

Neotropical jewels in the moss: biodiversity, distribution and evolution of the genus *Barbaria* (Heterotardigrada: Echiniscidae)

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The genus *Barbaria*, recently established to accommodate the former *Echiniscus bigranulatus* group, is a tardigrade group emblematic for the South American tardigrade fauna. This unappendaged echiniscid lineage is widely recognized for the so-called ‘double’ sculpturing composed of endocuticular pillars and pseudopores or pores in the dorsal cuticle. The phylogenetic relationships in the genus have so far been completely unknown, but the discovery of two new species (*B. paucigranulata* sp. nov. and *B. weglarskae* sp. nov.), together with new genetic data for further six species (*B. bigranulata*, *B. charrua* comb. nov., *B. danieli*, *B. jenningsi*, *B. madonnae* and *B. ollantaytamboensis*), create an opportunity not only to uncover phyletic relationships, but also to reconstruct morphological evolution in the genus. To achieve this, we sequenced five genetic markers (18S rRNA, 28S rRNA, ITS1, ITS2, *COI*) for multiple populations of eight species of *Barbaria* (two-thirds of all known species) collected in Alabama (USA), Argentina and the Antarctic, and we analysed them in tandem with detailed morphological data. Our phylogenetic analysis and the reconstruction of evolution of morphological traits suggests that the ancestor of the genus inhabited the Neotropics, and it was morphologically most similar to *B. bigranulata*. We also analyse literature records of *Barbaria* and conclude that the genus is most likely limited to the Neotropics, Antarctica and southern parts of the Nearctic. The findings are discussed in the context of the phylogeny of the *Echiniscus* evolutionary line.

ADDITIONAL KEYWORDS: cuticle – integrative taxonomy – morphometry – phylogeny – species delineation – tardigrades – trait evolution.

INTRODUCTION

The phylum Tardigrada comprises over 1350 species described to date (Degma *et al.*, 2009–21). Three main morphotypes can be distinguished within this metazoan group: (1) soft-bodied, unappendaged and generally limnoterrestrial eutardigrades (Bertolani *et al.*, 2014); (2) soft-bodied, appendaged and marine arthrotardigrades (Fontoura *et al.*, 2017); and (3) usually plated, mostly appendaged and generally limnoterrestrial echiniscoideans (Kristensen, 1987).

The remarkably sclerotized dorsum (and sometimes also the venter) covered with richly ornamented plates is a distinguishing feature of the largest echiniscoidean family, the Echiniscidae (Thulin, 1928; Kristensen, 1987), which inhabit all continents, from the tropical rainforests to the polar regions. Many echiniscid genera are cosmopolitan (e.g. *Bryodelphax* Thulin, 1928, *Echiniscus* Schultze, 1840, *Hypechiniscus* Thulin, 1928 or *Pseudechiniscus* Thulin, 1911), but numerous cases of distributions restricted to particular continents or climate zones are also known (e.g. Western Palaearctic *Parechiniscus* Cuénot, 1926 or tropical *Kristenseniscus* Gašiorek *et al.*, 2019). One of the genera exhibiting a limited geographic range is *Barbaria* Michalczyk *et al.*, 2019 (Gašiorek *et al.*, 2019). The vast majority of species in this genus have been recorded in the Neotropics (see Michalczyk & Kaczmarek, 2006, 2007 for the most

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comprehensive summaries), with only two species known from adjacent zoogeographic realms: *Barbaria jenningsi* (Dastych, 1984) in Antarctica and the neighbouring sub-Antarctic archipelagos (e.g. Dastych, 1984; McInnes, 1995), and *Barbaria danieli* (Meyer et al., 2017) in the southern Nearctic.

The first species of *Barbaria* was described more than a hundred years ago as *Echiniscus bigranulatus* (Richters, 1908). The specific epithet, referring to two types of granulation, is probably an unfortunate result of misinterpretation of the dorsal plate structure. The light and electron microscopy analyses in the first revision of *Barbaria* (the *Echiniscus bigranulatus* group then) showed that the dorsal plates of *B. bigranulata* do not exhibit any cuticular granulation (Michalczyk & Kaczmarek, 2006). What Richters (1908) interpreted as two types of granules and what has been termed as ‘double sculpture’, ‘bigranular sculpture’ or ‘double granulation’ are, in fact, endocuticular pillars (appearing, similarly to epicuticular granules, as dark dots in a light microscope, hence sometimes they are referred to as ‘pseudogranulation’; Michalczyk & Kaczmarek, 2006) and cuticular pores (visible as bright dots in a light microscope). Confusing the intra- and epicuticular elements of armour and the limited original description led to many false reports of *Barbaria bigranulata* throughout the world. Perhaps the best-known case is the New Zealand endemic echiniscid with eminent epicuticular granules and the endocuticular sponge layer or fine pillars, *Zealandiscus palmai* (Dastych, 1997), originally recorded as *Echiniscus bigranulatus* by Horning et al. (1978). In summary, when identifying echiniscids, some authors have interpreted the co-occurrence of epicuticular pores and endocuticular pillars as two types of granulation (e.g. Richters, 1908), some have understood this term as the co-occurrence of epicuticular granules and an endocuticular sponge layer or pillars (e.g. Horning et al., 1978), whereas others have correctly separated cuticular pores from endocuticular pillars and did not refer to them as ‘granulation’ (e.g. Ramazzotti, 1964). Currently, a wider spectrum of sculpturing variability is recognized in the genus, because there are species with fully developed pillars and pores [*Barbaria bigranulata*, *B. danieli*, *B. ollantaytamboensis* (Nickel et al., 2001), *B. ranzii* (Ramazzotti, 1964)], with pillars and pseudopores [*B. jenningsi*, *B. madonnae* (Michalczyk & Kaczmarek, 2006)], with poorly developed pillars and pores [*B. ganczareki* (Michalczyk & Kaczmarek, 2007)], and with plate portions devoid of pillars and pores [*B. hanna* (Roszkowska et al., 2019)].

Here, we present the broadest study to date that encompasses the phylogeny, morphological evolution and species delineation in *Barbaria*. We address these issues by DNA sequencing of five genetic markers, light microscopy analyses and statistical morphometry of numerous *Barbaria* populations from the Americas and

the Antarctic. One new species, *Barbaria paucigranulata* sp. nov., is described using minute sculpturing differences regarding its congeners, and the second new species, *Barbaria weglarskae* sp. nov., is established for echiniscids with peculiarly elongated cirri A. Conclusions related to the sculpturing evolution in the *Echiniscus* evolutionary line are drawn and discussed.

MATERIAL AND METHODS

SAMPLING AND DATA COLLECTION

Abundant populations of *Barbaria* were found in moss/lichen samples collected by Witold Morek and Bartłomiej Surmacz in Argentina, and samples provided by our collaborators from the USA and Antarctica (see Table 1 for populations used for DNA barcoding and Supporting Information, Material S1 for all examined populations from Argentina). Air-dried material was placed in paper envelopes and subsequently re-hydrated using tap or distilled water, vigorously shaken in beakers and the supernatant was transferred to measuring cylinders. Excess of water was first discarded, then sediments comprising bryophilous and lichenophilous animals, soil and plant particles were poured on to Petri dishes and searched for tardigrades using stereomicroscopes. Each specimen was drawn into a glass pipette and placed in distilled water. After extraction, the animals were divided into two groups destined for different analyses: (1) qualitative and quantitative morphology investigated with phase contrast microscopy (PCM) and (2) DNA sequencing. Due to the scarcity of specimens representing new species, only four specimens of *B. paucigranulata* (sample AR.303) were analysed in scanning electron microscopy (SEM). These animals were dried out in a CO₂ critical point, sputter-coated with gold and observed in Versa 3D DualBeam SEM at the ATOMIN facility of the Jagiellonian University.

COMPARATIVE MATERIAL AND TERMINOLOGY

We examined the following species that we loaned from various museums and universities: *Barbaria jenningsi* paratypes (King George Island, Antarctic; Dastych, 1984; University of Hamburg) and its additional representatives (Signy Island, Antarctic; McInnes, 1995; University of Hamburg), *B. madonnae* paratypes (Peru; Michalczyk & Kaczmarek, 2006; Jagiellonian University), *B. pseudowendti* (Dastych, 1984) paratypes (Enderby Land; Dastych, 1984; University of Hamburg), *B. ranzii* syntypes (Chile; Ramazzotti, 1964; Museo Civico di Storia Naturale in Verona and University of Modena and Reggio Emilia) and *Echiniscus quitensis* Pilato, 2007 topotypes (Ecuador, not examined by Pilato, 2007 in the species description, but coming from the same sample

Table 1. List of populations used in integrative analyses. Types of analyses: (PCM) imaging and morphometry in PCM, (DNA) DNA sequencing. Number in each analysis indicates how many specimens were utilised in a given method (f, females; m, males; a, adults of unidentified sex; j, juveniles; l, larvae)

Species	Sample code	Coordinates and altitude	Locality	Sample type	Collection date	Analyses	
						PCM	DNA
<i>Barbaria bigranulata</i>	AR.049	48°25'37"S 71°44'45"W 782 m asl	Argentina, Patagonia, Santa Cruz Province, vicinity of La Florida	lichen from tree trunk	18.02.2019	13f	8a
	AR.143	46°51'49"S 71°53'34"W 1169 m asl	Argentina, Patagonia, Santa Cruz Province, vicinity of Monte Zeballos	moss from rock	20.02.2019	2f	1a
	AR.144	46°51'49"S 71°53'34"W 1169 m asl	Argentina, Patagonia, Santa Cruz Province, vicinity of Monte Zeballos	lichen from rock	20.02.2019	3f	1a
<i>Barbaria charrua</i>	AR.325	26°52'9"S 65°25'31"W 861 m asl	Argentina, Tucumán Province, vicinity of San Miguel de Tucumán	moss + lichen from tree branch	01.03.2019	11f + 2j	5a
	AR.364	27°46'10"S 55°4'30"W 95 m asl	Argentina, vicinity of Río Uruguay	moss + lichen from tree branch	05.03.2019	11f	2a
	AR.447	26°17'3"S 53°46'30"W 741 m asl	Argentina, vicinity of Campina de América	lichen from tree trunk	06.03.2019	4f	2a
<i>Barbaria danieli</i>	US.086	33°12'51"N 87°34'17"W 43 m asl	USA, Alabama, Tuscaloosa, campus of the University of Alabama	lichen from rock boulder	17.02.2020	5f	4a
<i>Barbaria jenningsi</i>	AQ.033	62°4'43"S 58°25'29"W 2 m asl	Antarctica, Admiralty Bay, Mackellar Inlet, Keller Peninsula	moss from soil	20.12.2020	25f + 21m + 11j	10a
<i>Barbaria madonnae</i>	AR.184	44°10'22"S 71°33'58"W 696 m asl	Argentina, Patagonia, vicinity of Río Pico	lichen from rock	21.02.2019	30f + 4l	4a
<i>Barbaria ollantaytamboensis</i>	AR.292	24°47'17"S 65°43'28"W 2149 m asl	Argentina, Rosario de Lerma Department, Salta	lichen from rock	28.02.2019	11f + 8j + 6l	4a
	AR.303	24°47'14"S 65°43'30"W 2150 m asl	Argentina, Rosario de Lerma Department, Salta	lichen from rock	28.02.2019	8f + 1j	10a
<i>Barbaria paucigranulata</i>	AR.303	24°47'14"S 65°43'30"W 2150 m asl	Argentina, Rosario de Lerma Department, Salta	lichen from rock	28.02.2019	8f + 4j	3a
<i>Barbaria weglarskiae</i>	AR.059	48°25'42"S 71°44'48"W 803 m asl	Argentina, Patagonia, Santa Cruz Province, vicinity of La Florida	lichen from rock	18.02.2019	3f	2a

as the type series; Museo Civico di Storia Naturale in Verona and University of Modena and Reggio Emilia). The Ramazzotti and Maucci collections were inspected in order to verify species identifications present in literature (McInnes, 1994). In addition, unpublished microphotographs of the type series of *Barbaria danieli* (courtesy of Harry Meyer, McNeese State University, USA) were analysed, and microphotographs of the type series of *B. hanna*e (courtesy of Milena Roszkowska and Łukasz Kaczmarek, Adam Mickiewicz University, Poland).

Terminology for sclerotized structures follows Kristensen (1987). Primary spurs are placed on internal claws and are directed downwards; secondary spurs are on external claws and are directed upwards. The dorsal plate naming system is consistent with that of Jørgensen *et al.* (2011). Epicuticular pores are holes in the epicuticle and appear in PCM as bright dots with sharp edges, whereas epicuticular pseudopores are depressions in the epicuticle and appear in PCM as less bright dots with fuzzy edges (Michalczyk & Kaczmarek, 2006). The characterization of elements of dorsal plate sculpturing follows Gašiorek *et al.* (2019). The term ‘appendaged’ describes species with at least one pair of cuticular appendages on the trunk in any of the positions *B–E*, whereas the term ‘unappendaged’ describes species with cuticular appendages limited to the head (i.e. cephalic cirrus internus and externus and cirri in the position *A*; Gašiorek *et al.*, 2019). Claws are classified as homomorphic/isonych when claws and spurs I–III and IV are similar in size and shape (e.g. *B. pseudowendti*; Dastych, 1984), and as heteromorphic/heteronych when claws IV are typically more robust and with differently formed spurs compared to claws I–III (e.g. *B. jenningsi*; Dastych, 1984).

MICROSCOPY, IMAGING AND MORPHOMETRY

Specimens for light microscopy and morphometry were mounted in Hoyer’s medium and examined under Olympus BX53 light microscope with phase contrast (PCM), associated with an Olympus DP74

digital camera. All figures were assembled in Corel Photo-Paint X8. For deep structures that could not be fully focused on a single PCM photograph, a series of images were taken every *c.* 0.1 μm of vertical focusing and then assembled manually in Corel Photo-Paint into a single deep-focus image. Structures were measured only when oriented properly and not broken or deformed. Body length was measured from the anterior to the posterior end of the body, excluding the hind legs. The *sp* index is the ratio of the length of a given structure to the length of the scapular plate (Dastych, 1999). Morphometric data were handled using the Echiniscoidea v.1.3 template available from the Tardigrada Register, <http://tardigrada.net/register> (Michalczyk & Kaczmarek, 2013).

GENOTYPING AND PHYLOGENETICS

A Chelex 100 resin (Bio-Rad) method was used for DNA extraction (Casquet *et al.*, 2012; Stec *et al.*, 2020). Hologenophores (Pleijel *et al.*, 2008) were obtained after DNA extraction for all species, except for *B. weglarskae* **sp. nov.** Five DNA fragments were sequenced: the small ribosome subunit 18S rRNA, the large ribosome subunit 28S rRNA, the internal transcribed spacers ITS1 and ITS2 and the cytochrome *c* oxidase subunit I (*COI*). All fragments were amplified and sequenced according to the protocols described in Stec *et al.* (2020); primers and original references for specific PCR programmes are listed in Supporting Information, Material S2. GenBank accession numbers for all utilized species with fully complementary marker datasets are provided in Table 2. All markers were aligned with sequences from *Diploechiniscus oihonnae* (Richters, 1903) and *Testechiniscus spitsbergensis tropicalis* Gašiorek *et al.* 2018 as outgroups (Supporting Information, Material S2), using the Q-INS-i strategy in MAFFT v.7 (Katoh *et al.*, 2002; Katoh & Toh, 2008) or ClustalW Multiple Alignment tool (Thompson *et al.*, 1994) implemented in BioEdit (Hall, 1997) for both ITS markers. The aligned fragments were edited and checked manually

Table 2. GenBank accession numbers for the *Barbaria* spp. sequenced in this work

Species	18S rRNA	28S rRNA	ITS1	ITS2	<i>COI</i>
<i>B. bigranulata</i>	MZ820792–5	MZ820810–3	MZ820828–31	MZ822376–9	MZ820846–9
<i>B. charrua</i> comb. nov.	MZ820796–9	MZ820814–7	MZ820832–5	MZ822380–3	MZ820850–2
<i>B. danieli</i>	MZ820800	MZ820818	MZ820836	MZ822384	MZ820853
<i>B. jenningsi</i>	MZ820801–2	MZ820819–20	MZ820837–8	MZ822385–6	–
<i>B. madonnae</i>	MZ820803	MZ820821	MZ820839	MZ822387	MZ820854
<i>B. ollantaytamboensis</i>	MZ820804–7	MZ820822–5	MZ820840–3	MZ822388–91	MZ820855–8
<i>B. paucigranulata</i> sp. nov.	MZ820808	MZ820826	MZ820844	MZ822392	MZ820859
<i>B. weglarskae</i> sp. nov.	MZ820809	MZ820827	MZ820845	MZ822393	MZ820860

in BioEdit with remaining gaps left intact. Beside the newly sequenced species, two previously published *COI* sequences (HM193406 labelled as *Barbaria bigranulata* in Jørgensen *et al.*, 2011 and KP013596 identified as *B. jenningsi* in Velasco-Castrillón *et al.*, 2015) were used to calculate uncorrected pairwise distances in MEGA7 (Kumar *et al.*, 2016).

The sequences were concatenated to generate a matrix of 3372 bp in SequenceMatrix (Vaidya *et al.*, 2011). Using PartitionFinder v.2.1.1 (Lanfear *et al.*, 2016) with applied Bayesian information criterion (BIC) and greedy algorithm (Lanfear *et al.*, 2012), the best substitution model and partitioning scheme was chosen for posterior phylogenetic analysis. As the best-fit partitioning scheme, PartitionFinder suggested six partitions characterized by various evolution models (I: 18S rRNA + 28S rRNA – GTR+I+G; II: ITS1 – GTR+G; III: ITS2 – TVM+G; IV: the first coding position of *COI* – GTR+G; V: the second coding position of *COI* – TVM; VI: the third coding position of *COI* – HKY+G). Bayesian inference (BI) marginal posterior probabilities were first calculated using MrBayes v.3.2 (Ronquist & Huelsenbeck, 2003). Random starting trees were used, and the analysis was run for 10 million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of < 0.01 was used as a guide to ensure that the two independent analyses had converged. TRACER v.1.3 (Rambaut *et al.*, 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct ‘burn-in’ for the analysis i.e. the first 10% of generations. The effective sample size values were greater than 200 and the consensus tree was obtained after summarizing the resulting topologies and discarding the ‘burn-in’.

ModelFinder (Kalyaanamoorthy *et al.*, 2017) was used to choose the best-fit models in maximum likelihood (ML): I: 18S rRNA + 28S rRNA – K2P+I; II: ITS1 – TIM2e+G4; III: ITS2 – TPM2u+F+G4; IV: the first coding position of *COI* – TN+F+G4; V: the second coding position of *COI* – F81+F; VI: the third coding position of *COI* – HKY+F+G4, chosen according to the Bayesian information criterion. W-IQ-TREE was used for ML reconstruction (Nguyen *et al.*, 2015; Trifinopoulos *et al.*, 2016). A thousand ultrafast bootstrap (UFBoot) replicates were applied to provide support values for branches (Hoang *et al.*, 2018).

MORPHOLOGICAL EVOLUTION

A set of Bayesian phylogenetic trees needed for ancestral state reconstruction analyses was obtained using BEAST (Drummond & Rambaut, 2007). Four clock and tree prior combinations were chosen and

run in parallel: (1) random local clock (Drummond & Suchard, 2010) with the coalescent tree prior, (2) random local clock with the speciation: Yule process as the tree prior, (3) strict clock (Ferreira & Suchard, 2008) with the coalescent tree prior and (4) strict clock with the speciation: Yule process as the tree prior. Tree searches were run for 10 million generations, sampling a tree each 1000 steps. These trees were summarized with the TREEANNOTATOR software (distributed with BEAST) removing the first 1000 trees. TRACER v.1.3 (Rambaut *et al.*, 2014) was then used to ensure Markov chains had reached stationarity. The effective sample size values were greater than 200 and the consensus tree was obtained after summarizing the resulting topologies and discarding the ‘burn-in’. All final consensus trees were viewed and visualized using FigTree v.1.4.3 available from <https://tree.bio.ed.ac.uk/software/figtree>.

Consensus trees constructed according to combinations of parameters a–d, described in the previous paragraph, shared identical topologies, congruent with the MrBayes tree. A set of 1000 most credible trees was used in ancestral state reconstruction analyses in BayesTraits (Pagel *et al.*, 2004) implemented in RASP (Yu *et al.*, 2020) to include phylogenetic uncertainty in calculations. We chose to reconstruct the evolution of four key morphological traits used or potentially usable in the delineation of species of *Barbaria*: dorsal plate sculpturing, pedal plate I–III sculpturing (pedal plate IV is typically better developed in echiniscids than the remaining ones, thus more homogeneous between pairs of closely related species), the shape of papillae IV and claw isomorphy (claws homomorphic/isonych vs. heteromorphic/heteronych).

RESULTS

TAXONOMIC ACCOUNT

Species transfers

Gašiorek *et al.* (2019) transferred *Echiniscus pseudowendti* to *Barbaria*, because the dorsal sculpturing was characterized in the original description of the species by Dastych (1984) as ‘double’, which is a historical term used to describe the *bigranulata*-type sculpturing. However, our examination of *B. pseudowendti* paratypes revealed that the dorsal sculpturing is of the *wendti*-type [see Degma *et al.* (2021) for an amended definition of the morphotype], and the species is similar to *Claxtonia wendti* (Richters, 1903), as originally reported (Dastych, 1984). Therefore, here, we transfer the species to *Claxtonia* and designate it as ***Claxtonia pseudowendti* (Dastych, 1984) comb. nov.**

Based on drawings in the original description of *Echiniscus charrua* Claps & Rossi, 1997, Kaczmarek *et al.* (2015) hypothesized that the species could represent *Barbaria* (the *Echiniscus bigranulatus* group then). Finding numerous representatives of *E. charrua* in northern Argentina and subsequent genetic (Fig. 5) and morphological (Figs 7B, 8B, 9B, 10B) analyses in this contribution confirmed that it indeed belongs in *Barbaria*. Therefore, it is placed here in that genus as ***Barbaria charrua* (Claps & Rossi, 1997) comb. nov.**

Finally, since the dorsal sculpturing of *Echiniscus quitensis* is of the *Barbaria*-type (Figs 7J, 8J, 9J, 10J), it is also transferred to *Barbaria* as ***Barbaria quitensis* (Pilato, 2007) comb. nov.** The specimen that became the holotype of *B. quitensis* was first identified by Maucci as *Viridiscus rufoviridis* (du Bois-Reymond Marcus, 1944). Thus, Pilato (2007) hypothesized that *B. quitensis* could exhibit green pigment. However, the dorsal sculpturing of *B. quitensis* is clearly of the *bigranulata*-type and the pigment colour needs to be verified with live individuals.

Descriptions of new species

BARBARIA PAUCIGRANULATA WILAMOWSKI, VONČINA, GAŚIOREK & MICHALCZYK, SP. NOV.

(Figs 1–3, 7I, 8I, 9I, 10I, 11K, L; TABLES 3, 4)

Zoobank registration: urn:lsid:zoobank.org:act:A11E6BDF-7E2A-4F0A-BD6A-9BC87FCE147A

Morphometric data: www.tardigrada.net/register/0109.htm

Type material: Holotype (adult female, slide AR.303.02) and 12 paratypes (eight adult females on slides AR.302.02, AR.303.01, 3, 5–6 and four juveniles on slides AR.303.03–4). Found together with *B. ollantaytamboensis*.

Type locality: 24°47'14"S, 65°43'30"W, 2150 m asl: Argentina, Salta Province, Rosario de Lerma Department, vicinity of Río Rosario; lichen on rock in a shrubland (see also Table 1).

Etymology: From Latin *paucus*, few, and *granulatus*, grained, alluding to the scarcity of epicuticular granulation on the dorsal plates. An adjective in nominative singular.

Description: Adult females (i.e. from the third instar onwards, measurements in Table 3). Dark orange body with large red eyes; the pigment and eyes dissolve quickly after mounting in Hoyer's medium. Body massive with stumpy limbs (Figs 1A, B, 2). Cylindrical,

Echiniscus-type cephalic papillae (secondary clavae) and (primary) clavae; cirri embedded in bulbous cirrophores (Fig. 3D). Cirrus A is short (< 20% of the body length) and with evident, but small cirrophore (Fig. 1A, B).

Dorsal plate sculpturing of the *bigranulata*-type, comprising minute, poorly developed pillars (pseudogranulation) and pores (Figs 1C, 2, 3A–C, 7I, 8I, 9I, 10I). Pillars are densely packed and additionally interconnected by thin *striae* in the scapular (Fig. 7I) and caudal (terminal) plates (Fig. 10I). Pores are slightly larger in the scapular plate, median plate 1, posterior portion of median plate 2 and centroposterior portions of paired segmental plates compared with posterolateral portions of paired segmental plates and the caudal plate (Figs 1, 2); pores of similar diameter are rarely present in the entire dorsum. Pores are always absent in the anterior portion of median plate 2 (Fig. 8I), paired segmental plates (Fig. 9I) and the entirety of median plate 3; and all these areas are covered with epicuticular, multangular granules. Lateralmost portions of the scapular and paired segmental plates can be poreless or with single minute pores (Figs 1A, B, 2). The cephalic plate is large, with a pronounced chalice-shaped anterior incision and with only pillars in the posterior portion of the plate. A broad and strongly sclerotized cervical plate is divided into sculptured anterior portion and smooth posterior portion bordering with the scapular plate (Figs 1A, 2A). Lateral sutures in the scapular plate demarcate lateralmost, trapezoidal portions (Figs 1A, B, 2B). Median plates 1 and 3 are unipartite (the latter with strongly developed granules), median plate 2 is bipartite (Fig. 8I). Paired segmental plates are without transverse unsculptured bands, as epicuticular granules of anterior portion transition gradually into pillars of the posterior portions (Fig. 9I). The caudal plate with short, poorly sclerotized incisions and no signs of faceting (Figs 1A, 2, 10I). Ventral cuticle with minute endocuticular pillars (Fig. 11K) distributed evenly throughout the entire venter, lacking plates, beside of rarely developed subcephalic plates (Fig. 3D). Sexpartite gonopore placed anteriorly to a trilobed anus between legs IV.

Pedal plates without pores, their sculpturing consists of poorly developed endocuticular pillars formed as belts in the central portions of the legs (Figs 1A, B, 11K, L). Thick pulvini on outer side of all legs (Figs 1A, B, 2B). Dentate collar IV has numerous irregular short teeth (Fig. 11L). A small, elongated spine I and a tubby papilla IV (Figs 1B, 2B, 11K, L). Claws slightly heteronych with claws IV (Fig. 3F) higher and more robust than claws I–III (Fig. 3E). Internal claws IV have needle-like spurs

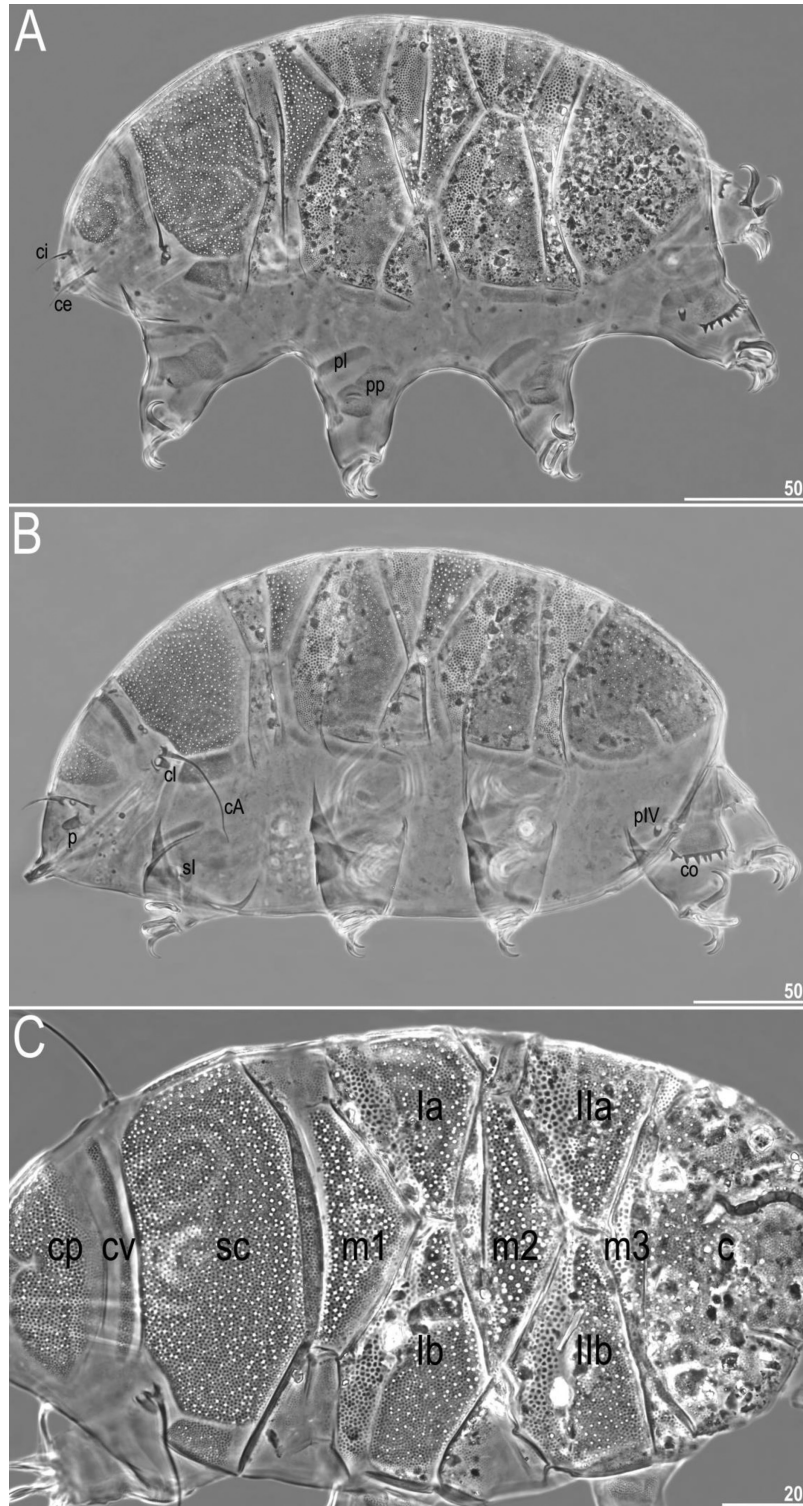


Figure 1. Habitus of *Barbaria paucigranulata* (PCM): A, holotype (female) in dorsolateral view; B, paratype (female) in lateral view; C, dorsal sculpturing in close-up. Abbreviations: Ia–IIb, paired segmental plates; c, caudal plate; cA, cirrus A; ce, cirrus externus; ci, cirrus internus; cl, (primary) clava; co, dentate collar IV; cp, cephalic plate; cv, cervical plate; m1–3, median plates; p, cephalic papilla (secondary clava); pIV, papilla IV; pl, pulvinus; pp, pedal plate; sl, spine I; sc, scapular plate. Scale bars in μm .

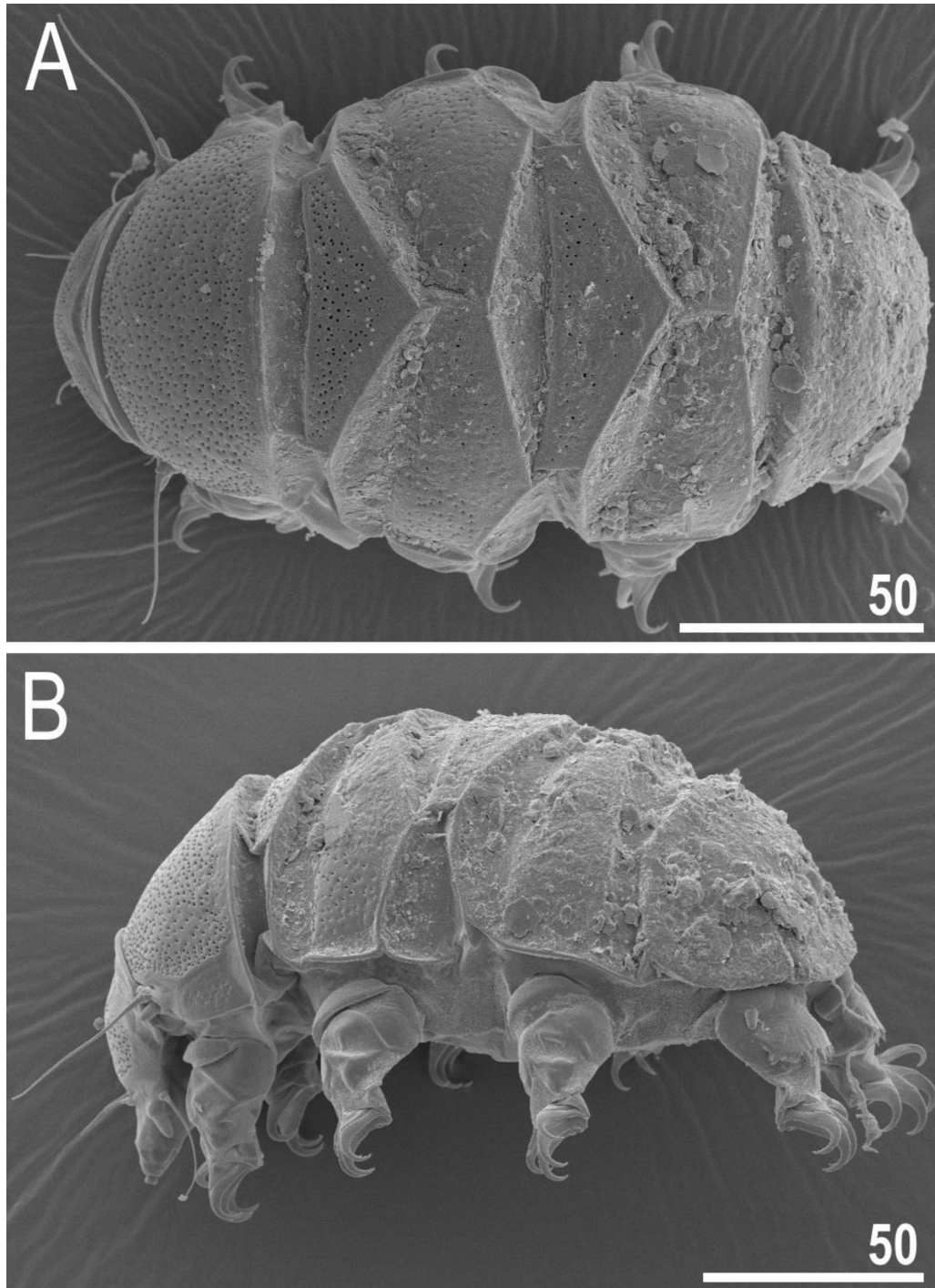


Figure 2. Habitus of *Barbaria paucigranulata* (SEM): A, paratype (sex undetermined) in dorsal view; B, paratype (female) in lateral view. Scale bars in μm .

more divergent from branches compared to spurs I–III (Fig. 11K, L). Cuticular bars below claw bases on the inner side of legs present. Buccal apparatus with a rigid tube and round pharynx containing placoids. Flexible stylet supports present.

Juveniles (i.e. the second instar, measurements in Table 4). Clear morphometric gap between juveniles and adult females. Qualitatively alike adult females, excluding the lack of gonopore.

Adult males, larvae or eggs not found.

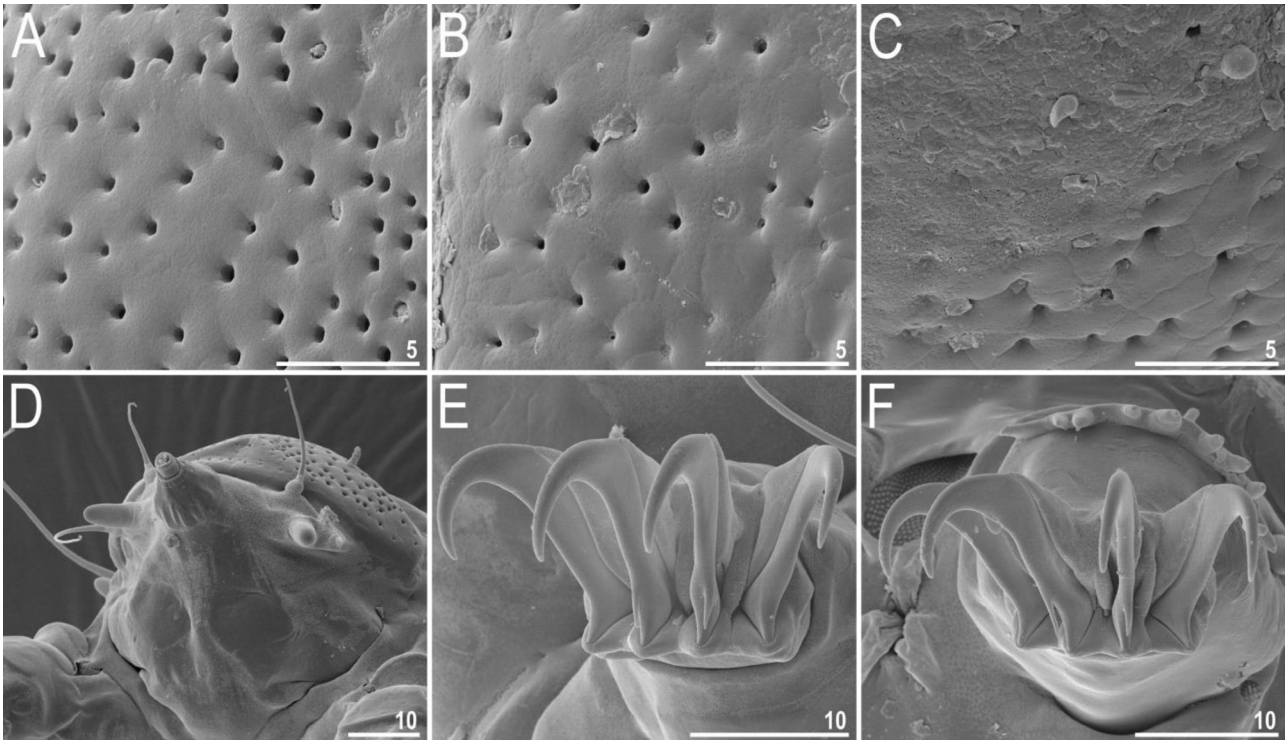


Figure 3. Details of *Barbaria paucigranulata* (SEM): A, pores in the scapular plate; B, pores in the posterior portion of the paired segmental plate II; C, pores in the caudal plate; D, cephalic appendages and a pair of subcephalic plates; E, claws I; F, claws IV. Scale bars in μm .

Differential diagnosis: The new species *B. paucigranulata* is distinguished from its congeners (alphabetically):

- *Barbaria bigranulata*, by the distribution of pores in plates [absent in the anterior portion of median plate 2 (Fig. 8I), paired segmental plates (Fig. 9I) and the entirety of median plate 3 in *B. paucigranulata* vs. present in the anterior portion of m2 (Fig. 8A), paired segmental plates (Fig. 9A) and the entirety of m3 in *B. bigranulata*], the shape of papilla IV [tubby in *B. paucigranulata* (Fig. 11L) vs. elongated in *B. bigranulata* (Fig. 11B)] and the primary spur morphology [needle-like and adjacent to the claw branch in *B. paucigranulata* (Fig. 11K, L) vs. robust, hook-shaped and divergent from the claw branch in *B. bigranulata* (Fig. 11A, B)].
- *Barbaria charrua*, by the shape of papilla IV [tubby in *B. paucigranulata* (Fig. 11L) vs. elongated in *B. charrua* (Fig. 11D)] and by the primary spurs [present in *B. paucigranulata* (Fig. 11K, L) vs. typically absent in *B. charrua* (Fig. 11C, D)].
- *Barbaria danieli*, by the shape of papilla IV [tubby in *B. paucigranulata* (Fig. 11L) vs. elongated in *B. danieli* (Fig. 12)] and by the primary spurs

[present in *B. paucigranulata* (Fig. 11K, L) vs. absent in *B. danieli* (Fig. 11O)].

- *Barbaria ganczareki*, by the dorsal sculpturing [both pillars and pores easily identifiable in *B. paucigranulata* (Figs 7I, 8I, 9I, 10I) vs. pillars so poorly developed that pores become the dominant element of the sculpture in *B. ganczareki* (Figs 7C, 8D, 9C, 10C)], the pores in the subcephalic region (absent in *B. paucigranulata* vs. present in *B. ganczareki*) and in pedal plates (absent in *B. paucigranulata* vs. present in *B. ganczareki*) and by the primary spur morphology [needle-like and adjacent to the claw branch in *B. paucigranulata* (Fig. 11K, L) vs. robust, hook-shaped and divergent from the claw branch in *B. ganczareki*, fig. 21–22 in Michalczyk & Kaczmarek (2007)].
- *Barbaria hanna*, by the dorsal sculpturing [no smooth plate portions in *B. paucigranulata* (Figs 8I, 9I, 10I) vs. thickened plate portions devoid of sculpturing present in *B. hanna* (Figs 8E, 9D, E, 10D, E)] and the primary spur morphology [needle-like and adjacent to the claw branch in *B. paucigranulata* (Fig. 11K, L) vs. robust, hook-shaped and divergent from the claw branch in *B. hanna* (Fig. 11P)].
- *Barbaria jenningsi*, by the cirrus A length (< 20% of the body length in *B. paucigranulata* vs. > 50% of the

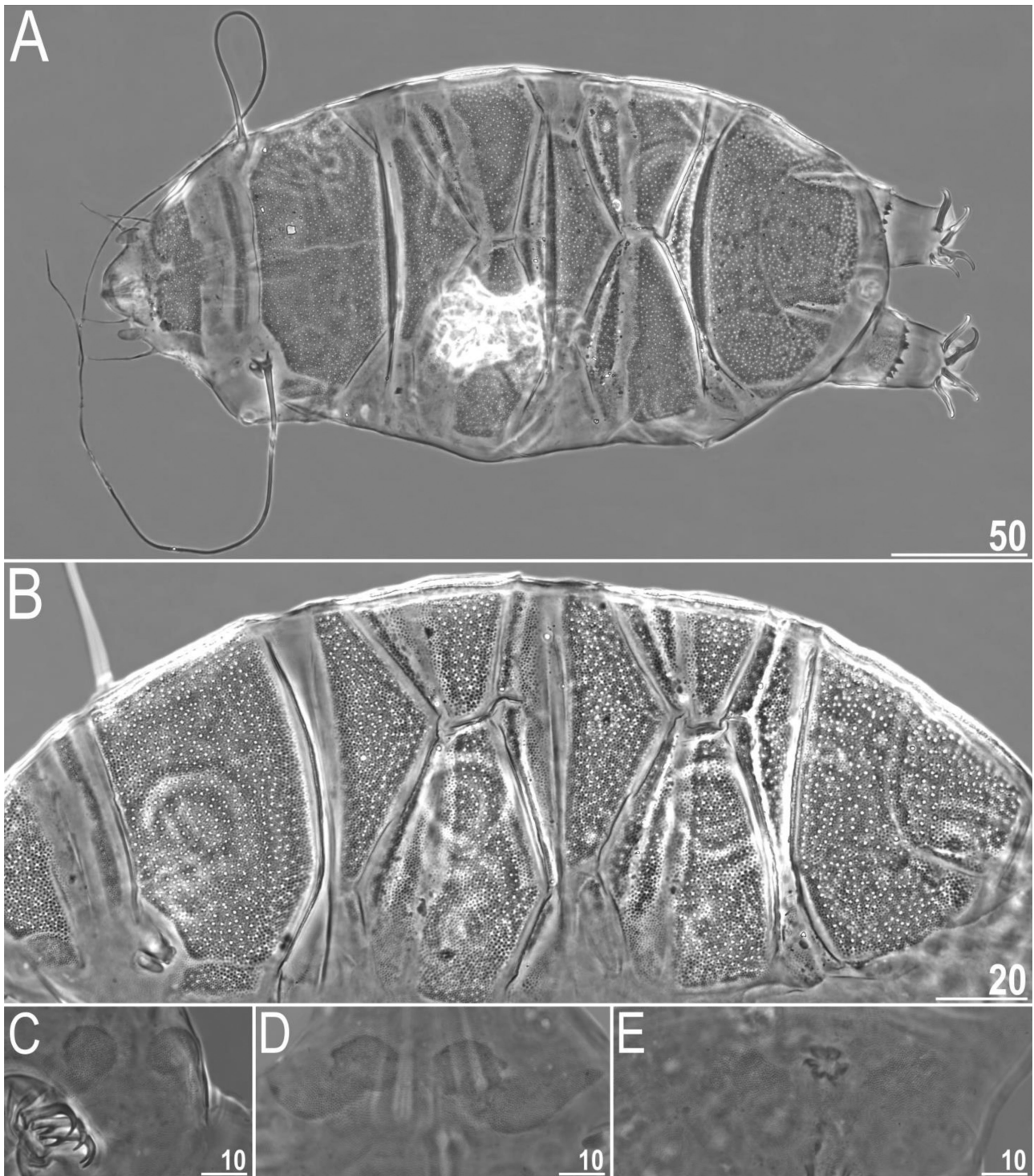


Figure 4. Habitus of *Barbaria weglarskae* (PCM): A, holotype (female) in dorsal view; B, dorsal sculpturing in close-up (paratype); C, subcephalic plates (paratype); D, subcephalic plates (holotype); E, gonoporal area (holotype). Scale bars in μm .

body length in *B. jenningsi*), the type of perforation in the dorsal plates [pores in *B. paucigranulata* (Fig. 7I) vs. pseudopores in *B. jenningsi* (Fig. 7E, F)] and by the primary spur morphology [slightly

heteronych, needle-like and adjacent to the claw branch in *B. paucigranulata* (Fig. 11K, L) vs. fully heteronych, robust, hook-shaped and divergent from the claw branch in *B. jenningsi* (Fig. 11E, F)].

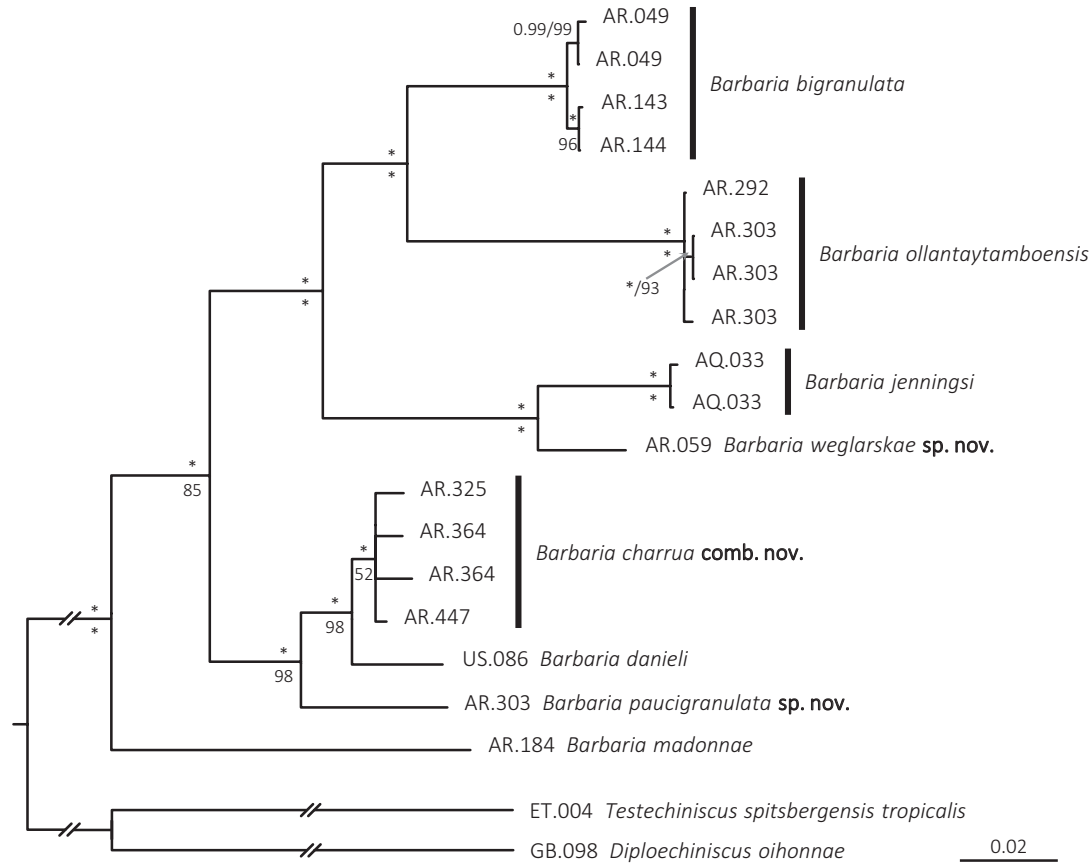


Figure 5. Phylogeny of the genus *Barbaria* based on five concatenated markers conducted in MrBayes and W-IQ-TREE (values above the nodes signify posterior probabilities, whereas bootstraps are provided below the nodes; maximal supports are denoted with asterisks). *Diploechiniscus oihonnae* and *Testechiniscus spitsbergensis tropicalis* were used as an outgroup. The scale bar represents 0.2 substitutions per nucleotide position and refers to the Bayesian inference.

- *Barbaria madonnae*, by *striae* between pillars in the scapular and the caudal plate [present in *B. paucigranulata* (Figs 7I, 10I) vs. absent in *B. madonnae* (Figs 7G, 10G)], the shape of papilla IV [tubby in *B. paucigranulata* (Fig. 11L) vs. elongated in *B. madonnae* (Fig. 11H)] and the primary spur morphology [needle-like and adjacent to the claw branch in *B. paucigranulata* (Fig. 11K, L) vs. robust, hook-shaped and divergent from the claw branch in *B. madonnae* (Fig. 11G, H)].
- *Barbaria ollantaytamboensis*, by the distribution of pores in plates [absent in the anterior portion of median plate 2 (Fig. 8I), paired segmental plates (Fig. 9I) and the entirety of median plate 3 in *B. paucigranulata* vs. present in the anterior portion of m2 (Fig. 8H), paired segmental plates (Fig. 9H) and the entirety of m3 in *B. ollantaytamboensis*] and claw isomorphy [slightly heteronych (heteromorphic) in *B. paucigranulata* (Fig. 11K, L) vs. isonych (homomorphic) in *B. ollantaytamboensis* (Fig. 11I, J)].
- *Barbaria quitensis*, by the type of perforation in the dorsal plates [pores in *B. paucigranulata* (Figs 7I, 8I, 9I, 10I) vs. pseudopores in *B. quitensis* (Figs 7J, 8J, 9J, 10J)] and the distribution of pores/pseudopores [scarcer on lateralmost portions of the caudal plate (Fig. 10I) in *B. paucigranulata* vs. roughly equally distributed in all portions of the caudal plate (Fig. 10J) in *B. quitensis*].
- *Barbaria ranzii*, by the cirrus A length (< 20% of the body length in *B. paucigranulata* vs. > 50% of the body length in *B. ranzii*), the distribution of pores in plates [absent in the anterior portion of median plate 2 (Fig. 8I), paired segmental plates (Fig. 9I) and the entirety of median plate 3 in *B. paucigranulata* vs. present in the anterior portion of m2 (Fig. 8K), paired segmental plates (Fig. 9K) and the entirety of m3 in *B. ranzii*] and the by secondary spurs directed upwards on external claws IV [absent in *B. paucigranulata* (Fig. 11L) vs. present in *B. ranzii*, Fig. 11R].
- *Barbaria weglarskae*, by the cirrus A length (< 20% of the body length in *B. paucigranulata*

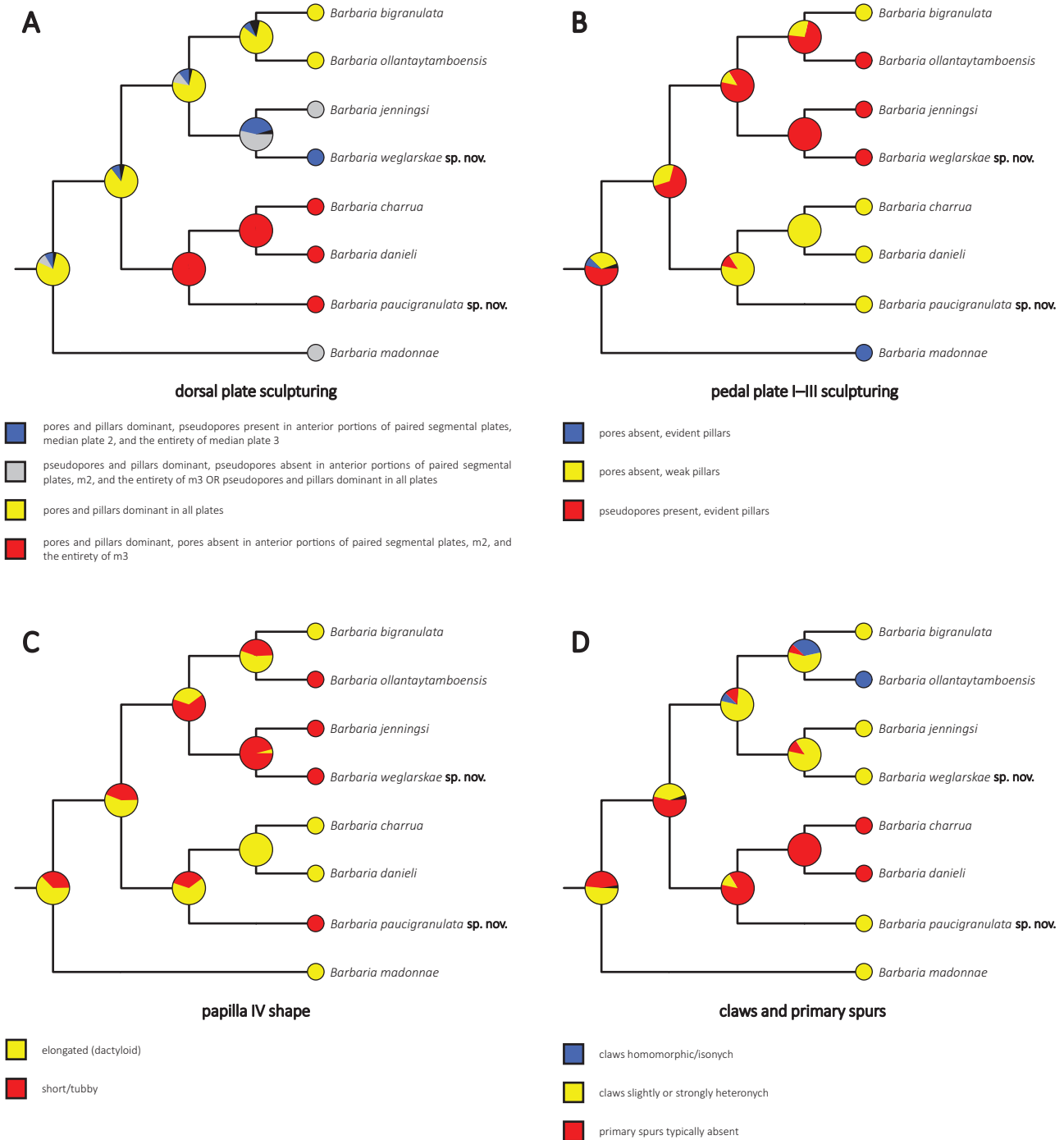


Figure 6. Ancestral state reconstruction in the genus *Barbaria* conducted in BEAST on identical dataset as in MrBayes (species are represented by single lineages for simplicity): A, dorsal plate sculpturing; B, pedal plate I–III sculpturing; C, papilla IV shape; D, claw isomorphy. Outgroup character states were coded as independent from the *Barbaria* matrix.

vs. > 50% of the body length in *B. weglarskae*) and by the primary spur morphology [needle-like and adjacent to the claw branch in *B. paucigranulata* (Fig. 11K, L) vs. robust, hook-shaped and divergent from the claw branch in *B. weglarskae* (Fig. 11M, N)].

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(FIGS 4, 7L, 8L, 9L, 10L, 11M, N; TABLE 5)

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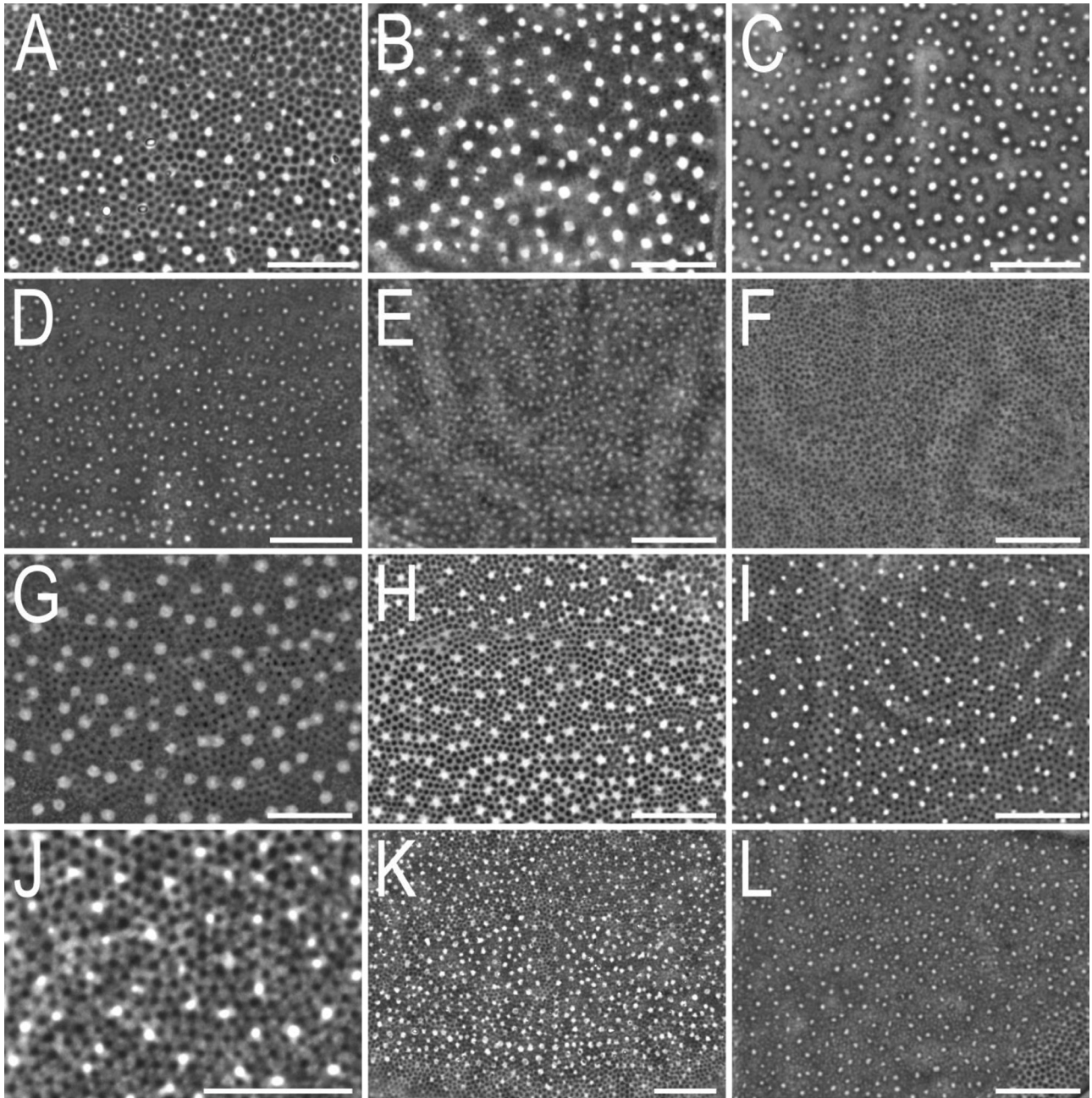


Figure 7. Variability of the scapular plate sculpturing (PCM): A, *Barbaria bigranulata*; B, *Barbaria charrua*; C, *Barbaria ganczareki*; D, *Barbaria hanna*; E, F, *Barbaria jenningsi* at two focus levels; G, *Barbaria madonnae*; H, *Barbaria ollantaytamboensis*; I, *Barbaria paucigranulata*; J, *Barbaria quitensis*; K, *Barbaria ranzii*; L, *Barbaria weglarskae*. Scale bars = 10 μ m.

Morphometric data: www.tardigrada.net/register/0110.htm

Type material: Holotype (adult female, slide AR.059.04) and two paratypes (adult females, slides AR.059.01, 05).

Type locality: 48°25'42"S, 71°44'48"W, 803 m asl: Argentina, Patagonia, Santa Cruz Province, Río Chico Department, vicinity of La Florida; lichen from on in the Andean Patagonian forest (see also [Table 1](#)).

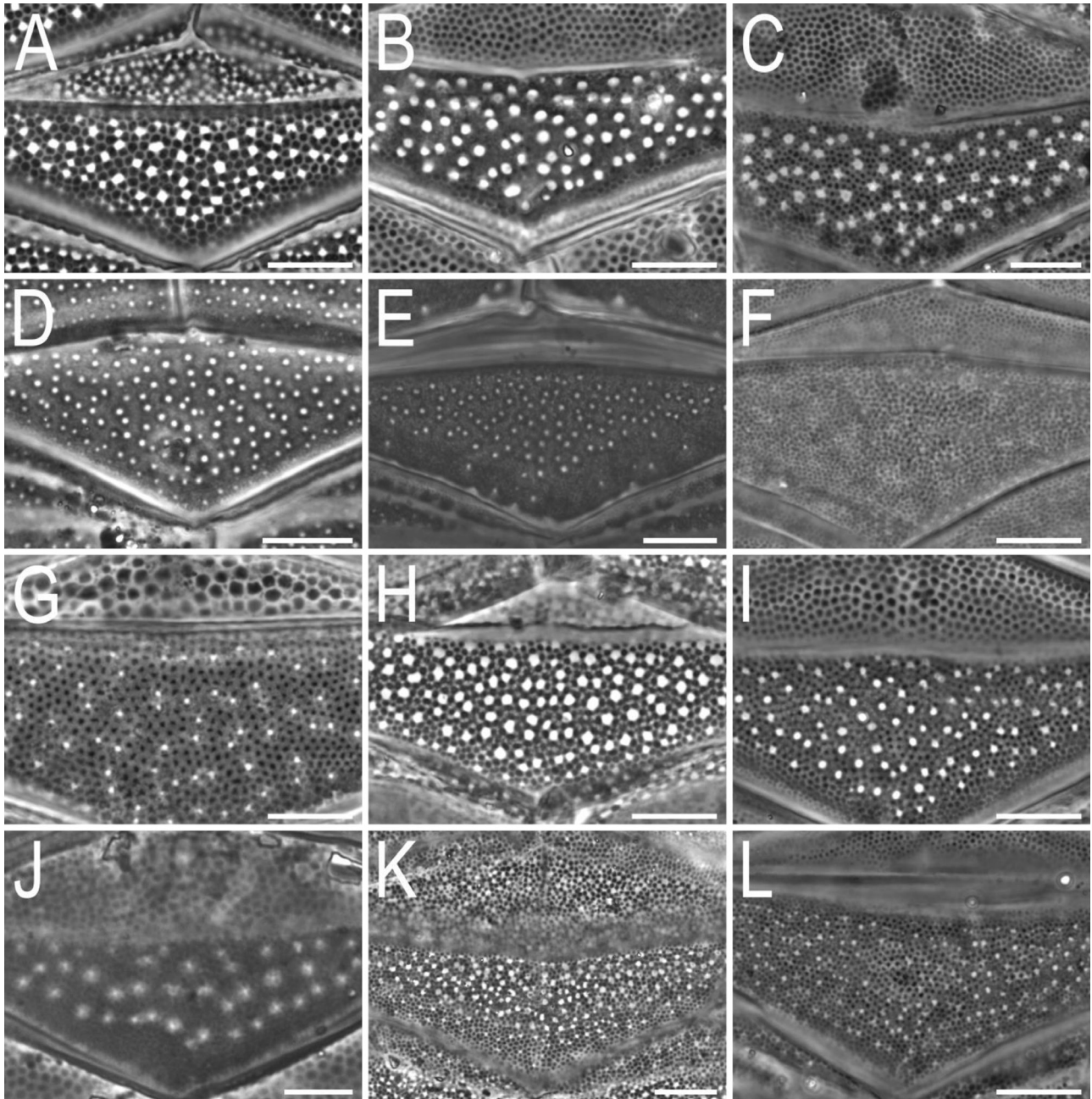


Figure 8. Variability of the median plate 2 sculpturing (PCM): A, *Barbaria bigranulata*; B, *Barbaria charrua*; C, *Barbaria danieli*; D, *Barbaria ganzareki*; E, *Barbaria hanna*; F, *Barbaria jenningsi*; G, *Barbaria madonnae*; H, *Barbaria ollantaytamboensis*; I, *Barbaria paucigranulata*; J, *Barbaria quitensis*; K, *Barbaria ranzii*; L, *Barbaria weglarskae*. Scale bars = 10 μ m.

Etymology: A patronym honouring Professor Barbara Weglarska, 20.02.1922–02.10.2020, whose death left a void in the community of tardigradologists. A noun in the genitive case.

Description: Adult females (i.e. from the third instar onwards, measurements in Table 5). Orange body

with large, red eyes; the entire pigment and eyes dissolve quickly after mounting in Hoyer's medium. Body massive (Fig. 4A). Cylindrical, *Echiniscus*-type cephalic papillae (secondary clavae) and (primary) clavae; cirri embedded in bulbous cirrophores. Cirrus A is long (> 50% of the body length) and with evident, conical cirrophore (Fig. 4A, B).

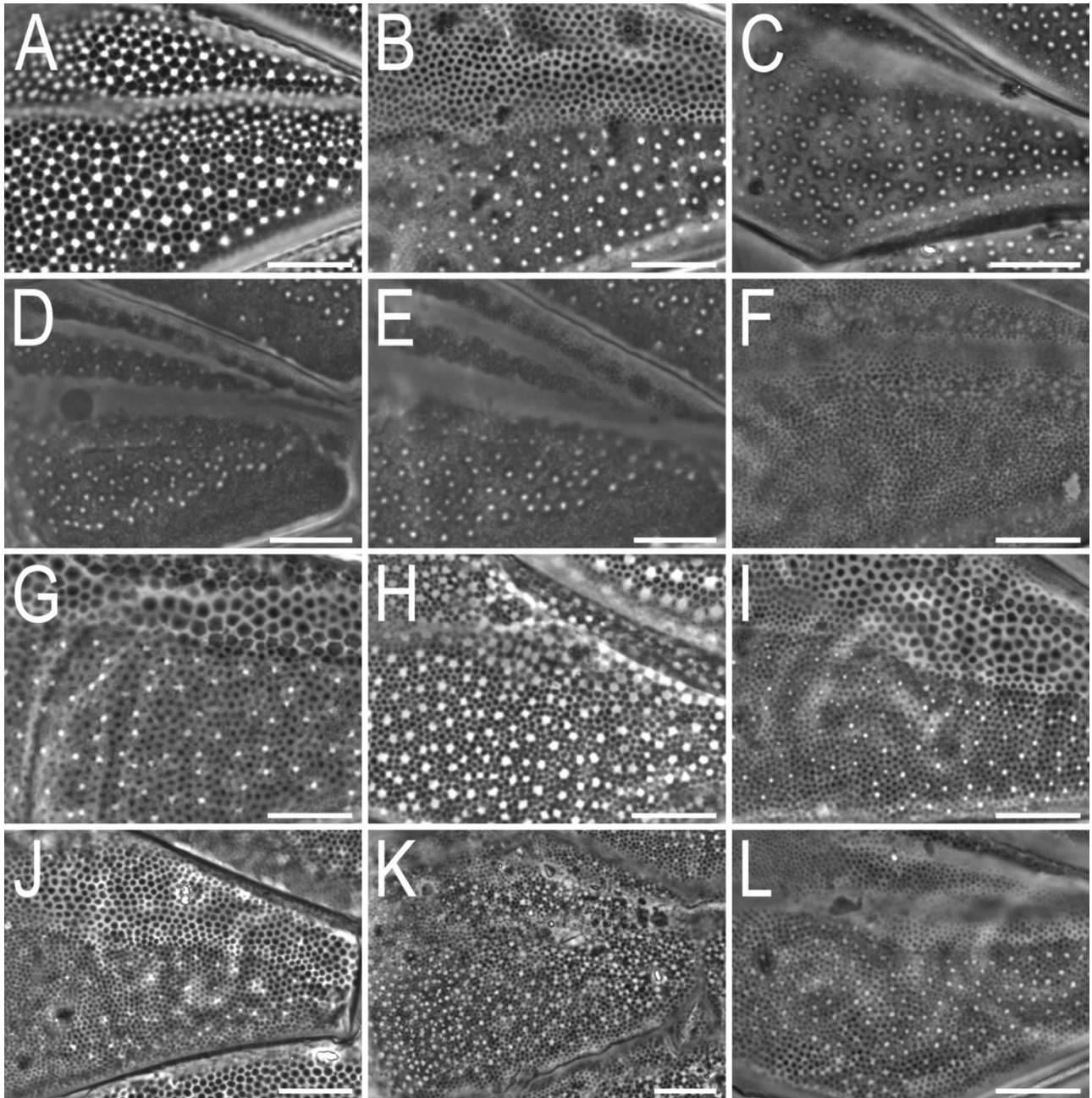


Figure 9. Variability of the paired segmental plate II sculpturing (PCM): A, *Barbaria bigranulata*; B, *Barbaria charrua*; C, *Barbaria ganzareki*; D, E, *Barbaria hanna*; F, *Barbaria jenningsi*; G, *Barbaria madonnae*; H, *Barbaria ollantaytamboensis*; I, *Barbaria paucigranulata*; J, *Barbaria quitensis*; K, *Barbaria ranzii*; L, *Barbaria weglarskae*. Scale bars = 10 μ m.

Dorsal plate sculpturing of the *bigranulata*-type, composed of pillars present in all plate portions (pseudogranulation) and pores and pseudopores present in different elements of armour (Figs 4A, B, 7L, 8L, 9L, 10L). Pseudopores can be present or absent exclusively in the anterior portion of the median plate 2 and paired segmental plates and the entirety of median plate 3 (Fig. 4A, B). Minute pores of equal

size are regularly distributed in the remaining plate portions (Figs 4A, B, 7L, 8L, 9L, 10L). The cephalic plate large, with a pronounced chalice-shaped anterior incision and lateral sutures demarcating roughly triangular lateralmost portions of the plate (Fig. 4A, B). Thin cervical plate with developed pillars and pseudopores. Lateral sutures in the scapular plate demarcate lateralmost, rectangular portions

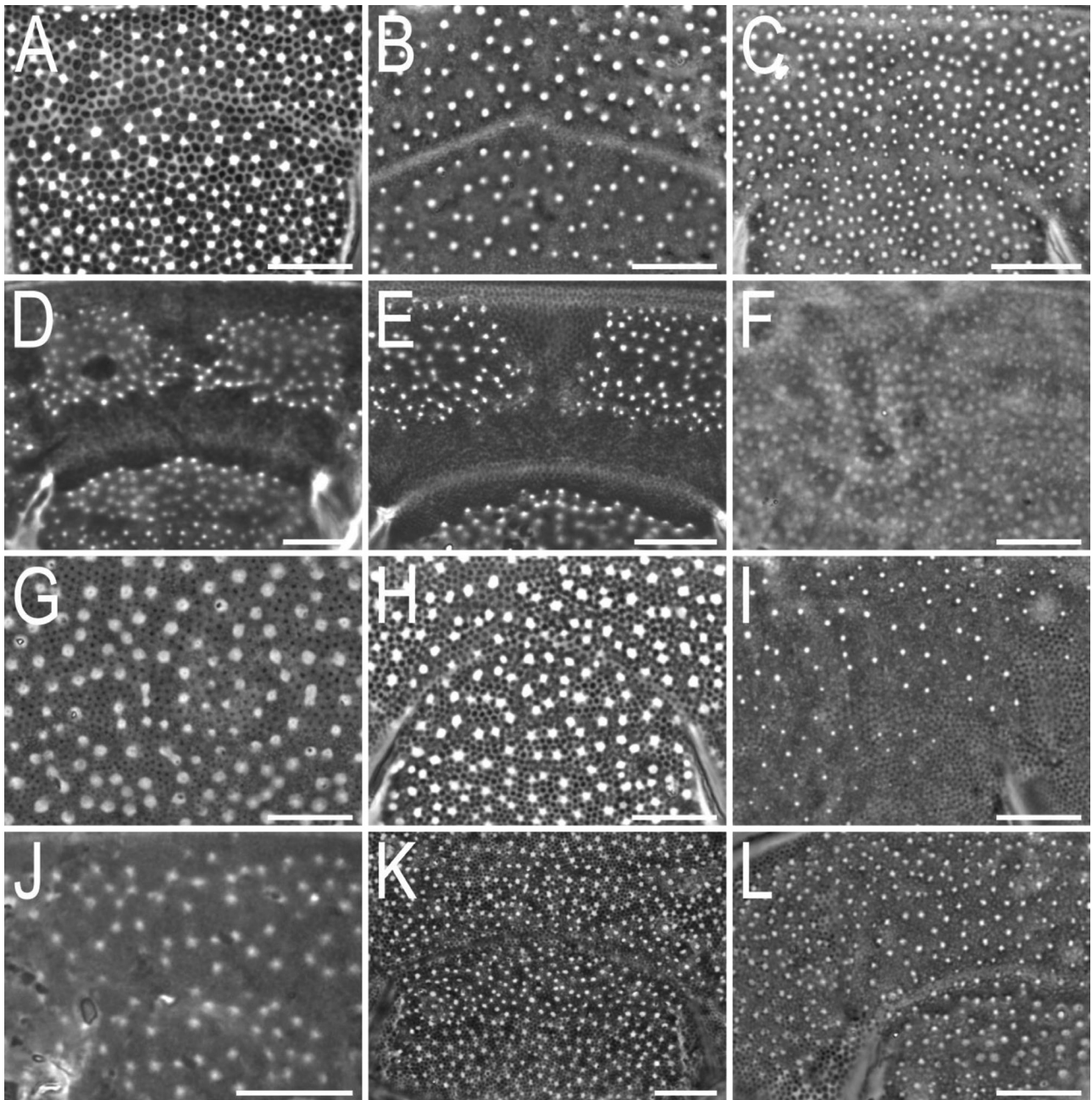


Figure 10. Variability of the caudal (terminal) plate sculpturing (PCM): A, *Barbaria bigranulata*; B, *Barbaria charrua*; C, *Barbaria ganzareki*; D, E, *Barbaria hanna*; F, *Barbaria jenningsi*; G, *Barbaria madonnae*; H, *Barbaria ollantaytamboensis*; I, *Barbaria paucigranulata*; J, *Barbaria quitensis*; K, *Barbaria ranzii*; L, *Barbaria weglarskae*. Scale bars = 10 μ m.

with identical sculpturing as on the rest of the plate (Fig. 4A, B). Median plates 1 and 3 unipartite (the latter strongly reduced and partially covered by the caudal plate), median plate 2 bipartite (Figs 4A, B, 8L). Paired segmental plates with broad, transverse, unsculptured bands (Figs 4A, B, 9L). The caudal plate with short, poorly sclerotized incisions clearly joined by a transversal suture (Figs 4A, B, 10L). Ventral

cuticle with minute endocuticular pillars distributed evenly throughout the entire venter; a pair of small, subcephalic plates present (Fig. 4C–D). Sexpartite gonopore (Fig. 4E) placed anteriorly to a trilobed anus between legs IV.

Pedal plates I–IV with evident pillars and pseudopores (Figs 4A, 11M, N). Evident pulvini on outer sides of all legs. Dentate collar IV with numerous

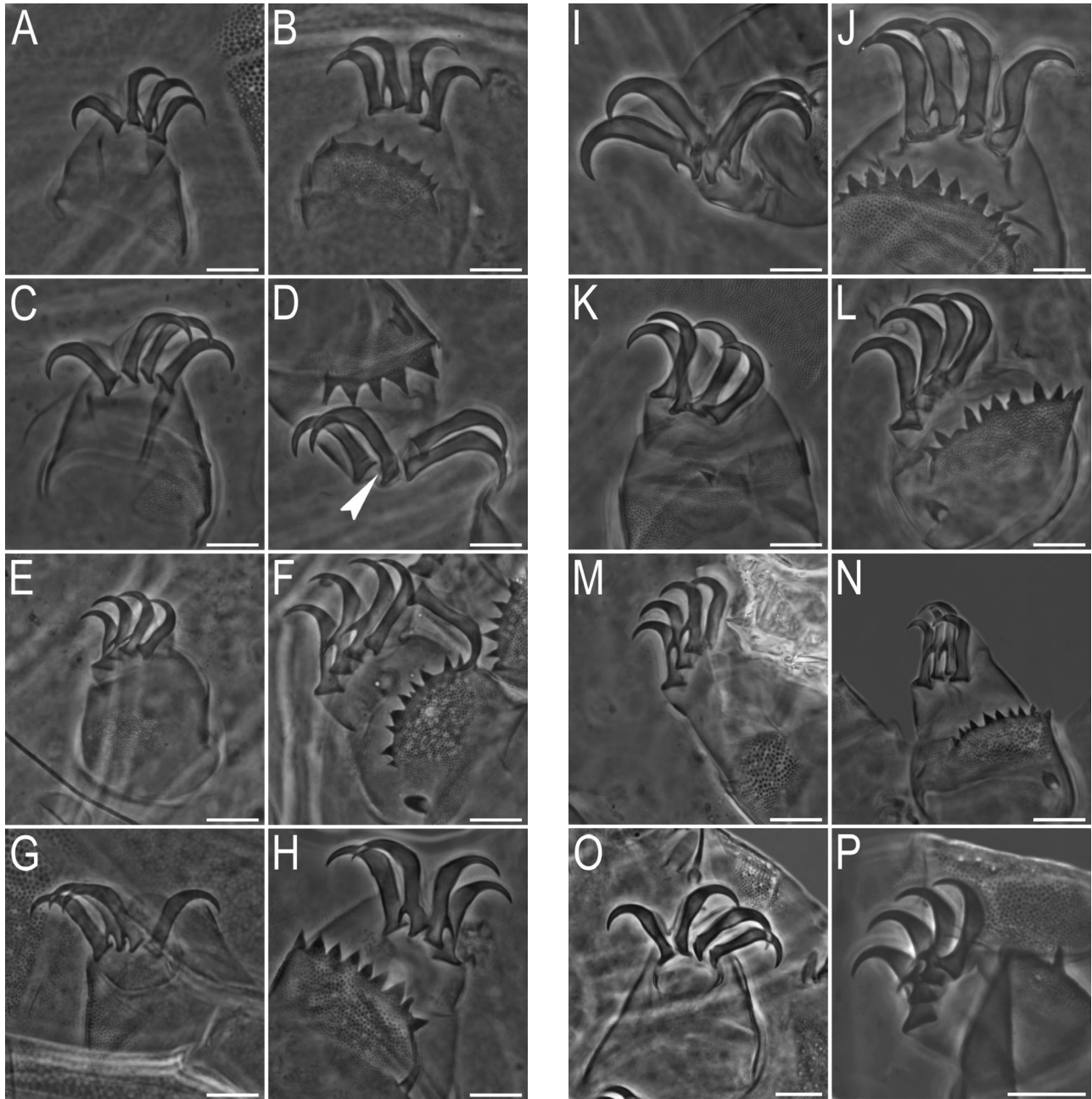


Figure 11. Claws and leg structures (PCM): A, claws III of *Barbaria bigranulata*; B, claws IV of *Barbaria bigranulata*; C, claws II of *Barbaria charrua*; D, claws IV of *Barbaria charrua*; E, claws II of *Barbaria jenningsi*; F, claws IV of *Barbaria jenningsi*; G, claws III of *Barbaria madonnae*; H, claws IV of *Barbaria madonnae*; I, claws II of *Barbaria ollantaytamboensis*; J, claws IV of *Barbaria ollantaytamboensis*; K, claws I of *Barbaria paucigranulata*; L, claws IV of *Barbaria paucigranulata*; M, claws II of *Barbaria weglarskae*; N, claws IV of *Barbaria weglarskae*; O, claws I of *Barbaria danieli*; P, claws I of *Barbaria hanna*; Q, claws III of *Barbaria quitensis*; R, claws IV of *Barbaria ranzii*. Arrowheads indicate asymmetrically developed primary spurs. Scale bars = 10 μ m.

Figure 11. Continued.

irregular short teeth (Fig. 11N). A tiny spine I and a tubby papilla IV (Fig. 11N). Claws slightly heteronych, because primary spurs on internal claws IV are positioned higher than those on claws I–III (Fig. 11M, N). The shape and angle at which spurs diverge from branches are almost identical on all limbs. Cuticular bars below claw bases on the inner side of legs present. Buccal apparatus with a rigid tube and round pharynx containing placoids. Lacking stylet supports.

Adult males, juveniles, larvae or eggs not found.

Differential diagnosis: There are only two other species of *Barbaria* with a cirrus A/body length ratio > 50% [the titles and values in the last five rows of table 1 in Michalczyk & Kaczmarek (2007) are mismatched in the case of sexes treated separately, but the ratio statistics for all measured specimens of *B. ganczareki* stands valid: min = 15%, max = 23%, mean = 19%]: *B. jenningsi* and *B. ranzii*, but *B. weglarskae* can be distinguished from:

- *Barbaria jenningsi*, by the type of perforation in the dorsal plates [dominant pores in *B. weglarskae* (Fig. 7L) vs. pseudopores in *B. jenningsi* (Figs 7E, F, 8F, 9F, 10F)] and claw isomorphy [anisonych/slightly heteronych in *B. weglarskae* (Fig. 11M, N) vs. strongly heteronych in *B. jenningsi* (Fig. 11E, F)].
- *Barbaria ranzii*, by the pedal plate sculpturing [with evident pillars in *B. weglarskae* (Fig. 11M, N) vs. without pillars in *B. ranzii* (Fig. 11R)], the shape of papilla IV [tubby in *B. weglarskae* (Fig. 11N) vs. elongated in *B. ranzii*] and by the presence of secondary spurs directed upwards on external claws IV [absent in *B. weglarskae* (Fig. 11N) vs. present in *B. ranzii* (Fig. 11R)].

MOLECULAR PHYLOGENY

Phylogeny based on the five concatenated markers brought fully resolved relationships between all eight analysed species of *Barbaria*, which form clades characterized by low intraspecific and large interspecific genetic variability (Fig. 5). The topology is as follows: *B. madonnae* is a sister-species to all other sequenced *Barbaria* spp., which are clustered in two clades: the first comprising (*B. paucigranulata* (*B. danieli* + *B. charrua*)) and the second grouping ((*B. weglarskae* + *B. jenningsi*) + (*B. ollantaytamboensis* + *B. bigranulata*)).

INTRA- AND INTERSPECIFIC GENETIC VARIABILITY

Regarding *COI* sequences deposited in GenBank, the data are available only for *B. bigranulata* and *B. jenningsi*. *COI* *p*-distances between populations

of *B. bigranulata* and the previously published data for a population from Chile (HM193406; Jørgensen *et al.*, 2011) ranged between 2.6 and 2.9% (alignment length = 585 bp). Analogous index for the pair *B. weglarskae*–*B. jenningsi* (KP013596; Velasco-Castrillón *et al.*, 2015) was 18.9% (alignment length = 472 bp).

More than one haplotype per marker has been found for all markers, but only in a few species. The intraspecific *p*-distances are as follows: 18S rRNA: 0.1% (in *B. bigranulata* and *B. charrua*); 28S rRNA: 0.1–0.4% (*B. bigranulata*, *B. ollantaytamboensis*); ITS1: 0.5% (*B. bigranulata*), 0.1–1.0% (*B. charrua*); ITS2: 0.2% (*B. bigranulata*), 0.2–0.4% (*B. ollantaytamboensis*); *COI*: 0.1–2.3% (*B. bigranulata*) and 0.7% (*B. charrua*).

Interspecific *p*-distances in the analysed dataset are as follows:

- **18S rRNA:** 0.0–2.5% (1.2% on average), with the most similar being *B. charrua* (MZ820796) and *B. danieli* (MZ820800); and the least similar being *B. madonnae* (MZ820803) and *B. ollantaytamboensis* (MZ820804).
- **28S rRNA:** 0.0–4.3% (2.4% on average), with the most similar being *B. charrua* (MZ820814) and *B. danieli* (MZ820818); and the least similar being *B. madonnae* (MZ820821) and *B. ollantaytamboensis* (MZ820823).
- **ITS1:** 0.3–10.3% (5.5% on average), with the most similar being *B. charrua* (MZ820833) and *B. danieli* (MZ820836); and the least similar being *B. madonnae* (MZ820839) and *B. bigranulata* (MZ820828).
- **ITS2:** 1.6–10.8% (8.1% on average), with the most similar being *B. charrua* (MZ822380) and *B. danieli* (MZ822384); and the least similar being *B. danieli* + *B. madonnae* (MZ822384, MZ822387) and *B. ollantaytamboensis* (MZ822388–91).
- **COI:** 9.1–20.4% (16.0% on average), with the most similar being *B. charrua* (MZ820850) and *B. danieli* (MZ820853); and the least similar being *B. ollantaytamboensis* (MZ820855) and *B. weglarskae* (MZ820860).

MORPHOLOGICAL EVOLUTION

Mapping morphological traits on to the phylogeny suggests that the ancestor of *Barbaria* was most probably covered with uniform dorsal sculpturing comprising both pillars and pores (Fig. 6A) and well-delimited pedal plates on legs I–III with densely packed pillars and pseudopores (Fig. 6B). Its papillae on legs IV were elongated (Fig. 6C), meaning that they were much longer than wide. Internal claws were exhibiting heteronychy or lacking primary spurs (Fig. 6D). In

Table 3. Measurements (in μm) of selected morphological structures of adult females of *Barbaria paucigranulata* (type series) mounted in Hoyer's medium. Abbreviations: *N*, number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD, standard deviation; *sp*, the proportion of the length of a given structure to the length of the scapular plate

Character	<i>N</i>	RANGE		Mean		SD		Holotype	
		μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>
Body length	9	258 – 327	482 – 539	283	513	22	17	302	539
Scapular plate length	9	49.0 – 62.0	–	55.1	–	3.7	–	56.0	–
Head appendage lengths									
Cirrus internus	9	13.5 – 20.0	26.0 – 32.7	16.3	29.5	2.2	2.7	16.6	29.6
Cephalic papilla	9	7.5 – 9.5	14.1 – 19.0	8.5	15.5	0.7	1.6	9.5	17.0
Cirrus externus	9	23.0 – 32.0	46.0 – 51.6	26.6	48.3	2.5	2.3	26.0	46.4
Clava	9	5.2 – 6.2	9.0 – 11.7	5.8	10.5	0.3	1.0	5.5	9.8
Cirrus A	8	41.8 – 51.9	74.7 – 93.5	45.3	82.5	3.0	5.9	?	?
Cirrus A/Body length ratio	8	14% – 18%	–	16%	–	1%	–	?	–
Body appendage lengths									
Spine on leg I length	9	3.1 – 4.2	5.8 – 7.9	3.7	6.7	0.4	0.7	4.2	7.5
Papilla on leg IV length	9	4.0 – 5.4	7.1 – 9.4	4.4	8.1	0.4	0.8	4.7	8.4
Number of teeth on the collar	9	8 – 13	–	10.4	–	1.8	–	13	–
Claw I heights									
Branch	9	15.0 – 18.5	24.2 – 34.9	17.4	31.7	1.0	3.1	18.0	32.1
Spur	8	1.9 – 2.5	3.3 – 5.1	2.3	4.2	0.2	0.6	2.5	4.5
Spur/branch height ratio	8	11% – 15%	–	13%	–	2%	–	14%	–
Claw II heights									
Branch	9	14.8 – 18.4	28.5 – 33.5	16.7	30.3	1.0	1.6	16.9	30.2
Spur	9	1.5 – 2.6	2.8 – 4.6	2.2	4.0	0.3	0.6	2.0	3.6
Spur/branch height ratio	9	9% – 16%	–	13%	–	2%	–	12%	–
Claw III heights									
Branch	9	15.5 – 17.8	27.7 – 34.3	16.7	30.4	0.8	2.0	17.5	31.3
Spur	8	2.1 – 2.5	3.7 – 5.1	2.3	4.1	0.1	0.5	2.2	3.9
Spur/branch height ratio	8	12% – 15%	–	13%	–	1%	–	13%	–
Claw IV heights									
Branch	9	17.6 – 20.4	31.2 – 37.9	19.1	34.8	0.9	2.0	18.9	33.8
Spur	6	2.0 – 3.2	3.8 – 5.9	2.6	4.7	0.5	0.9	2.4	4.3
Spur/branch height ratio	6	11% – 16%	–	13%	–	2%	–	13%	–

other words, among the extant species, *B. bigranulata* is morphologically overall the most similar to the last common ancestor of the analysed species.

DISCUSSION

MORPHOLOGICAL EVOLUTION

Our phylogenetic analysis (Fig. 5), based on as many as eight out of the 12 known *Barbaria* species, allows for initial hypothesizing on evolutionary trends in the genus (Fig. 6). One of the crucial taxonomic characters in Echiniscidae is the dorsal sculpturing pattern that has been demonstrated to bear a phylogenetic signal

(e.g. Guil *et al.*, 2013; Vicente *et al.*, 2013; Gąsiorek *et al.*, 2019). The analysis indicated that the ancestor of *Barbaria* probably had both well-developed pillars and pores in all elements of the dorsal armour (Fig. 6A), as currently exhibited by *B. bigranulata*, *B. ollantaytamboensis* and *B. ranzii* (e.g. Figs 7A, H, K, 10A, H, K). This suggests that *B. ranzii* could be directly related to the two former species. Moreover, the development of pseudopores either in the entire dorsum, as in *B. madonnae* (e.g. Figs 7G, 8G, 9G) and *B. quitensis*, or in some plate portions, as in *B. jenningsi* (e.g. Fig. 7E, F), is a secondary change in the light of this hypothesis. Analogously, the dominance of epicuticular pores associated with the miniaturization of endocuticular pillars in *B. ganzareki* (e.g. Figs

Table 4. Measurements (in μm) of selected morphological structures of juveniles of *Barbaria paucigranulata* (type series) mounted in Hoyer's medium. Abbreviations: *N*, number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD, standard deviation; *sp*, the proportion of the length of a given structure to the length of the scapular plate

Character	<i>N</i>	RANGE		Mean		SD	
		μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>
Body length	4	227 – 236	472 – 540	231	512	4	29
Scapular plate length	4	42.0 – 50.0	–	45.3	–	3.4	–
Head appendage lengths							
Cirrus internus	4	10.7 – 15.6	24.3 – 31.2	12.9	28.4	2.2	3.3
Cephalic papilla	4	6.2 – 7.3	13.8 – 16.2	6.8	15.0	0.5	1.0
Cirrus externus	4	16.3 – 24.8	37.0 – 50.7	20.9	45.9	3.7	6.2
Clava	4	3.6 – 5.8	8.0 – 12.4	4.6	10.0	1.1	2.3
Cirrus A	4	32.3 – 41.8	73.4 – 98.1	37.6	83.3	4.6	10.7
Cirrus A/Body length ratio	4	14% – 18%	–	16%	–	2%	–
Body appendage lengths							
Spine on leg I length	3	3.5 – 3.8	8.0 – 9.0	3.6	8.3	0.2	0.6
Papilla on leg IV length	4	3.4 – 4.4	7.7 – 8.9	3.9	8.6	0.4	0.6
Number of teeth on the collar	4	8 – 10	–	9.3	–	1.0	–
Claw I heights							
Branch	4	12.2 – 14.0	24.4 – 31.4	13.2	29.3	0.7	3.3
Spur	3	2.0 – 2.3	4.2 – 5.2	2.1	4.6	0.2	0.5
Spur/branch height ratio	3	14% – 17%	–	16%	–	2%	–
Claw II heights							
Branch	4	12.9 – 15.8	28.7 – 33.8	14.0	31.0	1.3	2.2
Spur	4	1.5 – 2.4	3.6 – 4.8	1.9	4.2	0.4	0.6
Spur/branch height ratio	4	11% – 15%	–	14%	–	2%	–
Claw III heights							
Branch	4	12.5 – 15.6	28.4 – 32.6	13.7	30.3	1.3	1.9
Spur	4	1.4 – 2.6	3.3 – 5.2	2.0	4.4	0.5	0.9
Spur/branch height ratio	4	10% – 18%	–	15%	–	3%	–
Claw IV heights							
Branch	4	14.8 – 17.5	32.9 – 40.2	16.1	35.7	1.3	3.2
Spur	3	2.0 – 2.6	4.0 – 5.8	2.2	4.9	0.3	0.9
Spur/branch height ratio	3	11% – 18%	–	14%	–	3%	–

7C, 8D) and in *B. hanna*e (Fig. 7D) is an apomorphy. Furthermore, the presence of wide epicuticular ridges on dorsal plates in *B. hanna*e (Figs 8E, 9D, E, 10D, E; Roszkowska *et al.*, 2019) is another apomorphic condition. Of the remaining species, *B. charrua*, *B. danieli*, *B. paucigranulata* and *B. quitensis* (Figs 7B, I, J, 8C) share a similar sculpturing pattern, as noted by Pilato (2007) for *B. charrua* and *B. quitensis*. Thus, considering that *B. charrua*, *B. danieli* and *B. paucigranulata* form a clade in congruence with dorsal plate sculpturing (Fig. 6A), we hypothesize that *B. quitensis* could be directly related with these three species.

Pedal plate sculpturing has started to be used in echiniscid taxonomy only recently [e.g. Michalczyk & Kaczmarek (2007) in the case of *Barbaria*; Pilato *et al.* (2008) in the case of the *Echiniscus spinulosus*

complex, and further proposed as an important taxonomic criterion by Gaśiorek & Degma (2018)]. The present analysis shows that the last common ancestor of *Barbaria* exhibited weakly sculptured pedal plates on legs I–III, i.e. with endocuticular pillars and pseudopores (Fig. 6B). Pedal plate IV, equipped with the dentate collar, is usually clearly sculptured and more homogeneous within the genus. This is typical for many Echiniscidae, and it should be stressed that the pores in pedal plates I–III of *B. ganczareki* (Michalczyk & Kaczmarek, 2007) are an autapomorphic state.

The differentiation of papillae IV into two morphotypes: elongated and tubby (barrel-like), was recently underlined as a discriminant trait in the delineation of closely related species of *Pseudechiniscus* (Tumanov, 2020). Our analysis is in accordance with this suggestion, as some similar species can be easily

Table 5. Measurements (in μm) of selected morphological structures of adults females of *Barbaria weglarskae* (type series) mounted in Hoyer's medium. Abbreviations: *sp*, the proportion of the length of a given structure to the length of the scapular plate;?, unknown

Character	Holotype		Paratype 1		Paratype 2	
	μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>
Body length	291	549	246	559	207	410
Scapular plate length	53.0	–	44.0	–	50.5	–
Head appendage lengths						
Cirrus internus	18.9	35.7	18.1	41.1	?	?
Cephalic papilla	10.6	20.0	8.2	18.6	7.9	15.6
Cirrus externus	24.5	46.2	19.8	45.0	?	?
Clava	7.3	13.8	6.2	14.1	6.3	12.5
Cirrus A	222.0	418.9	125.4	285.0	157.0	310.9
Cirrus A/Body length ratio	76%	–	51%	–	76%	–
Body appendage lengths						
Spine on leg I length	?	?	1.6	3.6	3.1	6.1
Papilla on leg IV length	4.7	8.9	4.0	9.1	?	?
Number of teeth on the collar	13.0	–	12.0	–	12.0	–
Claw I heights						
Branch	15.3	28.9	13.3	30.2	14.8	29.3
Spur	4.2	7.9	3.8	8.6	3.9	7.7
Spur/branch height ratio	27%	–	29%	–	26%	–
Claw II heights						
Branch	14.2	26.8	12.3	28.0	14.8	29.3
Spur	4.0	7.5	3.6	8.2	3.4	6.7
Spur/branch height ratio	28%	–	29%	–	23%	–
Claw III heights						
Branch	14.4	27.2	11.1	25.2	13.5	26.7
Spur	?	?	3.0	6.8	4.2	8.3
Spur/branch height ratio	?	–	27%	–	31%	–
Claw IV heights						
Branch	16.6	31.3	14.2	32.3	17.0	33.7
Spur	?	?	4.1	9.3	?	?
Spur/branch height ratio	?	–	29%	–	?	–

distinguished based on the shape of papillae IV (Fig. 6C). On the other hand, this fact implies re-examination of many type series as the morphotype of papillae was seldom reported in earlier taxonomic contributions since researchers usually noted the presence/absence of these receptors, without their further morphological description (Ramazzotti & Maucci, 1983).

Perhaps the most intriguing ancestral state reconstruction is the claw morphotype, as the analysis did not unambiguously indicate whether the ancestor of *Barbaria* was heteronych or spurless (Fig. 6D). In the first scenario of ancestrally heteronych claws in *Barbaria*, spurless claws of *B. charrua* and *B. danieli* are autapomorphic. The second scenario involves acknowledging that spurless claws of *B. charrua* and *B. danieli* are a retained plesiomorphy and this is supported by another character, not reported in the original description (Meyer *et al.*, 2017), i.e. the presence

of rudimentary spines/papillae on legs II–III (Fig. 12), which are a plesiomorphy in Echiniscidae in general [present also in *Echiniscus perarmatus* Murray, 1907 or *Hypechiniscus papillifer* (Robotti, 1972); see Gąsiorek *et al.* (2021a) for details]. Since one of the ancestral echiniscid traits are internal claws equipped with primary spurs (Kristensen, 1987), the re-acquisition of spurs in other members of *Barbaria* would be an example of evolutionary reversal. A contrasting pattern was recently hypothesized for *Pseudechiniscus* (Gąsiorek *et al.*, 2021b), whose representatives have typically reduced and small primary spurs, thus the likelihood of spur loss is high in the course of evolution in that genus (some *Pseudechiniscus* spp. are spurless; Tumanov, 2020; Gąsiorek *et al.*, 2021b). Similar to the papillae IV, pairs of closely related species of *Barbaria* can be readily separated by the claw morphotype. Overall, our analyses augment what was revealed



Figure 12. Habitus of *Barbaria danieli* (PCM, female from Alabama). Arrowheads indicate rudimentary papillae on legs II–III. Scale bar in μm .

for *Hypechiniscus* (Gašiorek *et al.*, 2021a), i.e. that detailed examination of dorsal plate sculpturing and spur morphology may significantly affect taxonomic and phylogenetic inference.

BIOGEOGRAPHY

As many as 83% *Barbaria* species have a Neotropical distribution (Kaczmarek *et al.*, 2014; Kaczmarek *et al.*, 2015; Roszkowska *et al.*, 2019) (Fig. 13). Only two of the 12 known species, *B. danieli* and *B. jenningsi*, were found outside this zoogeographic realm: in the southern Nearctic (south-eastern USA; Meyer *et al.*, 2017) and the Antarctic Peninsula and adjacent islands (McInnes, 1995), respectively. Given that the greatest *Barbaria* diversity is found in South and Central America, and because the Nearctic and Antarctic are adjacent to the Neotropics, it is most likely that these two species (or their ancestors) dispersed to the north and the south, respectively. The fact that both species are nested among Neotropical species (Fig. 5), suggests that the dispersal was recent relative to the age of the genus.

The single record of *B. jenningsi* from South America, reported from high elevation in Bolivia (Garitano-Zavala, 1995), is a misidentification (Kaczmarek *et al.*, 2015). Because *B. weglarskae* is morphologically similar to *B. jenningsi*, we hypothesize that the Bolivian record represents *B. weglarskae* or another new species.

Of all known species in the genus, *B. bigranulata* has the longest list of records, but also the largest percentage of erroneous and dubious reports. Being the first and for decades the only described species without trunk appendages and with cuticular sculpturing of the *Barbaria* type, *B. bigranulata* was probably mistaken for most congeners in the Neotropics. The ambiguous original description contributed further to misidentifications with various echiniscids in other parts of the world [see Table 6 for the results of our examination of the Ramazzotti and Maucci collections and falsified records from Italy (Ramazzotti, 1945) and Austria (Maucci, 1974)]. Thus, the only confirmed records of this species are the following reports from the Neotropics: the original record (Argentina in Richters, 1908), the redescription

TAXONOMIC KEY

After taxonomic re-shuffling (see above), the genus currently comprises 12 species. Since females are currently known for all species of *Barbaria*, but we still lack data on immature instars and the presence of males in some species, this key uses exclusively qualitative traits of sexually mature females.

1. Secondary spurs on external claws IV present* *B. ranzii*
 – Secondary spurs on external claws IV absent 2
 2(1). Internal claws spurless** 3
 – Internal claws with primary spurs 4
 3(2). Pores of similar diameter in all dorsal plates *B. charrua*
 – Pores of the caudal (terminal) plate clearly smaller than in the remaining dorsal plates *B. danieli*
 4(2). Pores present in pedal plates and in ventral subcephalic cuticle *B. ganzzareki*
 – Pores absent in pedal plates and in ventral subcephalic cuticle 5
 5(4). Cirrus A/body length ratio > 50% (typically >> 50%) 6
 – Cirrus A/body length ratio < 50% (typically < 25%) 7
 6(5). Pseudopores present, claws strongly heteronych *B. jenningsi*
 – Pores present, claws slightly heteronych *B. weglarskae*
 7(5). Pronounced thick and poreless ridges on the caudal plate present *B. hanna*
 – Pronounced thick and poreless ridges on the caudal plate absent 8
 8(7). Pores dominant 9
 – Pseudopores dominant 11
 9(8). Pores absent in anterior portion of median plate 2 and anterior portion of paired segmental plates and in the entirety of median plate 3 *B. paucigranulata*
 – Pores present in anterior portion of median plate 2 and anterior portion of paired segmental plates and in the entirety of median plate 3 10
 10(9). Papilla IV elongated, claws slightly heteronych *B. bigranulata*
 – Papilla IV tubby, claws isonych *B. ollantaytamboensis*
 11(8). Papilla IV elongated, primary spurs robust and hook-shaped *B. madonnae*
 – Papilla IV tubby, primary spurs delicate and needle-like *B. quitensis*

* The examination of syntypes (slides 154, 157 in the Ramazzotti collection) revealed that secondary spurs may be occasionally present also on claws I–III.

** *Barbaria charrua* may rarely have asymmetrically developed, needle-like spurs on one of the internal claws (Fig. 11D).

(Argentina, Chile and Costa Rica in Michalczyk & Kaczmarek, 2006) and reports verified against the redescription [Chile in Binda & Pilato (1999) and in Jørgensen *et al.* (2011); Ecuador in Pilato *et al.* (2001) and Roszkowska *et al.* (2019); and Argentina in Maucci (1988), Rossi *et al.* (2009), Roszkowska *et al.* (2016) and in the present study]. The above-mentioned records pre-dating the redescription were verified either by Michalczyk & Kaczmarek (2006) or in the present study, and the COI sequence from Jørgensen *et al.* (2011) was verified herein. Whereas the following records of *B. bigranulata* from the Neotropics should be treated as *Barbaria* sp., because they could represent almost any species of the genus (even though some of them may represent *B. bigranulata*, their exact identity remains unverified): Richters (1911); Marcus (1939); Iharos (1963); Riggin (1963); Mehlen (1969); Rossi & Claps (1980, 1989); Claps & Rossi (1981, 1984, 1988); Garitano-Zavala (1995,

1996). Finally, all *B. bigranulata* records outside the Neotropics should be considered erroneous or highly dubious: the Nearctic [Kansas in Lehmann *et al.*, 2007], Palaearctic [Italy in Ramazzotti (1956) and Arcidiacono (1964); China in Yang (2007); Spain in Rodríguez-Candela *et al.* (2016)], Afrotropics [South Africa mentioned in Heinis (1928); Cameroon in Iharos (1969); Botswana in Middleton (2003)], Orient [Indonesia in Heinis, 1928] and Australasia [New Zealand in Horning *et al.*, 1978]. Specifically, the New Zealand example turned out to represent a new species, *Z. palmai* (Dastych, 1997), the Italian records were invalidated by Ramazzotti & Maucci (1983), the record from Spain is a misidentified *Pseudechiniscus* sp. [see figs 2–3 in Rodríguez-Candela *et al.* (2016) and Kaczmarek (2021)], and the Chinese and the African reports are highly unreliable, most likely representing *Echiniscus* species (McInnes *et al.*, 2017). Even recently, *Barbaria* was reported from India (Bhakare



Figure 13. The extant verified distributions of all known species of *Barbaria*: the main map – Neotropic; the upper insert – south-eastern Nearctic; the lower insert – the Antarctic Peninsula. See the Discussion for discarded records. Maps from www.freeworldmaps.net.

& Pai, 2021), but the specimens found represent an undescribed species of *Viridiscus* (Bhakare, personal communication based on microphotographs). In fact, there is no sound evidence for any *Barbaria* species outside the Nearctic, Neotropic and Antarctic realms. Thus, *Barbaria* is a good example of how misidentifications led to false records for the genus, which, in consequence, provided false support for the

‘everything is everywhere’ (‘EiE’) hypothesis (Baas-Becking, 1934).

Despite the numerous invalid records, *B. bigranulata* still has the widest documented geographic range of all species in the genus, extending from Tierra del Fuego in the south to Costa Rica in the north (Fig. 13). Importantly, together with *B. madonnae* and *B. weglarskae*, they seem to be the only *Barbaria*

Table 6. A list of verified records of *Barbaria bigranulata* based on the examination of slides from the Ramazzotti and Maucci collections

References (chronologically)	Slide number	Collection date	Locality and sample type	Remarks	Correct identification
Ramazzotti (1945)	14/II-76	02.08.1945	Europe, Italy, Alta Val Bognanco, Piemonte, Inferiore Lago Paione; 2002 m asl; moss from rock	mounted in Faure's medium; invalidated in Ramazzotti & Maucci (1983) , but without new identification of specimens	<i>Claxtonia</i> aff. <i>wendtii</i>
Ramazzotti (1962)	131	08.08.1962	South America, Chile, Cerro El Roble; ca. 1900 m asl; lichens from rock	mounted in Faure's medium	<i>Barbaria ollantaytamboensis</i>
Horning et al. (1978)	C.T.6846 (NZ564)	01.1971	Australasia, New Zealand, South Island, shores of Lake Te Anau	collected by J.L. Burnip; invalidated in Dastych (1997)	<i>Zealandiscus palmai</i>
Maucci (1974)	C.T.2972-4, 86, 90	29.08.1973	Europe, Austria, the border of Carinthia and Tyrol, Großglockner; 2120 m asl; moss from rock	mixed with specimens determined as <i>E. arctomys</i> , but all representing the same species <i>Claxtonia</i> aff. <i>wendtii</i>	<i>Claxtonia</i> aff. <i>wendtii</i>
Maucci (1988)	C.T.13009-32	11.02.1987	South America, Argentina, Santa Cruz, Calafate, La Bandera	–	<i>Barbaria madonnae</i>
–	C.T.13059-60	14.02.1987	South America, Chile, Magallanes, Última Esperanza	–	<i>Barbaria bigranulata</i>
–	C.T.13371-2	14.02.1987	South America, Chile, Torres del Paine	–	<i>Barbaria bigranulata</i>
–	C.T.13128-9, 13144-9, 13150-1, 13172, 13226, 13312-4, 8-9	11.02.1987	South America, Argentina, Santa Cruz, Lago Argentino	–	<i>Barbaria bigranulata</i>
–	C.T.13760	10.11.1987	South America, Chile, Los Ríos, Valdivia, Parque Saval	–	<i>Barbaria bigranulata</i>
–	C.T.13830-8	30.03.1988	South America, Ecuador, Cordillera Oriental	–	<i>Barbaria bigranulata</i> + <i>Barbaria ollantaytamboensis</i>

species inhabiting Patagonia, but further sampling is needed to confirm this. Such a vast geographic range, spanning more than 7000 km longitudinally, with elevations ranging from sea level to c. 4500 m asl and across several climate types (from tropical rainforest in Costa Rica, through temperate with hot summer and without dry season in Argentina, to polar tundra in Chile; classification according to [Peel *et al.*, 2007](#)), suggest that *B. bigranulata* is eurytopic. *Barbaria madonnae* also has a broad distribution ranging from the Colombian Sierra Nevada de Santa Marta ([Lisi *et al.*, 2014](#)) to southern Patagonia ([Maucci, 1988](#)). Furthermore, *B. charrua* is known from many locales in northern Argentina ([Claps *et al.*, 2008](#)) and Uruguay ([Kaczmarek *et al.*, 2015](#)). New records of *B. ollantaytamboensis* in north-western Argentina, northern Chile and Ecuador are the first reports of the species outside Peru ([Table 6](#); [Fig. 13](#); [Nickel *et al.*, 2001](#)). The remaining species of the genus are known exclusively from their type localities (*B. ranzii* from Chile, *B. hanna*e and *B. quitensis* from Ecuador, *B. paucigranulata* and *B. weglarskae* from Argentina) or from several close locales (*B. ganczareki* from Costa Rica, *B. jenningsi* from Antarctica, *B. danieli* from USA). The limited geographic ranges of *Barbaria* species, and of the genus itself, go against the EiE hypothesis. This is in concordance with recent discoveries in other tardigrade lineages, such as two clades representing the Western Palaearctic and Orient in the echiniscid genus *Bryodelphax* ([Gašiorek *et al.*, 2020](#)), and an overwhelming percentage of species restricted to single geographic realms in the echiniscid genus *Pseudechiniscus* ([Gašiorek *et al.*, 2021b](#)) and in the apochelan genus *Milnesium* Doyère, 1840 ([Morek *et al.*, 2021](#)). Thus, the present study provides further evidence supporting the hypothesis that widely distributed tardigrade species are an exception rather than the rule.

FUTURE DIRECTIONS

With two-thirds of the known species that have been analysed under the integrative taxonomy framework, *Barbaria* is now one of the few tardigrade genera with such a high proportion of integratively analysed species. Nevertheless, there are still many unknowns about the natural history of the genus. As shown above, *Barbaria* is common and widely distributed in the Neotropics. However, the numerous uncertain species records, combined with a recent influx of new taxa in the genus [as many as eight of 12 (67%) species have been described since the beginning of the present century], may suggest that there could be many more species awaiting discovery. If this is the case, then adding the known and yet undescribed species to the current dataset is likely to clarify or change some of the conclusions about

the morphological evolution of the genus presented in this study. Other issues are the dubious records of *Barbaria* outside the Neotropics, southern Nearctic and Antarctica, as well as the northern boundary of the geographic range of the genus. Hopefully, with the increasing use of integrative methods, future records of *Barbaria* will be easily verifiable and genetically confirmed geographic ranges of species in the genus will allow for more reliable reconstructions of historical biogeography of *Barbaria*.

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DATA AVAILABILITY

Raw morphometric data for both new species are deposited in the Tardigrada Register. Raw morphometric data for *Barbaria jenningsi* from Signy Island are presented as [Supporting Information, Material S3](#). All DNA sequences were uploaded to GenBank ([Table 2](#)).

CONFLICTS OF INTEREST

The Authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Material S1. The list of all examined *Barbaria* populations from Argentina.

Material S2. Primers and references for specific protocols for amplification of the five DNA fragments sequenced in the study. GenBank accession numbers for outgroup taxa used in phylogenetics.

Material S3. Morphometric data for *Barbaria jenningsi* from Signy Island (sub-Antarctic zone).