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Posted Date: 18 July 2023

doi: 10.20944/preprints202307.1250.v1

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Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 July 2023 doi:10.20944/preprints202307.1250.v1

Review

A Review on Genus *Paramacrobiotus*

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Abstract: *Paramacrobiotus* species has been described from almost every corner of the world. To date 45 species have been reported from this genus. The species' presence in different climatic conditions and habitat provides evidence of their adaptation to various harsh environments. In this review, we provide a concise summary of changes observed due to various cryptobiotic conditions in many species of this genus, geographical distribution of all the species, feeding behaviour, life history, microbiome community, *Wolbachia* endosymbiont identification, reproduction, phylogeny and general taxonomy of the species from genus *Paramacrobiotus*. Furthermore, we provide a new diagnostic key to the genus *Paramacrobiotus* based on the morphological and morphometric characters of adults and eggs.

Keywords: tardigrade; reproduction; taxonomy; distribution; microbiome

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1. Introduction

Tardigrade, also called water bears, is a phylum consisting of *ca.* 1,500 species [1–4] that inhabit terrestrial and aquatic environments throughout the world[5]. They are mostly found in mosses, lichens, soil, leaf litter, sediments and on aquatic plants[5–7]. The phylum consists of two classes, i.e., Heterotardigrada and Eutardigrada[5]. Eutardigrada is further divided into two limnoterrestrial orders, i.e., Apochela and Parachela. Moreover, order Parachela consists of various superfamilies and families, one of them being Macrobiotidae (Thulin, 1928)[8] with genus *Paramacrobiotus* Guidetti, Schill, Bertolani, Dandekar and Wolf, 2009[9]. The genus was erected in 2009 from the genus *Macrobiotus* and till date 45 species have been described: *Paramacrobiotus alekseevi* (Tumanov, 2005)[10], *Pam. arduus* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. areolatus* (Murray, 1907)[12], *Pam. beotiae* (Durante Pasa & Maucci, 1979)[13], *Pam. celsus* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. centesimus* (Pilato, 2000)[14], *Pam. chieregoi* (Maucci & Durante Pasa, 1980)[15], *Pam. corgatensis* (Pilato, Binda & Lisi, 2004)[16], *Pam. csotiensis* (Iharos, 1966)[17], *Pam. danielae* (Pilato, Binda, Napolitano & Moncada, 2001)[18], *Pam. danielisae* (Pilato, Binda & Lisi, 2006)[19], *Pam. depressus* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. derkai* (Degma, Michalczyk & Kaczmarek, 2008)[20], *Pam. experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020[21], *Pam. fairbanksi* Schill, Förster, Dandekar & Wolf, 2010[22], *Pam. filipi* Dudziak, Stec & Michalczyk 2020[23], *Pam. gadabouti* Kayastha, Stec, Mioduchowska and Kaczmarek 2023[24], *Pam. garynahi* (Kaczmarek, Michalczyk & Diduszko, 2005)[25], *Pam. gerlachae* (Pilato, Binda & Lisi, 2004)[16], *Pam. halei* (Bartels, Pilato, Lisi & Nelson, 2009)[26], *Pam. hapukuensis* (Pilato, Binda & Lisi, 2006)[19], *Pam. huziori* (Michalczyk & Kaczmarek, 2006)[27], *Pam. intii* Kaczmarek, Cytan, Zawierucha, Diduszko & Michalczyk, 2014[28], *Pam. kenianus* Schill, Förster, Dandekar & Wolf, 2010[22], *Pam. klymenki* Pilato, Kiosya, Lisi & Sabella, 2012[29], *Pam. lachowskae* Stec, Roszkowska, Kaczmarek & Michalczyk, 2018[30], *Pam. lorenae* (Biserov, 1996)[31], *Pam. magdalenae* (Michalczyk & Kaczmarek, 2006)[27], *Pam. metropolitanus* Sugiura, Matsumoto & Kunieda, 2022[32] *Pam. palaui* Schill, Förster, Dandekar & Wolf, 2010[22], Pam. peteri (Pilato, Claxton & Binda, 1989)[33], *Pam. pius* Lisi, Binda & Pilato, 2016[34], *Pam. priviterae* (Binda, Pilato, Moncada & Napolitano, 2001)[35], *Pam. richtersi* (Murray, 1911)[36], *Pam. rioplatensis* (Claps & Rossi, 1997)[37], *Pam. sagani* Daza, Caicedo, Lisi & Quiroga, 2017[38], *Pam. savai* (Binda & Pilato, 2001)[39], *Pam. sklodowskae* (Michalczyk, Kaczmarek & Węglarska, 2006)[40], *Pam. spatialis* Guidetti, Cesari, Bertolani, Altiero & Rebecchi, 2019[11], *Pam. spinosus*

Kaczmarek, Gawlak, Bartels, Nelson & Roszkowska, 2017[41], *Pam. submorulatus* (Iharos, 1966)[17], *Pam. tonollii* (Ramazzotti, 1956)[42], *Pam. vanescens* (Pilato, Binda & Catanzaro, 1991)[43], *Pam. walteri* (Biserov, 1997/98)[44] and *Pam. wauensis* (Iharos, 1973)[45]. Furthermore, the genus is divided into two species groups, i.e., *richtersi* group with presence of microplacoid within the pharynx, and *areolatus* group without microplacoid within the pharynx. In turn, Kaczmarek *et al.*[41] proposed to separate subgenera for which specific names were clarified by Marley *et al.*[46]. However, the two subgenera are not valid according to Guidetti *et al.*[11] and Stec *et al.*[47].

In this paper we summarise the data on taxonomy, distribution, mode of reproduction, microbiome study, feeding behaviour, life history, morphological taxonomy, phylogeny and cryptobiotic studies along with new key for species identification in genus *Paramacrobiotus*.

2. Cryptobiosis

A stage of an organism's life known as cryptobiosis is one in which no activity is apparent[48]. Many organisms go through cryptobiosis to survive the harsh environmental conditions they encounter [49–51]. These conditions can include anhydrobiosis (lack of water), anoxybiosis (lack of oxygen), cryobiosis (low temperature), or osmobiosis (change in osmotic conditions). Tardigrades have a remarkable capacity for undergoing and surviving several types of cryptobiosis [48,52]. The majority of anhydrobiosis, or absence of water, has been studied in the species of the genus *Paramacrobiotus*, although there has also been research on famine, freezing, and bet-hedging[53–57]. Reuner *et al.*[53] studied how the influence of starvation and anhydrobiosis affects the size and number of storage cells in *Paramacrobiotus tonollii* to understand the energetic side of anhydrobiosis. Starving *Pam. tonollii* for seven days led to reduction in storage cell size by 46.41% but no significant reduction in storage cell number was observed. Furthermore, when storage cells size and number were investigated after inducing anhydrobiosis for seven days where no significant changes in storage cell size and number of *Pam. tonollii* was observed. Also, the mortality was checked using prolonged starvation and *Pam. tonollii* reached 50% mortality after 30 days. Likewise, Rizzo *et al.*[54] investigated antioxidant defenses (capable of counteracting reactive oxygen species (ROS)) in *Pam. richtersi* in both active and dehydrated states. Activity of several antioxidant enzymes, the fatty acid composition and heat shock protein (Hsp) expression were compared in these two states. The increase in both antioxidant enzyme (superoxide dismutase due to induction of both glutathione peroxidase and glutathione during desiccation) and the fatty acid composition (polyunsaturated fatty acids and the amount of thiobarbituric acid reactive substances) were observed in desiccated *Pam. richtersi* specimens but no significant differences in the relative level of heat shock proteins were observed (Hsp70 and Hsp90). In addition, Tsujimoto *et al.*[55] performed a study where the production of reactive oxygen species and involvement of bioprotectants during anhydrobiosis in *Pam. spatialis* was investigated. The study provides evidence of increase in ROS production relative to time spent in anhydrobiosis which is due to oxidative stress in the animals. Using RNA interference, involvement of bioprotectants, including those combating ROS was assessed. As Rizzo *et al.*[54] concluded the role of glutathione peroxidase in desiccation in *Pam. richtersi*, this gene was targeted and what was observed is that glutathione peroxidase gene compromised survival during drying and rehydration of *Pam. spatialis*. This furthermore strengthened the evidence that glutathione reductase and catalase play important roles during desiccation and rehydration. Also, involvement of aquaporins 3 and 10 during rehydration of *Pam. spatialis* was observed. And recently Roszkowska *et al.*[57] study the length that different tardigrades survive in the anhydrobiotic state including *Pam. experimentalis*. The study concludes that anhydrobiotic competence is dependent on habitat instead of nutritional behavior and the time taken to return to activity after anhydrobiosis is dependent upon the length of the anhydrobiosis.

3. Distribution

The distribution of species from this genus shows worldwide distribution. The distribution of all 45 species in genus *Paramacrobiotus* till date is presented in SM.01 and Figure 1.

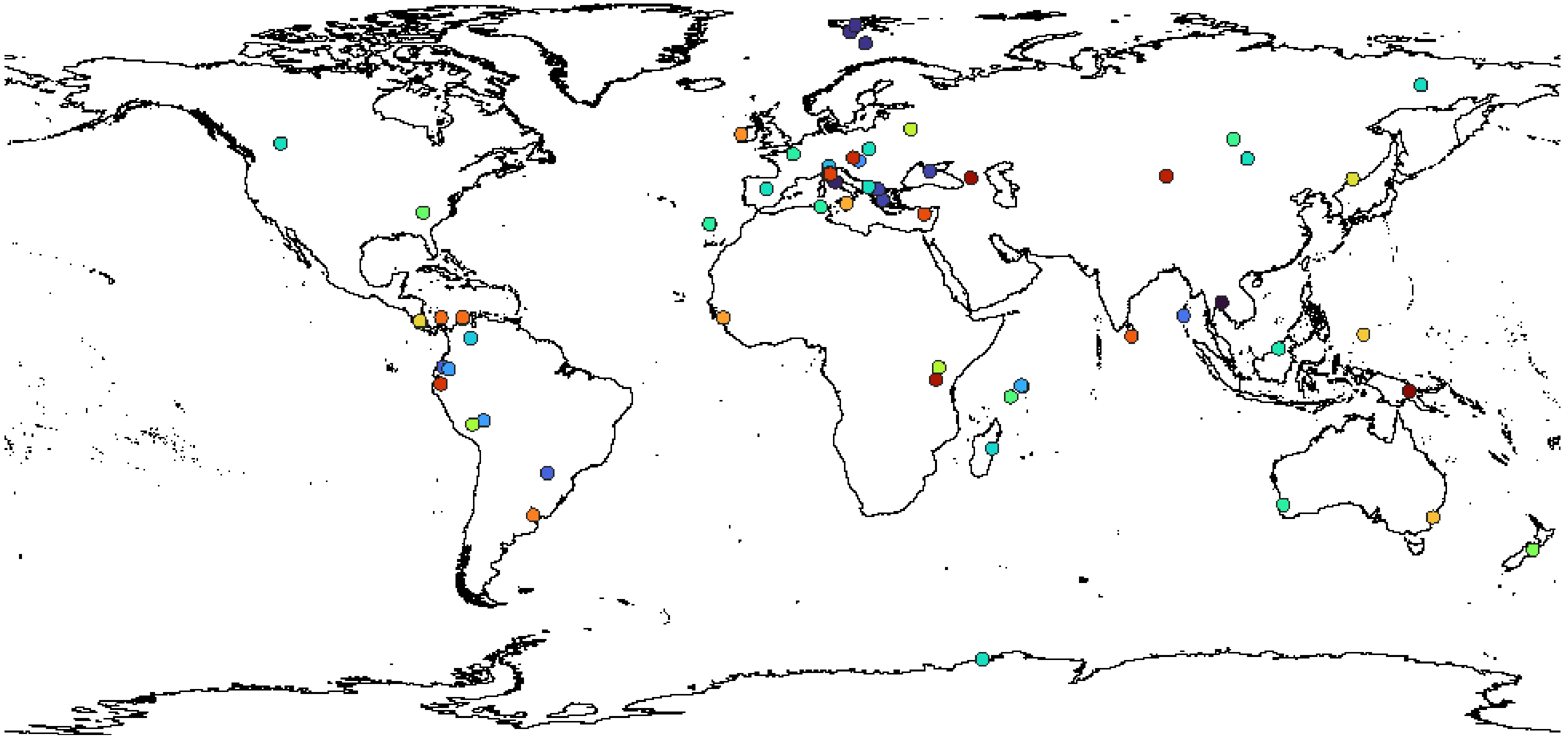

Figure 1. Distribution of all the species in genus *Paramacrobiotus* (co-ordinates present in SM.01). (Map prepared using QGIS ver. 3.28.0-Firenze).

4. Feeding behaviour

The tardigrade species *Paramacrobiotus* are omnivorous, consumes a variety of organisms, including certain cyanobacteria, algae, and fungi, as well as the rotifer *Lecane*, the nematode *Caenorhabditis*, and small juvenile tardigrades. Additionally, the diet of adults and juveniles eat is different: adults favour rotifers and nematodes, whereas juveniles favour unicellular green algae. Moreover, juveniles suck out all of them, including algal cells, animal food, and fungal cells, in contrast to adults who only consume entire fungal and algal cells[58].

5. Life history

Life history refers to total life span, development, reproduction and death of an organism[59]. The life history list in case of tardigrades consists of age at first oviposition, clutch size, fecundity, hatching percentage, hatching success, lifespan, moulting number and total number of ovipositions[60,61]. The lifespan differs from species to species in case of tardigrades[62]. The life history of only a few *Paramacrobiotus* species have been reported till date. Namely, *Pam. fairbanski* with an average lifespan of 137.3 ± 136.4 days and 194.9 ± 164.4 days respectively and age at first oviposition of 70.7 ± 19.4 days and 76.9 ± 16.4 days respectively[63]; *Pam. kenianus* with average lifespan of 125 ± 35 days and 141 ± 54 days respectively, maximum life span of 204 days and 212 days respectively and age at first oviposition of 10 days and10 days respectively[60]; *Pam. metropolitanus* with juveniles hatching in 12–20 days, first oviposition in 11–13 days after hatching[64]; *Pam. palaui* with average lifespan of 97 ± 31 days, maximum life span of 187 days and age at first oviposition of 10 days[60]; *Pam. richtersi* with age at first oviposition of 64.2 ± 1.7 days[65]; *Pam. tonollii* with average lifespan of 69.0 ± 45.1 days and maximum life span of 237 days and age at first oviposition 24.4 ± 4.4 days[62].

6. Microbiome

The microbiome represents the entire community of microorganisms, including fungi, protists, bacteria, archaea, as well as viruses, that inhabit all known metazoan species. The bacterial component of the microbiome community plays crucial roles in multiple aspects of ecdysozoan host life, such as behavior, metabolism, development, immunity, or pathogen defense, thereby regulating the functioning of the entire organism[66,67]. Conversely, it has also been demonstrated that the host's phylogeny[68] and diet[69] have significant impacts on the overall microbial composition. Indeed, many metazoan species appear to harbor their own specific microbiome community[70]. However, our understanding of the microbiome composition of Tardigrada, based on next-generation sequencing methods (NGS) targeting the standard 16S rRNA bacterial barcoding gene fragment, is limited to a very small number of published articles[71–77].

In the case of species from the genus *Paramacrobiotus,* the microbiomes of a few species have been studied to date. In 2018, Vecchi *et al.*[71] described the bacterial communities associated with six limno-terrestrial tardigrade taxa, one of which was *Pam. areolatus*. The study revealed that the microbial community was mainly composed of Proteobacteria and Bacteroidetes. Interestingly, certain classified Operational Taxonomic Units (OTUs) showed variations among species from geographically distant samples, indicating the presence of specific bacterial communities in each species. However, in all the investigated species' microbiome profiles, the order Rickettsiales was consistently identified. This order belongs to the class Alphaproteobacteria and is characterized by both pathogens and intracellular mutualists[78]. There were two distinct patterns in the diversity observed between tardigrades and their substrates, indicating significantly less microbial diversity in tardigrades compared to their substrates. This phenomenon may be attributed to tardigrades selectively associating with specific microbial communities that promote the growth of certain bacterial species while inhibiting others. Another hypothesis suggests that substrates, being complex matrices with wide surface areas and volumes, can support a high bacterial biomass, resulting in a vast and complex microbial community.

Similarly, Kaczmarek *et al.*[21] conducted a microbiome analysis on two populations of *Pam. experimentalis* from Madagascar and their laboratory culture environment. These populations of *Pam.*

experimentalis had been maintained in laboratory culture for two years. The most abundant phylum in all samples was Proteobacteria. Firmicutes was the second most dominant phylum in both *Pam. experimentalis* populations, while Bacteroides was the second most dominant phylum in the laboratory habitat. With the exception of the phyla Verrucomicrobia and Saccharibacteria, which were not found in the tardigrade microbiome, all identified taxa in the *Pam. experimentalis* microbiome community and laboratory culture environment were widespread and had comparable abundances. This confirms that the tardigrade microbiome significantly differs in composition from the bacteria inhabiting their environment. Moreover, within the microbiome of *Pam. experimentalis*, Operational Taxonomic Units (OTUs) classified as potential endosymbionts belonging to the order Rickettsiales were identified. The absence of Rickettsiales OTUs in the environment of the studied species further supports the close association of these bacteria with their host.

Furthermore, Mioduchowska *et al.*[73] conducted a study to investigate whether tardigrade species are infected with bacterial endosymbionts belonging to the genus *Wolbachia*. The analysis included *Pam. fairbanksi* and *Paramacrobiotus* sp. In the study Proteobacteria, Firmicutes, and Actinobacteria as the three most prevalent phyla among the analyzed tardigrades, including species outside the genus *Paramacrobiotus*, were identified. However, the focus of the study was on potential tardigrade endosymbionts, particularly Operational Taxonomic Units (OTUs) from the order Rickettsiales and the genus *Wolbachia*. Both Rickettsiales and *Wolbachia* were detected in adult *Paramacrobiotus* sp., while only Rickettsiales were found in *Pam. fairbanksi* eggs. Adult *Pam. fairbanksi* did not have either *Wolbachia* or Rickettsiales infections. The genus *Wolbachia* is an intracellular bacterium belonging to the order Rickettsiales and it infects various invertebrates, particularly terrestrial insects[79]. However, recent studies have identified infections of this bacterial endosymbiont in various freshwater invertebrate species[77,80,81]. Generally, this bacterium is transmitted vertically from mother to offspring and/or through horizontal transfer directly from the environment or between different hosts[82]. Subsequently, *Wolbachia* manipulates host reproduction by inducing parthenogenesis, feminization, male killing, or cytoplasmic incompatibility[83,84].

In 2023, Mioduchowska *et al.*[77] described new molecular and bioinformatic tools for detecting *Wolbachia* in freshwater invertebrates. In this study, *Wolbachia* was detected in *Pam. experimentalis*, which were the same isolates analysed by Kaczmarek *et al.*[72]. Phylogenetic analysis of the obtained bacterial sequences allowed for their classification within the differentiated supergroup A of the genus *Wolbachia*. The discovery of *Wolbachia* in tardigrades opens new frontiers in understanding the *Wolbachia*-driven biology and ecology of Tardigrada.

7. Reproduction

Reproduction refers to the process where every organism known produces offspring either sexually or asexually. In case of tardigrades, they reproduce only through gametes via many different patterns i.e. dioecious (separate male and female), hermaphroditic (single animal with both male and female reproductive parts) or parthenogenetic (form of asexual reproduction)[85]. The genus *Paramacrobiotus* consists of both bisexual and unisexual species/populations. *Pam. richtersi* is both bisexual and unisexual from Italy; according to modern taxonomy they probably constitute a distinct species, *Pam. areolatus* population from Italy is bisexual and population from Svalbard is unisexual, *Pam. tonolli* from the USA is bisexual, *Pam. fairbanksi* is unisexual from various locations as Antarctic, Italy, Poland, Spain and USA, *Pam. kenianus* from Kenya is unisexual and *Pam. palaui* from Micronesia is unisexual, *Pam. depressus* from Italy is bisexual, *Pam. celsus* from Italy is bisexual, *Pam. spatialis* from Italy is bisexual, *Pam. arduus* from Italy is bisexual, *Pam. experimentalis* from Madagascar is bisexual and *Pam. gadabouti* is unisexual from various locations in Portugal, Australia, France and Tunisia. Out of 45, mode of reproduction for only 18 species are known (SM.01). Also, Guidetti *et al.*[11] suggests the mode of reproduction being related to constrained or wide distribution of the species. The amphimictic species displays a very constrained or punctiform distribution, in contrast to the parthenogenetic species' extremely extensive spread and presence over multiple continents. The difference in the ability for dispersal linked to the two modes of reproduction can be used to explain why apomictic and amphimictic populations are distributed differently.

8. Morphological taxonomy

The genus *Paramacrobiotus* is divided into two morphologically distinct species groups: *areolatus* (species without a microplacoid or with rudimentary structures in the place of microplacoid in the pharynx) and *richtersi* (species with a microplacoid in the pharynx)(e.g.[23,28]). It was suggested that initially the microplacoid was present, however it was lost in some species from the *areolatus* group. But, the opposite situation, in which the microplacoid gradually appears, is also possible[41]. For example, in Pam vanescens the microplacoid suggests a gradual reduction. In turn, in *Pam. areolatus* and *Pam. centesimus* the microplacoid is generally absent, but a thin cuticular thickening is present in the place where microplacoid should be normally present and can be considered as rudimentary microplacoid[14,47]. Although, the presence or absence of microplacoid seems to be a clear morphological character dividing genus *Paramacrobiotus* into two separate phylogenetic lineages (which was suggested by Kaczmarek *et al.*[41]) the genetic studies did not confirm this[11,47].

At present 45 species are formally attributed to the genus *Paramacrobiotus* and 13 belong to *areolatus* group and 32 *richtersi* group. They can be further divided into smaller groups based on egg types. In total, seven types of eggs were identified. However, two of them (*areolatus* and *richtersi* types) are the most common and occur in 37 species (*ca.* 82%). In the next two species *huziori* type of eggs are present (*ca.* 5%). The other types of eggs (i.e. *beotiae*, *chieregoi*, *csotiensis*, *tonollii* and *submorulatus*) were identified only in single taxa (for details of egg morphology see Kaczmarek *et al.*[41]). What is more, eggs are unknown for one species i.e. *Pam. wauensis*.

In recent years two very important for taxonomy of the entire genus, species *Pam. areolatus* and *Pam. richtersi* were integratively redescribed[11,47]. Another species *Pam. fairbanksi* described based, mostly, on genetic data was also morphometrically well characterized few years ago[21]. However, a few *Paramacrobiotus* species still need a redescription based on type material or on additional material from type localities. Descriptions of *Pam. beotiae, Pam. chieregoi, Pam. csotiensis, Pam. rioplatensis*, *Pam. submorulatus*, *Pam. tonollii* and *Pam. wauensis* are inaccurate and some important morphological information are lacking.

Another two species, i.e., *Pam. kenianus* and *Pam. palaui* are cryptic taxa described mostly based on genetic data without morphological differential diagnosis[22].

Descriptions of the other *Paramacrobiotus* species more or less complete, but in most of them exact morphometric data of claws, buccal tubes placoids and above all genetic data are lacking (see Table 1 and SM.01). Based on all the abovementioned doubts, 3 species, i.e., *Pam. kenianus*, *Pam. palaui* and *Pam. wauensis* were not included to the key.

Table 1. Selected morphological characters of the known species of genus *Paramacrobiotus*.

Species	**Cuticle**	**Oral Cavity Armature**	**Eyes**	**Lunules IV**	**Granulation on Legs**	**Type of Egg**	**Egg process height (min. and max. values in μm)**	**Egg process base width (min. and max. values in μm)**	**Egg process shape**	**Number of processes on circumference**
Paramacrobiotus alekseevi	smooth	I–III	absent	dentate	IV	*richtersi*	11.8–21.8	13.3–22.9	with cap	10–12
Paramacrobiotus arduus	smooth	I–III	absent	smooth	I–IV	*richtersi*	12.1–18.3	10.4–16.3	conical	16–21
Paramacrobiotus areolatus	smooth	I–III	present	crenate	I–IV	*areolatus*	20.0–28.0	19.0–22.0	conical	?
Paramacrobiotus beotiae	smooth	I–III	absent	dentate	?	*beotiae*	up to 16.0	?	spines	?
Paramacrobiotus celsus	smooth	I–III	absent	smooth	I–IV	*richtersi*	15.2–19.1	14.3–18.2	conical (jagged)	15–19
Paramacrobiotus centesimus	smooth	I–III	absent	smooth	I–IV	*areolatus*	7.0–11.0	?	conical	11–12
Paramacrobiotus chieregoi	smooth	I–III	absent	smooth	?	*chieregoi*	?	?	elongated	14
Paramacrobiotus corgatensis	sculptured	I–III	present	dentate	?	*richtersi*	20.0–25.0	18.0–24.0	conical (jagged)	8–11
Paramacrobiotus csotiensis	smooth	II–III	present	?	?	*csotiensis*	?	?	blunt	?
Paramacrobiotus danielae	sculptured	I–III	present	smooth	?	*areolatus*	14.5	24.7	conical	?

Paramacrobiotus danielisae	sculptured	I–III	absent	smooth	?	*richtersi*	17.3–23.0	17.5–20.0	conical	9–10
Paramacrobiotus depressus	smooth	I–III	absent	smooth	IV	*richtersi*	9.3–12.4	12.4–15.2	conical	16–23
Paramacrobiotus derkai	smooth	I–III	present	smooth	I–IV	*huziori*	8.0–17.1	12.5–28.3	conical	12–16
Paramacrobiotus experimentalis	smooth	I–III	absent	smooth	IV	*areolatus*	10.3 – 14.9	13.8 – 19.4	conical	10–12
Paramacrobiotus fairbanksi	smooth	I–III	absent	smooth	I–IV	*richtersi*	10.9 – 14.9	10.9 – 20.8	conical (jagged)	?
Paramacrobiotus filipi	granulation	I–III	absent	smooth	I–IV	*richtersi*	17.8–25.2	11.7–21.7	elongated with disc	10–11
Paramacrobiotus gadabouti	smooth	I–III	absent	smooth	IV	*richtersi*	12.1–23.7	15.0–25.5	truncated cones	11–13
Paramacrobiotus garynahi	with pores	I–III	absent	smooth	I-IV	*areolatus*	18.0–30.0	20.0–42.0	with cap	10–13
Paramacrobiotus gerlachae	smooth	I–III	absent	smooth	IV	*richtersi*	11.8–14.5	16.8–18.7	blunt	?
Paramacrobiotus halei	sculptured	I–III	absent	?	I-IV	*richtersi*	11.0–14.0	22.0–23.5	blunt	11
Paramacrobiotus hapukuensis	smooth	I–III	absent	smooth	absent	*areolatus*	15.7–21.1	14.8–16.6	elongated	10
Paramacrobiotus huziori	smooth	I–III	present	smooth	I–IV	*huziori*	20.0–33.0	20.0–30.0	conical	9–11

Paramacrobiotus intii	smooth	II–III	present	dentate	I–IV	*areolatus*	15.4–24.4	22.0–34.0	conical	9–10
Paramacrobiotus kenianus	smooth	?	present	?	?	*richtersi*	13.5 ± 1.9	19.7 ± 2.7	conical	17.7 ± 3.6
Paramacrobiotus klymenki	smooth	I–III	absent	dentate	I–IV	*areolatus*	14.5–18.5	16.4–18.2	conical	10–11
Paramacrobiotus lachowskae	smooth	I–III	present	smooth	I–IV	*areolatus*	17.6–32.1	8.1–17.7	dome with filaments	8–14
Paramacrobiotus lorenae	smooth	I–III	absent	smooth	I-IV	*richtersi*	25.0–42.2	17.8–23.0	elongated	?
Paramacrobiotus magdalenae	smooth	I–III	present	smooth	IV	*richtersi*	13.0–25.0	16.2–21.0	conical	10–12
Paramacrobiotus metropolitanus	smooth	I–III	absent	smooth	IV	*areolatus*	7.4–14.6	9.8–21.1	conical	10–15
Paramacrobiotus palaui	smooth	?	present	?	?	*richtersi*	10.2 ± 1.3	13.4 ± 1.3	conical	15.4 ± 1.4
Paramacrobiotus peteri	smooth	I–III	absent	smooth	?	*areolatus*	10.0–14.0	9.0–12.0	conical (jagged)	?
Paramacrobiotus pius	smooth	I–III	absent	smooth	I-IV	*richtersi*	up to 12.3	19.5–24.7	conical (jagged)	10
Paramacrobiotus priviterae	smooth	I–III	present	smooth	I-IV	*richtersi*	11.8–15.0	12.9–16.3	conical (jagged)	?
Paramacrobiotus richtersi	smooth	I–III	absent	smooth	I-IV	*richtersi*	17.1–22.1	17.2–22.2	conical	13–17

Paramacrobiotus rioplatensis	smooth	I–III	present	smooth	?	*areolatus*	ca. 4.6	?	elongated	17-19
Paramacrobiotus sagani	granulation	I–III	present	smooth	I-IV	*richtersi*	9.4–13.2	14.6–22.4	cylindrical, indented apices	10–13
Paramacrobiotus savai	smooth	I–III	present	smooth	IV	*areolatus*	12.0–18.0	16.7–18.5	blunt	?
Paramacrobiotus sklodowskae	smooth	I–III	present	smooth	I-IV	*richtersi*	16.0–17.5	20.5–23.5	blunt	10
Paramacrobiotus spatialis	smooth	I–III	absent	smooth	I-IV	*richtersi*	13-16	15.2–20.4	conical	15–23
Paramacrobiotus spinosus	smooth	I–III	absent	smooth	I–IV	*richtersi*	22.1–42.2	17.3–26.0	elongated (jagged)	10–11
Paramacrobiotus submorulatus	smooth	II–III	present	?	?	*submorulatus*	7.0–8.3	17.5–20.4	blunt	13
Paramacrobiotus tonollii	smooth	?	present	smooth	?	*tonollii*	32.0–35.0	?	conical	8–10
Paramacrobiotus vanescens	smooth	I–III	absent	?	I-IV	*richtersi*	16.0–17.0	24.0–25.0	blunt (jagged)	9–12
Paramacrobiotus walteri	smooth	I–III	present	dentate	I–IV	*areolatus*	10.0–17.0	9.0–20.0	conical (jagged)	?
Paramacrobiotus wauensis	smooth	I– III	absent	?	?	?	?	?	?	?

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9. Molecular taxonomy

Molecular markers serve as valuable tools for species identification. In the integrative taxonomy of Tardigrada, four DNA fragments with different mutation rates are commonly used: two conservative nuclear ribosomal subunit genes, namely 18S rRNA (the small ribosome subunit) and 28S rRNA (the large ribosome subunit), the noncoding nuclear ITS2 fragment (the internal transcribed spacer-2) with high evolution rates, and the protein coding mitochondrial COI barcode gene (the cytochrome oxidase subunit I) with an intermediate effective mutation rate (e.g., Kaczmarek *et al.*[72]). The COI mtDNA molecular marker, in particular, has been recommended for DNA barcoding purposes (http://www.barcodinglife.org), such as rapid species identification, discrimination between cryptic species, and resolving phylogenetic relationships among closely related species[86,87]. To gain additional insights into the phylogenetic relationships within the genus *Paramacrobiotus*, an analysis based on COI mtDNA was conducted. This analysis was performed to supplement the information obtained from previous studies using four molecular markers[24].

Due to ongoing revisions and redescriptions of *Paramacrobiotus* species, studies are becoming more accessible, leading us to anticipate that the species diversity within the genus is greatly underestimated[11,23]. One significant challenge that needs to be addressed in future studies is the lack of available barcodes. Despite the designation of 45 species to the genus *Paramacrobiotus*, not all species have available barcode sequences. In this study, we aimed to estimate the phylogenetic relationships among all *Paramacrobiotus* species (including taxa designated as "cf." – meaning "compare with" and "aff." – meaning "similar to") for which COI barcode sequences are available in the GenBank database. We used the COI sequence of *Milnesium berladnicorum* Ciobanu, Zawierucha, Moglan & Kaczmarek, 2014[88] as outgroups to construct the most reliable evolutionary tree. To determine the most appropriate model of sequence evolution, we applied jModelTest v. 2.1.4[89] with both the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC)[90]. The GTR + G (Time Reversible model with gamma distributed rate heterogeneity) was selected as the best-fit evolutionary model. The phylogenetic tree was constructed using Bayesian inference (BI) analysis with the program MrBayes 3[91], following the settings described by Mioduchowska *et al.*[92]. The alignment of COI barcode sequences resulted in 574 characters, with 270 variable sites and 241 parsimony informative sites. Uncorrected pairwise distances (p-distances) were calculated using MEGA X[93].

The binary model of phylogenetic relationships, which involves reconstructing gene trees from sequence data, allows us to gain insights into the speciation history of species[94]. However, in our analysis of barcode sequences, we observed speciation events that resulted in polytomies within the phylogeny of the genus *Paramacrobiotus* (Figure 2). This means that more than two descendants were observed from certain nodes[95]. The presence of unresolved nodes in a polytomic multifurcating tree indicates a lack of signal in the data to resolve relationships within the genus *Paramacrobiotus*. This observation is partially consistent with previous studies, where both groups of *richtersi* and *areolatus* were described as polyphyletic[11,47]. However, in the work by Kayastha *et al.*[24], the interrelationships of the genus *Paramacrobiotus* were not depicted as a polytomy when two conservative coding nuclear molecular markers (18S rRNA and 28S rRNA) and a noncoding nuclear marker with high evolution rates (ITS2) were included in the analysis. As a result, the phylogenetic relationships within the genus *Paramacrobiotus* were resolved. Interestingly, other examples of polytomies in Tardigrada gene trees based on nuclear molecular markers have also been observed[96].

The genetic p-distances between the analyzed COI barcode sequences of *Paramacrobiotus* species ranged from 16% to 27%, indicating different species (Table 2). However, it was shown that there are very low genetic differences, i.e., a p-distance of 0.3%, between *Pam*. aff. *richtersii* from Tunisia (GenBank: MH676016) and *Pam. gadabouti* from Portugal (GenBank: OP394113), suggesting they belong to the same species (Table 2). This finding is consistent with the work by Kayastha *et al.* [24], where both species were described as *Pam. gadabouti*. No genetic differences were found between *Pam*. aff. *richtersi* from Madagascar (GenBank: MH676008) and *Pam. experimentalis* from Madagascar (GenBank: MN097836) (Table 2). Both sequences represented *Pam. experimentalis*, which is also consistent with the previous study (Kayastha *et al.*[24]). Moreover, we found very low genetic differences, i.e., a p-distance of 2.1%, between *Pam. arduus* from Italy (GenBank: MK041020) and *Pam.* aff. *arduus* from Italy (GenBank: MK041022), indicating the same species (Table 2).

The text continues here (Figure 2 and Table 2).

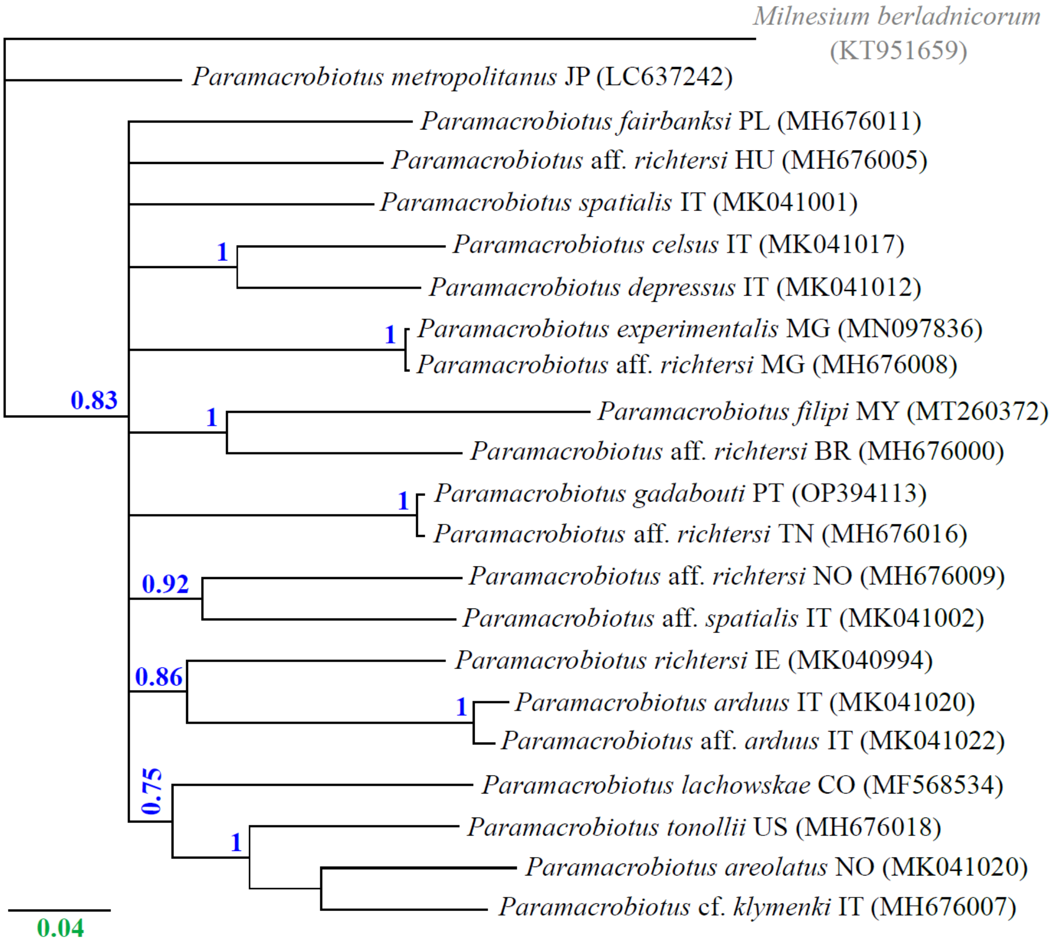

Figure 2. Phylogenetic relationships of the genus *Paramacrobiotus* constructed based on the COI barcode sequences obtained from the GenBank database. The GenBank accession numbers are given in parentheses. In turn, locations of identified species given in abbreviations: JP – Japan; PL – Poland; HU – Hungary; IT – Italy; MG – Madagascar; MY – Malaysia; BR – Brazil; PT – Portugal; TN – Tunisia; NO – Norway; IE – Ireland; CO – Colombia; US – United States. The numbers at the branches represent Bayesian posterior probabilities. The COI sequence of *Milnesium berladnicorum* was used as an outgroup.

Table 2. Estimates of evolutionary divergence between COI barcode sequences based on p-distances.

	1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**	**13.**	**14.**	**15.**	**16.**	**17.**	**18.**	**19.**	**20.**	**21.**
1. *Pam. arduus* (IT; MK041020)																					
2. *Pam.* aff. *arduus* (IT; MK041022)	**0,021**																				
3. *Pam. areolatus* (NO; MK041020)	0,244	0,240																			
4. *Pam. celsus* (IT; MK041017)	0,206	0,206	0,232																		
5. *Pam. depressus* (IT; MK041012)	0,209	0,204	0,228	0,141																	
6. *Pam. experimentalis* (MG; MN097836)	0,253	0,249	0,207	0,239	0,218																
7. *Pam. fairbanksi* (PL; MH676011)	0,218	0,213	0,207	0,186	0,179	0,213															
8. *Pam. filipi* (MY; MT260372)	0,268	0,270	0,253	0,228	0,258	0,232	0,260														
9. *Pam. gadabouti* (PT; OP394113)	0,207	0,207	0,220	0,195	0,192	0,239	0,195	0,240													
10. *Pam.* cf. *klymenki* (IT; MH676007)	0,226	0,223	0,176	0,242	0,223	0,211	0,233	0,230	0,235												
11. *Pam. lachowskae* (CO; MF568534)	0,226	0,221	0,199	0,242	0,233	0,199	0,216	0,267	0,237	0,192											
12. *Pam. metropolitanus* (JP; LC637242)	0,233	0,232	0,192	0,228	0,230	0,190	0,204	0,228	0,221	0,223	0,204										
13. *Pam. richtersi* (IE; MK040994)	0,199	0,192	0,216	0,192	0,186	0,204	0,188	0,237	0,214	0,213	0,226	0,209									
14. *Pam.* aff. *richtersi* (HU; MH676005)	0,206	0,193	0,211	0,193	0,183	0,225	0,192	0,240	0,186	0,200	0,190	0,209	0,200								
15. *Pam.* aff. *richtersi* (NO; MH676009)	0,199	0,193	0,232	0,186	0,176	0,225	0,188	0,268	0,214	0,230	0,237	0,230	0,181	0,211							
16. *Pam.* aff. *richtersi* (BR; MH676000)	0,225	0,218	0,239	0,202	0,193	0,202	0,214	0,225	0,202	0,230	0,226	0,207	0,190	0,204	0,216						
17. *Pam.* aff. *richtersi* (TN; MH676016)	0,209	0,207	0,220	0,195	0,192	0,239	0,195	0,240	**0,003**	0,235	0,237	0,221	0,214	0,186	0,214	0,202					
18. *Pam.* aff. *richtersi* (MG; MH676008)	0,253	0,249	0,207	0,239	0,218	**0,000**	0,213	0,232	0,239	0,211	0,199	0,190	0,204	0,225	0,225	0,202	0,239				
19. *Pam. spatialis* (IT; MK041001)	0,214	0,216	0,228	0,195	0,193	0,197	0,178	0,240	0,206	0,213	0,209	0,213	0,190	0,185	0,197	0,230	0,207	0,197			
20. *Pam.* aff. *spatialis* (IT; MK041002)	0,193	0,183	0,225	0,202	0,176	0,206	0,195	0,263	0,197	0,221	0,211	0,221	0,206	0,192	0,176	0,233	0,197	0,206	0,185		
21. *Pam. tonollii* (US; MH676018)	0,221	0,214	0,179	0,213	0,207	0,185	0,207	0,230	0,218	0,157	0,206	0,186	0,204	0,202	0,216	0,207	0,218	0,185	0,185	0,213	

10. Key for species identification

1. Microplacoid present (*richtersi* group) **2**

–. Microplacoid absent (*areolatus* group) **31**

2. Cuticular pattern on dorsal side of the body present and visible in LM (PCM and/or DIC) **3**

–. Cuticle on dorsal side of the body smooth or cuticular pattern not visible in LM (PCM and/or DIC) **7**

3. Eggs of *areolatus* type. . . . . . . ***Pam. danielae***

–. Eggs of *richtersi* type **4**

4. Eyes present, lunules under claws IV dentate . . . . . . ***Pam. corgatensis***

–. Eyes absent, lunules under claws IV smooth **5**

5. Dorsal cuticle covered with very small circular or elongated tubercles, egg processes less than 14.5 µm height . . . . . . ***Pam. halei***

–. Dorsal cuticle covered with small dots (granules) or small polygons, egg processes more than 15.5 µm height **6**

6. Dorsal cuticle covered with small dots (granules) . . . . . . ***Pam. vanescens***

–. Dorsal cuticle covered with small polygons . . . . . . ***Pam. danielisae***

7. Areolation between egg processes absent **8**

–. Areolation between egg processes present **9**

8. Lunules under claws IV dentate, eggs of *beotiae* type . . . . . . ***Pam. beotiae***

–. Lunules under claws IV smooth, egg of *chieregoi* type . . . . . . ***Pam. chieregoi***

9. Eggs of *submorulatus* type . . . . . . ***Pam. submorulatus***

–. Eggs of *richtersi* or *areolatus* type **10**

10. Eggs of *richtersi* type **11**

–. Eggs of *areolatus* type **25**

11. Only five or six areoles present around each egg process **12**

–. The number of areoles around each egg process larger than six **18**

12. Eyes present. . . . . . . ***Pam. priviterae***

–. Eyes absent. **13**

13. Granulation on leg I-III present. **14**

–. Granulation on legs I-III absent. . . . . . . ***Pam. depressus***

14. The *pt* values of the macroplacoid length less than *43.5*. . . . . . . ***Pam. pius***

–. The *pt* values of the macroplacoid length more than *49.0*. **15**

15. Egg process jagged **16**

–. Egg process not jagged **17**

16. Egg processes height less than 15.0 µm and parthenogenetic mode of reproduction. . . . . . . ***Pam. fairbanksi***

–. Egg processes height more than 15.1 µm and bisexual mode of reproduction. . . . . ***Pam. celsus***

17. Egg diameter without processes less than 62.5 µm. . . . . ***Pam. arduus***

–. Egg diameter without processes more than 65.0 µm. . . . . ***Pam. spatialis***

18. Eyes present **19**

–. Eyes absent **21**

19. Granulation on legs I-III present **20**

–. Granulation on legs I-III absent . . . . ***Pam. magdalenae***

20. Egg bare diameter less than 87.9 µm, egg process height more than 15 µm, egg processes hemispherical with blunt terminal part . . . . ***Pam. sklodowskae***

–. Egg bare diameter more than 92.0 µm, egg process height less than 13.5 µm, egg processes hemispherical with cylindrical indented apices . . . . ***Pam. sagani***

21. Lunules under claws IV dentate. . . . . ***Pam. alekseevi***

–. Lunules under claws IV smooth. **22**

22. Egg processes with cap-like vesicular structures on the top **23**

– Egg processes without cap-like vesicular structures on the top. **24**

23. Egg processes with elongated terminal portion, second macroplacoid length less than 6.5 µm, *pt* values of second macroplacoid length less than *14.0*, *pt* values of macroplacoid row length less than *59.0*, placoid row length less than 34.5 µm and *pt* values of placoid row length less than *74.0*. . . .. ***Pam. filipi***

– Egg processes without elongated terminal portion, second macroplacoid length 7.0 µm or more, *pt* values of second macroplacoid length more than *15.0*, *pt* values of macroplacoid row length more than *60.0*, placoid row length more than 34.9 µm and *pt* values of placoid row length more than *77.5*. . . . . ***Pam. gadabouti***

24. Egg processes with long, thin and flexible terminal portion and egg process height more than 24.5 µm . . . . ***Pam. lorenae***

– Egg processes without long, thin and flexible terminal portions and egg process height less than 22.5 µm . . . . ***Pam. richtersi***

25. Cuticle with oval pores, egg processes with cap-like structure on the top and clearly narrower under caps . . . . ***Pam. garynahi***

–. Cuticle without oval pores, egg processes without cap-like structure on the top and without narrowing at the top **26**

26. Egg processes hemispherical with blunt apex not divided and without elongated terminal part . . . . ***Pam. savai***

–. Egg processes different **27**

27. Egg processes with long flexible spines on the top . . . . ***Pam. rioplatensis***

–. Egg processes without long flexible spines on the top **28**

28. Egg processes base width less than 12.5 µm. . . . . ***Pam. peteri***

–. Egg processes base width more than 13.0 µm. **29**

29. Granulation on IVth pair of legs absent and egg processes height more than 15.5 µm . . . . ***Pam. hapukuensis***

–. Granulation on IVth pair of legs present and egg processes height less than 15.0 µm . **30**

30. Presence of wrinkled surface on the egg areolae and the absence of cuticular bulge on inner surface of claws I–III. . . . .***Pam. experimentalis***

–. Lack of wrinkled surface on the egg areolae and the presence of cuticular bulge on inner surface of claws I–III. . . . . ***Pam. metropolitanus***

31. Egg of *csotiensis* type . . . . ***Pam. csotiensis***

–. Eggs of *areolatus*, *huziori*, *tonollii* or *richtersi* type **32**

32. Eggs of *tonollii* type . . . .***Pam. tonollii***

–. Eggs of *areolatus*, *huziori* or *richtersi* type **33**

33. The egg areolation of the *huziori* type **34**

–. Eggs of *richtersi* or *areolatus* type **35**

34. Only one row of larger teeth present in the second band in the oral cavity, the distances between all macroplacoids are approximately the same, accessory points well developed but not protruding high above the primary branch, diameter of bases of egg processes approximately equal to or slightly smaller than their height, 9–11 processes on egg circumference . . . . ***Pam. huziori***

–. A row of larger teeth and a posterior band of small granules/conical teeth present in the second band of teeth in the oral cavity, the second macroplacoid situated closer to the first than to the third macroplacoid, accessory points extremely well developed, protruding high above the primary branch, diameter of bases of egg processes greater than their height, 12–16 processes on egg circumference . . . . ***Pam. derkai***

35. Eggs of *richtersi* type . . . . ***Pam. spinosus***

–. Eggs of *areolatus* type **36**

36. The first/anterior band of teeth visible under PCM **37**

–. The first/anterior band of teeth absent or not visible under PCM . . . .***Pam. intii***

37. Lunules under claws IV smooth. **38**

–. Lunules under claws IV dentate **39**

38. Eyes present, macroplacoid length sequence 2<3<1, full egg diameter more than 93.0 µm and egg process height more than 17.5 µm. . . . .***Pam. lachowskae***

–. Eyes absent, macroplacoid length sequence 2<1<3, full egg diameter less than 92.0 µm and egg process height less than 11.5 µm . . . .***Pam. centesimus***

39. Eyes present, macroplacoid length sequence 2<1<3 and egg processes elongated **40**

–. Eyes absent, macroplacoid length sequence 2<3<1 and egg processes short. . . . . ***Pam. klymenki***

40. Egg process height more than 26.5 µm and egg process surface smooth . . . . ***Pam. areolatus***

–. Egg process height less than 17.5 µm and egg process surface apically covered by irregular granulation . . . . ***Pam. walteri***

5. Conclusions

The genus *Paramacrobiotus* shows cosmopolitan distribution with presence of both bisexual and parthenogenetic species. Although the integrative descriptions and redescriptions are improving the overall situation and allowing for fresh opportunities for detailed study, the phylogeny of the genus *Paramacrobiotus* seems to be unresolved. Also, there are many other studies regarding life-history, cryptobiotic abilities and microbiome community as well as bacterial endosymbiont infections identification, which are lacking, and such studies are required for the advancement of tardigrade knowledge in general.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. SM.01 Locations, mode of reproduction and presence of genetic data for all the *Paramacrobiotus* species.

Author Contributions: Conceptualization, P.K. and Ł.K.; methodology, P.K. and M.M.; formal analysis, P.K. and M.M.; investigation, P.K.; data curation, P.K.; writing—original draft preparation, P.K.; writing—review and editing, P.K., M.M. and Ł.K; visualization, P.K. and M.M.; supervision, Ł.K. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: All the DNA sequences data are from GenBank.

Acknowledgments: Studies have been partially conducted in the framework of activities of BARg (Biodiversity and Astrobiology Research group). PK is scholarship holder of Passport to the future - Interdisci-plinary doctoral studies at the Faculty of Biology, Adam Mickiewicz University, Poznań POWR.03.02.00-00-I006/17. The work of MM was supported by National Science Centre, Poland, grant no. 2021/43/D/NZ8/00344 and grant no. 1220/146/2021 from the Small Grants Pro-gramme of the University of Gdansk (i.e., Ugrants-first competition). Tomasz Bartylak for helping with QGIS.

Conflicts of Interest: The authors declare no competing interests.

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