Presence of *Notahypsibius pallidoides* (Tardigrada: Hypsibiidae) in the fauna of Russia confirmed with the methods of DNA barcoding

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Abstract

A brief report is given on the tardigrade species *Notahypsibius pallidoides*, discovered in a moss cushion in the vicinity of Saint Petersburg. Morphological identification of the species is supported with the analysis of obtained sequences of nuclear and mitochondrial DNA barcode genes (ITS-2 and COI). The current state of knowledge of Russian fauna of Tardigrada is characterised, and all available data on the distribution of *Notahypsibius pallidoides* and its genetic variability are summarised. The data obtained in this study by methods of DNA barcoding demonstrated that studied specimens of the tardigrada population isolated in Russia belong to the same species (*Notahypsibius pallidoides*) as the control specimens isolated in Austria though represent another haplotype thus confirming the presence of *Notahypsibius pallidoides* for the fauna of Russia.

Keywords: distribution, zoogeography, genetic variability, COI, ITS-2, Pilatobiinae

Introduction

Phylum Tardigrada Doyère, 1840 is a group of microscopical multicellular animals. Tardigrades are widely distributed in nature, inhabiting marine and freshwater basins as well as terrestrial environments which contain (constantly or temporarily) free water (Nelson, Bartels and Guil, 2018). More than 1300 species of tardigrades have been described to date (Degma, Bertolani and Guidetti, 2009–2020), but the real taxonomic richness of this group is still underestimated because of the small number of active taxonomists and methodological difficulties (Guil and Cabrero-Sañudo, 2007; Bartels, Apodaca, Mora and Nelson, 2016).

Tardigrade fauna of Russia remains poorly investigated. Most of the published works were devoted to the European part of the territory (Biserov, 1991 and references within; Biserov, 1996a, 1997–98, 1999; Tumanov, 1997a, b, 2003, 2018; Kiosya and Inshina, 2008; Kiosya, 2009). Only a few publications were devoted to the Asian part of Russia (Biserov, 1992, 1996b, c, 1998; Abe, 2004; Kaczmarek, Michalczyk and Diduszko 2005, 2006; Biserov, Pilato and Lisi, 2011). In all abovementioned publications, taxonomical data were received using the traditional morphological approach. Until now there are only two published works, for the entire territory of Russia, in which the zoogeographical records are supported with DNA barcoding. One of them is primarily devoted to the phylogeny of the genus *Milnesium* (Morek and Michalczyk, 2019) and only confirms the presence of the species *Mil. tardigradum* Doyère, 1840 in Russia using an integrative taxonomy approach, i.e., with the analysis of both morphology and gene sequences.

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The other paper (Drohvalenko et al., 2019) is a perfunctory investigation, not focused specifically on Tardigrada, and its results are impossible to interpret unambiguously. So, until now, the presence of only one tardigrade species is confirmed in Russia with the methods of DNA barcoding.

Since most of the faunistic records were published, tardigrade taxonomy has undergone significant changes. Numerous forms, previously accepted as widely distributed and polymorphic species (e.g., *Macrobiotus hufelandi* C. A. S. Schultze, 1834, *Paramacrobiotus richtersi* (Murray, 1911), *Mesobiotus harmsworthi* (Murray, 1907), *Richtersius coronifer* (Richters, 1903), *Ramazzottius oberhaeuseri* (Doyère, 1840), *Hypsibius dujardini* (Doyère, 1840), *Mil. tardigradum*, *Pseudechiniscus suillus* (Ehrenberg, 1853)), have now been proven to be complexes of similar species, poorly demarcated morphologically, but well-distinguishable with the methods of DNA barcoding (Bertolani, Rebecchi, Giovannini and Cesari, 2011; Gąsiorek et al., 2016, 2018; Kaczmarek et al., 2018; Stec, Morek, Gąsiorek and Michalczyk, 2018; Guidetti et al., 2019; Morek and Michalczyk, 2019; Cesari et al., 2020; Grobys et al., 2020; Roszkowska et al., 2020; Stec, Krzywański, Arakawa and Michalczyk, 2020; Tumanov, 2020a). Also, the presence of true cryptic species, completely undistinguishable in morphology, has been demonstrated in tardigrades (Bertolani, Rebecchi, Giovannini and Cesari, 2011; Guidetti et al., 2019). As the result, most of the zoogeographical records of such species should be considered as doubtful for the Russian territory, and it means that the investigation of tardigrade fauna of Russia should be started *de novo*, using molecular methods.

Still little is known on the genetic diversity of tardigrade species with confirmed wide distribution (Jørgensen, Mobjerg and Kristensen, 2007; Jørgensen et al., 2013; Cesari, Bertolani, Rebecchi and Guidetti, 2009; Cesari et al., 2016; Gąsiorek et al., 2016, Gąsiorek, Blagden and Michalczyk, 2019; Gąsiorek et al., 2019; Zawierucha et al., 2018; Jackson and Meyer, 2019; Morek et al., 2019; Kaczmarek et al., 2020; Pogwizd and Stec, 2020; Sugiura, Arakawa and Matsumoto, 2020; Sugiura, Minato, Matsumoto and Suzuki, 2020).

Recently, the tardigrade species *Notahypsibius pallidoides* (Pilato et al., 2011) was redescribed using an integrative approach, and its phylogenetic position was revised (Tumanov, 2020b). This species was originally described from Kherson Oblast, South Ukraine (Pilato et al., 2011). Later it was recorded in Minsk Oblast, Central Belarus (Pilato, Kiosya, Lisi and Sabella, 2012) and Sicily (Lisi, 2015). Moreover, distribution of this species was extended to Northwest Russia and Croatia (Tumanov, 2020b). For the integrative redescription, a newly revealed population from Austria was used. Also, the presence of this species is suspected for Poland (Tumanov, 2020b) and Ireland (Erica De Milio, unpublished data). But whereas only the Austrian population has been characterized genetically so far (Tumanov, 2020b), it is possible that other records can represent cryptic species.

In summer 2020, 10 specimens and 2 exuvia with eggs of a species morphologically identical to *Not. pallidoides* were found in a moss sample from Pushkin city (vicinity of Saint Petersburg), close to the locality where the single specimen noted in the previous study (Tumanov, 2020b) was found. In order to determine the taxonomical status of this species, sequences of two fast-evolving genetic markers (mitochondrial — COI gene and nuclear — ITS-2) were obtained and compared with the available data on *Not. pallidoides*.

Materials and methods

Sampling

The moss sample was collected from stone in Pushkin city, vicinity of Saint Petersburg, Russia (59°43′30,9″N 30°23′32″E), on November 21, 2019, and July 3, 2020. Material was stored within paper envelopes at room temperature. Tardigrade specimens were extracted from rehydrated samples using the standard technique of washing them through two sieves (first with ≈ 1 mm mesh size and second with 35 μm mesh size; Tumanov, 2018). The contents of the finer sieve were examined under a Leica M205C stereomicroscope.

Microscopy and imaging

The tardigrades found in the moss sample were fixed with acetic acid and mounted on slides in Hoyer's medium. Light microscopy (LM): resulting permanent slides were examined under a Leica DM2500 microscope equipped with phase contrast (PhC) and differential interference contrast (DIC). Photographs were made using a Nikon DS-Fi3 digital camera with NIS-Elements™ software (Nikon, Japan).

Genotyping

DNA was extracted from four individual animals using QuickExtract™ DNA Extraction Solution (Lucigen Corporation, USA) using the modified protocol kindly provided by Torbjørn Ekrem, Norwegian University of Science and Technology.

1) Tardigrades were sorted in water and specimens were rinsed individually in ddH_2O.
2) Each individual specimen was transferred by pipette into a PCR-tube containing 70 μl QuickExtract™.
3) PCR-tubes were vortexed well, spun down (5 min at 3500 RPM), then placed in TS-100C Thermo-Shaker (30 °C; 300 RPM) for 1 hr.

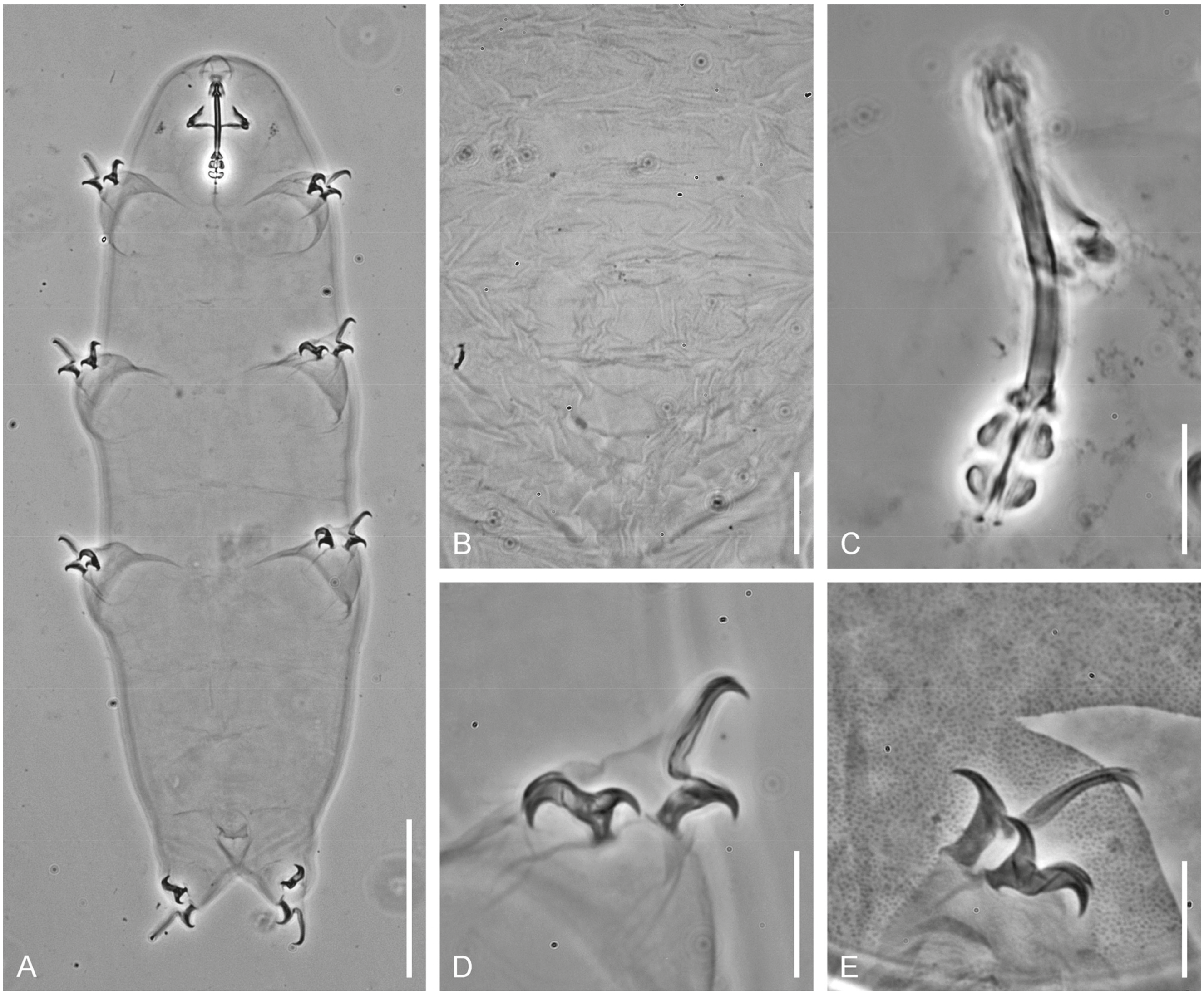
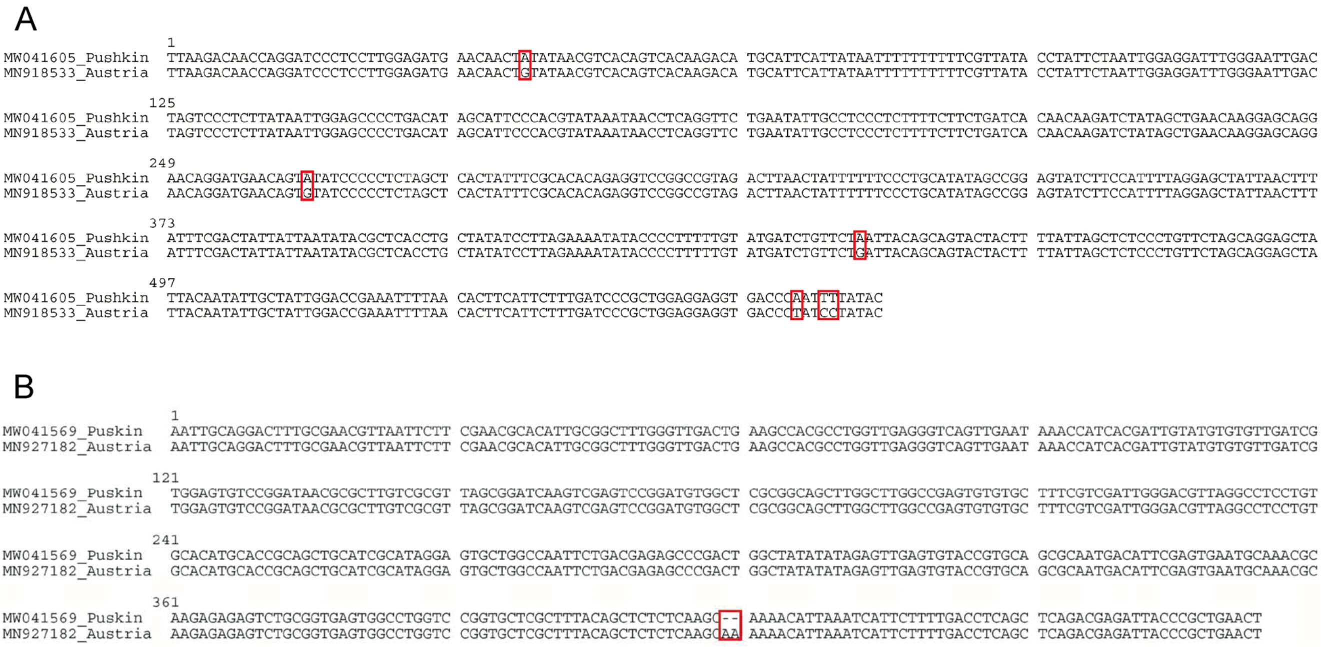


Fig. 1. *Notahypsibius pallidoides*, details of morphology (PhC). A — total view; B — dorsal cuticular sculpture; C — buccal-pharyngeal apparatus, lateral view; D — claws of leg II; E — claws of leg III and fragment of the egg chorion. Scale bars: A — 50 μm, .B–E — 10 μm.

Table 1. Primers and PCR programs used for amplification of the gene fragments sequenced in the study

Gene	Primer name	Primer direction	Primer sequence (5'–3')	Primer source	PCR programme
COI	COI_Mes.rev_Ff	forward	AATTTGAGCTGCAACAGTAGG	Gąsiorek et al., 2016	Michalczyk, Wełnicz, Frohme, and Kaczmarek, 2012
	COI_Mes.rev_Rr	reverse	GAATAAGTGTTGGTATAAAATTGG		
ITS-2	ITS2_Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	Stec, Morek, Gąsiorek and Michalczyk, 2018	Stec, Morek, Gąsiorek and Michalczyk, 2018
	ITS2_Eutar_Rr	reverse	TGATATGCTTAAGTTCAGCGG		



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                    1
MW041605_Pushkin  TTAAGACAACCAGGATCCCTCCTTGGAGATG AACAACTATATAACGTCACAGTCACAAGACA TGCATTCATTATAATTTTTTTTTTCGTTATA CCTATTCTAATTGGAGGATTTGGGAATTGAC
MN918533_Austria  TTAAGACAACCAGGATCCCTCCTTGGAGATG AACAACTGTATAACGTCACAGTCACAAGACA TGCATTCATTATAATTTTTTTTTTCGTTATA CCTATTCTAATTGGAGGATTTGGGAATTGAC

                125
MW041605_Pushkin  TAGTCCCTCTTATAATTGGAGCCCCTGACAT AGCATTCCCACGTATAAATAACCTCAGGTTC TGAATATTGCCTCCCTCTTTTCTTCTGATCA CAACAAGATCTATAGCTGAACAAGGAGCAGG
MN918533_Austria  TAGTCCCTCTTATAATTGGAGCCCCTGACAT AGCATTCCCACGTATAAATAACCTCAGGTTC TGAATATTGCCTCCCTCTTTTCTTCTGATCA CAACAAGATCTATAGCTGAACAAGGAGCAGG

                249
MW041605_Pushkin  AACAGGATGAACAGTATATCCCCCTCTAGCT CACTATTTCGCACACAGAGGTCCGGCCGTAG ACTTAACTATTTTTTCCCTGCATATAGCCGG AGTATCTTCCATTTTAGGAGCTATTAACTTT
MN918533_Austria  AACAGGATGAACAGTGTATCCCCCTCTAGCT CACTATTTCGCACACAGAGGTCCGGCCGTAG ACTTAACTATTTTTTCCCTGCATATAGCCGG AGTATCTTCCATTTTAGGAGCTATTAACTTT

                373
MW041605_Pushkin  ATTTCGACTATTATTAATATACGCTCACCTG CTATATCCTTAGAAAATATACCCCTTTTTGT ATGATCTGTTCTAATTACAGCAGTACTACTT TTATTAGCTCTCCCTGTTCTAGCAGGAGCTA
MN918533_Austria  ATTTCGACTATTATTAATATACGCTCACCTG CTATATCCTTAGAAAATATACCCCTTTTTGT ATGATCTGTTCTGATTACAGCAGTACTACTT TTATTAGCTCTCCCTGTTCTAGCAGGAGCTA

                497
MW041605_Pushkin  TTACAATATTGCTATTGGACCGAAATTTTAA CACTTCATTCTTTGATCCCGCTGGAGGAGGT GACCCAATTTTATAC
MN918533_Austria  TTACAATATTGCTATTGGACCGAAATTTTAA CACTTCATTCTTTGATCCCGCTGGAGGAGGT GACCCTATCCTATAC
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                    1
MW041569_Puskin    AATTGCAGGACTTTGCGAACGTTAATTCTT CGAACGCACATTGCGGCTTTGGGTTGACTG AAGCCACGCCTGGTTGAGGGTCAGTTGAAT AAACCATCACGATTGTATGTGTGTTGATCG
MN927182_Austria   AATTGCAGGACTTTGCGAACGTTAATTCTT CGAACGCACATTGCGGCTTTGGGTTGACTG AAGCCACGCCTGGTTGAGGGTCAGTTGAAT AAACCATCACGATTGTATGTGTGTTGATCG

                 121
MW041569_Puskin    TGGAGTGTCCGGATAACGCGCTTGTCGCGT TAGCGGATCAAGTCGAGTCCGGATGTGGCT CGCGGCAGCTTGGCTTGGCCGAGTGTGTGC TTTCGTCGATTGGGACGTTAGGCCTCCTGT
MN927182_Austria   TGGAGTGTCCGGATAACGCGCTTGTCGCGT TAGCGGATCAAGTCGAGTCCGGATGTGGCT CGCGGCAGCTTGGCTTGGCCGAGTGTGTGC TTTCGTCGATTGGGACGTTAGGCCTCCTGT

                 241
MW041569_Puskin    GCACATGCACCGCAGCTGCATCGCATAGGA GTGCTGGCCAATTCTGACGAGAGCCCGACT GGCTATATATAGAGTTGAGTGTACCGTGCA GCGCAATGACATTCGAGTGAATGCAAACGC
MN927182_Austria   GCACATGCACCGCAGCTGCATCGCATAGGA GTGCTGGCCAATTCTGACGAGAGCCCGACT GGCTATATATAGAGTTGAGTGTACCGTGCA GCGCAATGACATTCGAGTGAATGCAAACGC

                 361
MW041569_Puskin    AAGAGAGAGTCTGCGGTGAGTGGCCTGGTC CGGTGCTCGCTTTACAGCTCTCTCAAGC-- AAAACATTAAATCATTCTTTTGACCTCAGC TCAGACGAGATTACCCGCTGAACT
MN927182_Austria   AAGAGAGAGTCTGCGGTGAGTGGCCTGGTC CGGTGCTCGCTTTACAGCTCTCTCAAGCAA AAAACATTAAATCATTCTTTTGACCTCAGC TCAGACGAGATTACCCGCTGAACT
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Fig. 2. Comparison of the sequences of barcode genes of different populations of *Notahypsibius pallidoides*. A — COI; B — ITS-2. Variable positions are marked with red frames.

4) PCR-tubes were incubated at 65 °C for 15 min in a PCR machine, vortexed every 5 min and spun down.
5) PCR-tubes were incubated at 98 °C for 2 min.
6) PCR-tubes were spun down (10 min at 4000 RPM).
7) 60 µl of the extract supernatant were transferred into a new, sterile PCR tube. The supernatant was collected in order to avoid the exoskeleton remaining at the bottom. The PCR-tubes containing collected extract supernatant were then stored at −20 °C for later use in PCR.
8) 70 µl ddH_2O were added to the tube with the exoskeleton and mixed well with the pipette to wash the exoskeleton.
9) Water and exoskeleton were transferred to a glass staining block with ddH_2O. The exoskeleton was collected and mounted on a microscope slide in Hoyer's medium and retained as the hologenophore (Pleijel et al., 2008).

Two barcode markers were sequenced from four specimens: internal transcribed spacer (ITS-2, nuclear), and the cytochrome oxidase subunit I gene (COI, mitochondrial). The primers and PCR programs used are provided in Table 1. COI sequences were translated to amino acids using the invertebrate mitochondrial code, implemented in SeaView 4.0 (Gouy, Guindon and Gascuel, 2010), in order to check for the presence of stop codons and therefore of pseudogenes.

PCR products were visualized in 1.5 % agarose gel stained with ethidium bromide. All amplicons were sequenced directly using ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 310 Genetic Analyzer in the Core Facilities Center "Centre for Molecular and Cell Technologies" of St. Petersburg State University. Sequences were edited and assembled using ChromasPro software (Technelysium, USA).

Previously obtained sequences (Tumanov, 2020b) of the same markers for the Austrian population of the species *Not. pallidoides* (GeneBank accession numbers: MN927182 for ITS-2 and MN918533 for COI) were used for the comparison. Sequences were automatically aligned using the Muscle algorithm (Edgar, 2004) as implemented in SeaView 4.0. Uncorrected pairwise distances were calculated using MEGA7 (Kumar S, Stecher and Tamura, 2016) with gaps/missing data treatment set to "complete deletion".

Abbreviations of the tardigrade genera names are given according to Perry, Miller and Kaczmarek (2019).

Results and discussion

Specimens of the population investigated were morphologically identical to the Austrian population on *Not. pallidoides* (Fig. 1).

Sequences of both barcode markers (COI gene (GenBank accession number MW041605) and ITS-2 (GenBank accession number MW041569)) were obtained from four specimens. For both markers all obtained sequences were identical.

Comparison of fragments of the COI gene (573 bp length) and ITS-2 marker (474 bp length) with the sequences of the same markers of *Not. pallidoides* from Austria revealed close similarity of both populations.

Calculated uncorrected pairwise distance for COI gene was 1.2 %. The nucleotide substitutions were: A/G transitions in three nucleotide positions, T/C transition in two adjacent positions and A/T transversion in one position (Fig. 2A). Translated amino acid sequences were identical for both haplotypes. Calculated uncorrected pairwise distance for ITS-2 was 0 %, but the sequences were actually not identical, containing one short indel (incertion/deletion) (Fig. 2B).

The genetic distances revealed for both analysed barcode markers lie within the intraspecific variability range currently accepted for tardigrades (Bertolani, Rebecchi, Giovannini and Cesari, 2011; Morek et al., 2019; Stec, Krzywański, Arakawa and Michalczyk, 2020). Evident similarity indicates that both populations (from Austria and from Russia), although represented by different haplotypes, should be attributed to the same species. Concluding, *Not. pallidoides* is now the second tardigrade species whose presence is confirmed for the fauna of Russia with the methods of DNA barcoding.

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