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New records of tardigrade species of the *Macrobiotus hufelandi* group (Tardigrada: Eutardigrada: Macrobiotidae) from Ukraine

Ye.O. Kiosya, V.V. Shuba, Ye.D. Matsko

Species of the *Macrobiotus hufelandi* group are among the most well known and commonly found tardigrades. They are reported in almost every study of Tardigrada diversity in terrestrial habitats. In spite of this, zoogeographical data on this group remain ambiguous and insufficient. Since most species of the *M. hufelandi* group have been described in the last three decades, many older records need to be confirmed or revised. In this study we aimed to investigate, which species may be present in Ukraine, since most Ukrainian records of this group are outdated. We studied samples of mosses and lichens collected from different regions of Ukraine (mainly in the East and South of the country) in 2007–2019. Tardigrades were extracted from samples and mounted on permanent slides in Faure's medium. After primary identification of genera and species groups we focused on 13 samples containing local populations of the *M. hufelandi* group. Details of animal and egg morphology were studied under high magnifications of phase contrast light microscopy. Species were identified based on morphology of the oral cavity armature, egg shell morphology, animal cuticle granulation, as well as some morphometric characters of claws and bucco-pharyngeal apparatus. This research revealed the presence of at least six different species in Ukraine: *M. glebkai* Biserov, 1990; *M. hufelandi* C.A.S. Schultze, 1834; *M. macrocalix* Bertolani & Rebecchi, 1993; *M. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003; *M. sottilei* Pilato, Kiosya, Lisi & Sabella, 2012; *M. vladimiri* Bertolani, Biserov, Rebecchi & Cesari, 2011. Three of them: *M. macrocalix*, *M. sottilei,* and *M. vladimiri* are reported in this study from Ukraine for the first time. Adding three more species recorded in earlier studies: *M. diversus* Biserov, 1990; *M. persimilis* Binda & Pilato, 1972, and *M. sapiens* Binda & Pilato, 1984 – gives the total count of at least 9 species of the group in Ukraine. However, according to the recent studies on species complexes the real species diversity is very likely to be higher. Nevertheless, more sampling and obtaining molecular data from different Ukrainian populations of the *M. hufelandi* group from Ukraine are required.

Key words: *water bears, zoogeography, fauna, biodiversity.*

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Introduction

Macrobiotus hufelandi C.A.S. Schultze, 1834 was the first species of tardigrades formally described in a scientific paper. The name refers to Christoph Wilhelm von Hufeland, an adept of naturopathic medicine who promoted so called "macrobiotics" – the art of prolonging human life by special diet and healthy lifestyle (Bertolani et al., 2011b). Due to priority of description this species later became the type species (the nominal species) of the genus *Macrobiotus* which in turn became the type genus of the family Macrobiotidae.

Since then a lot of tardigrade species were described within the genus *Macrobiotus* – making it one of the most speciose genera of tardigrades. Most of these species were later found to be not so similar and not so closely related to *M. hufelandi* and transferred to the other newly erected genera: *Minibiotus* R.O. Schuster, 1980; *Paramacrobiotus* Guidetti, Schill, Bertolani, Dandekar & Wolf, 2009 – former "*Macrobiotus richtersi-areolatus* group"; *Tenuibiotus* Pilato & Lisi, 2011 – former "*Macrobiotus tenuis* group"; *Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi & Guidetti, 2016 – former "*Macrobiotus harmsworthi* group", and some other. Nevertheless, even revised *Macrobiotus* remains quite a large genus listing more than 100 species (Degma et al., 2019).

Among the species left within *Macrobiotus* nearly a half are attributed to the "*Macrobiotus hufelandi* group". Originally this group used to include only the species closely resembling *M. hufelandi* in details of egg morphology: eggs with processes in the shape of truncated cones usually terminated with a disc, commonly referred as "inverted-goblets" shape (Fig. 1 C), but later the criteria of inclusion were modified shifting the weight to animal morphology characters and molecular data (see the definition of the group in

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Guidetti et al., 2013; the review of the included species in Kaczmarek & Michalczyk, 2017; the updated phylogenies in Stec et al., 2018a,b; Coughlan & Stec, 2019; Kayastha et al., 2020).

Species of the *M. hufelandi* group are among the most commonly found tardigrades, reported in almost every study of Tardigrada diversity in terrestrial habitats. However, zoogeographical data on this group remain ambiguous and insufficient. Most of the records from all over the world are of the nominal species (*M. hufelandi*), while many of the other species are only known from their type localities. Since the great majority of species of the *M. hufelandi* group are quite similar and hard to distinguish, many records (especially the old ones) need to be confirmed or revised and more sampling is required.

In this study we aimed to investigate, which species of *M. hufelandi* group are present in Ukraine, since most Ukrainian records of this group are rather old and outdated (see the review in Pilato et al., 2011).

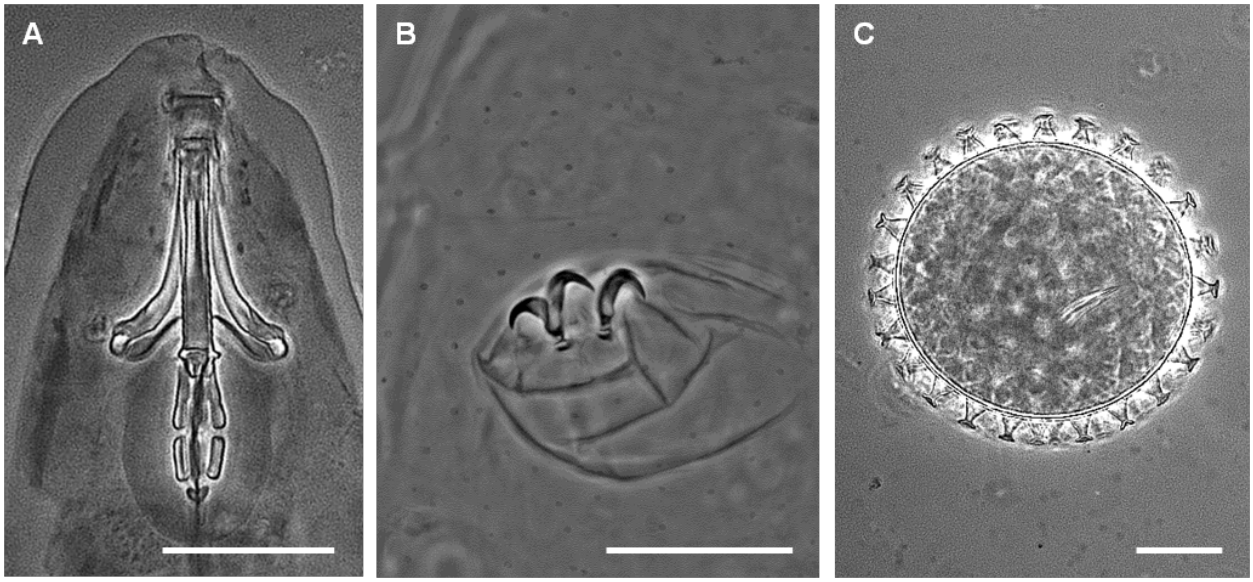

Fig. 1. Typical morphological characters of tardigrades of the *M. hufelandi* group, phase contrast, ×400 (scale bars – 25 μm): A – bucco-pharyngeal apparatus with two macroplacoids and a closely positioned microplacoid, B – "V-shaped" double claws on legs and pores (pearls) in the cuticle, C – ornamented egg with "inverted goblet"-shaped processes

Methodology

Tardigrades were extracted from samples which had been collected in 2007–2019 from different regions of Ukraine. Several hundreds of small pieces of mosses and lichens were taken from soil, tree trunks, branches, rocks and other substrates, then air-dried, labeled and stored in the lab in paper envelopes at room temperature.

To process a sample, a part of it (about 1 g of dry weight) was soaked in distilled water for 1–2 hours, then shaken and squeezed. The water was drained from a sample into a Petri dish and examined under a stereomicroscope (×40 magnification, dark field). Tardigrades and their eggs were collected from water and put on the glass slides with a very fine microspatula. If tardigrades were still alive and active, they were fixed with 40 % chloral hydrate solution in acetic acid. Animals and eggs were then mounted in the Faure's medium and covered with a coverslip. After several weeks of air-drying coverslips were sealed with nail polish – to prevent excessive drying and oxidation.

We used ×400 magnification of a light microscope (phase contrast) for primary identification of tardigrades found. This was sufficient to discriminate species of the *M. hufelandi* group from other eutardigrades based on their common morphological features (Fig. 1). Using the results of this primary identification, we have chosen several "referent samples" containing at least a dozen of animals and eggs of the *M. hufelandi* group species for a more detailed investigation (Table 1). We focused on these samples, because it had been shown, that studying series of many tardigrades from the same population allows more reliable measurements and identification, than studying individual specimens (Stec et al., 2016).

Fine morphology of animals and eggs from the referent samples was studied with the use of ×1000 magnification of a light microscope (phase contrast, oil immersion). Species were identified based on the diagnostic key by Kaczmarek & Michalczyk (2017), and further compared with the original species descriptions. The list of characters used for identification included, but was not limited to: morphology of oral cavity armature, egg shell morphology, animal cuticle granulation, as well as some morphometric characters of claws and bucco-pharyngeal apparatus. Measurements and photomicrographs were taken at the Łukasz Michalczyk's Lab in Jagiellonian University (Kraków, Poland) and Łukasz Kaczmarek's Lab in Adam Mickiewicz University (Poznań, Poland). Permanent slides with animals and eggs from the referent samples are stored in Kiosya's collection in the V.N.Karazin Kharkiv National University.

Table 1.

List of referent samples investigated

Region	No.	Sampling locality	Sample type	Date, collector	Original sample code
Crimea AR	1	44.4229N, 33.8539E, 600 m a.s.l., Crimean Mountains: forest nearby Shaytan-Merdven mountain pass	Moss from a stone	15.08.2009, N. Khudaeva (N. Kantsedal)	БКГ-13
Donetsk Oblast	2	47.0492N, 38.1314E, 2 m a.s.l., Meotyda national nature park, Kryva spit	Lichen from a tree trunk	30.06.2009, N. Khudaeva (N. Kantsedal)	ДМБ-11-1
Kharkiv Oblast	3	49.1017N, 37.4542E, 80 m a.s.l., pine forest in the vicinity of Studenok village	Lichens from bush branches	15.07.2007, Ye. Kiosya	ИЗ-38
	4	49.6863N, 36.4657E, 85 m a.s.l., lake Chernychne shore	Moss from a branch protruding from water	04.11.2018, Ye. Kiosya	ZMS-33
Kherson Oblast	5	46.4625N, 33.8777E, 25 m a.s.l., arboretum of Askania-Nova biosphere reserve	Moss from a stone	20.06.2012, Ye. Kiosya	ASK-14
	6	47.2162N, 33.8536E, 60 m a.s.l., vicinity of Havrylivka village	Moss from a tree trunk	09.08.2019, O. Nahimova	KHG-1B
Lviv Oblast	7	48.9722N, 23.4745E, 500 m a.s.l., vicinity of Hrebeniv village	Moss from soil	05.08.2016, Ye. Kiosya	SKGR-1
Poltava Oblast	8	49.9974N, 34.0237E, 90 m a.s.l., floodplain in the vicinity of Maly Pereviz village	Lichen from tree trunk	10.07.2011, Ye. Kiosya	HT-A-62
	9	50.0294N, 34.0616E, 125 m a.s.l., pine forest in the vicinity of Kovalivka village	Moss from soil	11.07.2011, Ye. Kiosya	HT-B-76
	10		Moss from soil	11.07.2011, Ye. Kiosya	HT-B-95
	11	49.9976N, 34.0429E, 120 m a.s.l., oak forest in the vicinity of Maly Pereviz village	Moss from tree trunk	13.07.2011, Ye. Kiosya	HT-C-118
	12		Moss from soil	13.07.2011, Ye. Kiosya	HT-C-127
Zaporizhzhia Oblast	13	47.7715N, 35.1785E, 20 m a.s.l., Zaporizhzhia, vicinity of a rowing canal	Moss from tree trunk	26.04.2009, Ye. Kiosya	Z-31

Results and discussion

This study revealed the presence of at least six different species in Ukraine: *M. glebkai* Biserov, 1990; *M. hufelandi* C.A.S. Schultze, 1834 (diagnosis in Bertolani, Rebecchi, 1993); *M. macrocalix* Bertolani & Rebecchi, 1993; *M. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003; *M. sottilei* Pilato, Kiosya, Lisi & Sabella, 2012; *M. vladimiri* Bertolani, Biserov, Rebecchi & Cesari, 2011. Three of them: *M. macrocalix*,

M. sottilei, and *M. vladimiri* are reported here from Ukraine for the first time. Most of these species had typical "*hufelandi*-like eggs" and could only be differentiated based on small details of animal and egg morphology. The only species strikingly dissimilar in egg morphology was *M. glebkai*. In this species the processes of the egg chorion are quite large (around 20 µm in height) and have elongated tips rather than terminal discs (Fig. 2). Adding three more species recorded in earlier studies: *M. diversus* Biserov, 1990; *M. persimilis* Binda & Pilato, 1972 and *M. sapiens* Binda & Pilato, 1984 – gives the total count of 9 species of the group in Ukraine (see the list in Table 2, the map of the records in Fig. 3).

Table 2.

List of species of the *Macrobiotus hufelandi* group recorded in Ukraine

Species	Type locality	Ukrainian localities*	References	Specimens found in this study**
M. diversus	Russia: Bashkortostan	Crimea AR: Karasivka	Pilato et al., 2011	-
M. glebkai	Russia: Dimitrovgrad	Crimea AR: unsp. localities	Pilato et al., 2011	-
		Kharkiv Obl.: Studenok	Pilato et al., 2011	-
		Kherson Obl.: sample 6	This study	30A + 6E
		Poltava Obl.: sample 8	This study	40A + 6E
		Zaporizhzhia Obl.: sample 13	This study	28A + 6E
M. hufelandi	Germany: Freiburg	Poltava Obl.: sample 9	This study	26A + 8E
M. macrocalix	Italy: Gaianello	Lviv Obl.: sample 7	This study	43A + 26E
		Poltava Obl.: sample 11	This study	15A + 7E
M. persimilis	Italy: Sicily	Crimea AR: unsp. localities	Pilato et al., 2011	-
M. polonicus	Poland: Poznań	Crimea AR: sample 1	This study	45A + 30E
		Crimea AR: Mtn. Opuk	Pilato et al., 2011	-
		Donetsk Obl.: sample 2	This study	13A +12E
		Kherson Obl.: sample 5	This study	14A + 4E
M. sapiens	Italy: Sicily	Crimea AR: unsp. localities	Pilato et al., 2011	-
		Kyiv Obl.: Chornobyl	Pilato et al., 2011	-
M. sottilei	Belarus: Zhodino	Kharkiv Oblast: sample 3	This study	32A + 3E
		Poltava Oblast: sample 12	This study	30A + 17E
M. vladimiri	Italy: Andalo	Kharkiv Oblast: sample 4	This study	54A + 7E
		Poltava Oblast: sample 10	This study	40E + 6E

Notes:
** Referent sample numbers are given according to Table 1.*
*** A – number of animals (adult or juvenile tardigrades) found, E – number of eggs found.*

All the species recorded in Ukraine had been described from other European localities. Here we present a brief overview of their known distribution outside Ukraine.

M. diversus was described from Bashkortostan, Russia (Biserov, 1990b) and later reported only from Poznań, Poland (Rost-Roszkowska et al., 2018).

M. glebkai is currently known outside Ukraine only from its type locality in Ulyanovsk Oblast, Russia (Biserov, 1990a).

M. hufelandi was reported from nearly all parts of the world, but after the redescription in 1993 only the records from neotype locality in Germany (Bertolani, Rebecchi, 1993) and Pomerania Province, Poland (Kaczmarek et al., 2018) can be treated as reliable, as they were based on the DNA barcoding and/or detailed morphological investigation with phase contrast microscopy and scanning electron microscopy. The rest of the reports of *M. hufelandi* or *M.* cf. *hufelandi* are ambiguous and should be reevaluated.

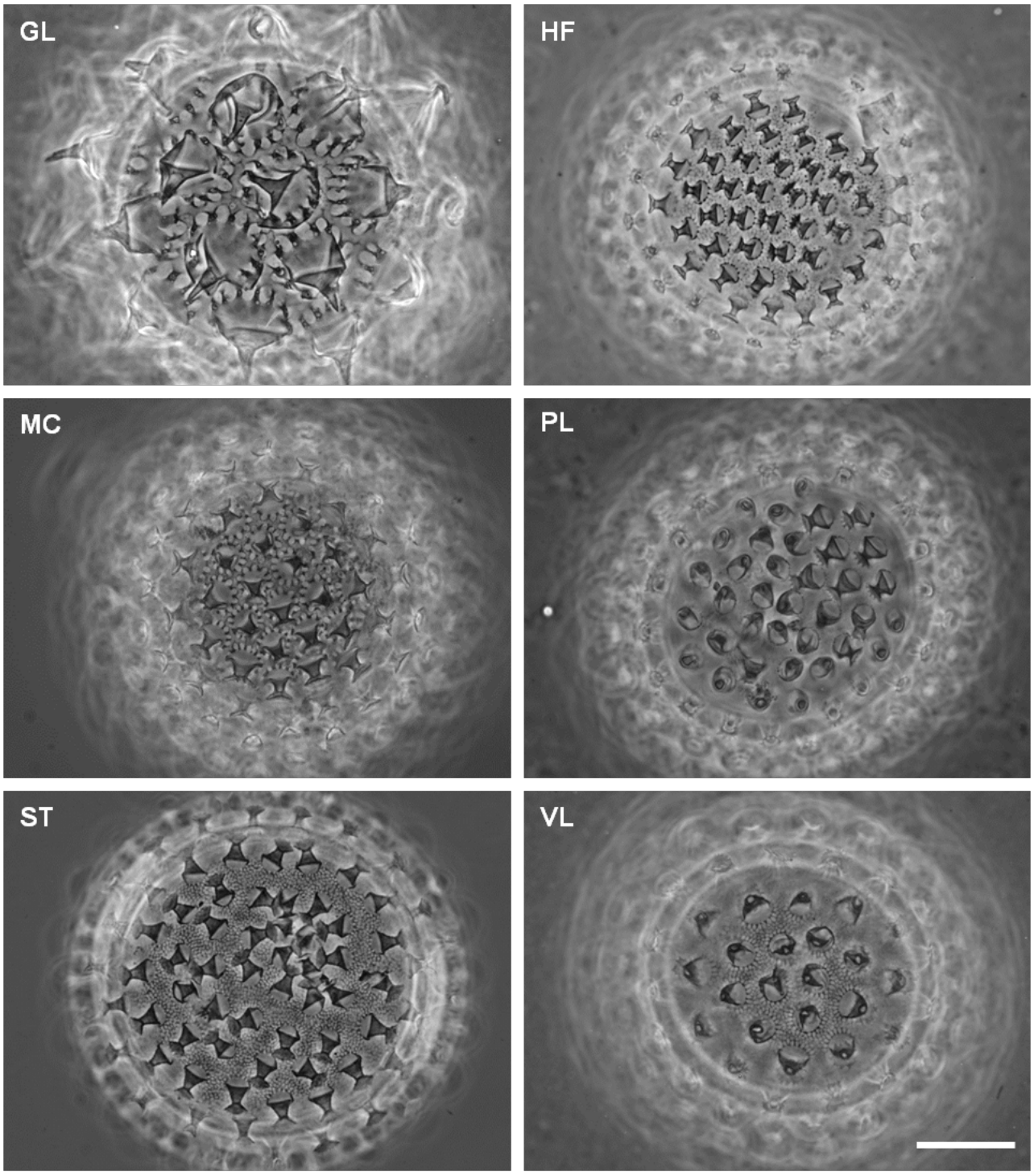

Fig. 2. Egg shell morphology of six newly found species of the *M. hufelandi* group, phase contrast, x1000, oil immersion (scale bar – 25 µm, all photos are in the same scale): GL – *M. glebkai* (8), HF – *M. hufelandi* (9), MC – *M. macrocalix* (7), PL – *M. polonicus* (1), ST – *M. sottilei* (3), VL – *M. vladimiri* (4). Note the differences in shape and size of the processes and the details of egg shell surface between the processes

Note: numbers given in brackets after species names are referent samples from Table 1.

M. macrocalix was identified with a molecular approach in Italy (Cesari et al., 2009), Sweden (same ref.), Poland (Stec et al., 2018b) and Portugal (Vicente et al., 2013), while specimens identified based exclusively on morphology were also reported from Austria (Michalczyk, Kaczmarek, 2003), Albania (Vargha, 2011), Spain (Guil, 2008), and Seychelles Islands (Pilato et al., 2002).

M. polonicus was earlier reported only from its type locality in Wielkopolska Province in Poland (Pilato et al., 2003) and Pomerania Province (Kaczmarek et al., 2018), also in Poland.

Fig. 3. Records of species of the *M. hufelandi* group in Ukraine (corresponds with Table 2): DV – *M. diversus*, GL – *M. glebkai*, HF – *M. hufelandi*, MC – *M. macrocalix*, PL – *M. polonicus*, PR – *M. persimilis*, SP – *M. sapiens*, ST – *M. sottilei*, VL – *M. vladimiri*. Old records of *M. hufelandi* and records of *M.* cf. *hufelandi* are not included

Note: aggregation of overlapping symbols in Poltava Oblast and Kharkiv Oblast represent species found in the same locality or very close localities, while the aggregation of overlapping symbols in Crimea represent species found in several distant localities in Crimean Mountains.

M. sottilei described from Zhodino, Belarus (Pilato et al., 2012) was later reported from Pomerania Province, Poland (Kaczmarek et al., 2018) and also Trento and Olbia-Tempio Provinces, Italy (Roszkowska, 2019).

M. vladimiri described from Andalo, Italy (Bertolani et al., 2011a) was also reported from several other localities in Italy (Roszkowska et al., 2019), Germany (Bertolani et al., 2011b), and Poland (Nowak, Stec, 2017; Kaczmarek et al., 2018).

As for *M. persimilis* and *M. sapiens* described from Sicily, Italy (Binda, Pilato, 1972, 1984), the situation is complicated. Although they were repeatedly recorded not only in Europe outside their *locus typicus*, but also in Asia (Biserov, 1999), Africa (McInnes et al., 2017) and even America (Kaczmarek et al., 2014, 2016), these records have to be treated with special caution, as the original descriptions (especially

the one of *M. sapiens*) lack some vital taxonomic information (Kaczmarek, Michalczyk, 2017). If these two species are redescribed in future, the records of them in Ukraine will also need confirmation.

To summarize, this study extends the known distribution range of *M. macrocalix*, *M. polonicus*, *M. sottilei*, and *M. vladimiri* to the East. Although as we shown at least nine *M. hufelandi* species can be found in Ukraine, the real species diversity in the country is very likely to be higher. More sampling in different regions and ecosystems is needed to evaluate the species richness and patterns of the species distribution properly. Noteworthy, it is not just about poor knowledge of tardigrades in Ukraine; similar situation was already reported for Poland (Kaczmarek et al., 2018), Belarus (Pilato et al., 2012), Romania (Ciobanu et al., 2014), Bulgaria (Yankova et al., 2016) and some other neighboring countries. The distribution ranges of many tardigrade species are not quite clear even in the regions where intense research had been performed in the past. As more and more species are being described and redescribed, it gets necessary to reevaluate the older records. In this study we show, that some species can be morphologically recognized – even on the slides which are more than 10 years old. However, tardigrades have many species complexes which are almost cryptic or pseudocryptic, so implementation of molecular tools would be crucial for proper assessment of species boundaries and their distribution ranges (e.g. Guidetti et al., 2019; Stec et al., 2020).

Conclusion

The diversity of tardigrades of *M. hufelandi* group in Eastern Europe is poorly understood. There are a lot of reports of *Macrobiotus hufelandi* or *Macrobiotus* cf. *hufelandi* from many different localities, but in fact most of them may represent some other species of the group. This study confirmed the presence of the nominal species in Ukraine, but also revealed several similar species. The total count of species of the group recorded in Ukraine is now elevated to 9. It is very likely that the total number of species of the *M. hufelandi* group is much higher, however, more sampling and studies that use integrative taxonomy approach are needed to elucidate the true species diversity of the *M. hufelandi* group in our country.

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Нові знахідки видів тихоходів роду *Macrobiotus* групи *hufelandi* (Tardigrada: Eutardigrada: Macrobiotidae) в Україні

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Тихоходи роду *Macrobiotus* групи *hufelandi* – одні з найбільш відомих та поширених тихоходів. Їх знаходять майже в кожному дослідженні різноманіття тихоходів у наземних оселищах. Незважаючи на це, зоогеографічні дані щодо цієї групи лишаються сумнівними та неповними. Оскільки більшість видів групи *M. hufelandi* описані в останні декілька десятиліть та їх важко розрізняти, багато старих знахідок потребують підтвердження або перегляду. У цьому дослідженні ми взяли за мету вивчити, які види групи *M. hufelandi* можна знайти в Україні, оскільки більшість даних щодо поширення тихоходів цієї групи в Україні є застарілими. Досліджували проби мохів та лишайників, зібраних у різних регіонах України (головним чином – на сході та півдні країни) у 2007–2019 роках. Тихоходів добували з проб та виготовляли з них постійні мікропрепарати з використанням рідини Фора. Після попереднього визначення родів та груп видів ми сфокусувалися на 13 пробах, які містили тихоходів якихось видів групи *M. hufelandi* у достатній кількості. Деталі морфології тихоходів та їх яєць вивчали методом світлової мікроскопії великого збільшення з використанням фазового контрасту. Види визначали за морфологічними ознаками ротової арматури, морфологією хоріона яєць, характером грануляції кутикули, а також на підставі певних морфометричних характеристик кігтиків та ротоглоткового апарату. Дослідження виявило наявність в Україні щонайменше шести різних видів: *M. glebkai* Biserov, 1990; *M. hufelandi* C.A.S. Schultze, 1834; *M. macrocalix* Bertolani & Rebecchi, 1993; *M. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003; *M. sottilei* Pilato, Kiosya, Lisi & Sabella, 2012 і *M. vladimiri* Bertolani, Biserov, Rebecchi & Cesari, 2011. Три з них: *M. macrocalix*, *M. sottilei* й *M. vladimiri* було вперше знайдено в Україні. З урахуванням ще трьох видів, вказаних у більш ранніх дослідженнях: *M. diversus* Biserov, 1990; *M. persimilis* Binda & Pilato, 1972 і *M. sapiens* Binda & Pilato, 1984, – загальна кількість видів, відомих в Україні, складає щонайменше дев'ять. Реальна кількість видів в Україні, ймовірно, є вищою. Потрібні подальші збори проб та отримання молекулярно-генетичних даних від різних видів тихоходів групи *M. hufelandi* з України.

Ключові слова: *тихоходи, зоогеографія, фауна, біорізноманіття.*

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Новые находки видов тихоходок рода *Macrobiotus* группы *hufelandi* (Tardigrada: Eutardigrada: Macrobiotidae) в Украине

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Тихоходки рода *Macrobiotus* группы *hufelandi* – одни из наиболее известных и распространенных тихоходок. Их находят практически в каждом исследовании разнообразия тихоходок в наземных местообитаниях. Несмотря на это, зоогеографические данные по этой группе остаются сомнительными и неполными. Поскольку большинство видов группы *M. hufelandi* описаны в последние десятилетия и их тяжело различать, многие старые находки нуждаются в подтверждении или пересмотре. В этом исследовании мы поставили цель выяснить, какие виды группы *M. hufelandi* можно найти в Украине, поскольку большинство данных по распространению тихоходок этой группы в Украине устарели. Исследовали пробы мхов и лишайников, собранных в разных регионах Украины (главным образом – на востоке и юге страны) в 2007–2019 годах. Тихоходок извлекали из проб и изготовляли из них постоянные микропрепараты на основе жидкости Фора. После предварительного определения родов и групп видов мы сфокусировались на 13 пробах, с достаточно высокой численностью тихоходок какого-либо вида группы *M. hufelandi.* Детали морфологии тихоходок и их яиц изучали методом световой микроскопии большого увеличения с использованием фазового контраста. Виды определяли по морфологическим признакам ротовой арматуры и хориона яиц, характеру грануляции кутикулы, а также по определённым морфометрическим характеристикам коготков и ротоглоточного аппарата. Наше исследование выявило присутствие в Украине не менее шести разных видов: *M. glebkai* Biserov, 1990; *M. hufelandi* C.A.S. Schultze, 1834; *M. macrocalix* Bertolani & Rebecchi, 1993; *M. polonicus* Pilato, Kaczmarek, Michalczyk & Lisi, 2003; *M. sottilei* Pilato, Kiosya, Lisi & Sabella, 2012 и *M. vladimiri* Bertolani, Biserov, Rebecchi & Cesari, 2011. Три из них: *M. macrocalix*, *M. sottilei* и *M. vladimiri* были впервые найдены в Украине. С учетом ещё трёх видов, указываемых в более ранних исследованиях: *M. diversus* Biserov, 1990; *M. persimilis* Binda & Pilato, 1972 и *M. sapiens* Binda & Pilato, 1984, – общее число видов, известных в Украине, составляет, как минимум, девять. Реальное число видов в Украине, вероятно, выше. Нужны дальнейшие сборы проб и получение молекулярно-генетических данных от разных видов тихоходок группы *M. hufelandi* из Украины.

Ключевые слова: *тихоходки, зоогеография, фауна, биоразнообразие.*

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