



An evolutionary timescale for terrestrial isopods and a lack of molecular support for the monophyly of Oniscidea (Crustacea: Isopoda)

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Abstract The marine metazoan fauna first diversified in the early Cambrian, but terrestrial environments were not colonized until at least 100 million years later. Among the groups of organisms that successfully colonized land is the crustacean order Isopoda. Of the 10,000 described isopod species, ~3,600 species from the suborder Oniscidea are terrestrial. Although it is widely thought that isopods colonized land only once, some studies have failed to confirm the monophyly of Oniscidea. To infer the evolutionary relationships among isopod lineages, we conducted phylogenetic analyses of nuclear *18S* and *28S* and mitochondrial *COI* genes using maximum-likelihood and Bayesian methods. We also analyzed a second data set comprising all of the mitochondrial protein-coding genes from a smaller sample of isopod taxa. Based on our analyses using a relaxed molecular clock, we dated the origin of terrestrial isopods at 289.5 million years ago (95% credibility interval 219.6–358.9 million years ago). These predate the known fossil record of these taxa and coincide with the formation of the supercontinent Pangaea and with the diversification of vascular plants on land. Our results suggest that the terrestrial environment has been colonized more than once by isopods. The monophyly

of the suborder Oniscidea was not supported in any of our analyses, conflicting with classical views based on morphology. This draws attention to the need for further work on this group of isopods.

Keywords Isopods · Oniscids · Phylogeny · Molecular clock

Introduction

The marine metazoan fauna first diversified in the early Cambrian around 540 million years (Myr) ago, but continental (terrestrial and freshwater) environments were not colonized by the descendants of these taxa until at least 100 Myr later (Labandeira 2005; Wilson 2010). The crustacean order Isopoda is one of the groups of organisms that have successfully colonized multiple environments, including continental environments. Modern isopods represent a wide range of morphological forms and include more than 10,000 described species. They are found in virtually all environments, including the deep sea, land, and freshwater (Wilson and Hessler 1987; Poulin 1995). About 57% of extant isopod species are found in marine environments, whereas about 34% of the species (~3600 species in the suborder Oniscidea) are terrestrial (Schmalfuss 2003; Wilson 2008; Sfenthourakis and Taiti 2015). Although oniscids typically rely on habitats with high moisture levels, some species are found in arid and semi-arid regions (Warburg 1992, 1995). Other species, such as those in the genus *Ligia* Fabricius, 1798, are amphibious, living in the supralittoral zone.

Colonization of the terrestrial environment by oniscids was once thought to have taken place via freshwater, but some studies have argued that the colonization of these two environments occurred independently from marine environments (Carefoot and Taylor 1995; Tabacaru and

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Danielopol 1996; Schmidt 2008). Species found in supralittoral environments are considered to represent transitional forms because of their intermediate adaptations to water restrictions. However, not all amphibious species have their most recent ancestor in the oceans but are instead secondarily amphibious, such as *Haloniscus* Chilton, 1920 (Wilson 2008). Species of the genus *Ligia* are considered as the prototypes of land isopods because of features such as undifferentiated sac-like pleopods for gas exchange, an open marsupium for egg incubation (“amphibious type”), and an open-type water-conducting system (Carefoot et al. 2000).

The colonization of continental environments by other arthropods, such as insects and arachnids, is relatively well understood (Friedrich and Tautz 1995; Grimaldi and Engel 2005; Labandeira 2006), but the timing of the transition to land in isopods is uncertain. This is partly because of the paucity of fossil data for the group, which is a consequence of terrestrial isopods lacking a waxy epicuticle and thus having low preservation probability (Broly et al. 2013).

Until recently, oniscids were thought to be a recently evolved group because of the lack of fossils older than the Eocene (Schmidt 2008; Broly et al. 2013). The oldest oniscid fossil, preserved in amber, has been dated to the Albian (Lower Cretaceous; Neraudeau 2008; Broly et al. 2015). The first formal description of a Cretaceous terrestrial isopod was published only recently (Broly et al. 2015).

The phylogenetic relationships within Isopoda have been discussed extensively (see Brandt and Poore 2003) but have not been resolved with confidence. In particular, the suborder Oniscidea has a complex taxonomic history (summarized by Schmidt 2008). The evolutionary relationships within the group have mainly been defined by morphological evidence. The monophyly of oniscids is supported by several apomorphies, including the complex water-conducting system present in all members of this group (Wägele 1989; Brusca and Wilson 1991; Tabacaru and Danielopol 1996; Erhard 1997; Schmidt 2008). Other studies, however, have suggested that different lineages of terrestrial isopods evolved independently, which would render Oniscidea polyphyletic (Vandel 1943, 1964). Although some molecular phylogenetic studies have included oniscids (Michel-Salzat and Bouchon 2000; Wetzler 2002), the monophyly of the suborder has not yet been tested.

In this study, we examined the evolution of Isopoda using all available genetic data for the order. Our phylogenetic analyses were based on a data supermatrix of one mitochondrial and two nuclear genes and on a second data set comprising sequences of all of the protein-coding genes from mitochondrial genomes. We specifically tested the placement of oniscids within the isopod tree. Using a molecular clock, we estimated the timing of terrestrial colonization by these organisms.

Materials and methods

Data sets

We assembled two data sets for phylogenetic analysis: a data set derived from complete or near-complete mitochondrial genomes (both at the nucleotide and amino acid levels) and a mito-nuclear supermatrix consisting of nucleotide sequences from one mitochondrial protein-coding gene (*COI*) and two nuclear rRNA genes (*18S* and *28S*). For both data sets, we obtained sequence data from GenBank (Supplemental Table 1). Most sequences were obtained from studies by Michel-Salzat and Bouchon (2000), Dreyer and Wägele (2001), and Raupach et al. (2004, 2009).

The mitogenome data set included sequences from 17 isopod species and one outgroup species (the amphipod *Gammarus duebeni* Lilljeborj, 1852). To minimize the impact of indels and mutational saturation, we limited our analyses to the amino acid sequences and the first and second nucleotide codon positions of the 13 protein-coding genes. Preliminary analyses of the mitochondrial ribosomal RNA genes revealed an abundance of indels and a high degree of mutational saturation, leading to their exclusion from our analyses. Thirteen isopod taxa had complete sequences, but *Janira maculosa* Leach, 1814, was missing four protein-coding genes, *Oniscus asellus* Linnaeus, 1758, was missing two, and *Bathynomus* sp. A. Milne-Edwards, 1879, and *Porcellio dilatatus* Brandt, 1831, were each missing one protein-coding gene. An alignment of the amino acid sequences was first performed in the Muscle v3.8.31 (Edgar 2004), and the result was used to align the corresponding nucleotide sequences in Pal2Nal (Suyama et al. 2006). The resulting data sets comprised a total of 4136 amino acids and 8272 nucleotide sites (with the third codon positions removed). A chi-squared test for compositional heterogeneity, conducted using PAUP* 4.0 (Swofford 1998), revealed no significant variation in nucleotide composition among taxa.

The mito-nuclear data set included sequences from 192 isopods and an outgroup species from the tanaid genus *Parapseudes* Sars, 1882. Owing to the potential problem of mutational saturation, we did not include more distant outgroup taxa even though they would have allowed us to implement a larger number of calibration constraints for molecular dating. All species had sequences for *18S*, whereas sequences of *28S* and *COI* were available only for a subset of taxa. Taxon sampling was aimed at including most of the families of Isopoda. When sequences were available for multiple species from the same genus, we included only the species for which the largest number of markers was available. This approach was taken in order to minimize the amount of missing data. The sequences of *Paragnathia formica* (Hesse, 1864) and *Paranthura japonica* (Richardson, 1909) were not included in our analysis because our preliminary

investigations revealed that their estimated relationships were highly problematic: the sequence of *P. formica* did not group with those of the other isopods, whereas the inferred placement of *P. japonica* within Phreatoicoidea Stebbing, 1893, is unusual and possibly a result of contamination. To examine the influence of missing data in the analysis of the mito-nuclear supermatrix, we performed a separate phylogenetic analysis of the *18S* sequences (which were available for all species; Supplemental Material). Complementary analyses were performed with only one species from each family to avoid over-representing some families in the analysis (Supplemental Material).

For the mito-nuclear supermatrix, nucleotide sequences of the three genes were aligned using the Muscle with the default settings. Highly variable regions were identified and removed using Gblocks 0.91b (Castresana 2000), with the following settings: minimum number of sequences for a conserved position was 50% of the sequences +1; and for a flank position 85% of the sequences; a maximum number of contiguous non-conserved positions of 8; and a minimum block length of 10. The resulting data set comprised a total of 5056 sites (1726 sites from *18S*, 3004 sites from *28S*, and 326 sites from *COI*). A chi-squared test for compositional heterogeneity, conducted using the PAUP*, revealed no significant variation in nucleotide composition among taxa.

Phylogenetic analyses

For all analyses, we assigned an independent model of nucleotide substitution to each gene, chosen using the PartitionFinder (Lanfear et al. 2012): GTR + G (*18S* and *COI*) and SYM + G (*28S*). The GTR + I + G model was selected for each of the two codon positions in the mitogenome data set. We performed maximum-likelihood and Bayesian analyses of all of the data sets. Likelihood analyses were performed using RAxML 8.0.14 (Stamatakis 2014) with 100 random starts for the heuristic search. Node support was estimated using 1000 rapid bootstrap replicates.

Bayesian phylogenetic analyses were performed using the BEAST v1.8.4 (Drummond et al. 2012). Rate variation among branches was modeled using uncorrelated log-normal relaxed clocks (Drummond et al. 2006), with separate models for nuclear and mitochondrial markers. A birth-death process was used for the tree prior. Posterior distributions of parameters, including the tree, were estimated via the Markov Chain Monte Carlo (MCMC) sampling. Three replicate MCMC runs were performed, with the tree and parameter values sampled every 10^4 steps over a total of 10^8 steps. For the analyses of the mito-nuclear supermatrix and the *18S* data set, we performed seven independent replicates and combined the samples after removing an appropriate proportion as burn-in (see the Supplemental Material for further detail). For the mitogenome data set, we performed a Bayesian phylogenetic for 7×10^8

steps, with the tree and parameter values sampled every 10^4 steps. Sufficient sampling was confirmed by inspection of effective sample sizes of parameters.

We inferred divergence times using the mito-nuclear data set, which contained a greater number of taxa and provided the opportunity to use a variety of fossil calibrations. The molecular clock was calibrated using several minimum age constraints. These were based on fossils from the ingroup Cirolanidae Dana, 1852, 150.8 Myr (Polz 2005), Amphisopidae, 237 Myr (Fu et al. 2010), Phreatoicoidea, 307 Myr (Schram 1970), and Oniscidea, 93.5 Myr (Broly et al. 2013). Based on the oldest crustacean fossils, from Branchiopoda, we specified a maximum age constraint of 499 Myr for the root (Walossek 1993). These constraints were implemented as uniform priors on the ages of the relevant nodes (Ho and Phillips 2009). We chose not to specify an informative prior for the substitution rate based on previous studies, because estimates of this parameter can be sensitive to taxon sampling and to the choice of data-partitioning scheme and substitution model.

Results

Our Bayesian and maximum-likelihood analyses of the mitogenomic data set (4136 amino acids and 8272 nucleotide sites) yielded similar estimates of the tree topology, regardless of whether analyses were performed at the nucleotide or amino acid level (Fig. 1 and Supplemental Fig. S1). There was strong support for a polyphyletic Oniscidea in all analyses, with the species *Ligia oceanica* (Linnaeus, 1767) grouping with species of the suborders Valvifera, Sphaeromatidea, and Cymothoida (pp 1, bs 86–92; Fig. 1 and Supplemental Fig. 1).

Our Bayesian analyses of the mito-nuclear and *18S* data sets also identified the suborder Oniscidea as polyphyletic (Fig. 1 and Supplemental Figs. 2–4), although with low support (pp < 0.5, bs < 50). In the tree inferred from the mito-nuclear data set, the family Ligiidae Leach, 1814, is paraphyletic with respect to Tylidae Dana, 1852, with species of the genus *Ligia* more closely related to species of Tylidae than to *Ligidium hypnorum* (Cuvier, 1792) (Fig. 2 and Supplemental Fig. 2). In the analysis of the *18S* data, species of the family Ligiidae grouped with the suborder Phreatoicoidea (Supplemental Fig. 2), whereas species of the family Tylidae grouped with the families Bopyridae Rafinesque, 1815, and Dajidae Giard & Bonnier, 1887, from the suborder Cymothoida in a clade that also includes the sphaeromatid *Cymodoce tattersalli* Torelli, 1929. Similarly, the maximum-likelihood analyses did not identify Oniscidea as a monophyletic group (Supplemental Figs. 5–8).

The molecular-clock analysis showed that the minimum estimated age of Isopoda was 447.9 Myr (95% CI 380.4–502.9 Myr; Fig. 2). The minimum estimated timing for the

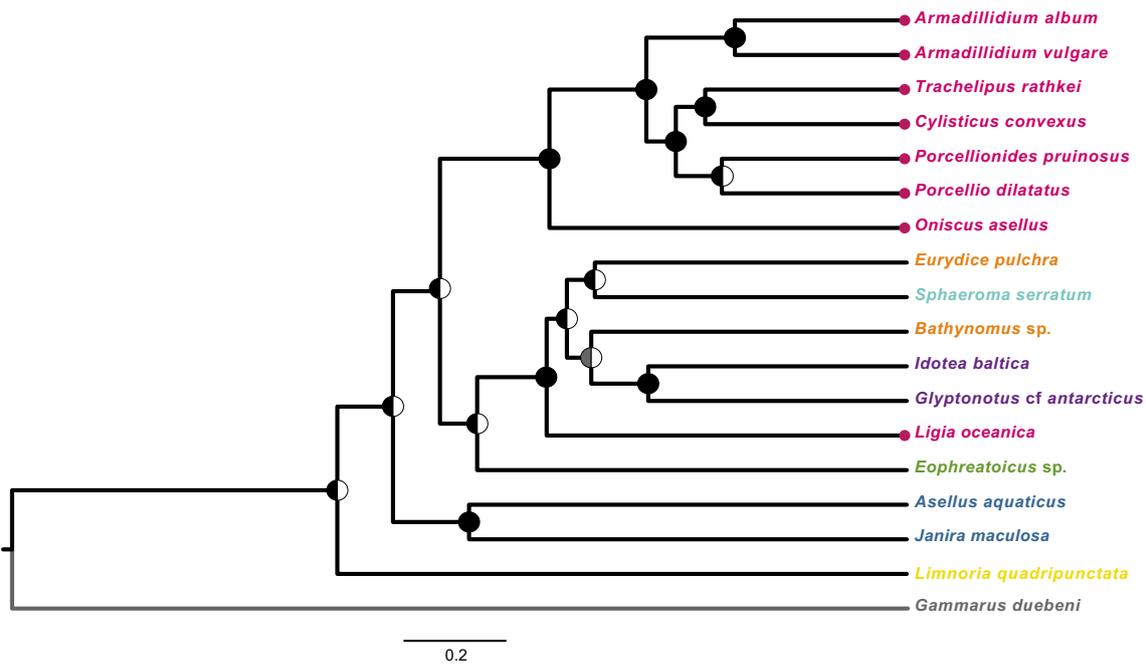


Fig. 1 Bayesian estimate of the isopod phylogeny, based on amino acid sequences of 13 mitochondrial protein-coding genes. Circles at internal nodes show posterior probabilities (left semi-circle) and likelihood bootstrap values (right semi-circle). Black denotes values equal to or greater than 0.9 (posterior probability) and 90% (bootstrap); gray denotes values

equal to or greater than 0.5 (posterior probability) and 50% (bootstrap); and white denotes values below 0.5 (posterior probability) and 50% (bootstrap). Species names are colored by suborder, with species of the order Oniscidea indicated by red circles at the tips

colonization of land by the main group of terrestrial isopods (excluding Tylidae and Ligiidae), inferred from the mitonuclear data set, was 289.5 Myr ago (95% CI 219.6–358.9 Myr ago; Fig. 1). Our analysis of *18S* sequences yielded a younger minimum age estimate of 215.9 Myr (95% CI 145.3–250.2 Myr; Supplemental Fig. 2) for this event. Owing to the relatively small number of taxa in the mitogenome data set, it was not used to infer the evolutionary timescale.

Discussion

Monophyly of Oniscidea

Our phylogenetic analyses of multiple data sets failed to support the monophyly of Oniscidea. The polyphyly of oniscids was strongly supported by our analyses of mitochondrial genomes. Analyses of the mitonuclear and *18S* data sets revealed the presence of three divergent oniscid lineages, although support values uniting oniscids with non-oniscid taxa were not strong.

These results go against the generally accepted hypothesis of oniscid monophyly, which is based on morphological data (Wägele 1989; Brusca and Wilson 1991; Erhard 1995; Tabacaru and Danielopol 1996) or a combined analysis of molecular and morphological data (Wilson 2009). Instead,

the oniscid families Ligiidae and Tylidae were placed outside the main oniscid clade. Most species in these two families live in near-shore environments, such that they have been regarded as representing a transitional stage in the conquest of terrestrial habitats by isopods (Edney 1968; Schmalzfuss 1989; Schmidt 2008). This idea is often linked to the early divergence of these families within Oniscidea. As noted by Carefoot and Taylor (1995), however, the fact that these species are well-adapted to this amphibious lifestyle does not necessarily imply that they represent an early-branching group within Oniscidea.

On its own, the strength of our evidence is not sufficient to reject conclusively the monophyly of the suborder Oniscidea. However, our results draw attention to a possible scenario that should be further explored, whereby this diverse group could have colonized the land on more than one occasion. With the diversification of plants on land providing a suitable environment to detritivores and herbivores to thrive in this environment and escape the predatory pressures in the ocean, it is possible that lineages of isopods, as with other groups, have convergently adapted to land. In this scenario, the families Tylidae and Ligiidae would have evolved from lineages other than the main group of Oniscidea (Crinocheta, Mesoniscidae, and Synocheta). The monophyly of Oniscidea is very well-supported by morphological characters such as the complex water-conducting system and reduced first antenna with only three articles. Although the reduction of the first antenna has been regarded as a prominent synapomorphy for Oniscidea

(Wägele 1989; Schmidt 2008), this reduction has been discussed as different and possibly convergent in *Tylos* Audouin, 1826, and *Ligia* (Schmidt 2008). Similarly, the water-conducting systems within Oniscidea are not all the same. Hornung (2011) found two structurally different types of water-conducting systems, the “*Ligia* type” and the “*Porcellio* type,” with the former being considered an ancient open system. Thus, the two main characters that have been used to support the monophyly of Oniscidea might actually be convergent responses to the challenges posed by the terrestrial environment.

Previous studies also presented evidence of Oniscidea not being a monophyletic group. Vandel (1943, 1964, 1965) proposed that the order is polyphyletic, with the oniscid family Tylidae being closely related to Valvifera. Other molecular phylogenetic studies have produced varied estimates of the relationships of Tylidae and Ligiidae, depending on the method of analysis (Michel-Salzat and Bouchon 2000; Wetzer 2002; Lins et al. 2012). Vandel (1943, 1964, 1965) and Michel-Salzat and Bouchon (2000) did not use a phylogenetic approach in their studies (Schmidt 2008), whereas the study by Wetzer (2002) was based on only mitochondrial markers and included only a few isopod taxa. Collectively, the variation in phylogenetic estimates reflects uncertainty over the monophyly of Oniscidea. Further analyses of the phylogenetic relationships of this suborder with larger numbers of genetic markers will help to resolve these relationships with confidence.

Colonization of land by isopods

Our study presents the first evidence that oniscids have an ancient origin, with the earliest colonization of the terrestrial environment being placed in the Permian. This result corroborates the predictions of other studies, based on the present-day cosmopolitan distribution of oniscids, that this group probably evolved in the Late Paleozoic (Hornung 2011; Broly et al. 2013). Indeed, the worldwide distribution of some oniscid species suggests that some of their ancestors were already on land when the continents separated. Our results lend support to the idea that this highly diverse clade has a much older origin in continental environments than implied by the fossil record (Schmidt 2008; Wilson 2009).

The colonization of land by isopods occurred later than the parallel events in hexapods, which are estimated to have taken place from the Ordovician to the Devonian (Rota-Stabelli et al. 2013; Tong et al. 2015). Our results are consistent with the claim that the increase in vascular plant diversity during the Upper Carboniferous to the Permian (around 320–245 Myr ago) played a key role in the diversification of insects and the terrestrial colonization of oniscids (Labandeira 2006; Broly et al. 2013).

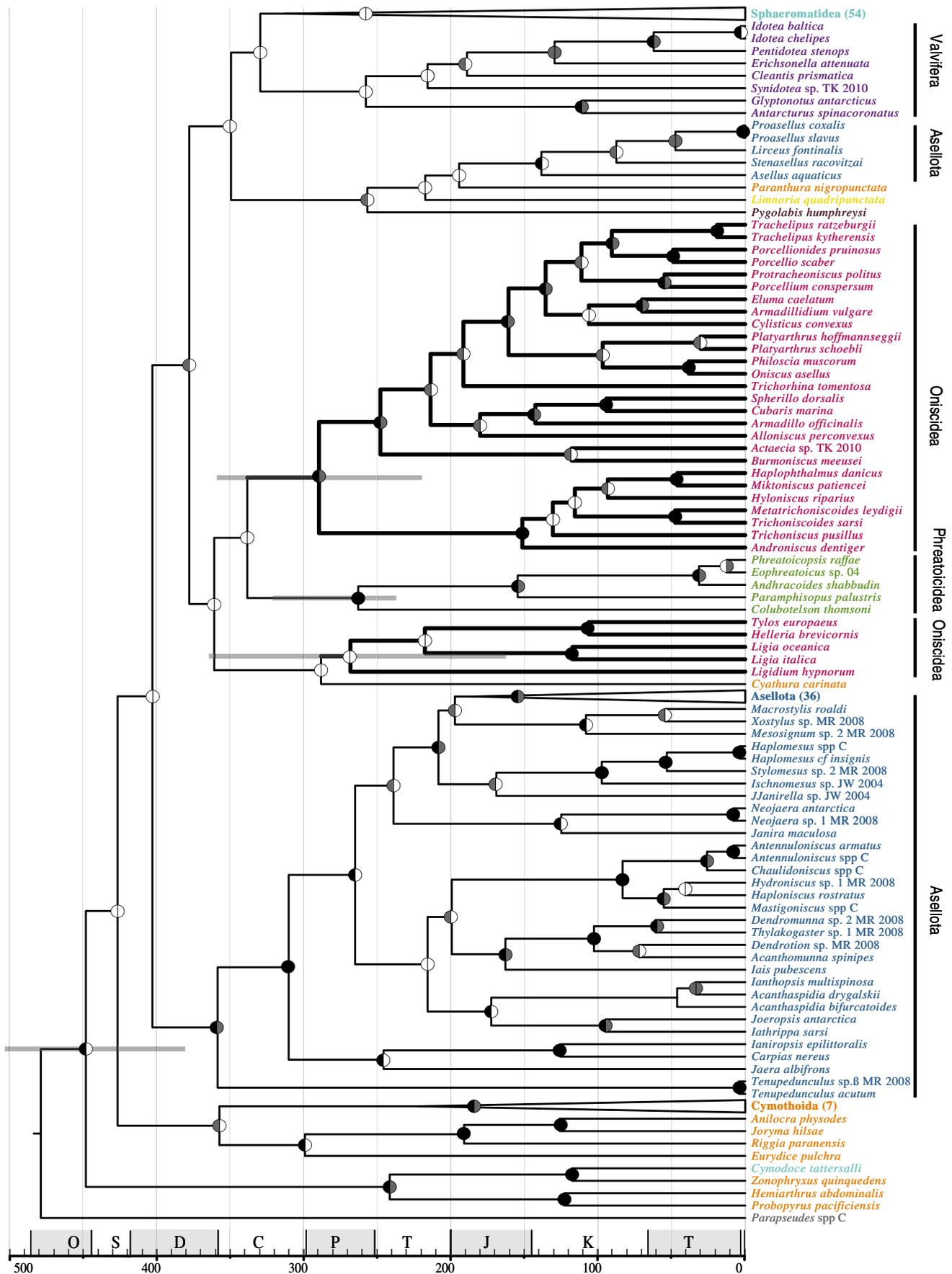
We also found evidence for multiple, independent colonizations of land by isopods. Further taxon sampling is required to achieve a better understanding of the evolutionary timescale of these families and their transition to land. Therefore, our results suggest that the supralittoral (also known as “supratidal”) taxa might not necessarily be descendants of the same ancestor that gave rise to most of the Oniscidea and question the “ligid ancestor” model (Schmalfuss 1989; Carefoot and Taylor 1995). Further phylogenetic analyses that include sequence data from additional species of Ligiidae and Tylidae are required before any changes in classification can be proposed.

The main oniscid clade, which contains taxa that are well-adapted to habitats away from the ocean (e.g., *Armadillidium vulgare*), was not found to cluster with either the supralittoral or the freshwater lineage. Thus, the mode of transition of this clade from marine environments to land remains unclear. Given the evolution of supralittoral taxa from marine taxa on at least two occasions (i.e., in Ligiidae and Tylidae) and the absence of closely related freshwater and terrestrial taxa in our analyses, the supralittoral route might be considered more likely (Hornung 2011). This mode of transition is thought to have occurred in terrestrial amphipods (Friend and Richardson 1986), which are close relatives of isopods.

The characteristic of direct development provides an explanation for how isopods could have colonized land directly from the marine environment. Isopod offspring develop within the female brood pouch (marsupium), in contrast with many other marine organisms that have aquatic larvae that disperse via marine currents (Sutton 1972). The development of offspring in close association with the adult female might have more easily facilitated the transition to living on land. Similarly, true land crabs also contain directly developing larvae, although their ancestors colonized land via the freshwater route (Burggren and McMahon 1988; Diesel et al. 2000).

Conclusion

The multiple analyses presented in our study challenge the notion of monophyly of the Oniscidea. In the case of the mitogenome data set, although only 17 ingroup taxa were analyzed, strong support was found for *L. oceanica* being more closely related to non-oniscid taxa. We anticipate that analyses involving additional isopod mitochondrial genomes will produce similar results. Our more taxon-rich mito-nuclear analyses also failed to recover the monophyly of Oniscidea, although the inferred relationships were less strongly supported than in the analysis of mitochondrial genomes. Future studies should aim to combine character-rich data sets with extensive taxon sampling to address this issue in more detail.



◀ **Fig. 2** Bayesian estimate of the isopod phylogeny, based on a mitochondrial data set comprising nucleotide sequences of *18S*, *28S*, and *COI* from 192 species. Branch lengths are proportional to time, and the tree shows the timing of colonizations of terrestrial habitats. Circles at internal nodes show posterior probabilities (left semi-circle) and likelihood bootstrap values (right semi-circle). Black denotes values equal to or greater than 0.9 (posterior probability) and 90% (bootstrap); gray denotes values equal to or greater than 0.5 (posterior probability) and 50% (bootstrap); and white denotes values below 0.5 (posterior probability) and 50% (bootstrap). Gray bars at nodes represent the 95% credibility intervals of the age estimates for key nodes. Thicker branches correspond to terrestrial Oniscidea species. Species names are colored by suborder. Some branches of the suborders Asellota, Valvifera, and Sphaeromatidea were collapsed (numbers of species are given in parentheses)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Only invertebrates were used in this study, and ethical approval is not needed.

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