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Tardigrades from Iztaccíhuatl Volcano (Trans-Mexican Volcanic Belt), with the Description of *Minibiotus citlalium* sp. nov. (Eutardigrada: Macrobiotidae)

Alba Dueñas-Cedillo ¹, Evelyn Martínez-Méndez ¹, Jazmín García-Román ¹,
Francisco Armendáriz-Toledano ^{2,*} and Enrico Alejandro Ruiz ^{1,*} 

¹ Laboratorio de Ecología, Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prolongación de Carpio y Plan de Ayala s/n, Ciudad de Mexico C.P. 11340, Mexico; aduenasc1600@alumno.ipn.mx (A.D.-C.); lageneal@gmail.com (E.M.-M.); lgarcia0706@alumno.ipn.mx (J.G.-R.)

² Colección Nacional de Insectos, Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Cto. Zona Deportiva S/N, C.U., Ciudad de Mexico C.P. 04510, Mexico

* Correspondence: farmendariztoledano@ib.unam.mx (F.A.-T.); eruizc@ipn.mx (E.A.R.)

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Abstract: The study of tardigrade diversity in Mexico is at early stage of development, to date, 56 extant species have been reported. To identify the tardigrade fauna associated with mosses in the Iztaccíhuatl volcano, we performed a systematic sampling along an altitudinal and multi-habitat gradient. A total of 57 moss samples were collected, 233 adults, 20 exuviae, and 40 free-laid tardigrade eggs were extracted from them. Five species were identified, and three putative species were determined. *Diphascion mitrense* and *Minibiotus sidereus* represents new records for Mexico and North America, while *Adropion scoticum* is a new record for Mexico. Additionally, one new species, *Minibiotus citlalium* sp. nov. was discovered; it resembles to *Min. constellatus*, *Min. sidereus* and *Min. pentannulatus* by the presence of a similar distribution pattern of star-shaped pores in the dorsal cuticle arranged in 11 transverse rows, which become double in the segments of the legs I–III, and by a very large star-shaped pore (5–6 tips) on each leg of the fourth pair. *Minibiotus citlalium* sp. nov. differs from other *Minibiotus* species mainly by macroplacoid length sequence, presence of both small and large star-shaped pores on the external surface on all legs, and by egg processes with inconspicuous ornamentation.

Keywords: multi-habitat gradient; new records; systematic sampling; water bears

1. Introduction

Tardigrades are ubiquitous micrometazoans present in every biome on Earth, as they can be found in marine, freshwater, and terrestrial habitats [1]. Ecological studies dedicated to tardigrades are scarce, especially those that depend on replicated quantitative samples [2] (pp. 163–210). A patch distribution has been suggested even in apparently identical microhabitats. Therefore, a high number of samples (from hundreds to more than 1000) are necessary to reduce the standard error of population estimates and to obtain valid data for testing ecological hypotheses [2–5]. Tardigrade taxonomy is currently the subject of investigations, most of them relying on increasingly complex molecular datasets and extensive taxonomic contexts [6–8]; nonetheless, the taxonomic studies on Mexican tardigrades are at early stage. These include exploration, intensive collections, detailed observations, description, and denomination of species [9]. Previous studies on tardigrades in Mexico have recorded and described

species from occasional and unsystematic collection events, which in turn have yielded scattered records in very few locations with more than a third of the country remaining unexplored [10].

Currently, 56 limno-terrestrial tardigrade species have been recorded in Mexico; however, one of them (*Hypsibius pallidus* Thulin, 1911) has no records of locality [11]. The remaining inhabit five of the seven ecoregions described for the country [12] (pp. 87–108). Seven species have been documented in at least two of the five ecoregions [10,13–17]: 1 in deserts of North America [15,18,19], 9 in great plains [15], 12 in warm-humid forests [10,16,20], 10 in warm-dry forest [10,13,14], and 31 in temperate mountains [14–17,19,21,22].

The temperate mountains, volcanoes, and high sierras of the Trans-Mexican Volcanic Belt (TMVB) belong to one of those ecoregions and are located between 19° and 20° N in central Mexico. TMVB includes the highest peaks in the country, with elevations up to 5650 m asl [23], and eight types of landscapes from sub-humid to arid [24] (pp. 39–55). These features lead to the recognition of the TMVB as a centre of diversification, endemism, and biogeographic transition for a wide variety of taxa [25].

Sierra Nevada is located within the central region of the TMVB, between the states of Puebla and Mexico, with the Popocatepetl and the Iztaccíhuatl volcanoes as main peaks. The predominant landscapes are temperate and semi-cold plains, hillsides, and sierras, while vegetation consists of pine and oak forests, with fragments of thorn scrub and low montane rainforest [24] (pp. 39–55). This kind of landscape with humid, temperate, and cold conditions, from low to high altitudes, provides suitable habitats for many species of tardigrades [26–28]. To our knowledge, the tardigrades from the Sierra Nevada have only been studied previously in 1972 [14], at the Popocatepetl volcano, with eight species recorded. These are (following the current taxonomic nomenclature and the system of the genera names abbreviation presented by Perry et. al. [29]): *Echiniscus kerguelensis* Richters, 1904, *Milnesium tardigradum* Doyère, 1840, *Macrobotus echinogenitus* Richters, 1904, *Macrobotus furcatus* Ehrenberg, 1859, *Macrobotus hufelandi* C.A.S. Schultze, 1834, *Ramazottius baumanni* Ramazzotti, 1962, *Ramazottius oberhaeuseri* Doyère, 1840, and *Pilatobius nodulosus* Ramazzotti, 1957.

Most of these records are considered doubtful and must be confirmed taxonomically. For example, *Macrobotus hufelandi*, *Ramazottius oberhaeuseri*, and *Milnesium tardigradum* were amongst the earliest formally described tardigrade species. Under these names plenty of species were registered worldwide and were also subsequently reported as terrestrial cosmopolitan eutardigrades in the literature [30]. The most recent revision of these species complex indicates the presence of cryptic species; therefore, the previously published records must be corroborated, including Beasley [14]. Other case, corresponding to Mexican record of *Macrobotus echinogenitus*, was published before the development of rigorous standards of specific diagnosis by Guidetti and Bertolani [31]. Therefore, previous records must be considered doubtful unless confirmed [32].

Access to this volcano was restricted by the Mexican federal government in 1994, due to increased volcanic activity. Therefore, further studies in this area have not been performed. On the other hand, even though the Iztaccíhuatl volcano is nearby and accessible from Popocatepetl, to date no tardigrades from this region were reported.

The goal of the present study was to explore the tardigrade fauna, using a systematic sampling in the Iztaccíhuatl volcano, along an altitudinal (2700–4500 m asl) and multi-habitat gradient, moss growing on rocks, tree bark, and soil; across *Pinus* L., 1753, *Cupressus* L., 1753, and *Quercus* L., 1753; mixed forest, and *Abies religiosa* (Kunth) Scheleschetendahl et Chamisso, 1830; and *Pinus hartwegii* Lindley, 1839 forest, scrub, and alpine tundra. The study includes a description of the new eutardigrade species *Minibiotus citlalium* sp. nov. *Diphascion mitrense* Pilato, Binda and Quattieri, 1999 and *Minibiotus sidereus* Pilato, Binda and Lisi, 2003 represent new records for Mexico and North America, while *Adropion scoticum* (Murray, 1905) is a new record for Mexico.

2. Materials and Methods

2.1. Study Site

Fifty-seven moss samples were collected in the southwestern slope of Iztaccíhuatl volcano (Sierra Nevada, Trans Mexican Volcanic Belt; Figure 1a) in January 2018. The mosses were sampled in 12 geographical stations (S1–S12) distributed along an altitudinal gradient (2700–4500 m asl; Figure 1b) and in at least five different vegetation types, including: *Pinus-Cupressus-Quercus* mixed forest; *Abies religiosa*, and *Cupressus* sp. forest; *Pinus hartwegii* forest; alpine scrub; and alpine tundra (Figure 1c, Table 1). In each station, different moss morphotypes were identified and a square of 2 × 2 cm was sampled from each moss cushion and characterized depending on the collecting site, vegetation, and substrate type (rock, tree bark, and soil).

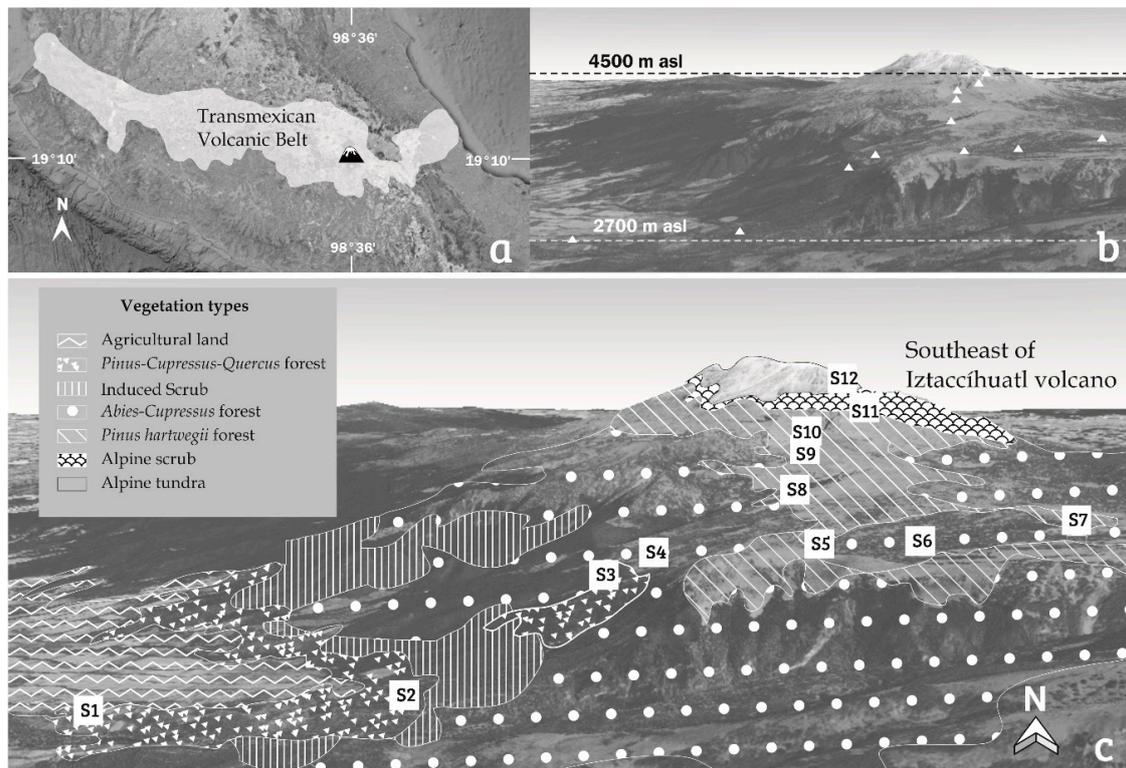


Figure 1. Map of the study area. (a) Trans Mexican Volcanic Belt (TMVB), Mexico; (b) altitudinal gradient sampled (2700–4500 m asl); and (c) geographical stations (S1–S12) with vegetation types.

Table 1. Station, sample number, geographic coordinates, vegetation, and substrate type, of the mosses sampled in the southwestern slope of Iztaccíhuatl volcano.

Station	Sample Number	Latitude (N)	Longitude (W)	Altitude (m asl)	Vegetation Type	Substrate Type
S1 La Comunidad	I–IV	19°04'24"	98°42'48"	2700	<i>Pinus</i> sp., <i>Cupressus</i> sp., <i>Quercus</i> sp.	tree bark
S2 Barranco Palomas	V–XI	19°04'24"	98°41'45"	3030	<i>Pinus</i> sp., <i>Cupressus</i> sp., <i>Quercus</i> sp.	tree bark (V–X), soil (XI)
S3 Cañada Cueva del Negro	XII–XIV	19°05'20"	98°40'54"	3278	<i>Abies religiosa</i> , <i>Cupressus</i> sp.	tree bark (XII–XIII), soil (XIV)
S4 Cañada Palo Rechino 1	XV–XIX	19°05'30"	98°40'38"	3411	<i>Abies religiosa</i> , <i>Cupressus</i> sp.	tree bark (XV–XVI), soil (XVII–XIX)
S5 Cañada El Paraje *	XX *–XXII	19°05'14"	98°40'03"	3498	<i>Abies religiosa</i> , <i>Cupressus</i> sp.	tree bark (XX), soil (XXI–XXII)
S6 Cañada Palo Rechino 2	XXIII–XXVIII	19°04'52"	98°39'40"	3613	<i>Abies religiosa</i> , <i>Cupressus</i> sp.	tree bark (XXVII–XXVIII), soil (XXIII–XXVI)

Table 1. Cont.

Station	Sample Number	Latitude (N)	Longitude (W)	Altitude (m asl)	Vegetation Type	Substrate Type
S7 Paso de Cortés	XXIX–XXXII	19°05′06″	98°38′49″	3700	<i>Pinus hartwegii</i>	tree bark (XXX, XXXII), soil (XXIX, XXXI)
S8 Subestación Eléctrica	XXXIII–XXXVII	19°06′13″	98°38′43″	4007	<i>Pinus hartwegii</i>	tree bark (XXXIII–XXXV), soil (XXXVI–XXXVII)
S9 Refugio Altzomoni	XXXVIII–XLII	19°07′31″	98°39′11″	3957	<i>Pinus hartwegii</i>	soil (XXXVIII–XXXIX), rock (XL–XLII)
S10 Parador La Joya	XLIII–XLVII	19°08′10″	98°38′56″	4126	<i>Pinus hartwegii</i>	soil (XLVII), rock (XLIII–XLVI)
S11 Primer Portillo	XLVIII–LII	19°08′31″	98°38′30″	4235	alpine scrub	soil (XLVIII), rock (XLIX–LII)
S12 Segundo Portillo	LIII–LVII	19°08′42″	98°38′19″	4500	alpine scrub	soil (LIII–LIV), rock (LV–LVII)

* type locality of *Minibiotus citlalium* sp. nov.

2.2. Sample Processing

Each moss sample was stored inside a paper envelope and dried. In the laboratory, the samples were rehydrated with 20 mL of tap water for 48 h. Later, samples were shaken and rinsed, and the supernatant were filtered using two stacked sieves with a decreasing mesh diameter (100 µm and 74 µm). The retained contents on the 74 µm sieve were washed into a Petri dish for examination under a stereoscopic microscope using dark field illumination at 45 × magnification.

All specimens for light microscopy were mounted individually onto microscope slides in Heinze polyvinyl alcohol (PVA) medium. Observations and photographs were taken using phase contrast microscopy (PCM) (ZEISS Axioskop with digital camera AxioCam ERC 55); for each species, images were recorded at successive focal depths and automatically combined into a single sharp image (i.e., focus stacking).

2.3. Morphometrics and Morphological Nomenclature

Structures were measured provided their orientations were suitable; body length was measured from the anterior to the posterior end of the body, excluding the hind legs. Terminology for the structures within the bucco-pharyngeal apparatus follows that from Pilato [33–35], Dastych [36], Kaczmarek and Michalczyk [37]. Macroplacoid length sequence is presented according to Kaczmarek et. al. [38]. Claws of Hypsibioidea were measured with the protocols as indicated in Beasley et. al. [21], whereas claws of Macrobiotoida were measured with the protocols as indicated in Kaczmarek and Michalczyk [37]. The *pt* ratio (which is the ratio of the length of a given structure to the length of the buccal tube), is expressed as a percentage [33] and its values are always provided in italics. This was done to distinguish them from absolute measurements in micrometers. Morphometric data were compiled using the Parachela Excel template version 1.6, which is available from the Tardigrada Register (<http://www.tardigrada.net/register/submit.htm>) [39].

Species were identified using the keys provided by Ramazzotti and Maucci [11], Claxton [40], Fontoura and Pilato [41], and the original descriptions/redescriptions [35,42–54].

2.4. Description of *Minibiotus citlalium* sp. nov.

The identity of specimens of genus *Minibiotus* was based on the description of Schuster et. al. [55] amended by Claxton [40]; both included in the definition of this genus by Pilato and Binda [56]. The characters observed in our specimens and diagnostic of this genus were presence of bucco-pharyngeal apparatus of the *Macrobiotus* type, *Minibiotus* variant: an antero-ventral mouth, a rigid, short and narrow buccal tube, short ventral lamina (*pt* < 62), stylet supports inserted at 73% or less of the buccal tube length, pharyngeal apophyses and placoids present, stylet furcae typically shaped, short macroplacoid row length (*pt* < 42), and claws of the *hufelandi* type with lunules present. The identity

of the *Minibiotus citlalium* sp. nov. was based on a character matrix of all described species of genus *Minibiotus*, which was built from the information contained in the original published descriptions. The characters used for the matrix were eyespot pigmentation, cuticular sculpturing, presence/absence of pores, pore shape, arrangement of pores, and pore size. Furthermore, the specimens were compared with the original descriptions of the most similar species: *Minibiotus vinciguerrae* Binda and Pilato, 1992; *Min. sidereus* Pilato et. al., 2003; *Min. constellatus* Michalczyk and Kaczmarek, 2003; *Min. eichhorni* Michalczyk and Kaczmarek, 2004; *Min. pseudostellarus* Roszkowska, Stec, Ciobanu, and Kaczmarek, 2016; and *Min. pentannulatus* Londoño, Daza, Lisi, and Quiroga, 2017. Given that *Min. sidereus*, *Min. constellatus* and *Min. pentannulatus* present a similar star-shaped pore sculpture pattern, compared to *Minibiotus citlalium* sp. nov., we requested photographs of these species to the authors of their descriptions for the comparison of their diagnostic characteristics.

To obtain more specimens and eggs of *Minibiotus citlalium* sp. nov. new moss samples were collected from the same stations and substrates (Table 1) where *Minibiotus* spp. were found (XX, Table 1). The samples were processed following the method described by Stec et. al. [57]. Ornamented eggs were individually placed in Petri dishes with mineral water at room temperature while waiting upon hatching.

Hatched specimens and eggs were used for light microscopy and processed as previously described; the eggs were measured based on Kaczmarek and Michalczyk [37]. For further refinement of initial observations, a subset of these material from the new species were processed for scanning electron microscopy (SEM). Briefly, specimens were first boiled in absolute ethanol and transferred to cold absolute ethanol. This was repeated three times. Then they were boiled again in absolute ethanol until complete evaporation. Finally, the specimens were mounted on metal plates and covered with gold. Specimens were examined in a Hitachi Scanning Electron Microscope S-2469N.

The description of *Minibiotus citlalium* sp. nov. was based on 16 animals and three eggs, one of them still hatching. In the description of *Min. citlalium* sp. nov. the diagnosis of the species and the differential diagnosis are included; both contain ranges of variation for some characters, while for others, the specific information obtained from the descriptions or from the images of the holotypes shown in the respective descriptions is included.

Information about diagnostic characters was consulted with the authors of the most similar species [58] (G. Pilato for *Min. sidereus*, Ł. Michalczyk for *Min. constellatus*, and R. Londoño for *Min. pentannulatus* pers. comm.)

Size Effect on Morphometric Data

Measurement and *pt* index of 29 morphological characters of animals corresponding to type series of *Minibiotus citlalium* sp. nov. are showed in Table 2. Because *pt* index only satisfactorily eliminates body size effects for isometric traits but do not eliminates from allometric ones [59,60]; we evaluate growth relation (isometric vs. allometric) in the continuous traits respect to body size in *Minibiotus citlalium* sp. nov., following the methodology proposed by Bartels et. al. [60]. The effect of “body size”, measured as buccal tube length (BTL), respect to 26 of 29 continuous body traits was evaluated, by mean a linear regression in each (Supplementary Material Table S1). Regressions were performed from log transformed values, omitting certain individuals if their orientation was unsuitable. The isometric or allometric trend was determinate in each trait, comparing the slope of each linear regression with a slope of 1; to do this, we performed *t*-tests ($t = (b-1)/SE$ of the slope, $df = n-20$) [61]. Regression analysis were carried out using PAST ver. 4.03 [62].

We provide supporting data for growth trends, as well the slope (b) and the Y intercept (a) for each trait analyzed. It has been suggested that *pt* index for each body trait should be reported together with the slope (b) and the Y intercept (a), both obtained from the regression of logBS (body size) vs. logY (Y = body trait). Whith these parameters Thorpe’s normalization can be performed yield any quantitative trait size-independent [60].

Table 2. Measurements and *pt* values of selected morphological structures of *Minibiotus citlalium* sp. nov. mounted in polivinil lactofenol medium. The individuals measured correspond to serial type (N- number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; and SD—standard deviation).

CHARACTER	N	RANGE		MEAN		SD		HOLOTYPE	
		µm	<i>pt</i>	µm	<i>pt</i>	µm	<i>pt</i>	µm	<i>pt</i>
Body length	15	142–250	507–925	195	746	37	121	142.4	507
Buccal tube									
Buccal tube length	16	20.2–28.7		26.1	-	2.4	-	28.1	-
Stylet support insertion point	16	12.1–17.0	57.7–61.5	15.6	59.9	1.5	1.0	17.0	60.2
Buccal tube external width	16	1.5–2.4	5.7–8.6	2.0	7.5	0.3	0.7	2.0	7.7
Buccal tube internal width	16	0.7–1.5	2.4–5.3	1.1	4.1	0.3	0.8	1.0	3.7
Ventral lamina length	15	9.2–13.4	44.6–49.1	12.1	46.3	1.3	1.3	12.9	45.9
Placoid length									
Macroplacoid 1	15	1.2–2.8	5.6–9.9	2.0	7.7	0.4	1.3	2.5	8.9
Macroplacoid 2	15	1.1–2.3	5.1–8.4	1.7	6.6	0.3	1.0	2.0	7.1
Macroplacoid 3	15	1.3–2.3	5.1–8.0	1.7	6.6	0.3	0.8	2.0	7.1
Microplacoid	15	0.5–1.1	1.9–3.8	0.7	2.8	0.2	0.6	0.9	3.0
Macroplacoid row	15	4.7–7.6	21.5–27.2	6.3	24.6	0.8	1.7	7.6	27.2
Placoid row	15	5.9–9.0	26.5–33.4	7.6	29.3	0.8	1.9	9.0	32.0
Claw 1 height									
External primary branch	15	3.1–5.8	11.8–20.7	4.6	17.4	0.9	2.8	5.8	20.7
External secondary branch	15	2.0–4.4	7.4–16.3	3.3	3.1	0.9	2.8	4.3	15.3
Internal primary branch	15	3.5–5.8	12.4–21.6	4.8	8.1	0.8	2.2	5.7	20.1
Internal secondary branch	15	2.9–4.9	10.4–18.2	3.9	14.8	0.7	2.1	4.2	15.1
Claw 2 height									
External primary branch	13	3.8–6.2	18.6–21.1	5.2	20.1.9	0.7	1.2	6.2	22.1
External secondary branch	12	2.5–5.3	12.1–19.6	4.0	15.3	0.9	2.4	4.7	16.9
Internal primary branch	13	3.3–6.0	14.8–21.5	4.8	18.7	0.9	2.3	6.0	21.5
Internal secondary branch	12	2.3–5.0	10.3–17.9	3.9	14.8	0.8	2.1	4.5	16.0
Claw 3 height									
External primary branch	14	3.8–5.7	17.0–22.1	4.9	18.8	0.5	1.5	4.8	17.0
External secondary branch	13	2.9–4.6	13.0–17.4	3.7	14.5	0.5	1.2	4.1	14.2
Internal primary branch	14	3.6–5.9	16.7–22.9	4.9	19.1	0.7	1.7	5.0	17.8
Internal secondary branch	13	2.7–5.3	12.7–18.6	3.9	15.2	0.8	1.8	4.5	16.0
Claw 4 length									
Anterior primary branch	13	3.3–6.9	14.0–25.8	5.6	21.3	1.1	3.0	5.5	19.5
Anterior secondary branch	12	2.5–5.3	10.7–20.5	4.4	16.6	1.1	3.3	4.6	16.5
Posterior primary branch	11	3.1–7.0	13.2–26.3	5.7	21.57	1.3	3.8	-	-
Posterior secondary branch	11	2.1–5.9	10.4–21.8	4.0	15.2	1.1	3.2	-	-

3. Results

Tardigrades were observed in 32 out of the 57 samples examined (ca. 56%). In total, 233 tardigrades, 20 exuviae, and 40 free-laid eggs were found. Only 13 eggs were identified at a specific level. The tardigrades belong to seven genera within three eutardigrade families: Calohypsibiidae: *Calohypsibius* cf. *ornatus* ($n = 5$ individuals); Hypsibiidae: *Diphascon mitrense* ($n = 2$), *Diphascon pingue* ($n = 11$), *Hypsibius* cf. *microps* ($n = 7$), *Hypsibius* cf. *pallidus* ($n = 10$), *Adropion scoticum* ($n = 51$), and *Pilatobius nodulosus* ($n = 32$); and Macrobiotidae: *Macrobiotus* spp. ($n = 24$), *Minibiotus sidereus* ($n = 50$), and *Minibiotus citlalium* sp. nov. ($n = 40$).

Figures of habitus, buccal apparatus, claws, and specific details of cuticle for each species are presented in Supplementary Material (Figures S1–S7).

Taxonomic Accounts

Family: Calohypsibiidae Pilato, 1969 [63].

Calohypsibius cf. *ornatus* [64].

Supplementary Material (Figure S1).

Material examined: five specimens XLV (5).

Remarks: The specimens correspond to the key proposed for the genus by Michalczyk and Kaczmarek [65] and the most recent revision of Calohypsibiidae by Gąsiorek et. al. [8]. We identified the presence of eight transverse lines of dorsal spines; however, we could not assign the specimens to *Calohypsibius ornatus sensu stricto*, because this taxon has a high intraspecific variability. The presence of several species has been suggested under this name but there is no detailed redescription [8,65]. *Calohypsibius ornatus sensu lato* has been reported from North America, in Greenland, Canada, and USA [66], and from South America, in Bolivia, Argentina, and Colombia [67].

Family: Hypsibiidae Pilato, 1969 [63].

Subfamily: Diphasconinae Diphasconinae Dastych, 1992 [68].

Genus: *Diphascon* Plate, 1888 [69].

Diphascon mitrense Pilato, Binda, and Qualtieri, 1999 [48].

Supplementary Material (Figure S2a,b).

Material examined: IV (1) and X (1).

Remarks: The specimens correspond well to the key proposed by Fontoura and Pilato [41]. *Diphascon mitrense* belongs to the *pingue*-species group, which is a complex of 10 morphologically homogeneous species [41].

This species has been only recorded in Argentina [67]; therefore, the specimens described in this study is the first record from Mexico and North America.

Diphascon pingue (Marcus, 1936) [70] *sensu lato*

Supplementary Material (Figure S2c,d)

Material examined: IV (1), IX (1), X (2), XXXI (3), XXXIII (1), and XXXVI (3).

Remarks: It is believed that *Diphascon pingue sensu lato* has a cosmopolitan distribution [71]. This species has been reported from North America in Greenland, Canada, and USA [66]; from Central America in Costa Rica [72]; and from South America, in Argentina, Bolivia, Brazil, and Ecuador [67]. From Mexico, it has been recently reported in temperate mountains of Sierra Madre Oriental [15].

Subfamily: Hypsibiinae Pilato, 1969 [63].

Genus: *Hypsibius* Ehrenberg, 1848 [73].

Hypsibius spp.

Supplementary Material (Figure S3).

Remarks: Based on the characters: absence of cuticular bars on legs I–III, the *pt* of stylet support insertion point, the external and posterior primary claw branches length, the *pt* of the external buccal tube width, and the *pt* of the septulum, and following the key by Gąsiorek et. al. [44], *Hypsibius dujardini* (Doyère, 1840) (Figure S3a–c) and *Hypsibius exemplaris* Gąsiorek, Stec, Morek, and Michalczyk, 2018 (Figure S3d–f), were recognized. However, few specimens were found, which prevents a comparison of its morphological variation for an identification. Both species are a member of the *dujardini*-species group.

Hypsibius dujardini sensu lato is a species group [44] with an apparent global distribution [71]; it has been reported from North America, in Canada, Greenland, and USA [66]; from Central America in Costa Rica [72]; and from South America, in Argentina, Bolivia, Chile and Uruguay [67]. On the other hand, *Hys. exemplaris* has only been recorded from England [44].

Hypsibius cf. *microps*.

Supplementary Material (Figure S3g–i).

Material examined: IX (1), XVI (1), XXXI (3), and XXXVI (2).

Remarks: All collected specimens showed a short claw base and therefore the primary branches seemed to be attached closely to the base of the secondary claw (Figure S3i), characters that allow to differentiate this species from *Hypsibius pallidus* Thulin, 1911, (amended by Kaczmarek and Michalczyk, 2009) [42]. However, they differ in the ranges of other body measurements (body length, buccal tube length, macroplacoid 1 length, and base and branches of claw 3) provided in the re-description of *Hypsibius microps* Thulin, 1928 (amended by Kaczmarek and Michalczyk, 2009) [42]. Additionally, we observed presence of the minute dot-like septula at the end of the placoids row (Figure S3h). A robust morphometric analysis is necessary to evaluate the morphological variation of this species and confirm the specific identity of the specimens.

Hypsibius microps is a member of *convergens* species group [42,44]. This species *sensu lato* is largely Holarctic [71] and has been recorded from North America, in Canada, Greenland, and USA [66]; from Central America in Costa Rica [72]; and from South America, in Argentina, Brazil and Uruguay [67].

Hypsibius cf. pallidus.

Supplementary Material (Figure S3j–l).

Material examined: IX (2), XXXI (3), XXXIII (1), XXXV (1), and XXXVI (3).

Remarks: All collected specimens show a long claw base and the primary branches seem to be connected at a high level of the secondary claw (Figure S3l), characters that allow to differentiate this species from *Hys. microps* [42]. However, they differ in the ranges of other body measurements (body length, macroplacoid 1 length, macroplacoid row length, and base and branches of claw 1 and 4) provided in the redescription of this species by the same authors. Additionally, we observed presence of the minute dot-like septula at the end of the placoids row (Figure S3k). A morphometric analysis is necessary to evaluate the morphological variation of this species and confirm the specific identity of the specimens.

Hypsibius pallidus is a member of *convergens* species group [42,44]; this species *sensu lato* has been reported from North America, in Canada, Greenland, USA and Mexico [11,66]; and from South America, in Argentina, Bolivia and Chile [67].

Subfamily: Itaquasconinae Rudescu, 1964 [74].

Genus: *Adropion* Pilato, 1987 [34].

Adropion scoticum [75] (Murray, 1905) *sensu lato*.

Supplementary Material (Figure S4a–e).

Material examined: XXIX (41), XXXI (2), XL (3), XLII (2), and XLVII (3).

Remarks: The collected specimens showed the characters proposed by Murray [75] and Li and Liu [76]. *Adropion scoticum sensu lato* is a cosmopolitan complex of remarkably similar species, which needs an integrative taxonomic review [66]. Additionally, there is no known type material of *Adr. scoticum sensu stricto* [66,77]; therefore, a redescription is required. This species has been reported from North America, in Canada, Greenland and USA [66]; from Central America in Costa Rica [72]; and from South America in Argentina, Bolivia, Brazil, Chile, Colombia, Peru, and Uruguay [67]. In this study, we present a new record from Mexico, which expands its distribution into North America.

Subfamily: Pilatobiinae Bertolani, Guidetti, Marchioro, Altiero, Rebecchi, and Cesari, 2014 [7].

Genus: *Pilatobius* Bertolani, Guidetti, Marchioro, Altiero, Rebecchi, and Cesari, 2014 [7].

Pilatobius nodulosus (Ramazzotti, 1957) [78].

Supplementary Material (Figure S5a–c).

Material examined: XX (7), XXX (2), XXXI (7), XXXIII (4), XXXIV (4), and XXXV (8).

Remarks: All collected specimens display the characters mentioned for this species in Ramazzotti [78]. *Pilatobius nodulosus* has been recorded from North America, in Canada, USA, and Mexico [66,72]. The Mexican record corresponds to Beasley [14], in the Popocatepetl volcano, near the tree line in open pine forest, and in temperate mountains in northern Mexico [15].

Family: Macrobiotidae Thulin, 1928 [79].

Genus: *Macrobiotus* Schultze, 1834 [80].

Macrobiotus spp.

Supplementary Material (Figure S6a–h).

Material examined: IV (2), XV (1), XLV (2), XLVI (7), XLIX (1), L (1), and LI (10).

Remarks: Based on the revision of the *hufelandi* group by Kaczmarek and Michalczyk [37], two groups of specimens were recognized based on the presence of two types of oral cavity armature (OCA): the *maculatus* and the *patagonicus* types, in the *maculatus* morphotype only the third band of teeth could be observed (Figure S6b,c), while in the *patagonicus* morphotype the second and the third bands of teeth can be recognized (Figure S6f,g). Within both groups, the specimens showed conspicuous morphological differences (Figure S6a,e), in the sculpture of the cuticle, size of the claws, and presence and ornamentation of lunules (Figure S6d,h). This suggests the occurrence of more than one species sampled.

Species identification can only be made through observation of the eggs; unfortunately, the eggs found could not be identified at a specific level, due to their poor condition.

Genus: *Minibiotus* Schuster, 1980 [55].

Minibiotus sidereus Pilato, Binda and Lisi, 2003 [49].

Supplementary Material (Figure S7a–i).

Material examined: XX (4), XXX (4), XXXIII (5 and 5 eggs), XL (10), XLVIII (15), LIII (7 and 5 eggs), and LIV (6).

Remarks: All collected specimens show the characters of *Min. sidereus* proposed by Pilato et. al. [49]. Cuticle with three types pores (“pearls”, *sensu* Pilato et. al. [49]): circular, elliptical, and star-shaped pores (Figure S7b–d). The largest star-shaped pores are visible on the head and on the legs; prominent large star-shaped pores, with many arms (5–7) are present on each leg of the fourth pair, which should be noted as a specific character (Figure S7g).

Minibiotus sidereus has only been recorded in Ecuador [49]; therefore, the specimens described in this study are the first record from Mexico and North America.

Minibiotus citlalium sp. nov. Dueñas-Cedillo and García-Román

<http://zoobank.org/urn:lsid:zoobank.org:pub:115FFBE7-B7B7-4A8E-9AD6-DFDEB1648F2C>

(Figures 2–9 Tables 2–4)

Diagnosis

Macroplacoid length sequence $1 > 2 = 3$ (Figure 2a–d); abundant pores (rounded and star-shaped), more than 250 on the entire dorsal cuticular surface. Each leg with two star-shaped pores on external surface, one of them conspicuously larger; the larger ones three times larger than the smaller ones (Figure 3a–d). Under PCM egg processes with inconspicuous ornamentation, and under SEM egg processes with four or five barely visible annulations (see egg section).

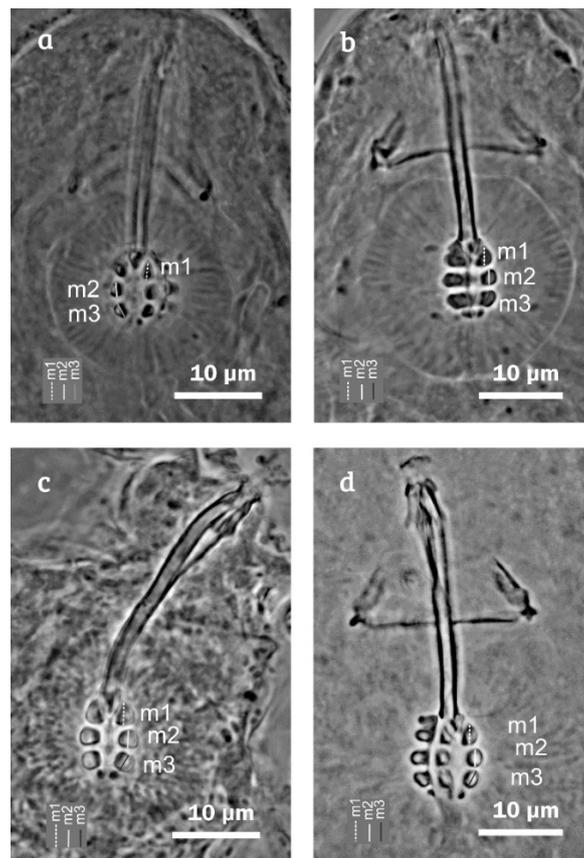


Figure 2. *Minibiotus citlalium* sp. nov. bucco-pharyngeal apparatus. (a) Paratype 2 (dorsal view, phase contrast microscopy (PCM)); (b) paratype 11 (dorsal view, PCM); (c) paratype 10 (dorsal view, PCM); and (d) paratype 12 (dorsal view, PCM), m1 macroplacoid 1, m2 macroplacoid 2, and m3 macroplacoid 3.

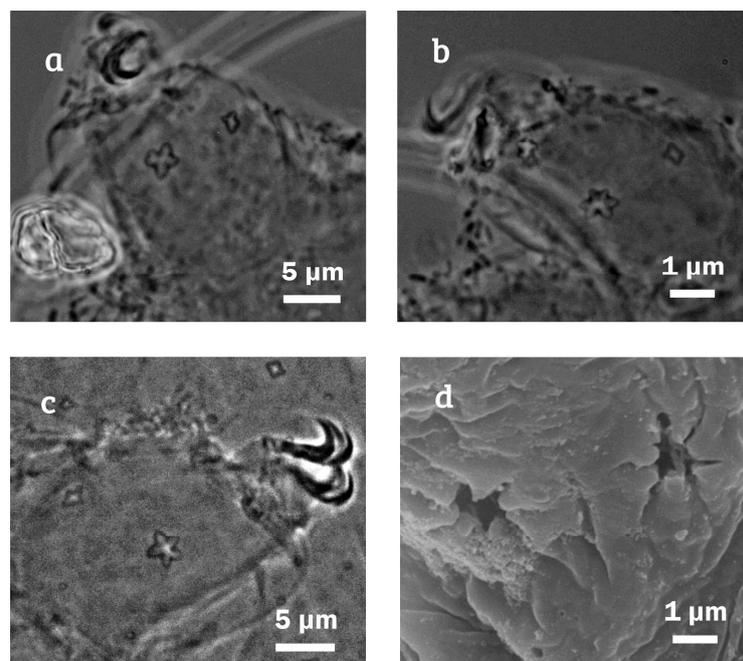


Figure 3. *Minibiotus citlalium* sp. nov. pair of star-shaped pores, one of them conspicuously larger. (a) Leg II, paratype 1 (PCM); (b) leg III, paratype 1 (PCM); (c) leg II, paratype 11 (PCM); and (d) leg III with both star-shaped pores on external surface (SEM).

Type Locality

Iztaccíhuatl volcano, 19°05'14" N, 98°40'03" W, ca. 3498 m asl, *Abies religiosa* and *Cupressus lusitanica* Mill. forest, Cañada El Paraje, moss on tree bark, January 2018, coll. Dueñas-Cedillo and Armendáriz-Toledano.

Type Material and Type Repository

Holotype (slide: TAR/001), 15 paratypes (slides: TAR/002–TAR/16) and 14 simplex specimens. Holotype and nine paratypes (hatched animals) and two eggs (one of them hatching) were deposited in the Colección Nacional de Insectos, Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México. Additionally, six paratypes and one egg were deposited in Laboratorio de Ecología, Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional.

Etymology

The specific epithet *citlali* is a substantive in genitive that refers to the Náhuatl word *citlali*, meaning star, due to the presence of star-shaped pores throughout the body. The Náhuatl is an original language spoken by ancient Mexicans in central Mexico from the fifth century AD to present.

Description of Holotype

Measurements and statistics are shown in Table 2. **Animals:** Body length 142.4 µm [507] (Figure 4), eye spots visible in three specimens after fixation in PVA medium (Figure 4a). The entire cuticle is smooth and exhibits numerous pores (including the legs) with variable shape (Figure 4), such as rounded (Figure 5a,b), multi-lobated (4 tips) (Figure 5c,d) and star-shaped (5–6 tips) (Figure 5e,f). In the holotype, up to 278 pores on the entire dorsal cuticular surface were observed (Figure 4a). Additionally, pores increasing in abundance from anterior (48 pores) to the posterior (116 pores) portions of the body (Figure 4a,b); 83% of the pores correspond to rounded, 10% to multi-lobated (4 tips), and 7% to star-shaped (5–6 tips); the abundance of last two pores types are from 6–10 and from 2–10 respectively (Table 3). The rounded ones are smaller (0.41–0.86 µm; Figure 5a,b) than the multi-lobated (0.92–2.38 µm; Figure 5c,d) or star-shaped ones (2.19–3.5 µm; Figure 5e,f). The star-shaped pores are larger at the cephalic and caudal regions of the body, these star-shaped pores are arranged in the cuticle in 11 transverse rows, which become double in the segments of the legs I–III (Figure 4). In the ventral region, the star-shaped pores are less numerous than in the dorsal region and are distributed in eight transversal rows from segment of legs I up to before legs IV (Figure 4c,d).

Table 3. Number of star-shaped pores of four and five tips, the most abundant, on dorsal cuticle of *Minibiotus citlali* sp. nov. mounted in polivinil lactofenol medium. The individuals measured correspond to type series (N—number of specimens measured, RANGE refers to the smallest and the largest structure among all measured specimens; and SD—standard deviation).

CHARACTER	N	RANGE	MEAN	SD
Number of pores on cuticle				
Multi-lobated pores				
1. up to legs I	3	6	6	
2. up to legs II	3	5–8	6.67	1.11
3. up to legs III	3	8–14	10.67	2.22
4. up to legs IV	3	8–9	8.67	0.44
Star-shaped pores				
1. up to legs I	3	6–10	7.67	1.56
2. up to legs II	3	5–8	6.33	1.11
3. up to legs III	3	2–4	3.67	1.11
4. up to legs IV	3	7–10	8.33	1.11

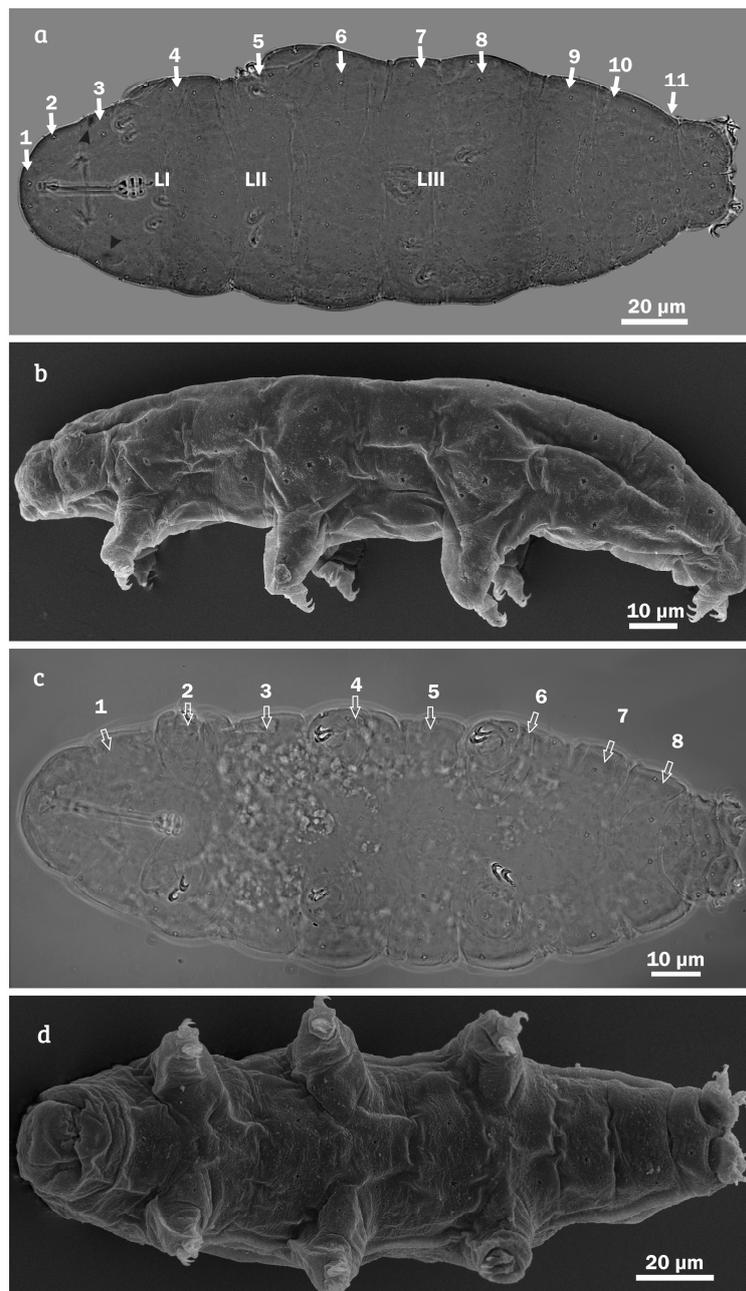


Figure 4. *Minibiotus citlalium* sp. nov. habitus. (a) Dorsal view, showing 11 transverse rows of pores (holotype, PCM), full arrows indicate the 11 transverse rows of pores (dorsal view); LI leg I, LII leg II, LIII leg III, black arrowhead indicate eyes spots; (b) lateral view (SEM); (c) ventral view, showing eight transverse rows of pores (holotype, PCM), empty arrows indicate the eight transverse rows of pores (ventral view); and (d) ventral view (SEM).

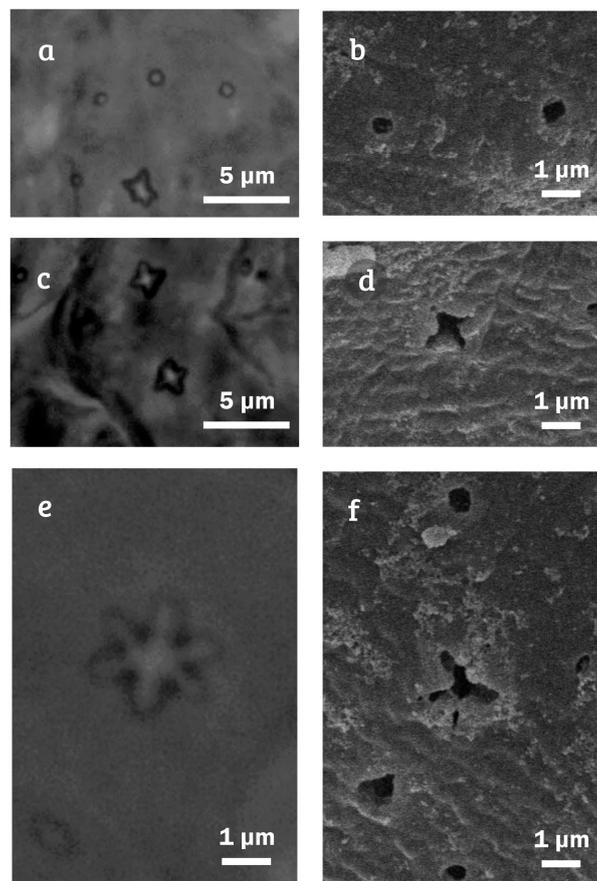


Figure 5. *Minibiotus citlalium* sp. nov. pores type on cuticle: (a) dorsal cuticle with rounded pores (holotype, PCM); (b) dorsal cuticle with rounded pores, with (SEM); (c) dorsal cuticle with multi-lobated (4 tips) pores (holotype, PCM); (d) dorsal cuticle with multi-lobated (4 tips) pores (SEM); (e) dorsal cuticle with star-shaped (5–6 tips) pores (holotype, PCM); and (f) dorsal cuticle with star-shaped (5–6 tips) pores (SEM).

Table 4. Measurements [in μm] of selected morphological structures of eggs of *Minibiotus citlalium* sp. nov. mounted in polivinil lactofenol medium (N—number of eggs/structures measured, RANGE—refers to smallest and largest structure among all measured specimens; and SD—standard deviation).

CHARACTER	N	RANGE	MEAN	SD
Diameter of egg without processes	3	48.8–60.8	54.6	6.0
Diameter of egg with processes	3	57.4–66.0	60.9	4.5
Process height	9	3.6–5.1	4.4	0.5
Process base width	8	1.8–2.6	2.1	0.3
Process base/height ratio	8	37–57%	48%	6%
Distance between processes	9	1.2–2.6	1.8	0.4
Number of processes on the egg circumference	2	28–33	30.5	3.5

Bucco-pharyngeal apparatus of the *Minibiotus* type, with five pores around the mouth and ten peribuccal papulae (Figure 6a–c). Oval pharyngeal bulb with triangular apophyses (near to first macroplacoid and about the same size), three macroplacoids and a small microplacoid. Macroplacoid shapes were drop-shaped (m1), granular (m2), and granular or almost quadrangular (m3); macroplacoid length sequence $1 > 2 = 3$ (Figure 2; Table 2). The oral cavity armature is composed of two bands of teeth, which are not visible under PCM and at least the first band of teeth is well visible under SEM (Figure 6d).

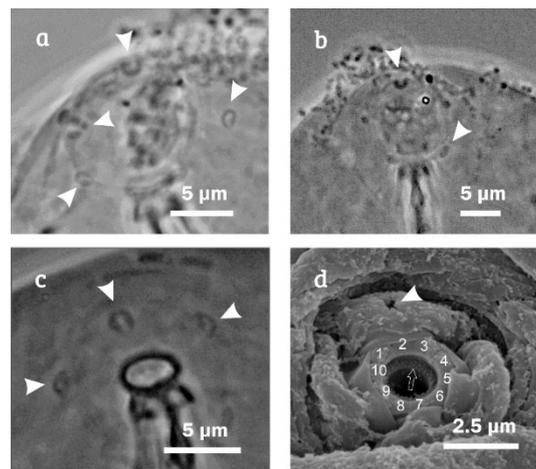


Figure 6. *Minibiotus citlalium* sp. nov. detail of the buccal opening with five pores around mouth: (a) paratype 15 (PCM); (b) paratype 13 (PCM); (c) holotype (PCM), white arrowhead indicates the buccal pores; and (d) detail of the buccal opening (ventral view, SEM) showing a buccal pore, the peribuccal papulae are show numbered 1 to 10, empty arrow indicates the teeth.

Legs show no cuticular bars and other thickenings (Figure 4); each leg with both small and large star-shaped pores on external surface, the larger ones three times larger than the smaller ones (Figure 3). Fine granulation exclusive on legs IV, on dorsal surface (Figure 7a,b,e) and a large star-shaped pore (5–6 tips) present in each leg (Figure 7c,d), with diameter of 2.4 μm in the holotype. Claws are short and robust, like the *hufelandi* type with conspicuous accessory points (Figure 7a–f). Lunules under all claws with smooth margins (Figure 7f).

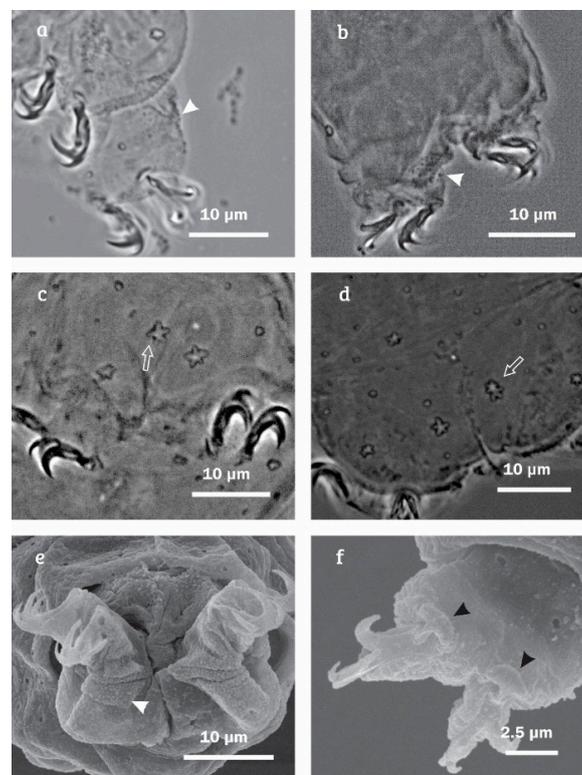


Figure 7. *Minibiotus citlalium* sp. nov. detail of legs IV: (a) granulation (paratype 14, PCM); (b) granulation (paratype 5, PCM); (c) star-shaped pores (paratype 13, PCM); (d) star-shaped pores (holotype, PCM, empty arrow indicate the pair of star-shaped pores); (e) granulation on dorsal surface (SEM, white arrowheads indicate granulation); and (f) claw IV with smooth lunules (SEM, black arrowheads).

Eggs. (measurements and statistics in Table 4): freely laid, colorless, spherical, smooth surface (Figure 8a,b) under PCM processes with inconspicuous ornamentation, only visible in few instances; if visible, it showed between three to four annulations (Figure 8a), under SEM processes with four annulations, five in some cases barely visible (Figure 8c); processes longer than width (37–57%), tipped processes flexible (1.53–2.08 μm , Figure 8a,b), and entire base process (Figure 8c). Among the eggs, one from a specimen hatching was observed (Figure 8d). On this individual, the oral apparatus and the star-shaped pores were observed (Figure 8e), as well as a pair of star-shaped pores, one of them conspicuously, on external surface of the legs III (Figure 8f), both diagnostic characters of *Minibiotus citlalium* sp. nov.

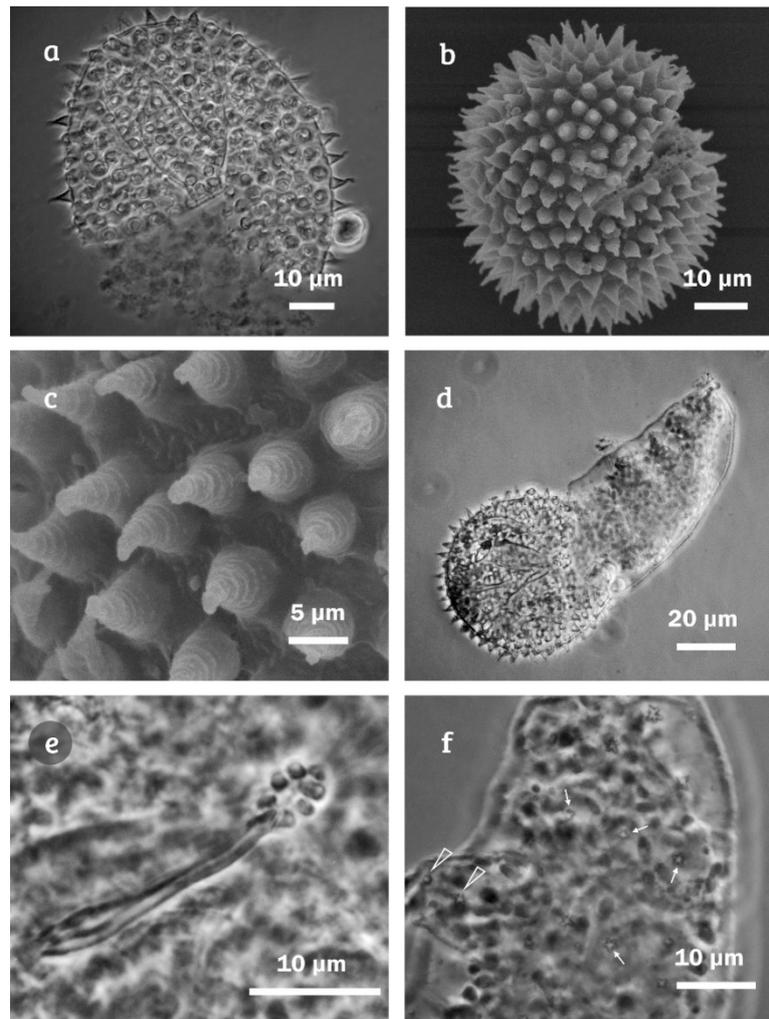


Figure 8. *Minibiotus citlalium* sp. nov. eggs: (a) egg surface (PCM); (b) egg surface (SEM); (c) egg processes showing four annulations (SEM); (d) hatching specimen (PCM); (e) detail of the embryo showing buccal apparatus; and (f) detail of cuticle of hatching specimen, arrows indicates a star-shaped pores, a pair of star-shaped pores, one of them conspicuously larger.

Differential diagnosis.

By the presence of star-shaped pores in the cuticle, *Minibiotus citlalium* sp. nov (Figure 9a) is similar to *M. pseudostellarus*, *M. eichhorni*, *M. constellatus*, *M. sidereus* and *Min. pentannulatus*, but differs from the first two species by the presence of eleven transverse rows of star-shaped pores, which are also present in *M. constellatus* (Figure 9b), *M. sidereus* (Figure 9c) and *Min. pentannulatus* (Figure 9d). The star-shaped pores in *M. pseudostellarus* are randomly distributed [81], and in *M. eichhorni* are arranged in six transverse rows [82]. The new species differs specifically from *M. constellatus*, *M. sidereus*, and *M.*

pentannulatus by two attributes, (1) different macroplacoid sequence $1 > 2 = 3$, and (2), each leg (I–III) with two star-shaped pores on external surface, one of them conspicuously larger; the larger ones three times larger than the smaller ones. In *M. constellatus* the macroplacoid sequence is $1 > 2 > 3$, in *M. sidereus* and *M. pentannulatus* is $1 > 2 < 3$; in *M. constellatus* each leg with almost two star-shaped pores of similar size, in *M. sidereus* about three star-shaped pores in each leg, and in *M. pentannulatus* almost four star-shaped pores in each leg. Additionally, *Min. citlalium* sp. nov. differs from:

(1) *Minibiotus constellatus*, only recorded from Peru [51], by absence of granulation on legs I–III (granulation present on all legs in *M. constellatus*).

(2) *Minibiotus sidereus*, only recorded from Ecuador [49], by shorter placoid row (5.9–9.0 μm (26.5–33.4) in *M. citlalium* sp. nov. vs. 9.6 μm (36.4) in *M. sidereus*); smaller star-shaped pores of legs IV (2.9 μm in *M. citlalium* sp. nov. vs. 6.4 μm in *M. sidereus*); smaller diameter egg including processes (57.4–66.0 μm in *M. citlalium* sp. nov. vs. 73–83.5 μm in *M. sidereus*) and excluding them (48.8–60.8 μm in *M. citlalium* sp. nov. vs. 62–69 μm in *M. sidereus*); shorter processes (4.4 μm in *M. citlalium* sp. nov. vs. up to 9.5 μm in *M. sidereus*), inconspicuous annulations or absent on most processes. When these are present, 3–4 can be recognized in *M. citlalium* sp. nov., while 6–7 conspicuous annulations are present on the most processes in *M. sidereus*.

(3) *Minibiotus pentannulatus*, recorded from Colombia [54] and Tanzania [83] by the pattern of star-shaped pores arranged in 11 transverse rows (double in the segments of the legs I–III) was present in all specimens, which ranged from 142.0–250.0 μm in length, whereas in *M. pentannulatus* the pattern of star-shaped pores can only be recognized in the smaller animals from 144–203 μm . *Minibiotus citlalium* sp. nov. also differ from *M. pentannulatus* by the *pt* of some claws, as follows: of the claw 1, by lower *pt* of the external primary branch (11.8–20.7 in *M. citlalium* sp. nov. vs. 21.3–24.8 in *Min. pentannulatus*); of the claw 2, by lower *pt* of the internal primary branch (14.8–21.5 in *M. citlalium* sp. nov. vs. 22.1–26.0) in *M. pentannulatus*, also of the claw 2 by lower *pt* of the internal secondary branch (10.3–17.9 in *Min. citlalium* sp. ov. vs. 18.0–21.2 in *M. pentannulatus*); and of claw 4 by lower *pt* of the anterior primary branch (14.0–25.8 in *M. citlalium* sp. nov. vs. 26.2–29.2 in *M. pentannulatus*), also of claw 4 by lower *pt* of the posterior primary branch (13.2–26.3 in *M. citlalium* sp. nov. vs. 27.4–28.9 in *M. pentannulatus*); egg with smaller diameter with processes (60.9 μm in *M. citlalium* sp. nov. vs. 74.5 μm in *M. pentannulatus*), and by the pattern of annulations in the processes (see comparison with *M. sidereus*), 4 to 6 conspicuous annulations are present on the processes in *M. pentannulatus*.

Stec et. al. [83] registered a population of *Minibiotus pentannulatus* in Tanzania; these authors add characters to those proposed by Londoño et. al. [54] for *M. pentannulatus*. These characters also support the differences between this species and *M. citlalium* sp. nov. These characters are granulation on legs I to IV, differ from *M. citlalium* sp. nov. which only presents granulation on legs IV (Figure 7a–c); *M. pentannulatus* presents up to three star-shaped pores of different size, on external surface of legs I–III, the smaller star-shaped pore always present in the centre of the above mentioned granulation patch, meanwhile *M. citlalium* sp. nov. present a pair of star-shaped pores (one smaller than the other one on external surface of legs I– III), and both pores are above smooth cuticle (Figure 3); and in *M. pentannulatus* a cuticular fold with a pore in the centre is present just above the mouth opening and visible well under PCM (only in laterally positioned specimens) and under SEM; meanwhile, in *M. citlalium* sp. nov. five pores around the mouth are present (Figure 6).

Size effect on morphometric data.

Linear regressions of 19 out of 26 continuous body traits of *Minibiotus citlalium* sp. nov. showed significant correlation with BTL (Supplementary Material Table S1), most of them with r^2 values higher than 0.8. Placoid lengths were uncorrelated with BTL. Of the 19 correlated traits, t-tests from slopes supported that 12 were isometric (slope value not significantly different from 1) and seven were allometric (slope value significantly different from 1) (SM Table S1), the relationship between the trait (Y) and BTL (=body size) (i.e., b-slope) and theoretical values of traits adjusted to mean BTL (Y-intercept) of this species are provided in SM Table S1.

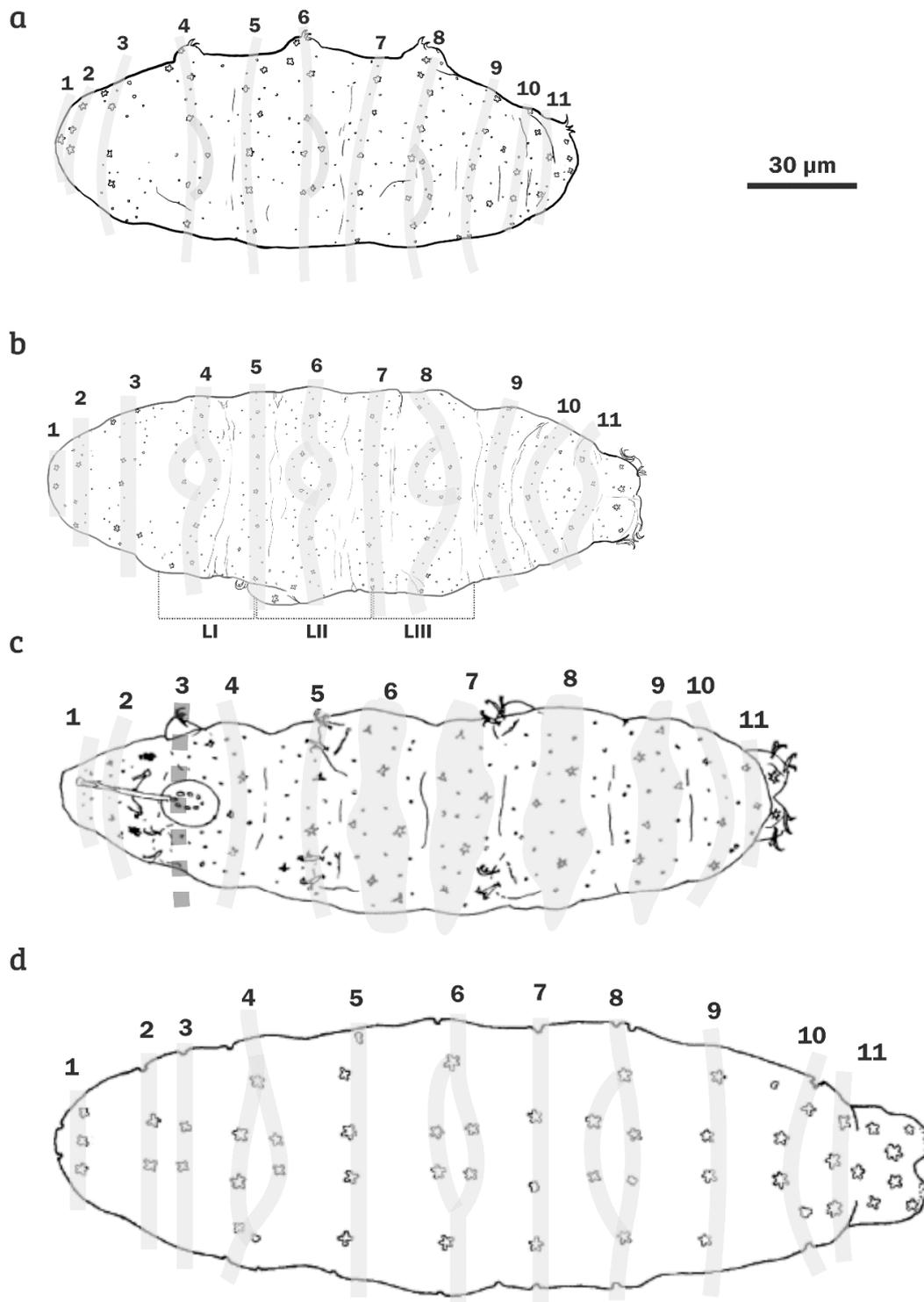


Figure 9. A semi-schematic drawing of a dorsal positioned animal showing a pattern of the dorsal cuticle of star-shaped pores arranged in 11 transverse rows (Arabic numbers), which become double in the segments of the legs I–III: (a) *Min. citlalium* sp. nov.; (b) *Min. constellatus*; (c) *Min. sidereus*; and (d) *Min. pentannulatus*. LI = segment of leg I, LII: segment of leg II, and LIII = segment of leg III.

4. Discussion

As noted in the introduction, the tardigrades of Mexico require more extensive taxonomic studies, and currently, only a few faunistic surveys have been published (e.g., [7,13–22]). Most of the previous

studies have provided an inventory of tardigrade fauna but from non-systematic samplings. Moreover, the number and size of samples and collection sites were rarely mentioned.

In the present study, from a systematic sampling of 57 moss samples, along an altitudinal gradient, including three moss substrates and four vegetation types, five tardigrade species were recorded (*Dip mitrense*, *Dip. pingue*, *Adr scoticum*, *Pil. nodulosus*, *Min sidereus*), three putative species could be associated (i.e., records with insufficient data to complete the identification: *Cal* cf. *ornatus*, *Hys* cf. *microps*, *Hys* cf. *pallidus*, and *Macrobiotus* spp.), and a new taxon *Min. citlalium* sp. nov., was described. For each of these taxa, description and illustrations were provided.

Geographic records

Our study confirms the presence of *Diphyscon pingue* and *Pilatobius nodulosus* species previously recorded in Mexico and provides three new records in the country expanding their distribution in America and in some cases connecting the previous records among North, Central and South America. The presence of *Dip. mitrense*, *Min. sidereus*, and *Adr. scoticum* in Iztaccíhuatl volcano constitute three new Tardigrada records from Mexico, the first two species also new records for North America. *Diphyscon mitrense* and *Min. sidereus* had only been recorded in their respective type localities, Argentina, and Ecuador [48,49]; therefore, this study provides the second record in America for both taxa, expanding their distribution to North America. On the other hand, *Adr. scoticum* record connects the previous records in North America [66], with Central and South America [67,72]. Iztaccíhuatl records of *Diphyscon pingue* and *Pil. nodulosus* demonstrate their occurrence in Mexico, since both taxa have been already recorded from Nuevo León state, [14,15] and in the Sierra Nevada in Popocatepetl volcano [14], respectively.

Additionally, the majority of examined species (*Cal. cf. ornatus*, *Hys. cf. microps*, *Hys. cf. pallidus*) belong to taxonomically difficult groups, or in some cases to species complexes as *Hys. dujardini* and *Dip. pingue*. These species groups and complex groups display high levels of morphological intra-specific variation from apparently wide geographic ranges in America and other areas around the world [66,67,72], which makes it difficult to interpret the records of this survey in a geographical context. Further studies considering both morphological and molecular data will clarify the taxonomic identity and distribution of their members.

Tardigrades community.

As mentioned above, Popocatepetl and Iztaccíhuatl volcanoes are the main physiographic elements of the Sierra Nevada; they present similar environmental conditions, landscapes, climate, seasonal rain regime, and same vegetation types [24]. Given these common characteristics and geographic proximity of both volcanoes, a similar species composition pattern between Iztaccíhuatl and that recorded previously for Popocatepetl [14] was expected. Although we found an equivalent number of species in both volcanoes, eight in Popocatepetl (*Echiniscus kerguelensis*, *Minesium tardigradum*, *Macrobiotus echinogenitus*, *Mac. furcatus*, *Mac. hufelandi*, *Ramazottius baumannii*, *Ram. oberhaeuseri*, and *Pilatobius nodulosus*), and five and three putative species in Iztaccíhuatl volcano, the species composition was dramatically different, because only one species, *Pil. nodulosus*, was present in both volcanoes. In the present study, *Pil. nodulosus* was found in moss growing on tree bark and soil, from 3400 to 4000 m asl, across the *Abies religiosa* and *Pinus hartwegii* forests. In the Popocatepetl volcano, it was found in the lichen *Pseudevernia intensa* (Nyl.) Hale and W. L. Culb., growing on bark at 4000 m asl, near the timberline in open *Pinus hartwegii* forest. This species has already been registered in *Pinus* and *Abies* forests, in soil, lichen, leaf litter, and moss growing on bark, and soil in North America [66,71]). Unfortunately, biogeographic or biological comparisons cannot be made because most of the records documented for the Popocatepetl are currently considered doubtful by other authors, as they were supported by a single collection event and the identification was based on the original descriptions available, which in turn was vague and barely detailed [10,14,37,84–86]. Despite that the taxa recorded in the Popocatepetl have been found in different regions of the world [71], recent studies based on morphological and molecular data have shown that they belong to taxonomically difficult species complexes [37,44,87–89].

To adequately compare the tardigrades from both volcanoes, it would be necessary to carry out an appropriate sampling in the Popocatepetl volcano and include both morphological and molecular data. This will allow the clarification of its tardigrade diversity and to propose additional ecological studies that include environmental variables to understand the differences in tardigrade communities, since it is common that tardigrade assemblages display ecological structure across mountain ranges. For example, in the Sierra de Guadarrama (Spain), Guil et. al. [27] found that the richness and abundance of tardigrades were associated with environmental variables at both macro (altitude, vegetation structure, climate, and soil characteristics) and micro (leaf litter type and moss weight) scales.

Systematic considerations.

Minibiotus citlalium sp. nov. share with *Min. constellatus*, *Min. sidereus* and *Min. pentannulatus* abundant star-shaped pores in anterior and posterior parts of the body, the distribution pattern of these ornamentations in dorsal cuticle arranged in eleven rows, and similar morphology of eggshell with ringed processes. They also share a holotropical distribution in American and African continent: *Min. constellatus* recorded in Peru, *Min. sidereus* in Ecuador and Mexico, and *Min. pentannulatus* in Colombia and Tanzania. The above characteristics support that these taxa conform a species-group, within which *Minibiotus citlalium* sp. nov. and *Min. sidereus* are the most similar in morphology; they present larger stars in the anterior and posterior regions of the body, show a clearly larger pair of stars in the fourth pair of legs, as well as a very similar eggshell. Furthermore, in the present study both species were found in the same samples, so apparently, they also share habitat characteristics. A phylogenetic reconstruction via molecular markers will allow help to clarify their evolutionary relationships.

Allometry in morphometric traits of *Min. citlalium* sp. nov.

In Eutardigrada taxonomy, many continuous traits display correlations with body size [90–92]. A considerably proportion of these characters grow proportionally with this trait (i.e., isometric traits), while in others the growth is not proportional (i.e., allometric ones), which in turn makes them unsuitable for taxonomic purposes [93]. As in other Eutardigrada members that display many allometric quantitative traits with respect to BTL [60], our regression analyses of morphometric data from *Min. citlalium* sp. nov., support that seven traits were allometric and 11 traits were isometric relative to BTL (SM Table S1), indicating that this trend is extensive in eutardigrades.

Ratios (*pt* indexes) are widely used to eliminate body size effects [33,92,93]; however, their use only successfully eliminate this effects in traits that increase proportionally to body size, but not in those that increase unproportionally (i.e., allometric ones) [59]. To overcome this problem, in this study a protocol [60] that provide parameters from regressions was performed, to obtain the slope (*b*) and *Y* intercept (*a*) in each analyzed trait. This in turn will allow to perform Thorpe normalization and to obtain size-effect free traits, independently of their correlation trend respect to body size (isometric or allometric).

5. Conclusions

Based on a systematic sampling across a multi-habitat gradient in a temperate mountain of the Trans Mexican Volcanic Belt, we found five tardigrade species, three putative species, one record to genus level, and a new species *Min. citlalium* sp. nov., which we described. Of them, three are new records for Mexico: *Dip. mitrense*, *Adr. scoticum* and *Min. sidereus* (the first two new for North America). This raises the current number of tardigrade taxa in Mexico from 56 to 61, and in the temperate mountains of the Trans Mexican Volcanic Belt from 8 to 13.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/7/271/s1>. Figure S1. *Calohypsibius* cf. *ornatus*. a—habitus (latero-dorsal view), showing eight transversal parallel rows of cuticular spines (**sp** spines, **rw** row); b—buccal apparatus of the *Calohypsibius* type, with one bend in the posterior portion. Figure S2. *Diphyscon mitrense* and *Dip. pingue*. a—*Dip. mitrense* habitus, b—bucco-pharyngeal apparatus, c—*Dip. pingue* habitus, and d—bucco-pharyngeal apparatus (buccal tube length, macroplacoid row, first macroplacoid, **m2** s macroplacoid, **m3** third macroplacoid, and white arrow indicates thickening drop shape). The length of the buccal tube is expressed as a percentage on a vertical line. It also stands for length comparison to other structures. Figure S3. *Hypsibius* cf. *dujardini*, *Hys.* cf. *exemplaris*, *Hys.* cf. *microps* and *Hys.* cf. *pallidus*. a—*Hys.*

cf. *dujardini* habitus, b—buccal apparatus, c—claws IV; d—*Hys.* cf. *exemplaris* habitus, e—buccal apparatus, f—claws III; g—*Hys.* cf. *microps* habitus, h—buccal apparatus, i—claws IV; j—*Hys.* cf. *pallidus* habitus, k—buccal apparatus, and l—claws IV. The white arrow indicates the minute dot-like septula at the end of the placoids row. Figure S4. *Adropion scoticum*. a—habitus, b—bucco-pharyngeal apparatus, c—claws I–II; d—claws III, and e—claws IV. (**ph** pharynx length, **m1** first macroplacoid, **m2** s macroplacoid, **m3** third macroplacoid, **cI** claws I, **cII** claws II, **cIII** claws III, **cIV** claws IV, and white arrow indicates cuticular bars on legs I–III). Figure S5. *Pilatobius nodulosus*. a—habitus, b—bucco-pharyngeal apparatus, c—posterior part of the body. (**ph** pharynx length, **m1** first macroplacoid, **m2** s macroplacoid, **m3** third macroplacoid, **tb** tubercles, and white arrow indicates thickening drop shape). Figure S6. *Macrobiotus* sp. a—habitus, b—buccal apparatus, c—oral cavity armature *maculatus* type, d—claws IV of *hufelandi* type; *Macrobiotus* sp. e—habitus, f—buccal apparatus, g—oral cavity armature *patagonicus* type, h—claws IV of *hufelandi* type. White arrow indicates II and III band of teeth. Figure S7. *Minibiotus sidereus*. a—habitus (dorsal view), b—cuticular small circular pores, c—large star-shaped pores with 3–8 arms (4–5 most common), d—big star-shaped pore in each leg of fourth pair, e—buccal apparatus of *Minibiotus* type, macroplacoid length sequence $1 > 2 < 3$, f—cuticular bar on leg III, g,h—egg, i processes. (**m1** first macroplacoid, **m2** s macroplacoid, **m3** third macroplacoid, white arrow indicates cuticular bar). Table S1. Results of linear regressions of morphometric characters measured in relation to buccal tube length in *Minibiotus citlalium* sp. nov. Data log–log transformed. N = sample size; b = slope; a^* = Y intercept; r^2 = coefficient of correlation; p (r^2) = probability associated to “ r ”; t = Student’s T test for slopes; $p(t)$ = probability that b differs from slope of 1; and traits with $p < 0.05$ are allometric and are indicated in bold.

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