e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 68 (2020), No 2
https://doi.org/10.3409/fb_68-2.08

New Records of *Dactylobiotus parthenogeneticus* Bertolani, 1982 Provide Insight into Its Genetic Variability and Geographic Distribution

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Accepted June 10, 2020 *Published online June 26, 2020* *Issue online June 30, 2020*

Original article

POGWIZD J., STEC D. 2020. New Records of *Dactylobiotus parthenogeneticus* Bertolani, 1982 provide insight into its genetic variability and geographic distribution. Folia Biologica (Kraków) **68**: 57-72.

In sediment samples collected from three distinct European locations (United Kingdom, France, Poland), populations of *Dactylobiotus parthenogeneticus* were found. The original description of this species was based solely on the morphology observed with light microscopy and later supplemented by some additional SEM data of the buccal apparatus and DNA sequences of 18S rRNA and COI. Here we provide an updated description of the species by means of integrative taxonomy. The description comprises a comprehensive set of morphometric and morphological data from light and scanning microscopy as well as nucleotide sequences of three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI) fragments. Our analysis of haplotype diversity confirmed our morphological identification and showed that *D. parthenogeneticus* is widely distributed in Europe.

Key words: aquatic tardigrade, biodiversity, DNA barcodes, freshwater meiofauna, taxonomy.

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Tardigrades, known also as water bears, are a phylum of micro-invertebrates that inhabit freshwater and marine habitats but also limno-terrestrial environments with at least temporary moisture (NELSON *et al.* 2015). Tardigrade taxonomy started almost two centuries ago and currently over 1300 nominal taxa are recognized within the phylum (GUIDETTI & BERTOLANI 2005; DEGMA & GUIDETTI 2007; DEGMA *et al.* 2019).

The genus *Dactylobiotus* Schuster, 1980 was erected by SCHUSTER *et al.* (1980), and presently it comprises 18 species, which are known to be exclusively aquatic (KIHM *et al.* 2020). In this study, we focus on *Dactylobiotus parthenogeneticus* Bertolani, 1982 which has been recorded numerous times from several European countries such as Italy, Greece, Poland and Spain (BERTOLANI 1982a,b; BINDA & GUGLIELMINO 1982; BERTOLANI 1988; GUIL 2002; GUIDETTI *et al.* 2006b; POPRAWA *et al.* 2015) but also from Argentina, Bolivia and Mexico (MEYER 2013; KACZMAREK *et al.* 2015; MORENO-TALAMANTES *et al.* 2015). The genus *Dactylobiotus* was established 40 years ago, and so far none of its species were described using an integrative approach except recently discovered species *Dactylobiotus ovimutans* KIHM *et al*., 2020. Therefore, the morphological data for the majority of *Dactylobiotus* taxa were collected only with light microscopy and were not associated with genetic markers. This limitation and scarce genetic data are clearly visible in GenBank, where DNA sequences are provided for only three species: *Dactylobiotus ambiguus* (Murray, 1907) (28S rRNA and 18S rRNA), *Dactylobiotus octavi* Guidetti *et al.*, 2006a (28S rRNA and 18S rRNA), and *D. parthenogeneticus* (18S rRNA and COI). In our work, we analysed three European populations of *D. parthenogeneticus* from Poland, France and Great Britain. We provide a new description of *D. parthenogeneticus* based on a detailed morphological examination with light and scanning electron microscopy as well as DNA sequences of the four standard molecular markers used in tardigrade taxonomy (18S rRNA, 28S rRNA, ITS-2 and COI). Based on our multifaceted approach and especially genetic comparisons, we confirmed that *D. parthenogeneticus* is distributed in aquatic habitats in at least three countries in Europe.

Material and Methods

Sample processing

Sediment samples, in which two populations of the studied species were previously discovered, were collected: 1) in a pond in the Botanic Garden of the Jagiellonian University (Kraków, Poland; 50°03′45′′N, 19°57′27′′E; coll. Artur Oczkowski and Bartłomiej Surmacz; 17 September 2017), and 2) in a pond in a park (Fontainebleau, France; 48°24′05′′N, 2°42′13′′E; coll. Daniel Stec; 11 March 2017). The third population analysed in this study came from a clonal laboratory strain of *Dactylobiotus dispar* (Murray, 1907) that was originally established on 13th November 1987 by Robert MCNUFF from a female collected from rotting leaves in a pond in Darcy Lever, Bolton, Lancashire, England (53°33′32′′N, 2°23′48′′W) (Robert McNuff, pers. com). Commercial cultures of this strain are made available by Sciento (under catalogue number Z160). This population was confirmed in our study to be *D. parthenogeneticus* and not *D. dispar*.

The samples were examined for tardigrades using the protocol by DASTYCH (1980) with modifications described in detail in STEC *et al*. (2015). For taxonomic analysis, animals and eggs isolated from the samples were separated into three groups for specific analyses: morphological analysis with phase contrast light microscopy, morphological analysis with scanning electron microscopy, and DNA sequencing (for details please see Table 1).

Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer's medium and secured with a cover slip, according to the protocol by MOREK *et al.* (2016). Slides were examined under an Olympus BX53 light microscope with phase contrast (PCM), connected with an Olympus DP74 digital camera. In order to obtain clean and extended specimens for scanning electron microscopy, tardigrades were processed according to the protocol by STEC *et al.* (2015). In short, specimens were first subjected to a 60 °C water bath for 30 min to obtain fully extended animals, then to a water/ethanol and an ethanol/acetone series, then to CO_2 critical point drying and finally sputter coated with a thin layer of gold. Bucco-pharyngeal apparatuses were extracted according to the protocol of EIBYE-JACOBSEN (2001) as modified by GĄSIOREK *et al.* (2016). Specimens were examined under high vacuum in a Versa 3D Dual-Beam Scanning Electron Microscope at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. All figures were assembled in Corel Photo-Paint X6, ver. 16.4.1.1281. For structures that could not be satisfactorily focused in a single light microscope photograph, a stack of 2-6 images were taken with an equidistance of ca. 0.2 μm and assembled manually into a single deep-focus image in Corel Photo-Paint X6, ver. 16.4.1.1281.

Morphometrics and morphological nomenclature

All measurements are given in micrometres (μm). Sample size was adjusted based on recommendations by STEC *et al*. (2016). Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. The terminology used to describe the oral cavity armature and egg shell morphology is given in MICHALCZYK and KACZMAREK (2003) and KACZMAREK and MICHALCZYK (2017), respectively. Macroplacoid length sequence is given according to the method in KACZMAREK *et al.* (2014). Buccal tube length and the level of the stylet support insertion point were measured according to PILATO (1981). The *pt* index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (PILATO 1981). Buccal tube width was measured according to KACZMAREK and MICHALCZYK (2017) as the external and internal diameter at the level of the stylet support insertion point. Claws were measured according to BINDA and

Table 1

Details of *Dactylobiotus parthenogeneticus* Bertolani, 1982 populations analysed in the study. Note: PCM – number of animals (A) and eggs (E) prepared for phase contrast microscopy examination, SEM – number of animals and eggs prepared for scanning electron microscopy examination, DNA – number of animals used for DNA sequencing

Population	Locality	Coordinates	Collector	PCM	SEM	DNA
GB.003	pond in Darcy Lever, Bolton, Lancashire, England	53°33′32′′N, 2°23′48′′W	Robert McNuff	88 A+ 13E	5 A+ 0 E	4A
FR.149	pond in park, Fontainebleau, France	48°24′05′′N, 2°42′13′′E	Daniel Stec	17 A+ 8E	0A+ 0E	2A
PL.317	pond in Botanic Garden, Kraków, Poland	50°03′45′′N, 19°57′27′′E	Artur Oczkowski & Bartłomiej Surmacz	114 A+ 48E	10 A+ 10E	4A

PILATO (1999). Distance between egg processes was measured as the shortest line connecting base edges of the two randomly chosen closest processes (KACZMAREK & MICHALCZYK 2017). Morphometric data were handled using the "Parachela" ver. 1.7 template available from the Tardigrada Register (MICHALCZYK & KACZMAREK 2013). Raw morphometric data for the analysed species are provided as supplementary materials (Suppl. Mat. 1). Tardigrade taxonomy follows GUIL *et al.* (2019).

Additional comparative material

For morphological comparison we used the original description as well as photomicrographs of the type series of *D. parthenogeneticus* deposited in the Roberto Bertolani collection taken by Piotr Gąsiorek and Witold Morek (both of Jagiellonian University, Poland), thanks to the courtesy of Roberto Guidetti and Roberto Bertolani (University of Modena, Italy). Four additional SEM photomicrographs of the British population of *D. parthenogeneticus* were kindly provided by Łukasz Michalczyk and assembled within the figure plates (Figs 2 C-D and 8D, G).

Genotyping

The DNA was extracted from individual animals following a Chelex® 100 resin (Bio-Rad) extraction method by CASQUET *et al.* (2012) with modifications described in detail in STEC *et al.* (2020a). We sequenced four DNA fragments: the small ribosome subunit (18S rRNA, nDNA), the large ribosome subunit (28S rRNA, nDNA), the internal transcribed spacer (ITS-2, nDNA), and the cytochrome oxidase subunit I (COI, mtDNA). All fragments were amplified and sequenced according to the protocols described in STEC *et al.* (2020a); primers and original references for specific PCR programs are listed in Table 2. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of the Jagiellonian University, Kraków, Poland. Sequences were processed in BioEdit ver. 7.2.5 (HALL 1999) and submitted to GenBank.

Comparative molecular analysis

Since there are no published sequences of ITS-2, only the sequences of 18S rRNA, 28S rRNA and COI markers for species in the genus *Dactylobiotus* were downloaded from GenBank (GUIDETTI *et al.* 2005; SANDS *et al.* 2008; CHEN *et al.* 2009, unpublished; JØRGENSEN *et al.* 2010; BERTOLANI *et al.* 2014; GUIL *et al.* 2019). However, nine 18S rRNA sequences (GQ925678-9, EF632436-42) and the only two 28S rRNA sequences (GQ849049 and MH079500) were not homologous with fragments sequenced in our study and thus excluded from further analysis. The sequences of each DNA marker were aligned separately using the AUTO (in the case of ITS-2 and COI) and the Q-INS-I strategy (in the case of ribosomal markers: 18S rRNA, 28S rRNA) of MAFFT version 7 (KATOH *et al.* 2002; KATOH & TOH 2008) and manually checked against non-conservative alignments in BioEdit. Then, the aligned sequences were trimmed to: 763 (18S rRNA), 769 (28S rRNA), 414 (ITS-2), 534 (COI) bp. All COI sequences were translated into protein sequences in MEGA7 version 7.0 (KUMAR *et al.* 2016) to check against pseudogenes. Uncorrected pairwise distances were calculated using MEGA7 and are provided as supplementary materials (Suppl. Mat. 2).

Networks of haplotypes of *D. parthenogeneticus* from four distinct populations (three populations from this study and one population from Italy; the only COI sequence was GenBank AY598771) were prepared using PopARTver.1.7 (http://popart.otago.ac.nz) with the implementation of Median-Joining method (BANDELT *et al*. 1999). For this purpose, single sequences of each haplotype present in each population were used (N = 3 for 28S rRNA, N = 3 for ITS-2 and N = 5 for COI). Sequences were aligned as described above and cut to the shortest available alignment.

Table 2

PCR primers for amplification of the four DNA fragments sequenced in the study

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source
18S rRNA	18S_Tar_1Ff	forward	AGGCGAAACCGCGAATGGCTC	STEC *et al.* 2017
	18S_Tar_1Rr	reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA	28S_Eutar_F	forward	ACCCGCTGAACTTAAGCATAT	GĄSIOREK *et al.* 2018 MIRONOV *et al.* 2012
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	
ITS-2	Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	STEC *et al.* 2018
	Eutar_Rr	reverse	TCCTCCGCTTATTGATATGC	
COI	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	FOLMER *et al.* 1994
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	

Results

Taxonomic account

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Richters, 1926

Order: Macrobiotoidea Guil *et al.*, 2019

Family: Murrayidae Guidetti *et al.*, 2005

Genus: *Dactylobiotus* Schuster, 1980
(in SCHUSTER *et al.* (1980))

Dactylobiotus parthenogeneticus Bertolani, 1982

Slide and SEM stubs depositories:
Polish population: 124 animals (slides: PL.317.*, with the asterisk substituted by any of the following numbers 01-26; SEM stub: 19.17) and 58 eggs (slides: PL.317.*: 27-33; SEM stub: 19.17);
British population: 93 animals (slides: GB.003.*: 01-20, 22-24; SEM stubs with buccal apparatuses: 6.072-6) and 13 eggs (slides: GB.003.*: 21, 25); French population: 17 animals (slides: FR.149.*: 01-17) and 8 eggs (slides: FR.149.*: 18, 19).
All are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland.

Updated description of the species

Animals

Body transparent in juveniles and whitish in adults, but transparent after fixation in Hoyer's medium (Fig.1A). In live specimens, eyes are present but they dissolve in Hoyer's medium. Cuticle without pores but clearly

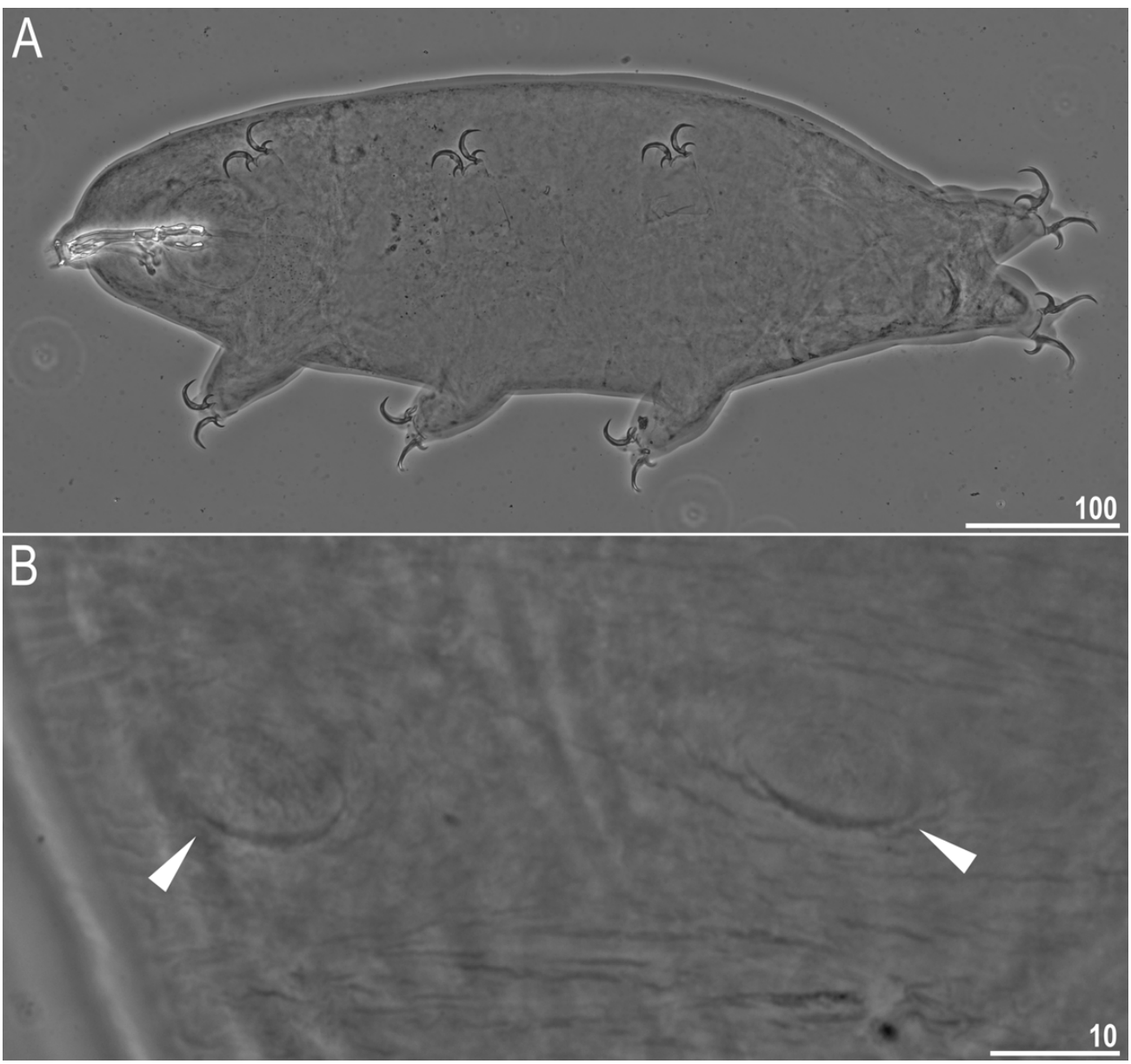

Fig. 1. *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland – habitus and dorsal cuticle (PCM): A – dorso-ventral view; B – dorsal cuticle with two flat, oval papillae present on dorsum between legs III-IV. Arrowheads indicate dorsal papillae. Scale bars in μm.

wrinkled with two flat, oval papillae present on the dorsum between legs III and IV in adults and juveniles (Figs 1B and 2A-F). Granulation absent on all legs.

Claws of the *Dactylobiotus* type with short basal portion and primary branches with distinct accessory points (Fig. 3A-D). Lunules absent but under PCM

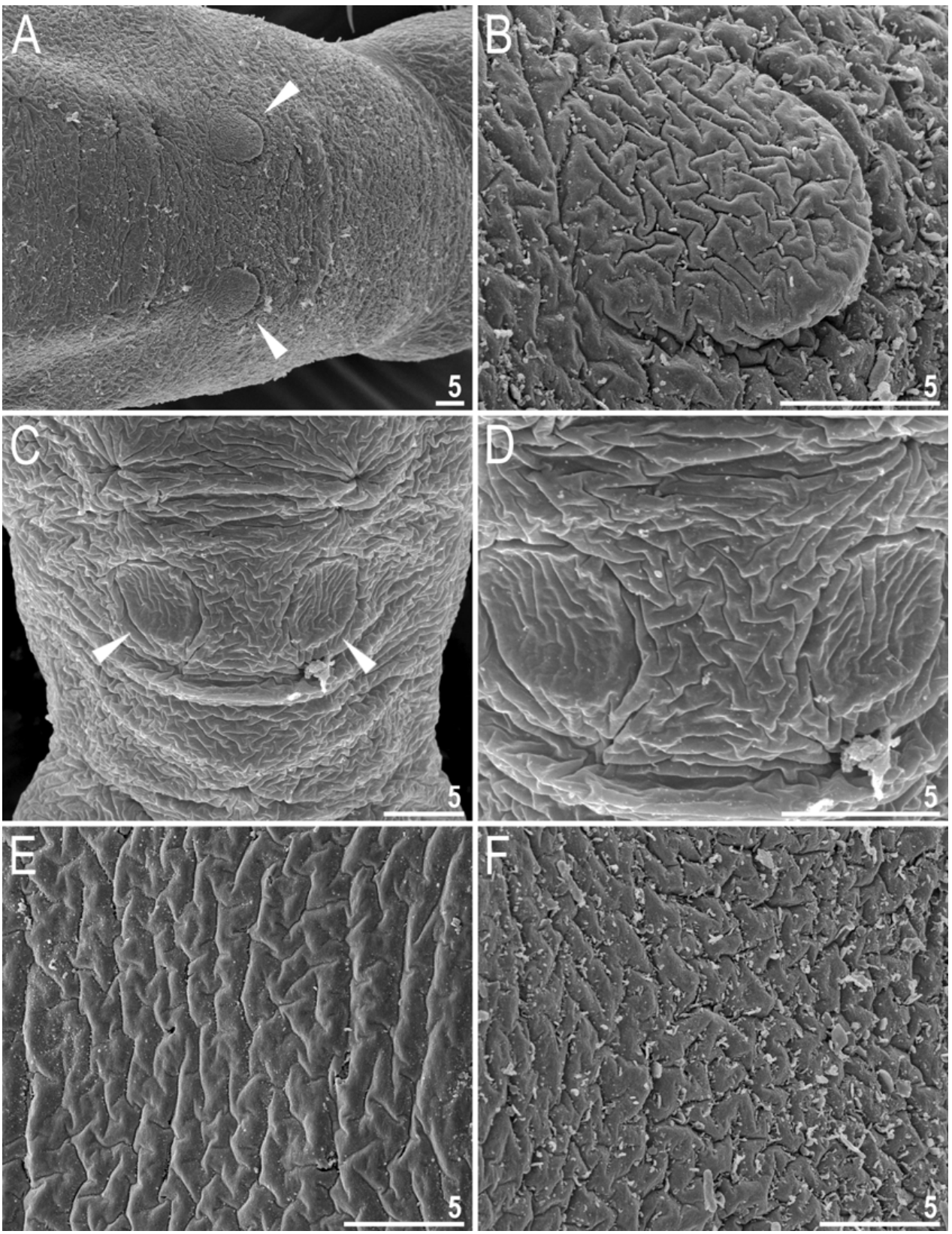

Fig. 2. *Dactylobiotus parthenogeneticus* Bertolani, 1982 – dorsal cuticle (SEM): A – dorsal cuticle between leg III-IV with two papillae (adult, Poland); B – magnification of one dorsal papilla (adult, Poland); C – dorsal cuticle between leg III-IV with two papillae (juvenile, United Kingdom); D – magnification of dorsal papillae (juvenile, United Kingdom); E-F – magnification of a fragment of dorsal cuticle of the adult specimen between legs II-III (C) and III-IV (D) (Poland). Arrowheads indicate dorsal papillae. Scale bars in μm.

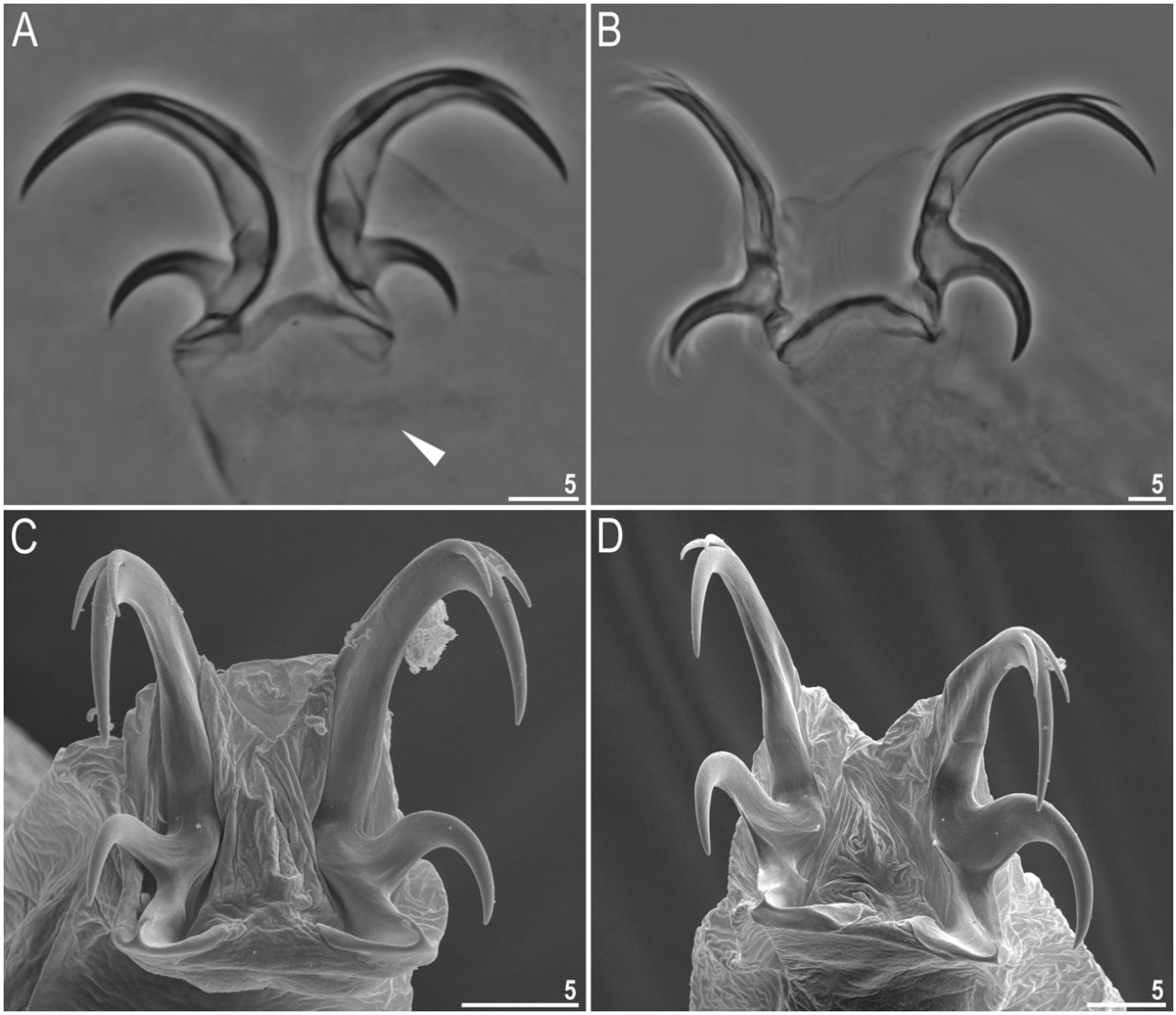

Fig 3. *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland – claws: A-B – claws III (A) and IV (B) seen in PCM; C-D – claws II (C) and IV (D) seen in SEM. Arrowhead indicates faintly visible double muscle attachments under the claws. Scale bars in μm.

a robust semilunar cuticular connection is present between external/posterior and internal/anterior claws (Fig. 3A-B). Under SEM this connection is visible as discontinuous, being composed of extended lunulae-like thickenings under the claws on the lateral sides whereas its median portion is located within or under cuticle (Fig. 3C-D). Claws on the first three pairs of legs similar in size but obviously longer on the hind legs.

Mouth antero-ventral followed by ten short peribuccal lamellae, bucco-pharyngeal apparatus of the *Macrobiotus* type (Figs 4A-G, 5A-H and 6A-B). Under PCM, the oral cavity armature comprises only the second and the third band of teeth (Fig. 4B-C). However, in SEM three bands of teeth are clearly visible with the first band being situated at the base of peribuccal lamellae and composed of 4-5 rows of scattered small conical teeth arranged around the oral cavity (Figs 5E and 6A-B). The second band of teeth is situated below the ring fold, and comprises 4-6 rows of small cone-shaped teeth which are larger than those of the first band and increase in size towards the third band of teeth (Figs 4B-C, 5E and 6A-B). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the buccal tube opening (Figs 4B-C, 5E and 6A-B). The third band of teeth is discontinuous and divided into dorsal and the ventral portions. Under PCM, the dorsal teeth are seen as three distinct transversal ridges whereas the ventral teeth appear as two separate lateral transverse ridges, between which a roundish median tooth is visible (Fig. 4B-C). In SEM, both dorsal and ventral teeth are also clearly distinct (Figs 5E and 6A-B). Under SEM, the dorsal teeth are sharpened at the end (Figs 5E and 6A-B), whereas the ventral portion of the third band of teeth comprises also several smaller additional teeth (Figs 5E and 6A-B). Under PCM in the lateral view of the buccal apparatus a strengthening bar (ventral lamina) with an incision determining a ventral

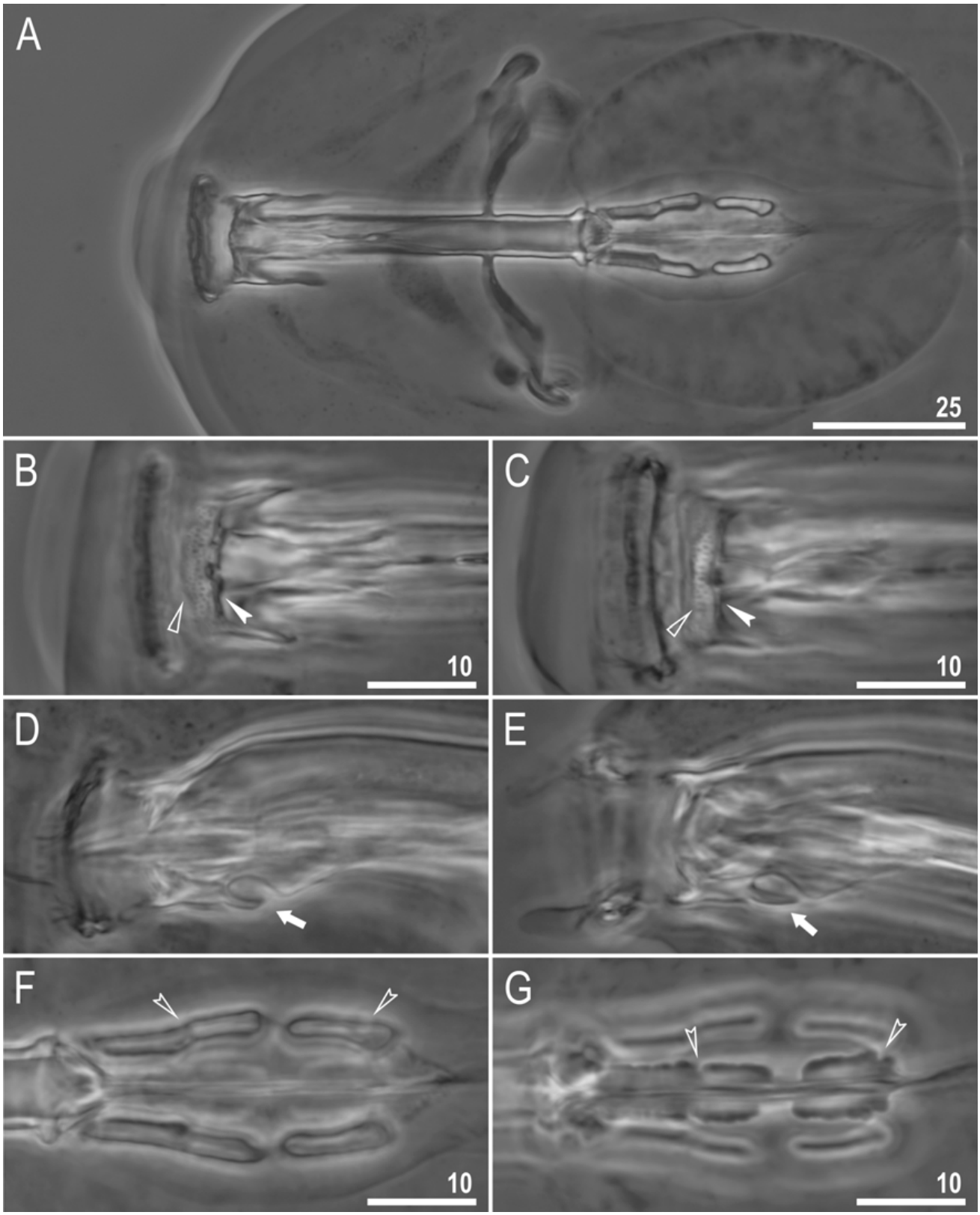

Fig 4. *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland – buccal apparatus and the oral cavity armature seen in PCM: A – dorso-ventral view of the buccal apparatus; B-C – oral cavity armature seen in dorsal (B) and ventral (C) view; D-E – lateral view of the anterior portion of the buccal apparatus with ventral lamina and the incision determining the presence of a ventral hook; F-G – placoid morphology, dorsal (D) and ventral (E) view. Empty flat arrowheads indicate the second band of teeth, filled indented arrowheads indicate the third band of teeth, arrows indicate ventral hook, empty indented arrowheads indicate constrictions in macroplacoids. Scale bars in μm.

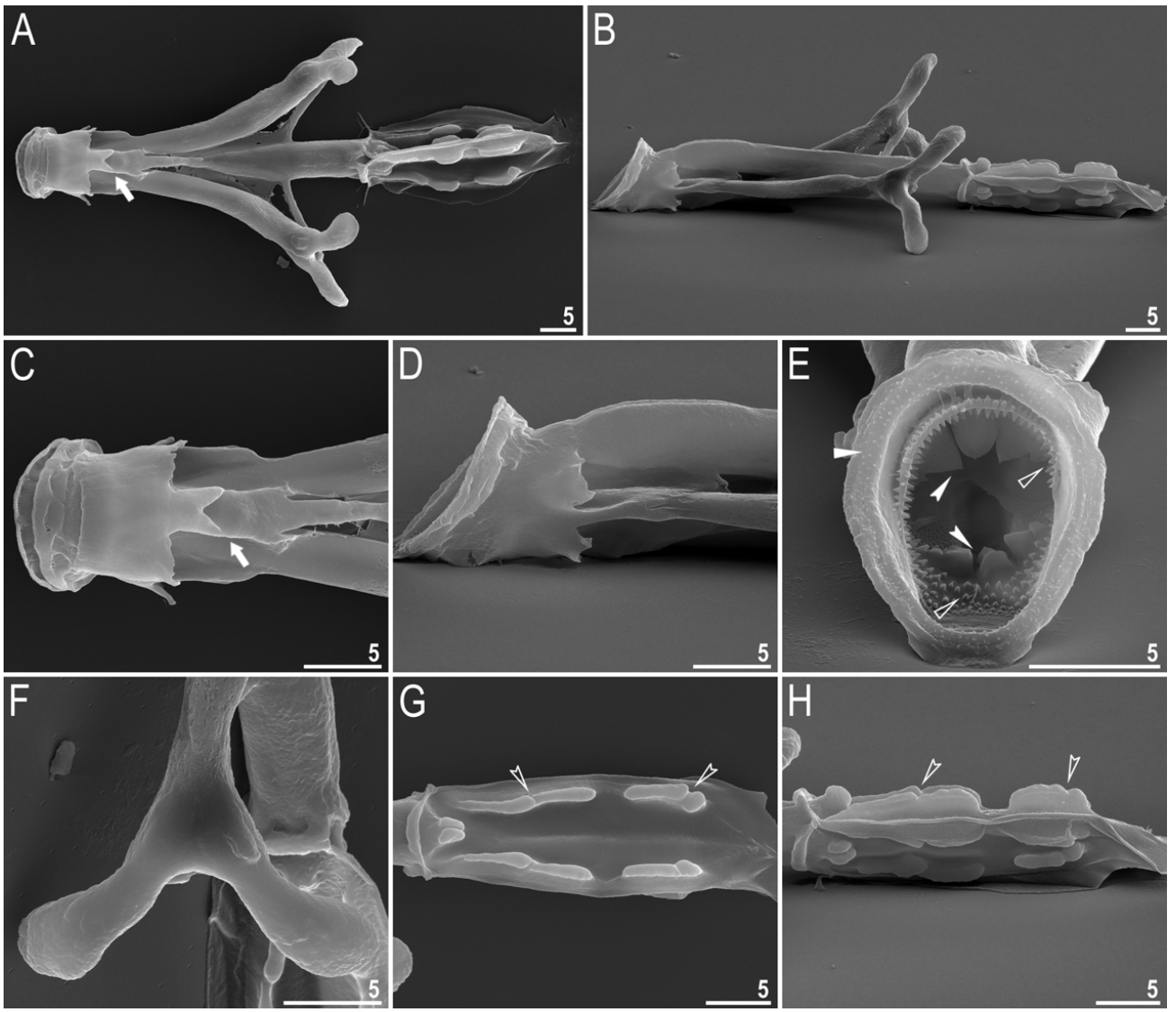

Fig 5. *Dactylobiotus parthenogeneticus* Bertolani, 1982 from United Kingdom – buccal apparatus and the oral cavity armature seen in SEM: A-B – entire buccal apparatus seen in ventral (A) and lateral (B) view; C-D – magnification of buccal crown seen in ventral (C) and lateral (D) view; E – oral cavity armature; F – magnification of stylet's furca; G-H – placoid morphology, dorsal (G) and ventral (H) view. Filled flat arrowhead indicates the first band of teeth, empty flat arrowheads indicate the second band of teeth, filled indented arrowheads indicate the third band of teeth, arrows indicate invagination in the ventral lamina that starts with branches of bifurcation and opening and is followed by a common tract of the bifurcation, empty indented arrowheads indicate constrictions in macroplacoids. Scale bars in μm.

hook is clearly visible (Fig. 4D-E). The hook is visible as an invagination in the ventral lamina when observed from lateral view under SEM that starts with bifurcation visible clearly only in ventral view (Fig. 5A, C) and develops further below the common tract of the bifurcation. The ventral portion of the hook visible in PCM (Fig. 4D-E) is actually constituted by a common tract of the bifurcation and the branches of the bifurcation (Fig. 5A, C), while the opening of the hook under PCM (Fig. 4D-E) is the opening of the invagination visible under SEM (Fig. 5A, C). The hook is not visible under SEM because the invagination has lateral walls that at certain focus are not visible under PCM but are always visible with SEM. Pharyngeal bulb spherical, with triangular apophyses, two rod-shaped macroplacoids which sometimes have jagged edges (Figs 4F-G and 5G-H). The macroplacoid length sequence 2<1. The first macroplacoid has a central constriction, whereas the second macroplacoid is constricted sub-terminally (Figs 4F-G and 5G-H). Measurements and statistics are given in Table 3.

Eggs

Laid freely, whitish, spherical (Figs 7A and 8A). Processes in the shape of short and wide cones with apexes divided into multiple (typically three to six) short, nodular, finger-like apices (Figs 7B-E and 8B-G, I). Under SEM, apices usually covered with microgranulation (Fig. 8I). Egg surface between the

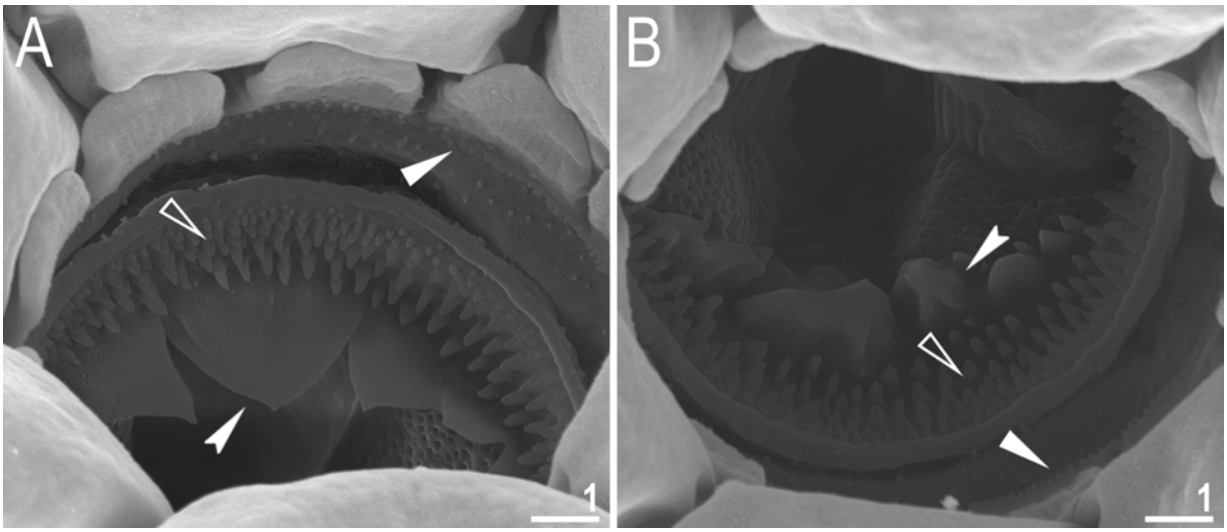

Fig 6. *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland – oral cavity armature seen in SEM: A-B – the oral cavity armature seen from different angles, dorsal (A) and ventral (B) view, respectively. Filled flat arrowhead indicates the first band of teeth, empty flat arrowheads indicate the second band of teeth, filled indented arrowheads indicate the third band of teeth. Scale bars in μm.

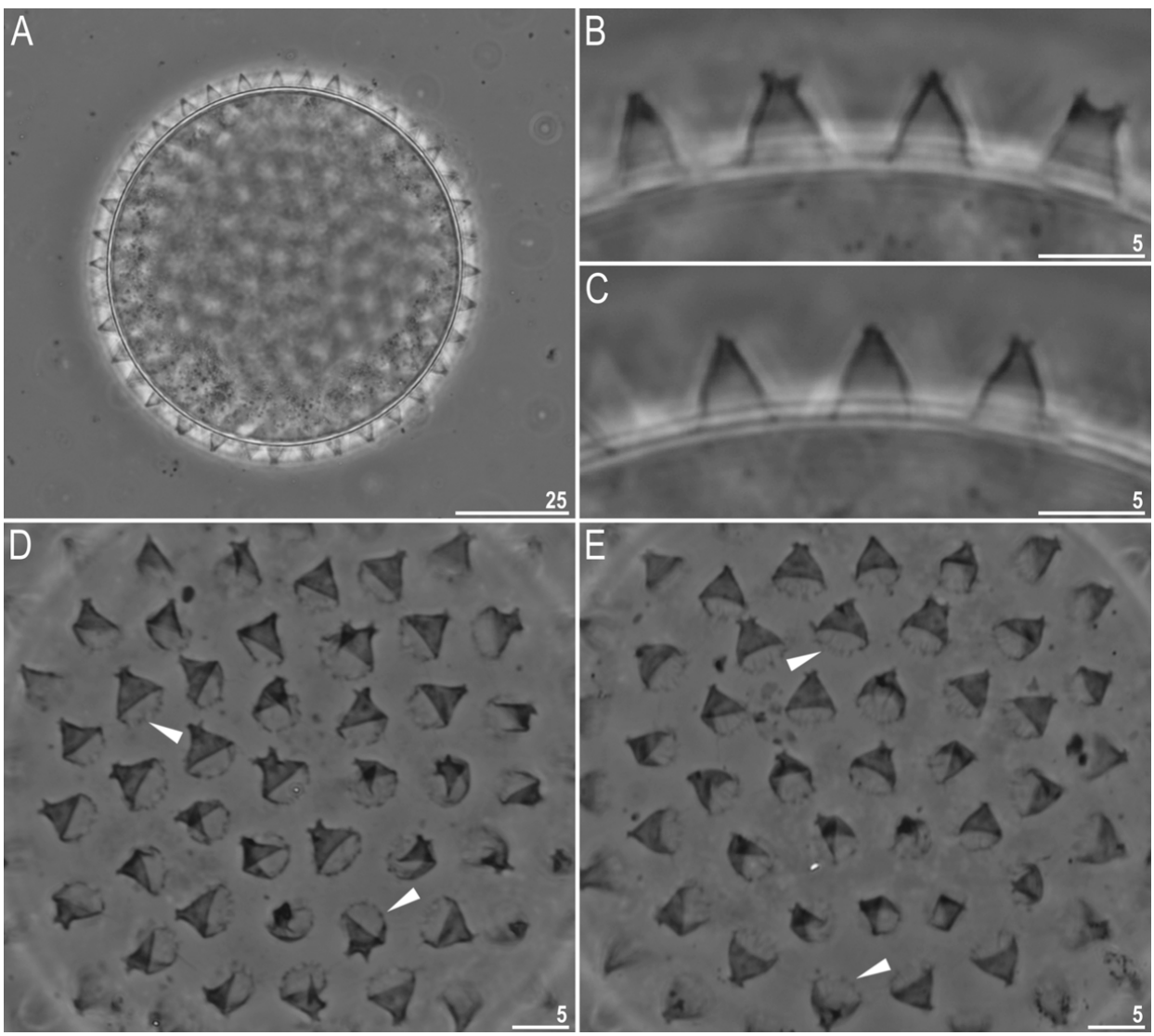

Fig 7. *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland – egg chorion morphology viewed in PCM: A – midsection under 400× magnification; B-C – midsections under 1000× magnification; D-E – egg surface under 1000× magnification. Filled flat arrowheads indicate crowns of small thickenings/projections around the bases of the processes. Scale bars in μm.

Table 3

Measurements (in µm) of selected morphological structures of individuals of *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland mounted in Hoyer's medium (N – number of specimens/structures measured, range refers to the smallest and the largest structure among all measured specimens; SD – standard deviation). The *pt* index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage

Character	N	Range		Mean		SD	
		µm	*pt*	µm	*pt*	µm	*pt*
Body length	26	374 – 679	*757 – 1170*	521	*979*	81	*111*
Buccal tube							
Buccal tube length	26	46.8 – 64.0	–	53.0	–	3.8	–
Stylet support insertion point	26	33.6 – 46.4	*69.5 – 72.8*	38.0	*71.6*	2.7	*0.8*
Buccal tube external width	26	5.3 – 7.9	*10.8 – 13.8*	6.5	*12.3*	0.7	*0.8*
Buccal tube internal width	26	3.4 – 6.2	*6.9 – 9.9*	4.4	*8.3*	0.6	*0.8*
Ventral lamina length	25	19.1 – 27.3	*37.1 – 46.5*	22.8	*43.1*	2.3	*3.0*
Placoid lengths							
Macroplacoid 1	26	12.0 – 20.4	*25.6 – 32.6*	15.2	*28.6*	1.9	*2.0*
Macroplacoid 2	26	6.9 – 11.2	*14.0 – 17.9*	8.7	*16.3*	1.1	*1.2*
Macroplacoid row	26	20.7 – 35.4	*44.2 – 55.3*	26.0	*48.9*	3.3	*3.0*
Claw 1 heights							
External primary branch	22	17.7 – 27.6	*32.1 – 46.9*	21.8	*41.4*	2.4	*3.1*
External secondary branch	22	6.5 – 9.1	*13.2 – 17.2*	7.6	*14.5*	0.7	*0.9*
External secondary/primary branch	22	30.9 – 42.4	–	35.2	–	2.7	–
Internal primary branch	22	15.3 – 26.5	*31.1 – 46.2*	20.4	*38.5*	2.7	*4.0*
Internal secondary branch	23	5.5 – 9.6	*11.2 – 16.1*	7.4	*13.9*	1.1	*1.3*
Internal secondary/primary branch	22	27.6 – 43.6	–	36.5	–	4.7	–
Claw 2 heights							
External primary branch	23	18.4 – 27.3	*34.3 – 48.3*	22.2	*42.1*	2.1	*3.0*
External secondary branch	23	6.5 – 10.4	*13.2 – 18.7*	8.1	*15.4*	1.0	*1.2*
External secondary/primary branch	23	31.6 – 44.1	–	36.7	–	2.8	–
Internal primary branch	23	15.3 – 22.6	*31.1 – 43.2*	19.9	*37.7*	1.7	*2.9*
Internal secondary branch	23	5.8 – 9.5	*11.1 – 17.2*	7.4	*14.0*	1.0	*1.4*
Internal secondary/primary branch	23	28.6 – 46.1	–	37.3	–	4.5	–
Claw 3 heights							
External primary branch	23	17.3 – 28.3	*35.2 – 49.1*	21.9	*41.0*	2.7	*3.8*
External secondary branch	23	6.8 – 10.3	*13.6 – 18.5*	8.4	*15.7*	1.0	*1.4*
External secondary/primary branch	23	32.9 – 45.5	–	38.6	–	3.6	–
Internal primary branch	21	17.1 – 26.9	*30.9 – 46.7*	20.1	*37.7*	2.1	*3.3*
Internal secondary branch	21	6.0 – 9.9	*11.9 – 17.2*	7.3	*13.6*	1.0	*1.2*
Internal secondary/primary branch	21	32.2 – 43.4	–	36.2	–	3.0	–
Claw 4 heights							
Anterior primary branch	19	26.0 – 33.0	*51.1 – 59.8*	29.2	*55.1*	2.1	*2.3*
Anterior secondary branch	19	9.2 – 14.4	*16.8 – 25.3*	11.7	*22.0*	1.2	*1.9*
Anterior secondary/primary branch	19	31.0 – 46.8	–	40.1	–	3.4	–
Posterior primary branch	12	23.9 – 33.9	*46.7 – 59.4*	29.1	*55.1*	3.0	*4.0*
Posterior secondary branch	11	10.2 – 14.6	*20.8 – 24.6*	12.1	*22.7*	1.2	*1.2*
Posterior secondary/primary branch	11	35.9 – 52.7	–	41.8	–	4.9	–

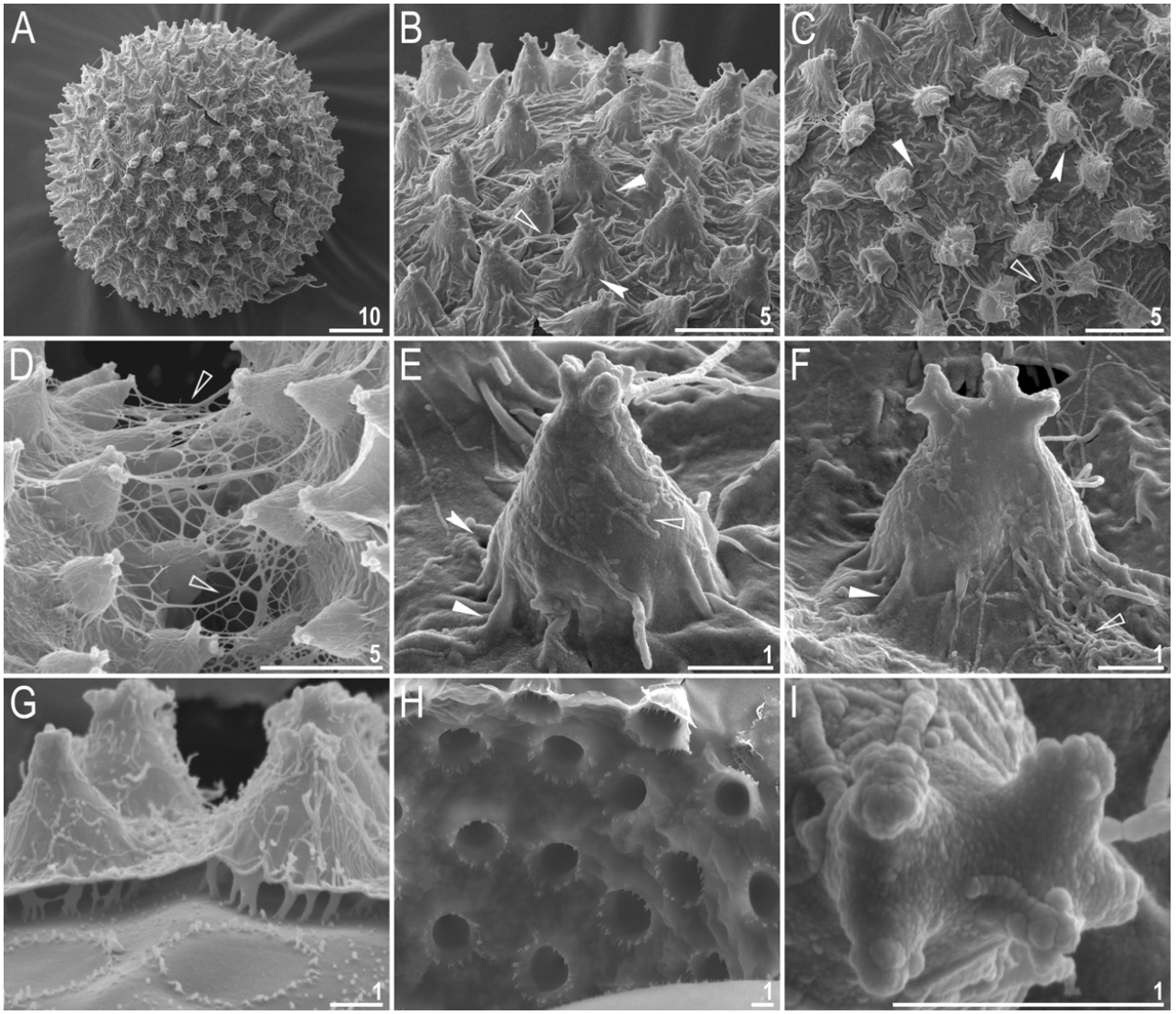

Fig 8. *Dactylobiotus parthenogeneticus* Bertolani, 1982 – egg chorion morphology seen in SEM: A – entire egg; B-D – magnification of the egg surface; E-F – egg processes; G-H – strengthening internal structures that stabilize the processes within the chorion; I – details of the apices of the egg processes. Photomicrographs A, B, C, E, F, H, I come from the Polish population, whereas D and G are from the British population. Filled flat arrowheads indicate vertical cuticular thickenings on the external surface of the bases of the egg processes, filled indented arrowheads indicate micropores, empty flat arrowheads indicate filamentous remains of mucus. Scale bars in μm.

processes seems to be smooth under PCM (Fig. 7D-E), whereas under SEM it is clearly wrinkled (Fig. 8B-F). Under PCM, the margins of processes bases seem to be serrated and surrounded by a crown of small thickenings/projections (Fig. 7D-E), which are internal strengthening structures stabilising the processes within the chorion, clearly visible under SEM when the chorion is broken (Fig. 8G-H) or vertical thickenings present on basal portions of processes walls (Fig. 8B-C, E-F). Sometimes, micropores are present on the egg surface near the processes' base but they are visible only under SEM (Fig. 8B-C, E). Eggs are sticky because they are covered by mucus which most likely enhances their adhesion to the substrate and maybe has also a protective function. This mucus is clearly visible under SEM as a web of flexible filaments that cover the egg surface (Fig. 8B-F) but is only faintly visible under PCM (Fig. 7D-E). Measurements and statistics are given in Table 4.

Remarks

In comparison with the original description the following morphological characters are newly reported for the species: the presence of the first band of teeth in the oral cavity armature visible only under SEM and the presence of constrictions in the first and second macroplacoids. Furthermore the updated description provides much more detailed morphological characterisation of the claws, oral cavity armature as well as egg ornamentation. Finally we did not notice any obvious variation in the observed morphological characters between specimens from the three distinct population examined in our study.

Table 4

Measurements (in µm) of selected morphological structures of the eggs of *Dactylobiotus parthenogeneticus* Bertolani, 1982 from Poland mounted in Hoyer's medium (N – number of eggs/structures measured, range refers to the smallest and the largest structure among all measured specimens; SD – standard deviation)

Character	N	Range	Mean	SD
Egg bare diameter	24	75.1 – 93.4	83.0	5.0
Egg full diameter	24	84.6 – 101.1	91.8	4.5
Process height	72	3.2 – 4.9	4.0	0.4
Process base width	72	3.1 – 5.2	3.8	0.4
Process base/height ratio	72	73% – 139%	96%	11%
Inter-process distance	72	2.0 – 4.8	2.8	0.5
Number of processes on the egg circumference	24	34 – 38	36.7	1.0

DNA sequences

For each of the three examined populations we obtained sequences for all four of the above-mentioned DNA markers which are as follows:

British population (GB.003): MT373693 (18S rRNA; 1016 bp), MT373699 (28S rRNA; 782 bp), MT374190 (ITS-2; 414 bp), MT373803 (COI; 658 bp);

French population (FR.149): MT373694 (18S rRNA; 826 bp), MT373700 (28S rRNA; 769 bp), MT374191 (ITS-2; 414 bp), MT373804 (COI; 658 bp);

Polish population (PL.317): MT373695 (18S rRNA; 1021 bp), MT373701 (28S rRNA; 782 bp), MT374192 (ITS-2; 414 bp), MT373805–6 (COI; 658 bp);

Genetic comparisons

Genetic distances showed small differences between the three *Dactylobiotus* populations examined in this work. All populations share the same 18S rRNA haplotype, whereas each population exhibits distinct 28S rRNA and ITS-2 haplotypes (Fig. 9A-B). The genetic distances are: 0.13-0.26% for 28S rRNA and 0.24-0.97% for ITS-2. The comparison with other 18S rRNA sequences from GenBank also shows very low genetic differences that range from 0.00% to 0.26%. Similarly, for COI all populations examined in this study exhibited at least one distinct, population-specific haplotype, however one of the two haplotypes in the Polish population is identical with the haplotype present in the French population (Fig. 9C). Moreover, comparisons with other COI sequences from GenBank confirmed that the three newly found populations represent *D. parthenogeneticus* as genetic distances between the haplotypes and the COI sequence of AY598771 are very small

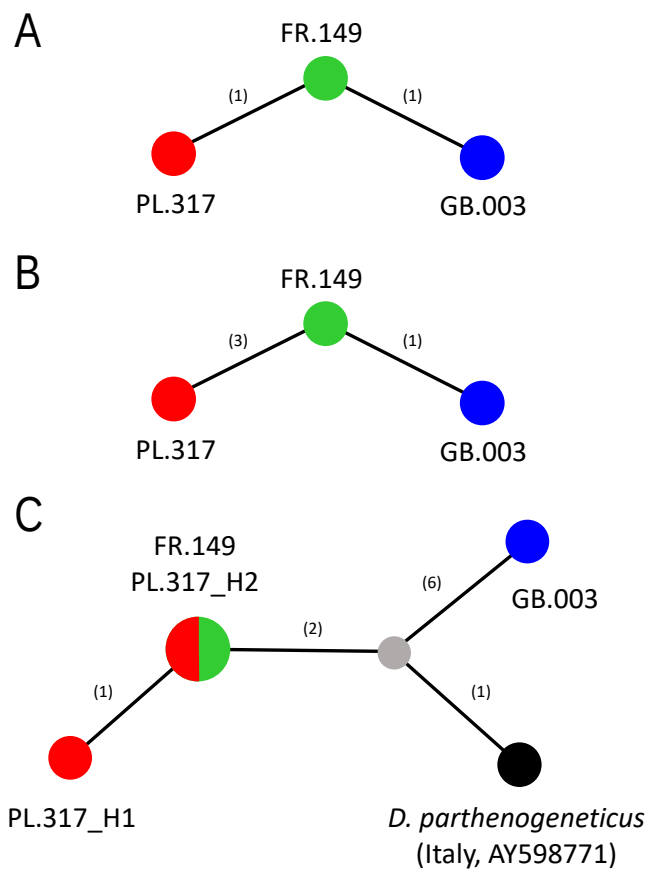

Fig 9. Haplotype Median Joining networks for nuclear and mitochondrial markers of *Dactylobiotus parthenogeneticus* Bertolani, 1982: A – 28S rRNA; B – ITS-2; C – COI. Haplotypes are represented by coloured circles. The size of circles is proportional to the number of populations in which a particular haplotype is present. Populations are listed in Table 1. Grey circles without a number indicate a hypothetical intermediate haplotype linking observed haplotypes of *D. parthenogeneticus*. Numbers in brackets indicate the numbers of mutations between the haplotypes.

and range from 0.37% to 0.75%. The COI sequence of AY598771 comes from a pond located ca. 6 km from the pond where the type population of *D. parthenogeneticus* was found, thus it can be considered as a barcode reliably representing this species. The peat bog where the species was originally discovered (type locality) has been destroyed (Roberto Bertolani, pers. com.). Moreover, the comparison also showed that other COI sequences labelled as *Dactylobiotus* sp. (EF632523-9; The South Shetland Islands, Antarctica) belong to a different species as they all differ from all haplotypes of *D. parthenogeneticus* in more than 17% (please see Suppl. Mat. 2 for detailed matrices with genetic distances calculated between all analysed sequences).

Discussion

Our study provides detailed morphological and genetic data on an aquatic tardigrade species, *Dactylobiotus parthenogeneticus*, collected from three distinct localities and analysed with integrative taxonomy approach. These results will enhance future species identifications but also will contribute to studies on tardigrade phylogeny with the set of four molecular markers. The DNA sequences and haplotype analysis confirmed our initial morphological identification and affirmed that this species is most probably very common in Europe.

To date, there are only a few studies that have investigated the distribution of a single tardigrade species using genetic data in Europe (e.g. CESARI *et al.* 2009; JØRGENSEN *et al.* 2007, 2013; GĄSIOREK *et al.* 2016, 2019b; MOREK *et al.* 2019a) as well as on other continents (e.g. CESARI *et al.* 2016; ZAWIERUCHA *et al.* 2018; GĄSIOREK *et al.* 2019c; JACKSON & MEYER 2019; KACZMAREK *et al.* 2020; SUGIURA *et al.* 2020). However, none of these studies was conducted on an exclusively aquatic, freshwater-dwelling tardigrade species. The most similar of all of these to our research in terms of tardigrade habitat were studies conducted by CESARI *et al.* (2016) and ZAWIERUCHA *et al.* (2018). The first one focused on the distribution of *Acutuncus antarcticus* (RICHTERS, 1904), the most abundant and common tardigrade species in Antarctica, which lives in freshwater ecosystems and terrestrial microhabitats in soil, grass, algae, moss and lichen in non-glacial areas (MURRAY 1910; DASTYCH 1991). The second one analysed the geographic distribution pattern of *Cryoconicus kaczmareki* ZAWIERUCHA *et al.*, 2018, a dark-pigmented tardigrade inhabiting cryoconite holes in mountain glaciers in China and Kyrgyzstan. Thus, our work can be considered as the first small-scale phylogeographic study on an exclusively aquatic tardigrade, which could have different dispersal modes compared to terrestrial species due to weak or absent anhydrobiotic abilities and – at the same time – the encystation capability of aquatic tardigrades (e.g. GUIDETTI *et al.* 2006b; JANELT & POPRAWA 2020). For example, epizoochory, which was suggested for some terrestrial tardigrades (MOGLE *et al.* 2018; ROBERTSON *et al.* in press), may play a vital role in species transmission between water bodies by aquatic birds and mammals both on the intra- and inter-continental scale. This seems to be relevant as *D. parthenogeneticus* has already been reported from Argentina, Bolivia and Mexico (see the Introduction section). Since some recent works have demonstrated or suggested the existence of cryptic/pseudocryptic taxa in tardigrades (e.g. FAURBY *et al.* 2008; FONTOURA & MORAIS 2011; GUIDETTI *et al.* 2016; STEC *et al*. 2018; GUIDETTI *et al.*2019; MOREK *et al.* 2019b; SURMACZ *et al.* 2019; STEC *et al.* 2020a, 2020b), these reports of *D. parthenogeneticus* from outside Europe and based only on morphological observations must be regarded with a dose of scepticism until genetically confirmed. Conversely, since the comparison of morphometric data obtained in our study with data presented by MORENO-TALAMANTES *et al.* (2015) showed no differences between Polish and Mexican populations, this suggests an extremely wide distribution range. This would not be very surprising especially since *D. parthenogeneticus* is a parthenogenetic species, and recent works have already demonstrated such an extensive distribution for asexual tardigrades in distinct genera, e.g. *Paramacrobiotus* Guidetti *et al*., 2009, *Richtersius* Pilato and Binda, 1989 and *Echiniscus* Schultze, 1840 (see GĄSIOREK *et al.* 2019d; GUIDETTI *et al.* 2019; KACZMAREK *et al.* 2020; STEC *et al.* 2020a; STEC *et al.* 2020b).

As mentioned in the introduction, almost all *Dactylobiotus* species were originally described using only traditional morphological techniques with light microscopy and often with small sample sizes of animals and eggs. Previously KACZMAREK *et al.* (2008) and MORENO-TALAMANTES *et al.* (2015) listed three species with uncertain taxonomic positions, but we also noted a fourth species. The first of these is *Dactylobiotus macronyx* (Dujardin, 1851), whose validity was questioned by many taxonomists due to the very inadequate original description and the lack of a modern redescription (CUÉNOT 1932; MARCUS 1936; RAMAZZOTTI & MAUCCI 1983; BINDA & PILATO 1999; GUIDETTI *et al.* 2006a; KACZMAREK *et al.* 2008). The description states that the species lays smooth unornamented eggs within exuviae, which is atypical not only for the genus, but also for the entire order Macrobiotoidea. Thus, following also previous recommendations by BINDA and PILATO (1999) and GUIDETTI *et al.* (2006b), we formally designate this species as *nomen dubium*: *Dactylobiotus macronyx* (Dujardin, 1851) nom. dub. Similarly, *Dactylobiotus kansae* Beasley *et al.*, 2009 was described as a species that lays unornamented eggs within exuviae. The photomicrographs of animals provided by BEASLEY *et al.* (2009) indeed show a *Dactylobiotus* species. However, Fig. 2D in this work clearly shows that the claws

of the exuviae belong to the recently established isohypsibioid aquatic genus *Grevenius* Gąsiorek *et al.*, 2019a. Thus, considering that the description is based on animals and eggs that represent different tardigrade orders, and that the species identification without eggs is almost impossible in the genus *Dactylobiotus*, here we also designate this species as *nomen dubium*: *Dactylobiotus kansae* Beasley *et al.*, 2009 nom. dub. Two other *Dactylobiotus* species with highly insufficient descriptions are *Dactylobiotus aquatilis* Yang, 1999 and *Dactylobiotus henanensis* Yang, 2002. These descriptions do not contain any information on egg morphology, which is crucial for species identification within the genus; they lack detailed descriptions and/or measurements of other taxonomically important characteristics, such as cuticle morphology, claws and buccal apparatus; and they do not contain a proper differential diagnosis with other similar taxa. Since a correct identification of these species is impossible, we also propose to designate them as *nomina dubia*: *Dactylobiotus aquatilis* Yang, 1999 nom. dub. and *Dactylobiotus henanensis* Yang, 2002 nom. dub.

Acknowledgments

We are very grateful to Bartłomiej SURMACZ and Artur OCZKOWSKI, who collected the sediment samples from Botanical Garden in Kraków. We would also like to thank to Piotr GĄSIOREK, Witold MOREK, Roberto BERTOLANI and Roberto GUIDETTI for the photomicrographs of animals and eggs from the type series of *Dactylobiotus parthenogeneticus*. We are also grateful to Łukasz MICHALCZYK for the buccal-apparatus extraction as well as for English improvements and suggestions to the manuscript and for providing us additional SEM photomicrographs that were taken during his SYNTHESYS project (grant no. DK-5510). We are also highly indebted to Diane NELSON and two anonymous reviewers whose comments substantially improved our manuscript and to Roberto GUIDETTI and Roberto BERTOLANI for consultation on the morphology of the buccal apparatus and the literature regarding the studied tardigrade. The study was supported by the by the Jagiellonian University (subsidy no. DS/D/WB/IZiBB/16/2019 awarded to DS). Some of the analyses were carried out with the equipment purchased from the Sonata Bis programme of the Polish National Science Centre (grant no. 2016/22/E/NZ8/00417 to Łukasz MICHALCZYK).

Author Contributions

Research concept and design: D.S.; Collection and/or assembly of data: J.P., D.S.; Data analysis and interpretation: J.P., D.S.; Writing the article: D.S.; Critical revision of the article: D.S.; Final approval of article: J.P., D.S.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary Materials to this article can be found online at: http://www.isez.pan.krakow.pl/en/folia-biologica.html

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