Genetic evidence against monophyly of Oniscidea implies a need to revise scenarios for the origin of terrestrial isopods

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Among the few crustacean taxa that managed to inhabit terrestrial environments, Oniscidea includes the most successful colonizers in terms of species richness and abundance. However, neither morphological traits nor molecular markers have definitively resolved phylogenetic relationships among major Oniscidea clades or established the monophyly of the taxon. Herein, we employed the highly conserved, nuclear protein-coding genes Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK), along with the traditionally used 18 s and 28 s ribosomal RNA genes, in an attempt to clarify these questions. Our dataset included sequences representing all major Oniscidea clades and closely related aquatic taxa, as suggested by previous studies. We applied Bayesian Inference and Maximum Likelihood methods and produced a robust and fully resolved phylogenetic tree that offers strong evidence against the monophyly of Oniscidea. The amphibious genus Ligia appears to be more closely related to representatives of marine suborders, while the phylogenetic pattern of the remaining Oniscidea implies a complex history of the transition from the marine environment to land. With the exception of the basal clade, all other established major clades have been recovered as monophyletic, even though relationships within these clades call for a revised interpretation of morphological characters used in terrestrial isopod taxonomy.

Among the 11 suborders currently identified in Isopoda, Oniscidea is the only terrestrial suborder and by far the richest, comprising more than 3,700 described species.2,3 Despite their generally limited dispersal abilities and their ancestors' dependence on aquatic environments, they managed to extend their presence all over the globe and inhabit most types of habitats, including deserts.4

According to current taxonomy, terrestrial isopods are divided into five main clades, with the more basal ones exhibiting behavioural, ecological and morphological traits related to aquatic environments.5,6 The more apical clades are generally more species-rich and more diverse, reflecting acquisition of vital adaptations to terrestrial environments that allowed them to conquer a wide range of habitats. According to the most widely accepted phylogeny based on morphological traits, proposed by Erhard, Oniscidea are divided in five major clades based on their morphological adaptations to terrestrial life and, hence, their dependence on the aquatic environment. In more detail, Diplocheta, is the most basal clade, exhibiting a series of morphological characters that suggest the form of the possible marine ancestor.6 The two apical sister-clades are Crinocheta and Synocheta, while Microcheta constitutes their very species-poor sister-clade and Tylida have a more basal position in-between Microcheta and the 'less terrestrial' basal Diplocheta. Schmidt proposed a more elaborate classification, reflecting assumed phylogenetic relationships, according to which there is a basal split into Ligiiidae and Holoverticata, which in turn split into Tylidae and Orthogonopoda, which consists of Mesoniscus Carl, 1906 and Euoniscoidea. The latter comprises the two major clades Synocheta and Crinocheta. Some of the most important characters that differ among taxa belonging to the major basal clades of Oniscidea are shown in Figs. 1–4. In particular, Figs. 1 and 2 show characters of the major genera in Ligiidae, Fig. 3 shows one of the two genera in Tylidae, and Fig. 4 shows the only genus in Microcheta.

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The phylogenetic position of Oniscidea within Isopoda has been based mainly on morphological characters with controversial results so far, even regarding their monophyly. Brusca and Wilson proposed Calabozoidea as sister group of Oniscidea, while Tabacaru and Danielopol suggested Valvifera as the sister group. Dreyer and Wägele conducted a molecular phylogeny based on one nuclear DNA marker and proposed Scutocoxifera as a monophyletic clade including Oniscidea, Valvifera, Sphaeromatidea, Anthuridea and Cymothoida, with Oniscidea as the basal clade in the group.

The monophyly of Oniscidea has been supported by several, presumably well-documented synapomorphies. The most important of these are: (1) the water conducting system, formed by scales on the ventral side of coxal plates, (2) the relatively short pleotelson, (3) an antennula with less than four articles, (4) the absence of the mandibular palp, (5) the occurrence of setae on the mandible, (6) the presence of only one moveable sclerite on the basis of the second maxilla, (7) a single coxal sclerite on the maxilliped, (8) a non-subchelate first pereopod, and (9) the occurrence of scale-setae on tergites. Nevertheless, Michel-Salzat and Bouchon, based on mtDNA markers and a similarity-based tree, suggested that Ligia Fabricius, 1798 (Diplocheta, Ligiidae) is closer to Valvifera, and Tylos Audouin, 1826 (Tylida) to Sphaeromatidea than to the other Oniscidea. A more recent study by Lins et al. arrived at similar conclusions, using a Bayesian Inference approach in the analysis of two datasets, one consisting of 18 s and 28 s rRNA and COI sequences, and one comprising 13 mitochondrial protein-coding genes, but for a limited number of specimens. In both cases, Ligia and Tylos (included only in the first dataset) were not included in the statistically well-supported group formed by the rest of Oniscidea. Unlike Tylos, represented by Helleria Ebner, 1868, whose close evolutionary relationship has strong statistical support, the monophyly of Ligidae is not well supported.

Furthermore, based solely on morphological characters, Vandell had proposed a repetitive invasion of isopods from aquatic to terrestrial environments that happened at least three times. More specifically, Vandell had suggested that terrestrial isopods should be divided into three lineages: (i) "Tylienne" (= Tylida - restricted to coastal areas), (ii) "Trichoniscienne" (= Trichonisidae + Styloniscidae - restricted to humid micro-habitats), and (iii) "Ligienne", which includes all remaining taxa that originated from an ancestor similar to the modern amphibious genus Ligia. The hypothesis that Tylos is more closely related to aquatic ancestors than the rest of Oniscidea was also supported by Tabacaru and Danielopol. Nevertheless, this hypothesis was based exclusively on a single morphological character (i.e., clearly distinct coxal plates from tergites, see Fig. 3A). Overall, it is
widely believed that the transition from marine to terrestrial environment was direct, without an intermediate freshwater stage.19–21.

Herein, we aim to investigate the phylogenetic relationships among major clades of Oniscidea, in order to evaluate the validity of current taxonomy and discuss issues related to the origins of terrestrial isopods. For this purpose, in addition to the traditionally used 18s and 28s ribosomal RNA genes, we also targeted the highly conserved, thus suitable for the resolution of deep phylogenies, protein-coding Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK)22–24 genes.
Results

Extracted DNA concentration was >15 ng/μl in all cases, with the A260/A280 purity rate over 1.5. Attempts to amplify and sequence all targeted loci were successful for almost all samples. The final compiled aligned dataset after Gblocks treatment consisted of 1,984 base pairs (bp). The initial alignment lengths and numbers of conserved, variable and parsimony-informative sites are shown in Table 1 for all sequenced loci separately. Among the tested models, the highest Akaike weight values, indicating the best fit to data, were exhibited by TIM2ef + I + G for 18 s, TIM3 + G for 28 s, TIM2 + I + G for NAK, and GTR + G for PEPCK.

Prior to calculation of genetic divergence, available sequences were grouped at the suborder level and those of Oniscidea were further grouped into the five known major subclades. *Ligia* specimens were grouped separately from the rest of the Diplocheta, as they appear to form a separate clade on the produced phylogenetic tree (Fig. 5). Genetic distances between examined taxa appeared to be constantly higher for ribosomal genes compared to the protein-coding ones. Genetic variation ranged between 6.6–30.2% in the case of 18 s, 33.3–71.6% for 28 s, 16.7–30.6% for NAK and 19.3–29.5% for PEPCK. The minimum and maximum genetic divergence values were not constantly found between the same groups for all genetic markers. More specifically, the maximum genetic distance was found between Tylida-Crinocheta, Sphaeromatidae-Crinocheta, Asellota-Valvifera and Asellota-Crinocheta, whereas the minimum values were identified between Asellota-Phreatoicidea, Tylida-Mesoniscus, Ligia-Sphaeromatidae and Valvifera-Diplocheta (excluding *Ligia*) in the case of 18 s, 28 s, NAK and PEPCK genes, respectively. All within- and between-group p-distances are given in Supplementary Material.

The Bayesian Inference (BI) and Maximum Likelihood (ML) trees exhibited largely congruent topologies. Nevertheless, in some cases, high BI posterior probabilities did not coincide with high ML bootstrap values (>80). This can be attributed to the fact that, in contrast to BI, the ML method implemented in available softwares (e.g. RAxML, PhyML, IQ-TREE) perceives gaps (−) and missing data (given as N or ? in DNA alignments) as

| Gene   | Alignment length (bp) Before Gblocks Treatment | After Gblocks Treatment | Conserved sites | Variable sites | Parsimony informative sites |
|--------|-----------------------------------------------|------------------------|----------------|---------------|---------------------------|
| 18 s   | 1031                                          | 532                    | 373            | 479           | 287                       |
| 28 s   | 1857                                          | 297                    | 221            | 1,055         | 666                       |
| NAK    | 639                                           | —                      | 303            | 256           | 639                       |
| PEPCK  | 516                                           | —                      | 247            | 261           | 214                       |

Table 1. Aligned bases length, before and after GBlocks treatment (for ribosomal genes), conserved, variable and parsimony-informative sites for all genes used in this study.

Figure 4. *Mesoniscus alpicola* (Heller, 1858) from San Martino cave, Varese, Lombardy, Italy, ♂: (A) adult specimen, dorsal; (B) uropod, (C) pereopod 7; (D) pleopod 1; (E) pleopod 2. Figures drawn by Taiti using the method by Montesanto52,53.
unknown characters that do not provide additional information for the resolution of phylogenetic relationships. Two out of four targeted loci are coding rRNAs whose three-dimensional structure is dependent on highly conserved regions which are interrupted by variable regions accumulating mutations, including indels. These regions are not under strong evolutionary pressure and, hence, mutations can explain the occurrence of gaps in final alignments. On the other hand, the BI approach takes into account insertion and deletion events that contain phylogenetically useful information. Therefore, only the BI tree is presented herein (Fig. 5).

Holoverticata (sensu Schmidt) is recovered as a well-supported clade, containing the traditionally recognised sub-clade structure: Crinocheta and Synocheta form two well-supported, monophyletic sister clades, and Microcheta is the intermediate clade of these and the more basal, monophyletic Tylida. Nevertheless, Diplocheta (hence, also Ligiidae) appear to be polyphyletic, with Ligia being the sister taxon of Valvifera + Sphaeromatidea, and the genera Ligidiidae, 1833, Tararoligidiidae Bourtzky, 1950 and Typhloligidiidae Verhoeff, 1918, traditionally grouped in Ligiidae, forming a well-supported monophyletic group, as the sister clade of Holoverticata. The monophyly of Oniscidea as currently defined is questioned, and could be saved if Ligia is excluded from the taxon. The basal position of Colubotelson Nicholls, 1944 (Phreatoicidea) and Asellus Geoffroy, 1762 (Asellota), as well as the statistically supported retrieval of Valvifera and Sphaeromatidae within the ‘Onisicidea’ clade, indicates the closer relationship of terrestrial isopods with these two suborders. Phylogenetic relationships inside Crinocheta also show some interesting patterns with important implications for oniscidean taxonomy. Porcellionidae form a well-supported clade with Trachelipodidae and part of Agnaridae (as the latter appear to be polyphyletic), while Armadillidiidae, traditionally considered sister-group of the Porcellionidae, is grouped with representatives of other families (e.g., Cyclidae and part of Agnaridae). Also, Platyarthridae Brandt, 1833 and Trichorhina Budde-Lund, 1908, presently included in the family Platyarthridae, do not seem to be related, and the representative of the most diverse family Armadillidae appears in a more basal position within Crinocheta. Within Synocheta, the monophyly of Trichoniscidae is not supported, as Styloniscus Dana, 1852, type-genus of Styloiscidae, seems to fall within the former. Moreover, no support for the monophyly of the subfamilies Trichonisca and Haplothalminadae could be found.

**Discussion**

This is the first time that nuclear protein-coding genes are used to resolve phylogenetic relationships among major groups of Oniscidea. The fact that this study is so far the only one that produced a fully resolved and robust molecular phylogeny of all five major oniscidean clades, proves the advantages of using these markers. NAK has been used before in terrestrial isopod phylogenetics, but at a lower taxonomic level. Of course, given the depth of phylogeny attempted herein, the use of mitochondrial genes, with their high mutation rates and, hence, saturation effects, is not appropriate. Also, the use of untreated nuclear ribosomal genes sequences, such as of 18s and/or 28s, might have led to biased or insufficiently supported results, as they contain regions that evolve at very different rates. Gblocks treatment was recruited to overcome possible issues that may arise due to the properties of these regions. Herein, we managed to produce a robust and sufficiently inclusive phylogeny of terrestrial isopods.
using a more reliable data set of nuclear DNA markers. This phylogeny has important implications for oniscidean systematics, as it undermines the validity of several morphological characters traditionally used in terrestrial isopod taxonomy. The transition of isopods from the marine to the terrestrial environment might also need to be reevaluated in light of the new evidence.

A number of unique adaptations to terrestrial life have led authors to assume that Oniscidea underwent only one transition from marine to land\textsuperscript{4,27}. However, the low number of studies using molecular data in the past failed to confirm the monophyly of Oniscidea\textsuperscript{5,16}, but also failed to provide a consistent phylogenetic pattern\textsuperscript{28,29}. According to the results of our analysis, the monophyly of Oniscidea, as currently defined, is not supported, since the genus \textit{Ligia}, generally considered as con-familiar with \textit{Ligidium} and a small number of other related taxa, none of which exploit littoral environments, appears to be a closer relative of a group of marine isopods, such as the Valvifera and Sphaeromatidae. The monophyly of Oniscidea could be saved if \textit{Ligia} is excluded. The assumed synapomorphies of ‘Ligidae’, such as the residual maxillipeds segment at the back of the cephalon, are rather symplesiomorphies, as has been previously suspected. \textit{Ligidium} and related genera of the polyphyletic family Ligidae could be assigned to a new family (we propose Ligidiidae, from the most speciose genus \textit{Ligidium}) that can be more safely defined by more reliable synapomorphies, such as the shape of the uropods with the endopod inserted distally compared to the exopod (cf. Figs. 1B and 2B). The genus \textit{Ligidioides} Währberg, 1922 (not included in our analysis) has a uropod more similar to that of \textit{Ligia}, i.e., with the insertions of the endopod and exopod at the same level\textsuperscript{30}, and might remain in the family Ligidae, but this has to be investigated by a future molecular analysis that also includes this genus. Lins \textit{et al.}\textsuperscript{31} came to similar conclusions regarding the relationships of \textit{Ligia} with marine taxa, but these authors did not include other Ligidae in their analysis, so they could not discuss the monophyly of the family. A common evolutionary history of the mitochondrial genomes of \textit{Ligia} and \textit{Idotea} Fabricius, 1798 was highlighted also by Kilpela\textit{et al.}\textsuperscript{32}. The high genetic divergence between \textit{Ligia} and \textit{Ligidium} was also evident from their distant position in the phenetic tree presented by Michel-Salza\textit{et al.}\textsuperscript{33}. Our findings are in agreement with all of these studies, a fact that further corroborates our hypothesis.

In view of the new phylogeny, the critical question regarding the transition from the marine environment to land should be addressed by taking into account the ecology of species in the major clades and, most importantly, the fact that the relevant event(s) happened sometime in the middle or even lower Mesozoic\textsuperscript{27}, so that a large number of crucial forms might have been extinct without leaving any fossils of ancestral lineages. In fact, the oldest fossil Oniscidea are much younger and consist of highly derived forms\textsuperscript{34}, while coastal marine or amphibious forms of animals that do not have hard skeletons, shells or teeth, are rarely fossilized anyway.

Considering that: (a) the most basal clade (Diplocheta, excluding \textit{Ligia}) consists of freshwater-related taxa, (b) the subsequent clade (Tylida) includes taxa mostly living along marine coasts (even though the genus \textit{Helleria} is fully terrestrial), and with a divergent morphology compared to other Oniscidea (at least regarding the form of the cephalon, the distinct epimera on most thoracic segments, and the unique type of respiratory structures on pleopods, not connected to those of other taxa, see Fig. 3), and (c) Microcheta are fully terrestrial (albeit dependent on very high humidity) and they exhibit an overall morphology closer to that of the more derived Oniscidea (see Fig. 4), one might consider revisiting scenarios regarding the transition of isopods form the marine environment to land. Even though most \textit{Ligia} species are amphibious, there are some species that live inland\textsuperscript{33}–\textsuperscript{36}. This means that we might envision a similar but independent transition that led to the common ancestor of ‘Ligidae’, given that this group consists today of species mostly living in close connection to freshwater. On the other hand, Tylidae might represent another transition, since they exhibit many characters that are difficult to recreate via a plausible transformation series from Diplocheta-type characters (cf. Figs. 1, 2 and 3). If this proves true, the next clade, Microcheta, which is basal to all Orthogonopoda, connected to very humid, freshwater-related habitats and with a more differentiated morphology than Tylida in many characters (cf. Figs. 3 and 4), would represent a third invasion to land, maybe using a freshwater path. Of course, this would undermine the actual monophyly of Oniscidea.

On the basis of current evidence, this is only a tentative hypothesis that has to be evaluated through careful elaboration of physiological traits and, hopefully, further fossil findings. Obviously, the very old origins of the Oniscidea\textsuperscript{27}, coupled with the difficulty of fossilization of these organisms, might have led to the permanent loss of crucial information from several basal clades representing possible direct ancestors of terrestrial forms. The phylogenetic reconstruction based on modern forms cannot recover such extinct clades, except in the case of some exceptional, but highly unlikely, fossils being found in the future.

The monophyly of Crinocheta and Synocheta seems to be unambiguous. The hypothesis by Tabacaru and Danielopol\textsuperscript{37} that Synocheta is a sister taxon with Mesoniscidae cannot be supported. The phylogenetic relationships inside the two major clades reveal that certain morphological characters that have been considered important in oniscidean taxonomy, such as the type and form of pleopodal lungs, the ornamentation of tergites or the shape of uropods, might not be very useful. In particular, Porcellionidae and Armadillidiidae, even though they seem to share a similar type of pleopodal lung, at least in comparison with that in Trachelipodidae, appear to belong to distant clades; the former related to Trachelipodidae and part of Agnaridae (the monophyly of which is not supported), and the latter to Cylistidae and other families. This is in agreement with the recent findings by Dimitriou \textit{et al.}\textsuperscript{38}. In turn, Cylisticidae appears to be closer to Armadillidiidae, even though they have styliform uropods. Within Synocheta, the traditional distinction between Trichoniscinae and Haplophthalminae, based largely on the presence of ornamentation on tergites, does not seem to be supported since \textit{Calconiscellus Verhoeff, 1927}, a member of Haplophthalminae, appears to be the sister-taxon of \textit{Caucasosethes Verhoeff, 1932} and nested within other genera of Trichoniscinae. Furthermore, the status of Styloniscidae as a separate family from Trichoniscidae is also undermined. More detailed analyses, using more extensive taxonomic sampling inside these clades, are necessary to clarify these issues.

The closer relationship of terrestrial isopods with Valvifera and Sphaeromatidae than with Asellota or Phreatoicidea, revealed by our analysis, agrees with the hypothesis of Brusca and Wilson\textsuperscript{39}. Using a more detailed phylogenetic analysis, future studies should also address the question of the monophyly of \textit{Phreatoidea}, considering that \textit{Phreatoidea} is known to include both terrestrial and semi-aquatic species. However, the recent molecular results of \textit{Phreatoidea}\textsuperscript{38} demonstrated that \textit{Phreatoidea} is not a monophyletic group and that the closest relative of \textit{Phreatoidea} is not \textit{Phreatoidae}. Therefore, a more detailed study of the relationships inside Synocheta and the status of Phreatoidea is needed.
In conclusion, Oniscidea should not be considered monophyletic. Systematics in this very old group, which presents an amazing case of animal invasions to land, are in urgent need of extensive revision, taking into account robust molecular evidence. New techniques, such as whole genome sequencing, transcriptomics and ultra-conserved elements, should be applied to the whole range of terrestrial isopod taxa, in order to resolve the complete phylogenetic history of the group and shed light on crucial questions regarding the evolution of terrestriality in this taxon. Modern terrestrial isopoda is probably the only animal taxonomic group lower than Class that includes representatives of most steps of the transition from aquatic environments to almost all terrestrial environments, despite the presumed large number of extinct forms. Furthermore, considering the fact that these animals have evolved structures analogous to the complex organs of terrestrial vertebrates, such as lungs (pleopodal lungs) and the placenta (marsupial, egg-feeding ‘cotyledons’), a detailed phylogenetic reconstruction can provide invaluable information on many exciting aspects of evolutionary biology, but also physiology, behaviour, ecology, and several other fields.

Methods

Sample collection. Using both field collecting, deposited and loaned material, we compiled a data set including 34 Oniscidea species, representing 30 genera and 14 families. Moreover, non-Oniscidea specimens of Valvifera (Idotea), Sphaeromatidea (Sphaeroma Bosc, 1801) and Asellota (Asellus) were also included. Colleagues that kindly sent us material are mentioned in the Acknowledgements. Freshly collected specimens, as well as the majority of available museum specimens were placed in 96% ethanol until further laboratory procedures, but we also managed to retrieve genetic data from specimens preserved in 70% alcohol for a relatively long period. Detailed information about specimens is given in Table 2.

Amplification of targeted loci. Total genomic DNA was extracted from available specimens using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer’s proposed protocol. Quality and quantity control of extracted DNA was performed with NanoDrop 2000/200c (Thermo Fisher Scientific Inc., USA). The final concentration was measured in ng/μl and purity was verified with A260/A280nm absorption ratio.

The non-coding nuclear genetic markers 18s and 28s, and the protein-coding Sodium-Potassium Pump (NAK) and Phosphoenolpyruvate Carboxykinase (PEPCK) genetic loci were targeted with common PCR procedures using gene specific primers. Desired regions were successfully amplified using 18Aimod/700 R primer pair for 18s26, 28s/28sb for 28s39, NAK for-b/NAK rev 2 or NAK for-b/NAK 638 R for NAK24,25 and PEPCKfor/PEPCKrev24 and the newly designed PEPCK 545 R (5′-CCRAAGAANGGYSTCATNGC-3′) for PEPCK. All PCR reactions were carried out in a Veriti thermal cycler (Applied Biosystems, USA). Taking into account the genetically diverse samples, we used a touchdown PCR approach to eliminate aspecific products and save time, opposed to using multiple reactions, specific for different taxa. This way we managed to increase specificity, sensitivity and yield40. In each case, the final reaction volume was adjusted to 20μl, including 0.5 U of Kapa Taq DNA Polymerase, 3 mM MgCl2, 1X of Kapa PCR buffer A, 0.3 mM dNTP (Kapa) 0.3 μl, including 0.5 U of Kapa Taq DNA Polymerase, 3 mM MgCl2, 1X of Kapa PCR buffer A, 0.3 mM dNTP (Kapa) 0.3 μM of each primer and >20 ng of DNA template. The reactions’ thermal profile followed Dimitriou et al.25. Amplicons were purified with a Qiaquick Purification Kit (Qiagen, Germany) following the proposed instructions. The final products were sent for sequencing of both DNA strands at Macrogen facilities (Amsterdam, The Netherlands).

Data processing. CodonCode Aligner (v. 3.7.1; CodonCode Corp., USA) was used to manually inspect chromatograms, generate assemblages and make edits, where necessary. Our final dataset also included sequences of additional Ligia spp. and Colubotelson thomsoni Nicholls, 1944 (Phreatoicidea) retrieved from NCBI GenBank. The latter was included to serve as an additional outgroup. In the case of the genus Ligia, apart from the data generated in the framework of the present study, a chimeric sequence combining data from all targeted genes from the congenic species L. oceanica (Linnaeus, 1767), L. hawaiensis (Dana, 1853) and L. exotica Roux, 1828 was included in our analyses. In this way, we manage to verify the phylogenetic position of the genus in the produced tree in a robust way. Accession numbers of all sequences used herein are given in Table 2. Sequences from each targeted gene were separated in different files and multiple sequence alignments were performed with MAFFT v.741. MEGA v.642 was used to calculate genetic distances for each alignment. Relatively longer sequences with no overlapping fragments for the majority of the samples were trimmed prior to further data elaboration.

Given that ribosomal genes consist of multiple conserved and flanking hypervariable regions, related to their functional three-dimensional structure after gene expression, alignment might be challenging44. In order to test the sensitivity of produced alignments and remove possibly aligned regions for 18s and 28s genes, we used Gblocks v.0.91b44 through the Gblocks server available at http://molevol.cimm.cscs.es/castresana/Gblocks_server.html. The analysis was run allowing smaller final blocks, less strict flanking and gap positions. The positive effects of removing divergent and ambiguously-aligned blocks in phylogenies are discussed by Talavera and Castresana45.

Phylogenetic analyses. The optimal nucleotide substitution model for each loci was selected according to Akaike’s Information Criterion (AIC)46 using jModeltest v.2.1.147. Phylogenetic reconstructions were conducted with BI and ML methods implemented in MRBAYES v.3.2.648 and RAxML-NG web server49 respectively.

The concatenated data set was fed as partition blocks to MrBayes. Metropolis-Inference analysis was run with the selected model of nucleotide evolution for each gene, under the default settings for within-partition among-site rate variation, allowing rate heterogeneity between partitions. BI, applying Metropolis-coupled Markov Chain Monte Carlo algorithms, was set to run four independent times with eight chains per run for 20 million generations and a sampling frequency of 100. Stationarity and convergence among runs, were ensured by monitoring the standard deviation of split frequencies of the four simultaneous and independent runs in MrBayes.
| Species                              | Family            | Suborder           | Section      | Origin          | Genes/Acc. number |
|-------------------------------------|-------------------|--------------------|--------------|-----------------|------------------|
| Ligia italica Fabricius, 1798       | Ligidae           | Oniscidea          | Diplocheta   | Cyprus          | MN171516         |
| Ligia oceanica Linnaeus, 1767       | Ligidae           | Oniscidea          | Diplocheta   | Galicia (Spain) | AF25698          |
| Ligia hawaiiensis Dana, 1853        | Ligidae           | Oniscidea          | Diplocheta   | Hawaii          | KF546702         |
| Ligia exotica Roux, 1828            | Ligidae           | Oniscidea          | Diplocheta   | Kanagawa (Japan)| MG676443         |
| Ligia exotica Roux, 1828            | Ligidae           | Oniscidea          | Diplocheta   | China           |                 |
| Ligidium ghigii Arcangeli, 1928     | Ligidae           | Oniscidea          | Diplocheta   | Greece          | MN171506         |
| Tauroniscus cf. stygium Borutzky, 1950 | Ligidae           | Oniscidea          | Diplocheta   | Crimea          | MN171509         |
| Typhlodigalidium coecum (Carl, 1904) | Ligidae           | Oniscidea          | Diplocheta   | Crimea          | MN171507         |
| Typhlodigalidium coecum             | Ligidae           | Oniscidea          | Diplocheta   | Caucasus        | MN171508         |
| Heterothea brevicornis Ehmer, 1868  | Tylidae           | Oniscidea          | Tylola       | France          | MN171518         |
| Trichorhina heterophthalma          | Ligidae           | Oniscidea          | Tylola       | Cyprus           | MN171519         |
| Platyarthrus schoblii              | Mesoniscidae      | Oniscidea          | Microcheta   | Italy            | MN171513         |
| Stylogeniscus magellanicus Dana, 1853 | Stylogeniscida    | Oniscidea          | Synocheta    | Argentina        | MN171512         |
| Androniscus roseus (C. Koch, 1838)  | Trichoniscidae    | Oniscidea          | Synocheta    | The Netherlands  | MN171501         |
| Calconiscellus karawankiansus (Verhoeff, 1908) | Trichoniscidae | Oniscidea          | Synocheta    | Croatia          | —                |
| Caucasomethes sp.                  | Trichoniscidae    | Oniscidea          | Synocheta    | Caucasus        | MN171502         |
| Tauronethes lebedinskyi Borutzky, 1949 | Trichoniscidae    | Oniscidea          | Synocheta    | Crimea          | MN171505         |
| Trichoniscus provisorius Racovitza, 1908 | Trichoniscidae   | Oniscidea          | Synocheta    | Cyprus           | MN171503         |
| Agrana madagascariensis (Budde—Lund, 1885) | Agnaridae        | Oniscidea          | Crinocheta   | U.A.Emirates    | MG887977         |
| Hemilepistus klugii (Brandt, 1833)  | Agnaridae        | Oniscidea          | Crinocheta   | Iran            | MG887978         |
| Hemilepistus schirazi Lincoln, 1970 | Agnaridae        | Oniscidea          | Crinocheta   | Iran            | MG887979         |
| Hemilepistus roseum (Milne-Edwards, 1840) | Agnaridae        | Oniscidea          | Crinocheta   | Tunisia          | MN171500         |
| Armadillo officinalis Dumeril, 1816 | Armadillidae     | Oniscidea          | Crinocheta   | Greece          | MN171494         |
| Armadillidium vulgar (Latreille, 1804) | Armadillidae     | Oniscidea          | Crinocheta   | Cyprus           | MN171488         |
| Cyphodilidium absolan (Strouhal, 1934) | Armadillidae     | Oniscidea          | Crinocheta   | Croatia          | MN171495         |
| Typhlarmadillidium sp.              | Armadillidae     | Oniscidea          | Crinocheta   | Croatia          | MN171484         |
| Cylisticus convexus (De Geer, 1778) | Cylisticidae     | Oniscidea          | Crinocheta   | Greece          | MN171493         |
| Ononisicus dalmaticus Strohul, 1937 | Oniscidae        | Oniscidea          | Crinocheta   | Croatia          | MN171486         |
| Platyarthus schoblii Budde-Lund, 1885 | Platyarthridae   | Oniscidea          | Crinocheta   | Cyprus           | MN171492         |
| Trichoniscus heterophthalma Lemos de Castro, 1964 | Platyarthridae | Oniscidea          | Crinocheta   | The Netherlands (greenhouse) | MN171496         |
| Agabiformis excavatus Verhoeff, 1941 | Porcellionidae   | Oniscidea          | Crinocheta   | Cyprus           | MG887969         |
| Porcellio nasutus Strohul, 1936     | Porcellionidae   | Oniscidea          | Crinocheta   | Cyprus           | MG887980         |
| Porcellionides cyprius (Strohul, 1968) | Porcellionidae   | Oniscidea          | Crinocheta   | Cyprus           | MN171488         |
| Porcellionides pruinus (Brandt, 1833) | Porcellionidae   | Oniscidea          | Crinocheta   | Cyprus           | MN171489         |
| Actaea eucharis Dana, 1853          | Scyphacidae      | Oniscidea          | Crinocheta   | New Zealand      | MG887985         |
| Levantonicus maleri Cardoso, Taiti and Stentouhrakis, 2015 | Trachelipodidae | Oniscidea          | Crinocheta   | Cyprus           | MN171490         |
| Levantonicus bicostalatus Cardoso, Taiti and Stentouhrakis, 2015 | Trachelipodidae | Oniscidea          | Crinocheta   | Cyprus           | MN171491         |
| Trachelipus nitzebargi (Brandt, 1833) | Trachelipodidae | Oniscidea          | Crinocheta   | Germany          | MN171497         |
| Asellus aquaticus (Linnaeus, 1758). | Asellidae        | Asellota           | Greece       | MN171511         |
| Colubroceph thorsoni Nicholls, 1944  | Phreaticidae     | Phreaticidae       | —            | —                |
| Sphaeroma serratum (Fabricius, 1787) | Sphaeromatidae   | —                  | Italy         | MN171520         |
| Idotea chelipes (Pallas, 1766)      | Idoteidae        | Valvifera          | —            | Italy            | MN171515         |

| Gene                    | Accession number |
|-------------------------|------------------|
| 18 s                    | MN171516
| 28 s                    | MN174838
| NAK                     | MN234250
| PEPPC                   | MN234312

Table 2. Species, locality of origin and GenBank accession numbers of individuals used in the molecular phylogenetic analyses. (√ will be replaced with accession numbers when available).
Furthermore, likelihood values, as well as all other parameters estimated as indicators for the convergence among runs were monitored using Tracer v 1.5.30. From the sampled trees, 10% were discarded as the burn-in phase and a 50% majority-rule consensus tree was constructed from the remaining trees in MrBayes.

Maximum Likelihood trees were constructed under the same partitioning scheme and nucleotide substitution models. The reliability was tested by bootstrapping with 1,000 replicates.

Data availability
Genetic data used in the present study are deposited at Genbank and publicly accessible through the provided accession numbers.

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Author contributions

A.C.D. and S.S. conceived the idea and wrote the first M.S. draft. S.T. contributed to the final draft of the text and prepared the Figs. 1–4. A.C.D. performed all laboratory and phylogenetic analyses. All authors revised and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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