaquasconinae. Therefore, we proposed the erection of a new genus *Arctodiphascon* **gen. nov.**

Status of *Diphascon alpinum*

The validity of the species *Diphascon alpinum* was a subject of a long-lasting discussion (see Pilato & Binda, 1977, 1998; Dastych, 1988, 1989, 2015). Based on the incomplete and contradictory description of this species, Dastych (1988, 1989) considered *D. alpinum* as a species that "probably should be recognized as 'nomen nudum'"* while Pilato & Binda (1998) based on the specimen from Siberia considered *D. alpinum* as a rare but valid species. Later on, after re-investigation of this material Pilato re-assigned the Siberian material as *D. tenue* and noted that he has never found a specimen surely attributable to the species *D. alpinum* (Pilato, pers. comm., 25 October 2017). In our opinion, *D. alpinum* is very likely a senior synonym of *A. tenue*, **comb. nov.**, but due to the incompleteness of its description should be considered a *nomen dubium*.

Phylogeny and taxonomic structure of the Itaquasconinae, with comments on the relevance of some morphological characters

Comparison of the phylogenetic tree obtained in our analysis with the most recent publications (Gąsiorek & Michalczyk, 2020; Tumanov, 2020; Zawierucha et al., 2023) revealed the evident similarities in general topology of the clades.

For the first time, we included in the analysis some recently described as well as yet undescribed taxa: *Diphascon wuyingensis*, *Meplitumen* sp., *Astatumen* sp. and *Astatumen*(?) sp.

Diphascon wuyingensis is recently described from China (Sun et al., 2020). It is morphologically similar to *Arctodiphascon tenue*, **comb. nov.** in having three elongate macroplacoids with no microplacoids or septula and a drop-like apodeme of the bucco-pharyngeal tube. Our results revealed that *D. wuyingensis* belongs to the subfamily Itaquasconinae but not to the same clade as *A. tenue*, **comb. nov.** *Diphascon wuyingensis*

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* This statement is evidently incorrect, because the Murray's description of *D. alpinum* meets the criteria of availability according to the Code (ICZN, 1999).
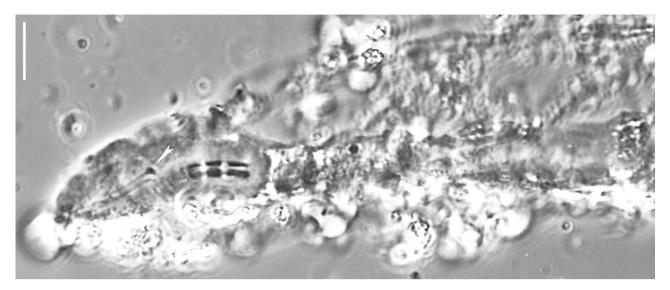
Fig. 4. *Arctodiphascon tenue* (Thulin, 1928), **comb. nov.**, syntype (ZMUC-TAR-774). White arrowhead indicates dorsal drop-like apodeme of bucco-pharyngeal tube (PhC). Scale bar: 10 μm.

forms a moderately supported monophyletic clade with the genus *Guidettion*. Unfortunately, only the 18S rRNA sequence of this species was available for the analysis, so the result obtained cannot be regarded as final. Anyway, this species clearly belongs to the Itaquasconinae and represents the second case of presence of the drop-like apodeme of the bucco-pharyngeal tube within this clade (see discussion below). In our opinion, this species should undoubtedly be excluded from the genus *Diphascon* as belonging to a different phylogenetic lineage and is here provisionally assigned to the genus *Arctodiphascon* **gen. nov.** as *A. wuyingensis*, **comb. nov.** If future phylogenetic analysis of this species including additional gene sequences supports its separate position from *A. tenue*, a new genus should be established for *A. wuyingensis*, **comb. nov.**

The genus *Meplitumen* was established for the species *M. aluna* Lisi et al., 2019 described from Colombia (Lisi et al., 2019). It is similar to the genus *Platicrista* in most of morphological characters except for the distribution of annulation of the bucco-pharyngeal tube. In *Platicrista* the annulation is present from the insertion point of the stylet supports to the caudal end of the pharyngeal tube, while in *Meplitumen* incomplete annulation is visible on the rigid buccal tube anterior to the insertion point of the stylet supports (Lisi et al., 2019; Massa et al., 2021). From the time of the

description of *Meplitumen* until now, no molecular data were available for the genus. We obtained the sequences of both 18S and 28S rRNA gene fragments for the undescribed tardigrade species that demonstrates a complete set of characters typical for the genus *Meplitumen*: the presence of the annulation of the buccal tube anterior to the stylet supports insertion points, non-swollen apices of the stylet furcae, and caudal processes of AISM pointed laterally (Fig. 7A). In the phylogeny obtained, this *Meplitumen*-like species clearly falls within the genus *Platicrista*, being a sister taxon to *Platicrista* aff. *angustata* from Norway (Fig. 6). We carried out a thorough investigation of the buccal tube structure of this species and revealed that the pattern described as "annulation" present anterior to the stylet support insertion point is rather a system of parallel grooves on the external surfaces of dorsal and ventral zones of thickened wall of the buccal tube (Fig. 7B). Moreover, a comparative examination of a specimen of *Platicrista* aff. *angustata* from Spitsbergen revealed the presence of the same structure on the buccal tube wall, but it was extremely poorly visible (Fig. 7C, D). Thus, instead of the distinct differences in the buccal tube structure between the genera *Meplitumen* and *Platicrista*, a gradual transition between the states of a binary character is shown. No other discriminating morphological characters are available for these genera. Taking
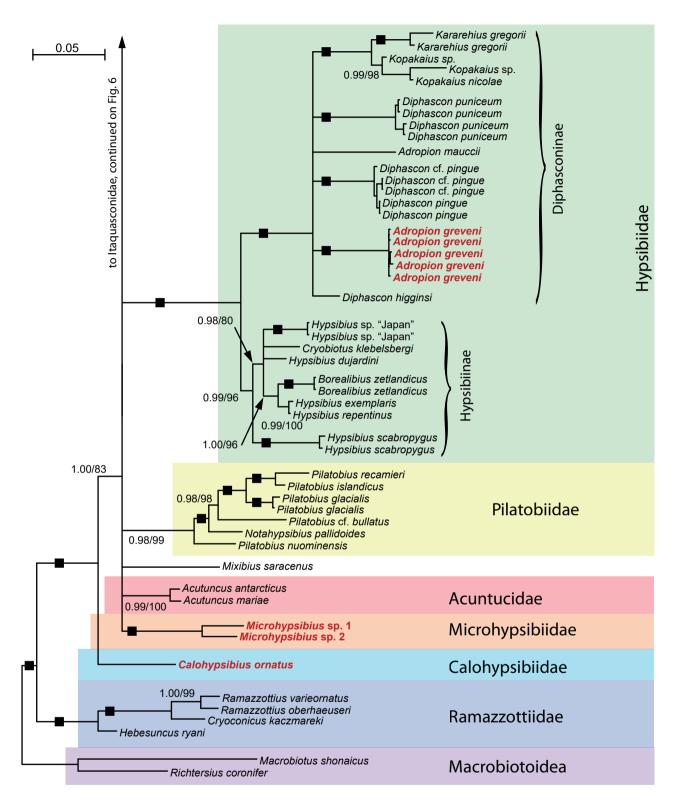
Fig. 5. Phylogeny of Hypsibioidea based on concatenated 18S + 28S rRNA sequences. Numbers at nodes indicate Bayesian posterior probability values (BI, first values) and bootstrap values (ML, second values). Black squares indicate the nodes supported by values of 1.0/100% with both methods. Branches with support below 0.9 in BI (70% in ML) were collapsed. Scale bar and branch lengths refer to the Bayesian analysis. Specimens with sequences produced in this study are given in red.
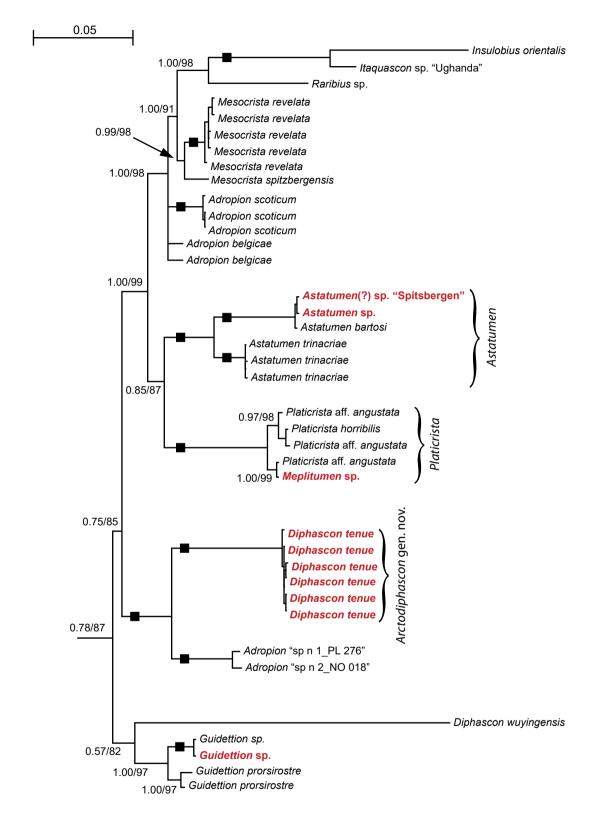
Fig. 6. Phylogeny of Itaquasconidae based on concatenated 18S + 28S rRNA sequences. Numbers at nodes indicate Bayesian posterior probability values (BI, first values) and bootstrap values (ML, second values). Black squares indicate the nodes supported by values of 1.0/100% with both methods. Branches with support below 0.9 in BI (70% in ML) were collapsed. Scale bar and branch lengths refer to the Bayesian analysis. Specimens with sequences produced in this study are given in red.

into account the close genetic similarity of the *Meplitumen*-like species to the genus *Platicrista*, we consider the generic name *Meplitumen* to be a junior synonym of the generic name *Platicrista* (**syn. nov.**). Two species are currently attributed to the genus *Meplitumen*: *M. aluna* (type species) and *M. itaquasconoide* (Durante Pasa et Maucci, 1975) recently transferred to this genus by Massa et al. (2021). The new designation for the first species should be *Platicrista aluna*, **comb. nov.** and the second species should be transferred back as *Platicrista itaquasconoide*, **stat. resurr.**

The current diagnosis of the genus *Astatumen* (Pilato, 1997; Pilato & Binda, 2010) postulates three key characters: (1) pharynx lacking apophyses and placoids or provided with a long, undivided placoid, (2) stylet supports absent, and (3) rigid part of the bucco-pharyngeal tube reduced to the most anterior region bearing the AISM. Species with the same configuration of pharyngeal structures, but with stylet supports present and with the longer rigid part of the bucco-pharyngeal tube are assigned to the genus *Itaquascon*. Until now, all species currently attributed to the *Astatumen / Itaquascon* complex clearly matched one of these character sets, so that the morphological gap between these two genera was evident. The few exceptions were the inadequately described old species: *Itaquascon enckelli* (Mihelčič, 1971), *I. simplex* (Mihelčič, 1971) and *I. umbellinae* de Barros, 1939. The situation is also unclear for *I. placophorum* Maucci, 1973, as the available reports on the re-examination of the type material contradict each other (Guidetti et al., 1999; Tibbs et al., 2016; Massa et al., 2021). These four species urgently require complete redescriptions based on the material from the type localities in order to include them in the analysis.

During our investigation of the Spitsbergen fauna, we found a single tardigrade specimen with a unique combination of characters of the bucco-pharyngeal apparatus, demonstrating the absence of stylet supports, strongly reduced stylet furcae (*Astatumen* characters) and a long rigid buccal tube (*Itaquascon* character) simultaneously (Fig. 7E, F). We were able to obtain the fragments of both the 18S and 28S rRNA gene sequences for this specimen and in our phylogenetic tree this species [indicated here as *Astatumen*(?) sp.] clear-ly falls within the genus *Astatumen*, being a sister taxon to the typical *Astatumen* sp. and closely related to *A. bartosi* (Fig. 6). The presence of a form with such combination of morphological and molecular characters raises the question of the boundaries of the genus *Astatumen*. Taking into account the case of *Meplitumen / Platicrista* (see discussion above), where the annulation-like pattern is proved to be present or absent in the anterior part of the bucco-pharyngeal tube in closely related species, we can assume that the *Astatumen* diagnosis needs to be widened to incorporate the forms with a long rigid buccal tube. Here we provide the emended diagnosis of this genus in order to facilitate identification by other researchers in the future:

Genus *Astatumen* Pilato, 1997

Diagnosis (*emended*). Claws of *Hypsibius*-type; bucco-pharyngeal tube subdivided into anterior, rigid portion (buccal tube) and posterior, flexible portion (pharyngeal tube) provided with spiral thickening. Stylet supports absent. Apophyses for insertion of muscles of stylets in shape of "wide and flat ridge" symmetrical with respect to frontal plane; caudal processes of those apophyses thin and pointing laterally. Pharyngeal bulb lacking apophyses and placoids or provided with a long, undivided placoid. Peribuccal lamellae and peribuccal papulae absent. Furcae of stylets reduced; their branches short and tapering at their apices. No cuticular thickening present between buccal tube and pharyngeal tube. Lunules absent. Eggs smooth, laid in exuviae.

Phylogenetic position of *Adropion greveni* and composition of the genus *Diphascon*

Our phylogenetic analysis revealed the presence of a fully supported clade that incorporates all currently investigated genera of Diphasconinae (*Diphascon*, *Kararehius* and *Kopakaius*) together with some species currently belonging to the genus *Adropion*: *A. greveni* and *A. mauccii* (Dastych et McInnes, 1996) (Fig. 5). Within this monophyletic clade, six fully supported subclades with poorly resolved relationships were revealed: a clade comprising the genera *Kararehius* and *Kopakaius*, a clade comprising two species of the *Diphascon pingue* group (*D. pinque* and *D. cf.*
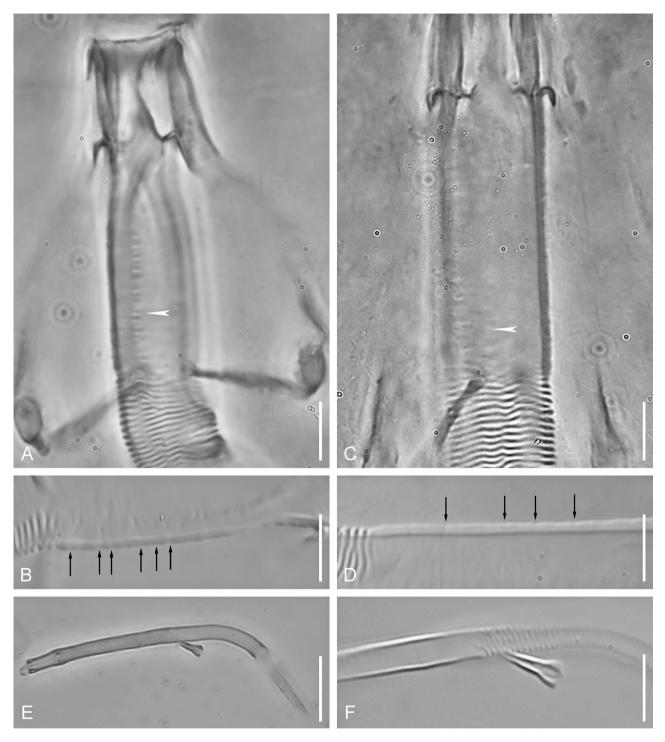
Fig. 7. Buccal tube of different taxa of Itaquasconinae. **A**, **B**, *Meplitumen* sp.; **C**, **D**, *Platicrista* aff. *angustata* (Murray, 1905); **E**, **F**, *Astatumen*(?) sp. **A**, **C**, buccal tube, white arrowhead indicates thickened zone on dorsal side (PhC); **B**, **D**, optical section of buccal tube wall in thickened zone on ventral side, black arrows indicate the grooves in tube wall (DIC); **E**, total view of bucco-pharyngeal apparatus laterally (PhC); **F**, transition zone between the rigid buccal tube and flexible pharyngeal tube with *Astatumen*-type spoon-like stylet furca visible (DIC). Scale bars: A–D, F – 5 μm, E – 10 μm.
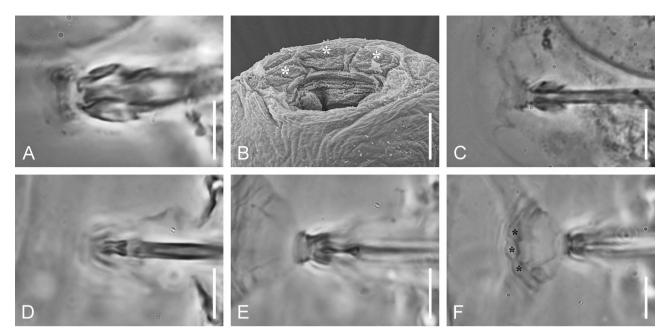
Fig. 8. Anterior part of bucco-pharyngeal apparatus of different species of *Diphascon* (subfamily Diphasconinae). **A**, **B**, *Diphascon greveni* Dastych, 1984, **stat. resurr.**; **C**, *D.* cf. *pingue* Marcus, 1936; **D–F**, *D. sanae* Dastych, Ryan et Watkins, 1990. **A**, **C**, lateral view of the AISM (PhC); **B**, **F**, mouth opening, asterisks indicate the peribuccal papulae (SEM); **D**, dorsal AISM (PhC); **E**, ventral AISM (PhC). Scale bars: A–F – 5 µm.

pinque), *D. puniceum* (Jennings, 1976), *D. higginsi* Binda, 1971, *Adropion greveni* and *A. mauccii.*

It is interesting to note that most species within the clade (except for *D. pingue* group) do not have a developed dorsal apodeme on the bucco-pharyngeal tube: this structure is either absent or extremely poorly developed. This is in contrast with the current diagnosis of the subfamily Diphasconinae (Bertolani et al., 2014) which specifically states that the dorsal apodeme is present. Such an ambiguity was often a source of taxonomic difficulties in the past (see Bertolani, 1982; Dastych, 1984, 1988; Dastych et al., 1990; Pilato & Binda, 1988, Bertolani et al., 2014; Zawierucha et al., 2023). A thorough investigation of all available species belonging to this complex (*A. greveni* and *Diphascon sanae* closely related to *D. puniceum*) and an analysis of the published descriptions (Dastych, 1984; Dastych et al., 1990) revealed some further differences from the "typical" *Diphascon* morphotype. In both of these species the AISM are asymmetrical with respect to the frontal plane, with the dorsal apophyses being distinctly higher, shorter and thicker than the ventral ones (Fig. 8A, D, E; see also Dastych, 1984: p. 415, fig. 23 for *D. puniceus*, and Dastych et al., 1990: p. 64, fig. 11 for *D. sanae*).

Species of this complex also seem to have peribuccal papulae, which are discernible in SEM images of *A. greveni* (Fig. 8B, asterisks) and are probably present in *D. sanae* (Fig. 8F, asterisks). Such a combination of characters clearly distinguishes this species complex from the genus *Diphascon*, which is defined by the symmetrical AISM (Pilato, 1987; Pilato & Binda, 2010; Fig. 8C) and the absence of peribuccal papulae. On the other hand, these characters (asymmetrical AISM and presence of the peribuccal papulae) seem to be plesiomorphic at least within the Hypsibioidea, being widely distributed within the clade and therefore cannot be used to unite these taxa. The overall organisation of the bucco-pharyngeal apparatus is also very different in the *D. puniceus / D. sanae* complex and *A. greveni*. Taking into account the unresolved phylogenetic relationships between the clades within the Diphasconinae, we can assume the paraphyletic nature of the genus *Diphascon* in its current composition.

Following the results of the phylogenetic analysis, we propose to transfer *Adropion greveni* and *A. mauccii* to the genus *Diphascon* as *D. greveni* Dastych, 1984, **stat. resurr.** and *D. mauccii* Dastych et McInnes, 1996, **stat. resurr.**

Phylogenetic analysis of Hypsibiinae

Recently, an undescribed species attributed to the genus *Hypsibius* Ehrenberg, 1848 from Mt. Gassan (Japan) was recognised as representing a new, sister lineage to all other species of Hypsibiinae (Ono et al., 2022). Our analysis did not confirm this result. In the obtained phylogenetic tree (Fig. 5), this species (designated here as *Hypsibius* sp. "Japan") was positioned close to *Cryobiotus klebelsbergi* (Mihelčič, 1959), albeit with weak support. The close affinity of *H.* sp. "Japan" to the genus *Cryobiotus* Dastych, 2019 is also supported by the evident similarity in the AISM morphology (see Ono et al., 2022, p. 11, fig. 8a, insert, and Dastych, 2019, p. 103, fig. 15). In lateral view, both taxa have a low hook-like AISM with a sharp caudal process separated from the anterior ridge-like part by a saddle-like depression. It is interesting to note the ecological similarity of these taxa: all members of *Cryobiotus* are obligate glacier dwellers, while *H.* sp. "Japan" was found in the snow algae blooming area in the mountains.

In our cladogram (Fig. 5), a well-supported monophyletic clade comprising members of the genera *Hypsibius*, *Cryobiotus* and *Borealibius* Pilato, Guidetti, Rebecchi, Lisi, Hansen et Bertolani, 2006 consists of two subclades. The first is the *Hypsibius scabropygus* Cuénot, 1929 subclade, which is a sister group to the well-supported complex of *Hypsibius*, *Cryobiotus* and *Borealibius* species. Within this complex, the group of *Borealibius zetlandicus* (Murray, 1907) + (*Hypsibius exemplaris* Gąsiorek, Stec, Morek et Michalczyk, 2018 + *H. repentinus* Tumanov et Avdeeva, 2021) forms a well-supported subclade, while the relationships between all other taxa [*Cryobiotus klebelsbergi*, *Hypsibius dujardini* (Doyère, 1840), and *H.* sp. "Japan"] remain poorly resolved (Fig. 5).

Thus, the results of our phylogenetic analysis confirm the paraphyletic nature of the genus *Hypsibius* in its current composition. The genera *Cryobiotus* and *Borealibius* do not represent separate evolutionary lineages within the Hypsibiinae, but seem to be specialised apical groups within the *Hypsibius* clade. A similar situation was recently revealed for the genus *Xerobiotus* Bertolani et Biserov, 1996 (Macrobiotoidea, Macrobiotidae), which was proved to be a specialised morphological group within the monophyletic genus *Macrobiotus* (Stec et al., 2021). In our opinion, the future integrative revision of the Hypsibiinae clade may lead to a change in the taxonomic status of both the genera *Cryobiotus* and *Borealibius*, but taking into account the evident scarcity of the molecular data for the genus *Hypsibius* we prefer not to change their status now.

Evolution of morphological characters within Hypsibioidea and the taxonomic structure of the group

Since Pilato's (1987) revision of the genus *Diphascon*, two morphological characters have been routinely used in the taxonomy of Hypsibiidae: (1) the presence or absence of the flexible annulated pharyngeal tube and (2) the presence or absence of the dorsal drop-like apodeme on the bucco-pharyngeal tube. Phylogenetic reconstructions using molecular data (Sands et al., 2008; Bertolani et al., 2014; Guil et al., 2019; Gąsiorek & Michalczyk, 2020; Zawierucha et al., 2023; present investigation) revealed that none of these characters can be considered exclusive to any of the large phylogenetic clades of Hypsibiidae.

Within Itaquasconinae, most species lack a drop-like apodeme on the bucco-pharyngeal tube, but this structure is present in the genus *Arctodiphascon* **gen. nov.** In most cases, species of this group have the flexible annulated pharyngeal tube, but in the genera *Parascon* Pilato et Binda, 1987 and *Sarascon* Guil, Rodrigo et Machordom, 2015 the pharyngeal tube is absent. Within the Pilatobiinae, two morphotypes of the bucco-pharyngeal apparatus are known: (1) with a long flexible annulated pharyngeal tube and a drop-like apodeme (the genus *Pilatobius*) and (2) with an undivided rigid buccal tube without a drop-like apodeme (the genus *Notahypsibius* Tumanov, 2020). The third large clade comprises two subfamilies, Hypsibiinae and Diphasconinae, and includes forms with a flexible annulated pharyngeal tube bearing a drop-like apodeme (the genus *Diphascon*) or without it (the genera *Diphascon*, *Kararehius* and *Kopakaius*), together with species that have an undivided rigid buccal tube (the genera *Borealibius*, *Cryobiotus* and *Hypsibius*).

Evidently, none of the large phylogenetic clades of Hypsibiidae can currently receive a comprehensive morphological diagnosis, moreover it is not

possible to give a morphological diagnosis for the family Hypsibiidae itself, as there are no characters or sets of characters that can be considered as characterising exclusively this group. Even the claw morphology is much more variable than what can be described by the term *"Hypsibius*-type claw" (e.g. *Ramazzottius*-like claws of *Notahypsibius*; Tumanov, 2020).

In this situation, molecular phylogenetic analysis provides the only reliable basis for the modern taxonomy of the Hypsibioidea clade. We propose that instead of adhering to the current megadiverse family Hypsibiidae (whose monophyly is poorly supported in our analysis), each of the large well-supported subclades should be elevated to family rank. Thus, the clades Pilatobiinae and Itaquasconinae form the families Pilatobiidae Bertolani et al., 2014, **status promotus**, and Itaquasconidae Bartoš in Rudescu, 1964, **status promotus**. The subfamilies Diphasconinae and Hypsibiinae form a fully supported monophyletic clade that should be accepted as the family Hypsibiidae Pilato, 1969 with two well-delimited subfamilies, Hypsibiinae Pilato, 1969 and Diphasconinae Dastych, 1992.

Addenda

Electronic supplementary material 1. Primers and PCR programs used for amplification of four DNA fragments sequenced in the study. File format: PDF.

Electronic supplementary material 2. Complete list of sequences used in the molecular phylogenetic analysis. File format: PDF.

Electronic supplementary material 3. Final alignment used for the phylogenetic analyses. File format: FST, as 7Z archive.

Electronic supplementary material 4. Matrices of *p*-distances for species of Hypsibioidea. File format: XLSX.

All these materials are available from: https://doi.org/10.31610/zsr/2023.32.1.50

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