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

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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.

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. 2006; 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 M. McNuff, pers. com). Commercial cultures of this strain are made available by Sciento (under catalogue number Z160). This population was confirmed by DASTYCH (1980) with modifications.

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.
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 Gasiorek and Witold Murek (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 Michałczyk 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 method (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 pseudo-genes. 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.

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| 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 | AGGGCGAAGCCCGAAATGGCTC | STEC et al. 2017 |
| | 18S_Tar_1Rr | reverse | GCCCGCAGGCTCACTCTCGG | |
| 28S rRNA | 28S_Eutar_F | forward | ACCCGCTGAACTTAAGCATAT | GASIOREK et al. 2018 |
| | 28S_R0990 | reverse | CCTTGGTGCTCGTTTCTAGAC | MIKOVÁ et al. 2012 |
| ITS-2 | Eutar_F | forward | CTGTTACGTTAGTGCAGGAC | STEC et al. 2018 |
| | Eutar_Rr | reverse | TCTCTCCGCTTATGATATG | |
| COI | LCO1490 | forward | GGTCAACAAACATATAAGATATG | FOLMER et al. 1994 |
| | HCO2198 | reverse | TAAACTTCAGGGTGACAAAAATCA | |
Results

Taxonomic account

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Macrobiotoidea Gui et al., 2019
Family: Murrayidae Guidetti et al., 2005
Genus: Dactylobiotus Schuster, 1980
(in SCHUSTER et al. (1980))

Dactylobiotus parthenogeneticus Bertolani, 1982

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.
winkled 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.
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 lunulalike 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.
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 | 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 | –       | 3.8    | 3.0  |
| 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 | –       | 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 | –       | 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 | –       | 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 | –       | 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 | –       | 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 | –       | 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 | –       | 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 | –       | 4.9    | –    |
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.
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 AY 598771 are very small.

Table 4

| 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 |

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 A Y 598771 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, A ntarctica) belong to 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; Gasiorek et al. 2016, 2019a) as well as on other continents (e.g. Cesari et al. 2016; Zawierucha et al. 2018; Gasiorek et al. 2019c; Jackson & Meyer 2019; Kazmerek et al. 2020; Sugiera 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* (Richers, 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, epizochochory, 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. Faury et al. 2008; Fontoura & Moraes 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 parthenogenic 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 Schultz, 1840 (see Gasiorek et al. 2019d; Guidetti et al. 2019; Kazmerek 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 & Maucchi 1983; Binda & Pilato 1999; Guidetti et al. 2006a; Kazmerek 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* B easley et al., 2009 was described as a species that lays unornamented eggs within exuviae. The photomicrographs of animals provided by B easley 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 iso-
hypsibioid aquatic genus Grevenius Gasiorek et al.,
2019a. Thus, considering that the description is based
on animals and eggs that represent different tardi-
grade orders, and that the species identification with-
out 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 aq-
uatilis Y ang, 1999 and Dactylobiotus hennanensis
Y ang, 2002. These descriptions do not contain any in-
formation on egg morphology, which is crucial for
species identification within the genus; they lack de-
tailed descriptions and/or measurements of other
taxonomically important characteristics, such as cuti-
cle 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 Y ang,
1999 nom. dub. and Dactylobiotus hennanensis Y ang,
2002 nom. dub.

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Critical revision of the article: D.S.; Final approval
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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary Materials to this article can be found
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