

28 ecological opportunities that promoted radiation into many new niches. Our study also provides a
29 solid, time-calibrated, evolutionary framework to accelerate research on this overlooked, yet globally
30 important taxon.

31

32 **Keywords:** Amphipoda, Cenozoic, climatic cooling, ecological radiation, molecular phylogeny,
33 tectonic reconfiguration

34

35 **Introduction**

36 Global environmental changes shaped biodiversity patterns throughout Earth's history (Roelants *et al.*
37 2007; Hannisdal & Peters 2011; Condamine *et al.* 2013). Understanding the historical factors that
38 triggered large-scale evolutionary radiations or extinctions remains a central tenet in evolutionary
39 biology. Investigating the effects of these past changes at the planetary level requires suitable model
40 systems which can be represented by species rich taxonomic groups with a global distribution and
41 high ecological diversity.

42 The Amphipoda is among the most ecologically diverse and speciose crustacean orders,
43 encompassing over 10,000 species (Arfianti *et al.* 2018; Horton *et al.* 2019) inhabiting all aquatic
44 environments worldwide, from hadal depths to alpine freshwater streams, from lightless groundwater
45 to tropical forests, and from sea bottom sediments to the entrails of gelatinous plankton (Bousfield
46 1983; Barnard & Karaman 1991; Lowry & Myers 2017). Amphipods are highly abundant and have an
47 important function in structuring aquatic communities (Oliver *et al.* 1982; Duffy & Hay 2000;
48 González *et al.* 2008; Best & Stachowicz 2014). Furthermore, due to their omnivorous diet and
49 intermediary trophic position, they represent a key link between trophic levels, thus playing an
50 essential role in nutrient recycling (Dangles & Malmqvist 2004; Piscart *et al.* 2011; Machado *et al.*
51 2019). The dispersal abilities of amphipods are poor due to egg brooding, lack of free-swimming
52 larvae and extended parental care (Barnard & Karaman 1991; Thiel 1999; Väinölä *et al.* 2008).
53 Consequently, populations can easily become genetically isolated, leading to high species diversity
54 and biogeographical patterns which accurately reflect ancient historical events (Finston *et al.* 2007;

55 Hou *et al.* 2011; Bauzà-Ribot *et al.* 2012; Copilaş-Ciocianu & Petrusek 2017; Copilaş-Ciocianu *et al.*
56 2019). Lastly, amphipods are emerging model organisms for research on development, regeneration,
57 ecotoxicology and evolutionary biology (Fišer 2012; Weston *et al.* 2013; Kao *et al.* 2016; Naumenko
58 *et al.* 2017; Fišer *et al.* 2018).

59 Despite global distribution, high abundance, ecological significance, and importance as
60 emerging model organisms, only little is known about the evolutionary history of amphipods and the
61 factors that triggered their impressive radiation. This scarcity of knowledge is due to several critical
62 factors. Deep evolutionary relationships within the Amphipoda are uncertain. The most
63 comprehensive phylogenetic studies were based on morphology (Lowry & Myers 2013, 2017), which
64 is known to be highly homoplastic in amphipods (Berge *et al.* 2000). Indeed, molecular phylogenies at
65 lower taxonomic levels do not fully agree with the morphology-based systematics (Macdonald *et al.*
66 2005; Havermans *et al.* 2010; Hurt *et al.* 2013; Esmaeili-Rineh *et al.* 2015; Hou & Sket 2016; Mamos
67 *et al.* 2016; Verheye *et al.* 2016; Copilaş-Ciocianu *et al.* 2019). The age of the order is puzzling as
68 well. It has been suggested that amphipods already appeared in the Late Palaeozoic, when the lineages
69 of the superorder Peracarida split (Bousfield 1977, 1978; Schram 1986). Yet, unlike the rest of
70 peracaridan orders with pre-Cenozoic fossil record (Schram 1986; Wolfe *et al.* 2016), the handful of
71 amphipod fossil taxa are dated no earlier than Eocene and usually bear a modern appearance
72 (Derzhavin 1927; Bousfield & Poinar 1994; Coleman 2006; Kupryjanowicz & Jażdżewski 2010).
73 Hence, the temporal origin and main cladogenetic events of modern amphipods are not known and
74 cannot be paralleled to main global environmental changes.

75 The present view on the modern diversity of amphipods can be summarised by two competing
76 hypotheses. The first hypothesis, stating that most of the modern diversity has been attained by the end
77 of the Palaeozoic or Early Mesozoic, is based on the current distribution of superfamilies in
78 relationship to geochronology, cladistic relationships, or on patterns observed in related
79 malacostracans (Barnard & Barnard 1983; Lowry & Myers 2013). The alternative hypothesis suggests
80 that the diversity of amphipods could be much younger due to morphological continuity among higher
81 taxa, and due to lower taxonomic diversity in terrestrial and deep-sea habitats in comparison to the

82 closely related isopods (Bousfield 1978). Molecular phylogenetic studies tend to support this view
83 because they indicate that the onset of diversification of several major amphipod clades dates to the
84 Cretaceous/Palaeogene (Hou *et al.* 2014; McInerney *et al.* 2014; Corrigan *et al.* 2014; Verheye *et al.*
85 2017; Copilaş-Ciocianu *et al.* 2019). Apart from the recent fossil record and molecular timetrees,
86 several other independent lines of evidence point out to a more recent radiation of amphipods.
87 Amphipods are particularly cold adapted animals, exhibiting an inverse latitudinal richness gradient in
88 marine and freshwater habitats (Barnard 1976; Barnard & Barnard 1983; Barnard & Karaman 1991;
89 Väinölä *et al.* 2008; Rivadeneira *et al.* 2011; Copilaş-Ciocianu *et al.* 2019), and high diversity and
90 dominance in the cold, deep-sea benthic assemblages (De Broyer *et al.* 2004; Verheye *et al.* 2017;
91 Havermans & Smetacek 2018). This pattern is probably related to their generally low tolerance to
92 hypoxia, given that warmer water has a lower concentration of dissolved oxygen (Modig & Ólafsson
93 1998; Wiklund & Sundelin 2001; Wu & Or 2005; Vaquer-Sunyer & Duarte 2008). As such, it seems
94 unlikely that amphipods could have attained most of their current ecological disparity during the Early
95 to Middle Mesozoic (Triassic to Early Cretaceous), a period characterized by warm temperatures even
96 in the deep-sea, by weakly stratified oceans and frequent anoxic events that caused major extinctions
97 (Lear *et al.* 2000; Jacobs & Lindberg 2002; McClain & Hardy 2010). Therefore, we hypothesize that
98 amphipods ecologically radiated in the Late Mesozoic/Cenozoic, when large-scale continental
99 reconfiguration induced global climatic cooling, causing the oceans to transition to a thermohaline
100 (two-layered) circulation which, in turn, increased productivity and oxygenation levels (McClain &
101 Hardy 2010; Donnadieu *et al.* 2016; Mills *et al.* 2019). To test our hypothesis, we generated the first
102 large-scale, time calibrated molecular phylogeny of the Amphipoda and reconstructed the course of
103 diversification and ecological transitions. We focused on the timing of major cladogenetic events and
104 ecological transitions.

105

106

107 **Materials and Methods**

108 *Data collection and sequence alignment*

109 As a taxonomic backbone for data collection we used the classification on the World Register
110 of Marine Species database (WoRMS; <http://www.marinespecies.org/>) which is based on Lowry &
111 Myers (2017). All the data used in the present study is publicly available in GenBank
112 (www.ncbi.nlm.nih.gov/genbank) and originates from 63 published and 13 unpublished studies (a list
113 of the data sources is found in Appendix 1 and Table S1; data collection ended in January 2018). Taxa
114 were included in a way that we would cover as much phylogenetic and ecological diversity as
115 possible. Maximizing phylogenetic diversity diminishes the effect of long-branch attraction and
116 increases topological accuracy by dispersing homoplasy across the tree (Heath *et al.* 2008). In most
117 cases, we included one representative species per genus. We selected four molecular markers based on
118 their abundance and representativeness for all main clades: the mitochondrial cytochrome c oxidase
119 subunit I (COI), the nuclear ribosomal RNA for the large and small subunits (28S and 18S), and the
120 nuclear histone 3 (H3). All sequences were screened for contamination, presence of stop codons and
121 homology. Preliminary gene trees were constructed for each marker to identify and eliminate
122 unreliable sequences. To properly root the phylogeny, we included nine outgroups representatives: the
123 sister order Ingolfiellida, as well as other members of the Peracarida and Decapoda. The dataset
124 contained 210 (201 ingroup) terminals, representing 102 of the 226 recognized families (45%) (Table
125 S1).

126 The PhyRe python script (Plazzi *et al.* 2010) was used to assess the phylogenetic
127 representativeness of our dataset. The analysis was run at the genus level and the reference taxonomy
128 was obtained from the WoRMS database. Confidence intervals for the average and the variation in
129 taxonomic distinctness were calculated using 1000 random replicates.

130 The protein coding COI and H3 sequences were aligned with MUSCLE (Edgar 2004) in
131 MEGA 6 (Tamura *et al.* 2013) and amino acid translated to check for premature stop codons
132 (indicating pseudogenes). Following Copilaş-Ciocianu *et al.* (2018), we separately aligned the 18S and
133 28S rRNA sequences with SATé 2.2.7 (Liu *et al.* 2012). SATé simultaneously co-estimates the
134 alignment and phylogenetic tree, which makes it far more accurate than other alignment methods
135 (Mirarab *et al.* 2015). MAFFT 6.7 (Katoch *et al.* 2005) was used as the aligner and OPAL 1.0.3

136 (Wheeler & Kececioglu 2007) as the merger because this combination provides the highest
137 phylogenetic accuracy (Liu & Warnow 2014). For tree inference we used the maximum-likelihood
138 method implemented in FastTree 2.1.4 (Price *et al.* 2010) with the GTR+G20 substitution model. The
139 cycle of alignment and tree building was repeated ten times for each marker. The alignments with the
140 best likelihood score were used as input for final tree estimation and statistical support analyses (see
141 *Phylogenetic reconstruction*). Gblocks 0.9 (Talavera & Castresana 2007) was used to remove poorly
142 aligned regions with questionable homology in the 18S and 28S alignments. Minimum restrictive
143 settings were applied and regions with gap positions were allowed in the final alignment. The final
144 alignment length was 1741 bp for 18S, 883 bp for 28S, 436 bp for COI (third codon position removed;
145 see next section), and 327 bp for H3, totalling 3387 bp. Individual marker alignments were
146 concatenated using Sequence Matrix (Vaidya *et al.* 2011). The degree of missing data was 29%. The
147 alignment in NEXUS format is available on Figshare (doi available after acceptance).

148 Ecological data regarding habitat (marine, freshwater and terrestrial; benthic vs. pelagic),
149 mode of life (free vs. symbiotic), depth (littoral/epipelagic, shelf/mesopelagic, bathyal/bathypelagic,
150 abyssal/abyssalpelagic and hadal/hadalpelagic) and temperature (cold vs. warm) were gathered from
151 the relevant literature at the genus level (Bazikalova 1945; Laval 1980; Barnard & Barnard 1983;
152 Barnard & Karaman 1991; Vinogradov *et al.* 1996; de Broyer *et al.* 2007). Note that “symbiotic mode
153 of life” encompasses different types of symbiosis (commensalism, parasitism, and amensalism). The
154 depth zone was attributed on the mean depth value, obtained from the minimum and maximum values
155 for depth ranges of each taxon. A taxon was considered as cold water distributed when its
156 representatives occurred at high to temperate latitudes or deeper than 1000 m. Similarly, a taxon was
157 classified as warm water when its representatives were distributed in tropical to temperate waters
158 above 1000 m depth. All ecological data can be found in the supplementary information (Table S2).
159 To provide an overview of the geographic distribution of clades, we also gathered distribution data
160 which was obtained from Barnard & Karaman (1991). Terminals were assigned to 17 geographical
161 areas (Table S2).

162

163 *Phylogenetic reconstruction*

164 We evaluated the level of substitution saturation of each marker using the index of Xia et al. (2003)
165 implemented in DAMBE 5.3.10 (Xia & Xie 2003). Significant levels of saturation were detected at the
166 COI 3rd codon position ($I_{ss} > I_{ss,c}$, $p = 0.0001$), and, as such, these sites were not included in the
167 phylogenetic analyses. Variable and parsimony informative sites were calculated in MEGA. The
168 concatenated alignment contained 1917 parsimony informative out of 2333 variable sites (18S:
169 1006/1273; 28S: 575/667; COI: 209/251; H3: 127/142). Best-fitting evolutionary models were
170 selected using PartitionFinder 2 (Lanfear *et al.* 2017) under the Bayesian Information Criterion and
171 greedy search option.

172 Phylogenetic relationships were inferred using maximum likelihood (ML), Bayesian inference
173 (BI) and maximum parsimony (MP) methods. The ML analyses were conducted with IQTREE 1.6
174 (Nguyen *et al.* 2015) and RAxML HPC 8.2.10 (Stamatakis 2014). The IQTREE search was performed
175 under an edge-linked partitioned model (applied to each gene partition), using the GTR model with
176 free rate heterogeneity (+R) which relaxes the assumption of Gamma distributed rates and has a better
177 fit to large and complex datasets (Yang 1995; Soubrier *et al.* 2012). Statistical support for branches
178 was assessed using 1000 ultra-fast bootstrap replicates (UFBS; Hoang et al. 2018) and the Shimodaira-
179 Hasegawa approximate likelihood ratio test (SH-aLRT; Shimodaira and Hasegawa 1999, Guindon et
180 al. 2010). The RAxML analysis was run with the GTR+ Γ model applied to each gene partition. A
181 thorough ML search was performed and 1000 rapid bootstrap replicates (RBS) were used to assess
182 branch support. Bayesian analyses were performed with ExaBayes 1.5 (Aberer *et al.* 2014) under the
183 GTR+ Γ model applied to each gene partition. All parameters (except branch length) were unlinked
184 and rates were allowed to vary independently. The analysis was run for 10^7 iterations, with a thinning
185 of 500 and 50% burn-in. The value for the parsimony subtree pruning and regrafting (SPR) radius
186 parameter was set to 50 and the number of swaps per generation to 10. Maximum parsimony was
187 performed with PAUP* 4.0a164 (Swofford 2002), using heuristic searches with TBR branch swapping
188 and 1000 random taxon additions. Only phylogenetically informative sites were retained, gaps were
189 treated as missing data, and all characters were unordered and equally weighted. Nodal support was

190 estimated with 500 jackknifing replicates (JK) with 50% character removal. All phylogenetic analyses
191 were performed on the CIPRES Science Gateway v3.3 (Miller *et al.* 2010).

192

193 *Molecular dating*

194 Molecular dating was performed in BEAST 1.8.2 (Drummond *et al.* 2012) using the GTR+I+ Γ model
195 for COI and H3, and SYM+I+ Γ for 18S and 28S (as selected with PartitionFinder). The ML
196 phylogram from the IQTREE analysis was used as a starting tree in order to reduce computational
197 time. An uncorrelated relaxed clock with a lognormal distribution was applied to each partition and
198 speciation was modelled using the Birth-Death process. The MCMC chain was run for 10⁸
199 generations, with a sampling frequency of 2000. Convergence of parameters and effective sample size
200 were assessed with Tracer 1.6 (Rambaut *et al.* 2014) after discarding 20% of trees as burn-in. We
201 performed three independent runs, which gave the same result. As such, all runs were combined using
202 LogCombiner 1.8.2 and the maximum clade credibility tree was produced with TreeAnnotator 1.8.2,
203 both part of the BEAST package.

204 For divergence times estimation we employed the fossil calibration scheme described in detail
205 by Copilaş-Ciocianu *et al.* (2019), to which we added one more calibration point. Only fossil taxa that
206 are well studied and represented by several specimens were used for calibration. All calibration nodes
207 were assigned exponential prior distributions since they require fewer parameters and are more
208 appropriate when the fossil record of the focal group is poorly known (Ho & Phillips 2009). Briefly,
209 we used five calibration points, the youngest (node 1), representing the origin of the Ponto-Caspian
210 gammarid amphipod radiation, was set to a minimum age of 9 Ma (mean = 25, offset = 8, 95%
211 HPD = 9–83) based on Caucasian fossil specimens (Derzhavin 1927). The
212 Niphargidae/Pseudoniphargidae and Crangonyctidae/Pseudocrangonyctidae splits (nodes 2 and 3,
213 respectively) were set at a minimum of 35 Ma (mean = 60, offset = 35, 95% HPD = 38–215) based
214 on Eocene Baltic amber fossils (Coleman & Myers 2000; Coleman 2004, 2006; Kupryjanowicz &
215 Jazdzewski 2010). The additional calibration point (node 4) that we use in this study is based on

216 Miocene amber specimens of the family Talitridae from Central America (Bousfield & Poinar 1994,
217 1995). The minimum age was set to 25 Ma (mean = 30, offset = 21, 95% HPD = 22–111) and was
218 applied to the stem of the Talitridae because this family has an uncertain phylogenetic position within
219 the Talitroid clade of our phylogeny. Finally, the oldest calibration point (node 5) was placed as close
220 to the root as possible (following Duchêne *et al.* 2014), and represents the oldest known member of
221 Eumalacostraca, *Palaeopalaemon newberry* Whitfield, 1880 (minimum = 358 Ma, mean = 55, offset =
222 355, 95% HPD = 358–514) (Schram *et al.* 1978). The nodes do not seem misdated, given that
223 inconsistency between fossil ages and lineage history was not significant (Shapiro-Wilk normality test,
224 $W = 0.91$, $p = 0.51$, see Marshall 2008).

225 Due to the notable difference between the recent amphipod (ingroup) calibration points (9-35
226 Ma) and the old outgroup (358 Ma) calibration, we also ran two additional analyses, one only with
227 ingroup calibrations, and one only with the outgroup calibration to assess if they produce compatible
228 results.

229

230 *Diversification through time and ancestral state reconstruction*

231 A sliding window analysis was performed according to Meredith *et al.* (2011) to visually inspect the
232 tendency of diversification rates through time. The period between 180 and 10 Ma was divided into
233 sliding windows of 10 Ma, with a frequency of 2.5 Ma. The period prior to 180 Ma was not considered
234 due to the low number of lineages which would indicate an artificially inflated diversification rate. The
235 number of lineages originating in a particular sliding window was divided by the number of lineages
236 occurring prior to the start of that respective sliding window.

237 We reconstructed ancestral states for the five abovementioned ecological traits with the aim of
238 evaluating the temporal framework of the amphipod ecological transitions. As such, we used the time-
239 calibrated tree from the BEAST runs in subsequent analyses. Traits were treated as discrete and
240 analysed with the re-rooting method using maximum likelihood (ML) (Yang *et al.* 1995) as well as
241 stochastic character mapping (SCM) using Bayesian inference (Bollback 2006), both implemented in

242 R (v.3.5.2) package *phytools* (v.0.6-60) (Revell 2012; R Core Team 2018). ML ancestral state
243 reconstruction was performed with the *rerootingMethod* function, while SCM with the *make.simmap*
244 function (Revell 2012). In order to estimate the possible ancestral character states on internal nodes,
245 we ran 1000 simulations of stochastic character histories, using continuous-time reversible Markov
246 model parameters of trait evolution, estimated using default settings and the character states on the tips
247 of the phylogeny.

248 In order to evaluate the tempo of ecological disparification through time, we divided the
249 phylogenetic history in 10 MY time bins, and calculated the average number of all possible transitions
250 between states per time bin, as well as for the whole phylogeny. For the sake of clarity, we refer the
251 term “diversification” to the process of speciation, while we use the term “disparification” to the
252 process of ecological divergence.

253

254 **Results**

255 *Dataset*

256 The phylogenetic representative analysis indicated a highly representative taxon sampling. The
257 Average Taxonomic Distinctiveness (AvTD) and Variation in Taxonomic Distinctiveness (VarTD)
258 were above the highest AvTD and below the mean VarTD respectively (Fig. S1). Furthermore, von
259 Euler’s index of imbalance ($I_E=0.102$) was well below the recommended 0.25 threshold value,
260 indicating unbiased sampling (Plazzi *et al.* 2010) (Fig. S1).

261

262 *Phylogenetic reconstruction and molecular dating*

263 All five phylogenetic reconstruction methods yielded congruent results by recovering the same major
264 clades. All model based methods recovered similar topologies and discordance was observed mainly at
265 poorly supported nodes (Fig. 1, Figs. S2-S5). Altogether, we identified nine major clades which we
266 named informally: Gammaroids– mainly northern hemisphere taxa, with freshwater proclivity,
267 Lysianassoids–mostly deep-sea scavengers, Crangonyctoids– Holarctic freshwater species,
268 Corophioids– tube-building epifaunal/infaunal marine species, Eusiorids/Iphimedioids– ecologically

269 diverse cold-water marine species, Physosomatans and Physocephalata—exclusively commensal and
270 parasitic warm-water marine species, most of them pelagic, Talitroids—shallow water, ecologically
271 diverse containing the only terrestrial lineage, and Atylids—a morphologically plesiomorphic and
272 cosmopolitan marine group. The following groups were either weakly supported or polyphyletic, but
273 we retained them for the sake of brevity: the Miscellaneous clade was weakly supported and
274 comprised morphologically and ecologically disparate families, while the Hadzioids were
275 morphologically consistent but polyphyletic. The order Ingolfiellida was recovered as a sister to
276 Amphipoda only in the IQTREE analysis. All remaining analyses (ML with RAxML, BI, and MP)
277 recovered the order Spelaeogriphacea as a sister to amphipods with high support, while the order
278 Ingolfiellida was sister to (Amphipoda+Speleogriphacea) clade (Figs. S3-S5).

279 Molecular dating using all the calibration points or only the root calibration resulted in similar
280 estimations (10 to 20 Ma differences; Table S3). The calibration scheme that included only the recent
281 ingroup fossils resulted in expectedly younger estimates, however, the 95% HPD intervals of all
282 calibration schemes overlapped to some extent (Table S3). Altogether, these results contradict the
283 previous views that the modern diversity of the Amphipoda was already established by the Late
284 Palaeozoic/Early Mesozoic (Barnard & Barnard 1983; Lowry & Myers 2013). For evolutionary,
285 ecological and biogeographical interpretation we considered the complete calibration scheme since it
286 is the most balanced and informed. Accordingly, Amphipoda has split off from Ingolfiellida during the
287 Permian (~281 Ma) and started radiating shortly after the Permo-Triassic mass extinction (~240 Ma).
288 Crown ages of all major clades lie between the Early Cretaceous and Early Palaeogene (Fig. 2, Table
289 S3). A fully annotated chronogram is available as a supplementary figure (Fig. S6). An additional tree
290 with the geographical distribution of taxa mapped onto it is also available as supplementary
291 information (Fig. S7).

292

293 *Diversification through time and ancestral states reconstruction*

294 The sliding window analysis identified four main pulses of diversification: the first occurred during
295 the middle Jurassic, the second during the late Jurassic/early Cretaceous, the third during the mid-
296 Cretaceous, and the fourth in the late Cretaceous (Fig. 2).

297 The SCM (Fig. 3) and ML (Fig. S8) reconstructions of ancestral states were highly congruent
298 and revealed a dynamic evolution of Amphipod ecology. The analyses based on 1000 simulations
299 suggested that all ecological changes, except the shift to semiterrestriality, happened multiple times:
300 on average 5.7 times from free-living to symbiotic life-style, 7.4 times from marine to freshwater and
301 2.6 times vice versa, 12.9 times from cold to warm waters and 5.3 times back to cold water, and 9.5
302 times from benthic to pelagic habitat (Table 1). At least 100 changes of depth zones were estimated,
303 mostly from shallow waters to deep sea (Table 1). There was a presumably single shift to
304 semiterrestrial life from ancestors with uncertain salinity preference (Table 1, Fig. 3) (see Discussion).

305 All these ecological changes took place relatively late in the amphipod evolutionary history,
306 no earlier than 170Ma. The periods of intense ecological disparification corresponded to peaks of
307 lineage diversification. The ancestral amphipod was reconstructed as a cold-water, free-living, marine
308 animal, inhabiting shallow benthos. The first ecological shifts date to middle Jurassic, when the
309 ancestor of Hyperiid clade (Physosomatans+Physocephalatans) likely switched from cold-water and
310 free-living benthic species to warm-water and mostly pelagic symbiont mode of life (Table 1, Figs. 3-
311 S8). The ancestral salinity of Talitroids is uncertain because the ML analysis suggests a marine
312 ancestry while the SCM indicates a freshwater ancestry (see Discussion for details). The next pulse of
313 disparification took place in Late Cretaceous, when the caprellid lineage of the Corophioid clade
314 switched from cold-water free-living animals to warm water-symbionts. At the same time, at least two
315 transitions to freshwater occurred in Crangonyctoids and Niphargidae/Pseudoniphargidae clades.
316 Gammaroids presumably transitioned to fresh waters at least twice during the Palaeogene. Similarly,
317 transition to deep-sea niches (>1000 m) occurred predominantly during the Palaeogene, mainly in the
318 Lysianassoids, the Physosomatans and several lineages of the Eusiroid/Iphimediod clade. The third
319 pulse of ecological disparification took place during the last 50 Ma, with multiple ecological shifts all
320 over the phylogeny (Table 1, Fig3. 3-S8).

321

322 **Discussion**

323 Our study reveals an ancient Permian origin of amphipods, and their delayed diversification during the
324 Late Jurassic-Early Cretaceous followed by ecological disparification during the Cretaceous-
325 Palaeogene. These results refute the view that most of the modern amphipod diversity already existed
326 since the Late Palaeozoic-Early Mesozoic and help reconcile their old history with their absence in the
327 pre-Cenozoic fossil record. Below we discuss the main global events that may have affected their
328 diversification and ecological radiation. We focus on large-scale patterns and emphasize that although
329 many nested radiations likely occurred discussing them in detail is beyond the scope of this study.

330

331 *Global evolutionary and biogeographical patterns*

332 Our results indicate that amphipods split off from ingolfiellids during the Permian (~280 Ma).
333 This is in good agreement with previous studies which suggested a Late Palaeozoic age of the
334 Amphipoda based on the fact that peracarids went through an extensive phase of radiation and
335 dispersal during this time (Bousfield 1983; Schram 1986; Wolfe *et al.* 2016). Furthermore, the
336 Permian is also known for its peak in the diversification of marine invertebrates (Alroy *et al.* 2008).
337 Extant amphipod lineages started radiating after the major Permo-Triassic extinction, a catastrophic
338 event that wiped out up to 95% of marine taxa (Benton & Twitchett 2003). During this dramatic
339 extinction, the Panthalassic Ocean was largely anoxic, especially at higher latitudes or depths, and
340 only the Palaeotethys Ocean retained suitable levels of oxygenation (Penn *et al.* 2018), where hypoxia-
341 sensitive amphipods could survive and from where they subsequently radiated. This hypothesis is
342 concordant with tethyan distribution of the basal, shallow-water Hadzioid lineages in our phylogeny
343 (Barnard 1976; Stock 1993; Bauzà-Ribot *et al.* 2012).

344 Most of the deep splits in the phylogeny leading towards the extant major clades took place
345 during the Triassic and Early Cretaceous (Fig. 2). Also, three of the four main lineage diversification
346 peaks took place in this period. Assuming that amphipods were littoral inhabitants throughout this
347 time-frame, it is likely that the breakup of Pangaea followed by subsequent fragmentation of the

348 resulting Laurasia and Gondwana supercontinents (Seton *et al.* 2012) played an important vicariant
349 role, as seen in the Holarctic freshwater crangonyctoids (Copilaş-Ciocianu *et al.* 2019) or thalassoid
350 lineages with an amphi-Atlantic distribution (Bauzà-Ribot *et al.* 2012). We consider that more detailed
351 inferences are speculative due to the cosmopolitan distribution of many marine lineages and the lack
352 of pre-Cenozoic fossils. Exploring this issue further requires more data and in-depth phylogenetic and
353 biogeographical analysis of the cosmopolitan clades. Similarly, a detailed analysis of patterns of
354 diversification rates through time was not realistic due to the extremely scarce amphipod fossil record
355 and our incomplete dataset lacking half of the known families (Marshall 2017; Diaz *et al.* 2019).

356 The last major spike in the amphipod diversification history occurred ca. 90 Ma ago (Fig. 2).
357 This peak of diversification could be related to the globally high sea-level stands which resulted in
358 vast shallow epicontinental seas that provided a lot of habitat suitable for amphipods (Scotese 2014).
359 This is particularly visible in the warm, shallow-water Corophioid, Physocephalatan and
360 Physosomatan clades which started proliferating in this period. Furthermore, this diversification peak
361 coincides with a peak in diversity of marine invertebrates (Alroy *et al.* 2008) and also to the highest
362 levels of atmospheric oxygen during the last 250 Ma (Berner 2002).

363 Our estimated time-frame of overall amphipod diversification agrees well with recent
364 molecular studies which consistently indicate a Cretaceous-Palaeogene diversification of several major
365 clades, despite different approaches to calibrate divergence times (Hou *et al.* 2014; McInerney *et al.*
366 2014; Corrigan *et al.* 2014; Verheye *et al.* 2017; Copilaş-Ciocianu *et al.* 2019). Therefore, these
367 studies provide strong evidence to indicate that the diversity of extant amphipods is indeed relatively
368 recent, despite the old Permian origin of the group.

369

370 *Ecological radiation*

371 Our ancestral state reconstructions suggested that ancestral amphipods were free-living, marine,
372 littoral animals with an affinity for cold water for a large part of their evolutionary history (Permian to
373 Late Jurassic), and are in agreement with previous hypotheses (Bousfield 1983). The littoral, which is
374 the most oxygenated part of the ocean (Keeling *et al.* 2010) possibly acted as a long-term refugium for

375 hypoxia sensitive animals such as amphipods (Modig & Ólafsson 1998; Wiklund & Sundelin 2001;
376 Wu & Or 2005; Vaquer-Sunyer & Duarte 2008). This would suggest that the long Permian-to-mid
377 Mesozoic ecological stasis in littoral habitats was a result of environmental constraints, reflecting the
378 suboptimal conditions present in non-littoral parts of the ocean, such as high global temperatures
379 (especially in the deep sea), weak oceanic circulation, hypoxic conditions and frequent deep-sea
380 anoxia (McClain & Hardy 2010; Mills *et al.* 2019). Considering that the coastal environment is prone
381 to erosion rather than deposition (Wilke *et al.* 2016), our results support the view that the absence of a
382 pre-Cenozoic amphipod fossil record (Bousfield 1978; Schram 1986) is best explained by a
383 combination of long evolutionary confinement to active littoral habitats, small size and weakly
384 fossilizable cuticle (Bousfield 1983).

385 The overall evidence thus indicates that the ecological radiation of amphipods proceeded from
386 shallow marine waters. Most of the freshwater radiations emerged relatively recently (Late
387 Cretaceous/Cenozoic) after the substantial Late Cretaceous temperature drop (Figs. 2-3)(Mills *et al.*
388 2019). All of these freshwater clades (especially the Holarctic Crangonyctoids, Gammaroids and
389 Niphargids) descended from shallow-water ancestors and often exhibit a peculiar biogeographic
390 pattern with older lineages and higher phylogenetic diversity occurring in the northern part of their
391 ranges (McInerney *et al.* 2014; Copilaş-Ciocianu *et al.* 2017, 2019). This is likely a relict pattern
392 which reflects their more northwards pre-Pleistocene distribution, and consequently their affinity for
393 cold water. This hypothesis is supported by amber fossils clearly belonging to the Niphargidae clade,
394 found in the Baltic region, which lies northwards of the contemporary range of the group (Coleman &
395 Myers 2000; Kupryjanowicz & Jazdzewski 2010). Noteworthy, the ancestral salinity preference of the
396 Talitroid clade is uncertain in our analyses. The Bayesian reconstruction indicated a freshwater
397 ancestry (Fig. 3) while the ML reconstruction suggested a marine ancestry (Fig. S8). These contrasting
398 results are most likely an artefact of low sampling of marine Talitroid lineages due to limited sequence
399 availability. Most likely the group has a marine origin (Serejo 2004) and more fine scale studies also
400 showed a saline origin of some inland Talitrids (Yang *et al.* 2013).

401 All the colonizations of the deep-sea also took place throughout the cooling of the Late-
402 Cretaceous/Cenozoic, and postdate the Cenomanian-Turonian oceanic anoxic event which triggered
403 global faunal extinctions (Figs. 2-3)(80-100 Ma; Arthur *et al.* 1987). This time frame also corresponds
404 with the opening and deepening of the North Atlantic and Southern Oceans (Seton *et al.* 2012) which
405 are thought to have played an important role in the diversification of the Lysianassoid and
406 Eusiroid/Iphimedioid clades (Corrigan *et al.* 2014; Verheye *et al.* 2017). These relatively recent deep-
407 sea radiations agree well with the prevailing view that most of the deep-sea fauna is of Cenozoic
408 origin due to the hypoxic conditions during most of the Mesozoic (Lindner *et al.* 2008; McClain &
409 Hardy 2010; Vrijenhoek 2013; Herrera *et al.* 2015). At least for deep-sea amphipods, the tectonic-
410 induced climatic cooling, leading to a better ventilated ocean (Donnadieu *et al.* 2016), coupled with
411 the Cenomanian-Turonian extinction event not only dwindled the competitors but also created suitable
412 environmental conditions for amphipods to thrive and ecologically expand. Moreover, the inverse
413 latitudinal richness gradient observed in contemporary freshwater and marine amphipods (Barnard &
414 Barnard 1983; Väinölä *et al.* 2008; Rivadeneira *et al.* 2011) further emphasizes that low temperature is
415 somehow critical for the colonization of freshwater and deep-sea niches. However, we lack a precise
416 understanding of these processes, which are probably related to the generally low tolerance of
417 amphipods to hypoxic conditions (Modig & Ólafsson 1998; Wiklund & Sundelin 2001; Wu & Or
418 2005; Vaquer-Sunyer & Duarte 2008).

419 Transitions to a pelagic life-style occurred numerous times as well, and are especially
420 prevalent in deep-sea lineages (Fig. 3). In freshwater, this transition occurred only once, in the highly
421 specialized Baikal species *Macrohectopus branickii*. Like in the previous cases, the majority of these
422 pelagic transitions are relatively recent (Late Cretaceous to Cenozoic), mainly because these lineages
423 occur in the deep-sea. Exceptionally, the diverse Physocephalatan clade colonized warm shallow
424 epipelagic waters during the Early Cretaceous, earlier than all of the other pelagic lineages. Our results
425 reveal that Hyperiid (Physocephalatan+Physosomatans) colonized pelagic niches two times
426 independently and contradict the prevailing view of a single colonization of pelagial (Lowry & Myers
427 2017).

428 The expansion into symbiotic niches seems to not have been influenced that much by climatic
429 cooling. Both the large symbiotic Hyperiid clade as well as the caprellid lineage of the Corophioid
430 clade switched to this life-style during the warm Late Jurassic/Cretaceous (Figs. 2-3). These clades are
431 associated with an ancestral preference for warmer temperatures (Fig. 3). Therefore, it is likely that the
432 Cretaceous high sea-level which led to the formation of numerous shallow and warm epicontinental
433 seas (Scotese 2014) created vast amounts of suitable habitats which promoted diversification and
434 facilitated the formation of symbiotic relationships. The crown age of the exclusively symbiotic
435 Hyperiid clade (ca. 140 Ma) corresponds well with a peak of reef forming coral diversity at 150 Ma
436 (Simpson *et al.* 2011), an important habitat for the basal members of this clade (Barnard & Karaman
437 1991; Lowry & Myers 2009). These symbiotic clades often contain taxa with extreme ecological
438 specialization, and exhibit a highly modified morphology (Fig. 1) (Laval 1980; Ito *et al.* 2011; Hurt *et*
439 *al.* 2013).

440 The colonization of terrestrial habitats occurred only in the lineage of the family Talitridae of
441 the Talitroid clade. Although this is possibly the most radical ecological transition among amphipods
442 (Spicer *et al.* 1987), it is also one of the most recent, occurring during the Palaeogene (Figs. 2-3). It
443 has been previously proposed that the terrestrial transition of talitrids was dependent on the availability
444 of angiosperms (main food source), and thus, should not be older than the Cretaceous radiation of this
445 group of plants (Bousfield 1983; Barba-Montoya *et al.* 2018). Nevertheless, some authors suggest a
446 Pangaean origin of talitrids given their global distribution (Lowry & Myers 2019). Additional
447 evidence supporting a young age for this clade is its less advanced stage of terrestrial specialization
448 and far lower taxonomic diversity in comparison with the related oniscoidean isopods (Bousfield
449 1983; Spicer *et al.* 1987), which have colonized this habitat at least since the Early Cretaceous,
450 possibly even the Late Palaeozoic (Broly *et al.* 2013). The young age of the Talitridae implies that the
451 switch to semiterrestrial life likely took place multiple times within the Talitroid lineage, a hypothesis
452 that remains to be tested using more comprehensive taxon sampling.

453

454 *Systematic implications*

455 The main molecular clades recovered in our phylogeny correspond well in most cases with the
456 major morphological groups (Lowry & Myers 2017). Due to some unresolved basal nodes and
457 incomplete taxon sampling, the topology of our molecular phylogeny cannot refute the current
458 systematic view. Although we had data on four of the six recognized suborders (Hyperioidea and
459 Pseudoingolfiellidea were missing), our phylogeny generally supports the three main suborders,
460 Amphilochea, Hyperioidea and Senticaudata, but suggests some reshuffling. For example, the
461 parvorders Lysianassidira, as well as the polyphyletic Haustoriidira and Synopiidira should be placed
462 into the Senticaudata rather than Amphilochea. Similarly, the suborder Colomastigidea along with
463 the amphilochean families Amphilocheidae and Leucothoidae should be placed within Hyperioidea,
464 reinforcing a previously proposed close relationship between Hyperioidea and Amphilocheidae (Kim &
465 Kim 1993). We consider that a thorough systematic discussion is beyond the scope of this paper, and
466 suggest that a phylogenomic approach could prove invaluable for a better understanding of amphipod
467 evolution, as evidenced in a recent study on decapod crustaceans (Wolfe *et al.* 2019). However, any
468 evolutionary hypothesis needs to be tested by independent lines of evidence, and, as such, we consider
469 that phenotypic data plays a crucial role even in the modern era of phylogenomics (Lee & Palci 2015).

470

471 **Conclusion**

472 The Late Mesozoic is notable for its dramatic global changes which saw the rise and demise of
473 many organismal groups, leading towards the modern biota (Scott 1995; Roelants *et al.* 2007; Alroy *et al.*
474 *et al.* 2008; Schulte *et al.* 2010; Meredith *et al.* 2011; Barba-Montoya *et al.* 2018; Varga *et al.* 2019). In
475 the case of the Amphipoda, these changes brought an important turning point in their evolution. The
476 fortuitous coupling of several critical circumstances such as extinction of deep-sea competitors
477 followed by climatic cooling, oceanic deepening and increased oxygenation, created ecological
478 opportunities which allowed hypoxia-sensitive amphipods to overcome a long period of ecological
479 stasis and to radiate into many new niches. Such patterns of protracted stasis followed by extensive
480 ecological radiation due to global changes seem to be common throughout the tree of life (Mitchell &
481 Makovicky 2014; Halliday *et al.* 2019). Our results also emphasize the importance of molecular

482 phylogenetics in illuminating the evolutionary history of highly diverse clades which have an
483 extremely poor fossil record.

484

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490

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813

814 **Data accessibility**

815 Trees and alignments will be available at FigShare and Treebase.

816

817 **Author contributions**

818 DCC, CF and SB conceived the study, DCC collected data, performed phylogenetic and molecular
819 dating analyses, SB performed ancestral state reconstructions and diversification analyses, DCC lead
820 the writing, all authors contributed to and approved the final version of the manuscript.

821

822 Table 1: Average number of transitions (first column) and average number of transitions per time bin,
 823 from 1000 trees with mapped discrete character states. Cells with average changes ≥ 0.5 per time bin
 824 or average total change ≥ 1 are highlighted and maximum values per transition are bolded.

825

Time bins	Mean	Trias.	Jurassic		Cretaceous			Paleogene		Neo.
		>200	200-170	170-140	140-110	110-80	80-60	60-40	40-20	20-0
MODE OF LIFE										
free - symbiont	5.9	0	0	1	0.1	0.9	0.3	0.5	0.6	2.6
symbiont - free	0.2	0	0	0	0	0	0	0	0	0
HABITAT										
freshwater - marine	2.6	0	0	0	0	0	0	0.2	1.5	0.8
freshwater - semiterrestrial	1	0	0	0	0	0	0	0.5	0.5	0
marine - freshwater	7.4	0	0.4	0.3	0.2	0.9	1.8	1.3	1.8	0.6
marine - semiterrestrial	0	0	0	0	0	0	0	0	0	0
semiterrestrial - freshwater	0	0	0	0	0	0	0	0	0	0
semiterrestrial - marine	0	0	0	0	0	0	0	0	0	0
benthic - pelagic	9.5	0	0	0.2	1	0.7	0.5	1	3.1	3.1
pelagic - benthic	0.7	0	0	0	0.1	0.2	0.1	0.1	0.1	0.2
TEMPERATURE										
cold - warm	12.9	0.1	0.1	1	0.7	0.8	1.3	1.6	3.1	4.2
warm - cold	5.3	0.1	0	0.1	0.1	0.4	0.9	1	1.2	1.5
DEPTH										
[<0.2 km] - [0.2-1 km]	35.5	0.3	0.4	0.9	1.9	3.4	4.5	5.6	7.4	11
[<0.2 km] - [1-4 km]	5	0	0	0.1	0.2	0.4	1.1	1	0.9	1.3
[<0.2 km] - [>4 km]	0	0	0	0	0	0	0	0	0	0
[0.2-1 km] - [1-4 km]	19.6	0.1	0.1	0.1	0.5	1.4	2.7	3.7	4.5	6.5
[0.2-1 km] - [>4 km]	1.5	0	0	0	0	0	0.2	0.2	0.2	0.9
[1-4 km] - [>4 km]	5.5	0	0	0	0	0	0.1	0.4	1.9	2.9
[0.2-1 km] - [<0.2 km]	19.1	0.2	0.2	0.4	0.8	1.6	2.4	3.4	4.4	5.8
[1-4 km] - [<0.2 km]	1.7	0	0	0	0	0.1	0.1	0.3	0.5	0.7
[1-4 km] - [0.2-1 km]	12.5	0	0	0.1	0.2	0.6	0.8	2	3.2	5.6
[>4 km] - [0.2-1 km]	1.2	0	0	0	0	0	0	0	0	1.1
[>4 km] - [1-4 km]	1	0	0	0	0	0	0	0.1	0.3	0.5
[>4 km] - [<0.2 km]	0	0	0	0	0	0	0	0	0	0

826

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829

830 Fig. 1 Maximum likelihood phylogram obtained from the concatenated dataset (COI, H3, 28S and
831 18S) using IQTREE. Nodes receiving strong support in all analyses are indicated with green circles,
832 while nodes that received strong support in all analyses except parsimony are indicated with purple
833 circles. Bars indicate the strength of the support that each node received in each analysis. Nodes that
834 were weakly supported in all analyses are not annotated. Families are indicated with coloured font.
835 Photographs on the right indicate the extent of morphological diversity of the Amphipoda.
836 Photographs: Denis Copilas Ciocianu: a) *Dikerogammarus villosus*, b) *Chaetogammarus*
837 *warpachowskyi*, d) *Gammarus roeselii*, h) *Niphargus pannonicus*; Dante Fenolio (used with author's
838 permission, photos available at www.anotheca.com/photograph): n) *Stygobromus* sp., z) *Cystisoma*
839 sp., a') *Phronima* sp., b') *Streetsia* sp., c') *Scypholanceola* sp., d') *Scina* sp.; David Fenwick (used
840 with author's permission, photos available at www.aphotomarine.com): m) *Lysianassa ceratina*, p)
841 *Caprella tuberculata*, q) *Corophium volutator*, u) *Pontocrates arenarius*, g') *Orchestia gammarellus*,
842 h') *Melita hergensis*, j') *Ingolfiella britannica*; Hans Hillewaert (licensed under Creative Commons,
843 photos available at www.flickr.com/photos/bathyporeia/albums/72157639365477036): c) *Bathyporeia*
844 *pilosa*, e) *Ampelisca brevicornis*, f) *Stenothoe marina*, i) *Lepidepecreum longicorne*, j) *Urothoe*
845 *brevicornis*, o) *Cyamus boopis*, s) *Megaluropus agilis*, f') *Leucothoe incise*, i') *Nototropis*
846 *swammerdamei*, v) *Iphimedia nexa*; Russ Hopcroft (licensed under Creative Commons, photo
847 available at <https://en.wikipedia.org>): y) *Eusirus holmi*; Joanna Legeżyńska (licensed under Creative
848 Commons, photo available at www.marinespecies.org): g) *Stegocephalus inflatus*; Gustav Paulay
849 (licensed under Creative Commons, photo available at <https://calphotos.berkeley.edu/>): e') *Colomastix*
850 sp.; Martin Rauschert (licensed under Creative Commons, photo available at www.marinespecies.org):
851 r) *Paraceradocus* sp.; Alexander Semenov (used with author's permission, photos available at
852 www.coldwater.science): k) *Aristias timidus*, l) *Gammarellus homari*, t) *Acanthonotozoma* sp., x)
853 *Pleustes panopla*; Cédric d'Udekem d'Acoz (licensed under Creative Commons, photo available at
854 www.marinespecies.org): w) *Epimeria oxycarinata*;

855

856 Fig. 2 Evolutionary timescale of the amphipod radiation. The time-calibrated phylogeny was obtained
857 with BEAST using the concatenated dataset (COI, H3, 28S and 18S). Circles at nodes indicate
858 posterior probabilities (black ≥ 0.95 , grey ≥ 0.9 , white ≥ 0.8 , not shown if < 0.8) and blue bars indicate
859 the 95% HPD intervals of clade age (shown only for main and/or strongly supported nodes). Red bars
860 accompanied by numbers indicate node ages that were constrained using fossils. The fully annotated
861 chronogram can be found in the supplementary information (Fig. S6). Coloured boxes on the right
862 summarize the ecological traits of each terminal in the tree (see legend in the top left) and bars indicate
863 depth ranges. The line graphs below the phylogeny indicate (from top to bottom) the amphipod
864 diversification rate through time, the evolution of Cenozoic deep-sea bottom temperature (Lear *et al.*
865 2000), the evolution of Phanerozoic surface temperature (Mills *et al.* 2019), the evolution of
866 Phanerozoic invertebrate diversity (Alroy *et al.* 2008), and the evolution of the global Mesozoic-
867 Cenozoic sea level (Miller *et al.* 2005). The gradient between turquoise and white colours indicates the
868 transitions between thermohaline (two-layered ocean) and thermospheric (weakly stratified)
869 circulation (McClain & Hardy 2010).

870

871 Fig. 3 Ecological transitions of Amphipoda through time. Ancestral state reconstruction was estimated
872 using stochastic character mapping. Pie-charts at nodes indicate the probability of ancestral states. The
873 maximum likelihood results of the ancestral states are presented in the supplementary information
874 (Fig. S8).

875

876

877 **Appendix**

878 Reference list of all studies which generated the sequence data used in the current work

879 **Supporting Information**

880 Table S1 Sequence data used in this study and its origin

881 Table S2 Ecological and geographical data

882 Table S3 Comparison of clade ages under different calibration schemes

883 Fig. S1 Funnel plots resulting from the PhyRe analyses

884 Fig. S2 Maximum likelihood phylogeny obtained with IQTREE.

885 Fig. S3 Maximum likelihood phylogeny obtained with RAxML.

886 Fig. S4 Bayesian phylogeny obtained with ExaBayes

887 Fig. S5 Maximum parsimony phylogeny obtained with PAUP

888 Fig. S6 Fully annotated Bayesian chronogram.

889 Fig. S7 Geographical distribution of taxa used in the phylogenetic analysis.

890 Fig. S8 Maximum-likelihood reconstruction of ancestral states.

891

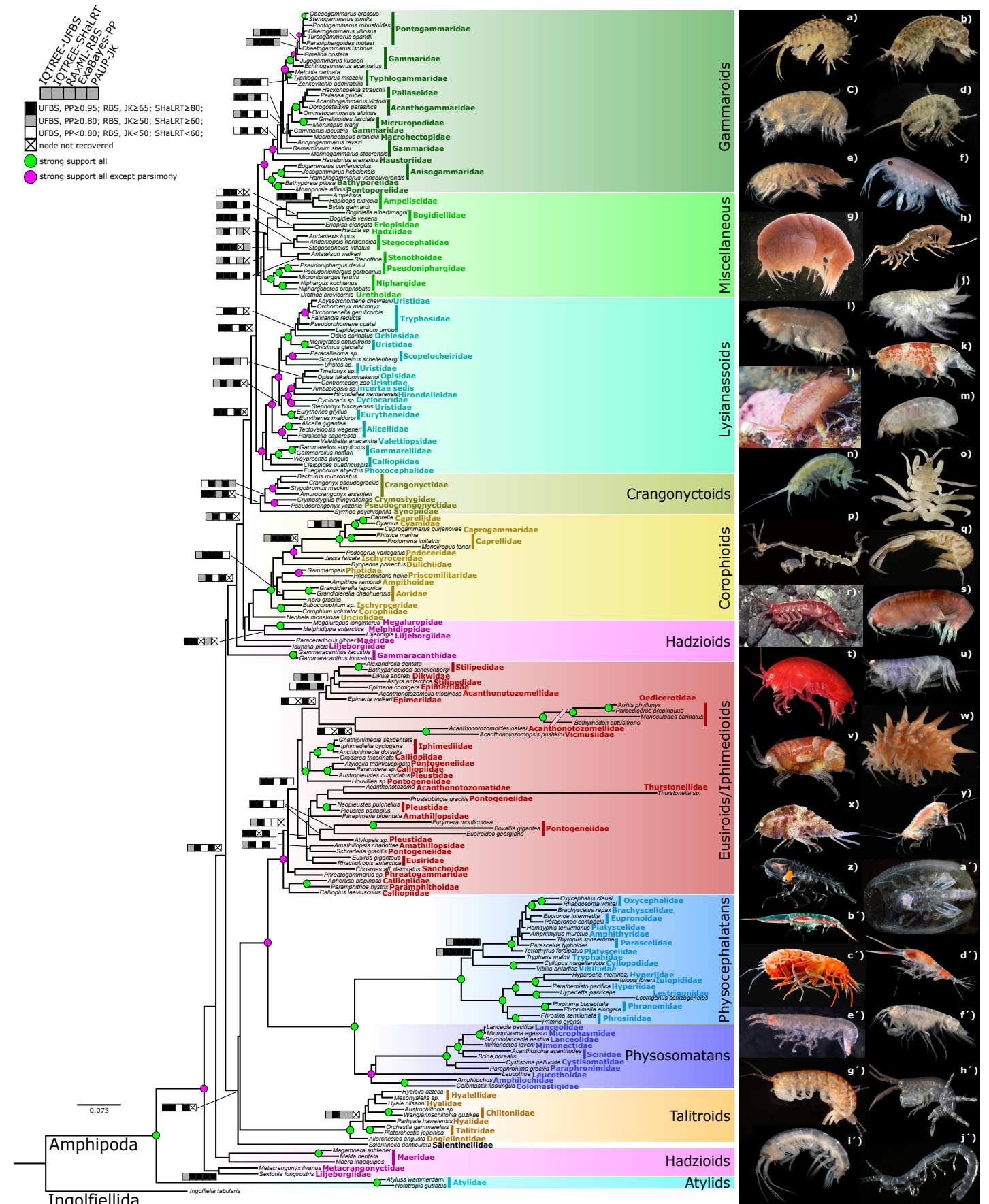


Fig. 1

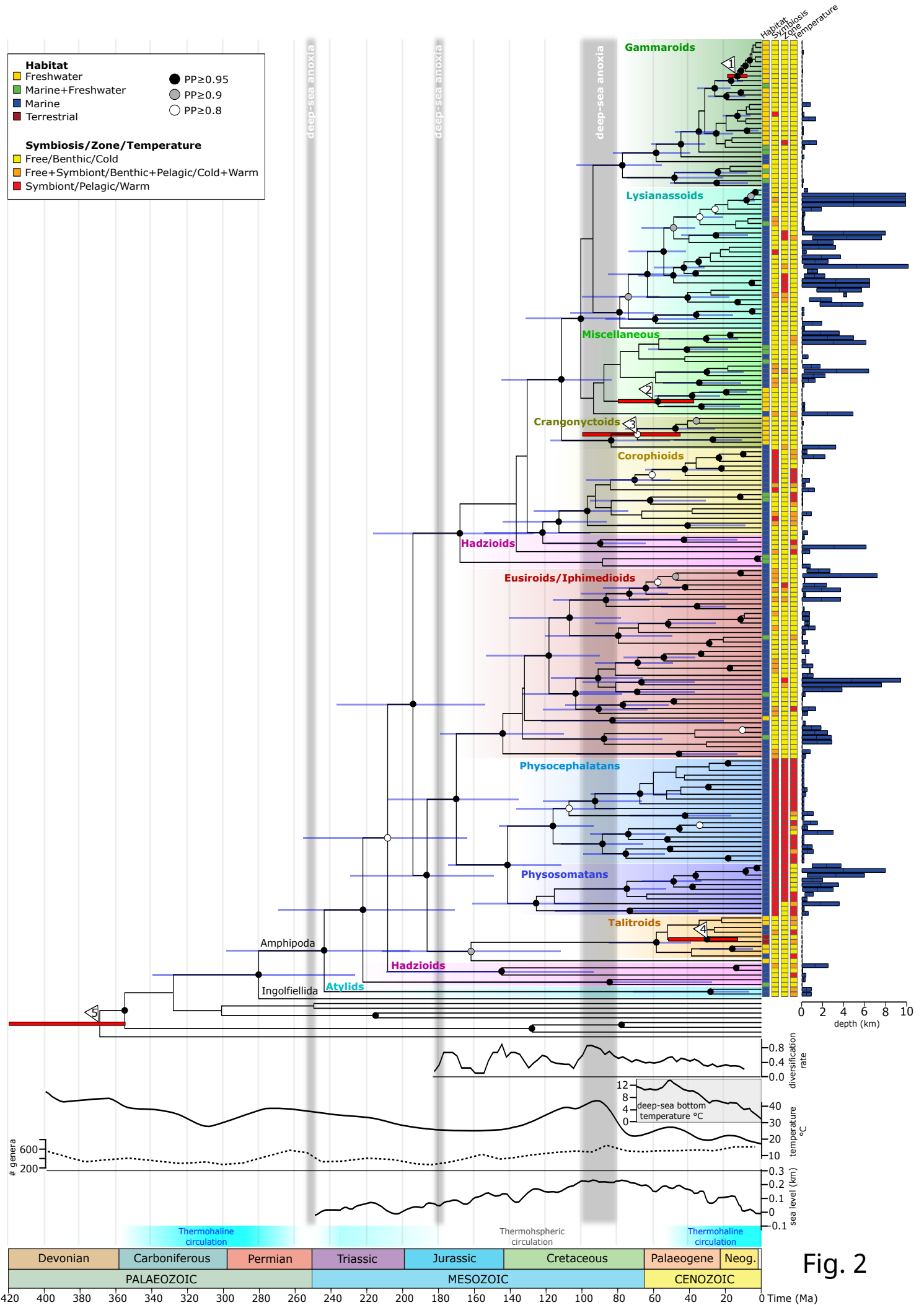


Fig. 2

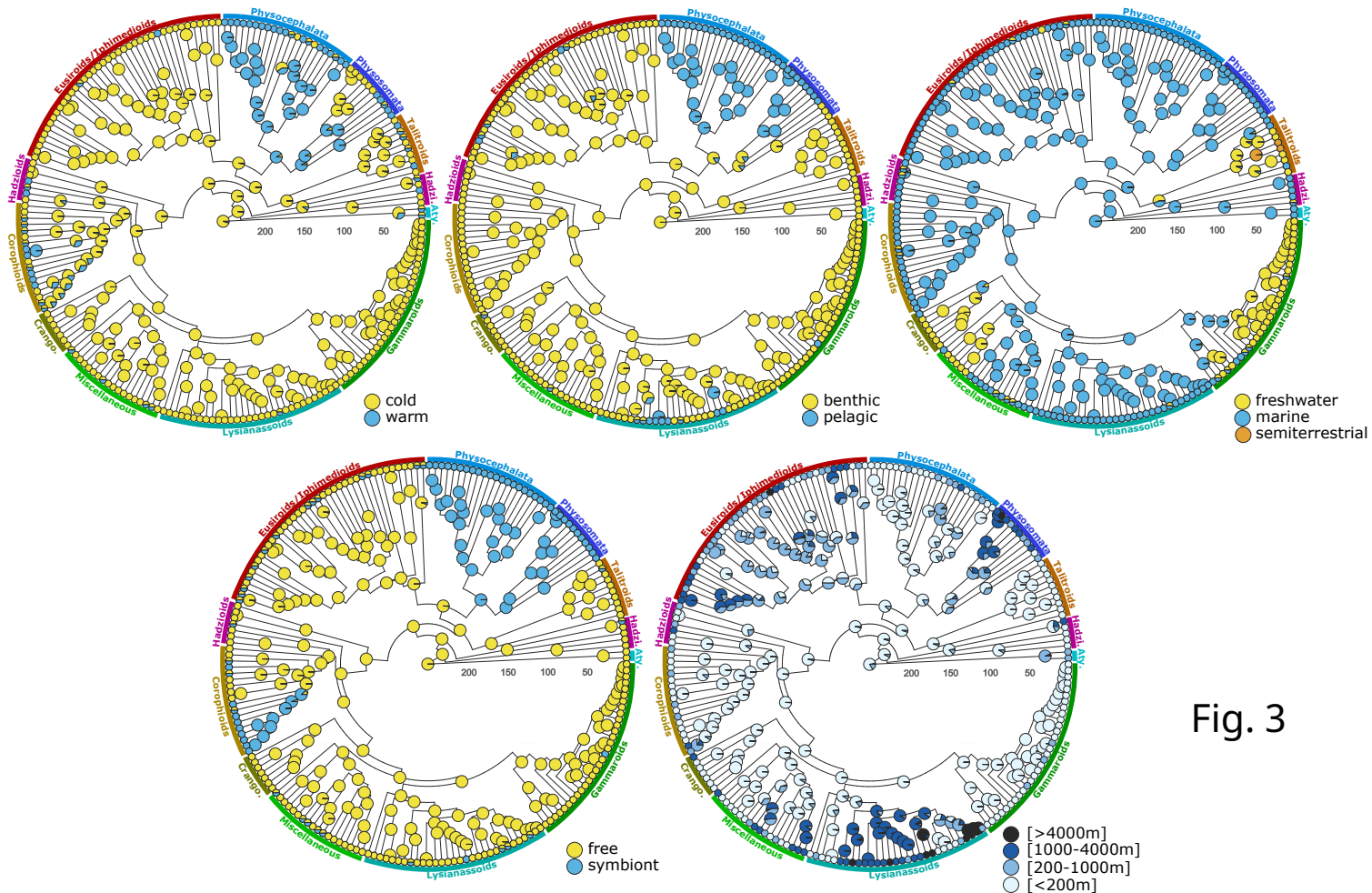


Fig. 3

Appendix

Reference list of all studies which generated the sequence data used in the current work

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