Whole-Genome Phylogenetic Reconstruction as a Powerful Tool to Reveal Homoplasy and Ancient Rapid Radiation in Waterflea Evolution

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SUPPLEMENTARY MATERIAL

CONTENT

SUPPLEMENTARY TEXT* ST1-2 & SUPPLEMENTARY REFERENCES SR1

ST2.4. *Final notes.*

Supplementary References SR1. A complete reference list accompanying this study, including all references of the main manuscript and the references of the supplementary material (all supplementary figures, tables and text).

*this text has been peer-reviewed, edited, corrected and approved. Originally this text was part of the main manuscript. We have kept these relevant sections here in the supplementary material due to the space limitations in the Spotlight Paper section.

Supplementary Text ST1.

A detailed overview of all Materials and Methods used in the study (supplementary to "Materials and Methods" in the manuscript).

MATERIALS AND METHODS

ST1.1. Taxa and genomic DNA extraction

We selected taxa belonging to the most speciose and widespread cladoceran families representing all four extant orders (Haplopoda, Onychopoda, Ctenopoda, Anomopoda) and examined them against six outgroup taxa representing the five large branchiopod orders: Anostraca (*Artemia* Leach, 1819), Notostraca (*Triops* Schrank, 1803 and *Lepidurus* Leach, 1819), Laevicaudata (*Lynceus* O.F. Müller, 1776), Spinicaudata (*Eulimnadia* Packard, 1874) and Cyclestherida (*Cyclestheria*) from available databases (Table S2). We consider the choice of non-cladoceran orders as outgroup taxa to the Cladocera as robust, in particular with the inclusion of the Cyclestherida.

Our criteria for the inclusion of taxa was based on the following: *i*) to provide a phylogenomic context for the Cladoceromorpha (Cladocera+Cyclestherida) and the Cladocera, including all (nine) living orders of the class Branchiopoda; *ii*) to include at least one representative of the most widespread and speciose cladoceran families, especially the most speciose order, the Anomopoda (Forró et al. 2008) which contains the model taxon *Daphnia*; *iii*) to aim for the maximum number of high quality single-copy orthologs for the phylogenomic analysis.

The latter criterion excluded several lower quality genomes that strongly decreased the resolution of the tree, as they reduced the number of shared single-copy orthologs. Subsequently, although representatives of the onychopod families Podonidae (*Podon* Lilljeborg, 1853), Cercopagidae (*Cercopagis*), the ctenopod family Holopediidae (*Holopedium* Zaddach, 1855) and the monotypic anomopod families Acantholeberidae (*Acantholeberis* Lilljeborg, 1853) and Ophryoxidae (*Ophryoxus* Sars, 1862) were collected, reared, sequenced and their genomes assembled, these taxa were excluded from our analysis because of the low-quality sequence data.

A collection of the taxa used to assemble the new genomes (voucher specimens from the monoclonal cultures, fixed in ethanol in separate glass tubes), is deposited at the Royal Belgian Institute of Natural Sciences in Brussels (RBIN) under the accession numbers R INV.159000-159008. In addition to eight *de novo* cladoceran genomes, we included representative assemblies of each genus and subgenus in the Daphniidae, one species of the Moinidae and the Bosminidae assembled by Cornetti et al. (2019), several transcriptomes from Schwentner et al. (2018) and other genomes from available databases; in total, we included 23 branchiopod taxa (Table S2) to provide the first phylogenomic analysis of all extant branchiopod orders. Our study includes first *de novo* high-quality genome assemblies for the families Sididae, Ilyocryptidae, Macrothricidae, Chydoridae, Eurycercidae, Podonidae and Leptodoridae, which represent the first genomes for three of the four cladoceran orders (Onychopoda, Ctenopoda and Haplopoda). Our analysis covers all orders, all suborders and 11 of the 20 extant families in the Cladocera of which seven (of 11) are in the Anomopoda sensu Kotov (2013). The taxon sampling is therefore comprehensive at the family level; the remaining families contain a

very small number of taxa (the majority contains only one genus with one species or a complex of species, e.g., Acantholeberidae, Ophryoxidae, Dumontiidae, Gondwanotrichidae, Pseudopenilidae). The families included in our study harbour a total diversity of at least 90% of the known species in the Cladocera, mainly found in the Daphniidae and the Chydoridae (Forró et al. 2008). All data matrices are submitted to GenBank (ENA study number ERZ1964691, project ID: PRJEB44293, accession numbers: OC977944-OC995744).

To harvest genomic DNA for extraction, we set up monoclonal cultures at the University of Basel (Switzerland) and Tvärminne Zoological Station (University of Helsinki, Finland) for each taxon, starting from a single individual collected live from the field. For all extractions, only parthenogenetic (asexual) females were used. To maintain an asexual population, each culture was kept under stable conditions in 200 mL glass beakers in bottled water under 20 °C, 14:10h light/dark cycle, fed daily with *Chlorella* Beijerinck, 1890 and transferred to new water every two weeks. Cultures (divided over different beakers to reduce the risk of gamogenetic stages or sudden population crashes) were maintained until large numbers of (>200) individuals could be collected, which were then divided into sterilized 40 mL glass vials with sterile mineral water (commercial bottled water) and submitted to the antibiotics protocol described in Cornetti et al. (2019) to reduce bacterial contamination. For the predatory cladocerans *Leptodora kindtii* (Focke, 1844) (Haplopoda) and *Evadne anonyx* Sars, 1897 (Onychopoda), culturing was not possible using the aforementioned conditions because the antibiotic treatment in combination with gut clearance using polymer beads or bentonite clay led to rapid mortality; for these taxa we isolated (through dissection) a live brood from parthenogenetic females, resulting in a batch of identical clones, and kept these alive in sterile conditions for 24 hours without feeding. We added the three different antibiotics (Cornetti et al. 2019) to reduce bacterial contamination, then fixed the samples in RNAlater (Sigma-Aldrich). For *Leptodora,* neonates from all individuals of a monoclonal culture reared to adults (one generation, fed on *Chydorus*) were pooled; for *Evadne,* all individuals were extracted from the broodpouch of a single parthenogenetic mother.

Genomic DNA was extracted using the QIAGEN Gentra Puregene Tissue Kit, including an RNaseA (100 mg/ml; Sigma) digestion step. Since the genomic DNA extraction did not yield enough material for high-throughput sequencing using non-Nextera kits, we performed a whole genome amplification step using the REPLI-g Single Cell Kit (Qiagen). This allowed us to carry out whole-genome Illumina paired-end sequencing (read length 125 bp) using a TruSeq library preparation. The sequencing was performed by the Genomics Facility service platform at the Department of Biosystem Science and Engineering (D-BSSE, ETH) in Basel, Switzerland, on an IlluminaHiSeq 2500 and NovaSeq 6000.

ST1.2. Genome and transcriptome assemblies

To assemble the *de novo* nuclear genomes (Table S2), we used MaSuRCA (Zimin et al. 2013), which combines the benefits of the deBruijn graph and Overlap-Layout-Consensus assembly, and followed the methodology described in Cornetti et al. (2019). In addition, three transcriptomes from Schwentner et al. (2018) were included in our phylogenomic analysis (Table S2). For transcriptome assembly, paired end (PE) Illumina RNAseq files were accessed via the European Nucleotide Archive (ENA), specifically of *Cyclestheria hislopi* Baird, 1859 (SRA518604;

PE 250), *Lynceus* sp. (SRA518604; PE 150bp), and *Polyphemus pediculus* (Linnaeus, 1758) (SRA518604; PE 150bp). These raw data were processed with a custom bash script to properly format read headers for downstream assembly.

De novo transcriptome assembly was completed in two different ways to attain the most complete BUSCO gene sets for the individual datasets. For *C. hislopi*, we applied a multi-kmer multi-assembler approach followed by an assembly merging step described as the Oyster River Protocol v. 2.2.2 (MacManes 2018). Briefly, this approach includes an adapter and quality trimming step using Trimmomatic (Bolger et al. 2014). Adapter and quality trimmed reads are then self-corrected using a kmer-based approach as implemented in the Rcorrector approach (Song and Florea 2015). Trimmed and corrected reads are subsequently used as the input for multiple RNAseq assemblers including Trinity (Grabherr et al. 2011; Haas et al. 2013), rnaSPAdes (Bushmanova et al. 2019) and Trans-ABySS (Robertson et al. 2010). We chose the latter combination of approaches to allow for the best reconstruction of transcriptomes based on short reads, accounting for potential transcripts from recently duplicated genes and alternatively spliced isoforms. The resulting assemblies are then merged, and a 'best' transcript is selected from among the separate assemblies using OrthoFuse (MacManes 2018), which relies on a modified version of OrthoFinder (Emms and Kelly 2015) and TransRate (Smith-Unna et al. 2016) to determine the most accurate assembly of individual transcripts. To collapse individual isoforms into distinct transcripts, we used Necklace (Davidson and Oshlack 2018).

For the transcriptome assembly of *Lynceus* sp. and *P. pediculus,* we attained the highest BUSCO completeness scores relying, as with *C. hislopi*, on the MacManes (2018) approach to trim and correct the raw data. These data were input into Trinity v.2.10 (Grabherr et al. 2011; Haas et al. 2013), and we then used the EvidentialGene package (Gilbert 2013) to collapse individual isoforms into distinct transcripts for these two assemblies.

We also assembled high-quality mitochondrial genomes of the newly sequenced Cladocera (insufficient quality assemblies were obtained for *Chydorus, Sida* Straus, 1820 and *Evadne* Loven, 1836) following the same method as in Cornetti et al. (2019). Mitogenomes completeness and annotation was performed with the MITOS webserver (Bernt et al. 2013).

ST1.3. Ortholog identification

All nuclear assemblies were assessed for biological completeness using BUSCOv3 which contain a set of conserved single-copy genes that are present across the arthropod Tree of Life (Benchmarking Universal Single-Copy Orthologs) (Simão et al. 2015; Waterhouse et al. 2018, 2019). The BUSCO genes (often used as a quality metric to assess the quality of the assembly) are part of ORTHODB (Kriventseva et al. 2019). A total of 1,066 single-copy arthropod genes were searched against each individual assembly. The genes identified as near-universal single-copy orthologues as "complete" were used to define ortholog groups across the genomes and to build the phylogenetic trees. High-confidence and gap-free sequence alignments were obtained for nucleotide and amino acid data using the approach outlined in Cornetti et al. (2019). Nucleotide alignments were computed with TranslatorX (Abascal et al. 2010) using the deduced ORF (open reading frame) for each protein-coding gene to

guide the alignment in MUSCLE v3.8.31 (Edgar 2004). Amino acid sequences were directly aligned with MUSCLE v3.8.31. We used Gblocks v.0.91b to remove ambiguously aligned positions (Castresana 2000) and to produce gap-free final alignments; the alignments for each single gene were then concatenated using Sequence Matrix (Vaidya et al. 2011).

ST1.4. Phylogenomic analysis

Once we identified the sets of orthologs (i.e., nucleotide and amino acid), we estimated the best substitution model for each individual gene using jModelTest v2.1.10 (Darriba et al. 2012) for the nucleotide alignments and ProtTest v3.4.2 (Darriba et al. 2011) for the amino acid alignments. We also obtained the best partitioning schemes for both datasets using PartitionFinder v2.1.1 (Lanfear et al. 2017), which identifies data blocks that should be combined into partitions with the same substitution model and parameters. For the partitioning of the nucleotide matrix, we also considered the codon positions of each independent gene. As the phylogenomic analyses were conducted using the *-raxml* mode (see below), we also ran PartitionFinder in *-raxml* mode using these settings for the nucleotide dataset: branch lengths = linked, models = all, search = rcluster, rcluster-max = 10000, and the following settings for the amino acid dataset: branch lengths = linked, models = LG, LG+G, LG+F, LG+G+F, WAG, WAG+G, WAG+F, WAG+G+F, BLOSUM62, BLOSUM62+F, BLOSUM62+G, BLOSUM62+G+F, DCMUT, DCMUT+F, DCMUT+G, DCMUT+G+F, JTT, JTT+F, JTT+G, JTT+G+F, search = rcluster, rcluster-max = 10000. We applied the Akaike Information Criterion (AIC) to estimate the best substitution model and partition.

After selecting and partitioning the model, we applied several of the few tree reconstruction programmes that are considered suitable for handling large multi-locus datasets and also based on reliable likelihood approaches (Kapli et al. 2020). The different features of each of these programmes (Kapli et al. 2020), provided a comprehensive approach given the complexity of the dataset (in particular, the large timespan of the clade). Several tree-building methods helped to assess the robustness of the phylogeny. First, we applied the maximum likelihood (ML) phylogenetic inference tool RAxML-NG (Kozlov et al. 2019)—a new and improved next generation version of RAxML (Randomized Accelerated Maximum Likelihood; Stamatakis et al. 2014)—which is specifically designed for large-scale phylogenomic analyses and provides single analysis of concatenated datasets, to build the best ML trees for the partitioned datasets suggested by PartitionFinder. Using RAxML-NG, which allowed us to incorporate the specific substitution model obtained for each gene/partition, we also produced individual nucleotide and amino acid gene trees. We used a bootstrap approach to test the reliability of the best tree inference and generate one hundred pseudo-replicates for the concatenated output. For the mitogenome tree, which represented a subset of the taxa, we used RAxML (Stamatakis et al. 2014) and the GTR+G model (the mitogenome tree was not the aim of this study).

The second ML-based method we applied to our dataset was ASTRAL (Accurate Species Tree Algorithm), a leading coalescent-based species-tree method that accounts for potential gene tree discordance by applying a two-step approach to infer species trees from gene trees (Zhang et al. 2018). ASTRAL has shown promising outputs for cladoceran phylogenomic analyses (Daphniidae; Cornetti et al. 2019), prompting us

to use ASTRAL-III v5.6.3 (Zhang et al. 2018) here in addition to the concatenated analysis, starting from individual gene trees as in Cornetti et al. (2019). We ran ASTRAL using default settings and, as input, used the gene trees obtained from nucleotide and amino acid sequences. We also used ASTRAL to score the best RaxML-NG species tree to compare the confidence of each node obtained via the different approaches.

Finally, we used PhyloBayes MPI (Lartillot et al. 2013), a Bayesian tree reconstruction programme to assess for site heterogeneity under models not yet included in RAxML-NG (Kozlov et al. 2019). Since our study includes distantly related lineages, we conducted a Bayesian inference using the CAT-GTR model available in PhyloBayes MPI (Lartillot et al. 2013) on the same datasets as for the RAxML-NG analysis to control for artefacts arising from potential long-branch attraction in the RAxML-NG approach. Two independent chains were run for the concatenated datasets, and their convergence was assessed using the *bpcomp* command in PhyloBayes. The PhyloBayes run was stopped after reaching convergence, which means when bpdiff was < 0.1.

Gene tree–species tree discordance was tested using a multidimensional scaling (MDS) approach (Duchêne et al. 2018; Roycroft et al. 2020). For both the nuclear and amino acid datasets, we calculated the pairwise Robinson-Foulds phylogenetic distances (Robinson and Foulds 1981) of all gene trees, including the best ML species trees obtained by RAxML-NG, using the function *multiRF* in the R package *phytools* (Revell 2012) and plotted them in two dimensions using *ggplot2* (Wickham 2016). This allowed us to visually compare the distances of each gene tree relative to each other and the species tree. We also included the average bootstrap value of the nodes calculated for each gene tree to evaluate gene tree support in the MDS plots and used this information to assess which dataset showed the highest statistical reliability. We applied the Wilcoxon rank sum test to evaluate the average bootstrap support between all individual nucleotide and amino acid gene trees.

ST1.5. Molecular clock and species divergence estimates

Using the nucleotide dataset as input for BEAST 2.4.5 (Bouckaert et al. 2014), we estimated the ages and confidence intervals of branching events between taxa. We applied eight fossil-calibrated uniform priors to key nodes in our branchiopod tree to estimate approximate divergence times among cladoceran lineages. Because reliable fossil data are lacking for the most common recent ancestor of the Cladocera, the Anomopoda and the majority of the cladoceran families (Van Damme and Kotov 2016; Dumont et al. 2020), we used the oldest fossil evidence of each lineage as a minimal age, and the age of the crown Branchiopoda as a maximum to determine the range of the uniform priors. For the latter, we used a maximum constraint of 521 Ma as suggested in Wolfe et al. (2016), since the earliest undisputed branchiopods fossils date back to the Cambrian (Harvey and Butterfield 2008). For the minimal ages, we used the following estimates based on reliably dated, taxonomically confirmed fossils (Van Damme and Kotov 2016; Wolfe et al. 2016): Anostraca (*Artemia*) – 405 Ma (Wolfe et al. 2016); Laevicaudata (*Lynceus*) – 386.9 Ma (Wolfe et al. 2016); Cyclestherida (*Cyclestheria*) – 212 Ma (Raymond 1946); Ctenopoda (*Sida*) – 176 Ma (Kotov 2007); Anomopoda, Moinidae (*Moina* Baird, 1850) – 145 Ma (Kotov and Taylor 2011); Anomopoda, Daphniidae (*Daphnia*) – 145 Ma (Kotov and Taylor 2011); Anomopoda, Daphniidae (*Ceriodaphnia* Dana, 1853) – 118 Ma (Hegna and Kotov 2016). *Ceriodaphnia* ephippia are probably also present in Khotont (Mongolia) around

145 Ma (Smirnov, 1992; Kotov, 2007), but the first unambiguous fossils are described in detail in Hegna and Kotov (2016). In addition, one uniform prior was added to an internal node in the Notostraca to help calibrate the tree, dated at a minimum of 121.8 Ma for *Lepidurus-Triops* (Wolfe et al. 2016). We did not apply any hypothetical priors based on evidence other than these currently available fossil data and timings.

We examined the level of saturation of the genes at the 1st, 2nd and 3rd codon positions and four-fold degenerate sites (4fds) by plotting the relationship between the raw pairwise genetic distances in an alignment against the model-corrected genetic distances (Fig. S2). Because the first and the second codon positions appeared to be less affected by saturation (compared to the third codon position and the 4fds), we considered them more reliable for further analyses. BEAST was run using an unpartitioned alignment of the first and second codon positions with a "GTR + GAMMA + Invariant sites" (GTR+G+I) substitution model, considering that about 70% of the genes showed GTR + GAMMA (GTR+G) or GTR + GAMMA + Invariant (GTR+G+I) sites as a best substitution model (using jModeltest; Fig. S3) and following the recommendations of Abadi et al. (2019). Bayesian analysis in BEAST was performed with a MCMC chain length of 30,000,000 after discarding the first 10% of the iterations as burn-in (parameter sampling every 5,000 generations). We examined the log files using Tracer v1.6 (Rambaut et al. 2014) to assess the convergence of the analysis and ensure that the effective sample size (ESS) of the parameters was greater than 200.

Supplementary Text ST2.

Additional discussion sections on the implications of our phylogenomic study for cladoceran evolution, focusing on major orders, suborders and families in detail. It contains four sections:

ST2.1. *Homoplasy in the predatory orders and implications for the Cladocera*
ST2.2. *Implications for the Anomopoda*
ST2.3. *Ages of the cladoceran lineages*
ST2.4. *Final notes*

DISCUSSION

ST2.1. Homoplasy in the predatory orders and implications for the Cladocera

Our analysis supported the general hypothesis that Cladocera are monophyletic and confirmed their sister relationship with the monotypic Cyclestherida, hence the monophyly of the Cladoceromorpha. We found no support for the Calyptomera concept, the sister relationship between Ctenopoda and Anomopoda (Fig. S1), which is accepted by several authors (Negrea et al. 1999; deWaard et al. 2006; Olesen 2009), nor for a sister relationship between the Ctenopoda and the remaining three cladoceran orders (Martin and Cash-Clark 1995; Schwenk et al. 1998; Swain and Taylor 2003). Our analysis shows a sister relationship between Anomopoda and Onychopoda and a relationship between the Haplopoda and Ctenopoda. Indeed, the ML trees (Fig. 1; Table S4; Figs S4-5) do not support two widely accepted hypotheses in cladoceran higher systematics: the Gymnomera and Calyptomera (Fig. S1), although these relationships are maximally supported in our protein and nucleotide trees using different approaches (RAxML-NG, ASTRAL-III, PhyloBayes; Table S4). The Gymnomera concept assumes a sister relationship between the pelagic predatory cladoceran orders Haplopoda (*Leptodora*) and Onychopoda. Monophyly of the Gymnomera is a generally accepted hypothesis (Olesen 1998; Forró et al. 2008; Kotov 2013; Table S1), based mainly on morphological features of a predatory lifestyle in the pelagic and often supported by molecular phylogenies using a few mitochondrial and/or nuclear markers (Olesen 1998; Schwenk et al. 1998; Braband et al. 2002; Swain and Taylor 2003; Kotov 2013). Stenderup et al. (2007) even placed *Leptodora* in the Onychopoda. We can observe a clade joining the Onychopoda and the Anomopoda independently in the transcriptomic study by Schwentner et al. (2018: Fig. 3), which, however, lacked *Leptodora*. Thus, two different approaches using whole genomes or transcriptomes support the same hypothesis, that the order Onychopoda, not Ctenopoda, appears as the sister clade to the Anomopoda.

There are no obvious shared morphological characters (synapomorphies) linking **Onychopoda and Anomopoda**, although there are many similarities, assumed by researchers as synapomorphies, linking Haplopoda and Onychopoda (Olesen 1998, 2009; Negrea et al. 1999; Kotov

2013), such as stenopodous raptorial trunk limbs and reductions such as the loss of maxillae, food groove, epipods, ocellus and the carapace (not *Evadne*), which developed into a special broodpouch (Martin and Cash-Clark 1995; Negrea et al. 1999; Olesen 1998, 2009). Remarkable adaptations in *Leptodora*, increase their efficiency catching prey and their swimming speed, supporting the predatory, pelagic lifestyle (Rivier 1998; Negrea et al. 1999).

A phylogenetic grouping is more likely to represent a true evolutionary phenomenon when supported by independent datasets. Although the lack of strong morphological support for the relationship we observe in our study between Onychopoda and Anomopoda versus Haplopoda and Ctenopoda may question the validity of the dataset, our study might, in fact, suggest that the morphological characteristics used to group cladocerans at higher levels should be reconsidered. Several authors have remarked that the predatory cladoceran orders may diverge more than is apparent, suggesting other possible relationships based on development and morphology (Boikova 2008; Korovchinsky and Boikova 2008). Olesen (1998), who reinstated the Gymnomera, mentioned a remarkable similarity between the antennal developments of the Onychopoda and some Anomopoda, carefully questioning the relationship between the raptorial orders, although he preferred to keep the Gymnomera as a grouping until new evidence suggested otherwise. In addition, ***Leptodora*** retains a number of autapomorphies (both advanced and "primitive" characteristics) that make it hard to place in the cladoceran tree (Fryer 1987b; Negrea et al. 1999). Fryer (1987b) suggested that limb type and a few other advanced adaptations in the predatory orders could result from convergence rather than common ancestry and observed that other features such as the antennal morphology and development clearly differed; importantly, the Haplopoda exhibit some features considered primitive among morphologists, such as six limbs and four-segmented antennae (Fryer 1987b; Negrea et al. 1999). A sister relation of the Haplopoda, as opposed to the other three orders (Eucladocera concept), has been hypothesized based on developmental differences including the unique metanauplius stage in *Leptodora* (Eriksson 1934; Bowman and Abele 1982; Negrea et al. 1999; Spears and Abele 2000); however, this theory is not commonly accepted (deWaard et al. 2006). Negrea et al. (1999) also proposed the potentially ancient origin of the Haplopoda as the first lineage to appear after the Cyclestherida, whereas Brooks (1959) stated that *Leptodora* is more likely an aberrant "conchostracan" rather than a derived cladoceran.

Historically, a few authors such as **Wesenberg-Lund** (1902, 1952) speculated that **Haplopoda and Ctenopoda are potentially sister lineages**. He proposed that Onychopoda and Anomopoda may share a common ancestry and that *Leptodora* could be a highly specialised ctenopod, pointing out morphological similarities in the male antennules with Sididae (Wesenberg-Lund, 1952). Lacking compelling evidence for the groupings, Wesenberg-Lund's Danish texts (Fryer 1987b; Olesen 1998) are not frequently cited, and his phylogenetic suggestions about cladoceran orders are largely forgotten. As there is relatively less oligomerization in Haplopoda and Ctenopoda (*Leptodora* has visible segmentation, six limbs and four-segmented antennae; Ctenopoda retained the six serial limbs) than in the Onychopoda and Anomopoda (strongly oligomerized and the sixth limb is reduced or absent; Fig. S10), these morphological features may contain a phylogenetic signal at the deeper level. Rivier (1998) notes several similarities between Anomopoda and Onychopoda; however, he equally lists similarities between the latter and Ctenopoda, suggesting that the onychopods, which include several secondary marine lineages, derived from freshwater filter-feeding

cladocerans and citing, in particular, a few Podonidae such as *Evadne* that show some similarities to anomopods (Rivier 1998). Our analysis supports the hypothesis that onychopods and anomopods may share a common ancestor, most likely a deposit-suspension feeder living in freshwater.

Whatever the true relationship between the four extant cladoceran orders may be, the genetic divergence between both raptorial cladoceran orders was of such an extent that these never grouped in a single clade in our analysis. We therefore consider the possibility that the pelagic predatory lifestyle occurred, independently, more than once in the evolutionary history of the Cladocera and that its morphological similarity is a result of macroevolutionary convergence. Reductions of the carapace (not in *Evadne*) and other typical adaptations in the predatory lineages may have happened independently and seem tied to the particular niche. Independent evolution of the predatory cladocerans would also mean that the transition from the phyllopodous to the stenopodous limb (Olesen et al. 2001) may have happened twice instead of once, which would be a highly unusual event in Arthropoda (homoplasy with the stenopodous limbs in non-branchiopod crustaceans). In addition, the pelagic carnivorous lifestyle in cladocerans implies other adaptations, such as the conspicuously large eye relative to body size and the ability to digest prey items. In the case of parallel evolution, the signature would be visible in the genomes, for example independent expansions in gene families may potentially occur as an adaptation to specific ecological specialisations; comparing the evolution of deeply divergent genomes of cladoceran lineages with similar ecological adaptations is a promising line of research that is now becoming possible for the first time. The Calyptomera are not supported in our analysis, which may suggest a potential convergence in body plans in deposit- and suspension-feeding cladocerans Ctenopoda and Anomopoda, for example between the filter-feeding *Daphnia* and *Sida*. Although the latter two orders are known to differ strongly (Fryer 1987b), they have often been grouped together. Indeed, not even all Ctenopoda are purely filter feeders, like the Black Sea endemic *Pseudopenilia* Sergeeva, 2004, which has serial non-filtering limbs (Korovchinsky and Sergeeva 2008).

Although we include no formal definition here, we suggest, for practical use and future reference, names for the clades supported by our genomic analysis. We suggest a superorder “Oligopoda” Van Damme et Ebert, derived from the Greek prefix *oligo* (“few”) and *poda* (“feet”) to contain the orders Onychopoda and Anomopoda. This name refers to the strong oligomerisation of body and limbs, with the sixth limb being strongly reduced or absent; even the fifth limb may be reduced (Fig. S10). The second proposed group would be the “Magipoda” Van Damme et Ebert from the Latin *magis* (“more”), which would entail the Haplopoda and Ctenopoda, referring to six serial limbs. Our divergence estimates situate the most recent common ancestor of the Magipoda and Oligopoda towards the end of the Early Carboniferous (Fig. 3).

A phylogeny based on the extant cladoceran orders will always remain fragmentary. We will never be able to assess the relations of extinct orders such as the Early Jurassic Cryptopoda†, containing the peculiar *Leptodorosida*† Kotov, 2007, based on anything other than morphology. The latter taxon had features of both haplopods and ctenopods and used filter-feeding, not raptorial limbs (Kotov 2007); perhaps *Leptodorosida*† , the name itself a contraction of *Leptodora* and *Sida*, is close to the true common ancestor of Ctenopoda and Haplopoda, or a stem group offshoot of their shared ancestral lineage. If more truly predatory cladoceran orders existed in the past, they would be hard to find; although other deep lineages with stenopodous limbs may have existed during the evolutionary history of the branchiopods, the soft body and the

reductions visible in extant cladoceran predators would hide the presence of such groups in the fossil record. All extant branchiopod orders have been retrieved as fossils except for the raptorial cladocerans (Van Damme and Kotov 2016).

ST2.2. Implications for the Anomopoda

Our current knowledge of the relationships between the anomopod families is mainly based on morphological rather than molecular studies. Molecular studies that used only a few markers did not resolve relationships between the families (Stenderup et al. 2007; Van Damme et al. 2007), while morphological datasets provide our only evolutionary framework in the most speciose Anomopoda (Elmoor-Loureiro 2004; Kotov 2013). In our first phylogenomic exploration of the **Anomopoda families**, we found no support for the two known suborders (Aradopoda and Radopoda), making the anomopod interfamilial relationships found in our study partly incongruent with limb morphology. The position of *Macrothrix* was a key difference between the morphological and phylogenomic analyses. In our phylogenomic analysis, the Macrothricidae *sensu stricto*, represented here by *Macrothrix,* consistently grouped with Moinidae and Daphniidae, mostly forming a clade with the Moinidae (Table S4). Whether the Macrothricidae form a sister lineage with the Moinidae or the Daphniidae is less relevant from an evolutionary history perspective than the observation that these three families clustered with high confidence. Therefore, the Macrothricidae s.str. appears in our study as a clade with the suborder Aradopoda instead of falling in the Radopoda.

Morphologically, the macrothricids are grouped with families that show typical mixed suspension-deposit feeding (scraping, collecting) traits. The limb adaptations are originally linked to a **littoral-benthic lifestyle** as in the Chydoridae and the secondary pelagic Bosminidae versus the specialised suspension- or filter-feeding Moinidae and Daphniidae (Behning 1941; Manuilova 1964; Fryer 1968, 1974, 1991; Kotov 1995; Dumont and Silva-Briano 1998; Elmoor-Loureiro 2004; Kotov 2013). The daphniid mode of filtration in Moinidae and Daphniidae is considered secondary, not likely to have derived from a “conchostracan”-ctenopod feeding mode. Daphniids and moinids use gnathobases for filtration, while “conchostracans” use exopodites (Kotov 2013). The Moinidae collect detritus and algae using daphniid-type limbs with large filtering screens, lacking the modified scraping setae found in the Macrothricidae (Fryer 1974, 1991; Kotov 2013). Based mainly on these structures, the Moinidae have been allocated to the Aradopoda together with the Daphniidae (Kotov 2013). The grouping we found of moinids and macrothricids remained consistent in most analyses, including the mitogenome-, the nucleotide- and the more conservative amino acid datasets withstanding tests of long-branch attraction (Table S4). Independently, Schwentner et al. (2018) also showed strong support for a clade grouping the Macrothricidae with Moinidae and Daphniidae using a different multi-locus approach, transcriptomes and different cladoceran taxa than the ones in our study, noting that this grouping was consistent in alternative topologies, although they did not discuss this relationship in detail.

As independent phylogenetic approaches using transcriptome- and genome data lead to the same conclusion, the potential sister relationship between anomopod families with different feeding mechanisms and morphologies, though surprising, is hard to ignore. If this represents an accurate evolutionary reconstruction, it implies that the specialised radopod body plan, functionally linked to the complex food

collection of cladocerans in littoral-benthic environments (except for the pelagic bosminids), evolved independently in the Anomopoda more than once from a more generalised feeding mechanism. If the **Macrothricidae** are indeed an "aradopod" lineage (or a basal offshoot of the aradopods), it marks an extreme example of homoplasy in cladoceran morphology, as the limbs are truly of the radopod (mainly deposit-feeding) type (Dumont and Silva-Briano 1998; Kotov 2013). In any case, the deep split of Aradopoda–Radopoda may not be as clear-cut as the morphology suggests.

Some morphological studies suggest a potential relationship between the **Macrothricidae** and the **Moinidae or Daphniidae**. Fryer (1991, 1995) remarked several similarities in features between *Moina* and Macrothricidae and later postulated that the Macrothricidae and the Daphniidae may be sister groups, stating that the latter family likely arose from a macrothricid-like stem (*Ophryoxus*) and showing how the daphniid filter screens could have realistically evolved from modified macrothricid limbs. However, at the time, *Ophryoxus* was still considered a macrothricid, although it was later moved to a monotypic family, where it has since been accepted (Dumont and Silva-Briano 1998). The Ophryoxidae were not included in our analysis due to a low retrieval of single-copy genes. A clade containing Moinidae, Daphniidae, Macrothricidae and Acantholeberidae, separate from other anomopod families, appears in the tree by Olesen (1998: Fig. 14) based on general morphology. Goulden (1968) also suggested that Moinidae and Macrothricidae share several features that may imply common ancestry. Whether these are truly sister lineages, should be further investigated, for example by including other macrothricid-like families like the Acantholeberidae, Ophryoxidae, Dumontiidae or Gondwanotrichidae, but also taking into account that the radopods may be polyphyletic. See also Wingstrand (1978).

Homoplasy should be considered possible, even likely, in a group as ancient as the Cladocera. As homoplasy is primarily assumed to occur in external body shapes, it has not previously been considered in internal limb arrangements in the Cladocera. For example, the extinct Prochydoridae† (Proanomopoda†) look identical to extant Chydoridae, yet have completely different limbs of the serial filter-feeding type, an example of macroevolutionary convergence in body shape (Kotov 2009; Van Damme and Kotov 2016). Homoplasy in feeding mechanisms as a result of ecological specialisations, which include morphological traits under strong selection, could mask underlying evolutionary paths in the Cladocera that independent datasets may reveal. Not all morphological traits we consider in cladoceran systematics at these levels are selectively neutral, as they cannot be disentangled from the niche. Hundreds of millions of years is a long time for homoplasy to occur. Along with morphology and genetics, other approaches, such as developmental data, should also be considered as a third major approach to studying homoplasy (Wake et al. 2011).

The remaining **radopod** families represented in our study (Chydoridae, Eurycercidae, Ilyocryptidae and Bosminidae) formed a well-supported group in the ML analyses and could be considered as Radopoda *sensu stricto*. The Bosminidae formed a sister group to the three other families (Ilyocryptidae, Eurycercidae and Chydoridae), although the BEAST tree showed a slight incongruence related to the position of the Bosminidae (Table S4).

A sister relationship of **bosminids** to the remaining radopods challenges the view that the benthic specialist family Ilyocryptidae, which exhibits unusual morphological features such as segmented antennules, forms a sister lineage to the Macrothricidae, Bosminidae, Eurycercidae and Chydoridae (Kotov 2013). According to morphology, both Ilyocryptidae and Bosminidae take relatively isolated positions in the Anomopoda because they show strong specialisations to benthic and planktonic lifestyles, respectively (Kotov 2013; Dumont 2016). Our analysis confirms the Bosminidae as a secondary planktonic lineage and its distance from the daphniids in the ML trees confirms that these typical planktonic grazers (bosminids and daphniids) adapted to this lifestyle independently.

Although the **bosminids** retain features close to some chydorids (Kotov 1995, 2013), our study showed them further from the Chydoridae than expected. Fryer's (1995) suggestion that the Bosminidae arose early in the evolution of the anomopods, branching off from a scraper-feeding chydorid-like ancestor before chydorids lost one antennal segment, is not rejected. One consistent grouping in the Anomopoda families made both by morphology and our analysis is the close relationship between the Eurycercidae and the Chydoridae, supporting the superfamily Eurycercoidea (Dumont and Silva-Briano 1998). Schwentner et al. (2018) shows the same clade (Eurycercoidea) using different taxa, though the study does not discuss it.

Adding anomopod taxa—such as the monotypic Ophryoxidae, Acantholeberidae, Dumontiidae and Gondwanotrichidae (which are morphologically grouped in the radopods (Kotov 2013)) and additional genera of other families such as the Bosminidae, Chydoridae and the polyphyletic Macrothricidae—to future phylogenomic analyses would better reveal the evolutionary relationships between these lineages. Suborders in the anomopods may have to be reconsidered. However, we think that this first cladoceran phylogeny using complete genomes represents enough anomopod families to allow for basic hypothesis testing, examining patterns, and assessing the approach.

ST2.3. Ages of the cladoceran lineages

Our divergence time estimate tree with eight fossil-calibrated nodes supports a Paleozoic origin for the Cladoceromorpha (Devonian) and the Cladocera (Carboniferous). Studies suggesting similar ages are reviewed in Van Damme and Kotov (2016). So far, molecular studies had been unable to assess the ages of the major cladoceran lineages. According to our analysis, the origins of the extant cladoceran orders, including the most common recent ancestor of the Anomopoda, are situated in the late Paleozoic (Carboniferous-Permian). This estimate strengthens the hypothesis that, although the earliest fossils of the Cladocera are known from the Mesozoic, the ancestor most likely appeared in the Paleozoic during a time of branchiopod mega-evolution (Van Damme and Kotov 2016). In general, our analysis shows that the late Paleozoic and early Mesozoic, in particular the Permian-Triassic (P/T) boundary, appear to be very important for the divergence of deeper cladoceran lineages (orders-families). In addition, the diversification of several lineages apparently happened over a relatively short time frame. For anomopod families, for example, our estimates show radiation occurring over a period of about 20 myr, remarkably brief compared to the long evolutionary history of the Cladocera. These ancient rapid radiations of the higher lineages could explain why unravelling the cladoceran relationships has

proven so difficult using only a few genes and morphology, which provide an unreliable or insufficient phylogenetic signal to disentangle ancient taxa that diversified rapidly. Whole-genome approaches may help to overcome such challenges by providing wider coverage (Whitfield and Lockheart 2007; Feng et al. 2017; Hime et al. 2021). In our case, the multigene approach supports potentially rapid ancient radiations in the Cladocera around the Paleozoic-Mesozoic boundary, events that are key to understanding the evolutionary history of the entire group.

Our molecular clock data suggests early Mesozoic roots for the anomopod families. The Bosminidae and Ilyocryptidae seem relatively older lineages, appearing directly after the end of the Permian. Therefore, it's plausible that a Mesozoic radiation occurred in anomopod lineages soon after the major global extinction event at the P/T boundary (Van Damme and Kotov 2016). Such an extinction event and its effect in freshwater is supported by our divergence time estimates.

Biogeographical arguments position the origin of **Ilyocryptidae** in the Mesozoic (Kotov and Elías-Guttiérez 2009), with Bosminidae also considered a relatively old group (Fryer 1995; Kotov 1995). Although some suggest a Paleozoic origin for the **Chydoridae**, perhaps diversifying as early as the Devonian (Sacherová and Hebert 2003), our estimates do not support this theory, pointing instead to the divergence of the two main chydorid subfamilies in the Mesozoic (Triassic). The latter is more realistic, considering that the appearance of new aquatic niches after the end of the Permian may have allowed chydorids to diversify (Van Damme and Kotov 2016).

While former studies may have overestimated the divergence of the Chydoridae, the origin of several other lineages seems underestimated according to our results. For example, divergence in the **Onychopoda families** Polyphemidae and Podonidae was thought to be a recent event, dating back about 8-11 Ma (Miocene) based on a general arthropod mitochondrial clock and linked to the evolution of the Ponto-Caspian Basin (Cristescu and Hebert 2002). Our analysis, however, shows the divergence of *Polyphemus* and *Evadne* not situated in the Cenozoic but the Paleozoic, around 273 Ma (Permian). The ancestors of the extant onychopod families, which include the marine representatives Podonidae, may, thus, indeed be older than previously assumed. These pelagic predatory cladocerans could have been present long before the evolution of the Ponto-Caspian Basin and the **Para-Tethys**, later finding refuge and radiating into inland seas. If so, it would imply that oceanic lineages may have derived from freshwater ancestors early in cladoceran evolution and may even have been living in the Paleo-Tethys Ocean in the late Paleozoic or early Mesozoic. More high-quality onychopod genomes should be explored to further examine this hypothesis. As we lack fossils for the Onychopoda, molecular clock estimates are currently our only method for assessing the timing of divergence in this order.

An important implication of our time estimates is that the Cladocera's basic **Bauplans**—the predatory and basic suspension/deposit-feeding models adapted to freshwater pelagic and littoral areas—must already have been present in the late Paleozoic. The pelagic predatory lifestyle, including the remarkable transition from phyllopodous to stenopodous limbs and reduction of the carapace, which we now know happened more than once independently, is likely an innovation dating back to the Paleozoic. For the non-predatory lifestyles, the ancestral lineages may have combined both deposit- and suspension-feeding in the pelagic and possibly also near the sediment (Kotov 2009, 2013), yet the highly-specialised limb arrangements we observe, such as in the Daphniidae and Chydoridae, seem to have a Mesozoic signature,

strengthening the theory that diversification of the successful herbivorous anomopod cladocerans is linked with the Lacustrine Mesozoic Revolution (Van Damme and Kotov 2016).

In Cladocera, the exploration of evolutionary relationships using complete genomes has just begun. Even if our phylogenomic study were influenced by analytical artefacts and taxon undersampling, it provides compelling evidence that the Cladocera harbour more homoplasy than hitherto known. Morphological affinities between several cladoceran lineages mask genetic distances and vice versa. In any case, the genetic distances between deep lineages, such as the two predatory orders and between deposit-feeding anomopod families, were larger than we expected. Potential homoplasy of the hyponeustonic body plan in morphologically similar daphniids *Scapholeberis* and *Megafenestra* Dumont & Pensaert, 1983 has been suggested in other studies using phylogenomic approaches (Cornetti et al. 2019). To understand the underlying genetic mechanisms that govern the diversity of cladoceran body plans, it will be useful to (re)visit existing and new data layers and consider homoplasy as an important phenomenon in the Cladocera Tree of Life. An approach that takes embryonic development into account may be a promising step forward and is still largely unexplored for cladocerans (Kotov 2017).

ST2.4. *Final notes*

Deep evolutionary history and highly diversified morphology are notoriously difficult to tackle, as we see here for the branchiopods, a group of lower crustaceans with a long scientific history and highly complex evolutionary relationships. Our study, based on the use of combined approaches, illustrates the importance of exploring new datasets to reconstruct evolutionary history. By providing a comprehensive phylogenomic reconstruction based on single-copy, orthologous genes, we hypothesize that several “similar” phenotypes in the cladocerans may have emerged independently, not through common ancestry. Morphology concealed larger genetic distances and diversity than expected. Identifying homoplasy and estimating the timing of origins in the Cladocera are key to understanding the group’s diversity and investigating the developmental and genetic mechanisms behind that diversity. It helps us understand how drastic morphological changes (that can lead to apparent similarities) may arise, as well as the constraints of morphological space. As such, our study provides great opportunities for understanding phenotypic evolution not only in the Cladocera but more generally in other organisms. Examining traits through state-of-the-art phylogenetic reconstruction presents their trait evolution and inferred niches in a new perspective and allows us to assess the role of homoplasy in diversification within a calibrated temporal context. The Cladocera, our case study for this method here, show several examples of a strong mismatch between morphological and phylogenetic similarity, underscoring that here, and likely in other taxa, homoplasies should be considered an important aspect of the evolution of biodiversity.

Specific to the evolution of the group, we noted incongruence between our phylogenomic trees and recent phylogenies in the Cladocera, leading to unexpected relationships between the families and orders. Although the presented phylogeny should be interpreted with the usual care, we suggest that homoplasies should be considered at deeper systematic levels in the Cladocera. Each of the major Cladocera forms with

predatory, suspension-feeding and deposit-feeding lifestyles may have evolved more than once independently in the Cladocera Tree of Life, as we observe unexpected large genetic distances between taxa such as the predatory Onychopoda and Haplopoda, as well as between the Macrothricidae and other deposit-feeding (radopod) anomopod families that are currently assumed to be closely related because of morphological similarities. Our analysis supports monophyly of the Cladocera and suggests ancient rapid diversification of major lineages. Our divergence time estimates support a Paleozoic origin for Cladoceromorpha and Cladocera orders and a Mesozoic rapid diversification of the anomopod families. We are careful to avoid overinterpretation of our results, yet the phylogenomic approach raises new questions that may help illuminate evolution in the group. As phylogenomic methods improve, more taxa are added and other important data layers (e.g., development) are explored, future analyses may shed further light on the relationships among cladoceran families, suborders and orders. This current study is therefore not an end-point but rather a first step to testing hypothetical relationships and timings of divergence in the deep cladoceran lineages using whole-genome data.

Supplementary Reference List SR1.

A complete reference list accompanying this study, including all references of the main manuscript and of the supplementary material (all supplementary figures, tables and text).

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