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Towards a time-tree solution for Branchiopoda diversification: a jackknife assessment of fossil age priors

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Abstract

An understanding of Branchiopoda's evolutionary history is crucial for a comprehensive knowledge of the Pancrustacea tree of life, given their close evolutionary relationship with Hexapoda. Despite significant advances in molecular and morphological phylogenetics that have resolved much of the branchiopod backbone topology, a reliable temporal framework remains elusive. Key challenges include a sparse fossil record, long-term morphological stasis, and past topological inconsistencies. Leveraging a Bayesian Inference approach and the most extensive phylogenomic dataset for branchiopod to date, encompassing 46 species and over 130 genes, we inferred a time-calibrated phylogenetic tree. Furthermore, to strengthen the confidence in our divergence times estimation, we assessed the impact of age priors, topological uncertainties, and gene trees which are discordant from the species trees. Our results are largely consistent with the fossil record and with previous studies, indicating that Branchiopoda originated between 400 and 500 million years ago, and the orders of large branchiopods diversified during the Mesozoic. Concerning Cladocera, results remain problematic, with a sharper uncertainty in the diversification time with respect to the fossil record. Though, the jackknife resampling of fossils and the other sensitivity analyses proved our calibration method to be robust, suggesting that the difficulties in obtaining a paleontological-consistent time tree may be hindered by the variability in branchiopod substitution rates and topological instability within certain clades.

Keywords: MCMCtree, node dating, Phyllopoda, Spinicaudata, Anostraca, Notostraca, Cladocera, time-tree calibration

Introduction

Branchiopoda are a class of Pancrustacea comprising more than 1,500 living species spread between nine orders (Bánki *et al.*, 2024). They mainly occur in freshwater habitats, especially in temporary ponds, although some may be found also in marine environments, salt pan, and semi-terrestrial ecosystems (Brendonck *et al.*, 2008; Forro *et al.*, 2008). From a taxonomic and systematic point of view, branchiopods have a contentious history, mainly due to the well-known, long-term morphological stasis exhibited by many taxa (Mathers *et al.*, 2013; Gueriau *et al.*, 2016). Nonetheless, the most recent molecular phylogenies (Oakley *et al.*, 2013; Schwentner *et al.*, 2018; Lozano-Fernandez *et al.*, 2019) largely agree with the morphological ones (Richter *et al.*, 2007; Olesen, 2009), and the phyletic relationships among the major clades now seem largely resolved. However, the placement of the orders within Cladocera (*i.e.*, Anomopoda, Ctenopoda, Haplopoda, and Onychopoda) is still unsettled, as both nuclear- and mitochondrial-based phylogenies tend to return conflicting topologies (Schwentner *et al.*, 2018; Xu *et al.*, 2021). Having a well-established phylogenetic backbone has allowed researchers to notably advance the study of branchiopods evolution. For example, by integrating comparative genomics analyses with a phylogenetic framework, it has been shown that morphological evolution in tadpole shrimps (Notostraca) is decoupled from molecular evolution, the former appearing static and the latter being more dynamic in terms of (i) gene family and (ii) transposable elements turnover (Luchetti *et al.*, 2021), as well as in terms of (iii) the rate of sequence evolution (Luchetti *et al.*, 2021; Nicolini *et al.*, 2023a). Similarly, phylogenetics allowed

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the identification of *Cyclestheria hislopi* as a complex of species rather than the only living species of the order Cyclestherida (Schwentner *et al.*, 2013).

However, despite the well-established phylogenetic history, Branchiopoda still lacks a comprehensive time-calibrated tree. The reason behind this knowledge gap is that, traditionally, the phylogenetic placement of fossil specimens has been hindered by the exceptional morphological stasis observed in several branchiopod lineages (Mathers *et al.*, 2013), first and foremost in Notostraca, but also in Anostraca and the former 'Conchostraca' group (Laevicaudata + Spinicaudata + Cyclestherida; Fryer, 1987; Ax, 1999; Olesen, 2000; Richter *et al.*, 2007). It must also be considered that the fossil record for Anostraca, Notostraca, and 'Conchostraca' is notably more abundant than that of Cladocera (Van Damme & Kotov, 2016), which is however the most ecologically successful, and species-rich clade within branchiopods (Van Damme & Kotov, 2016; Xu *et al.*, 2021). Furthermore, the availability of omics-level sequencing resources for branchiopods only began to increase significantly about a decade ago (Colbourne *et al.*, 2011; Baldwin-Brown *et al.*, 2018; Schwentner *et al.*, 2018; Lee *et al.*, 2019; Savojardo *et al.*, 2019; Luchetti *et al.*, 2021; Kieran Blair *et al.*, 2022; Van Damme *et al.*, 2022; Kieran Blair *et al.*, 2023a, b), thus preventing the development of a comprehensive and trustworthy phylogenetic hypotheses. Therefore, extensive time tree inferences have mostly relied on external calibration points or included a limited number of Branchiopoda species (*e.g.*, see Mathers *et al.*, 2013; Sun *et al.*, 2016; Van Damme *et al.*, 2022; Bernot *et al.*, 2023). The phylogenetic position of Branchiopoda within the larger Allotriocarida is still unclear, with Copepoda alternatively being placed in the group or within the Multicrustacea (Schwentner *et al.*, 2013; Bernot *et al.*, 2023). In addition, correct age estimations for Branchiopoda may be hindered also by such topological uncertainty, as substitution rates (and thus clock calibration) along erroneous branches may be misestimated (Carruthers *et al.*, 2022).

Inferring robust phylogenetic hypotheses and the corresponding time-calibrated trees is crucial for characterizing evolutionary processes in a comparative framework. Moreover, broad phylogenetic analyses have proven essential for investigating patterns of trait evolution, orthologous gene relationships, and speciation dynamics (*e.g.*, Recknagel *et al.*, 2018; Forni *et al.*, 2022; Grau-Bové *et al.*, 2022; Nicolini *et al.*, 2023b). However, reliable phylogenetic hypotheses and time-calibrated trees remain elusive for some neglected clades, such as branchiopods, due to limited sequencing efforts and/or sparse fossil records. Here, we provide the first time tree calibration of the Branchiopoda tree of life using extensive taxon sampling and only internal age priors, which have been chosen based on their phylogenetic justification. Our analyses encompass the majority of the branchiopod phyletic diversity with a nearly complete phylogenomic matrix, spanning more than 150 genes for 46 species in 8 (out of 9) distinct orders. To test the reliability of our calibration procedure, we also conducted several sensitivity tests, regarding both the calibration points themselves, and the topological uncertainties caused by unstable branches or by events of species tree/gene tree discordance. As a matter of fact, given the knowledge gap on the exact diversification times of Branchiopoda major clades, particularly Cladocera, our goal was to assess how the time-tree inference procedure might be affected by different priors, such as limited usage of unambiguous and paleontological-aware fossil calibrations or diverse molecular alignments.

Material and methods

Phylogenomic dataset construction

The dataset used for the phylogenomic and divergence times analyses was built leveraging both genomic and transcriptomic resources, in order to maximize the taxonomic sampling of Branchiopoda. Annotated genome and transcriptome assemblies were retrieved from NCBI (Suppl. Table S1), including the genomes of the damselfly *Ischnura elegans* and the springtail *Sinella curviseta*, which were used as outgroups. Duplicated sequences and isoforms were removed from transcriptomes initially using CD-HIT (v.4.8.1; sequence identity threshold and tolerance for redundancy of 1; Fu *et al.*, 2012), and subsequently with a Perl script from the Trinity package ('get-longestisoform-seq-per-trinity-gene.pl', Grabherr *et al.*, 2011). Gene sequences used in the phylogenomic analysis were retrieved by running BUSCO (v5.4.2; Simão *et al.*, 2015) with the 'arthropoda_odb10' dataset on genomes and transcriptomes, with default parameters. Single-copy genes retrieved by BUSCO and present in at least 90% of the species (43 out of 48) were then selected for downstream analyses.

Phylogenetic inference and tree calibration

Amino acid and nucleotide alignments of BUSCO genes were jointly inferred with TranslatorX (v1.1; Abascal *et al.*, 2010), then trimmed with trimAl (v1.4rev15; Capella-Gutiérrez *et al.*, 2009) with a heuristic selection of the automated trimming method (-automated1). Afterwards, IQ-TREE (v2.2.0, Nguyen *et al.*, 2015) was used to infer a maximum likelihood (ML) partitioned phylogenetic inference on amino acid alignments, with automatic model selection (Kalyaanamoorthy *et al.*, 2017) and 1000 ultrafast bootstrap replicates. Bayesian time-tree

TABLE 1. Fossil calibrations for the MCMCtree dating of the branchiopod phylogenetic tree. Node codes are the same as in Fig. 1.

Group	Node code	Fossil	Min. Age (Ma)	Max. Age (Ma)	Reference
Branchiopoda + Hexapoda (Allotriocarida)	0	*Rehbachiella kinnekullensis*	497.00	636.00	Wolfe *et al.*, 2016
Branchiopoda crown group	1	*Lepidocaris rhyniensis*	405.00	521.00	Wolfe *et al.*, 2016
Anostraca crown group	2	*Palaeochirocephalus rasnitsyni*	125.71	521.00	Wolfe *et al.*, 2016
Notostraca crown group	3	*Chenops yixianensis*	121.80	521.00	Wolfe *et al.*, 2016
Cladocera crown group	4	*Smirnovidaphnia smirnovi*	173.10	521.00	Wolfe *et al.*, 2016

was obtained on the concatenated nucleotide alignment (produced with a custom bash script) with MCMCtree from the package PAML (Yang, 2007). The independent-rate clock model and the HKY85 substitution model were used as priors. Age calibration priors were set on the root (Allotriocarida) and on the nodes of Anostraca, Branchiopoda, Notostraca, and Cladocera with a uniform distribution (Table 1). Age intervals and phylogenetic justifications were retrieved from Wolfe *et al.*, 2016, which provides the most extensively documented, paleontological- and phylogenetic-devised set of calibration points for the arthropod tree of life, at present (Van Damme & Kotov, 2016).

The alpha parameter was set to 2, while beta was estimated with R following the pipeline by Álvarez-Carretero (https://github.com/sabifo4/Tutorial_MCMCtree; accessed in February 2024). Branch lengths, the gradient, and the Hessian matrix were estimated following the approximate likelihood calculation (Reis & Yang, 2011) from the baseml PAML package. Five independent MCMCtree runs were performed to ensure convergence, with a total number of 20,000,000 iterations (sampfreq = 1000; nsample = 20000), and a burn-in of 100,000. Convergence of runs and effective sample size (ESS) were checked in Tracer (v1.7; Rambaut *et al.*, 2018).

Sensitivity analyses

In order to validate the consistency of our divergence times analysis, we performed several sensitivity tests. For each of them, we replicated the entire IQ-TREE ML inference and the MCMCtree analysis, with the same approach described previously. Firstly, (i) to check for the influence of each calibration point, we re-run the MCMCtree estimation after alternatively removing each of them (i.e., a jackknife resampling approach), except for the one in the root, which is necessary to estimate priors in the approximate likelihood calculation; furthermore, considering the extremely fragmented and problematic fossil record for Cladocera, we also run MCMCtree after alternatively removing two calibration points each time: Cladocera and Branchiopoda crown groups; Cladocera and Anostraca crown groups; Cladocera and Notostraca crown groups. Afterwards, (ii) considering the uncertain phylogenetic placement of *Lynceus* sp. (Laevicaudata; Sun & Cheng, 2023), we performed the divergence time analysis removing this species from the dataset. Eventually, (iii) to check for the impact of gene tree discordance from the species tree, we replicated the dating inference on two additional trees: one using only genes supporting the species tree topology (hereon referred to as 'concordant genes') and another using only genes not supporting it (hereon referred to as 'discordant genes'). In particular, we run a tree-topology test as implemented in IQ-TREE on each gene tree analysed, and then assigned them to the concordant or discordant gene category. Then, the two resulting sets were individually concatenated and used to infer ML phylogenetic trees.

Results

Branchiopod tree topology and time tree calibration

The dataset used for the species tree inference and calibration included 18 branchiopod genomes, 28 branchiopod transcriptomes and 2 outgroup genomes (Supp. Table S1). The percentages of BUSCO single-copy complete genes for genome assemblies were high (median value of 96.9%), while for transcriptomes it spanned from the 21.7% in *Streptocephalus* sp. to the 91.0% in *Moina* sp. (Supp. Table S1). Nonetheless, we successfully retrieved from 90 (*Streptocephalus* sp.) to 134 (*Triops cancriformis* ITA, *T. longicaudatus*, *Lepidurus packardi*, *Anchistropus emarginatus*, and *Sida crystallina*) BUSCO single-copy genes for the phylogenomic analysis (Supp. Table S1) and overall, the corresponding trimmed alignment consisted of 30,767 amino acid positions (92,301 nucleotide positions), with a degree of missing data of 5.9%. The resulting tree topology is strongly in agreement with previous phylogenetic analyses (*e.g.*, Olesen, 2009; Olesen & Richter, 2013; Schwentner *et al.*, 2018; Van Damme *et al.*, 2022): all the branchiopod orders represented in our dataset were retrieved as monophyletic with maximum bootstrap support values; the same holds true for higher-rank taxonomic clades, such as Phyllopoda, Diplostraca, Onychocaudata, Cladoceromorpha and Cladocera (Fig. 1). Altogether, these results show that branchiopod phylogenetic reconstruction based on nuclear genes is not generally affected by topological instability (at least across its deepest nodes), as instead is observed for mitochondrial phylogenies, which place *Lynceus* sp. (Laevicaudata) either as the sister-taxon to Notostraca (Xu *et al*., 2021) or to Phyllopoda (Sun & Cheng, 2023). As a matter of fact, the tree topology here obtained with ~30,500 amino acid positions completely agrees with that obtained with ~167,000 positions by Schwentner *et al*. (2018), suggesting that branchiopods phylogeny can be resolved in its backbone nodes with a relatively low number of nuclear markers (in this work, up to 134 genes). Regarding Cladocera, we retrieved Ctenopoda (comprising the only specimen *Sida crystallina*) as the sister-taxon to Onychopoda + Anomopoda, in accordance

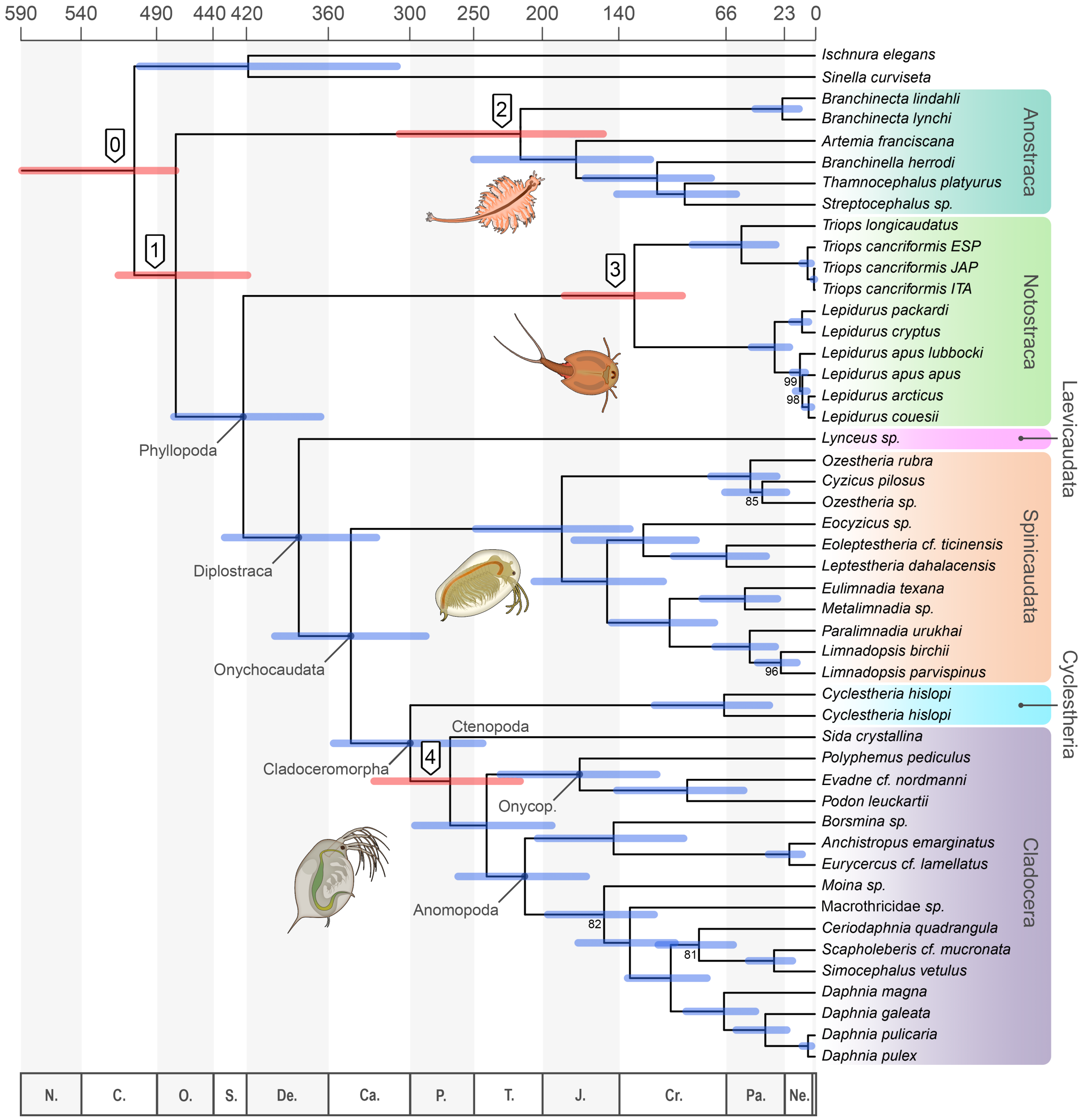

FIGURE 1. Time-calibrated phylogenomic tree of Branchiopoda and two Hexapoda outgroups. Bars represent confidence intervals for each split. Numbers and red bars indicate fossil calibrations (Table 1): (0) Branchiopoda + Hexapoda (Allotriocarida), (1) Branchiopoda crown group, (2) Anostraca crown group, (3) Notostraca crown group, and (4) Cladocera crown group. Major Branchiopod clades are highlighted either on the right in different colors or in nodes across the tree. Bootstrap values less than 100 are shown. The tree was plotted using the R package 'MCMCtreeR' (Puttick, 2019).

with Schwentner *et al*. (2018), Xu *et al*. (2021) and Van Damme *et al*. (2022). However, we were not able to include any representative of the order Haplopoda, as the assembled genome of the only sequenced species (*Leptodora kindtii*; Van Damme *et al*., 2022) is not available for downloading. Thus, we are not able to further speculate on the phylogenetic relationships within Cladocera, also considering the conflicting topologies returned by different phylogenetic approaches and using either nuclear or mitochondrial markers (Schwentner *et al*., 2018; Xu *et al*., 2021; Castellucci *et al*., 2022; Van Damme *et al*., 2022; Sun & Cheng, 2023).

The five MCMCtree independent calibration runs successfully converged (Supp. Fig. S1, S2), considering an ESS for the lnL greater than 200. Divergence times estimated using the full set of age calibration priors were generally in agreement with the fossil record distribution (see the next sections; for a complete and up-to-date reference list of the branchiopod fossil record, please refer to https://sites.google.com/site/thehegnalab/home/reference-lists/).

Age estimates for branchiopod deep nodes and early-branching orders

Our divergence time estimates returned an origin of crown Branchiopoda at around 475 million years ago (Mya), within the Ordovician, with a stem group extending back to the Cambrian. This is in agreement with calibrations provided by previous works (Mathers *et al*., 2013; Uozomi *et al*., 2021; Van Damme *et al*., 2022; Bernot *et al*., 2023), which all place the diversification of early branchiopods between the Cambrian and the Silurian (Fig. 2), as well as with the paleontological record, which considers *Lepidocaris rhyniensis* (410.8–407.6 Mya) the oldest known crown group branchiopod fossil specimen (Wolfe *et al*., 2016).

The occurrence of subsequent splits and, thus, the diversification of major branchiopod lineages (*i.e*., Phyllopoda, Diplostraca, Onychocaudata, and Cladoceromorpha), is estimated to take place in a time range of about 100 million years, between the Devonian and the Permian (Fig. 1; Suppl. Table S2), in line with findings by Mathers *et al*. (2013). However, the majority of previous works tend to return more ancient ages for these clades, suggesting for example that the diversification of Phyllopoda, Diplostraca, and Onychocaudata might have occurred during the Ordovician and early Silurian (Fig. 2; Sun *et al*., 2016; Uozomi *et al*., 2021; Van Damme *et al*., 2022), in what is widely recognized as a period of rapid diversification soon after the appearance of branchiopods. Concerning the oldest taxonomic orders (*i.e*., Anostraca, Notostraca, Laevicaudata, Spinicaudata, and Cyclestherida), which include the so-called large branchiopods, our analysis suggests a massive diversification during the Mesozoic, particularly within the Triassic and Jurassic periods, which agrees with the works by Mathers *et al*. (2013), Uozomi *et al*. (2021), and Van Damme *et al*. (2022), as well as with the fossil calibration of crown-Spinicaudata suggested by Wolfe *et al*. (2016). At present, the number of phylogenetic analyses investigating the origin of 'Conchostraca' orders are still limited, since extensive molecular resources have emerged only recently. The fossil record of this ancient portion of the branchiopod tree of life is particularly rich, especially for 'Conchostraca' (Novojilov, 1960; Tasch, 1969; Zhang *et al*., 1976; Chen & Shen, 1985; Gueriau *et al*., 2016; Van Damme & Kotov, 2016; Hegna & Astrop, 2020; Poschmann *et al*., 2024). Compared to the diversification times obtained in this work, the fossil record seems to suggest an older origin for the aforementioned branchiopod groups, which validates many previous time-tree calibration analyses (Sun *et al*., 2016; Uozomi *et al*., 2021; Van Damme *et al*., 2022). However, it must be taken into account that, despite being abundant, fossil specimens for conchostracans are represented almost by fossilized carapaces, whose morphology and characteristics are however poorly documented for most of the modern species (Sun *et al*., 2016; Hegna & Astrop, 2020; Poschmann *et al*., 2024). Additionally, conchostracan paleontology still faces challenges due to the lack of standardized methods to accurately place specimens within the appropriate taxonomic group, and distinguish between stem and crown lineages (Wolfe *et al*., 2016; Hegna & Astrop, 2020). Hence, the phylogenetic relationships with other fossil and extant branchiopods are unresolved, correct placement in higher-level taxonomic groups is problematic, and the placement within stem- and crown-groups are consequently difficult (Hegna, 2012; Hegna & Astrop, 2020).

Accurate time-tree calibrations should thus rely on fossil specimens with unambiguous taxonomic identity and phylogenetic placement (Wolfe *et al*., 2016). For example, discrepancies between ages estimated in this work and those in Sun *et al*. (2016) (which are systematically older, even among different calibration algorithms), may be traced back to the fact that the fossil placement into the corresponding calibration points may not have followed best-practice standards (Wolfe *et al*., 2016), and that the phylogenetic tree topology is not in agreement with most recent findings (Mendes & Hahn, 2016; Carruthers *et al*., 2022), at least concerning the placement of branchiopods in the wider context of Pancrustacea. As a matter of fact, Sun *et al*. (2016) used a phylogenetic tree where Branchiopoda is sister to Multicrustacea (Copepoda + [Malacostraca + Thecostraca]), which is in turn sister to Miracrustacea (Hexapoda + [Cephalocarida + Remipedia]). However, the most up-to-date and

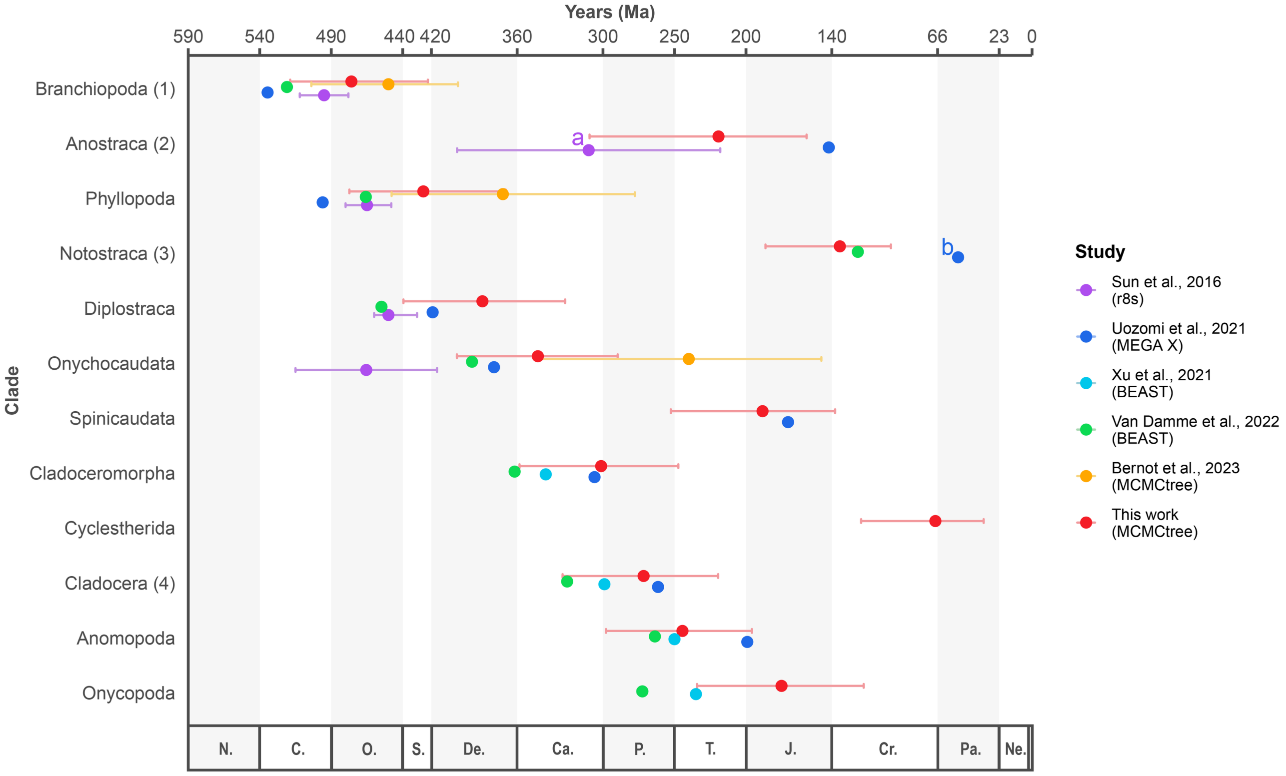

FIGURE 2. Age estimates for the major branchiopod clades as inferred in this study, compared with estimates from previous analyses. Numbers in parenthesis correspond to calibration points of this work (see also Fig. 1 and Supp. Table S1). Note that the time estimates for Anostraca by Sun *et al*., 2016 (a) and for Notostraca by Uozomi *et al*., 2021 (b) have been included despite being referred to different clades: the former is a time estimate for Artemiidae + Thamnocephalidae + Streptocephalidae (but not also Branchinectidae, as in this work), while the latter is a time estimate for *Triops* spp. (and not for both *Triops* and *Lepidurus*, as in this work). For Sun *et al*., 2016, we reported the most conservative time estimates (in terms of the youngest ages) of the used calibration strategies, while for Bernot *et al*., 2023 we reported the time estimates represented in the main figure of the manuscript. Confidence intervals from Uozomi *et al*., 2021 and Xu *et al*., 2021 are not present because they are not specified in the original manuscript. Confidence intervals for Van Damme *et al*., 2022 are present but not visible because they are very narrow. Calibration results from Oakley *et al*., 2013 and Mathers *et al*., 2013 are not present because they are not specified in the manuscripts. Plotted data can be found in Supp. Table S2.

accepted phylogenetic inference for Pancrustacea places Branchiopoda within the Allotriocarida (together with Hexapoda + Remipedia, and Cephalocarida), which is in turn sister either to Multicrustacea (Malacostraca + [Copepoda + Thecostraca]; Oakley *et al*., 2013; Schwentner *et al*., 2018; Lozano-Fernandez *et al*., 2019) or to Communostraca (Malacostraca + Thecostraca; Bernot *et al*., 2023).

Age estimates for Cladocera and downstream nodes

While large branchiopods account for the majority of the branchiopod fossil record and show a reduced diversity of extant species, the opposite is true for Cladocera, the so-called small branchiopods (Van Damme & Kotov, 2016; Xu *et al*., 2021). The clade comprises over half of the extant species diversity of branchiopods, but fossil species are known mainly for the Quaternary period (Van Damme & Kotov, 2016), though records of fossil ephippia and adults are also available from the Mesozoic (Kotov, 2007; Kotov, 2009a, b; Huang *et al*., 2018). Our estimates place the origin of Cladocera within the Permian, while the diversification of Anomopoda and Onychopoda is during the Triassic and Jurassic, respectively. Comparisons with previous time-tree calibration analyses is not straightforward for Cladocera and nearby nodes, as results are usually conflicting (Fig. 2). For example, the emergence of Cladocera is set in the Permian by this work and by analyses from Schwentner *et al*. (2013) and Uozomi *et al*. (2021). However, both Xu *et al*. (2021) and Van Damme *et al*. (2022) placed the diversification time for clades within Cladoceromorpha from the early Carboniferous period onward, thus older than our results (Figs 1, 2; Suppl. Table S2). This value is more in line with the fossil record (Kotov & Taylor, 2011; Van Damme &

Kotov, 2016), which places the origin of crown Cladocera between the Devonian and the Carboniferous (Van Damme & Kotov, 2016) and the split of the two *Daphnia* subgenera no later than the Cretaceous (Kotov & Taylor, 2011), here dated around the Cretaceous/Paleogene boundary. Similarly, older age estimates have been inferred also for Daphniidae and their sister taxa *Moina* sp. and Macrothricidae sp. (Schwentner *et al*., 2013; Xu *et al*., 2021; Van Damme *et al*., 2022). However, when comparing age estimates for Anomopoda, both Xu *et al*. (2021) and Van Damme *et al*. (2022) are more in line with our findings, and place its diversification within the late Permian/early Jurassic.

Overall, the difficulty in obtaining a solid time-tree calibration for Cladoceromorpha and its internal clades can be attributed to the use of different molecular markers to infer the phylogenetic tree, as Schwentner *et al*. (2013) used two markers of nuclear origin (EF1α and 28S) and one of mitochondrial origin (cox1), while Xu *et al*. (2021) used only mitochondrial genomes. Additionally, these inconsistencies may also be a reflection of the poor cladoceran fossil record (Kotov, 2009b), mainly restricted to the Quaternary period (Van Damme & Kotov, 2016). These limitations significantly impede our ability to construct accurate and paleontologically informed time-trees. On one side, the instability in the inferred relationships among the main Cladocera orders makes it difficult to establish a consistent evolutionary timeline (Xu *et al*., 2021), as topological differences between phylogenetic inferences affect the subsequent calibration analyses. On the other side, the scarcity of cladoceran fossils introduces additional uncertainty, as it provides few reliable calibration points (Schwentner *et al*., 2013). Overall, this situation suggests that more cladoceran research is necessary, both from a phylogenetic and paleontological perspective. As a matter of fact, our understanding of the evolutionary history of the clade has been changing rapidly during the last decades (Van Damme & Kotov, 2016), thanks mainly to the discovery of new fossils and the reinterpretation of old fossils. On the other hand, the sequencing effort has focused on the ecological and experimental key species of the genus *Daphnia*, overlooking other Cladocera orders, which are however of primary importance in such evolutionary studies because of their phylogenetic position. The fossil record and many time-tree estimations (Kotov & Taylor, 2011; Van Damme & Kotov, 2016; Xu *et al*., 2021) suggest an ancient origin (150–350 Mya) for Cladocera and its nested clades, placed even during the Paleozoic and Mesozoic, respectively. However, conflicting age estimates are returned for certain nodes (Schwentner *et al*., 2013; Xu *et al*., 2021), highlighting the intrinsic difficulties of obtaining a solid phylogenetic hypothesis for Cladocera.

Testing the impact of the dataset in branchiopod time-tree calibrations

With the attempt to overcome the discussed challenges in using the fossil record for time-tree calibration analyses, we obtained additional MCMCtree-calibrated trees using different sets of age priors. Divergence times inferred through the jackknife resampling (thus removing alternatively either one or two fossil calibrations) were largely overlapping with those having all the calibration points (Fig. 3; Suppl. Fig. S1–S3). This indicates that ages inferred in this work are not influenced by the given age priors (which are currently the most reliable calibration points for arthropods at high taxonomic levels; Van Damme & Kotov, 2016) but rather by the calibration algorithm itself and/or the phylogenetic signal of the alignment. As a matter of fact, even if both Uozomi *et al*. (2021) and Bernot *et al*. (2023) employed calibration points from Wolfe *et al*. (2016), they obtained very different divergence time estimations for Branchiopoda, Phyllopoda and Onychocaudata, in some cases with a difference of more than 100 Mya (Fig. 2). The same, but with smaller age differences, can be argued for calibration results of Cladoceromorpha taxa provided by Xu *et al*. (2021) and Van Damme *et al*. (2022), who employed age priors as indicated by Van Damme & Kotov (2016). Altogether, the jackknife analysis suggests that the molecular clock of the main branchiopod lineages may not perfectly match the paleontological framework, likely due to genomic characteristics and other life-history traits (for example, differences in cladogenetic timing [Mathers *et al*., 2013; Van Damme *et al*., 2022] and in substitution rates [Luchetti *et al*., 2021]). This is particularly true for Cladocera taxa whose divergence times estimated through time-tree calibrations are younger than those inferred from the fossil record. Consequently, if carefully devised calibration points for Cladocera internal nodes (such as Onychopoda, Anomopoda, and Daphniidae) are not employed, the age estimates may be inaccurate. As a matter of fact, in this study we calibrated only the Cladocera crown-group, which led to younger divergence times for its internal nodes than the fossil record, even when tested through the jackknife resampling. Therefore, these results proved to be methodologically robust and underline the importance of using paleontologically-derived calibrations for time trees, and not only information coming from molecular alignments and substitution rates (Van Damme & Kotov, 2016). The latest fossil calibrations for Cladocera and its subclades (Van Damme & Kotov, 2016; Van Damme *et al*., 2022) should thus be taken into account for future branchiopod time-tree inferences, though much work is still needed to understand the evolutionary underpinning of genome and molecular differences of the various branchiopod clades. Testing different calibration approaches for Cladocera, such as the usage of geological-based calibration points or a total evidence time-tree inference, would also be helpful to unravel the intricate evolutionary history of the clade. As a matter of fact, these methods might provide additional sources of information, through the integration of geological events and fossil specimens directly into the phylogenetic hypothesis.

In order to account also for the characteristics of the alignment and the possible role of phylogenetic biases, we tested the MCMCtree calibration with different starting trees, (i) one lacking the often-misplaced *Lynceus* sp. (Laevicaudata), (ii) one built after genes with gene trees concordant with the species tree, and (iii) one built after genes with gene trees discordant with the species tree. All of these proved to have a negligible impact on the resulting time tree (Fig. 3; Suppl. Figs S1–S3), suggesting that the phylogenetic biases affecting inference at the topological level do not impact the estimations of divergence times.

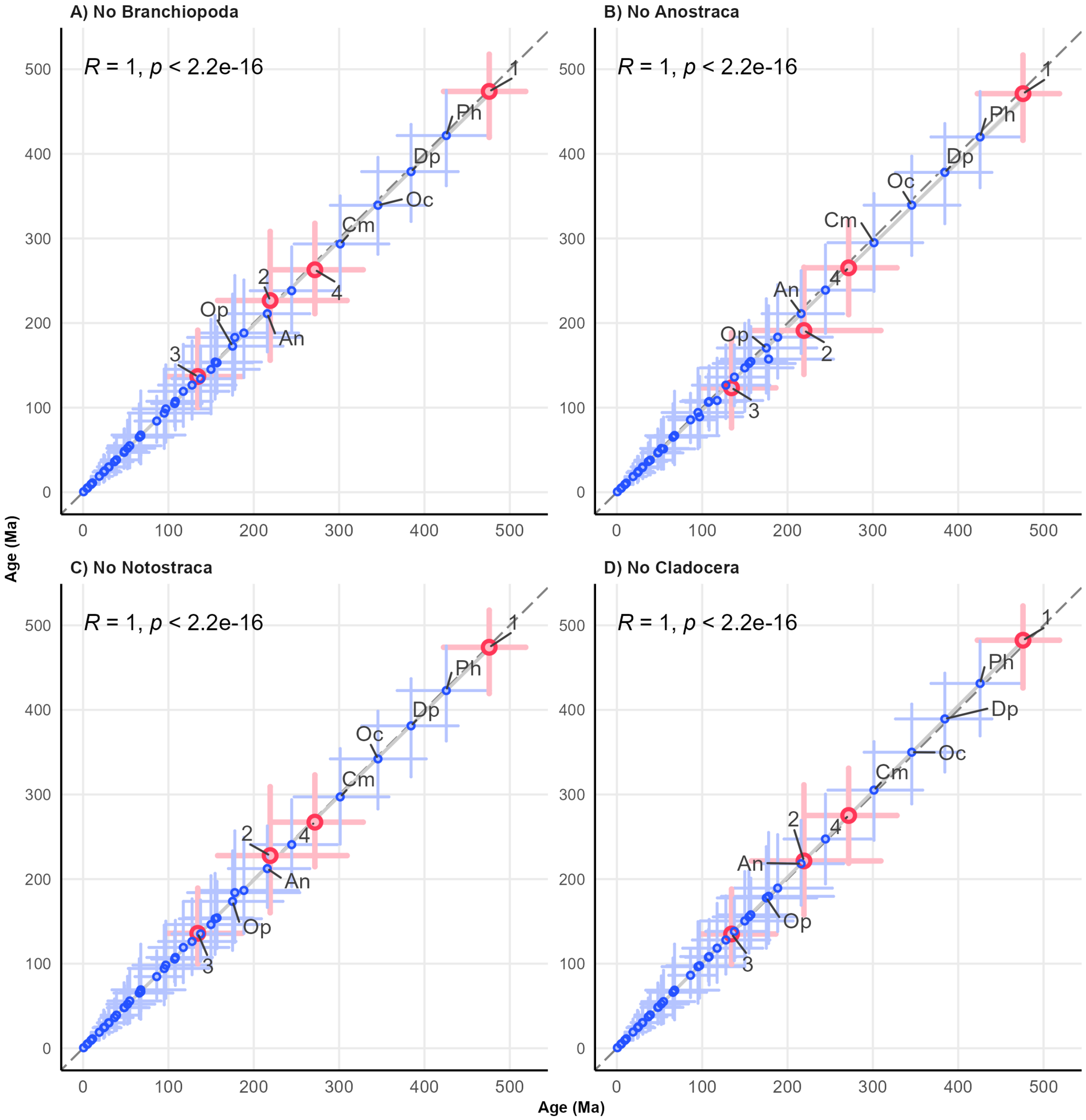

FIGURE 3. Correlations of age estimates between the timetree calibrated with all 5 age priors (Table 1) and time trees calibrated using the single jackknife resampling of age priors. Error bars are shown for each estimate. Red points indicate calibration nodes and numbers are the same as in Fig. 1 and Table 1. Dashed lines represent the bisector of the quadrant, while solid lines represent the linear regression of plotted data. The root node and the node of *Ischnura elegans* + *Sinella curviseta* (outgroups) were removed. Pearson's correlation coefficients (R) and the associated p-values are provided. Ph: Phyllopoda; Dp: Diplostraca; Oc: Onychocaudata; Cm: Cladoceromorpha; An: Anomopoda; Op: Onychopoda.

Conclusion

Understanding the evolutionary history of Branchiopoda is crucial for unraveling the evolutionary history of the Pancrustacea tree of life, given their close relationship to Hexapoda (Schwentner *et al*., 2018; Lozano-Fernandez *et al*., 2019; Bernot *et al*., 2023). Currently, thanks to both molecular and morphological phylogenetics, the topology of the backbone of branchiopod diversification is mostly resolved. Nonetheless, the sparse fossil record, the impact of various life history traits (such as the long-term morphological stability observed in certain taxa), and previous topological inconsistencies have traditionally impeded the ability to establish a reliable timeline for the emergence of branchiopods and their groups. Here, we assembled the most taxonomically comprehensive molecular dataset for branchiopod evolutionary studies so far and estimated their divergence times in a Bayesian framework leveraging multiple fossil calibration points. While doing so, we also focused on the impact of age calibration priors, topological misplacements, and gene tree inconsistencies. Overall, we showed that ages estimated for deeper nodes are concordant with the fossil record and with estimates coming from different approaches in literature. The origin of Branchiopoda is therefore estimated between 400 and 500 Mya, while the diversification of most orders and the establishment of their remarkable morphological stasis took place during the Mesozoic. The clade Cladocera is instead more puzzling: relationships within this clade remain uncertain, as does the timing of their diversification. As a matter of fact, while returning age estimates for large branchiopods coherent with the fossil records and previous works, our analysis provided younger diversification times for Cladocera and its internal orders. Such conflicting results are nonetheless confirmed by the jackknife resampling of age priors, suggesting that incorrect age estimates might have arisen from deceiving phylogenetic information at the molecular level. This challenge should be addressed by increasing sequencing resources for low-diversity cladoceran orders, especially Haplopoda, Ctenopoda, and Onychopoda, as well as by testing the performance of different calibration methods, such as those based on geological events or on total evidence, will be essential.

Data availability: Data and codes used in this paper can be found in the GitHub repository https://github.com/NiccoloRighetti/Branchio_Tree

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Supplementary materials:

SUPPLEMENTARY FIGURE S1. Tracer plots of lnL values for each MCMCtree analysis. Numbers for jackknife resampling are the same as in Fig. 1 and Supp. Table S1.

SUPPLEMENTARY FIGURE S2. Pairwise correlations of mean age estimates of the 5 runs for each MCMCtree analysis. Numbers of jackknife resampling are the same as in Fig. 1 and Supp. Table S1.

SUPPLEMENTARY FIGURE S3. Correlations of age estimates between the timetree calibrated with all 5 age priors (Table 1) and timetrees calibrated using the joint jackknife resampling of age priors (two nodes each time). Error bars are shown for each estimate. Red points indicate calibration nodes and numbers are the same as in Fig. 1 and Table 1. Dashed lines represent the bisector of the quadrant, while solid lines represent the linear regression of plotted data. The root node and the node of *Ischnura elegans* + *Sinella curviseta* (outgroups) were removed. Pearson's correlation coefficients (R) and the associated p-values are provided. Ph: Phyllopoda; Dp: Diplostraca; Oc: Onychocaudata; Cm: Cladoceromorpha; An: Anomopoda; Op: Onychopoda.

SUPPLEMENTARY FIGURE S4. Correlations of age estimates between the timetree calibrated with 5 age priors (Table 1) and timetrees calibrated after phylogenetic trees built with different alignments. Error bars are shown for each estimate. Red points indicate calibration nodes and numbers are the same as in Fig. 1 and Table 1. The root node and the node of *Ischnura elegans* + *Sinella curviseta* (outgroups) were removed. Pearson's correlation coefficients (R) and the associated p-values are shown. Ph: Phyllopoda; Dp: Diplostraca; Oc: Onychocaudata; Cm: Cladoceromorpha; An: Anomopoda; Op: Onychopoda.

SUPPLEMENTARY TABLE S1. Genomes and transcriptomes used in the phylogenetic analysis of Branchiopoda, including the two Hexapoda outgroups. For each sample, ID codes used in the analyses, taxonomic information, GenBank accession numbers (or source website), BUSCO statistics, and the number of BUSCO genes used in the ML tree inference, are reported.

SUPPLEMENTARY TABLE S2. Age estimates for the main Branchiopoda clades as obtained in this work and in previous analyzes. Where possible, 95% confidence intervals (95% CI) are reported. a: The calibration refers to Artemiidae + Thamnocephalidae + Streptocephalidae, and lacks Branchinectidae. b: The calibration refers to the genus *Triops*.