Protocol Article

An all-inclusive approach: A universal protocol for the successful amplification of four genetic loci of all Oniscidea

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ABSTRACT

Accounting more than 3,700 described species, Oniscidea is the largest and at the same time the only terrestrial isopod suborder inhabiting almost all terrestrial biomes. Despite the great effort dedicated on describing taxonomic diversity of Oniscidea, mainly employing morphology, there is still a considerable number of species/genera of uncertain generic/familiar assignment. Based on different morphological characters, alternative evolutionary relationships have been proposed to describe the diversity of Oniscidea at different phylogenetic levels. Accumulating morphological and genetic data are repeatedly challenging the monophyly of established taxa, undermining the validity of several morphological characters traditionally used in terrestrial isopod taxonomy, leading to frequent revisions of the current taxonomy of the Oniscidea. The use of genetic data facilitates the efforts to reconstruct the complex evolutionary history of the focal group by providing important data for the identification, delimitation, and description of species. The proposed protocol with universal PCR conditions and primers was used to successfully amplify COI, 16S, 28S and NAK loci in diverse Oniscidea taxa. The application of this protocol is anticipated to facilitate the generation of new genetic data and hence promote scientific research in Isopoda taxonomy, evolution, ecology, and other related fields.

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### SPECIFICATIONS TABLE

| Subject Area;          | Biochemistry, Genetics and Molecular Biology |
|------------------------|---------------------------------------------|
| More specific subject area; | PCR, Gene amplification                      |
| Protocol name;         | A universal protocol for the successful amplification of four genetic loci of all Oniscidea |
| Reagents/tools;        | PCR machine                                  |
|                        | Primer A                                     |
|                        | Primer B                                     |
|                        | dNTPs                                        |
|                        | MgCl₂                                        |
|                        | Taq polymerase                               |
|                        | Taq buffer                                   |
| Experimental design;   | Herein we propose a universal easy and cost-effective way to produce genetic data for all Oniscidea. Multiple combinations and newly designed primers were tested under the same amplification conditions at a wide variety of taxa. Using the same primers for all targeted taxa the same gene fragments are targeted and hence produced data could be used to generate new or enrich existing datasets. |
| Trial registration;    | N/A                                          |
| Ethics;                | N/A                                          |
| Value of the Protocol; | • Universal for all Oniscidea                |
|                        | • Simple and easy to follow protocol         |
|                        | • Sequencing of the most popular genetic markers for taxonomic and phylogenetic studies |
|                        | • Cost effective                             |

**Description of protocol:** Touchdown PCR was initially developed as a modification to traditional PCR aiming to increase the specificity and sensitivity of amplification [1–3]. The sensitivity of the proposed protocol was tested for four commonly targeted genetic loci for many taxonomically diverse taxa. The proposed protocol could be used to generate mitochondrial (16S, COI) and nuclear (28S, NaK) data.

**Required reagents and equipment**
- PCR thermal cycler
- PCR tubes
- Sterile 1.5 mL Eppendorf style microcentrifuge tubes
- Benchtop microcentrifuge
- Adjustable micropipettes (0.1–1,000 mL)
- Micropipette tips (10–1,000 mL)
- dNTPs
- MgCl₂
- Taq polymerase
- Taq Buffer
- Primers (Table 1)

**Table 1**
Proposed primers for each locus.

| Gene | Primer name | 5’-Sequence -3’ | Product size | Reference |
|------|-------------|-----------------|--------------|-----------|
| COI  | LCO1490     | GGT CAA CAA ATC ATA AAG ATA TTG G | ~560 | [4]         |
|      | IsoCoIRint  | GCC CCA GCC AAC ACA GGC ARD GA | | [5]         |
| 16S  | 16sTRF      | CTG ACT GTG CTA AGG TAG CA | ~375bp | This study |
|      | 16sTRH      | CGG TTY GAA CTC AGA TCA YGT GA | |             |
| 28S  | 28S a       | GAC CCG TCT TGA AAC AGC GA | 350bp - 800bp | [6]         |
|      | 28S b       | TCG GAA GAA ACC AGC TAC TA | |             |
| NaK  | NakForb     | ATG ACA GTT GCT CAT ATG TGG TT | ~600bp | [7]         |
|      | Nak638R     | GGD RGR TCR ATC ATD GAC AT | | [8]         |
Method details

The proposed final reaction volume is 20 μL and consists of 0.1 μL of Taq DNA Polymerase (5U/μL), 2.4 μL of 25 mM MgCl2, 1X of Taq buffer A, 0.6 μL of 10 mM dNTPs, 0.6 μL of each primer (10 μM; Table 1) and >2 ng of DNA template in case of COI and 16s or >10ng in case of 28s and NAK. In any case the use of more than 100ng as DNA template should be avoided. The proposed protocol works effectively with samples of high DNA purity i.e. A260/A280 rates over 1.5.

Thermocycling conditions for all four proposed genes should be as follows:

a) 94°C 10 min initial denaturation 1 cycle
b) 94°C 60s, 55°C 60s, 72°C 60s for 5 cycles
c) 94°C 60s, 50°C 60s, 72°C 60s for 5 cycles
d) 94°C 60s, 47°C 60s, 72°C 60s for 32 cycles
e) 94°C 60s, 42°C 60s, 72°C 60s for 7 cycles
f) 72°C 10 min final extension 1 cycle
g) Hold 10°C

The proposed protocol works efficiently even at very low DNA concentrations (<2ng), ill preserved or very old specimens. If aspecific products are amplified under these conditions an alternative, stricter thermocycling profile should be followed:

a) 94°C 10 min initial denaturation 1 cycle
b) 94°C 60s, 55°C 60s, 72°C 60s for 10 cycles
c) 94°C 60s, 50°C 60s, 72°C 60s for 10 cycles
d) 94°C 60s, 47°C 60s, 72°C 60s for 30 cycles
e) 72°C 10 min final extension 1 cycle
f) Hold 10°C

PCR products may be visualized using a common agarose gel electrophoresis. Successfully amplified samples should be purified using any commercial purification protocol following the manufacturer's instructions before proceeding with sequencing. Cycle sequencing can be performed with the same primers used for the initial PCR reaction.

Validation

The effectiveness of the proposed protocol was tested for all available specimens, representing 142 species in 97 genera and 22 families, representing a diverse cross-section of the Oniscidea. Beyond terrestrial isopods, the same protocol was also successfully applied for representatives of the isopod orders Valvifera, Sphaeromatidea and Asellota.

Generated data were deposited in NCBI GenBank (Table 2). A limited number of sequences produced from our group within the framework of other projects, using a very similar protocol, were already published within the framework of previews studies and their corresponding accession numbers are given in Table 1. [8,9]. Although the new protocol using proposed thermocycling profile and primers were successfully tested in these cases too.
| Isolate code | Species | 16S | COI | 28S | NaK | Origin |
|--------------|---------|-----|-----|-----|-----|--------|
| Oniscidea Crinocheta Agnaridae | | | | | | |
| 55z* | *Agnara madagascariensis* (Budde-Lund, 1885) | – | – | MG888003 | MG887924 | U.A.Emirates |
| 77Y | *Desertoniscus zaitsevi* Gongalsky, 2017 | ON219990 | ON212532 | – | – | Bolshoy Tsaryn (Russia) |
| 183z | | ON219989 | ON212533 | ON312023 | – | Bolshoy Tsaryn (Russia) |
| 184z | | ON219988 | ON212534 | ON312022 | – | Bolshoy Tsaryn (Russia) |
| 35h | *Hemilepistus elongatus* Budde-Lund, 1885 | ON220006 | ON212527 | – | – | Kerman Anar (Iran) |
| 23h | | ON220001 | ON212528 | ON312034 | – | Shirvan (Iran) |
| 34h | | ON220005 | ON212529 | – | – | Semnan Douzahir (Iran) |
| 31h | | ON220002 | ON212525 | – | – | Marand (Iran) |
| 32h | | ON220003 | ON212526 | – | – | Shahrood (Iran) |
| 33h | | ON220004 | ON212497 | – | – | Shahrood (Iran) |
| 24h* | *Hemilepistus reaumurii* (Milne-Edwards, 1840) | ON220016 | ON212514 | MN174828 | MN234258 | Medenine (Tunisia) |
| 27h | | ON220019 | ON212516 | – | – | Libya |
| 29h | | ON220021 | ON212519 | – | – | Jordan |
| 30h | | ON220022 | ON212520 | – | – | Syria |
| 25h | | ON220017 | ON212515 | – | – | Tunisia |
| 26h | | ON220018 | ON212517 | – | – | Jordan |
| 28h | | ON220020 | ON212518 | ON312036 | – | Syria |
| 2h* | *Hemilepistus schirazi* Lincoln, 1970 | – | – | MG888012 | MG887927 | Isfahan (Iran) |
| 6h | | ON220026 | ON212493 | ON312031 | – | Isfahan (Iran) |
| 8h | | ON220027 | ON212494 | ON312032 | – | Talkhuncheh (Iran) |
| 3h | | ON220023 | ON212496 | ON312028 | ON227363 | Zahedshahr Nasirabad (Iran) |
| 4h | | ON220024 | ON212492 | ON312029 | – | Neyriz (Iran) |
| 5h | | ON220025 | ON212495 | ON312030 | ON227366 | Arsanjan (Iran) |
| 36h | *Hemilepistus taftanicus* Kashani, Sari & Hosseini, 2010 | ON220028 | ON212498 | – | – | Kerman (Iran) |
| 37h | | ON220029 | ON212499 | – | – | Khash sistan (Iran) |

(continued on next page)
| Isolate code | Species | 16S | COI | 28S | NaK | Origin |
|-------------|---------|-----|-----|-----|-----|--------|
| 15h         | *Hemilepistus aphganicus* Borutzky, 1958 | ON219997 | ON212500 | – | – | Mashhad (Iran) |
| 16h         | ON219998 | ON212501 | ON312033 | ON227368 | ON227367 | Mashhad (Iran) |
| 17h         | ON219999 | ON212502 | – | – | ON227367 | Razavi Khorasan Saraks (Iran) |
| 18h         | *Hemilepistus klugii* (Brandt, 1833) | ON220000 | ON212503 | – | ON227370 | Torbate-Jam (Iran) |
| 7h          | ON220014 | – | ON312027 | ON227362 | – | Meymeh (Iran) |
| 9h          | ON220015 | – | – | ON227365 | – | Hosnijeh (Iran) |
| 19h         | ON220012 | ON212511 | – | – | – | Qazvin, Bouin-Zahra (Iran) |
| 1h∗         | MG887951 | MG887938 | MG888011 | MG887926 | – | Isfahan (Iran) |
| 7h          | – | ON212504 | – | – | – | Meymeh (Iran) |
| 9h          | – | ON212505 | – | – | – | Hasanjeh (Iran) |
| 10h         | ON220007 | ON212506 | ON312035 | ON227364 | – | Isfahan kashan Borzak (Iran) |
| 11h         | ON220008 | ON212509 | – | ON227369 | – | Zavieh (Iran) |
| 12h         | ON220009 | ON212508 | – | – | – | Komijan (Iran) |
| 13h         | ON220010 | ON212507 | – | – | – | Mahallat (Iran) |
| 14h         | ON220011 | ON212512 | – | – | – | Mahallat (Iran) |
| 64z         | *Mongoloniscus sinensis* (Dollfus, 1901) | – | – | ON312044 | – | Koko Nor Lake (China) |
| 3z          | *Orthometopon phaleronense* (Verhoeff, 1901) | ON220049 | – | ON312049 | – | Pertouli (Greece) |
| 142z∗       | *Protracheoniscus* af. *fossiliger* Verhoeff, 1901 | – | – | MN174817 | MN234281 | Greece |
| 181z        | *Protracheoniscus major* (Dollfus, 1903) | ON220053 | ON212531 | ON312053 | – | Bolshoy Tsaryn (Russia) |
| 182z        | ON220054 | ON212530 | ON312054 | ON227371 | – | Bolshoy Tsaryn (Russia) |
| 63z         | *Alloniscus oahuensis* Budde-Lund, 1885 | ON219960 | – | – | – | Bali (Indonesia) |
| Armadillidae | *Armadillo officinalis* Duméril, 1816 | ON219965 | – | MN174812 | MN234252 | Larnaca Salt Lake (Cyprus) |
| 47cy∗       | – | – | ON312067 | – | – | Agios Theodoros (Cyprus) |
| 48cy        | ON219966 | – | ON312066 | ON227376 | – | Agia Varvara (Cyprus) |
| 51cy        | – | – | ON312007 | – | – | Geri (Cyprus) |
| 56cy        | – | – | ON312008 | – | – | – | Austalia |

(continued on next page)
| Isolate code | Species                              | 16S  | COI  | 28S     | NaK   | Origin                      |
|--------------|--------------------------------------|------|------|---------|-------|-----------------------------|
| 59z          | Cubaris nepalensis (Vandel, 1973)    | –    | –    | ON312019| –     | Ilam (Nepal)                |
| 96z          | Sphaerillodillo pubescens (Budde-Lund, 1885) | –    | –    | ON312024| –     | Eastern Cape Transkei (South Africa) |
| 98z          | Laureola hiatus Barnard, 1960        |      |      | ON312037| –     | Haenertsburg (South Africa)  |
| 78z          | Lobodillo hebridarum (Verhoeven, 1926) | ON220041| –    | –       | –     | Vanuatu islands             |
| 99z          | Pseudodiploexochus sp.               | –    | –    | ON312055| –     | Moramanga (Madagascar)      |
| 100z         | Pseudolaureola hystrix (Barnard, 1958) | –    | –    | ON312056| –     | Moramanga (Madagascar)      |
| 83z          | Venezillo sp.                        | –    | –    | ON312087| –     | Caldera de Bandama (Canary Islands) |

**Armadillidiidae**

| Isolate code | Species                                | 16S  | COI  | 28S     | NaK   | Origin                      |
|--------------|----------------------------------------|------|------|---------|-------|-----------------------------|
| 52cy         | Armadillidium fallax Brandt, 1833      | ON219962| ON212577| –       | –     | Cape Greco (Cyprus)         |
| 87cy         | Armadillidium vulgar (Latreille, 1804) | ON219963| –    | –       | –     | Akrotiri (Cyprus)           |
| 34z          | Armadillidium vulgar (Latreille, 1804) | –    | –    | ON212545| –     | Limassol (Cyprus)           |
| 55cy         | Armadillidium vulgar (Latreille, 1804) | –    | –    | ON212537| –     | Limnatis (Cyprus)           |
| 66cy         | Armadillidium cavernarum Vandel, 1958  | ON219964| –    | –       | ON312005| –                           |
| 62T          | Armadillidium cavernarum Vandel, 1958  | –    | –    | –       | –     | Cave Katolygi, Crete (Greece) |
| 151z         | Cyphodillidium absoloni (Strouhal, 1934) | ON219983| ON212539| MN174814| MN234276| Saplunara (Croatia)         |
| 156z         | Echinarmadillidium frugali (Verhoeven, 1900) | ON219993| –    | –       | –     | Baška voda (Croatia)        |
| 157z         | Echinarmadillidium frugali (Verhoeven, 1900) | ON219994| ON212538| –       | –     | Lumbarda (Cyprus)           |
| 100cy        | Schizidium fissum (Budde-Lund, 1885)   | –    | –    | ON312057| –     | Pitargou (Cyprus)           |
| 100cy        | Schizidium fissum (Budde-Lund, 1885)   | –    | –    | ON312057| –     | Pitargou (Cyprus)           |
| 75T          | Troglarmadillidium halophilum Senthourakis, 1993 | –    | ON212540| MG888005| –     | Antikythera (Greece)        |
| 179z         | Typhlarmadillidium sp.                | ON220016| –    | MN174815| MN234273| Gromača (Croatia)           |
| 180z         | Typhlarmadillidium sp.                | ON220017| –    | –       | –     | Krvévine (Bosnia and Herzegovina) |
| 79z          | Australiodillo bifrons (Budde-Lund, 1885) | ON219967| –    | –       | –     | Alligator Creek (Australia) |

**Balloniscidae**

| Isolate code | Species                                     | 16S  | COI  | 28S     | NaK   | Origin                      |
|--------------|---------------------------------------------|------|------|---------|-------|-----------------------------|
| 49z          | Balloniscus sellowi (Brandt, 1833)         | –    | –    | ON312009| –     | Montevideo (Uruguay)        |

(continued on next page)
| Isolate code | Species | 16S | COI | 28S | NaK | Origin                  |
|--------------|---------|-----|-----|-----|-----|-------------------------|
| Cylisticidae |         |     |     |     |     |                         |
| 143z*        | Cylisticus convexus (De Geer, 1778) | –   | –   | MN174813 | MN234280 | Budapest (Hungary) |
| 72z          | Deto marina (Chilton, 1885) | ON219991 | – | – | – | Mount Agnew (Tasmania) |
| Detonidae    |         |     |     |     |     |                         |
| 11z          | Dioscoridillo melanoleucos Ferrara & Taiti, 1996 | ON219992 | – | – | – | Socotra (Yemen) |
| 86z          | Microcercus sp. | – | – | ON312041 | – | Lake Bogoria (Kenya) |
| 57z          | Periscyphis omanensis Taiti & Ferrara, 1991 | – | – | – | ON227374 | Dhofar (Oman) |
| Eubelidae    |         |     |     |     |     |                         |
| 99cy         | Halophiloscia sp. | ON219996 | – | – | – | Fontana Amorosa (Cyprus) |
| 120z         | Halophilosic zosterae (Verhoeff, 1928) | – | – | ON312025 | – | Zygi (Cyprus) |
| 44z          | Litterophiloscia tropicalis Taiti & Ferrara, 1986 | – | – | ON312039 | – | Tinos (Greece) |
| Oniscidae    |         |     |     |     |     |                         |
| 141z         | Oniscus asellus Linnaeus, 1758 | – | – | ON312047 | – | Hispaniola island |
| 169z         | Oroniscus dalmaticus Strouhal, 1937 | ON220046 | ON212548 | – | – | Jujnovici (Croatia) |
| 170z         | – | – | ON312026 | – | – | Antunovići (Croatia) |
| 171z         | – | – | ON312026 | – | – | Golubinjak (Croatia) |
| 171z         | – | – | ON312026 | – | – | Golubinjak (Croatia) |
| 39z          | Phalloniscus mateui Vandel, 1953 | – | – | ON312050 | – | Segovia (Spain) |
| 203z         | Rodoniscus anophthalmus Arcangeli, 1934 | ON220055 | ON212544 | – | ON227373 | Iraklia island (Greece) |
| Philosciidae |         |     |     |     |     |                         |
| 28z          | Benthana trinodulata Araujo & Lopes, 2003 | – | – | ON312010 | – | Bahia (Brazil) |
| 35T          | Chaetophiloscia cellaria (Dollfus, 1884) | ON219979 | – | – | – | Nychteridospilios, Crete (Greece) |
| 19cy         | Chaetophiloscia elongata (Dollfus, 1884) | – | – | ON312015 | – | Arakapas (Cyprus) |
| 8z*          | – | – | – | MG887929 | – | Sardinia |

(continued on next page)
| Isolate code | Species                                                                 | 16S | COI | 28S | NaK | Origin                        |
|--------------|-------------------------------------------------------------------------|-----|-----|-----|-----|-------------------------------|
| 105cy        | Chaetophiloscia lagoi (Arcangeli, 1934)                                  | –   | ON212543 | ON312016 | – | Larnaca (Cyprus)            |
| 93z          | Congophiloscia longiannamnata Schmalfuss & Ferrara, 1978                 | –   | ON212578 | –   | ON227375 | Kribi (Cameroon)             |
| 40z          | Ctenoscia minima (Dollus, 1892)                                         | ON220032 | ON212559 | –   | –   | Mallorca                     |
| 162z         | Lepidoscinus minutus (C. Koch, 1838)                                    | ON220033 | –   | ON312018 | – | Zapręśić (Croatia)          |
| 163z         | Phyllosca affinis Verhoeff, 1908                                         | ON220050 | –   | –   | –   | Pedra Longa (Sardinia)       |
| 27z          | Androdeloscia lejunei (Lemos de Castro & Souza, 1986)                  | –   | –   | ON312004 | – | Brazil                       |
| Platyarthridae |                                                           |     |     |     |     |                               |
| 32z*         | Platyarthrus schobli Budde-Lund, 1885                                    | –   | –   | MN174833 | MN234254 | Pitargou (Cyprus)           |
| 140z*        | Trichorhina heterophthalma Lemos de Castro, 1964                        | –   | –   | MN174845 | MN234282 | The Netherlands (greenhouse) |
| Porcellionidae |                                                       |     |     |     |     |                               |
| 18p*         | Acaeroplotes melanurus (Budde-Lund, 1885)                               | MG887960 | MG887945 | MG887991 | MG887912 | Punta Menga (Sardinia)       |
| 2p*          | Agabiformius excavatus Verhoeff, 1941                                    | MG887955 | –   | MG888009 | MG887921 | Pafos (Cyprus)              |
| 3p           | Agabiformius lentus (Budde-Lund, 1885)                                   | MG887956 | –   | MG887922 | –   | Pafos (Cyprus)              |
| 94cy         | Brevanus masandaranus Schmalfuss, 1986                                   | ON219958 | ON212535 | –   | –   | Kourio (Cyprus)             |
| 20p*         | Lucasius pallidus Budde-Lund, 1885                                       | –   | –   | MG888008 | MG87919 | Iran                         |
| 13p*         | Caeroplotes porphyriacus (Verhoeven, 1918)                               | –   | MG887932 | MG887990 | –   | Toulon (France)             |
| 8p           | Leptotrichus koss wig Strauchal, 1960                                   | MG887963 | –   | MG888013 | MG887915 | Pafos (Cyprus)              |
| 1KR          | Mica tardus (Budde-Lund, 1885)                                          | ON220034 | ON212536 | ON312040 | MG887917 | Kourio (Cyprus)             |
| 16p*         | Lucasius pallidus Budde-Lund, 1885                                       | –   | –   | ON312040 | MG887917 | Monte Arci (Sardinia)        |
| 17p*         | Mica tardus (Budde-Lund, 1885)                                          | MG887959 | –   | MG887996 | –   | Monte Coa Margine (Sardinia) |
| 4p*          | Porcellio laevis Latreille, 1804                                        | MG887957 | MG887936 | MG887993 | MG887913 | Limassol (Cyprus)           |
| 5p*          | Porcellio laevis Latreille, 1804                                        | MG887958 | MG887937 | MG887994 | MG887914 | Limassol (Cyprus)           |
|              | (continued on next page)                                               |     |     |     |     |                               |
| Isolate code | Species | 16S  | COI   | 28S   | NaK   | Origin                  |
|--------------|---------|------|-------|-------|-------|-------------------------|
| 10p          | *Porcellio nasutus* Strouhal, 1936 | ON220051 | -     | -     | -     | Mt.Parnon (Greece)      |
| 10p          | MG887953 | MG887944 | MG887998 | MG887910 | Mt.Parnon (Greece)      |
| 11p          | MG887954 | MG887999 | MG887911 | MG887911 | Mt.Parnon (Greece)      |
| 204z         | Porcellio sp. | ON200052 | ON212560 | -     | ON227372 | Chrysorogiatissa (Cyprus) |
| 15z          | Porcellionides sp. | -     | -     | -     | ON227358 | Larnaca (Cyprus)        |
| 18z          | -       | -     | -     | ON227359 | Larnaca (Cyprus)        |
| 21p          | *Porcellionides cicutius* (Verhoeff, 1918) | -     | -     | -     | ON227357 | Agios Sozomenos (Cyprus) |
| 21p          | -       | -     | -     | MG887909 | Agios Sozomenos (Cyprus) |
| 19z          | *Porcellionides pruinosa* (Brandt, 1833) | -     | -     | ON312052 | ON227361 | Limassol (Cyprus)       |
| 17z          | -       | -     | ON312051 | ON227360 | Limassol (Cyprus)       |
| 17z          | MG887950 | MG887935 | MG887989 | MG887908 | Larnaca (Cyprus)        |
| 1p           | *Proporcellio vulcanius* (Verhoeff, 1908) | MG887948 | MG887933 | MG887988 | MG887906 | Larnaca (Cyprus)        |
| 19p          | Soteriscus laouensis Taiti & Rossano, 2015 | MG887964 | MG887931 | MG887997 | MG887918 | Oued Laou valley (Morocco) |
| 15p          | Thermocellio sp. | MG887962 | -     | MG887995 | -     | Dar es salaam (Tanzania) |
| 12p          | Tura sp. | MG887966 | MG887946 | MG888001 | MG887920 | Mombasa (Kenya)         |
| 14p          | *Urama triangulifera* Budle-Dune, 1910 | MG887961 | -     | MG888002 | MG887923 | Aberdare (Kenya)        |

**Scleropactidae**

| 26z          | Circoniscus bezzii Arcangeli, 1931 | -     | -     | -     | -     | Brazil                  |
| 65T          | *Kithironiscus paragamiani* Schmalfuss, 1995 | ON212615 | -     | -     | -     | Cave Ag, Sofia Kythira (Greece) |

**Scyphacidae**

| 4z           | Actaeia euchroa Dana, 1853 | -     | -     | MG888007 | MG887930 | New Zealand            |
| 10z          | Scyphax ornatus Dana, 1853 | ON219955 | -     | ON312058 | -     | New Zealand            |

**Trachelipodidae**

| 24z          | Levantoniscus bicostatus Cardoso, Taiti & Sfenthourakis, 2015 | -     | -     | MN174811 | MN234257 | Pafos (Cyprus)         |
| 25z          | -       | -     | MG888000 | MG887928 | Pafos (Cyprus) |
| 22z          | Levantoniscus makrisi Cardoso, Taiti & Sfenthourakis, 2015 | -     | ON212546 | MN174810 | MN234260 | Pafos (Cyprus)         |
| 28cy         | Nagurus carinatus (Dollfus, 1905) | ON220044 | ON212541 | ON312046 | -     | Cedar valley (Cyprus)  |
| 93cy         | -       | ON220045 | ON212542 | -     | -     | Kykkos monastery (Cyprus) |
| 134z         | Trachelipus aegaes (Verhoeff, 1907) | -     | -     | ON312073 | MG887925 | Naxos (Greece)         |

(continued on next page)
| Isolate code | Species | 16S     | COI     | 28S | NaK    | Origin                                      |
|--------------|---------|---------|---------|-----|--------|---------------------------------------------|
| 63T          | Trachelipus cavaticus Schmalfuss, Paragamian & Sfenthourakis, 2004 | ON220079 | ON212614 | –   | –      | Cave Landaka Chania, Crete (Greece)          |
| 144z*        | Trachelipus ratzeburgii (Brandt, 1833) | –        | –       | MN174830 | MN234279 | Germany                                     |
| 64Tc         | Trachelipus sp.       | –        | –       | –   | –      | Cave Mougi Sisses, Crete (Greece)           |
| ?            | Exalloniscus brincki Manicastro & Argano, 1986 | ON219995 | –       | –   | –      | Sri Lanka                                   |
| 68T          | Cordioniscus sp.      | –        | ON212524 | –   | –      | Cave Spilia tis Agias at Katafygi (Peloponese) |
| 27T          |               | ON219981 | –       | –   | –      | Cave Dionysos Leonidion (Peloponnisos, Greece) |
| 29T          |               | ON219982 | –       | –   | –      | Cave Katofygi (Creta, Greece)               |
| 33Tc         |               | –        | ON212513 | –   | –      | Cave of Sava Vardaki Stroumboulas (Greece)  |
| 38T          |               | –        | ON212491 | –   | –      | Cave Katofygi, Creta (Greece)               |
| 42z*         | Styloniscus magellanicus Dana, 1853 | –        | –       | MN174832 | –      | Lago Argentino (Argentina)                 |
| Trichoniscidae|         |          |         |     |        |                                             |
| 145z         | Aegonethes cervinus (Verhoeef, 1931) | ON219956 | ON212562 | –   | ON227384 | Dugopolje (Croatia)                         |
| 146z         | Alistratia beroni Andreev, 2004 | ON219957 | ON212561 | –   | ON227385 | Dračevac (Croatia)                         |
| 61T          | Alpioniscus balthasari (Frankenberger, 1937) | ON219959 | –       | –   | –      | Cave Alistrati (Greece)                     |
| 147z         |                   | ON219961 | ON212551 | ON312003 | –      | Kistanje (Croatia)                         |
| 139z*        | Androniscus roseus (C. Koch, 1838) | –        | –       | MN174824 | MN234283 | The Netherlands                             |
| 201z         | Borutzkyella reavi (Borutzky, 1973) | ON219968 | ON212555 | ON312011 | –      | Abkhazia (Caucasus)                        |
| 202z         |                   | ON219969 | ON212554 | ON312012 | –      | Abkhazia (Caucasus)                        |

(continued on next page)
| Isolate code | Species | 16S   | COI   | 28S   | NaK   | Origin              |
|-------------|---------|-------|-------|-------|-------|---------------------|
| 149z        | Buddelundiella cataractae Verhoeff, 1930 | ON219971 | –     | –     | ON227382 | Šibenik (Croatia)   |
| 148z        | Buddelundiella sp. | ON219972 | –     | –     | –     | Trogir (Croatia)    |
| 150z*       | Calconiscellus karawankianus (Verhoeff, 1908) | ON219973 | ON212556 | MN174827 | MN234277 | Blato (Croatia)     |
| 196z        | Caucasocyphonethes cavaticus | ON219974 | ON212572 | ON312013 | ON227377 | Krasnodar (Caucasus) |
| 197z        | Borutzky, 1948 | ON219975 | ON212573 | ON312014 | ON227378 | Krasnodar (Caucasus) |
| 193z*       | Caucasocyphonethes sp. | ON219977 | ON212557 | MN174825 | MN234268 | Ochamchira (Abkhazia) |
| 194z*       | ON219976 | ON212558 | MN174826 | MN234269 | Ochamchira (Abkhazia) |
| 76Y         | Caucasonenetes sp. | ON219978 | –     | –     | –     | Ochamchira (Abkhazia) |
| 200z        | Colchidoniscus kutaissiamus Borutzky, 1974 | ON219980 | –     | ON312017 | ON227380 | Martvili (Georgia)  |
| 152z        | Cyphonethes herzegovinensis (Verhoeff, 1900) | ON219984 | ON212553 | ON312020 | –     | Začir (Montenegro)  |
| 153z        | ON219985 | ON312021 | –     | –     | –     | Osogjin (Croatia)   |
| 154z        | Cyphopleon kratochvili (Frankenberger, 1939) | ON219986 | ON212574 | –     | –     | Ključ (Bosnia and Herzegovina) |
| 155z        | ON219987 | –     | –     | –     | –     | Ključ (Bosnia and Herzegovina) |
| 160z        | Hyloniscus adonis Verhoeff, 1927 | ON220030 | –     | –     | –     | Slijeme (Croatia)   |
| 161z        | Hyloniscus sp. | ON220031 | ON212552 | –     | ON227381 | Samobor (Croatia)   |
| 76T         | Kosswigius delattini Verhoeff, 1941 | –     | ON212616 | –     | –     | Thraki, Sapka (Greece) |
| 132z        | Miktoniscus sp. | –     | –     | ON312043 | –     | Clohars-fouesnant (France) |
| 92z         | Miktoniscus patience Vandel, 1946 | –     | –     | ON312042 | –     | Madeira             |
| 164z        | Moserius percoi Strouhal, 1940 | ON220043 | –     | ON312045 | –     | Škocjan (Slovenia)  |
| 174z        | Tachysoniscus cf. austriacus (Verhoeff, 1908) | ON220057 | ON212550 | ON312059 | ON227391 | Lovranka (Croatia)  |
| 59Y         | Tauronethes cf. lebedinskyi | –     | –     | –     | –     | Morcheka (Crimea)   |
| 60Y         | Borutzky, 1949 | –     | ON212568 | –     | –     | Morcheka (Crimea)   |
| 63Y         | ON220074 | –     | –     | –     | –     | Morcheka (Crimea)   |
| 185z*       | Tauronethes lebedinskyi Borutzky, 1949 | ON220075 | –     | MN174831 | MN234272 | Baidarskaya (Crimea) |
| 186z        | ON220076 | ON212596 | ON312072 | –     | –     | Baidarskaya (Crimea) |

(continued on next page)
| Isolate code | Species | 16S | COI | 28S | NaK | Origin |
|-------------|---------|-----|-----|-----|-----|--------|
| 61Y         | *Tauronethes* sp. | ON220078 | –  | –  | –  | Bakhchsarai (Crimea) |
| 62Y         | *Thaumatoniscellus speluncae* | ON220077 | –  | –  | –  | Sevastopol (Crimea) |
| 176z        | Karaman, Bedek, & Horvatović, 2009 | –  | –  | –  | ON227383 | Skadanščina (Slovenia) |
| 42T         | *Trichonethes kosswigi* Strouhal, 1953 | ON220080 | ON212617 | –  | –  | Cave Karami Archangelo (Rhodes, Greece) |
| 66Y         | *Trichoniscus aphonicus abchasicus* Borutzky, 1977 | ON220081 | ON212570 | –  | –  | Sukhum (Abkhasia) |
| 75Y         | *Trichoniscus aphonicus abchasicus* Borutzky, 1977 | ON220083 | ON212569 | –  | –  | Sukhum (Abkhasia) |
| 64Y         | *Trichoniscus aphonicus codoricus* Borutzky, 1977 | ON220084 | ON212563 | –  | –  | Ochamchira (Abkhasia) |
| 65Y         | *Trichoniscus aphonicus codoricus* Borutzky, 1977 | –  | ON212566 | –  | –  | Ochamchira (Abkhasia) |
| 68Y         | *Trichoniscus aphonicus codoricus* Borutzky, 1977 | –  | ON212564 | –  | –  | Ochamchira (Abkhasia) |
| 69Y         | *Trichoniscus aphonicus codoricus* Borutzky, 1977 | ON220085 | ON212580 | –  | –  | Ochamchira (Abkhasia) |
| 70Y         | *Trichoniscus aphonicus codoricus* Borutzky, 1977 | ON220086 | ON212565 | –  | –  | Ochamchira (Abkhasia) |
| 47T         | *Trichoniscus cavernicola* Vandel, 1958 | ON220087 | ON212522 | –  | –  | Pathole Xepatomeni Latsiola, Crete (Greece) |
| 71Y         | *Trichoniscus cf. aphonicus abchasicus* Borutzky, 1977 | ON220088 | –  | –  | –  | Sukhum (Abkhasia) |
| 72Y         | *Trichoniscus cf. aphonicus codoricus* Borutzky, 1977 | ON220089 | ON212567 | –  | –  | Gudauta (Abkhasia) |
| 67Y         | *Trichoniscus cf. gudauticus* Borutzky, 1977 | ON220090 | ON212571 | –  | –  | Ochamchira (Abkhasia) |
| 73Y         | *Trichoniscus cf. gudauticus* Borutzky, 1977 | –  | ON212564 | –  | –  | Gudauta (Abkhasia) |
| 59T         | *Trichoniscus fragilis* Racovitza, 1908 | ON220091 | ON212521 | –  | –  | Varathro Stenou Logiou, Crete (Greece) |
| 1T          | *Trichoniscus intermedius* Vandel, 1958 | ON220092 | –  | –  | –  | Pothole Xylouri Tafkos, Crete (Greece) |
| 2T          | *Trichoniscus lindbergi* Vandel, 1958 | ON220095 | ON212523 | –  | –  | Pathole tafkos xepatomenos, Crete (Greece) |
| 20T         | –  | ON220093 | ON212613 | –  | –  | Pathole Dalamoutou, Crete (Greece) |
| 25T         | –  | ON220094 | –  | –  | –  | Pothole Tafkos Myristis, Crete (Greece) |
| 4T          | –  | ON220096 | ON212612 | –  | –  | Cave Geranion, Crete (Greece) |
| 5T          | –  | ON220097 | –  | –  | –  | Cave Kamilari Tylisos, Crete (Greece) |

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| Isolate code | Species | 16S | COI | 28S | NaK | Origin |
|--------------|---------|-----|-----|-----|-----|--------|
| 23z*         | Trichoniscus provisorius Racovitza, 1908 | –   | –   | MN174834 | MN234259 | Aphrodite’s baths (Cyprus) |
| 33z*         | –       | –   | MN174836 | MN234253 | Pitargou (Cyprus) |
| 21T          | Trichoniscus pusillus Brandt, 1833 | ON220098 | – | – | – | Cave Peristeri (Peloponese) |
| 8T           | ON220099 | – | – | – | – | Cave Perama (Greece) |
| 9T           | ON220102 | – | – | – | – | Pathole Votsos |
| 24T          | ON220100 | – | – | – | – | Pothole Megalos Votsos (Greece) |
| 6T           | ON220101 | – | – | – | – | Pathhole Maratholakou Liliano (Greece) |
| 107cy*       | –       | ON212549 | MN174835 | MN234286 | – | Afrodite’s baths (Pafos) |
| 74Y          | ON220103 | – | – | – | – | Gulipsh (Abkhasia) |
| 177z         | Troglocyphoniscus absoloni | ON220104 | ON212576 | – | – | Lumbarda (Croatia) |
| 178z         | Strouhal, 1939 | ON220105 | ON212575 | ON312074 | ON227379 | Blato (Croatia) |
| 6z*          | Mesoniscus alpicola (Heller, 1858) | ON220042 | – | MN174829 | MN234249 | Lombardia (Italy) |
| 133z*        | Helleria brevicornis Ebner, 1868 | – | ON212510 | MN174843 | MN234285 | Croix-Valmer (France) |
| 138z         | Tylus ponticus Grebnicki, 1874 | – | – | ON312075 | ON227388 | Konnos (Cyprus) |
| 37cy         | –       | – | ON312078 | ON227386 | – | Governor’s Beach (Cyprus) |
| 59cy         | –       | – | ON312079 | ON227387 | – | Konnos (Cyprus) |
| 210z         | –       | – | ON312076 | ON227389 | – | Cape Greco (Cyprus) |
| 211z         | –       | – | ON312077 | ON227389 | – | Cape Greco (Cyprus) |
| 212z*        | –       | – | MN174844 | MN234265 | – | Cape Greco (Cyprus) |
| Ligidiidae   |         |     |     |     |     |        |
| 51Y          | Ligidium cavaticum Borutzky, 1950 | ON220035 | – | – | – | Krasnodar (Caucasus) |
| 53Y          | ON220036 | – | – | – | – | Krasnodar (Caucasus) |
| 54Y          | ON220037 | ON212611 | – | – | – | Krasnodar (Caucasus) |
| 57Y          | ON220038 | – | – | – | – | Krasnodar (Caucasus) |
| 58Y          | ON220039 | – | – | – | – | Krasnodar (Caucasus) |
| 59Y          | ON220040 | – | – | – | – | Morcheka Mt. (Crimea) |

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| Isolate code | Species | 16S | COI | 28S | NaK | Origin          |
|-------------|---------|-----|-----|-----|-----|-----------------|
| 136z        | Ligidium ghigii Arcangeli, 1928 | –   | –   | –   | MN174818 | MN234284 | Naxos (Greece) |
| 61cy*       | Ligia italica Fabricius, 1798   | –   | –   | –   | MN174819 | MN234250 | Konnos (Cyprus)|
| 188z        | Tauroligidium cf. stygium       | –   | –   | –   | –   | –   | Baidarskaya (Crimea) |
| 189z*       | Borutzky, 1950                  | –   | –   | –   | MN174820 | MN234270 | Baidarskaya (Crimea) |
| 190z*       |                           | –   | –   | –   | –   | –   | Baidarskaya (Crimea) |
| 191z        |                           | –   | –   | –   | –   | –   | Baidarskaya (Crimea) |
| 21Y         |                           | ON220058 | ON212598 | ON312060 | –   | –   | Bakhchsarai (Crimea) |
| 22Y         |                           | ON220059 | ON212604 | –   | –   | –   | Bakhchsarai (Crimea) |
| 23Y         |                           | ON220060 | –   | ON312061 | –   | –   | Bakhchsarai (Crimea) |
| 24Y         |                           | –   | ON212603 | ON312062 | –   | –   | Bakhchsarai (Crimea) |
| 25Y         |                           | ON220061 | –   | ON312063 | –   | –   | Bakhchsarai (Crimea) |
| 26Y         |                           | ON220062 | ON212610 | –   | MN234256 | –   | Bakhchsarai (Crimea) |
| 27Y*        |                           | ON220063 | ON212607 | ON312064 | –   | –   | Bakhchsarai (Crimea) |
| 29Y         |                           | ON220064 | ON212597 | MN174821 | MN234255 | –   | Bakhchsarai (Crimea) |
| 31Y         |                           | ON220065 | –   | ON312065 | –   | –   | Bakhchsarai (Crimea) |
| 32Y         |                           | ON220066 | ON212609 | ON312066 | –   | –   | Bakhchsarai (Crimea) |
| 36Y         |                           | ON220067 | ON212606 | ON312067 | –   | –   | Bakhchsarai (Crimea) |
| 37Y         |                           | ON220068 | ON212608 | ON312068 | –   | –   | Bakhchsarai (Crimea) |
| 38Y         |                           | ON220069 | ON212605 | ON312069 | –   | –   | Bakhchsarai (Crimea) |
| 40Y         |                           | –   | ON212594 | –   | –   | –   | Bakhchsarai (Crimea) |
| 42Y         |                           | ON220070 | –   | –   | –   | –   | Bakhchsarai (Crimea) |
| 44Y         |                           | ON220071 | –   | –   | –   | –   | Bakhchsarai (Crimea) |
| 198z        | Tauroligidium stygium Borutzky, 1950 | ON220072 | ON212591 | ON312070 | –   | –   | Bakhchsarai (Crimea) |
| 199z        |                           | ON220073 | –   | ON312071 | –   | –   | Bakhchsarai (Crimea) |
| 39Y         | Typhloligidium cf. karabijajae | –   | ON212592 | –   | –   | –   | Belogorsk (Crimea) |
| 48Y         | Borutzky, 1962             | –   | ON212585 | –   | –   | –   | Belogorsk (Crimea) |

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| Isolate code | Species                                      | 16S  | COI            | 28S  | NaK  | Origin                  |
|--------------|---------------------------------------------|------|----------------|------|------|-------------------------|
| 192z         | *Typhloligidium coecum* (Carl, 1904)         | ON220108 | ON212588      | –    | –    | Simferopol (Crimea)     |
| 20Y          |                                             | ON220109 | ON212595      | ON312080 | –    | Simferopol (Crimea)     |
| 28Y          |                                             | –      | ON212586      | –    | –    | Simferopol (Crimea)     |
| 33Y          |                                             | ON220110 | ON212589      | ON312081 | –    | Simferopol (Crimea)     |
| 35Y*         |                                             | ON220111 | ON212584      | ON312082 | –    | Simferopol (Crimea)     |
| 45Y*         |                                             | ON220112 | ON212587      | MN174822 | –    | Simferopol (Crimea)     |
| 25Y          | *Typhloligidium karabijajae* Borutzky, 1962 | ON220113 | ON212601      | ON312083 | –    | Belogorsk (Crimea)      |
| 34Y          |                                             | ON220114 | ON212602      | ON312084 | –    | Belogorsk (Crimea)      |
| 41Y          |                                             | ON220115 | ON212590      | –    | –    | Belogorsk (Crimea)      |
| 43Y          |                                             | ON220116 | –             | –    | –    | Belogorsk (Crimea)      |
| 195z         | *Typhloligidium kovali* Gongalsky & Taiti, 2014 | ON220117 | ON212581      | ON312085 | –    | Kabardino-Balkaria (Caucasus) |
| 47Y          |                                             | ON220118 | ON212579      | –    | –    | Kabardino-Balkaria (Caucasus) |
| 19Y          | *Typhloligidium lithophagum* Turbanov & Gongalsky, 2016 | ON220119 | ON212582      | ON312086 | –    | Simferopol (Crimea)     |
| 46Y          |                                             | ON220120 | ON212583      | –    | –    | Simferopol (Crimea)     |
| 50Y*         | *Typhloligidium* sp.                        | ON220121 | ON212600      | MN174823 | MN234251 | Martvili (Georgia)     |
| 56Y          |                                             | ON220122 | –             | –    | –    | Martvili (Georgia)      |
| 49Y          |                                             | –      | ON212599      | –    | –    | Martvili (Georgia)      |
| A. Asellota  | *Asellus aquaticus* Linnaeus, 1758           | –    | –             | MN174846 | MN234267 | Kozani (Greece)        |
| Asellidae    |                                             | –    | –             | MN174840 | MN234263 | Ansedonia (Italy)      |
| 209z*        |                                             | –    | –             | MN174839 | MN234264 | Ansedonia (Italy)      |
| Valviferida  | *Idotea chelipes* Pallas, 1766              | –    | –             | MN174842 | MN234262 | Ansedonia (Italy)      |
| Idoteidae    |                                             | –    | –             | MN174841 | MN234261 | Ansedonia (Italy)      |
| 213z*        |                                             | –    | –             | MN174840 | MN234263 | Ansedonia (Italy)      |
| 214z*        |                                             | –    | –             | MN174839 | MN234264 | Ansedonia (Italy)      |
| Sphaeromatidea| *Sphaeroma serratum* Fabricius, 1787       | –    | –             | MN174842 | MN234262 | Ansedonia (Italy)      |
| Sphaeromatida|                                             | –    | –             | MN174841 | MN234261 | Ansedonia (Italy)      |
| 215z*        |                                             | –    | –             | MN174842 | MN234262 | Ansedonia (Italy)      |
| 216z*        |                                             | –    | –             | MN174841 | MN234261 | Ansedonia (Italy)      |
Conclusion

Taking advantage of the universal and cost-effective nature of the proposed protocol a new potential for the production of genetic data could be foreseen. Based on this protocol retrieved data could be included in multiple datasets given that the same gene regions are targeted. Beyond the data published within the framework of this study, scientific research in related fields such as Isopod systematics, evolution, phylogeny e.t.c. could be facilitated by the application of the proposed protocol. It is worth noting that the majority of available specimens were collected more than two decades ago and a considerable number of them were ill-preserved for a long time (i.e., in 70% alcohol or formaldehyde). Hence, the absence of data in some cases might be attributed to the bad quality of extracted DNA or the lack of effort since we did not aim to sequence all loci for all specimens.

Data availability

All data were deposited in NCBI Genbank. Accession numbers are provided in the main text.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data were deposited in NCBI Genbank. Accession numbers are provided in the main text.

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