Copenhagen, 20. November 2023

Review of PhD thesis submitted by
Pushpalata Kayastha, Adam Mickiewicz University in Poznań, Poland

The PhD thesis entitled "The genus *Paramacrobiotus* (Tardigrada): integrative taxonomy, biogeography and effects of stress factors on the selected species", submitted for evaluation by Pushpalata Kayastha, revolves around the eutardigrade genus *Paramacrobiotus*.

The thesis is based on five first authored articles dealing with various aspects of the genus *Paramacrobiotus*, *i.e.*, four published peer-reviewed articles (three original research articles and one review article) as well as a submitted manuscript, which is currently under review. The thesis includes co-author statements confirming that Kayastha performed a major part of the work presented in these articles. Specifically, the five publications involve 19 co-authors representing eight national and one foreign university/institution. The thesis, moreover, includes a Summary (in Polish), an Abstract and an Introductory Chapter.

In addition to the five articles formally included as part of the PhD thesis, the candidate further lists twelve co-authored publications, which do not form part of the thesis, but nevertheless were published during her PhD period, revealing an excellent to outstanding research output.

The thesis is generally well-written. The Abstract and the Introductory Chapter provide a summary of, and a very nice framework for, the five articles constituting the basis of the thesis. Specifically, the Introductory Chapter includes detailed schematic representations of the methods and protocols used in the original articles, emphasizing that the candidate has been deeply involved in designing and executing the experimental work underlying the original publications. While the figures are well made, the font size is sometimes too small, making the text within selected figures (Figs 7 & 8) difficult to read. The Introductory Chapter moreover presents an overview of tardigrade habitats and taxonomy with focus on Macrobiotidae to which *Paramacrobiotus* belongs. Within this chapter, the candidate also introduces "Integrative taxonomy", the "Everything is Everywhere" hypothesis, environmental niche modelling and selected abiotic stressors involved in *e.g.*, induction of cryptobiosis. While the chapter without doubt provides a good introduction to the five articles, I do find that there is too much overlap and repetition between the Abstract and Introductory Chapter as regards summarizing the

content of the five selected publications. On the other hand, I lack a brief explanation to and discussion of why the genus *Paramacrobiotus* was chosen for the investigations forming the basis of this thesis, and I note that referencing is not always adequate or correct. For example, on page 15 the candidate refers to Keilin (1959), when mentioning the five currently recognized forms of cryptobiosis. However, one of these forms (*i.e.*, chemobiosis) was not coined by Keilin, but introduced much later (see *e.g.*, Møbjerg et al. 2011: https://doi.org/10.1111/j.1748-1716.2011.02252.x).

The first of the five publications, forming the basis of the thesis, is entitled "Integrative taxonomy reveals new, widely distributed tardigrade species of the genus *Paramacrobiotus* (Eutardigrada:Macrobiotidae)". This article, which was published in the scientific journal *Scientific Reports*, includes a description of a new parthenogenetic and widely distributed species belonging to the genus *Paramacrobiotus, i.e.*, *Paramacrobiotus gadabouti* sp. nov.. The description of the new species is based on state-of-the-art methodology within integrative taxonomy, involving amplification of genetic markers as well as light and scanning electron microscopy of cultured adult specimens and eggs. Specifically, fragments from three genetic markers (18S rRNA, 28S rRNA and COI) were successfully amplified from three specimens originating from a population collected in Madeira and subsequently compared with sequences from other *Paramacrobiotus* specimens available in GenBank. Based on the current investigation of *Paramacrobiotus gadabouti*, the authors present solid arguments supporting the notion that the "Everything is Everywhere" hypothesis applies to at least some tardigrade species. They list a total of four tardigrade species known from more than one zoogeographic realm, however, without providing references to the distribution of the three additional species. In addition, I note an inconsistency in the ranking of the clade Macrobiotoidea. Specifically, in the current publication this clade is listed as an order, whereas the candidate in her Introductory Chapter (Fig. 1, page 16) places the family Macrobiotidae within the order Parachela.

The second publication entitled "Morphological and genetic variability in cosmopolitan tardigrade species—*Paramacrobiotus fairbanksi* Schill, Förster, Dandekar & Wolf, 2010" also published in *Scientific Reports* represents a comprehensive study of *P. fairbanksi* originally described from Fairbanks, Alaska (USA). Specifically, the authors report four new populations of this tardigrade (from Albania, Canada, Madeira and Mongolia), confirming that the species is widespread. For each of these new populations the authors provide morphometric data from adults and eggs, as well as sequences of three genetic markers (18S rRNA, 28S rRNA, COI).

They subsequently compare these data with data from five known populations of *P. fairbanksi* (from Antarctica, Spain, Italy, Poland and USA). With twelve identified COI haplotypes and uncorrected p-distances in the range 0.002-0.005, the data reveals high haplotype, but low sequence diversity, indicating a high dispersal potential. I note that the authors throughout the text list p-distances in percentages. However, these percentages do not reflect the values given in Figure 8. Hence, I would expect that the candidate can explain how she calculated the p-distances. The authors further show variation in selected morphological characters between specimens but conclude that this variation should not affect species identification. While the authors clearly state how they define a cosmopolitan species, *i.e.*, as a species that has been recorded in more than one zoogeographic realm (based on Gąsiorek et al. 2019), there is no reflection anywhere in this thesis on how the candidate defines a zoogeographic realm. I also note that the authors in the current publication list 19 species as being cosmopolitan, while only four species were listed in the first publication (see above). I further note that none of the mentioned species are marine. Hence, I would expect that the candidate can explain what a zoogeographic realm is and how many realms and cosmopolitan tardigrade species she currently recognizes.

The third publication entitled "Tolerance against exposure to solution of magnesium perchlorate in microinvertebrates" was published in *Zoological Journal of the Linnean Society.* This is an interesting publication addressing a very different topic as compared to the first two publications included in the thesis. Specifically, the current publication revolves around the potential survival of small invertebrates on Mars. Perchlorates are present at high concentrations in Martian regolith (mean of 0.6 wt%) and in the current publication the authors investigate tolerance towards high levels (0.25–1.00%) of magnesium perchlorate in the crustacean *Artemia salina*, the nematode *Caenorhabditis elegans*, the rotifer *Lecane inermis* and the three tardigrade species *Hypsibius exemplaris*, *Paramacrobiotus experimentalis* and *Milnesium inceptum*. Generally, the results show that the number of active specimens of all six species decrease with increasing magnesium perchlorate concentration and with exposure time, indicating that these invertebrates would not survive prolonged exposure to Martian conditions with regolith perchlorate concentrations in the range of those used in the current study. One could argue that the choice of tardigrade species for the current study was not optimal, *i.e.*, it is well-known that limno-terrestrial tardigrades are vulnerable to increase in external electrolyte concentrations, whereas marine species can be highly tolerant of both gradual and

acute changes in external salt concentrations. Hence, I lack a reflection on the choice of tardigrade species for the current study.

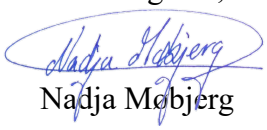
The fourth publication entitled "Elevated external temperature affects cell ultrastructure and heat shock proteins (HSPs) in *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa & Roszkowska, 2020" is the only of the five publications included in the thesis that has not, yet, been published. Specifically, this original research article is currently submitted to *Scientific Reports*. The article focusses on storage cell ultrastructure and heat shock protein abundance in *Paramacrobiotus experimentalis* following heat stress. The study is timely, and the manuscript is generally well-written, but referencing is not always adequate or correct and I would expect that the candidate can explain why she chose to investigate the abundance of the specific heat shock proteins. I lack reflections and references to previous studies dealing with heat stress and regulation of heat shock proteins in tardigrades. Also, the authors state that the eutardigrade *Ramazzottius varieornatus* exhibits tolerance to high temperature in both its active and anhydrobiotic states. However, previous studies have shown that the active state of this species is vulnerable to high temperatures, with an LD50 of around 37°C – a temperature which is not normally regarded as particularly high. As holds for the publication on tolerance towards magnesium perchlorate, I also note that the authors use the term "extremophile" – I would expect that the candidate can explain why she may think that selected animal species belong to this category of organisms. Also, the method section would benefit from some elaboration. Specifically, I lack an explanation to the choice of primary and secondary antibodies, temperatures and exposure times, as well as why a protocol for slow drying was applied. I also note that argumentation is not always easy to follow. As an example, in the introduction the authors state that "desiccated (tun-state), but not active, tardigrades are resistant to high temperatures", but in the Results section they conclude that the tuns apparently die following exposure to 37-42 °C. The latter conclusions seem somewhat contradictory and hence would benefit from further explanation.

The fifth and last publication formally included in the thesis is a review article with the title "A review on the genus *Paramacrobiotus* (Tardigrada) with a new diagnostic key" published in the MDPI journal, *Diversity*. This review is timely and summarizes current knowledge and worldwide distribution of the forty-five described *Paramacrobiotus* species. Focus is on defining morphological characters, such as presence or absence of a microplacoid, on life history traits, such as average lifespan and whether reproduction occurs bisexually or via

parthenogenesis as well as on diet and microbiome composition, including Wolbachia endosymbiont identifications. The review also summarizes current knowledge on cryptobiotic abilities of selected species within the genus and the authors further provide a new diagnostic key for identification of the different *Paramacrobiotus* species based on morphological characters of adults and eggs. The review is concluded with an indication for future research highlighting obvious gaps in current knowledge, including the lack of barcoding sequences for many *Paramacrobiotus* species leading to unresolved phylogenetic relationships within the genus. The authors furthermore call for more studies on various aspects of life history traits, including more comprehensive data on cryptobiotic abilities and microbiome compositions, which are lacking across tardigrade clades.

Without hesitation, I hereby confirm that the candidate, Pushpalata Kayastha, can proceed to the final stages towards the award of a doctoral degree.

Kind Regards,



Nadja Møbjerg