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Integrative description of a new Tunisian tardigrade species, *Macrobiotus azzunae* sp. nov. (Eutardigrada, Macrobiotidae, *hufelandi* group)

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Abstract. In this paper a new tardigrade species, *Macrobiotus azzunae* sp. nov., from Tunisia, is described. An integrative taxonomic approach was applied by combining morphological, morphometric and molecular data. In particular, light and scanning electron microscopy observations, and four genetic markers, three nuclear (18S rRNA, 28S rRNA and ITS-2) and one mitochondrial (COI) were used. The analysis showed that *M. azzunae* sp. nov. belongs to the *Macrobiotus hufelandi* group and is most similar to *Macrobiotus sandrae* Bertolani & Rebecchi, 1993. It differs from *M. sandrae* by a more pronounced constriction of the first macroplacoid (hardly visible in *M. sandrae*) and for the eggshell shape, with thinner wires of the reticulum and meshes around the processes larger than the inter-process meshes in *M. azzunae* sp. nov., while all meshes are similar in size in *M. sandrae*. The species is gonochoristic. With this discovery, there are 33 species of tardigrades identified in Tunisia, all non-marine. This result, compared with nearby Sicily, where more research has been conducted, indicates that there is a considerable potential for identification of new species. Further research will be most informative if multiple habitats are explored and if carried out with an integrated approach as done in this present work.

Keywords. Tardigrada, Ain Soltan Forest, Tunisia, *Macrobiotus azzunae*, new species.

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Introduction

Tardigrades are hygrophilous micrometazoans whose outstanding resistance enables most of them to inhabit a large variety of habitats from the greatest depths of oceans to the highest mountain peaks, as well as extreme environments such as cryoconite holes. In terrestrial environments they colonize mosses, lichens, leaf litter and soil which receive stochastic hydration (Nelson *et al.* 2015; Schill 2018). Much research on the diversity and distribution of tardigrade fauna has been carried out in recent years in various parts of the world, especially considering the terrestrial environment. This has led to a significant increase in the number of known species: 531 in 1983 (Ramazzotti & Maucci 1983), about 960 in 2005 (Guidetti & Bertolani 2005) and more than 1300 species in 2020 (Degma *et al*. 2020). Nevertheless, knowledge of the diversity and distribution of North African tardigrades is still very limited and in particular in Tunisia, for which only three studies have been conducted. The first report on Tunisian tardigrades comes from a survey by Iharos (1978) in Northern Tunisian regions in which eleven species of tardigrades were detected. Then, Binda & Pilato (1987) studied the tardigrade fauna of Salambo (Tunis), Tabarka and Ain Drahem (Jendouba), and recently Gąsiorek *et al*. (2017) studied the tardigrade fauna of Bni Mtir, Jendouba. Currently, the known Tunisian tardigrade fauna includes 32 species, of which one, *Bryodelphax maculatus* Gąsiorek, Stec, Morek, Marnissi, Michalczyk, 2017, was originally discovered from the same region considered here, namely the Kroumirie Mountains. The Kroumirie Mountains are located in Northern Tunisia near the Algerian boundary and are characterized by a climate which varies from sub-humid to humid in winter. The Kroumirie forests are covered especially by cork oaks (*Quercus suber* L.) and Algerian oaks (*Quercus canariensis* Willd.) (Stambouli-Essassi *et al*. 2007). The analysis of the tardigrade fauna of a moss collected at the Kroumirie Mountains led us to find a new eutardigrade species (*Macrobiotus azzunae* sp. nov.) belonging to the *Macrobiotus hufelandi* group, considered as one of the most common groups of limnoterrestrial tardigrades on the planet (McInnes 1994; Kaczmarek & Michalczyk 2017a; McInnes *et al*. 2017). In this study we combined classical morphological and morphometric methods with modern molecular techniques in an integrative approach as suggested by Cesari *et al.* (2009, 2011, 2020), Stec *et al.* (2018b) and Kayastha *et al* (2020). Using phase contrast (PhC) light microscopy (LM), differential interference contrast (DIC) and scanning electron microscopy (SEM), we were able to describe the phenotypic characteristics of the new species whereas the amplification of DNA markers (three nuclear, 18S rRNA, 28S rRNA and ITS-2, and one mitochondrial, COI) provided barcodes for the genetic identification of this new species.

Material and methods

Tardigrade collection

The moss containing the new species was collected in April 2017 by the first author from Ain Soltan forest in the North West of Tunisia (Fig. 1) on a trunk of an Algerian oak tree. The sample was stored dry in a labeled paper bag and then sent to the Laboratory of Evolutionary Zoology at the Department of Life Sciences, University of Modena and Reggio Emilia (UNIMORE), Italy. In order to extract tardigrades from the sample, a small portion of the collected substrate was placed and maintained in tap water at room temperature (20°C) for about half an hour. The sample was then sieved to separate tardigrades and their eggs from the substrate, and both animals and eggs were isolated using a glass pipette under a stereo microscope.

Microscopy and imaging

The collected specimens (eggs and animals) were mounted on slides in Faure-Berlese fluid (permanent slides) or fixed in Carnoy fixative (methanol: acetic acid, 3:1) and in the latter case then stained with a drop of acetic-lactic orcein (Rebecchi & Bertolani 1988) for LM observations (not permanent slides).

Observations and measurements of the sclerified parts of animals and eggshells were carried out with LM under PhC and DIC up to the maximum magnification (100× oil objective) with a Leica DM RB microscope equipped with a Nikon DS-Fi 1 digital camera, at the Department of Life Sciences, UNIMORE. The species was identified by comparing the specimens with the original descriptions of similar species of *Macrobiotus* (for references see Results: Differential diagnosis) and, when possible, with the original type material of some of those species. In particular, we have examined the following material from Maucci's collection (thanks to the Civic Museum of Natural History of Verona, Italy): slides CT14009 holotype and CT14015 egg of *Macrobiotus madegassus* Maucci, 1993; slide CT14701 one animal and one egg (paratypes) of *Macrobiotus personatus* Biserov, 1990; from Bertolani's collection of the Department of Life Sciences, UNIMORE, Italy: slide C460–S97 holotype of *Macrobiotus sandrae* Bertolani & Rebecchi, 1993 and for the same species slides from the type locality C442–S79 (Animal) and C450–S01 and C2346–S2 (eggs). These specimens and eggs were analyzed under LM.

Measurements of animal length, claws and buccal-pharyngeal apparatus details were taken according to Pilato (1981). The external buccal tube diameter was measured at the level of the stylet support insertion point. We calculated the *pt* index which is the percentage ratio between the length of a structure and the length of the buccal tube measured from the medio-dorsal ridge of the buccal armature to the base of the pharyngeal apophyses (Pilato 1981). Single measurements are available in Supp. file 1.

Fifteen additional specimens were prepared for SEM analyses according to Bertolani *et al.* (2014), and observed with a Philips SEM XL 40, available at the Centro Interdipartimentale Grandi Strumenti (CIGS) of UNIMORE.

Molecular analysis

Before molecular analysis, each specimen was identified and photographed in vivo with LM using the method described by Cesari *et al.* (2011) in order to obtain voucher specimens. Supp. file 2 shows the buccal-pharyngeal apparatus of a hologenophore voucher specimen, showing how it is possible to obtain good morphological information on alive animals even when the entire animal has to be used

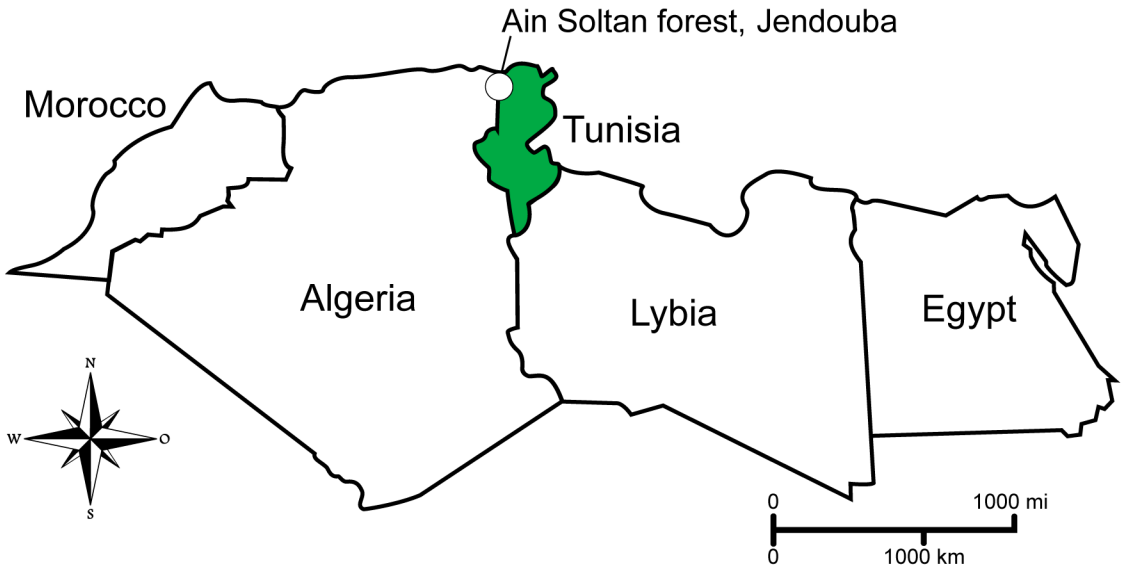

Fig. 1. Map of the sampling locality (circle) in the North-West of Tunisia.

Table 1. List of specimens of *M. azzunae* sp. nov. and *M. sandrae* Bertolani & Rebecchi, 1993 utilized for molecular analyses. Abbreviation: N/A = not available. * These specimens were analyzed for the COI gene in Bertolani *et al*. (2011b).

Specimen	18S	28S	ITS-2	COI
Macrobiotus azzunae				
C4218 V1	N/A	N/A	N/A	MW698695
C4218 V2	N/A	N/A	N/A	MW698696
C4218 V4	MW695447	MW695450	MW695454	MW698697
C4218 T5	MW695448	MW695451	MW695455	MW698698
C4218 T6	MW695449	MW695452	MW695456	N/A
C4218 V7	N/A	MW695453	MW695457	MW698699
Macrobiotus sandrae				
C2945 b	MW695445	N/A	N/A	HQ876573*
C2945 e	MW695446	N/A	N/A	HQ876577*

for molecular analysis. Table 1 lists the specimens utilized. Genomic DNA was extracted from six Tunisian single animals using a rapid salt and ethanol precipitation following the protocol described by Cesari *et al*. (2009). We amplified four DNA fragments: the small ribosome subunit (18S rRNA), the large ribosome subunit (28S rRNA), the internal transcribed spacer (ITS-2), and the cytochrome oxidase subunit I (COI), using the primers and protocols described by Bertolani *et al*. (2014), Cesari *et al*. (2016), Stec *et al*. (2018d) and Cesari *et al*. (2009), respectively. Additionally, a portion of the 18S gene was sequenced for two additional specimens of *M. sandrae* sampled in the locus typicus of the species (Black Forest, Germany). GenBank accession numbers of obtained sequences are listed in Table 1. The amplified products were gel purified using the Wizard Gel and PCR cleaning (Promega) kit, and fragments were sequenced according to the protocols described in Bertolani *et al*. (2014). All COI sequences were translated into protein sequences in MEGAX (Kumar *et al*. 2018) to check for the presence of stop codons, and therefore for the presence of pseudogenes. Sequences of the four genes pertaining to species of the *hufelandi* group were used for molecular comparisons (Table 2) and aligned with the newly produced sequences using the MAFFT algorithm (Katoh *et al.* 2002) as implemented in the MAFFT online service (Katoh *et al*. 2017) and checked by visual inspection (Supp. file 3, Supp. file 4, Supp. file 5, Supp. file 6). Uncorrected pairwise distances were computed using MEGAX (Supp. file 7, Supp. file 8, Supp. file 9, Supp. file 10). Furthermore, relationships between COI haplotypes pertaining to the clade A nested in the *M. hufelandi* complex sensu Stec *et al.* (2021) were estimated using a parsimony network, by applying the method described in Templeton *et al*. (1992), as implemented in TCS ver. 1.21 (Clement *et al*. 2000) and visualized using tcsBU (Múrias dos Santos *et al*. 2016). A 95% connection limit was employed, as it has been suggested as a useful general tool in species assignments and discovery (Hart & Sunday 2007). Putative species were also inferred by using the Poisson Tree Process (PTP; Zhang *et al.* 2013) and the Automatic Barcode Gap Discovery method (ABGD; Puillandre *et al.* 2012). PTP is a coalescent-based species delimitation method that uses non-ultrameric gene trees as input, and utilizes heuristic algorithms to identify speciation events relative to numbers of substitutions. The PTP method produces robust diversity estimates, in some cases more robust than those estimated under the generalized mixed Yule coalescent model (Tang *et al.* 2014). The starting gene tree was a maximum likelihood (ML) tree computed using RAxML ver. 7.2.4 (Stamatakis 2006), as implemented in CIPRES (Miller *et al*. 2010), under the GTR+I+G model, as inferred by using the Akaike Information Criterion on jModelTest2 (Guindon & Gascuel 2003; Darriba *et al*. 2012). A sequence of *Mesobiotus hilariae* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi & Guidetti, 2016 (GenBank accession number: KT226108) was used as outgroup. Bootstrap

resampling with 1000 replicates was undertaken via the rapid bootstrap procedure of Stamatakis *et al*. (2008) to assign support to branches in the ML tree. In order to consider different evolutionary models for the three COI codons, a Bayesian tree was computed using the following models, as inferred by MrModeltest ver. 2 (Nylander 2004): SYM+I+G for the first position of the codon, GTR+I+G for the second position of the codon and GTR+G for the third position of the codon. The Bayesian dendrogram was computed with the program MrBayes ver. 3.2.7a (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), as implemented in CIPRES. Two independent runs, each of four Metropolis-coupled Markov chains Monte Carlo method, were launched for 2×10^7 generations, and trees were sampled every 1000 generations. Convergence of runs was assessed by tracking average standard deviation of split frequencies between runs and by plotting the log likelihood of sampled trees in TRACER ver. 1.7 (Rambaut *et al.* 2018) and the first 2×10^6 sampled generations were discarded as burn-in. In the distance-based ABGD method, the sequences are sorted into hypothetical species based on the barcode gap (i.e., whenever the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species). The method first detects the barcode gap as the first significant gap beyond a model-based one-sided confidence limit for intraspecific divergence, and then uses it to partition the data. ABGD settings for the COI dataset were: prior minimum divergence of intraspecific diversity (Pmin) = 0.001; prior maximum divergence of intraspecific diversity (Pmax) = 0.1; Steps = 10 and gap width = 1.5. The analysis was performed on the ABGD website (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html).

Abbreviations

DIC = Differential Interference Contrast light Microscopy
LM = Light Microscopy
PhC = Phase Contrast light Microscopy
SEM = Scanning Electron Microscopy

Repository

UNIMORE = Department of Life Sciences, University of Modena and Reggio Emilia, via G. Campi 213/d, Modena, Italy

Results

Taxonomic account of the new species

Phylum Tardigrada Doyère, 1840
Class Eutardigrada Richters, 1926
Order Parachela Schuster, Nelson, Grigarick & Christenberry, 1980
Superfamily Macrobiotoidea Thulin, 1928 in Marley *et al.* 2011
Family Macrobiotidae Thulin, 1928
Genus *Macrobiotus* C.A.S. Schultze, 1834

Macrobiotus azzunae sp. nov.
urn:lsid:zoobank.org:act:933CCC06-F69D-49E2-AF4F-0C042D8F5C99
Figs 1–4, 5A, C, 7

Etymology

The new species is dedicated in honor of Atf Azzouna, professor in the Faculty of Mathematical, Physical and Natural Sciences of Tunis and supervisor of the PhD thesis of Jamila Ben Marnissi.

Table 2 (continued on next page). Sequences of species of the *Macrobiotus hufelandi* group used for molecular comparisons with *M. azzunae* sp. nov.

Species	Accession number				Country	Reference
	18S	**28S**	**COI**	**ITS2**		
Macrobiotus caelestis	MK737073		MK737922	MK737072	Kyrgyzstan	Coughlan *et al*. 2019
Macrobiotus canaricus	MH063922		MH057765–6	MH063928–30	Canary Island	Stec *et al*. 2018c
Macrobiotus crustulus	MT261912		MT260371	MT261907	French Guiana	Stec *et al*. 2020a
Macrobiotus engbergi	MN443039		MN444824–6	MN443036–7	Greenland	Stec *et al*. 2020b
Macrobiotus hannae	MH063922		MH057764	MH063923	Poland	Nowak & Stec 2018
Macrobiotus hufelandi	GQ849024				Greenland	Jørgensen *et al*. 2010
			HQ876584–88		Germany	Bertolani *et al*. 2011b
Macrobiotus cf. *hufelandi* sp. 1			HQ876589		Germany	
			HQ876590–4		Switzerland	Bertolani *et al*. 2011b
			HQ876595–7		Italy	
Macrobiotus group *hufelandi*	JX888491–8538				USA	Adams, unpublished
Macrobiotus joannae	HQ604974–5				Australia	Bertolani *et al*. 2014
Macrobiotus kamilae	MK737070		MK737920–1	MK737067	India	Coughlan & Stec 2019
Macrobiotus kristenseni	KC193577		KC193572–6		Argentina	Guidetti *et al*. 2013
Macrobiotus macrocalix	HQ604976		FJ176203–17	MH063931	Italy	Bertolani *et al*. 2014
	MH063926		MH057767		Poland	Stec *et al*. 2018c
Macrobiotus cf. *macrocalix*			JX683812–20		Portugal	Vicente *et al*. 2013
Macrobiotus cf. *nelsonae*	HQ604965–6				USA	Bertolani *et al*. 2014
Macrobiotus noongaris	MK737069		MK737919	MK737065–6	Australia	Coughlan & Stec 2019
Macrobiotus papei	MH063881		MH057763	MH063921	Tanzania	Stec *et al*. 2018b
Macrobiotus paulinae	KT935502		KT951668	KT935500	Kenya	Stec *et al*. 2015
Macrobiotus persimilis			EU244608		Germany	Schill, unpublished
Macrobiotus polonicus	HM187580			HM150647	Poland	Wełnicz *et al*. 2011
	MN888369		MN888317–19	MN888337–8	Austria	Stec *et al*. 2021
	MN888370		MN888320–21	MN888332–4	Slovakia	Stec *et al*. 2021

Table 2 (continued).

Species	Accession number				Country	Reference
	18S	**28S**	**COI**	**ITS2**		
Macrobiotus polypiformis	KX810008		KX810011–2	KX810010	Ecuador	Roszkowska *et al.* 2017
Macrobiotus cf. *recens*	MH063927		MH057768–9	MH063932–3	Spain	Stec *et al.* 2018
Macrobiotus sandrae			HQ876567–73		Italy	Bertolani *et al.* 2011b
			HQ876574–83		Germany	
Macrobiotus sapiens	DQ839601			GQ403680	Croatia	Schill *et al.* 2010
	HQ604971				Italy	Bertolani *et al.* 2014
Macrobiotus scoticus	KY797265		KY797267	KY797268	Scotland	Stec *et al.* 2017
Macrobiotus shonaicus	MG757132		MG757136–7	MG757134–5	Japan	Stec *et al.* 2018a
Macrobiotus cf. *shonaicus*			LC431582–90	LC431591–9	Japan	Sugiura *et al.* 2020
Macrobiotus terminalis			JN673958–62		Italy	Cesari *et al.* 2011
Macrobiotus cf. *terminalis*			AY598775		Italy	Guidetti *et al.* 2005
Macrobiotus vladimiri			JX683810–11		Portugal	Vicente *et al.* 2013
			HM136931–4		Italy	Bertolani *et al.* 2011a
	FJ435738–42	FJ435751–5			Spain	Guil & Giribet 2012
	MN888375		MN888327	MN888347	Finland	Stec *et al.* 2021
Macrobiotus wandae	MN435109–13		MN482681–7	MN435120–22	Nepal	Kayastha *et al.* 2020

Type material

Holotype

TUNISIA • spec. of unidentified sex; North-West Tunisia, Kroumiri Mountains, Ain Soltan forest, Jendouba; 36º31′21.788″ N, 8º19′57.741″ E; 893 m a.s.l.; Apr. 2017; Marnissi leg.; moss on trunk of *Quercus canariensis*; UNIMORE, slide code C4218–S32.

Paratypes

TUNISIA • 17 specs, sex unidentified; same collection data as for holotype; UNIMORE, slide codes C4218–S2 to C4218–S7, C4218–S9, C4218–S17, C4218–S30, C4218–S31, C4218–S33 to C4218–S35 • 3 eggs; same collection data as for holotype; UNIMORE, slide codes C4218–S10, C4218–S11, C4218–S25.

Type depositories

The holotype (slide: C4218–S32), 17 paratypes (slides C4218–S2 to C4218–S7, C4218–S17, C4128–S30, C4218–S31, C4218–S33 to C4218-S35), 3 eggs (slides: C4218–S10/11/25) and two vouchers (slides SP04 and SP07, corresponding to specimens C4218 V4 and C4218 V7, respectively) mounted in Faure-Berlese fluid, are deposited in the Bertolani collection at the Department of Life Science, UNIMORE, Modena, Italy.

Type locality

NW Tunisia, Kroumrie mountains, Ain Soltan forest, Jendouba, 36º31′21.788″ N, 8º19′57.741″ E. Altitude 893 m a.s.l.

Description

Adult specimens

Body white, transparent after mounting in Faure-Berlese, from 162.2 to 410.3 μm in length (Fig. 2A, Table 3; structures measured only in the animals more than 200 μm in length). Eye spots present, even after mounting. Cuticle smooth but with small round or oval pores, 1–1.5 μm in diameter (Fig. 2B), better visible after fixation in Carnoy and orcein staining (Fig. 3C), scattered randomly on the entire cuticle, including the dorsal surface of all legs. With SEM, pores look oval or in the shape of a seed (Fig. 3A, D) with the largest diameter of 0.7–0.8 μm. Weak cuticular granulation also present on the lateral surface of all legs and specially on legs IV (Fig. 2B, arrow). Only with SEM is it possible to define the shape of the granulation on the legs, which looks as a regular disposition of star-shaped protuberances (about 0.3 μm; Fig. 3F). Six buccal sensory lobes around the mouth, well recognizable with SEM. Mouth antero-ventral; buccal-pharyngeal apparatus of the *Macrobiotus* type (sensu Pilato & Binda 2010), with ventral lamina and ten small peribuccal lamellae (in the holotype, after mounting, separated from the mouth). Buccal armature, corresponding to oral cavity armature, OCA, according to Michalczyk & Kaczmarek (2003), without an anterior band of teeth visible, corresponding to the first band of teeth according to Michalczyk & Kaczmarek (2003), and to the anterior band of the buccal ring according to Guidetti *et al*. (2012); posterior band of teeth poorly visible, corresponding to second band of teeth, according to Michalczyk & Kaczmarek (2003), followed by three dorsal and three ventral crests, corresponding to third band of teeth according to Michalczyk & Kaczmarek (2003); the dorsal crests (Fig. 2D) are distinct transverse ridges, whereas the ventral crests (Fig. 2E) appear as two separate lateral transverse ridges and a roundish median tooth. The posterior band of teeth and the transverse ridges are part of the buccal tube, according to Guidetti *et al*. (2012). Buccal tube narrow; pharyngeal bulb spherical with triangular apophyses, two rod-shaped macroplacoids, relatively short, the first longer than the second and evidently but not deeply narrowed at its middle (Fig. 2C), the second with a not particularly evident subterminal constriction. Microplacoid present. Slender claws of the *hufelandi* type (sensu Pilato & Binda 2010); the external claw longer than the internal one and the posterior longer than

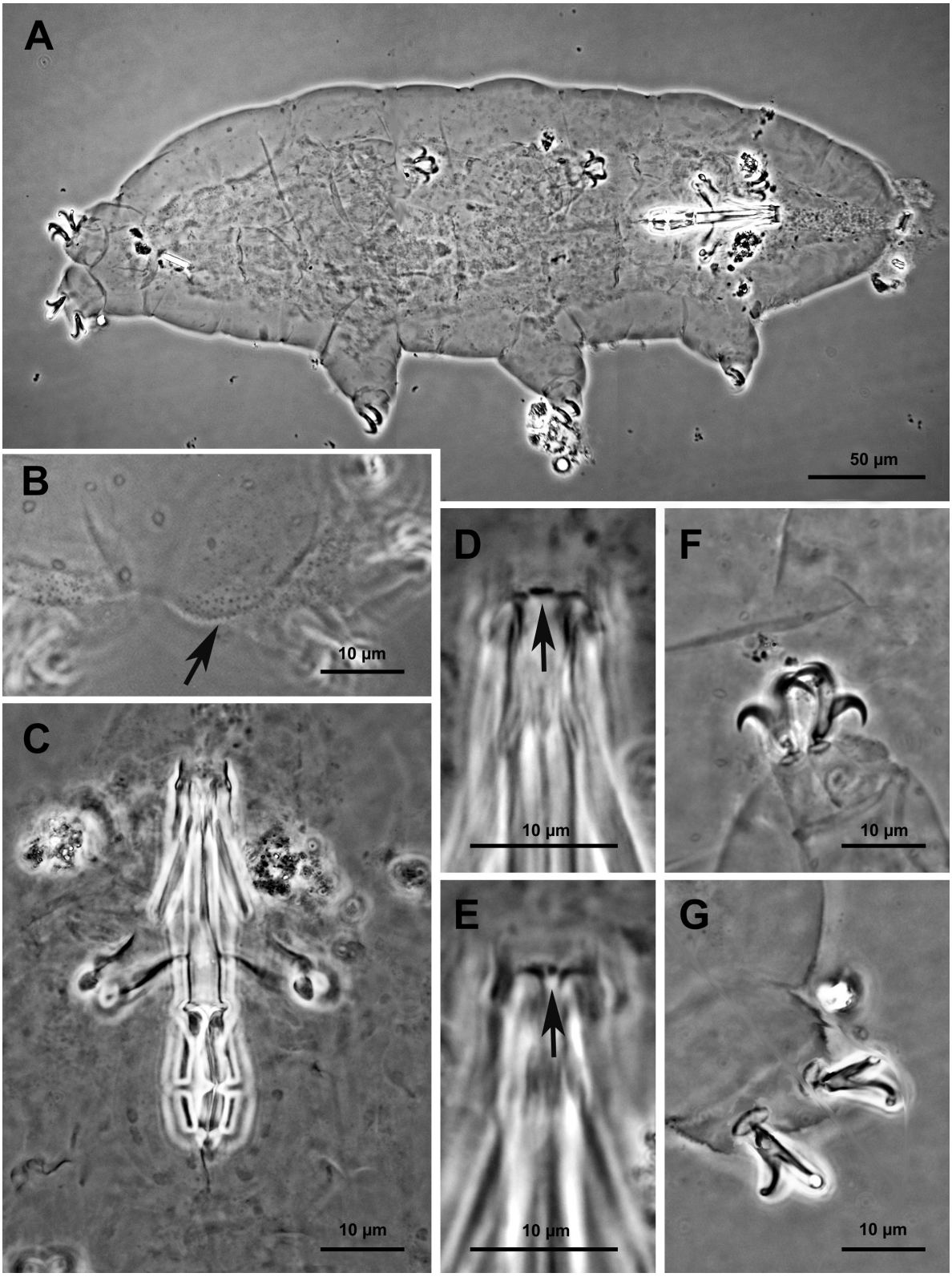

Fig. 2. *Macrobiotus azzunae* sp. nov., holotype (UNIMORE, slide C4218–S32). **A**. In toto animal. **B**. Cuticular pores and leg granulation (arrow) on the hind legs. **C**. Buccal-pharyngeal apparatus. **D**. Buccal armature: dorsal crests (arrow). **E**. Buccal armature: ventral crests (arrow). **F**. Claw and lunulae of the third pair of legs. **G**. Claw and lunulae of the fourth pair of legs. A–G: PhC.

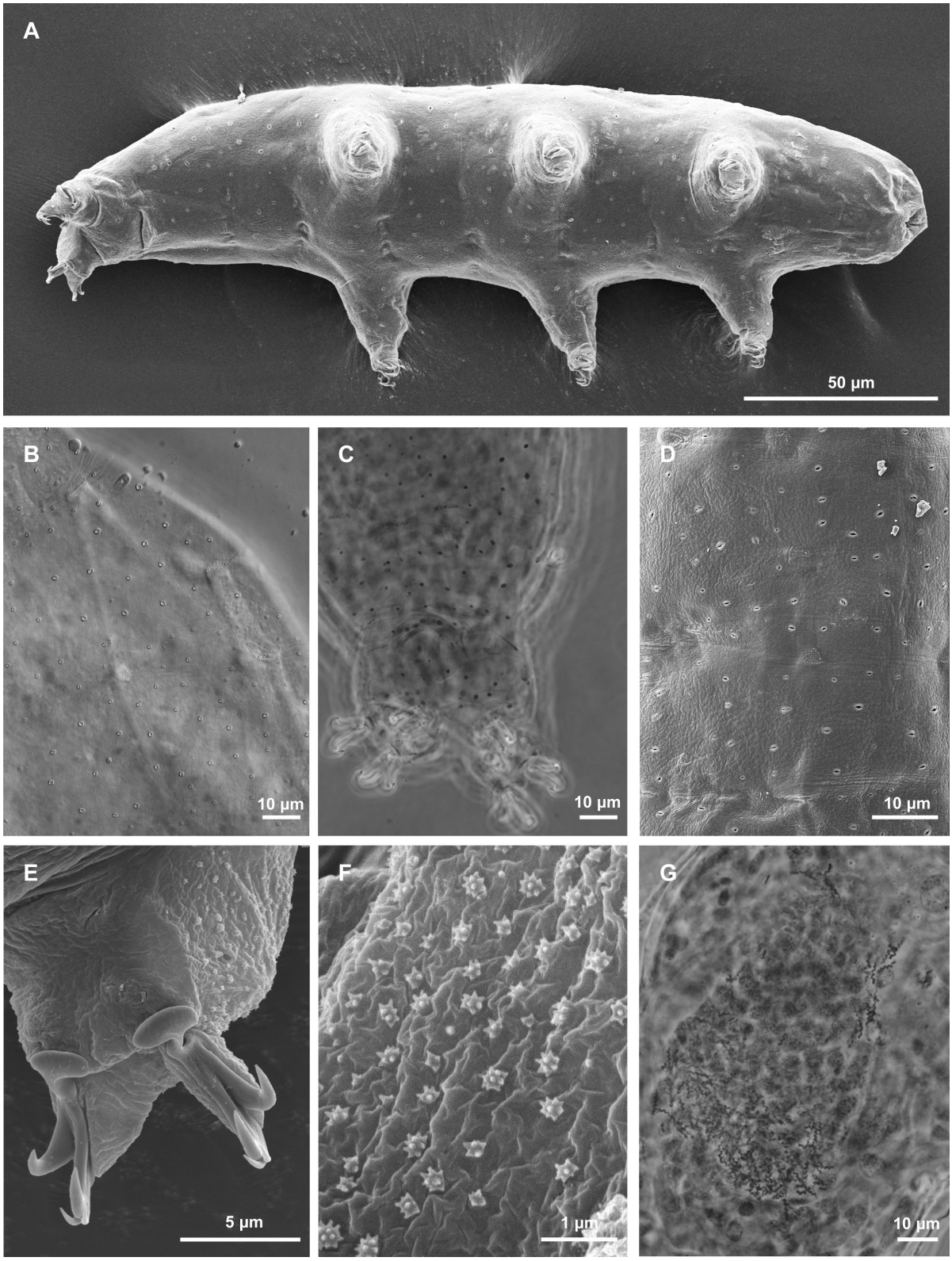

Fig. 3. *Macrobiotus azzunae* sp. nov., paratypes. **A**. In toto animal. **B–D**. Cuticular pores. **E**. Fourth pair of legs with smooth lunules and peculiar granulation on the legs. **F**. Granulation on the legs with a star-shaped organization. **G**. Male with testis full of mature spermatozoa with elongate, helicoidal nucleus. A, D–F: SEM (stub-C4218); B: in vivo DIC; C, G: orcein (not permanent slide TN02–04) PhC.

Table 3. Measurements (in µm) and *pt* of selected morphological structures of individuals of *Macrobiotus azzunae* sp. nov. mounted in Faure-Berlese. Abbreviations: Nr = number of specimens/structures measured; Range = smallest–largest value; SD = standard deviation. * Three animals below 200 µm in length were not considered in the analysis. Two were not measurable, while data of the only measurable animal below 200 µm is available in the supplementary material (Supp. file 1).

Character	Nr	Range µm	Mean ± SD	Holotype µm	
Body length	10*	246.0–410.3		374.0	
Buccal tube length	13	29.4–38.5		36.6	
		pt		µm	*pt*
Buccal tube external width	13	*11.6–15.9*	*13.3 ± 1.2*	5.8	*15.9*
Stylet support insertion point	13	*75.1–80.5*	*76.7 ± 1.5*	27.9	*76.1*
Placoid row	13	*50.1–57.7*	*51.8 ± 2.4*	18.9	*51.5*
Macroplacoid row	13	*37.7–48.7*	*44.6 ± 3.1*	15.7	*42.7*
First macroplacoid	13	*24.3–29.7*	*26.6 ± 2.2*	9.3	*25.5*
Second macroplacoid	13	*16.0–20.7*	*18.3 ± 1.5*	6.6	*18.1*
Microplacoid	13	*5.9–8.6*	*6.9 ± 1.0*	2.6	*7.0*
External Claws III main branch	13	*27.1–31.3*	*29.8 ± 1.3*	11.5	*31.3*
External Claws III secondary branch	13	*20.1–25.8*	*22.5 ± 1.6*	8.1	*22.1*
Internal Claws III main branch	13	*24.9–30.2*	*27.7 ± 1.6*	10.2	*27.9*
Internal Claws III secondary branch	13	*18.8–24.2*	*21.4 ± 1.7*	7.2	*19.7*
Posterior Claws IV main branch	9	*29.1–34.6*	*32.7 ± 1.8*	12.2	*33.2*
Posterior Claws IV secondary branch	9	*21.6–24.9*	*23.8 ± 1.6*	9.1	*24.9*
Anterior Claws IV main branch	10	*28.2–32.5*	*30.1 ± 1.9*	11.1	*30.3*
Anterior Claws IV secondary branch	10	*20.4–26.2*	*23.2 ± 1.7*	8.8	*24.0*

the anterior. Primary branches of each claw with distinct accessory points (Fig. 2F), a common tract of medium length (about a third of the total claw length) and an evident stalk connecting the claw to the lunule. Lunules under all claws, smooth, larger on the hind legs (Figs 2G, 3E). Cuticular bars under claws absent.

The population is dioecious (gonochoristic). Males were recognized using orcein staining, which revealed that the testis is filled with spermatozoa with a coiled head (Fig. 3G) and spermatids. No morphological secondary sexual dimorphism, such as gibbosities on legs IV in males, was identified.

Eggs

Eggs are laid freely, and are white, spherical or slightly oval. One egg containing a fully developed embryo showed the shape of the buccal-pharyngeal apparatus (Fig. 4A). Processes of the eggshell are in the shape of inverted goblets (Fig. 4B) with conical trunks and well-defined distal discs as large as the process bases (for measurements see Table 4). Distal discs concave, with a median small protuberance and, using PhC, with border often smooth, or sometimes slightly jagged, or slightly ragged (Fig. 4C),

Table 4. Measurements (in µm) of selected morphological structures of the eggs of *Macrobiotus azzunae* sp. nov. mounted in Faure-Berlese. Abbreviations: Nr = number of eggs/structures measured; Range = smallest–largest value; SD = standard deviation.

Character	Nr	Range	Mean ± SD
Egg diameter without processes	2	64.7–80.6	–
Egg diameter with processes	2	72.4–89.2	–
Process nr on egg circumference	2	29–33	–
Process height	10	4.2–6.4	5.4 ± 0.6
Process base width	10	3.2–5.2	4.2 ± 0.5
Distal disc width	10	3.2–5.2	4.4 ± 0.6
Inter process distance	10	2.7–4.8	3.5 ± 0.5

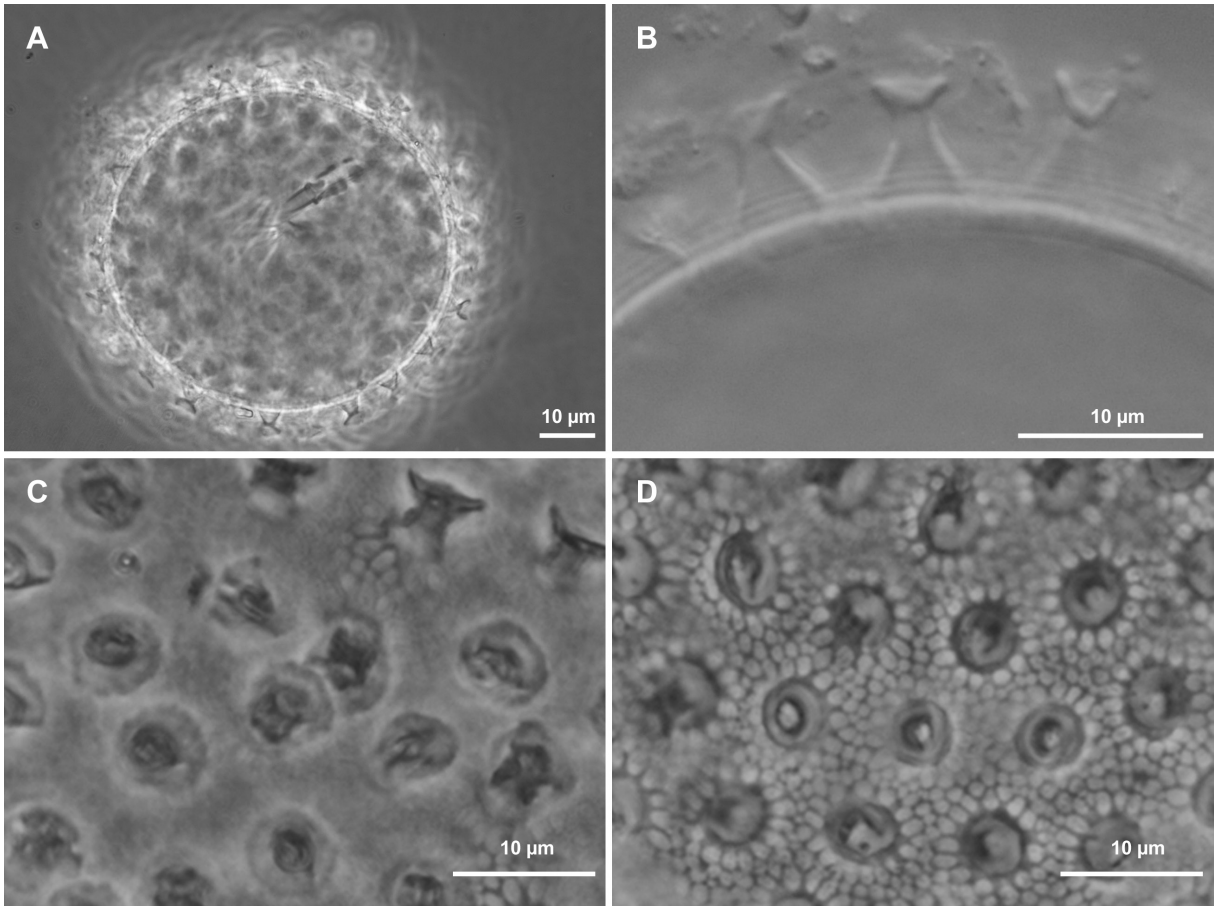

Fig. 4. Egg of *Macrobiotus azzunae* sp. nov., paratype (UNIMORE, slide C4218–S11). **A**. In toto egg with buccal-pharyngeal apparatus of its embryo at the end of development. **B**. Processes of the eggshell (midsection). **C**. Distal discs of the eggshell processes. **D**. Surface of the eggshell between processes. A, C–D: PhC; B: DIC.

but never clearly jagged, serrated or dentate. Surface among processes of the *hufelandi* type (sensu Kaczmarek & Michalczyk 2017a), i.e., covered by a very thin grid (Fig. 4D). Meshes around the process bases slightly larger and with slightly thicker wires compared with interbasal meshes. Mesh diameter around 0.5 µm.

Comparisons

Macrobiotus azzunae sp. nov. has eggs with processes as inverted goblets and a reticulate eggshell between the processes. Consequently, a comparison must be done with the *Macrobiotus* species listed by Kaczmarek & Michalczyk (2017a) with *hufelandi* type eggshells, excluding the species with processes that are not like inverted goblets, and adding the species with *hufelandi* type chorion eggs described after that publication. The species with *hufelandi* type chorion eggs that do not have processes as inverted goblets are *Macrobiotus acadianus* (Meyer & Domingue, 2011), *M. dariae* Pilato & Bertolani, 2004, *M. lissostomus* Durante Pasa & Maucci, 1979, *M. santoroi* Pilato & D'Urso, 1976, and *M. scoticus* Stec, Morek, Gąsiorek, Blagden & Michalczyk, 2017. Moreover, *M. azzunae* sp. nov. has egg processes with distal discs with a smooth or slightly jagged border, therefore it differs from all the species that have clearly indented, serrated or clearly jagged distal discs, such as: *Macrobiotus canaricus* Stec, Krzywański & Michalczyk, 2018, *M. crustulus* Stec, Dudziak & Michalczyk, 2020, *M. hannae* Nowak & Stec, 2018 (whose egg surface is more cribriform than reticulate), *M. hibiscus* de Barros, 1942, *M. horningi* Kaczmarek & Michalczyk, 2017b (which also has very high processes), *M. hufelandi* C.A.S. Schultze, 1834, *M. humilis* Binda & Pilato, 2001, *M. iharosi* Pilato, Binda & Catanzaro 1991, *M. joannae* Pilato & Binda, 1983, *M. julianae* (Meyer, 2012), *M. kamilae* Coughlan & Stec, 2019, *M. modestus* Pilato & Lisi, 2009, *M. noonragi*s Coughlan & Stec, 2019, *M. papei* Stec, Kristensen & Michalczyk, 2018 (with particularly long filaments starting from the distal disc), *M. paulinae* Stec, Smolak, Kaczmarek & Michalczyk, 2015, *M. polypiformis* Roszkowska, Ostrowska, Stec, Janko & Kaczmarek, 2017 (even with cog-teeth extended to form a long, thin, hair-like and flexible filament), *M. punctillus* Pilato, Binda & Azzaro, 1990, *M. sapiens* Binda & Pilato, 1984, *M. sottilei* Pilato, Kiosya, Lisi & Sabella, 2012.

For the shape of the egg *Macrobiotus azzunae* sp. nov. differs from *M. rawsoni* Horning, Schuster & Grigarick, 1978 because this species has only one strip of meshes around each egg process (see Kaczmarek & Michalczyk 2017b); it differs from *M. serratus* Bertolani, Guidi & Rebecchi, 1996 because in this species the egg surface is porous more than reticulated, with pores small and spaced from each other, and its egg processes have a large, often square, distal disc; it differs from *M. seychellensis* Biserov, 1994 because the distal disc of the egg processes of this species, even though not dentate, has long and very developed lobes.

The remaining nine species of the *hufelandi* group should be compared singularly.

***Macrobiotus almadai* Fontoura, Pilato & Lisi, 2008**

Macrobiotus azzunae sp. nov. differs from *M. almadai* in having a posterior band of teeth in the buccal cavity visible with LM (not visible in *M. almadai*), and distal disc with a jagged margin instead of very small teeth as in *M. almadai*.

***Macrobiotus canaricus* Stec, Krzywański & Michalczyk, 2018**

With LM the margin of the distal disc of *M. azzunae* sp. nov., never dentate in this species, looks similar to that of *M. canaricus*, but the SEM images of the eggs of the latter species evidence the presence of an almost dentate disc. Moreover, the peribasal meshes of the eggshell are larger than interbasal ones in the new species while they do not differ from the interbasal ones in *M. canaricus*; regarding the animals there are differences in the buccal armature: in *M. azzunae* sp. nov. the posterior band of teeth is visible with LM (even though poorly) and the three dorsal crests are distinct transverse ridges, while

in *M. canaricus* the posterior band of teeth is visible only with SEM and with LM the dorsal teeth form a transversal ridge weakly divided into three teeth.

***Macrobiotus madegassus* Maucci, 1993**

The new species differs from *M. madegassus* by the presence of the eye spots (absent in *M. madegassus*), pores on the cuticle (absent in *M. madegassus*), presence in the buccal armature of posterior band of teeth, even though weak (fully absent in *M. madegassus*), buccal tube much larger (*pt* of the holotypes 15.9 vs 7), insertion of the stylet supports on the buccal tube much more posterior (*pt* of the holotypes 76.1 vs 68), first and second macroplacoid longer (*pt* of the holotypes 25.5 and 18.1 vs 21.3 and 12.0), lunules on the hind legs without kerning (crenate in *M. madegassus*), eggshell processes with distal disc as large as the base (similar range 3.2–5.2 for both measurements) with respect to that of *M. madegassus* (disc vs base: 4.3–5.4 vs 2.3–2.6).

***Macrobiotus martini* Bartels, Pilato, Lisi & Nelson, 2009**

The cuticular pores in *M. azzunae* sp. nov. are much smaller than those of *M. martini*; the distal disc of the egg processes in *M. azzunae* sp. nov. has a diameter similar to that of the process base, while in *M. martini* the distal disc is much narrower than the base.

***Macrobiotus nebrodensis* Pilato, Sabella, D'Urso & Lisi, 2017**

Macrobiotus azzunae sp. nov. differs from *M. nebrodensis* by the absence of the cuticular bar near the lunules on the first three pairs of legs (a faint bar is present in *M. nebrodensis*). The egg processes of *M. azzuane* sp. nov. are in higher number on the circumference (29–33) with respect to those of *M. nebrodensis* (18). In the latter species there are some egg processes very high (up to 20.6 µm), while in the new species process height and shape are more uniform. The difference in the eggshell between meshes around the process base and the others is much less evident in *M. azzunae* sp. nov. than in *M. nebrodensis*.

***Macrobiotus personatus* Biserov, 1990**

The new species differs from *M. personatus* by the posterior band of the buccal armature less evident, the presence of a clear constriction in the first macroplacoid (Fig. 5A), in the paratype of *M. personatus* examined by us barely identifiable (Fig. 6A) and, according to Biserov (1990) usually absent in the type material of that species. The pores on the cuticle of *M. azzunae* sp. nov. are small, approximately 1 µm in diameter, while in *M. personatus* they are strongly elliptic and about 3 µm in length (Fig. 6B). Lunules on leg IV are always smooth in *M. azzunae* sp. nov., sometimes indented in *M personatus*. With respect to the eggs of *M. personatus* (Fig. 6C–D), the egg processes of *M. azzunae* sp. nov. (Figs 4C–D, 5C) are clearly shorter, 5.4 ± 0.6 vs 9.5 ± 0.5 (range 4.2–6.4 vs 9–10.5) and with a narrower base and distal disc (both 3.2–5.2 vs 7–10.5 and 7–9 respectively). Males are present in the new species, while in *M. personatus* only females were found (Biserov 1990), suggesting parthenogenesis in that species.

***Macrobiotus sandrae* Bertolani & Rebecchi, 1993**

The new species differs from *M. sandrae* for the eggshell shape, with thinner wires of the reticulum and meshes around the processes larger than the inter-process meshes in *M. azzunae* sp. nov. (Fig. 5C), all meshes similar in size in *M. sandrae* (Fig. 5D). Figure 5C–D also show a difference in the process base diameter, narrower in *M. azzunae* sp. nov.With regard to the animals, *M. azzunae* sp. nov. differs from *M. sandrae* by a constriction of the first macroplacoid much more pronounced (Fig. 5A; it is hardly visible in *M. sandrae*; Fig. 5B). Moreover, in animals of similar size the posterior band of the buccal armature is just less evident in the new species, and lunules on the hind legs are without hint of teeth (but teeth, present in the holotype of *M. sandrae*, are often difficult to identify in other specimens of that species).

***Macrobiotus terminalis* Bertolani & Rebecchi, 1993**

Macrobiotus azzunae sp. nov. differs from *M. terminalis* for the absence of granulation on the cuticle (noted only in the redescription of *M. terminalis*; see Cesari *et al*. 2011), for the absence of teeth on the lunules, especially evident on the hind legs of *M. terminalis*, and for the presence of males, absent in *M. terminalis* (see redescription by Cesari *et al*. 2011).

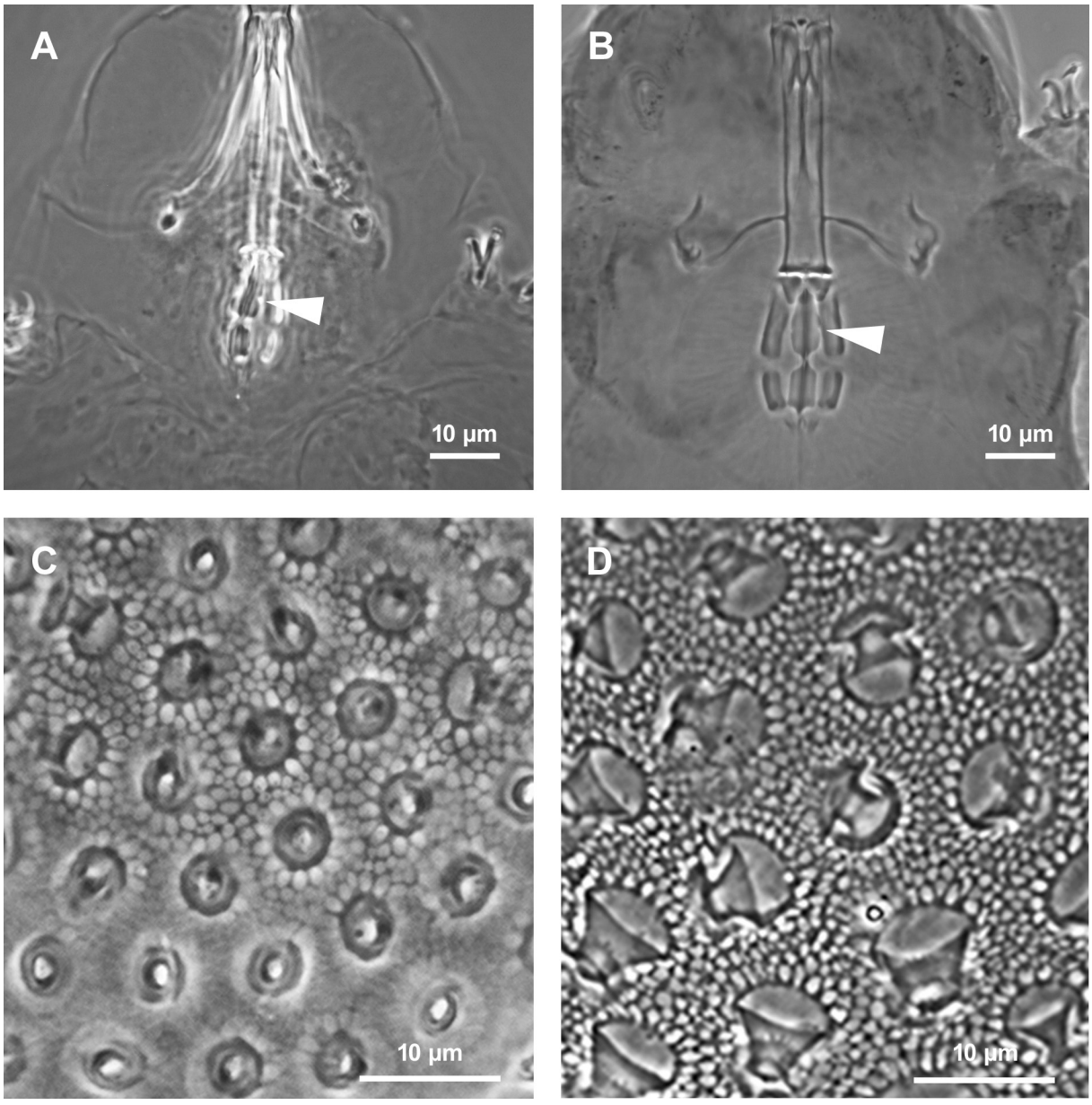

Fig. 5. Comparison between *M. azzunae* sp. nov. and *M. sandrae* Bertolani & Rebecchi, 1993. **A**. Placoids in *M. azzunae* sp. nov., paratype (UNIMORE, slide C4218–S30). **B**. Placoids in *M. sandrae* (UNIMORE, slide C442–S79); arrowheads evidence the different constriction depth of the first macroplacoid. **C**. Eggshell in *M. azzunae* sp. nov., paratype (UNIMORE, slide C4218–S4). **D**. Eggshell in *M. sandrae* (UNIMORE, slide C2346–S2); in *M. azzunae* sp. nov. there are smaller processes and reticulation with thinner wires and larger net around the processes than in *M. sandrae*. A–D: PhC.

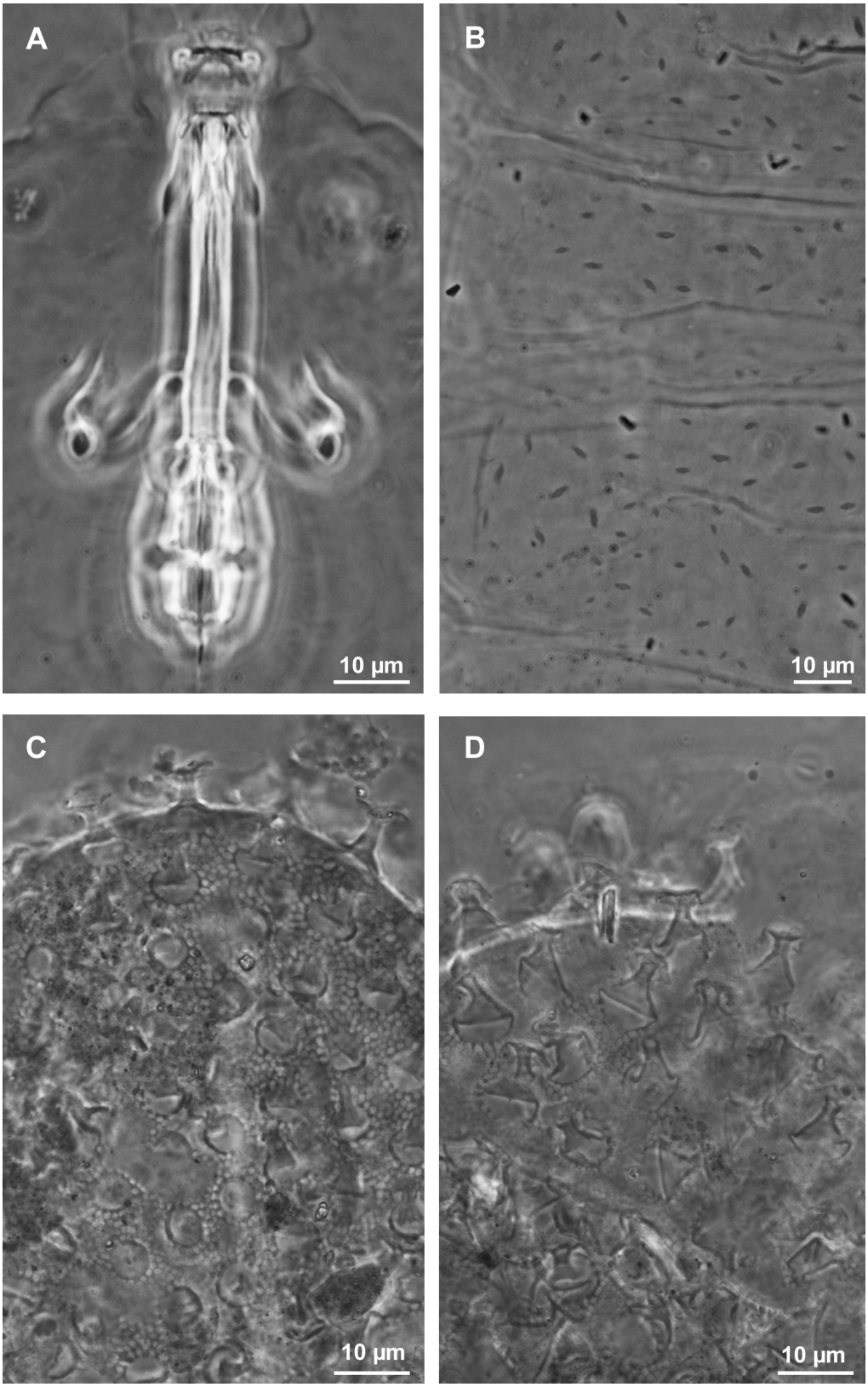

Fig. 6. *Macrobiotus personatus* Biserov, 1990, paratypes (Civic Museum of Natural History of Verona, Italy, CT14701). **A**. Buccal-pharyngeal apparatus with macroplacoids. **B**. Pores on the cuticle. **C**. Eggshell reticulation and egg processes. **D**. Egg processes. A–D: PhC.

***Macrobiotus vladimiri* Bertolani, Biserov, Rebecchi & Cesari, 2011**

With respect to *M. vladimiri*, animals of *M. azzunae* sp. nov. reach a shorter length (up to 410.3 µm vs 515.1 µm), in *M. azzunae* sp. nov. the posterior band of teeth of the buccal armature is less evident and the lunules on the hind legs are not indented. In *M. azzunae* sp. nov. the egg diameter without processes (64.7–80.6 µm) is less than that of the eggs of *M. vladimiri* (89.9–92.0 µm); the processes are shorter (4.2–6.4 µm in the new species vs 6.5–8 µm in *M. vladimiri*). In the new species the base process diameter is narrower (3.2–5.2 µm) than in *M. vladimiri* (5.1–7.3 µm), the distal disc is weakly or not jagged (clearly jagged in *M. terminalis*). In *M. azzunae* sp. nov. males are present, while they are absent in *M. vladimiri*.

Genetic distances

The ranges of uncorrected genetic p-distances between *M. azzunae* sp. nov. and the other species of the *M. hufelandi* group (Supp. file 7, Supp. file 8, Supp. file 9, Supp. file 10), are as follows:

18S 0.1–5.6%, with the most similar being *M. sandrae* from Germany (present paper)
28S 0.1%, with the only available *M. vladimiri* from Spain (FJ435751–5)
ITS-2 7.7–32.2%, with the most similar being *Macrobiotus vladimiri* (MN888347) from Finland
COI 6.3–25.6%, with the most similar being *Macrobiotus sandrae* (HQ876574, HQ876577, HQ876578, HQ876579, HQ876581) from Germany

The COI dataset is the most complete and informative for species delimitation investigation. Both phylogenetic reconstructions on the COI dataset resulted in the same topology, and thus the ML tree was utilized for the PTP analysis (Fig. 7, left), which shows 14 putative species clusters: *M. crustulus*, *M. hannae*, *M.* cf. *recens*, *M. canaricus*, *M. hufelandi*, *M.* cf. *hufelandi* sp.1, *M. terminalis*, *M.* cf. *terminalis*, *M. wandae*, *M. macrocalix*, *M.* cf. *macrocalix*, *M. vladimiri*, *M. sandrae* and *M. azzunae* sp. nov. This subdivision is further validated by both the ABGD and the haplotype network analysis (Fig. 7, centre and right). Present molecular data therefore confirms the validity of the erection of *M. azzunae* sp. nov.

Discussion

With the discovery of *M. azzunae* sp. nov. the species identified in Tunisia now comes to 33, all non-marine. This number is much lower than the number of non-marine species, 120, found in nearby Sicily (Pilato *et al.* 2017, 2019), an island extensively studied from a tardigradological point of view. This means that there is considerable potential for further discoveries that could come from the study of the various habitats colonized by tardigrades in Tunisia. Furthermore, the results will undoubtedly be greater and more informative if the research is carried out with an integrated approach, as done in this work.

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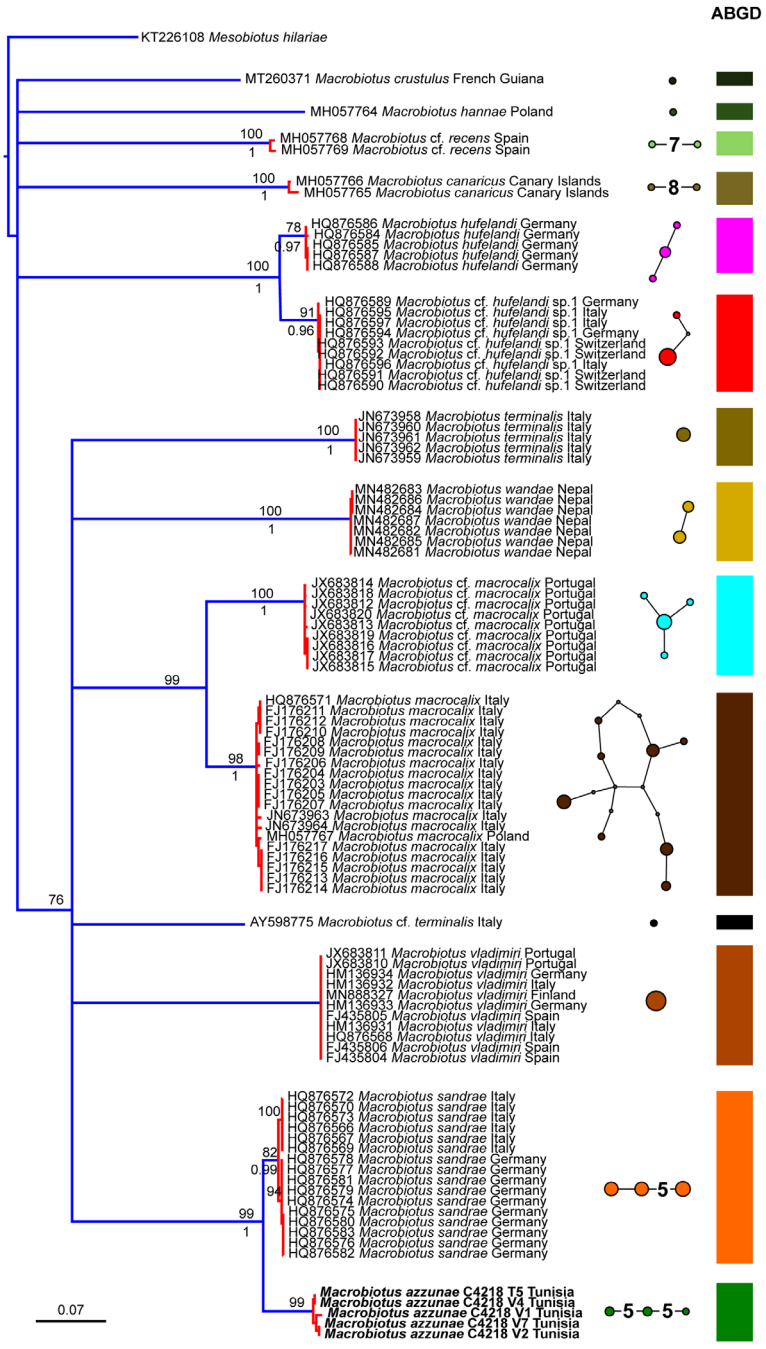

Fig. 7. Left: tree resulting from both the maximum likelihood analysis and the Bayesian inference of cytochrome c oxidase subunit I (COI) in *M. azzunae* sp. nov. specimens and sequences from GenBank. Values above branches point out bootstrap values, while values under branches represent posterior probability values. Results of the Poisson tree process analysis are provided using differently coloured branches: putative species are indicated using transitions from blue-coloured branches to red-coloured branches. Newly scored haplotypes are in bold. The scale bar shows the number of substitutions per nucleotide position. Centre: haplotype network of COI gene in *M. hufelandi* complex. Circles represent haplotypes, while circle surface denotes haplotype frequency. Networks falling below the value of the 95% connection limit are disconnected. Right: rectangles denote specimens grouped by ABGD analysis.

Contribution statement

The authors contributed equally to this work.

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Supp. file 1. Single measurements (in µm) and *pt* values of morphological structures of the holotype and paratypes of *Macrobiotus azzunae* sp. nov. https://doi.org/10.5852/ejt.2021.758.1429.4625

Supp. file 2. Buccal-pharyngeal apparatus of a hologenophore voucher specimen (C4218 V7) of *Macrobiotus azzunae* sp. nov. in vivo, PhC. https://doi.org/10.5852/ejt.2021.758.1429.4627

Supp. file 3. Alignment of all *Macrobiotus hufelandi* group sequences for the 18S gene. https://doi.org/10.5852/ejt.2021.758.1429.4629

Supp. file 4. Alignment of all *Macrobiotus hufelandi* group sequences for the 28S gene. https://doi.org/10.5852/ejt.2021.758.1429.4631

Supp. file 5. Alignment of all *Macrobiotus hufelandi* group sequences for the ITS-2 gene. https://doi.org/10.5852/ejt.2021.758.1429.4633

Supp. file 6. Alignment of all *Macrobiotus hufelandi* group sequences for the COI gene. https://doi.org/10.5852/ejt.2021.758.1429.4635

Supp. file 7. Genetic distances (p-distance) computed between species of the *Macrobiotus hufelandi* group for the 18S gene. https://doi.org/10.5852/ejt.2021.758.1429.4637

Supp. file 8. Genetic distances (p-distance) computed between species of the *Macrobiotus hufelandi* group for the 28S gene. https://doi.org/10.5852/ejt.2021.758.1429.4639

Supp. file 9. Genetic distances (p-distance) computed between species of the *Macrobiotus hufelandi* group for the ITS-2 gene. https://doi.org/10.5852/ejt.2021.758.1429.4641

Supp. file 10. Genetic distances (p-distance) computed between species of the *Macrobiotus hufelandi* group for the COI gene. https://doi.org/10.5852/ejt.2021.758.1429.4643