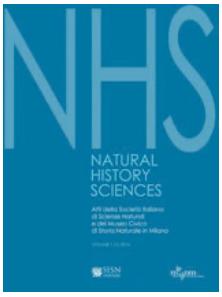

Natural History Sciences

https://sisn.pagepress.org/nhs

eISSN 2385-0922

Please cite this article as:

Loeffelholz JD, Raynor SJ, Morris ER, et al. **Tardigrades of North America: more new records of occurrence for species of green tardigrades (Heterotardigrada: Echiniscoidea: Echiniscidae: *Viridiscus*)**. *Natural History Sciences* doi: 10.4081/nhs.2025.898

Submitted: 4 April 2025
Accepted: 16 September 2025

Tardigrades of North America: more new records of occurrence for species of green tardigrades (Heterotardigrada: Echiniscoidea: Echiniscidae: *Viridiscus*)

Jacob D. Loeffelholz[1,2*], Seth J. Raynor[3,4], Erin R. Morris[5],
Erin A. Manzitto-Tripp[3,4], William R. Miller[5†]

[1]Department of Biological Sciences, University of Alabama, Tuscaloosa, AL, United States
[2]Education Department, The National Mississippi River Museum & Aquarium, Dubuque, IA, United States
[3]Museum of Natural History, UCB 350, University of Colorado-Boulder, Boulder, CO, United States
[4]Department of Ecology and Evolutionary Biology, University of Colorado-Boulder, Boulder, CO, United States
[5]Department of Biology and Chemistry, Baker University, Baldwin City, KS, United States

†Recently deceased

*Corresponding author: jacobloeffelholz2@gmail.com

Abstract - Green pigmented tardigrades known as *Viridiscus* (Heterotardigrada: Echiniscoidea: Echiniscidae) are typically found in limnoterrestrial habitats, such as moss and lichens. However, the distribution of this genus in North America has been historically understudied and remains elusive to microbiologists, ecologists, and tardigrade taxonomists. In the last couple years, new state records of *Viridiscus* spp. in the United States of America (USA) have been documented from Alabama, Florida, Indiana, Tennessee, and Texas. Here we acknowledge the recent reports along with providing new additional USA records to give an update of *Viridiscus* spp. distribution. Two species of green tardigrades, *Viridiscus perviridis* and *Viridiscus viridissimus*, are reported from six locations in Illinois, Iowa, Minnesota, Mississippi, Missouri, and South Dakota. This is the first report of these species from these states and the farthest west that *Viridiscus viridissimus* has been found in continental North America. Additionally, we utilized polymerase chain reaction, DNA barcoding of the cytochrome oxidase I (COI) mitochondrial marker, a maximum likelihood phylogenetic analysis, and ASAP analysis to confirm the existence of *Viridiscus viridissimus* in Missouri.
Key words: distribution, Iowa, Missouri, phylogeny, *Viridiscus*.

Riassunto - Tardigradi del Nord America: nuove segnalazioni di specie di tardigradi verdi (Heterotardigrada: Echiniscoidea: Echiniscidae: *Viridiscus*)
I tardigradi a pigmentazione verde, noti come *Viridiscus* (Heterotardigrada: Echiniscoidea: Echiniscidae), si trovano solitamente in habitat limnoterrestri come muschi e licheni. Tuttavia, la distribuzione di questo genere in Nord America è storicamente poco studiata e rimane vaga per microbiologi, ecologi e tassonomi. Negli ultimi due anni sono stati documentati nuovi record di *Viridiscus* spp. negli Stati Uniti d'America (USA) dagli stati di: Alabama, Florida, Indiana, Tennessee e Texas. Riportando i più recenti ritrovamenti e aggiungendo nuovi record, aggiorniamo la distribuzione di *Viridiscus* spp. negli USA. Le due specie di tardigradi verdi, *Viridiscus perviridis* e *Viridiscus*

viridissimus, sono ora segnalate per sei località in: Illinois, Iowa, Minnesota, Mississippi, Missouri e South Dakota. Questa segnalazione è la prima per queste specie in questi stati e rappresentano il punto più ad ovest in cui *Viridiscus viridissimus* sia stato mai rinvenuto in America continentale settentrionale. Inoltre, utilizzando la reazione a catena della polimerasi (PCR), il DNA barcoding del marcatore mitocondriale citocromo ossidasi I (COI), un'analisi filogenetica con maximum likelihood e un'analisi ASAP abbiamo confermato l'esistenza di *Viridiscus viridissimus* in Missouri.
Parole chiave: distribuzione, filogenesi, Iowa, Missouri, *Viridiscus*

Introduction

Tardigrades are microscopic, invertebrate animals that are commonly known to inhabit moss, lichens, liverworts, leaf litter, soils, freshwater systems, and marine environments. Murray (1910) described the first green tardigrade, *Echiniscus viridis* Murray 1910 from Oahu in the Hawaiian Islands of the United States of America (USA). By 1994, it had been documented from Europe, North and South America (McInnes, 1994). In 1944, the second green species, *Echiniscus rufoviridis* du Bois-Reymond Marcus 1944, was discovered in Brazil but only the posterior part of its body was green. *Echiniscus rufoviridis* has recently been reassigned to the genus *Barbaria* Gąsiorek and Michalczyk 2019. It is now considered as *Barbaria rufoviridis* and remains endemic to South America (Kaczmarek *et al*., 2015; Rocha *et al*., 2023). Twelve years after the second green species was described, *Echiniscus viridissimus* Péterfi 1956 was defined from Romania with a modest cirrus A length, lacking spurs on the internal claws, and having white pores on the cuticle. It has since been reported in both North and South America (Kaczmarek *et al*., 2015; Kaczmarek *et al*., 2016). Just three years later in Italy, *Echiniscus perviridis* Ramazzotti 1959 was added to the growing list of green species based on a long cirrus A and black circles on the cuticle (i.e., granulation), and it too has been found in North and Central America (Kaczmarek *et al*., 2014; Kaczmarek *et al*., 2016; Miller *et al*., 2020).

Forty-eight years passed before *Echiniscus viridianus* Pilato, Fontoura, & Lisi 2007 was described from Alabama, USA. The next year the same team re-described *E. viridis* in modern detail (Pilato *et al*., 2008). Three years later, *Echiniscus clavispinosus* Fontoura, Pilato, & Lisi 2011 was found on the Cape Verde Islands off the western coast of Africa. Later, Gąsiorek *et al*. (2019) erected the "*viridis"* group to its own genus *Viridiscus* Gąsiorek and Michalczyk 2019. The first paper of additional records and *Viridiscus* distribution within the USA was presented in 2020 (Miller *et al*., 2020).

Viridiscus perviridis has been previously reported in Tennessee, North Carolina, Kansas, Maine, and Pennsylvania. *Viridiscus viridianus* has been recorded from Alabama, New Mexico, and New Jersey. *Viridiscus viridis* has been reported from Hawaii, North Carolina, Tennessee and Oklahoma. *Viridiscus viridissimus* has been recorded from Alabama, Kansas, Maine, New Jersey, Tennessee, North Carolina, and West Virginia (Kaczmarek *et al*., 2016; Miller *et al*., 2020). The records of *V. viridis* in North America were called into question by Pilato *et al*. (2007, 2008), but Kaczmarek *et al*. (2016) accepts these records pending review of the original slides.

Since the occurrence of green echiniscids of the "v*iridis*" group (Ramazzotti & Maucci, 1983) was summarized across North America (Miller *et al*., 2020), a grade school class added a record of *V. perviridis* from Texas (Cotten & Miller, 2022), a new species, *Viridiscus celatus* Momeni, Gasiorek, Nelson, & Michalczyk 2023 was described from Tennessee, *V. perviridis* was documented in Alabama, *V. viridianus* was documented from Florida (Momeni *et al*., 2023), *V. viridianus* and *V. viridissimus* were

documented in the first report of tardigrade species from the state of Indiana (Massa & Vecchi, 2024), and *V. perviridis* and *V. viridissimus* were reported from Wisconsin (Loeffelholz *et al*., 2025).

The fifth-grade class of Hannah Cotten found *Viridiscus perviridis* on the roof of a gazebo on their campus in Austin, Texas (Cotten & Miller, 2022). Momeni *et al*. (2023) described *Viridiscus celatus* as a new species from Tennessee and also reported the first records of *V. perviridis* in Alabama and *Viridiscus viridianus* in Florida. Massa and Vecchi (2024) recently documented *V. viridianus* and *Viridiscus viridissimus* from moss samples on stone walls in the city of Bloomington, Indiana, which is the first record of *V. viridianus* from the Midwest region of the United States. Loeffelholz *et al.* (2025) reported *V. perviridis* from *Phaeophyscia adiastola* lichen and *V. viridissimus* from *Xanthomendoza ulophyllodes* lichen at Belmont Mound State Park in Belmont, Wisconsin.

The ecology of the genus *Viridiscus* has yet to be explored in depth and very little is known about their habitat preference (Nelson *et al*., 2020). Many past records of *Viridiscus* spp. do not mention the identifications of their respective cryptogams (Kaczmarek *et al*., 2014; Kaczmarek *et al*., 2016; Miller *et al*., 2020). Documenting the known distribution and habitats of this genus can aid future labs in rediscovering and isolating them for cultures to study their life cycles, cryptobiotic tolerance, and genetic/molecular processes.

The aim of our study was to update the known distribution of *Viridiscus* spp. in the USA, while also providing genus or species identifications of the lichen and moss samples they were extracted from. We hypothesized that obtaining DNA sequences of the cytochrome oxidase I (COI) mitochondrial gene would allow us to find genetic evidence of a species, *V. viridissimus*, identified in the current study. We also hypothesized that a maximum likelihood phylogeny and a delimitation algorithm known as the Assemble Species by Automatic Partitioning (ASAP) analysis would confirm and support the *V. viridissimus* identification. Thus, we collaborated to present this report of new records of occurrence of *Viridiscus* spp. from six USA locations.

Materials and Methods

Sample identification and processing

Each tardigrade researcher has their own unique process, but generally samples of moss or lichen were collected and stored in paper bags. Lichens were identified based on numerous keys written by regional specialists including Brodo (2016) and McMullin (2023). Moss identification was possible based on Crum (1973), Crum and Anderson (1981), and Klips (2022) as well as keys provided on the online Flora of North America (2007, 2014). Wet mounts of structures like ascospores of lichen and sporophytes of moss, often used for species identification, were viewed with an Olympus SZX16 dissecting microscope and an Olympus BX51 compound microscope. Standard spot tests (K, C, KC, P, UV) and methods following Brodo *et al.* (2001) were used for chemical analyses of lichen species.

A subsample of moss or lichen was soaked in spring water for 24 hours, and aliquots were examined with a dissecting microscope at 20-45X. Using an Irwin loop, tardigrades were fished out of the aliquot and placed in a drop of polyvinyl alcohol (PVA) or Hoyer's medium on a microscope slide, then covered with a glass coverslip. Each microscope slide was labeled with a tardigrade identification and a unique code corresponding to the sample code. After the mounting media dried, the coverslip edges were sealed with fingernail polish to prevent air bubbles from ruining the voucher specimens.

Examination was made with a light compound microscope such as an Olympus BX60 equipped with DIC (Differential Interference Contrast) optics and phase contrast. Tardigrades were imaged using the Olympus BX60, an Olympus CH30 light compound microscope, and an Invitrogen EVOS^{TM} FL Color fluorescence microscope, given the recent reports of autofluorescence in tardigrade species (Perry *et al*., 2015; Bartels, *et al*., 2024; Massa, *et al*., 2024). They were imaged using transmitted light, a DAPI filter (360 nm excitation, 447 nm emission), GFP filter (470 nm excitation, 525 nm emission), and RFP filter (530 nm excitation, 593 nm emission). The DAPI, GFP, and RFP filter images were joined together to create an overlay image. The guidelines of McInnes (2001) have been followed as to the use of digital images and they have only been modified for brightness and contrast necessary for clarity of printing.
Specimens were identified to species using Ramazzotti and Maucci (1983), Pilato *et al*. (2007, 2008), Fontoura *et al*. (2011), and Momeni *et al*. (2023). Nomenclature follows Guidetti and Bertolani (2005), Degma and Guidetti (2007), and Degma and Guidetti (2024). To ensure compatibility and ease of use, the new records of occurrence are reported per the format established by Kaczmarek *et al*. (2016) (Tab. 1). Preserved tardigrade specimens are deposited into the tardigrade collection at Baker University.

DNA barcoding and phylogenetic analyses

DNA barcoding of tardigrades was conducted to provide genetic evidence of species identification. The mitochondrial gene cytochrome oxidase I (COI) was chosen as the target sequence. DNA extraction methods, PCR cycles, and primers reported by Stec *et al*. (2015) were used. After gel electrophoresis, PCR products were purified and sent to the University of Missouri-Columbia for sequencing. Once sequences were obtained, they were analyzed using NCBI's BLAST tool (Altschul *et al*., 1990).
At least three COI sequences were chosen from multiple tardigrade taxa, including other *Viridiscus* spp., *Claxtonia wendti* Richters 1903, *Nebularmis reticulatus* Murray 1905, *Echiniscus quadrispinosus* Richters 1902, and *Milnesium pentapapillatum* Ciosek, Morek & Michalczyk 2020 (*Supplemental Table 1*). The *M. pentapapillatum* sequences were designated as the outgroup for the phylogenetic analysis, given the species is in a different class, order, and family compared to the other species in the analysis. As a result, 28 sequences were chosen for the phylogenetic analysis.
Sequences were aligned in Clustal Omega. Our sequence was trimmed accordingly, then sequences were realigned in Clustal Omega and downloaded as a *.fasta* file (*Supplemental File 1*). The *.fasta* file was uploaded to MEGA11 for maximum likelihood phylogenetic analyses (Tamura *et al*., 2021). The best fit model was selected based on which model presented the lowest Bayesian Inference Criterion (BIC) score. The Tamura-Nei model with rates among sites set to Gamma Distributed with Invariant Sites (TN93+G+I) was selected. The number of bootstrap replicates was set to 200. Gaps and missing data treatment was set to partial deletion with a 95% site coverage cutoff. The maximum likelihood heuristic method was set to nearest-neighbor-interchange (NNI).
Following the maximum likelihood phylogenetic analysis, we conducted the ASAP analysis by accessing the website SPART Explorer (https://spartexplorer.mnhn.fr/delimitation, accessed 17/06/2025; Brandoli *et al*., 2024). The *.fasta* file downloaded from Clustal Omega was uploaded to the SPART Explorer website. The ASAP analysis was selected. Under the section of Advanced options, the option of Simple Distance (p-distances) was selected, and other options were set to the default settings. After the analysis was conducted, visualization of the data was observed and downloaded. The lowest partition score was considered to be the best representation of species boundaries and most appropriate partition.

Results and Discussion

Biogeography records

We present seven new records of *Viridiscus* spp. spanning six states. The new records are from the states of Illinois, Iowa, Minnesota, Mississippi, Missouri, and South Dakota (Tab. 1 and Fig. 1). The new record of *V. viridissimus* from South Dakota is the farthest west that *V. viridissimus* has been recorded in the continental USA. This report also includes the first tardigrade species documented from the states of Minnesota and South Dakota (Kaczmarek *et al*., 2016; Miller & Perry, 2019).

From the specimens collected, *V. perviridis* was found in *Schistidium rivulare* (Brid.) Podp. moss on a roadside limestone rock boulder in Harpers Ferry, Iowa (Fig. 2), *V. perviridis* was recovered from *Xanthomendoza* sp. lichen on a rock wall in a public park in Caledonia, Minnesota, *V. perviridis* was found in moss from a *Ginkgo biloba* L. tree at the Mississippi University for Women in Columbus, Mississippi, *V. perviridis* and *V. viridissimus* were found in *S. rivulare* moss samples from a concrete wall at the University of Missouri-Columbia in Columbia, Missouri (Fig. 3), *V. viridissimus* was found in *Schistidium* sp. moss mixed with *Xanthomendoza weberi* (S.Y. Kondr. & Kärnefelt) L. Lindblom and *Physciella melanchra* (Hue) Essl. lichen on a tombstone in Galena, Illinois. *V. viridissimus* was also recovered from *Xanthomendoza* sp. lichen on a rock at Evans Cliff in Hot Springs, South Dakota (Fig. 4).

The lack of occurrence and documentation of this genus indicates it most likely has habitat preferences that are currently unknown. In the current report, one sample of unidentified moss, two samples of *Xanthomendoza* spp. lichen, one sample of a mixture of *Schistidium* sp. moss, *X. weberi* lichen and *P. melanchra* lichen, and three samples of *S. rivulare* moss were recorded as habitats of the *Viridiscus* spp. The unidentified moss sample was collected from a *Ginkgo biloba* L. tree trunk, and the rest of the samples were collected from rock and stone substrates. These are limited sporadic reports that do not indicate any significant habitat or substrate associations.

Nelson & Adkins (2001) found *Echiniscus* (=*Viridiscus*) *perviridis* and *Echiniscus* (=*Viridiscus*) *viridissimus* in moss identified as *Grimmia alpicola* (Brid.) Wahlenb. collected from concrete caps on brick fence posts. Nelson *et al*. (2020) found *V. perviridis* and *V. viridissimus* in *Grimmia* sp. moss on man-made cement structures in Tennessee. The genera *Schistidium* and *Grimmia* are in the same family of mosses (Grimmiaceae), lending solid evidence to a link between *Viridiscus* spp. and Grimmiaceae mosses on rock substrates. Further studies are recommended in which *Viridiscus* spp., other tardigrade species, and the taxonomic identity of their respective moss and lichen habitats are synchronously documented to gain insight into the symbiotic relationships of tardigrades and their cryptogams.

It is also noteworthy to point out that the genus *Viridiscus* is known to be common in the Southern nearctic and subtropical environments (Kaczmarek *et al.*, 2016; Nelson *et al*., 2020; Momeni *et al*., 2023), but this genus has recently been documented from the Northeastern and Midwestern states of the USA (Miller *et al*., 2020; Loeffelholz *et al*., 2025), where temperatures often reach below freezing (0 °C/32 °F) for multiple months during the winter season. It is clear this genus can tolerate a wide range of temperatures and environmental conditions (Loeffelholz *et al*., 2024).

These new records further elucidate the widespread occurrence of the genus *Viridiscus* in North America. Finding the presence of this genus in states such as Minnesota and South Dakota adds considerable change to our knowledge of its distribution. Many states of the USA still have no records of this genus,

particularly on the west coast (Kaczmarek *et al*., 2016; Miller *et al*., 2020), meaning there are likely still substantial gaps in our knowledge of the distribution of *Viridiscus* spp. in North America.

DNA barcoding and phylogenetic analyses

We obtained a COI sequence from a population of *V. viridissimus* from Columbia, Missouri. The BLAST results (Tab. 2) and maximum likelihood phylogenetic analysis (Fig. 5) featured in the current study clearly indicate genetic evidence of the species *V. viridissimus* from the *S. rivulare* moss sample from Columbia, Missouri. The maximum likelihood phylogeny conducted with the MEGA11 software shows our sequence as being supported and nested within the *V. viridissimus* clade. The ASAP analysis results revealed the best partition (i.e., lowest partition score) as having a partition score of one. The best partition demonstrates that the COI sequence featured in the current study is grouped with the other *V. viridissimus* sequences (Fig. 6).

Multiple phylogenetic analyses performed in the current study indicated that the COI sequence we obtained is most likely from the species *V. viridissimus*. This is the first molecular evidence of the species *V. viridissimus* in the state of Missouri. It is important to acknowledge that the species *V. viridissimus* was identified before DNA analyses, lending evidence to the idea that a tardigrade species can accurately be identified to the species level using morphological analyses prior to any DNA barcoding and phylogenetic analyses. However, many species of tardigrades have been described using molecular methods (Morek *et al*., 2020; Brandoli *et al*., 2024), therefore integrative approaches using morphological and molecular analyses are highly recommended. Further biogeography studies employing molecular methods will drastically improve our knowledge of tardigrade species that are native to North America.

Acknowledgements

We wish to acknowledge the National Science Foundation (NSF) for providing funding used in this study. We would like to thank Dr. Mark Levenstein at the University of Wisconsin-Platteville for allowing access to the Invitrogen EVOS™ FL Color fluorescence microscope. We would also like to thank Nicholas Merfeld of Dubuque, Iowa for getting permission and collecting the *Xanthomendoza* sp. lichen sample from Evans Cliff in Hot Springs, South Dakota. Lastly, we want to thank the peer reviewers for their suggestions that improved the quality of the manuscript.

Funding: NSF grant award #2002763 to Baker University and a Baker University internal grant were used for materials.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J., 1990 – Basic local alignment search tool. *Journal of Molecular Biology*, 215(3): 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bartels, P.J., Coffey, D.C., Pineau, M., Kaczmarek, Ł., & Nelson, D.R., 2024 – An exploration of autofluorescence in tardigrades (phylum Tardigrada). *Zoological Journal of the Linnean Society*, 200(1): 200-217. https://doi.org/10.1093/zoolinnean/zlad045

Brandoli, S., Cesari, M., Massa, E., Vecchi, M., Rebecchi, L., & Guidetti, R., 2024 – Diverse eggs, diverse species? Production of two egg morphotypes in *Paramacrobiotus bifrons*, a new eutardigrade species within the *areolatus* group. *The European Zoological Journal*, 91(1): 274-297.

Brodo, I.M., 2016 – *Keys to lichens of North America: revised and expanded*. Yale University Press.

Brodo, I.M., Sharnoff, S.D., & Sharnoff, S., 2001 – *Lichens of North America. New Haven (CT).* Yale University Press.

Cotten, H., & Miller, W.R., 2022 – Tardigrades in Texas: Fifth Graders collaborate to add three new records to the state. *Texas Journal of Science*, 74(1): 1-19.

Crum, H., 1973 – *Mosses of the Great Lakes forest.* University of Michigan Press.

Crum, H., & Anderson, L.E., 1981 – *Mosses of Eastern North America.* Columbia University Press.

Degma, P., & Guidetti, R., 2007 – Notes to the current checklist of Tardigrada. *Zootaxa*, 1579: 41-53.

Degma, P., & Guidetti, R., 2024 – Actual checklist of Tardigrada species (2009-2024: 01-07-2024). *Modena, Italy: Università di Modena e Reggio Emilia*. Accessed 10-03-2024.

Fontoura, P., Pilato, G., & Lisi, O., 2011 – Tardigrada from Santo Antão Island (Archipelago of Cape Verde, West Africa) with the description of a new species. *Zootaxa*, 2838(1): 30-40.

Flora of North America Editorial Committee, eds. 1993 onwards – *Flora of North America North of Mexico* [Online]. 25+ vols. *New York and Oxford.* vol. 27, 2007; vol. 28, 2014. <http://beta.floranorthamerica.org> (retrieved in May 2024).

Gąsiorek, P., Morek, W., Stec, D., & Michalczyk, Ł., 2019 – Untangling the *Echiniscus* Gordian knot: paraphyly of the "*arctomys* group" (Heterotardigrada: Echiniscidae). *Cladistics*, 35(6): 633-653.

Guidetti, R., & Bertolani, R., 2005 – Tardigrade taxonomy: an updated checklist of the taxa and a list of characters for their identification. *Zootaxa*, 845: 1-46.

Kaczmarek, Ł., Michalczyk, Ł., & McInnes, S.J., 2014 – Annotated zoogeography of non-marine Tardigrada. Part I: Central America. *Zootaxa*, 3763(1): 1-063.

Kaczmarek, Ł., Michalczyk, Ł., & McInnes, S.J., 2015 – Annotated zoogeography of non-marine Tardigrada. Part II: South America. *Zootaxa*, 3923(1): 1-107.

Kaczmarek, Ł., Michalczyk, Ł., & McInnes, S.J., 2016 – Annotated zoogeography of non-marine Tardigrada. Part III: North America and Greenland. *Zootaxa*, 4203(1): 1-249.

Klips, R., 2022 – *Common Mosses, Liverworts, and Lichens of Ohio: A Visual Guide*. Ohio University Press.

Loeffelholz, J., Meese, E., Giovannini, I., Ullibarri, K., Momeni, S., Merfeld, N., Wessel, J., Guidetti, R., Rebecchi, L., & Boothby, T.C., 2024 – An evaluation of thermal tolerance in six tardigrade species in an active and dry state. *Biology Open*, 13(10): bio060485. https://doi.org/10.1242/bio.060485

Loeffelholz, J. D., Raynor, S., Sánchez-Moreno, S., Momeni, S., & Manzitto-Tripp, E., 2025 – Tardigrada and Nematoda associations with lichen and bryophyte habitats from Southwest Wisconsin state parks, universities, and private land. *Biogeographia–The Journal of Integrative Biogeography*, 40(1): a045. https://doi.org/10.21426/B6.40056

du Bois-Reymond Marcus, E., 1944 – Sobre Tardigrados Brasileiros. *Comunicaciones Zoologicas del Museo de Historia Natural de Montevideo*, 1(13): 1-19.

Massa, E., Rebecchi, L., & Guidetti, R., 2024 – Composition and structural organization of tardigrades feeding apparatus focusing on chitin and other autofluorescent molecules. *Zoological Journal of the Linnean Society*, 200(1): 186-199.

Massa, E., & Vecchi, M., 2024 – Description of *Macrobiotus kathyae* sp. nov. (Parachela: Macrobiotidae) and first records of tardigrades from Indiana (USA). *Zootaxa*, 5471(3): 301-317.

McInnes, S.J., 1994 – Zoogeographic distribution of terrestrial/freshwater Tardigrades from Current Literature. *Journal of Natural History*, 28(2): 257-352.

McInnes, S.J., 2001 – Is it real?. *Zoologischer Anzeiger-A Journal of Comparative Zoology*, 240(3-4): 467-469.

McMullin, R.T., 2023 – *Lichens: Macrolichens of Ontario and the Great Lakes Region of the United States.* Firefly Books Limited.

Miller, W.R., & Perry, E.S., 2019 – Adjustments to the Annotated zoogeography of non-marine Tardigrada. Part III: North America and Greenland by Kaczmarek, Michalczyk & McInnes (Zootaxa 4203). *Zootaxa*, *4345*, 99-114.

Miller, W.R., McCowan, P.J., Perry, E.S., Schulze, S.L., Shannon, R.K., & Henry, C.S., 2020 – Tardigrades of North America: New records of occurrence for three species of green tardigrades (Heterotardigrada, Echiniscoidea, Echiniscidae, *Viridiscus*). *Transactions of the Kansas Academy of Science*, 123(1-2): 235-241.

Momeni, S., Gąsiorek, P., Loeffelholz, J., Chtarbanova, S., Nelson, D.R., Fletcher, R.A., Michalczyk, Ł., & Pienaar, J., 2023 – Green armoured tardigrades (Echiniscidae: *Viridiscus*), including a new species from the Southern Nearctic, exemplify problems with tardigrade variability research. *Scientific Reports*, 13(1): 16329.

Morek, W., Ciosek, J.A., & Michalczyk, Ł., 2020 – Description of *Milnesium pentapapillatum* sp. nov., with an amendment of the diagnosis of the order Apochela and abolition of the class Apotardigrada (Tardigrada). *Zoologischer Anzeiger*, 288: 107-117.

Murray, J., 1910 – Tardigrada. British Antarctic Expedition 1907 - 1909. *Reports on the Scientific Investigations*, 1(5): 83-187.

Nelson, D.R., & Adkins, R.G., 2001 – Distribution of tardigrades within a moss cushion: do tardigrades migrate in response to changing moisture conditions?. *Zoologischer Anzeiger-A Journal of Comparative Zoology*, 240(3-4): 493-500.

Nelson, D.R., Fletcher, R. A., Guidetti, R., Roszkowska, M., Grobys, D., & Kaczmarek, Ł., 2020 – Two new species of Tardigrada from moss cushions (*Grimmia* sp.) in a xerothermic habitat in northeast Tennessee (USA, North America), with the first identification of males in the genus *Viridiscus*. *PeerJ*, 8: e10251.

Perry, E.S., Miller, W.R., & Lindsay, S., 2015 – Looking at tardigrades in a new light: using epifluorescence to interpret structure. *Journal of Microscopy*, 257(2): 117-122.

Péterfi, F., 1956 – Contributioni la cunoasterea Tardigradelor din RPR Studii si Cercetari de Biologia. *Accademia RPR, Filiola Cluj*, 7: 149-155.

Pilato, G., Fontoura, P., & Lisi, O., 2007 – Remarks on the *Echiniscus viridis* group with the description of a new species (Tardigrada, Echiniscidae). *Journal of Limnology*, 66 (suppl 1): 33-39.

Pilato, G. Fontoura, P., & Lisi, O., 2008 – New description of *Echiniscus viridis* Murray, 1910 and remarks on the *viridis* group. *New Zealand Journal of Zoology,* 35: 85-92.

Ramazzotti, G., 1959 – Il gruppo dell' *Echiniscus viridis* con la nuova specie *E. perviridis* e *Macrobiotus pustulatus* altra nuova specie (Tardigrada). *Atti della Società Italiana di Scienze Naturali e del Museo Civico di Storia Naturale in Milano*, 98(4): 303-309.

Ramazzotti, G. and Maucci, W., 1983 – II Phylum Tardigrada, (CW Beasley, English Translation). *Memorie dell'Istitato idrobiologia*, 41: 1-985.

Rocha, A., Camarda, D., Ostertag, B., Doma, I., Meier, F., & Lisi, O., 2023 – Actual State of Knowledge of the Limno-Terrestrial Tardigrade Fauna of the Republic of Argentina and New Genus Assignment for *Viridiscus rufoviridis* (du Bois-Reymond Marcus, 1944). *Diversity*, 15(2): 222.

Stec, D., Smolak, R., Kaczmarek, Ł., & Michalczyk, Ł., 2015 – An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. *Zootaxa*, 4052(5): 501-526.

Tamura, K., Stecher, G., & Kumar, S., 2021 – MEGA11: molecular evolutionary genetics analysis version 11. *Molecular biology and evolution*, 38(7), 3022-3027.

Supporting information / Informazioni supplementari

Supplemental Table 1. COI sequences selected from GenBank for the maximum likelihood phylogeny in the current study. / Sequenze selezionate da GenBank nel presente studio per la ricostruzione filogenetica (maximum likelihood) di COI.

Supplemental File 1. FASTA file with COI sequences obtained from GenBank and used for the current study. / File FASTA con le sequenze COI ricavate da GenBank e impiegate per il presente studio.

Tab. 1 – New Records of Occurrence for *Viridiscus* spp. in North America. Genus Species Author, Year [Aquatic or Terrestrial]. Table format follows Kaczmarek, et al. (2016). / Nuove segnalazioni di *Viridiscus* spp. in Nord America. Genere Specie Autore, Anno [Acquatico o Terrestre]. La tabella segue la formattazione di Kaczmarek, et al. (2016).

Country (State):
Latitude, Longitude, Altitude; Locations, City, State, and Habitat on Substrate.

NORTH AMERICA

Viridiscus perviridis Ramazzotti, 1959 **[T]**

USA (Iowa):

- 43.200278 (43°12’1”N), -91.158333 (91°9’30”W), 194m asl; Harpers Ferry, IA; *Schistidium rivulare* moss on limestone rock boulder.

USA (Minnesota):

- 43.642222 (43°38’31”N), -91.497778 (91°29’52”W), 356m asl; North Park, Caledonia, MN; *Xanthomendoza* sp. lichen on a rock wall.

USA (Missouri):

- 38.946389 (38°56’47”N), -92.32583 (92°19’33”W), 232m asl; University of Missouri-Columbia campus, Columbia, MO; *Schistidium rivulare* moss on concrete wall.

USA (Mississippi):

- 33.495 (33°29’41”N), -88.41777 (88°25’4”W), 54m asl; Mississippi University for Women campus, Columbus, MS; Moss on *Ginkgo biloba* tree.

Viridiscus viridissimus Péterfi, 1956 **[T]**

USA (Illinois):

- 42.4155556 (42°24’56”N), -90.4450 (90°26’42”W), 238m asl; Greenwood Cemetery, Galena, IL; *Schistidium* sp. moss, *Xanthomendoza weberi* and *Physciella melanchra* lichens on tombstone.

USA (South Dakota):

- 43.441998 (43°26’31”N), -103.543439 (103°32’36”W), 1,229m asl; Evans Cliff, Hot Springs, SD; *Xanthomendoza* sp. on rock.

USA (Missouri):

- 38.946389 (38°56’47”N), -92.325833 (92°19’33”W), 232m asl; University of Missouri-Columbia campus, Columbia, MO; *Schistidium rivulare* moss on concrete wall.

Tab. 2 – Top five BLAST hits for the COI sequence featured in our study. / I primi cinque risultati BLAST per la sequenza COI analizzata nel nostro studio.

Description	Max Score	E-Value	Percent Identity	Acc. Length
Viridiscus viridissimus US.078.05	996	0.0	100.00%	611
Viridiscus viridissimus US.081.26	996	0.0	100.00%	611
Viridiscus viridissimus US.081.15	996	0.0	100.00%	611
Viridiscus viridissimus US.081.14	996	0.0	100.00%	611
Viridiscus viridissimus US.081.13	996	0.0	100.00%	611

Fig. 1 – USA map of new distribution of *Viridiscus perviridis* (black stars =★) and *Viridiscus viridissimus* (black circles = ●). / Mappa degli Stati Uniti raffigurante la nuova distribuzione di *Viridiscus perviridis* (stelle nere =★) e *Viridiscus viridissimus* (cerchi neri = ●).

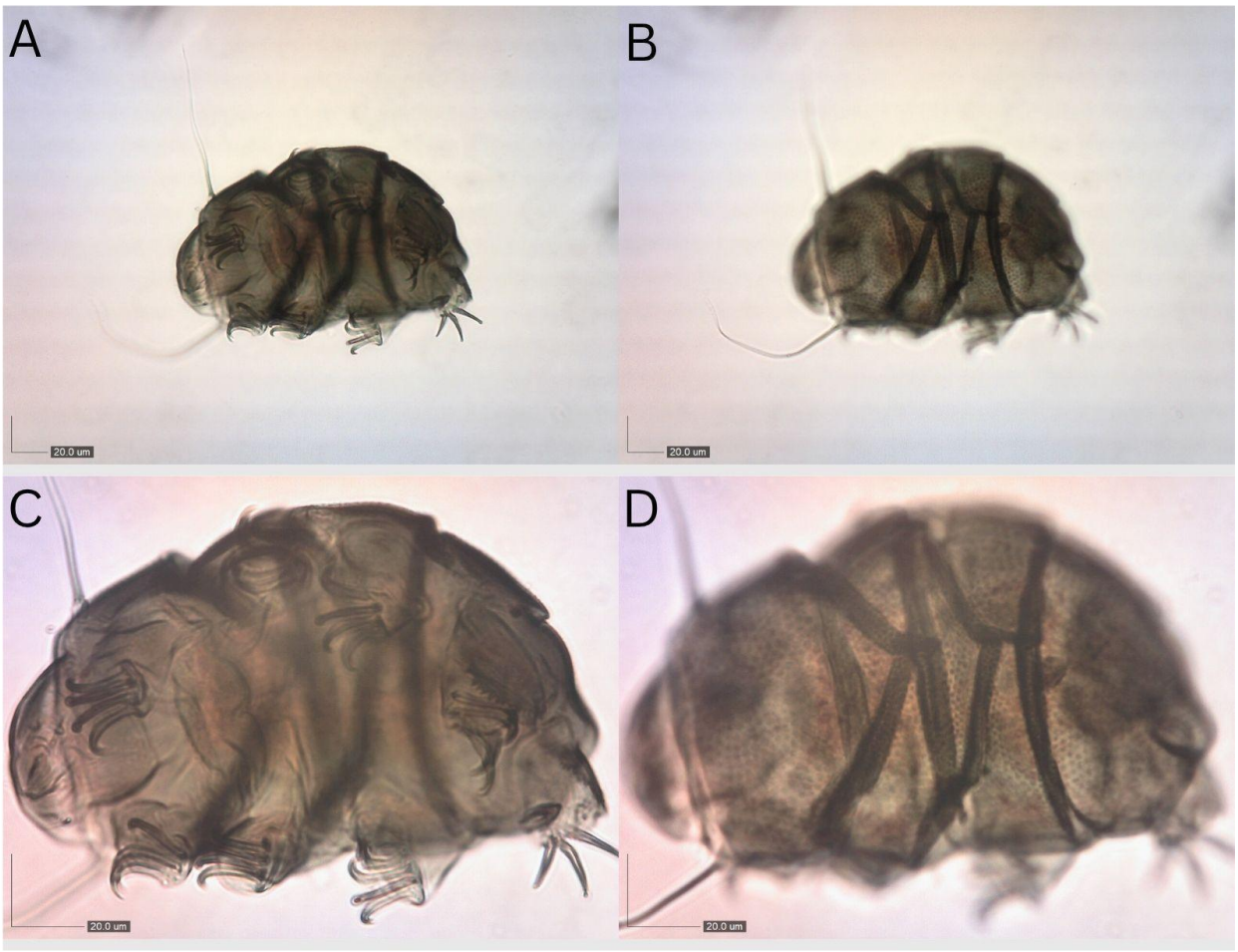

Fig. 2 – Light compound micrographs of *Viridiscus perviridis* from Harpers Ferry, Iowa. A) Focus on claws at 200X. B) Focus on granulation and dorsal plates at 200X. C) Focus on claws at 400X. D) Focus on granulation and dorsal plates at 400X. Scale bars are 20 micrometers. / Microfotografie in campo chiaro di *Viridiscus perviridis* da Harpers Ferry, Iowa. A) Messa a fuoco sulle unghie a 200X. B) Messa a fuoco su granulazione e placche dorsali a 200X. C) Messa a fuoco sulle unghie a 400X. D) Messa a fuoco su granulazione e placche dorsali a 400X. Barre di scala 20 micrometri.

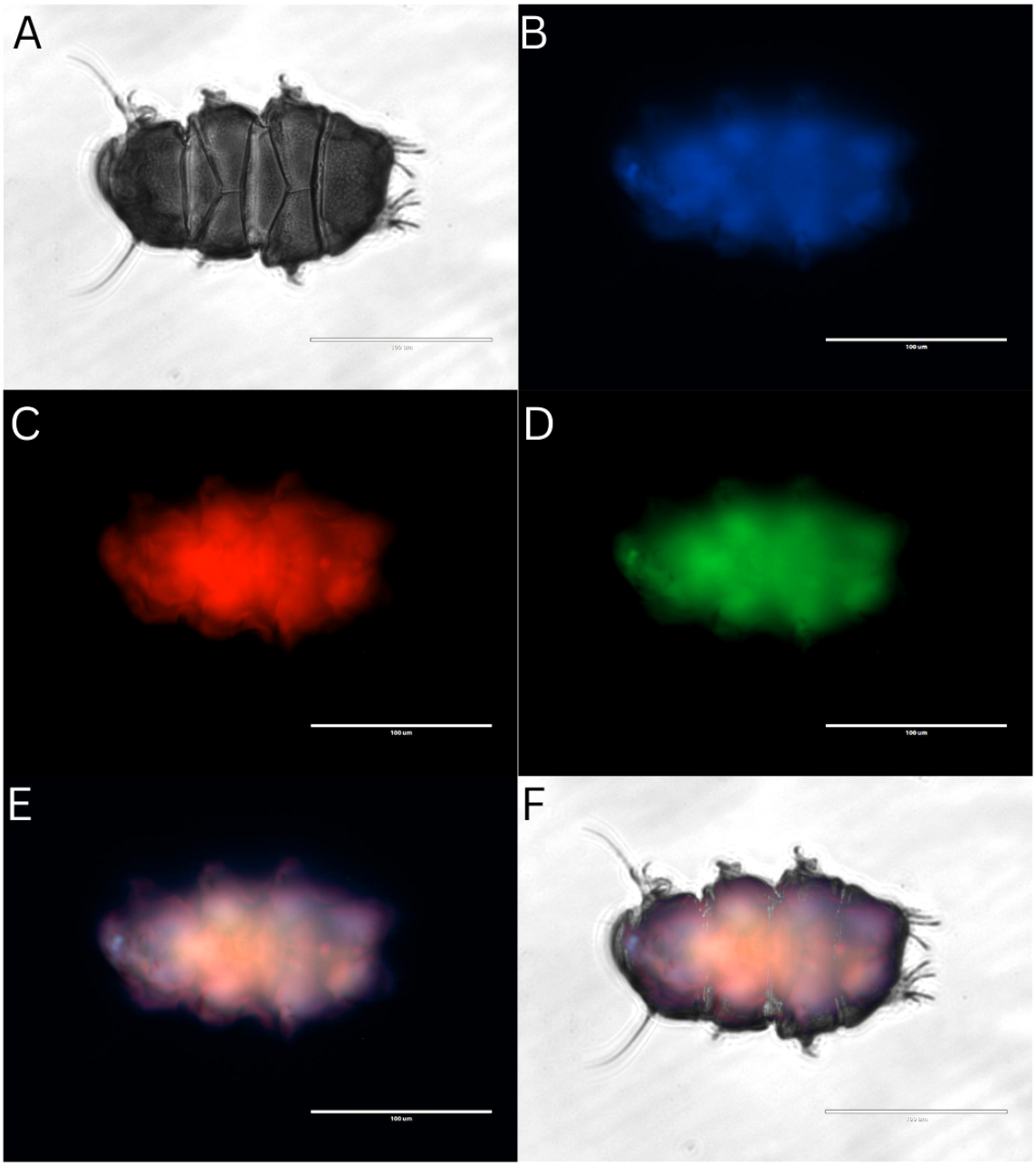

Fig. 3 – *Viridiscus viridissimus* at 400X from Columbia, Missouri. A) Black and white transmitted light micrograph. B) DAPI fluorescence filter. C) RFP fluorescence filter. D) GFP fluorescence filter. E) Overlay of fluorescence filters. F) Transmitted light and overlay of fluorescence filters combined. Scale bars are 100 micrometers. / *Viridiscus viridissimus* a 400X da Columbia, Missouri. A) Microfotografia a luce trasmessa in bianco e nero. B) Filtro di fluorescenza DAPI. C) Filtro di fluorescenza RFP. D) Filtro di fluorescenza GFP. E) Sovrapposizione di filtri di fluorescenza. F) Immagini con luce trasmessa e con i filtri di fluorescenza sovrapposte. Barre di scala 100 micrometri.

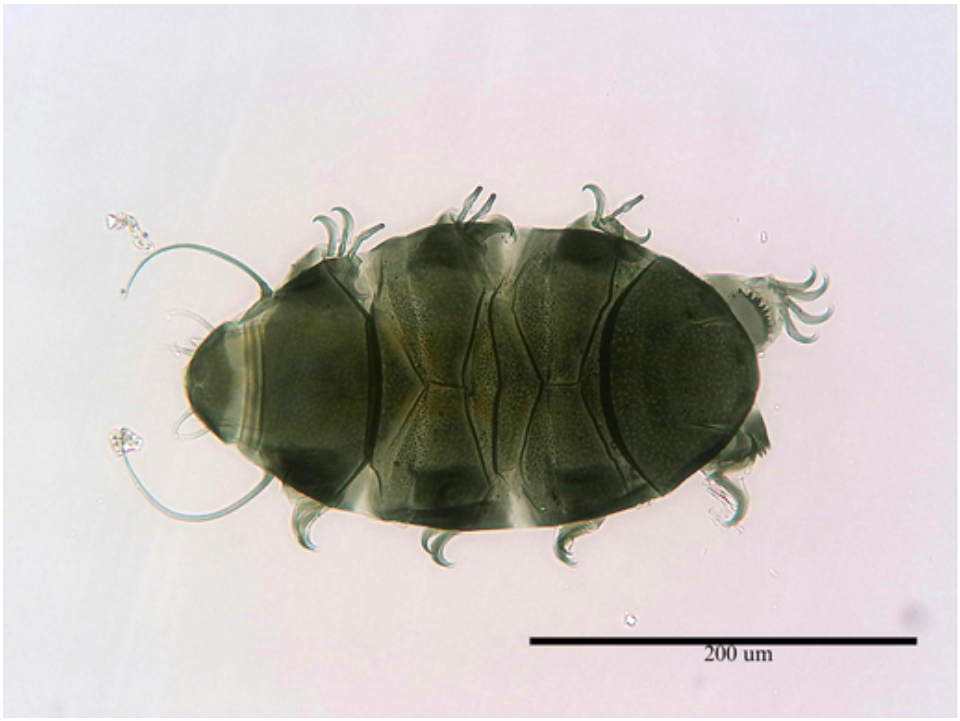

Fig. 4 – Light compound micrograph of *Viridiscus viridissimus* at 200X from Hot Springs, South Dakota. Scale bar is 200 micrometers. / Microfotografia in campo chiaro di *Viridiscus viridissimus* a 200X da Hot Springs, South Dakota. Barra di scala 200 micrometri.

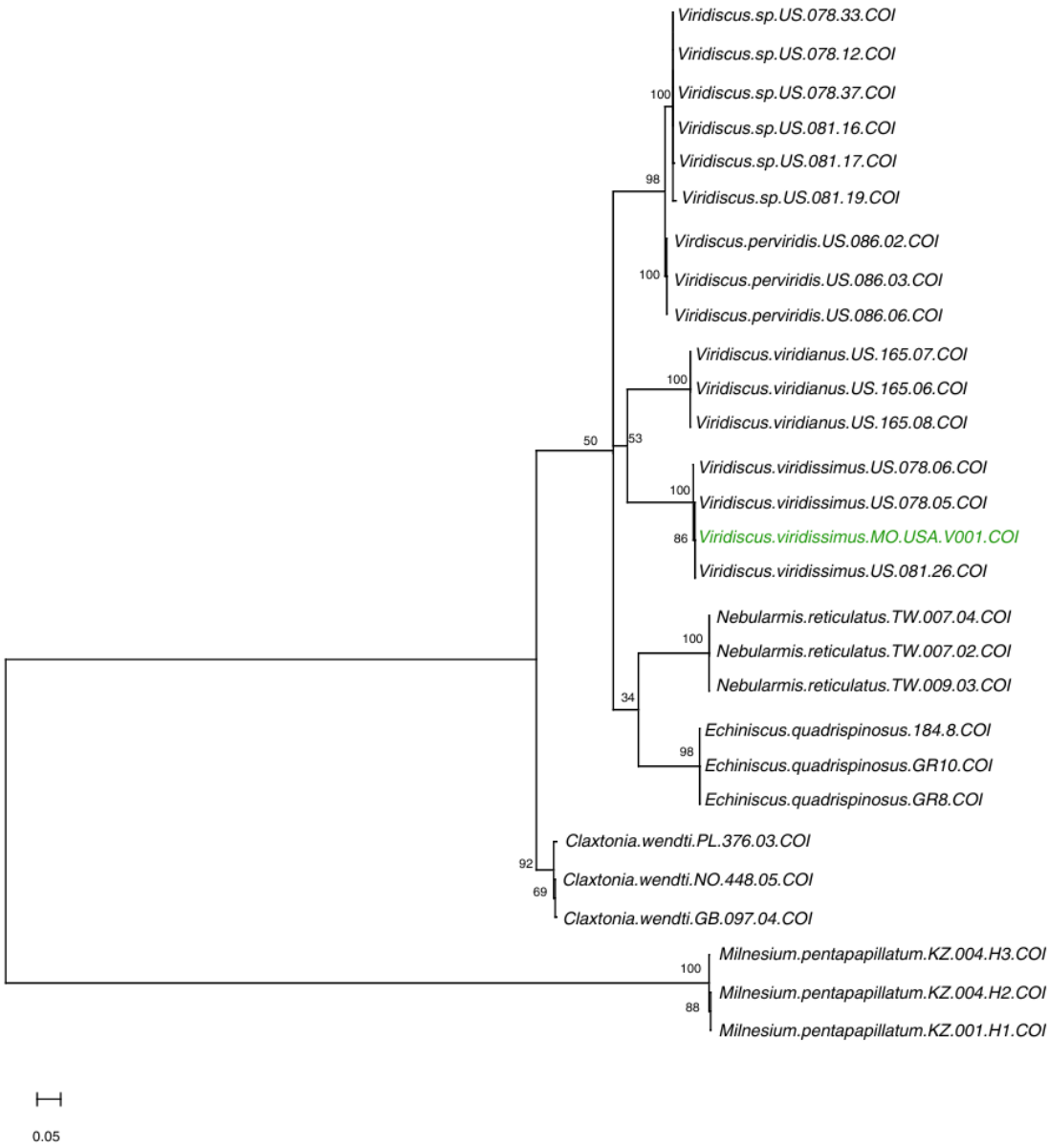

Fig. 5 – Phylogenetic result of the maximum likelihood analysis using a COI dataset. Numbers above or beside nodes represent bootstrap values. The name colored in green represents the *Viridiscus viridissimus* sequence obtained in the current study. / Risultato filogenetico dell'analisi di maximum likelihood utilizzando il dataset delle COI. I numeri sopra o accanto ai nodi rappresentano valori di bootstrap. Il nome colorato in verde rappresenta la sequenza di *Viridiscus viridissimus* ottenuta nel presente studio.

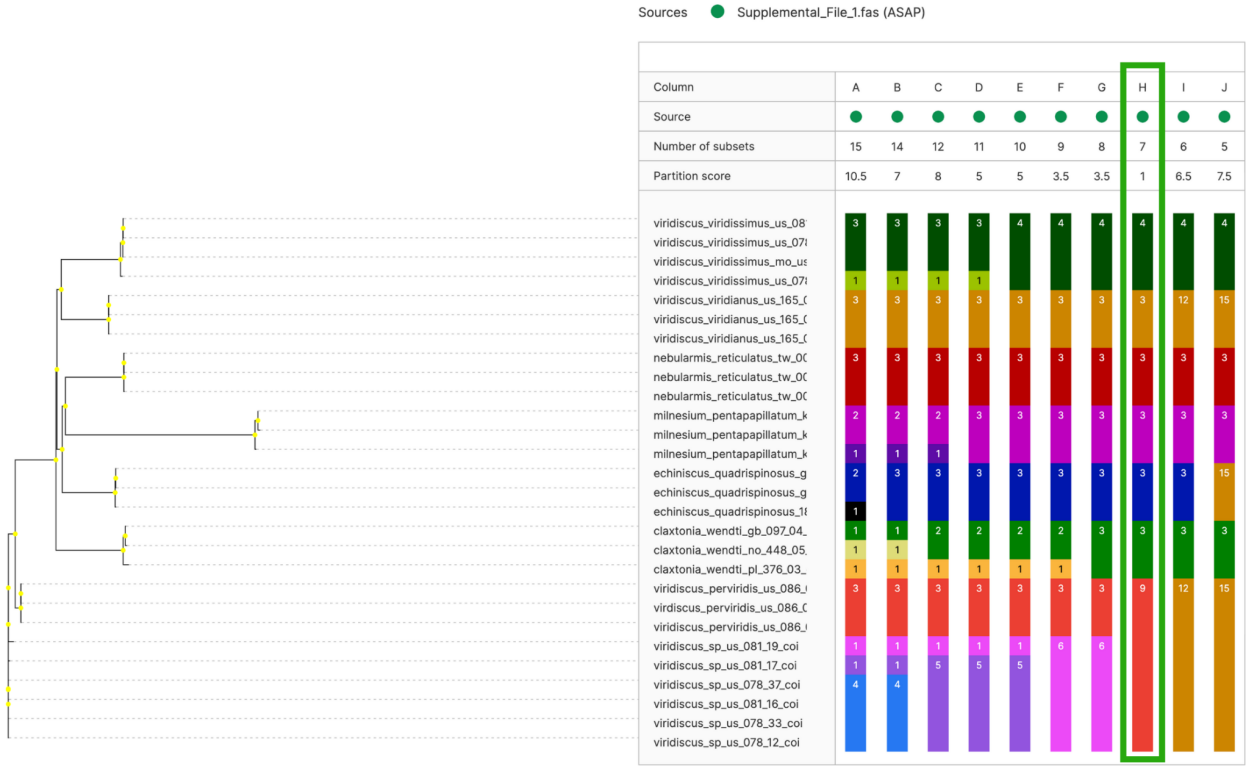

Fig. 6 – Results of the ASAP analysis indicating partition scores. The green box indicates the selected partition. / Risultati dell'analisi ASAP che indicano i punteggi di partizione. La casella verde indica la partizione selezionata.