Even one tardigrade is enough. Molecular methods revealed presence of pantropical species *Echiniscus lineatus* (Heterotardigrada, Echiniscidae) in the fauna of Seychelles

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Abstract

A brief report on the tardigrade species *Echiniscus lineatus* discovered in a moss cushion collected in Seychelles is given. The species represents a new pantropical element in the tardigrade fauna of Seychelles. With this record the number of echiniscid tardigrade species in Seychelles has reached five. Morphological identification of the species is supported with the analysis of obtained sequences of nuclear and mitochondrial DNA barcode genes (ITS-1, ITS-2 and COI). Analysis of the genetic data using the method of TCS haplotype networks revealed separated position of the Seychellian population of *E. lineatus* and supported the hypothesis of independent colonisation of Seychelles.

Keywords: distribution, zoogeography, genetic variability, COI, ITS-1, ITS-2, TCS haplotype networks, Echiniscidae

Introduction

Phylum Tardigrada Doyère, 1840 is a group of microscopical metazoans, widely distributed in nature. They inhabit marine and freshwater basins as well as terrestrial environments, which contain (constantly or temporarily) free water (Nelson, Bartels, and Guil, 2018). More than 1400 species of tardigrades are described up to date (Degma and Guidetti, 2023), but the real taxonomic richness of this group is still underestimated, because of the small number of active taxonomists as well as methodological difficulties (Guil and Cabrero-Sañudo, 2007; Bartels, Apodaca, Mora, and Nelson, 2016).

Tardigrades are well-known for their cryptobiotic abilities, especially for the possibility to endure desiccation of the inhabited substrates in anhydrobiotic state. This adaptation allowed them to populate habitats that temporarily contains water — cushions of moss and lichens, soil, and leaf litter. Most of the tardigrade's species diversity is connected with this kind of habitats (Nelson, Bartels, and Guil, 2018).

Extreme tolerance of the anhydrobiotic tardigrade stage was considered for the long time as the evidence of the high dispersal possibilities of this group (McInnes, 1994; Nelson, Bartels, and Guil, 2018). The dominating paradigm of pre-molecular period of tardigrade taxonomy was the presence of widely distributed polymorph species. Introduction of the molecular methods to the systematics of tardigrades revealed more complex situation. Most of "large" cosmopolitic or widely distributed old species were proved to be species complexes consisted of numerous local species, sometimes poorly morphologically differentiated (e. g. Kaczmarek et al., 2018; Guidetti et al., 2019; Grobys et al., 2020; Stec, Krzywański, Arakawa, and Michalczyk, 2020; Stec, 2023). On the other hand, some widely distributed species with limited morphological and genetic variability were also dis-

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covered (Jørgensen et al., 2013; Kaczmarek et al., 2020). Reasons for these differences of the species distribution are currently poorly understood.

One of the most intriguing cases of coexistence of different biogeographical patterns within a pair of cryptic species is the recently studied *Echiniscus lineatus*/*Echiniscus virginicus* species complex. These species are extremely close in morphology, being indistinguishable with light microscopy. The only distinctive morphological difference between these species (pores *vs* pseudopores on dorsal cuticular plates) may be detectable with SEM only (Gąsiorek et al., 2019). Thus the molecular method of species identification (DNA barcoding) is the most reliable way for the investigation of the species distribution. It was recently shown that *E. virginicus* Riggin, 1962 has limited distribution within temperate Nearctic locales, while *E. lineatus* Pilato, Fontoura, Lisi, and Beasley, 2008 is a species with pantropical distribution (Gąsiorek et al., 2019).

Seychelles is an archipelagic state in the Indian Ocean with tropical rain forest climate. Tardigrade fauna of Seychelles is relatively well known. The first data on the tardigrades of the archipelago were obtained by Biserov (Biserov, 1994; Biserov and Gerlach, 1998) and resulted in the description of three new species. Later, a series of publications of Italian specialists (Binda and Pilato, 1995; Pilato, Binda, and Lisi, 2002, 2004, 2006; Pilato and Lisi, 2009a, 2009b) devoted to the tardigrades of Seychelles was published. Current state of knowledge was summarized by Pilato with colleagues (Pilato, Lisi, and Binda, 2011). By now 34 tardigrade species are known for Seychelles, 19 of them are endemic. Four other species (*Paramacrobiotus richtersi*, *Minibiotus intermedius*, *Diphascon pingue*, and *Milnesium tardigradum*) need reinvestigation, using methods of molecular taxonomy, because the recent studies revealed hidden diversity within these morphological species (Guidetti et al., 2019; Morek, Surmacz, López-López, and Michalczyk, 2021; Tumanov, Androsova, Avdeeva, and Leontev, 2022).

In winter 2023, a single juvenile specimen of a species, belonging to the genus *Echiniscus* (Heterotardigrada, Echiniscidae) was found in a moss sample from Seychelles. In order to determine the taxonomical status of this species, sequences of three fast-evolving genetic markers (mitochondrial — COI gene and nuclear — ITS-1 and ITS-2) were obtained and compared with the available data on the genus *Echiniscus*.

Materials and methods

Sampling

The moss sample was collected by E. Korf and A. Runov from a tree trunk in Morne Seychelles National Park (Mahé island, Seychelles) (approx 4°39'06.2"S, 55°26'42.4"E), on February 11, 2023. Material was stored in a plastic container at room temperature. Tardigrade specimen was extracted from rehydrated samples using the standard technique of washing 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. The found tardigrade specimen was fixed in RNAlater® (Qiagen, Hilden, Germany) solution.

Genotyping and gene analisys

DNA was extracted from the single individual animal using QuickExtract™ DNA Extraction Solution (Lucigen Corporation, USA) using the protocol described in Tumanov, 2020. The exoskeleton was collected and mounted on a microscope slide in Hoyer's medium and retained as the hologenophore (Pleijel et al., 2008). Permanent slide is preserved in the tardigrades collection of the Department of Invertebrate Zoology, Faculty of Biology, Saint Petersburg State University, slide number 332(001).

Three barcode markers were sequenced: internal transcribed spacers (ITS-1 and ITS-2, nuclear), and the cytochrome oxidase subunit I gene (COI, mitochondrial). PCR reactions included 2 µl template DNA, 1 µl of each primer, 0.5 µl dNTP, 5 µl EncycloRed Buffer (5x), and 0.5 µl Encyclo Polymerase (Evrogen™) in a final volume of 25 µl. The primers and PCR programs used are provided in Table. The PCR products were visualised in 1 % agarose gel stained with ethidium bromide. All amplicons were sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the help of an ABI Prism 310 Genetic Analyzer in the Core Facilities Centre "Centre for Molecular and Cell Technologies" of St Petersburg State University. Sequences were edited and assembled using ChromasPro software (Technelysium, USA). The COI sequences were translated to amino acids using the invertebrate mitochondrial code, in order to check for the presence of stop codons and therefore of pseudogenes.

All available sequences of the same markers for the species *E. lineatus* available in GenBank were used for the comparison. Sequences were automatically aligned with the MAFFT algorithm (Katoh, Misawa, Kuma, and Miyata, 2002) with the software AliView version 1.27 (Larsson, 2014); the alignments were cropped to a length of 572 bp for COI, 625 bp for ITS-1, and 406 bp for ITS-2. Uncorrected pairwise distances were calculated using MEGA11 (Tamura, Stecher, and Kumar, 2021) with gaps/missing data treatment set to "pairwise deletion". Separate single-gene TCS haplotype networks (Clement et al., 2002) were generated in PopART ver.1.7 (Leigh and Bryant, 2015) for all three markers, using all available haplotypes.

Table. 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	LCO1490-JJ	forward	CHACWAAYCATAAAGATATYGG	Astrin and Stüben, 2008	Michalczyk, Wełnicz, Frohme, and Kaczmarek, 2012
	HCO2198-JJ	reverse	AWACTTCVGGRTGVCCAAARAATCA		
ITS-1	ITS1_Echi_F	forward	CCGTCGCTACTACCGATTGG	Gąsiorek et al., 2018	Stec, Morek, Gąsiorek, and Michalczyk, 2018
	ITS1_Echi_R	reverse	GTTCAGAAAACCCTGCAATTCACG		
ITS-2	ITS2_Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	Stec, Morek, Gąsiorek, and Michalczyk, 2018	
	ITS2_Eutar_Rr	reverse	TGATATGCTTAAGTTCAGCGG		
	ITS3	forward	GCATCGATGAAGAACGCAGC	Wełnicz et al., 2011	
	ITS4	reverse	TCCTCCGCTTATTGATATGC		

Microscopy and imaging

Permanent slide was 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). Trank appendages designations are given according Vicente et al. (2013).

Results

Molecular data

Sequences of all three barcode markers (COI gene (677 bp; GenBank accession number OR271985), ITS-1 (681 bp; GenBank accession number OR284830), and ITS-2 (406 bp; GenBank accession number OR345153) were obtained.

Homology comparison of the obtained COI sequence with the GenBank records (available on July 14, 2023) using BLASTn algorithm indicated high conformity to the species *E. lineatus*. Percent identity was 99.48 to 97.55 % (query coverage was 83 %, E value 0.00). The specimen analyzed in our study has unique COI haplotype, which do not match any of 28 mitochondrial haplotypes known for *E. lineatus*. Comparison of the obtained sequence with the full set of COI sequences for *E. lineatus* (obtained from GeneBank) using uncorrected *p*-distance value revealed distant position of the Seychellian material from the African haplotypes (Tanzania and Madagascar; *p*-distances 1.59–1.06 %) in relation to some Asian haplotypes (Taiwan, Vietnam, Indonesia, also Brazil and USA; *p*-distances 0.88–0.53 % (Supplementary material 1).

TCS haplotype network for the COI gene constructed in our study (Fig. 1) conforms to the results obtained by Gąsiorek et al. (2019). Analysis of the relationships of the Seychellian material confirmed the distant position from the “African” cluster of haplotypes, which includes sequences from Tanzania and Madagascar, as well as several sequences from Indonesia. Instead, the Seychellian specimen belongs to the haplotype cluster that incorporates sequences from Indonesia, Vietnam and USA.

Homology comparison of the obtained ITS-1 sequence with the GenBank records (available on 14 July 2023) using BLASTn algorithm indicated high conformity to the species *E. lineatus*. Percent identity was 99.84 to 99.52 % (query coverage was 91 %, E-value 0.00). Comparison of the obtained sequence with the full set of ITS-1 sequences for *E. lineatus* (kindly provided by Piotr Gąsiorek) using uncorrected *p*-distance value revealed full identity of the Seychellian material to the haplotypes from Taiwan, Northern and Central Sulawesi (Indonesia) (Supplementary material 1). Identity of our material with one of two haplotypes from Bali is an artifact associated with the inability of the PopART algorithm to take into account presence of indels in the compared sequences (haplotype from Bali have a small insert two basepairs long). “African” cluster of haplotypes (Tanzania and Madagascar, as well as several sequences from Indonesia) was among the most remote (*p*-distance = 0.48 %). The most remote haplotype came from Brazil (*p*-distance = 0.49 %).

TCS haplotype network for the ITS-1 marker constructed in our study (Fig. 2) conforms to the results obtained by Gąsiorek et al. (2019). Analysis of the relationships of the Seychellian material supported its distant position from the “African” cluster of haplotypes, and similarity to the Asian group of haplotypes. Our analysis also revealed some inaccuracies in the interpretation of the ITS-1 diversity presented by Gąsiorek et al. (2019). In the Figure 1A legend haplotypes from Northern Moluccas, Tidore were shown as belonging to the three groups of haplotypes, while in the Supplementary material 2 (Table S2.1) haplotypes from this locality were assigned to the groups B and C only. In fact, haplotypes from North-

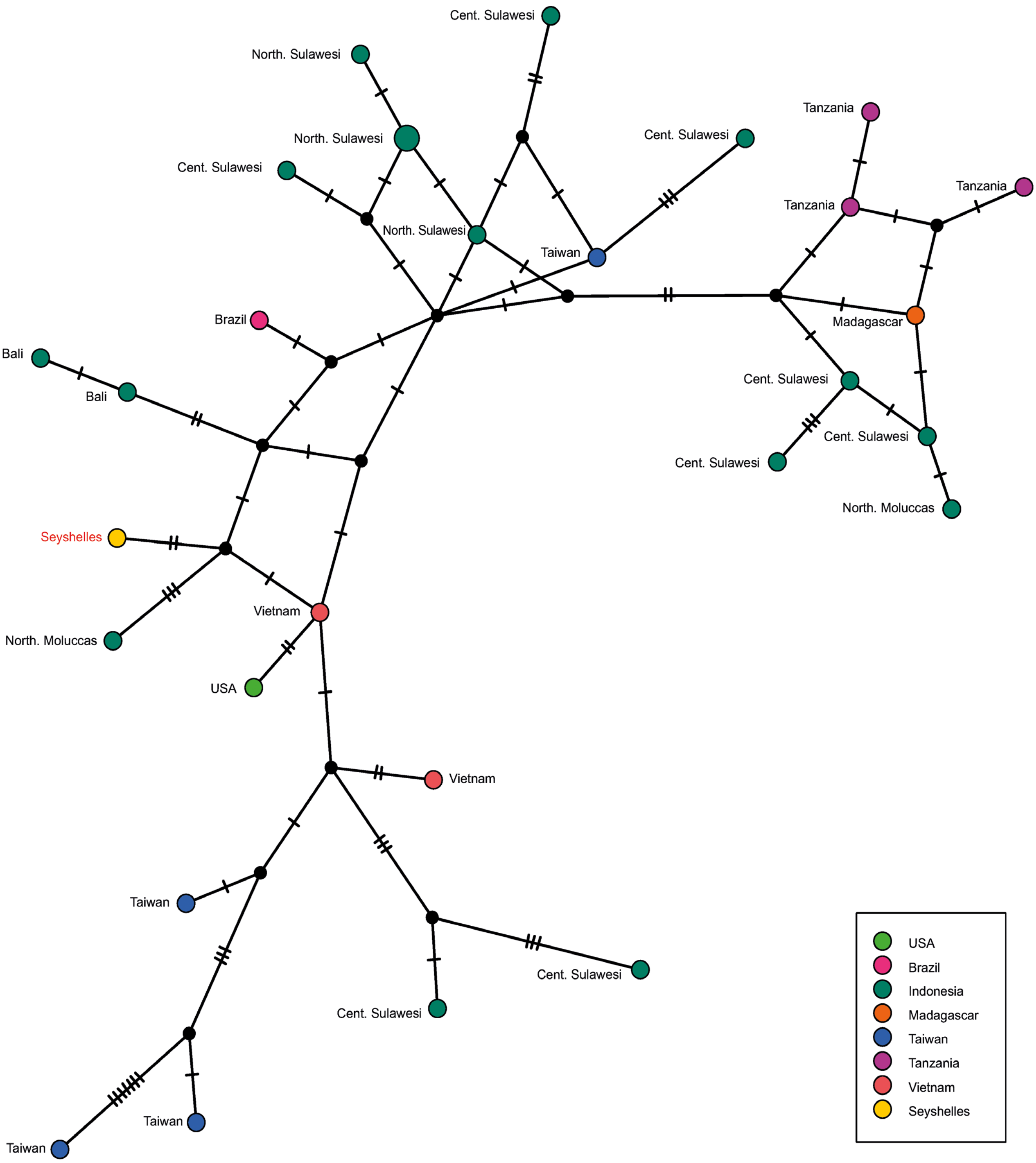

Fig. 1. TCS haplotype network for *Echiniscus lineatus*, COI marker (N = 29). Black circles represent putative haplotypes required to join the detected haplotypes. Transverse striae at connecting lines indicate mutations between the haplotypes.

ern Moluccas should be attributed to the two groups — group B (which includes also haplotypes from Central Sulawesi, Madagascar and Tanzania), and unnamed separate group which includes only single haplotype from Northern Moluccas. Also, the Bali haplotype attributed by Gąsiorek et al. (2019) to the largest C group (which includes haplotypes from Central and Northern Sulawesi, Taiwan, and Seychelles) should be in a separate group because the presence of the small insert (see above).

Homology comparison of the obtained ITS-2 sequence with the GenBank records (available on July 22, 2023) using BLASTn algorithm indicated high conformity to the species *E. lineatus*. Percent identity was 99.74 to 98.71 % (query coverage was 95 %, E-value 0.00).

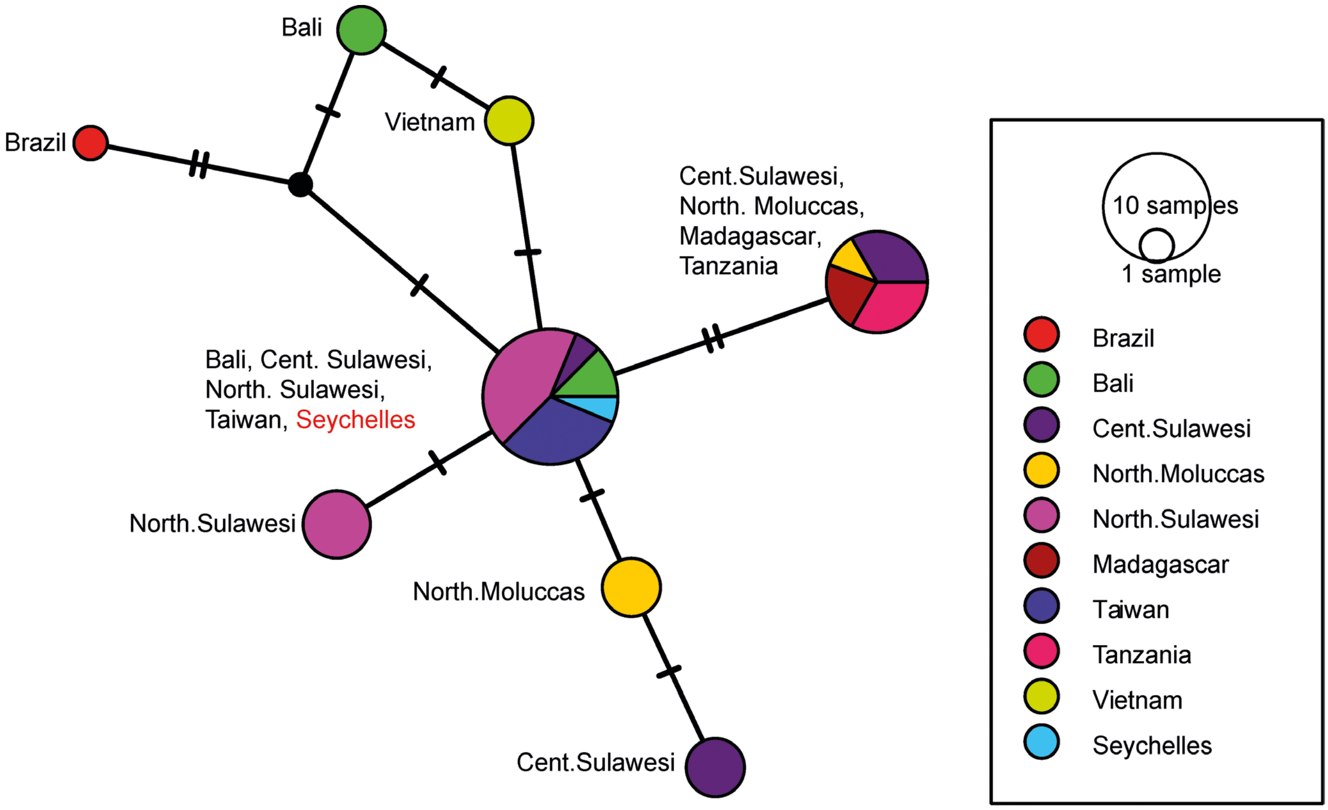

Fig. 2. TCS haplotype network for *Echiniscus lineatus*, ITS-1 marker (N = 40). The size of circles is correlated with the number of individuals representing a single haplotype. Black circles represent putative haplotypes required to join the detected haplotypes. Transverse striae at connecting lines indicate mutations between the haplotypes.

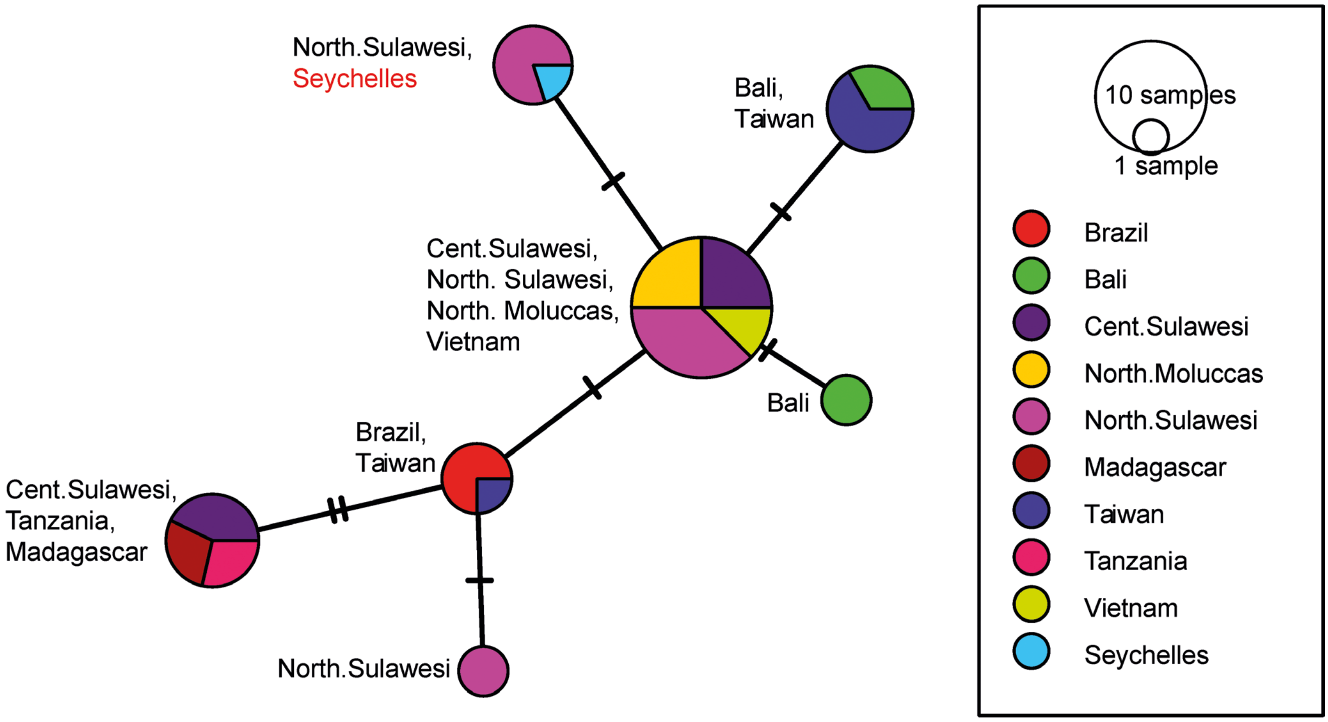

Fig. 3. TCS haplotype network for *Echiniscus lineatus*, ITS-2 marker (N = 42). The size of circles is correlated with the number of individuals representing a single haplotype. Transverse striae at connecting lines indicate mutations between the haplotypes.

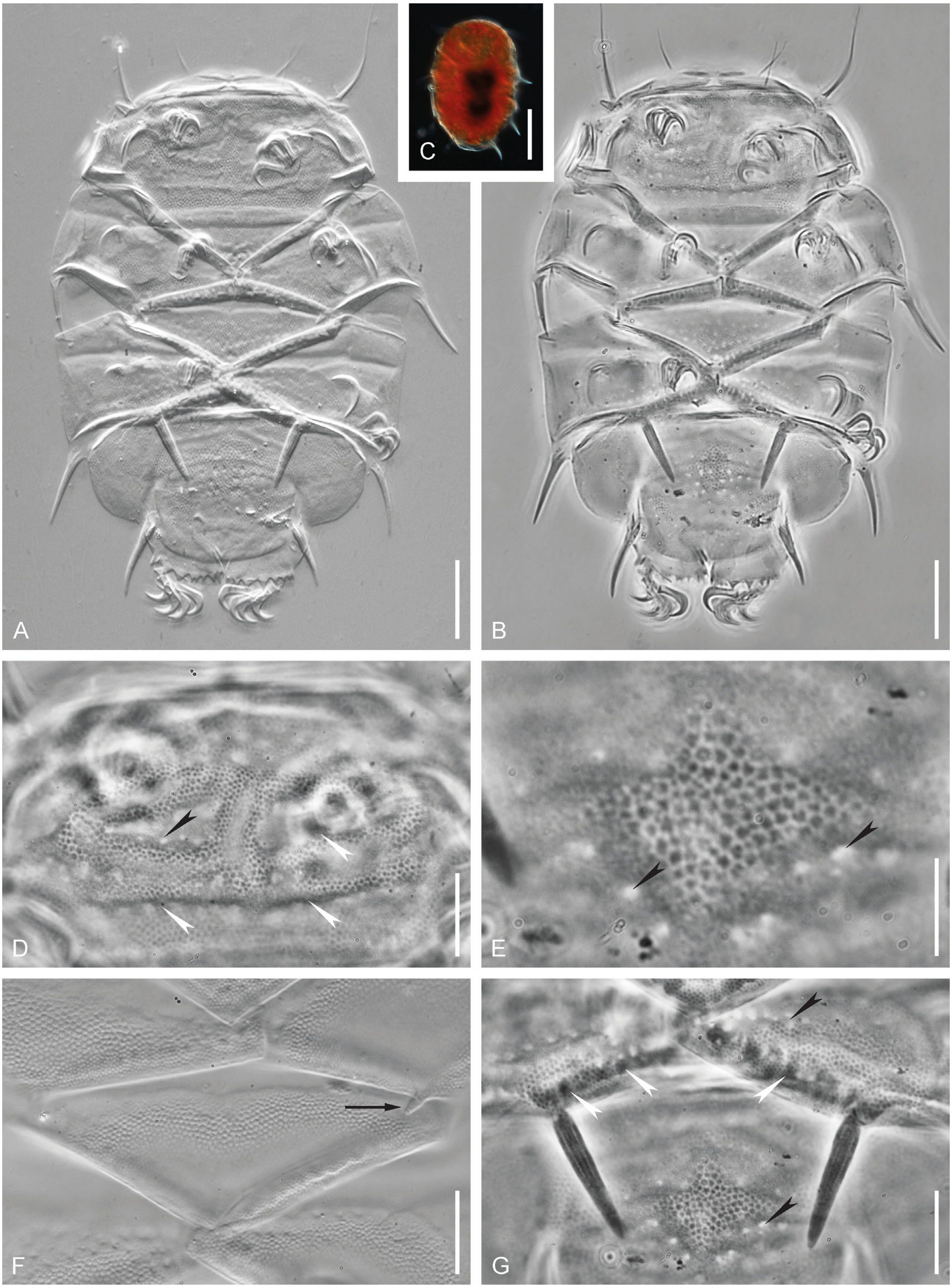

Fig. 4. *Echiniscus lineatus*, details of morphology. A — total view (DIC); B — total view (PhC); C — total view of the living specimen (dark field); D — cuticular sculpture of the scapular plate (PhC); E — cuticular sculpture of the caudal plate (PhC); F — median plate 2, black arrow indicates spine C^{d} (DIC); G — posterior margins of the paired segmental plates 2 with spines D^{d} (PhC). White arrrowheads indicate epicuticular elements, black arrowheads indicate pores/pseudopores. Scale bars: A, B — 20 μm, C — 50 μm, D, F, G — 10 μm, E — 5 μm.

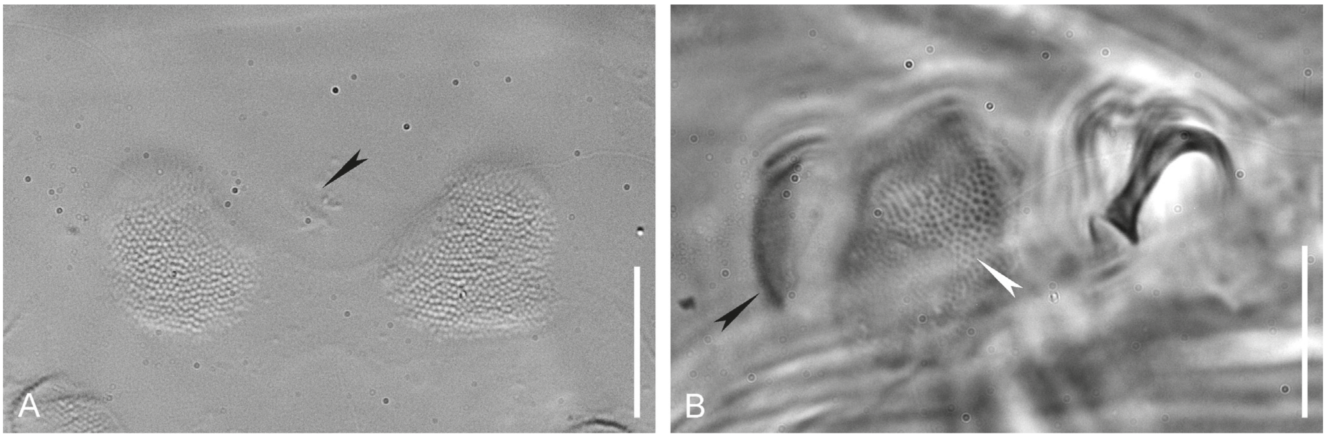

Fig. 5. *Echiniscus lineatus*, details of morphology. A — genital plates, black arrowhead indicates underdeveloped genital structures (DIC); B — leg plates, black arrowhead indicates coxal plate, white arrowhead indicates femoral plate (PhC). Scale bars: A–B — 10 µm.

Comparison of the obtained sequence with the full set of ITS-2 sequences for *E. lineatus* (kindly provided by Piotr Gąsiorek) using uncorrected *p*-distance value revealed full identity of the Seychellian material to the haplotypes from Northern Sulawesi (Indonesia) and the most remote position of the "African" group of haplotypes (Supplementary material 1).

TCS haplotype network for the ITS-2 marker constructed in our study (Fig. 3) also supported the results of Gąsiorek et al. (2019). Analysis of the relationships of the Seychellian material revealed the identity to the *E. lineatus* haplotypes from Kali Terjun, Northern Sulawesi and supported its distant position from the "African" cluster of haplotypes, and similarity to the Asian group of haplotypes (which includes haplotypes from Madagascar, Tanzania, and Central Sulawesi).

Morphological data

The obtained specimen was a premature female with underdeveloped rosette-like structure and without gonopore opening (Fig. 5A). Since no data on the morphology of immature studies of *E. lineatus* are currently available we provide here a brief description of our specimen.

Living specimen was intensively red with dark-green gut content (Fig. 4C). Dorsal cuticle with well-developed cuticular plates of *Echiniscus*-type, the third median plate was indeterminable because of the body contraction (Fig. 4A, B). Dorsal plates sculpture typical to the *E. virginicus* species complex (sensu Gasiorek, et al., 2019) with developed intracuticular pillars, visible in PhC as numerous dark dots or polygons all over the dorsal surface (Fig. 4D–G). In scapular and caudal plates, where the pillars are most developed, they often seem to be connected with thin striae being star-like in shape (Fig. 4E). The plates' surface bears three types of sculpture: (1) ridge-like elevations on the scapular and caudal plates (Fig. 4D); (2) epicuticular elements visible in PhC as partially fused dark granules arranged in lines along anterior and posterior margins of paired segmental plates 1 and 2 (Fig. 4D, G, white arrowheads); and (3) pores (or pseudopores, those structures can be differentiated with SEM only) developed on all dorsal plates (Fig. 4D, E, G, black arrowheads).

Our immature specimen differs from adults (see Pilato, et al., 2008) by complete absence of lateral appendages B (presence or absence of the spine B was also mentioned as polymorphous feature by Gąsiorek, Vončina, and Michalczyk (2020)). Lateral appendages C, D, E have form of thin sharp rigid spines (Fig. 4A, B, 15.01, 19.07, and 16.71 µm length respectively), spines C^d are very small (3.17 µm), poorly distinguishable (Fig. 4F, black arrow), spines D^d are massive, dagger-shaped with thinned base and well-visible internal structure (Fig. 4G, 17.32 µm). Contrary to the original description, two distinct genital plates are present on the ventral body surface (Fig. 5A). These structures were not previously mentioned for *E. lineatus*, but are known for two other species of *E. virginicus* species complex: *Echiniscus clevelandi* Beasley, 1999 (Gąsiorek, Vončina, Kristensen, and Michalczyk, 2021) and *E. masculinus* (Gąsiorek, Vončina, and Michalczyk, 2020).

Legs I–III with clearly delineated separate coxal and femoral plates (Fig. 5B, black and white arrowheads respectively). Both plates with developed intracuticular pillars, without epicuticular elements, and with rare small pores (or pseudopores) on femoral plates.

Other morphological details conforms to the original description (Pilato, Fontoura, Lisi, and Beasley, 2008).

Discussion

Our ideas about the pattern of tardigrade species distribution have undergone significant changes in the last decade. For a long time, presence of highly sustainable cryptobiotic stage in semiterrestrial tardigrades was considered as an argument for their easy distribution with air currents (Kinchin, 1994; Fontaneto, 2019). This hypothesis was a base for the taxonomical concept of presence of widely distributed polymorphous tardigrade species (Artois et al., 2011). Incorporation of molecular techniques into taxonomic and zoogeographic investigations in the last decade leaded to the shift of the paradigm. It was shown that most of widely distributed species of temperate zones and high latitudes are in fact complexes of morphologically similar (sometimes cryptic or semicryptic) species clearly separated genetically (e. g. Kaczmarek et al., 2018; Guidetti et al., 2019; Grobys et al., 2020; Stec, Krzywański, Arakawa, and Michalczyk, 2020; Stec, 2023). Only very few semiterrestrial tardigrade species have really wide or even cosmopolitic distribution (Jørgensen et al., 2013; Kaczmarek et al., 2020). Such pattern of species distribution questions the significance of the air flow for the long-range distribution of tardigrades.

On the other hand, study of the tropical fauna of semiterrestrial tardigrades revealed presence of species with pantropical distribution within several phylogenetically distant taxa (Gąsiorek et al., 2019; Gąsiorek, Vončina, Zając, and Michalczyk, 2021). Presence of such species was considered as evidence for the possibility of long-distance wind transport of cryptobiotic tardigrades by strong constant trade winds. This mode of distribution was supposed for *E. lineatus*, the first revealed pantropical tardigrade species (Gąsiorek et al., 2019). Our data provides new confirmation for this hypothesis. Despite the geographical proximity of Seychelles to Madagascar and Tanzania, the tardigrade specimen investigated by us is more closely related to Asian populations than to African ones. This seems to be likely the result of independent colonization of Seychelles by *E. lineatus* carried out by wind transfer.

Our finding adds a new species of the family Echiniscidae to the tardigrade fauna of Seychelles. Until now only 4 echiniscid species were recorded for the archipelago: *Claxtonia malpighii* (Biserov, 1994) (endemic species; Biserov, 1994), *Kristenseniscus tessellatus* (Murray, 1910) (species recorded from Australia and Bali; Murray, 1910; Pilato and Binda, 1990; Pilato, Lisi, and Binda, 2011), *Echinscus testudo* (Doyère, 1840) (widely distributed Palaearctic species, records from outside Palaearctic are possibly results of the anthropogenic introduction; Gąsiorek, Stec, Morek, and Michalczyk, 2017; Gąsiorek, Vončina, and Michalczyk, 2019), and *Echinicus tropicalis* Binda and Pilato, 1995 (endemic species; Pilato, Lisi, and Binda, 2011). Putting aside the endemic and possibly introduced species we once again observe the proximity of Seychellian tardigrade fauna to the fauna of tropical Asian regions. In our opinion it can be considered as additional evidence of the trade wind transfer hypothesis.

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References

Artois, T., Fontaneto, D., Hummon, W. D., McInnes, S. J., Todaro, M. A., Sørensen, M. V., and Zullini, A. 2011. Ubiquity of microscopic animals? Evidence from the morphological approach in species identification; pp. 244–283 in D. Fontaneto (ed.), Biogeography of microscopic organisms: Is everything small everywhere? Cambridge University Press, Cambridge. https://doi.org/10.1017/CBO9780511974878.014

Astrin, J. and Stüben, P. 2008. Phylogeny in cryptic weevils: Molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). *Invertebrate Systematics* 22(5):503–522. https://doi.org/10.1071/IS07057

Bartels, P. J., Apodaca, J. J., Mora, C., and Nelson, D. R. 2016. A global biodiversity estimate of a poorly known taxon: Phylum Tardigrada. *Zoological Journal of the Linnean Society* 178:730–736. https://doi.org/10.1111/zoj.12441

Binda, M. G. and Pilato, G. 1995. Remarks on tardigrades from the Seychelles, with a description of two new species. *Tropical Zoology* 8:1–6. https://doi.org/10.1080/03946975.1995.10539269

Biserov, V. I. 1994. Some tardigrades from the Seychelles with description of three new species. *Tropical Zoology* 7:181–189. https://doi.org/10.1080/03946975.1994.10539250

Biserov, V. I. and Gerlach, J. 1998. New records of Seychelles tardigrades. *Phelsuma* 6:85–87.

Clement, M., Snell, Q., Walke, P., Posada, D., and Crandall, K. 2002. TCS: Estimating gene genealogies. Proceedings 16th International Parallel and Distributed Processing Symposium, Ft. Lauderdale, FL, USA, 2002, p. 7. https://doi.org/10.1109/IPDPS.2002.1016585

Degma, P. and Guidetti, R. 2023. Actual checklist of Tardigrada species. 42nd ed. https://doi.org/10.25431/11380_1178608

Fontaneto, D. 2019. Long-distance passive dispersal in microscopic aquatic animals. *Movement Ecology* 7:10. https://doi.org/10.1186/s40462-019-0155-7

Gąsiorek, P., Jackson, K. J., Meyer, H. A., Zając, K., Nelson, D. R., Kristensen, R. M., and Michalczyk, Ł. 2019. *Echiniscus virginicus* complex: The first case of pseudocryptic allopatry and pantropical distribution in tardigrades. *Biological Journal of the Linnean Society* 128:789–805. https://doi.org/10.1093/biolinnean/blz147

Gąsiorek, P., Stec, D., Morek, W., and Michalczyk, Ł. 2017. An integrative redescription of *Echiniscus testudo* (Doyère, 1840), the nominal taxon for the class Heterotardigrada (Ecdysozoa: Panarthropoda: Tardigrada). *Zoologischer Anzeiger* 270:107–122. https://doi.org/10.1016/j.jcz.2017.09.006

Gąsiorek, P., Stec, D., Zawierucha, K., Kristensen, R. M., and Michalczyk, Ł. 2018. Revision of *Testechiniscus* Kristensen, 1987 (Heterotardigrada: Echiniscidae) refutes the polar-temperate distribution of the genus. *Zootaxa* 4472:261–297. https://doi.org/10.11646/zootaxa.4472.2.3

Gąsiorek, P., Vončina, K., and Michalczyk Ł. 2019. *Echiniscus testudo* (Doyère, 1840) in New Zealand: Anthropogenic dispersal or evidence for the 'Everything is Everywhere' hypothesis? *New Zealand Journal of Zoology* 46(2):174–181. https://doi.org/10.1080/03014223.2018.1503607

Gąsiorek, P., Vončina, K., and Michalczyk Ł. 2020. An overview of the sexual dimorphism in *Echiniscus* (Heterotardigrada, Echiniscoidea), with the description of *Echiniscus masculinus* sp. nov. (the *virginicus* complex) from Borneo. *Zoosystematics and Evolution* 96(1):103–113. https://doi.org/10.3897/zse.96.49989

Gąsiorek, P., Vončina, K., Kristensen, R. M., and Michalczyk, Ł. 2021. High mountain echiniscid (Heterotardigrada) fauna of Taiwan. *Zoological Studies* 60:70. https://doi.org/10.6620/ZS.2021.60-70

Gąsiorek, P., Vončina, K., Zając, K., and Michalczyk, Ł. 2021. Phylogeography and morphological evolution of *Pseudechiniscus* (Heterotardigrada: Echiniscidae). *Scientific Reports* 11:7606. https://doi.org/10.1038/s41598-021-84910-6

Guidetti, R., Cesari, M., Bertolani, R., Altiero, T., and Rebecchi, L. 2019. High diversity in species, reproductive modes and distribution within the *Paramacrobiotus richtersi* complex (Eutardigrada, Macrobiotidae). *Zoological Letters* 5:1. https://doi.org/10.1186/s40851-018-0113-z

Grobys, D., Roszkowska, M., Gawlak, M., Kmita, H., Kepel, A., Kepel, M., Parnikoza, I., Bartylak, T., and Kaczmarek, Ł. 2020. High diversity in the *Pseudechiniscus suillus-facettalis* complex (Heterotardigrada; Echiniscidae) with remarks on the morphology of the genus *Pseudechiniscus*. *Zoological Journal of the Linnean Society* 188:733–752. https://doi.org/10.1093/zoolinnean/zlz171

Guil, N. and Cabrero-Sañudo, F. 2007. Analysis of the species description process of a little known invertebrate group: The limnoterrestrial tardigrades (Bilateria, Tardigrada). *Biodiversity and Conservation* 16:1063–1086. https://doi.org/10.1007/s10531-006-9069-y

Jørgensen, A., Faurby, S., Persson, D. K., Halberg, K. A., Kristensen, R. M., and Møbjerg, N. 2013. Genetic diversity in the parthenogenetic reproducing tardigrade *Echiniscus testudo* (Heterotardigrada: Echiniscoidea). *Journal of Limnology* 72:136–143. https://doi.org/10.4081/jlimnol.2013.s1.e17

Kaczmarek, Ł., Mioduchowska, M., Kačarević, U., Kubska, K., Parnikoza, I., Gołdyn, B., and Roszkowska, M. 2020. New records of Antarctic Tardigrada with comments on interpopulation variability of the *Paramacrobiotus fairbanksi* Schill, Förster, Dandekar and Wolf, 2010. *Diversity* 12:108. https://doi.org/10.3390/d12030108

Kaczmarek, Ł., Zawierucha, K., Buda, J., Stec, D., Gawlak, M., Michalczyk, Ł., and Roszkowska, M. 2018. An integrative redescription of the nominal taxon for the *Mesobiotus harmsworthi* group (Tardigrada: Macrobiotidae) leads to descriptions of two new *Mesobiotus* species from Arctic. *PLoS ONE* 13:e0204756. https://doi.org/10.1371/journal.pone.0204756

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. 2002. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30(14):3059–3066. https://doi.org/10.1093/nar/gkf436

Kinchin, I. M. 1994. The Biology of Tardigrades. London, Portland Press.

Larsson, A. 2014. AliView: A fast and lightweight alignment viewer and editor for large data sets. *Bioinformatics* 30(22):3276–3278. https://doi.org/10.1093/bioinformatics/btu531

McInnes, S. J. 1994. Zoogeographic distribution of terrestrial/freshwater tardigrades from current literature. *Journal of Natural History* 28:257–352. https://doi.org/10.1080/00222939400770131

Leigh, J. W. and Bryant, D. 2015. PopART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6:1110–1116. https://doi.org/10.1111/2041-210X.12410

Michalczyk, Ł., Wełnicz, W., Frohme, M., and Kaczmarek, Ł. 2012. Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. *Zootaxa* 3154:1–20. https://doi.org/10.11646/zootaxa.3154.1.1

Morek, W., Surmacz, B., López-López, A., and Michalczyk, Ł. 2021. "Everything is not everywhere": Time-calibrated phylogeography of the genus *Milnesium* (Tardigrada). *Molecular Ecology* 30:3590–3609. https://doi.org/10.1111/mec.15951

Murray, J. 1910. Tardigrada. British Antarctic Expedition 1907–1909. Reports on the Scientific Investigations. Vol. 1 Biology (Part V):83–187, plates 14–21.

Nelson, D. R., Bartels, P. J., and Guil, N. 2018. Tardigrade Ecology; pp. 163–210 in R. O. Schill (ed.), Water Bears: The Biology of Tardigrades, Zoological Monographs. Book 2. Springer Nature Switzerland AG. https://doi.org/10.1007/978-3-319-95702-9_7

Pilato, G. and Binda, M. G. 1990. Notizie sui Tardigradi muscicoli di Bali (Indonesia). *Animalia* 17:209–218.

Pilato, G. and Lisi, O. 2009a. Description of three new species of Tardigrada from Seychelles. *Zootaxa* 2005:24–34. https://doi.org/10.11646/zootaxa.2005.1.2

Pilato, G. and Lisi, O. 2009b. Tardigrades from Seychelles Islands, with the description of three new species. *Zootaxa* 2124:1–20. https://doi.org/10.11646/zootaxa.2124.1.1

Pilato, G., Binda, M. G., and Lisi, O. 2002. Notes on tardigrades of the Seychelles with the description of two new species. *Bollettino delle sedute dell'Accademia Gioenia di Scienze Natutrali, Catania* 35:503–517.

Pilato, G., Binda, M. G., and Lisi, O. 2004. Notes on tardigrades of the Seychelles with the description of three new species. *Italian Journal of Zoology* 71:171–178. https://doi.org/10.1080/11250000409356569

Pilato, G., Binda, M. G., and Lisi, O. 2006. Three new species of eutardigrades from Seychelles. *New Zealand Journal of Zoology* 33:39–48. https://doi.org/10.1080/03014223.2006.9518429

Pilato, G., Lisi, O., and Binda, M. G. 2011. Phylum Tardigrada Spallanzani, 1776; pp. 70–111 in J. Gerlach (ed.), Crustacea, Platyhelminthes, Nematoda, Nemertea, Annelida, Rotifera and Tardigrada of the Seychelles islands. Siri Scientific Press.

Pilato, G., Fontoura, P., Lisi, O., and Beasley, C. 2008. New description of *Echiniscus scabrospinosus* Fontoura, 1982, and description of a new species of *Echiniscus* (Heterotardigrada) from China. *Zootaxa* 1856:41–54. https://doi.org/10.11646/zootaxa.1856.1.4

Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., Schander, C., Sundberg, P., and Thollesson, M. 2008. Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution* 48:369–371. https://doi.org/10.1016/j.ympev.2008.03.024

Stec, D. 2023. Integrative taxonomy helps to revise systematics and questions the purported cosmopolitan nature of the type species within the genus *Diaforobiotus* (Eutardigrada: Richtersiusidae). *Organisms Diversity & Evolution* 23:309–328. https://doi.org/10.1007/s13127-022-00592-6

Stec, D., Krzywański, Ł., Arakawa, K., and Michalczyk Ł. 2020. A new redescription of *Richtersius coronifer*, supported by transcriptome, provides resources for describing concealed species diversity within the monotypic genus *Richtersius* (Eutardigrada). *Zoological Letters* 6:2. https://doi.org/10.1186/s40851-020-0154-y

Stec, D., Morek, W., Gąsiorek, P., and Michalczyk, Ł. 2018. Unmasking hidden species diversity within the *Ramazzottius oberhaeuseri* complex, with an integrative redescription of the nominal species for the family Ramazzottiidae (Tardigrada: Eutardigrada: Parachela). *Systematics and Biodiversity* 16:357–376. https://doi.org/10.1080/14772000.2018.1424267

Tamura, K., Stecher, G., and Kumar, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution* 38:3022–3027. https://doi.org/10.1093/molbev/msab120

Tumanov, D. V. 2018. *Hypsibius vaskelae*, a new species of Tardigrada (Eutardigrada, Hypsibiidae) from Russia. *Zootaxa* 4399:434. https://doi.org/10.11646/zootaxa.4399.3.12

Tumanov, D. V. 2020. Integrative redescription of *Hypsibius pallidoides* Pilato et al., 2011 (Eutardigrada: Hypsibioidea) with the erection of a new genus and discussion on the phylogeny of Hypsibiidae. *European Journal of Taxonomy* 681:1–37. https://doi.org/10.5852/ejt.2020.681

Tumanov, D. V., Androsova, E. D., Avdeeva, G. S., and Leontev, A. A. 2022. First faunistic investigation of semiterrestrial tardigrade fauna of North-West Russia using the method of DNA barcoding. *Invertebrate Zoology* 19(4):452–474. https://doi.org/10.15298/invertzool.19.4.08

Vicente, F., Fontoura, P., Cesari, M., Rebecchi, L., Guidetti, R., Serrano, A., and Bertolani, R. 2013. Integrative taxonomy allows the identification of synonymous species and the erection of a new genus of Echiniscidae (Tardigrada, Heterotardigrada). *Zootaxa* 3613(6):557–572. https://doi.org/10.11646/zootaxa.3613.6.3

Wełnicz, W., Grohme, M. A., Kaczmarek, Ł., Schill, R. O., and Frohme, M. 2011. ITS-2 and 18S rRNA data from *Macrobiotus polonicus* and *Milnesium tardigradum* (Eutardigrada, Tardigrada). *Journal of Zoological Systematics and Evolutionary Research* 49:34–39. https://doi.org/10.1111/j.1439-0469.2010.00595.x