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ORIGINAL ARTICLE

Adrift across tectonic plates: molecular phylogenetics supports the ancient Laurasian origin of old limnic crangonyctid amphipods

Denis Copilaş-Ciocianu[1,2] · Dmitry Sidorov[3] · Andrey Gontcharov[3]

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Abstract

Crangonyctidae is a speciose and almost exclusively freshwater Holarctic family of amphipod crustaceans. Its members inhabit groundwater as well as epigean biotopes with groundwater connections, and often exhibit endemic, relict distributions. Therefore, it has been proposed that this poorly dispersing, yet intercontinentally distributed family must have ancient Mesozoic origins. Here, we test the hypothesis that Crangonyctidae originated before the final break-up of Laurasia at the end of the Cretaceous. We used molecular phylogenetic analyses based on mitochondrial and nuclear markers and incorporated six out of the seven recognized genera. We calculated divergence times using a novel calibration scheme based exclusively on fossils and, for comparison, also applied substitution rates previously inferred for other arthropods. Our results indicate that crangonyctids originated during the Early Cretaceous in a northerly temperate area comprising nowadays North America and Europe, supporting the Laurasian origin hypothesis. Moreover, high latitude lineages were found to be generally older than the ones at lower latitudes, further supporting the boreal origin of the group and its relict biogeography. The estimated substitution rate of 1.773% Ma^{-1} for the COI marker agrees well with other arthropod rates, making it appropriate for dating divergences at various phylogenetic levels within the Amphipoda. Furthermore, our extensive phylogeny reinforces the polyphyly of the intercontinental genera *Crangonyx*, *Stygobromus*, and *Synurella*, supports the monophyly of *Bactrurus*, and elucidates the position of *Lyurella*. We conclude that crangonyctids are an ideal model for testing continental-level vicariance hypotheses and should be in the focus of future phylogenomic studies.

Keywords Amphipoda · Continental breakup · Fossils · Laurasia · Molecular clock · Substitution rates

Introduction

Freshwater amphipod crustaceans and especially subterranean taxa often exhibit patchy and disjunct distribution patterns (Barnard and Barnard 1983; Bauzà-Ribot et al. 2012; Copilaş-Ciocianu and Petrusek 2017; Notenboom 1991; Sidorov et al. 2010). These organisms have limited dispersal ability due to the fact that they are bound to the discontinuous subterranean/freshwater environment and their biological features such as low fecundity, egg brooding, and lack or reduction of eyes and pigment (troglomorphy) (Väinölä et al. 2008) (but see Copilaş-Ciocianu et al. 2018a). As such, in the case of widespread taxa, it has been proposed that discontinuous geographical distributions originated through vicariant processes due to past geological events such as continental breakup (Holsinger 1994) and marine regressions (Notenboom 1991) rather than long-distance dispersal (Stock 1993).

Ideal model systems for testing continental breakup hypotheses are freshwater groups which are either distributed on both sides of the Atlantic (Tethyan distribution), or across the Holarctic (Laurasian distribution). Molecular phylogenetics and time-calibrated analyses concerning amphi-Atlantic distributed subterranean families such as Metacrangonyctidae Boutin

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✉ Denis Copilaş-Ciocianu
denis.copilas-ciocianu@gamtc.lt

[1] Laboratory of Evolutionary Ecology of Hydrobionts, Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania

[2] Department of Ecology, Faculty of Science, Charles University, Viničná 7, 12844 Prague, Czechia

[3] Federal Scientific Centre of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, 100-let Vladivostoku Av. 159, Vladivostok, Russian Federation 690022

& Messouli, 1988 and Pseudoniphargidae Karaman, 1993 have revealed that both ancient continental level vicariance (in the case of the former; Bauzà-Ribot et al. 2012) as well as more recent vicariance and long distance dispersal (in the case of the latter; Stokkan et al. 2018) were responsible for generating the observed disjunct distributional patterns. Both of these families are tightly linked to marine ancestors due to their distributions in areas formerly occupied by shallow seas as well as the existence of species that can live in saline conditions (Notenboom 1991; Stock 1993).

Crangonyctidae Bousfield, 1973 is another important group for testing continental level vicariance because of its wide Holarctic distribution and prevailing occurrence in freshwater habitats such as caves, springs, streams, swamps, and temporary ponds (Holsinger 1977). Due to its stygobitic/stygophilic affinity, the family is generally characterized by a tendency towards the reduction or complete loss of eyes and pigment (Fig. 1). Species richness is highest throughout North America, although many are also known from Eurasia and one endemic is even found in Iceland (Holsinger 1977; Kornobis et al. 2012; Sidorov and Holsinger 2007; Svavarsson and Kristjánsson 2006; J. Zhang and Holsinger 2003). Altogether, the family comprises more than 200 species classified into seven genera: *Amurocrangonyx* Sidorov & Holsinger, 2007 (monotypic, Far East), *Bactrurus* Hay, 1902 (eight species, North America), *Crangonyx* Spence Bate, 1859 (49 species, Holarctic), *Lyurella* Derzhavin, 1939 (two species, Caucasus), *Stygobromus* Cope, 1872 (142 species, Holarctic), *Stygonyx* Bousfield & Holsinger, 1989 (monotypic, North America), and *Synurella* Wrzesniowski, 1877 (20 species, Holarctic) (Holsinger 1974; Karaman 1974; Koenemann and Holsinger 2001; Özbek 2018; Sidorov 2015; Sidorov and Holsinger 2007; Sidorov and Palatov 2012; Svavarsson and Kristjánsson 2006; Taylor and Niemiller 2016; J. Zhang and Holsinger 2003).

According to current understanding, crangonyctids lack extant marine relatives (Lowry and Myers 2013, 2017; J. Zhang and Holsinger 2003). Molecular and morphological data indicate that the most closely related families are the East Asian Pseudocrangonyctidae Holsinger, 1989 and the Iceland endemic monotypic Crymostygidae Kristjánsson & Svavarsson 2004, both exclusively freshwater (Holsinger 1994; Kornobis et al. 2011; Kristjánsson and Svavarsson 2004; Sidorov and Gontcharov 2015). Along with several freshwater families mainly from the Southern Hemisphere, these three families belong to the superfamily Crangonyctoidea (Bousfield 1977; Holsinger 1994), a nevertheless disputed taxon (Holsinger 1992; Williams and Barnard 1988). However, some authors consider Crymostygidae as belonging to the Allocrangonyctoidea (Lowry and Myers 2013). Furthermore, fossil crangonyctids living in similar freshwater environments as extant species are known from Baltic amber of Eocene age, i.e., at least 35 Ma old (Coleman 2004, 2006). Thus, altogether, the evidence strongly suggests that this family has an old limnic origin. Consequently, Holsinger (1974, 1977, 1994) proposed that crangonyctids originated during the Mesozoic, because

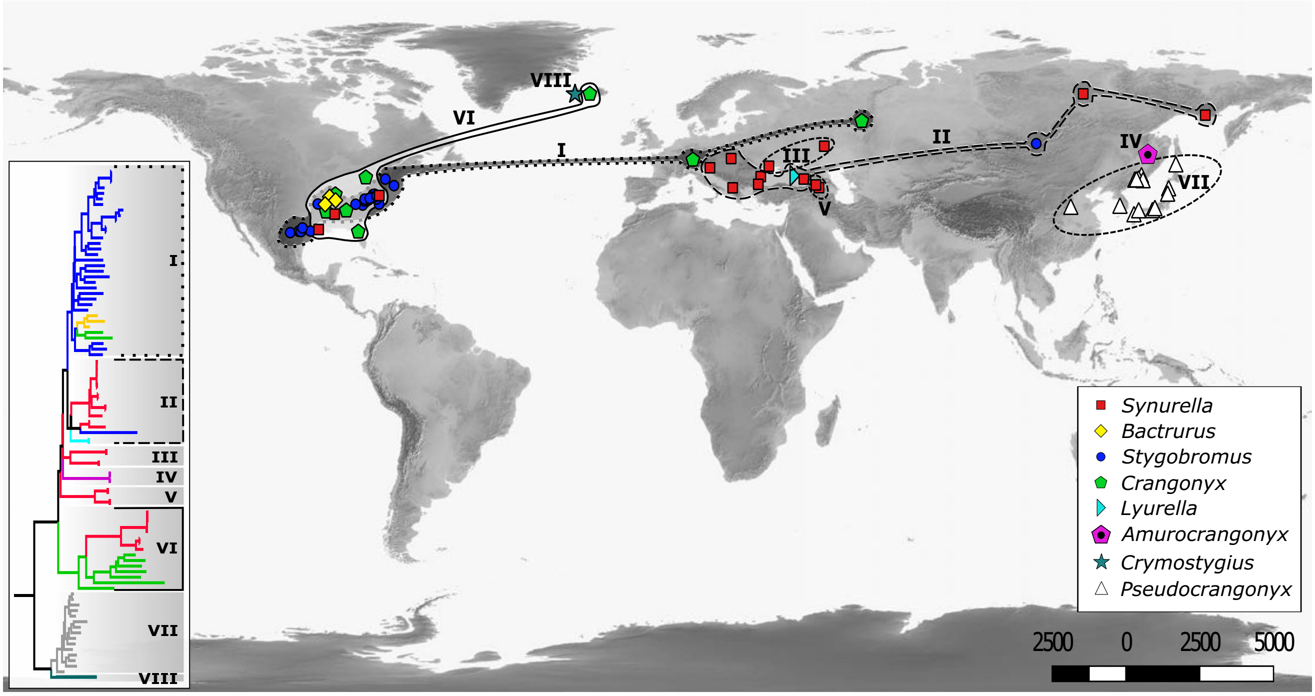

Fig. 1 Distribution of Crangonyctidae, Pseudocrangonyctidae, and Crymostygidae taxa used in the phylogenetic analyses. The inset shows a summary of the phylogenetic relationships presented in Fig. 2. The main clades are depicted with Roman numerals (I to VI = Crangonyctidae, VII = Pseudocrangonyctidae, and VIII = Crymostygidae). Colors on the maps correspond to those in the phylogeny

only a long period of time and a Laurasian continental configuration could explain how this Holarctic freshwater family has achieved such a wide transoceanic distribution, high species diversity, and ecological specialization, despite limited dispersal capability.

Crangonyctid amphipods have been in the focus of molecular phylogenetic analyses for almost two decades (Englisch et al. 2003; Ethridge et al. 2013; Koenemann and Holsinger 2001; Kornobis et al. 2011, 2012; Lee et al. 2018; Niemiller et al. 2018; Sidorov and Gontcharov 2015). However, despite these advancements and the biogeographic potential of the family, there have been no attempts at estimating its age. This issue was also likely exacerbated by the notorious scarcity of the amphipod fossil record (Karaman 1984; Schram 1986). Here, we aim to overcome these challenges and test the hypothesis that Crangonyctidae originated before the final separation of North America and Europe ca. 60–70 Ma ago (Eldholm and Thiede 1980; Mosar et al. 2002; Seton et al. 2012). Thus, we predict that the crown age of the family should not be younger than this time frame. We assembled a large dataset comprising six out of seven genera and estimated divergence times based on fossils and widely used molecular substitution rates. We propose a fossil calibration scheme as well as substitution rates that can be used in studying divergence times at various phylogenetic levels within the Amphipoda.

Material and methods

Sampling strategy

The prevailing majority of species of the family Crangonyctidae and especially the groundwater ones are narrowly distributed local endemics. Often, their dispersal is physically limited by the distribution of pore size in the sedimentary rocks, though this is still debated (Juberthie et al. 2016). Therefore, they are rare and difficult to sample. To better understand the phylogenetic structure of the family, we tried to cover as much as possible its wide area of distribution, as well as its taxonomic composition. We consider the final dataset representative for the family, comprising six out of the seven genera, but lacking the monotypic genus *Stygonyx* from North America. All important biogeographic regions are generally represented except North Africa (with the lack of *Crangonyx africanus* Messouli, 2006), and the southern range in Central Asia where attempts to collect species of *Stygobromus* (viz. *S. kazakhstanicus* Kulkina, 1992) were futile (Kulkina, pers. comm.). A couple of interesting species, *Lyurella hyrcana* Derzhavin, 1939, and an undescribed *Synurella* sp. from Wrangel Is., did not produce PCR products with universal primers. The material was collected from 15 different localities in several remote regions of the Caucasus, Urals, Siberia, the Far East, and southeastern Europe (see Table S1; Supporting information). Animals were sampled from a variety of aquatic habitats with a hand-net and were preserved in 96% ethanol in the field. In the laboratory, specimens were dissected in a mixture of glycerol and ethanol (1:1) and identified with the assistance of original author's descriptive diagnosis. Specimens sampled in areas far away from the *locus typicus* of known species or with doubtful identification were labeled with the modifier (cf.) "conformis." Temporary preparations were later transferred to permanent slides if necessary and stored in the zoological collection of the Far East Federal University, Vladivostok, Russia.

Specimens used for scanning electron microscopy (SEM) were dehydrated in acetone, critical point dried with a Bal-Tec CPD 030, and coated with palladium-gold before being photographed with a Carl Zeiss Evo 40 electron microscope.

DNA extraction, amplification, and sequencing

Total DNA was extracted from the abdomen and pereopod musculature with a DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's guidelines. Approximately, 548–674 bp fragments of the mitochondrial cytochrome c oxidase subunit I (COI) gene were amplified using the universal primers HCO2198 and LCO1490 (Folmer et al. 1994). The polymerase chain reaction (PCR) amplifications were performed in 10–12 μL, containing 0.15 mM dNTPs, 1× Taq buffer, 0.35 μM of primers, 0.5 unit of Taq polymerase (Fermentas/Thermo Fisher Scientific), and 10–100 ng of template DNA, with the following conditions: 95 °C for 5 min, followed by 37 cycles of 95 °C for 30 s, and an annealing temperature of 40 °C for 20 s. PCR products were directly sequenced using the ABI Big Dye Terminator v. 3.1 Cycle Sequencing Kit (according to the manufacturer's instructions) and the ABI 3130 genetic analyzer at the Federal scientific center of the East Asia terrestrial biodiversity FEB RAS, or at the Faculty of Science, Charles University. The resulting sequences were submitted to the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) nucleotide database (accession numbers HE794980–HE794994, LK028560–LK028569, MK044741–MK044745).

Dataset assembly and alignment

Our study includes for the first time sequences of the genus *Lyurella* (*L. shepsiensis* Sidorov, 2015), a Siberian species of *Stygobromus* (*S. anastasiae* Sidorov, Holsinger & Takhteev, 2010), eight species of *Synurella*, as well as additional sequences of *Amurocrangonyx arsenjevi* (Derzhavin, 1927) and *Synurella ambulans* (Müller, 1846) (see Table S1). The dataset of newly generated sequences was completed with additional COI, 28S rRNA, and 18S rRNA partial sequences downloaded from GenBank (see Table S1). These particular markers were chosen as they were the most commonly available. We aimed at obtaining the most complete multilocus dataset of Crangonyctidae available to date and included six out of the seven recognized genera that comprise this Holarctic family (Fig. 1, Table S1). To explore the crangonyctid phylogeny in a broader evolutionary and biogeographic context, we also included in the analyses its sister clade which comprises the north-east Asian family Pseudocrangonyctidae Holsinger, 1989 and the Iceland endemic monotypic Crymostygidae Kristijansson & Svavarsson, 2004 (Holsinger 1994; Kornobis et al. 2011; Lee et al. 2018; Sidorov and Gontcharov 2015). Progressively, more distantly related amphipod and other malacostracan outgroup taxa were also included to properly root the phylogeny and calibrate the molecular clock using fossils (see "Molecular dating" below). The dataset contained 54 Crangonyctidae, 15 Pseudocrangonyctidae, one Crymostygidae, and 15 outgroup taxa, totalling 85 taxa and 108 terminals. The concatenated matrix contained 50% missing data.

The protein-coding COI sequences were aligned using the MUSCLE algorithm (Edgar 2004) in MEGA 6 (Tamura et al. 2013). Ribosomal 18S and 28S sequences were aligned with MAFFT 7 (https://mafft.cbrc.jp/alignment/server/; Katoh and Standley 2013) using the Q-INS-i option (Katoh and Toh 2008). GBLOCKS 0.9 (Talavera and Castresana 2007) was used to identify and remove regions of ambiguous homology using minimum restrictive settings. The final alignment had a length of 4035 bp (COI, 668 bp; 28S, 1212 bp; 18S, 2155 bp) and contained 1876 parsimony informative out of 2579 variable sites (COI 365/413; 28S, 758/979; 18S,753/1187).

Substitution saturation at the COI locus was tested in DAMBE 5.3 (Xia and Xie 2003) using the test of Xia et al. (2003). Small levels of saturation were detected at the third codon position only when assuming a very unlikely asymmetric topology ($I_{ss} > I_{ss.cAsym}$; $p < 0.001$). Given that our phylogeny is not asymmetric (see the "Results" section), this is not considered problematic (Xia 2009). However, since saturation can potentially underestimate divergence times (Wilke et al. 2009), we ran the molecular dating analyses with and without the COI third codon position.

Phylogenetic reconstruction

Evolutionary models and the optimal partitioning scheme were selected with PartitionFinder 2 (Lanfear et al. 2017) using the greedy search option under the Bayesian Information Criterion. The TrNef+I + Γ model was used for 18S, GTR + Γ for 28S, SYM + I + Γ, GTR + I + Γ and HKY + I + Γ were used for the first, second, and third codon positions in COI, respectively.

Phylogenetic reconstruction was conducted under maximum-likelihood (ML) and Bayesian (BI) approaches. The ML analysis was performed on the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/; (Trifinopoulos et al. 2016) which implements the edge-linked partition model in IQ-TREE 1.6 (Chernomor et al. 2016; Nguyen et al. 2015). Branch support was obtained using 1000 ultrafast bootstrap replicates (UFBS; Hoang et al. 2018). Bayesian analyses were performed with MrBayes 3.2 (Ronquist et al. 2012). Two runs of four MCMC chains were run for 2.5×10^7 iterations, a thinning of 1000, and 50% of samples were discarded as burn-in. The temp parameter was set to 0.07. A 50% majority-rule consensus tree was built from the post burn-in sample. Convergence and mixing of runs were inspected with Tracer 1.6 (Rambaut et al. 2014). The BI analyses were performed on the CIPRES Science Gateway (Miller et al. 2010).

Molecular dating

Divergence times were calculated with BEAST 1.8.0 (Drummond et al. 2012). We used the same evolutionary models and partitions as in the previous section. A thinned dataset of one individual per taxon was used to improve accuracy and computational time. Since we are dealing with deep, interspecific time-scales, we employed an uncorrelated relaxed clock with a lognormal distribution (Drummond et al. 2006). For the tree model, we used a random starting tree, and speciation was modeled using the Birth-Death Process. The MCMC chain was run for 10^8 iterations, with a thinning of 1000. Effective sample sizes of parameters and convergence were checked with Tracer after discarding 20% of the trees as burn-in. We performed three independent runs which gave the same result. Thus, the resulting files were combined using LogCombiner 1.8 (Drummond et al. 2012), and the maximum clade credibility tree was produced using TreeAnnotator 1.8 (Drummond et al. 2012).

We dated the splits in the phylogeny by using (1) a calibration scheme based on the fossil record and (2) assuming COI substitution rates that were inferred for other crustacean or arthropod groups. For the first approach, we introduce a novel calibration scheme based entirely on fossils which can unambiguously be attributed to extant lineages. We opted for priors with exponential rather than lognormal distributions because they require fewer parameters and are more appropriate in cases where the fossil record of the focal group is poorly known (Ho and Phillips 2009). Three amphipod (ingroup) and one basal eumalacostracan (outgroup) fossil were used (Table 1). The first calibration point (1) is based on fossil remains in Baltic amber of Eocene age that can unambiguously be attributed to extant Crangonyctidae. Such fossil genera belong to *Synurella* (extant) or *Palaeogammarus* Zaddach, 1864 (extinct) (Coleman 2004, 2006; Coleman and Myers 2000; Holsinger 1977; Jazdzewski et al. 2014; Jazdzewski and Kulicka 2002; Just 1974). Given that extant *Synurella* is not a monophyletic genus (Kornobis et al. 2011; present study), we assign it as stem Crangonyctidae. Therefore, we constrain the split between Crangonyctidae and Pseudocrangonyctidae+Crymostygidae to a minimum age of 35 Ma (latest Eocene, Priabonian) with a mean of 60 and offset of 35 (resulting in a 95% HPD 38–215 Ma). For the second calibration point (2), we took into consideration casts of fossil *Niphargus* Schiödte, 1849 preserved in Baltic amber with the same stratigraphic age as for Crangonyctidae (Coleman and Myers 2000; Coleman and Ruffo 2002; Kupryjanowicz and Jażdżewski 2010). Since these fossils cannot be attributed to any extant species and considering the paraphyly of *Niphargus* (Fišer et al. 2008; Jurado-Rivera et al. 2017), we assign them as stem Niphargidae. Nevertheless, molecular data strongly supports the monophyly of Niphargidae and its sister relationship to Pseudoniphargidae (Jurado-Rivera et al. 2017). Thus, the split between these families was set to a minimum of 35 Ma and we applied the same parameter settings as for Crangonyctidae. The third calibration point (3) is based on the fossil remains of amphipods preserved in Upper Sarmatian (ca. 9 Ma) marls close to Grozny (Chechen Republic, Russian Federation) (Derzhavin 1927). These fossils have been classified into two genera, *Praegmelina*, Derzhavin 1927 and *Andrussovia*, Derzhavin, 1927, each containing two species. Although, only the former has clear affinities with the extant Ponto-Caspian *Gmelina* group, both genera can be confidently attributed to the Ponto-Caspian clade (Barnard and Barnard 1983; Derzhavin 1927; Karaman 1984), which represents a monophyletic radiation endemic to the Ponto-Caspian region (Hou et al. 2014; Hou and Sket 2016). Furthermore, extant *Gmelina* represents the earliest branching within the Ponto-Caspian clade, corroborating the fossil evidence (Hou et al. 2014). As such, we conservatively interpret these fossils as stem Ponto-Caspian amphipods. Considering that the sister taxon to the Ponto-Caspian Amphipoda is the Dinaric *Jugogammarus kusceri* (Karaman, 1953) (Hou et al. 2014), we constrained the split between these lineages to a minimum of 9 Ma (Upper Sarmatian), a mean of 25 and offset of 8 (95% HPD 9–83 Ma).

The fourth calibration point (4) was chosen as a maximum bound on divergence times and was based on the earliest known decapod fossil, *Palaeopalaemon newberry* Whitfield, 1880, from the Late Devonian, 358 Ma (Schram 2009; Schram et al. 1978; Wolfe et al. 2016). Following best practice rules, we placed this fossil constrain as close to the root as possible (Duchêne et al. 2014). Although, *P. newberry* belongs to the crown decapoda, it is considered the oldest known eumalacostracan (Wolfe et al. 2016). Thus, the split between *Hommarus gammarus* (Decapoda) and the rest of the taxa in our phylogeny (Peracarida) was set to a minimum of 358 Ma (Fammenian). The maximum age for this split was constrained at 514 Ma, which represents the oldest known mandibulate fossil, *Yicaris dianensis* Zhang, Siveter, Waloszek and Maas, 2007 (Wolfe et al. 2016; X. G. Zhang et al. 2007). The mean and offset values for the exponential distribution were set to 55 and 355, respectively (95% HPD 358–514) (Table 1).

The second approach for estimating divergence times was based on applying COI substitution rates inferred for other crustaceans or arthropods. Multiple substitution rates have been used for dating interspecific splits in crustaceans, e.g., 0.7% Ma^{-1} (Knowlton and Weigt 1998), 1.15% Ma^{-1} (Brower 1994), 1.25% Ma^{-1} (Ketmaier et al. 2003) and 1.77% Ma^{-1} (Papadopoulou et al. 2010). Given the subjectivity of choosing an appropriate rate and the relatively small differences among them, we preferred to calibrate the clock by incorporating these rates into a uniform prior with the interval ranging between 0.7 and 1.77% Ma^{-1} and a starting value of 1.2%, which represents the mean. The rates for the nuclear 28S and 18S markers were derived from the COI rate.

To visualize the tendency of lineage diversification through time of the three focal families, we constructed a lineages-through-time LTT) plot in Tracer using 2×10^4 post burn-in trees from the BEAST analysis. The relationship between species age and latitude was examined using a linear regression and calculating Pearson's correlation coefficient. Statistical significance was assessed using 2000 permutations. Additionally, we also tested whether the age of species which possess well developed eyes, vestigial eyes, or are eyeless significantly differs. For this, we used a Kruskal-Wallis test with Mann-Whitney pairwise comparisons and Bonferroni corrected p values. The analyses were performed in PAST 3 (Hammer et al. 2001).

To aid the interpretation of divergence times, we constructed paleogeographic maps using the Palaeomap Maker on the GPlates portal (http://portal.gplates.org/map/). The maps were created using the EarthByte Global Rotation Model 2012 (Seton et al. 2012) with a Mollweide projection. Over the obtained maps, we overlaid climatic reconstructions from Boucot et al. (2013).

Ancestral range reconstruction

This analysis was performed with the aim of uncovering the putative area of origin of the Crangonyctidae. For this purpose, we used a Bayesian implementation of the Dispersal-Extinction-Cladogeneis model (DEC; Ree et al. 2008) called S-DEC (Statistical-DEC or Bayes-Lagrange), implemented RASP 3.2 (Yu et al. 2015). The advantage of the S-DEC over the DEC model is that it can incorporate phylogenetic uncertainty. Therefore, we used 1000 post burn-in trees from the BEAST analysis which had the non-Crangonyctoidea taxa removed. The maximum number of ancestral areas was set to 3. Based on the current knowledge of the distribution of the family, we defined five distribution areas: (A) West Palaearctic, (B) East Palaearctic, (C)

Table 1 Fossil taxa, stratigraphic ages, and prior parameter settings used for calibrating the molecular clock

Calibration node	Taxa	Minimum stratigraphic age Ma (stage)	95% HPD of exponential age prior (Ma) (mean, offset values)	References
1	*Synurella* spp., *Palaeogammarus* spp.	35 (Priabonian)	38–215 (60, 35)	(Coleman 2004, 2006; Coleman and Myers 2000; Jazdzewski et al. 2014; Just 1974)
2	*Niphargus* spp.	35 (Priabonian)	38–215 (60, 35)	(Coleman and Myers 2000; Coleman and Ruffo 2002; Kupryjanowicz and Jażdżewski 2010)
3	*Praegmelina* spp., *Andrussovia* spp.	9 (Tortonian)	9–83 (25, 8)	(Barnard and Barnard 1983; Derzhavin 1927; Karaman 1984)
4	*Palaeopalaemon newberryi*	358.5 (Fammenian)	358–514 (55, 355)	(Schram 2009; Schram et al. 1978; Wolfe et al. 2016; X. G. Zhang et al. 2007)

Nearctic, (D) Iceland, and (E) the Japanese archipelago and Sakhalin Island.

Results

Phylogenetic reconstruction

The ML and BI methods recovered overall similar topologies, and disagreements were present only at poorly supported nodes (Fig. 2). Crangonyctidae was strongly recovered as monophyletic and its sister relationship with Pseudocrangonyctidae+ Crymostigidae was fully supported by both methods, corroborating previous results based on the 28S marker (Sidorov and Gontcharov 2015). In agreement with previous studies (Kornobis et al. 2011, 2012), the widespread genera *Synurella* and *Crangonyx* were recovered as polyphyletic (Fig. 2). Likewise, we further confirm the polyphyly of *Stygobromus* because (1) *Bactrurus* and two European *Crangonyx* species are nested within it and (2) the Siberian *Stygobromus anastasiae* is more closely allied with Eurasian *Synurella* (Fig. 2). *Bactrurus* is strongly supported as a monophyletic genus. *Lyurella* is strongly related with Eurasian *Synurella* and *Stygobromus*, while the monotypic *Amurocrangonyx* has an uncertain phylogenetic position within the family (Fig. 2).

Within Crangonyctidae, several well supported clades (I to VI) were recovered by both methods (Fig. 2). However, the relationships among these clades are obscure due to the very short internal branches which received low support. Clade I is the only one with an intercontinental distribution across North America and Europe (Fig. 1). It comprises North American species of *Stygobromus*, the genus *Bactrurus*, and two European species of *Crangonyx* (Fig. 2). Clade II is distributed across Eurasia (Fig. 1) and contains the genus *Lyurella*, several European and Siberian species of *Synurella*, as well as the Siberian species *Stygobromus anastasiae* (Fig. 2). The monophyly of clade III is supported by both methods, although not strongly (Fig. 2). This clade is distributed in Eastern Europe and comprises two species of *Synurella* (Figs. 1 and 2). Clade IV contains only the Far East monotypic *Amurocrangonyx* (Fig. 2). Clade V is restricted to the Caucasus region and consists of two *Synurella* species (Figs. 1 and 2). Clade VI occurs throughout North America and Iceland, and includes members of the genera *Crangonyx* and North American *Synurella* (Figs. 1 and 2). Clade VII is represented by the East Asian family Pseudocrangonyctidae, while Clade VIII represents the monotypic Icelandic Crymostygidae (Figs. 1 and 2).

Molecular dating

The results of both approaches to molecular dating (fossils and COI rates) were in agreement that the age of Crangonyctidae predates the final break-up of Laurasia ca. 70 Ma ago, which is in agreement with our hypothesis (Table 2). As expected, the COI rate analysis resulted in wider confidence intervals than the fossil calibration due to the wide uniform prior distribution. However, there is a notable discrepancy with respect to the inferred ages. The fossil calibration without the COI third codon position suggests Late Cretaceous age (ca. 80 Ma, Campanian) (Table 2); while with the COI third codon position it indicates an Early Cretaceous origin (ca. 146 Ma, Berriasian) (Fig. 3, Table 2). Dating with COI rates was the least conservative and indicated an Early Jurassic age (ca. 186 Ma, Pliensbachian) (Table 2). The split between Crymostygidae and Pseudocrangonyctidae occurred between 131 and 36 Ma, and the crown age of the Pseudocrangonyctidae ranges between 96 and 17 Ma (Fig. 3, Table 2). The timing and confidence intervals of the relevant splits in the phylogeny are shown in Table 2. For biogeographical interpretations, we mainly consider the approach which uses fossil calibration and includes all COI codon positions (see the "Discussion" section). This data set and

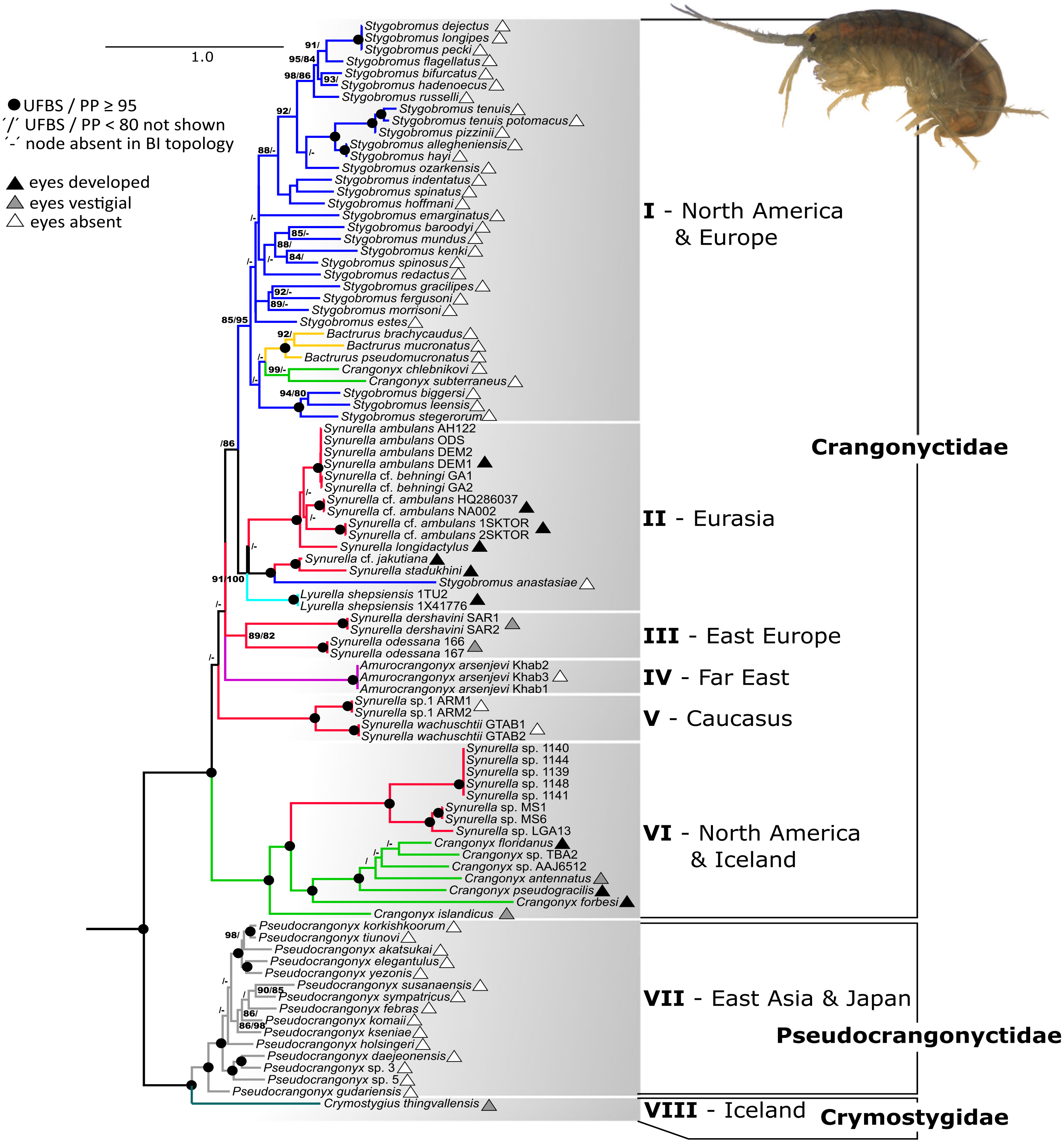

Fig. 2 Maximum-likelihood phylogeny of Crangonyctidae, Pseudocrangonyctidae, and Crymostygidae (outgroup not shown). Members of different genera are shown with distinctly colored branches, corresponding to Fig. 1. Black dots indicate strongly supported nodes with ultrafast bootstrap (UFBS) and Bayesian posterior probabilities (PP) values ≥95%. Values lower than 80% are not shown. The dash indicates that the respective node was not recovered in the Bayesian phylogeny. Triangles next to taxon names indicate the degree of eye loss (black = present, gray = vestigial, and white = absent). Inset image depicts an image of a female *Synurella ambulans*, a widespread European crangonyctid (Photograph: Denis Copilaş-Ciocianu)

corresponding chronogram have been deposited to Figshare (https://doi.org/10.6084/m9.figshare.7209617).

The substitution rate for COI (mean rate parameter) using the fossil calibration with the third codon position was estimated at 1.773% Ma^{-1} (SD 0.4) which results in a divergence rate of 3.546% Ma^{-1}(Table 3). For the 28S and 18S nuclear markers, the mean substitution rates derived from the fossil calibration were 0.16% Ma^{-1} (SD 0.036) and 0.068% (SD 0.016),

Table 2 Ages (median and 95% HPD intervals of three combined runs) of focal nodes obtained with fossil calibration (with and without the COI third codon position) or COI substitution rate (0.7 to 1.77% Ma^{-1})

Node	Fossil calibration (with COI 3rd)	Fossil calibration (without COI 3rd)	COI rate (with COI 3rd)
Stem Crangonyctoidea	157 (121–197)	97 (70–131)	207 (141–351)
Crown Crangonyctoidea	146 (109–184)	80 (56–109)	186 (125–315)
Crown Crangonyctidae	131 (96–166)	63 (43–86)	168 (114–279)
Crymostygidae	100 (66–137)	36 (17–65)	131 (79–222)
Crown Pseudocrangonyctidae	73 (47–102)	17 (9–29)	96 (60–165)

respectively (Table 3). A comparison of rates under different calibration assumptions is shown in Table 3. In general, the fossil calibration and COI rate approaches gave rather similar substitution rates for the nuclear markers. However, an almost

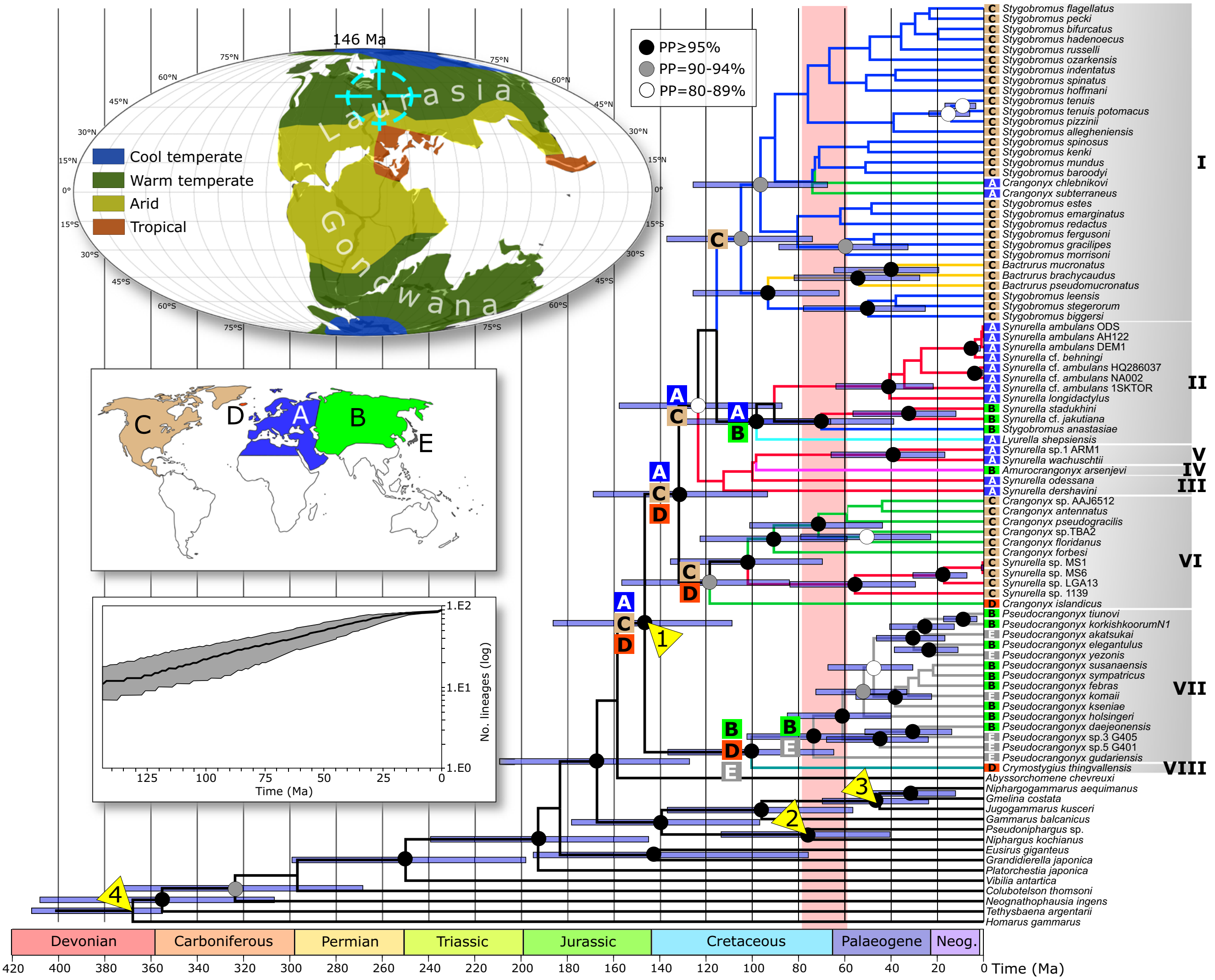

Fig. 3 Evolutionary timescale and historical biogeography of Crangonyctidae, Pseudocrangonyctidae, and Crymostygidae. The tree was time-calibrated using fossils and includes the COI third codon position. Clade support (PP) is shown by colored circles (black ≥ 95%, gray = 90–94% and white = 80–89%; not shown if < 80%), and blue bars indicate the 95% HPD interval of clade age (not shown if PP < 80%). The distribution ranges of present-day taxa are shown at the tips of the tree with colored boxes and letters that correspond to the map on the middle left. Likewise, the putative ranges of ancestors are shown with lettered boxes at relevant nodes. Numbered yellow triangles indicate the fossil calibration points and correspond with Table 1. The time interval of the final break-up between North America and Europe is indicated with pale red. The inset map at the upper left indicates the paleogeography of the World during the Upper Jurassic/Lower Cretaceous (obtained from http://portal.gplates.org/map/). The putative ancestral area of Crangonyctoidea is shown by the crosshairs. A palaeoclimatic reconstruction is overlaid over the landmasses (modified after Boucot et al. 2013). The inset in the lower left is an LTT plot which depicts the trend of diversification through time

twofold increase in the substitution rate of these markers was observed when using the fossil calibration scheme without the COI third codon position (Table 3).

The LTT plot of the clade containing Crangonyctidae, Pseudocrangonyctidae, and Crymostygidae indicated a rather constant accumulation of lineages through time with a slight decrease during the last 25 Ma (Fig. 3). A moderate, yet highly significant positive correlation was detected among species age and latitude ($r = 0.45$, $t = 4.18$, $p = 0.0004$) (Fig. 4a), which indicates that species at high latitudes tend to be older than the ones at lower latitudes. The age comparison among eyed, eyeless, and vestigial-eyed species revealed that the last group has significantly older species (median = 100 Ma, range = 43–118) than both of the other two groups (Fig. 4b; vestigial vs. eyed, $p = 0.03$, $U = 3$; vestigial vs. eyeless, $p = 0.007$, $U = 22$), which did not significantly differ (eyeless, median = 39 Ma, range = 9–98; eyed, median = 34, range = 27–98).

Ancestral range

The reconstruction under the S-DEC model indicated that the ancestral area of origin for Crangonyctidae and Crangonyctoidea was comprised of what are now North America, Europe, and Iceland, corresponding to a Laurasian origin. Widespread Clades I and VI most likely originated in North America, while Clade II in Eurasia (Fig. 3). The ancestor of Pseudocrangonyctidae and Crymostygidae was probably widely distributed across the Eastern Palaearctic (Fig. 3).

Discussion

Our estimations of divergence times based on fossil calibration indicate that the family Crangonyctidae most likely originated during the Early Cretaceous. Likewise, the ancestral range reconstruction reveals that the ancestor of this family was dispersed throughout North America and Europe. Taken together, these results strongly support the hypothesis that crangonyctids originated on the Laurasian supercontinent well before its final breakup. Below, we discuss the evolutionary, biogeographical, and systematic implications of these results.

Substitution rates and evolutionary timescale

The three different approaches to calibrating divergence times (fossil calibration with and without the COI third codon and COI rate) have resulted in significant differences with respect to the stem age of Crangonyctidae, indicating 146, 80, and 186 Ma, respectively (Table 2). Nevertheless, these results strongly agree that the age of the family does not post-date the final breakup of the Laurasian supercontinent (Mosar et al. 2002; Seton et al. 2012). We argue that the most justified approach is the one based on fossil calibration and includes the COI third codon position. First, this approach is superior to the one based on COI rates because it incorporates well-dated amphipod fossils; thus, it relies on tangible evidence rather than assumptions. Second, although signs of saturation have been detected at the COI third codon position, the potential effect of this shortcoming is the underestimation of divergence times (Wilke et al. 2009). However, given that saturation was supported only in the case of a very unlikely asymmetrical topology (not the case in our study), and parameter-rich evolutionary models were assigned to each codon position, we consider that this issue was at least partially mitigated (Wilke et al. 2009; Xia 2009; Xia et al. 2003). Completely removing the COI third codon from the analysis resulted in younger divergence times and a far less resolved tree (not shown), indicating that it contains a lot of phylogenetic signal. Thus, although all three approaches have their drawbacks, we consider that the fossil calibration and the inclusion of the COI third codon position give the most reasonable results.

The COI substitution rate obtained using the fossil calibration had a value of 1.773% Ma^{-1}. This result is in excellent agreement with the revised insect mitochondrial clock (1.77% Ma^{-1}; Papadopoulou et al. 2010) and with previous rates used to date divergences in amphipod crustaceans (1.65 to 1.8% Ma^{-1}; Copilaş-Ciocianu et al. 2018b; Copilaş-Ciocianu and

Table 3 Substitution rate estimates per site (mean ± 1SD) for each marker

Method	Gene region	Mean rate	ucld.mean	Coefficient of variance
Fossil calibration (with COI 3rd)	COI	0.01773 ± 0.00441	0.02091 ± 0.00619	0.5072 ± 0.0026
	28S	0.00161 ± 0.00036	0.00153 ± 0.00048	1.8379 ± 0.0172
	18S	0.00068 ± 0.00016	0.00067 ± 0.00023	1.8539 ± 0.0181
Fossil calibration (without COI 3rd)	COI	0.00244 ± 0.00062	0.00444 ± 0.00168	1.0751 ± 0.0050
	28S	0.00226 ± 0.00041	0.00225 ± 0.00061	1.0124 ± 0.0068
	18S	0.00091 ± 0.00015	0.00084 ± 0.00022	1.0091 ± 0.0073
COI rate (with COI 3rd)	COI	0.01286 ± 0.00364	0.01431 ± 0.00353	0.4496 ± 0.0020
	28S	0.00129 ± 0.00053	0.00131 ± 0.00063	1.9707 ± 0.0160
	18S	0.00058 ± 0.00025	0.00061 ± 0.00032	2.0004 ± 0.0215

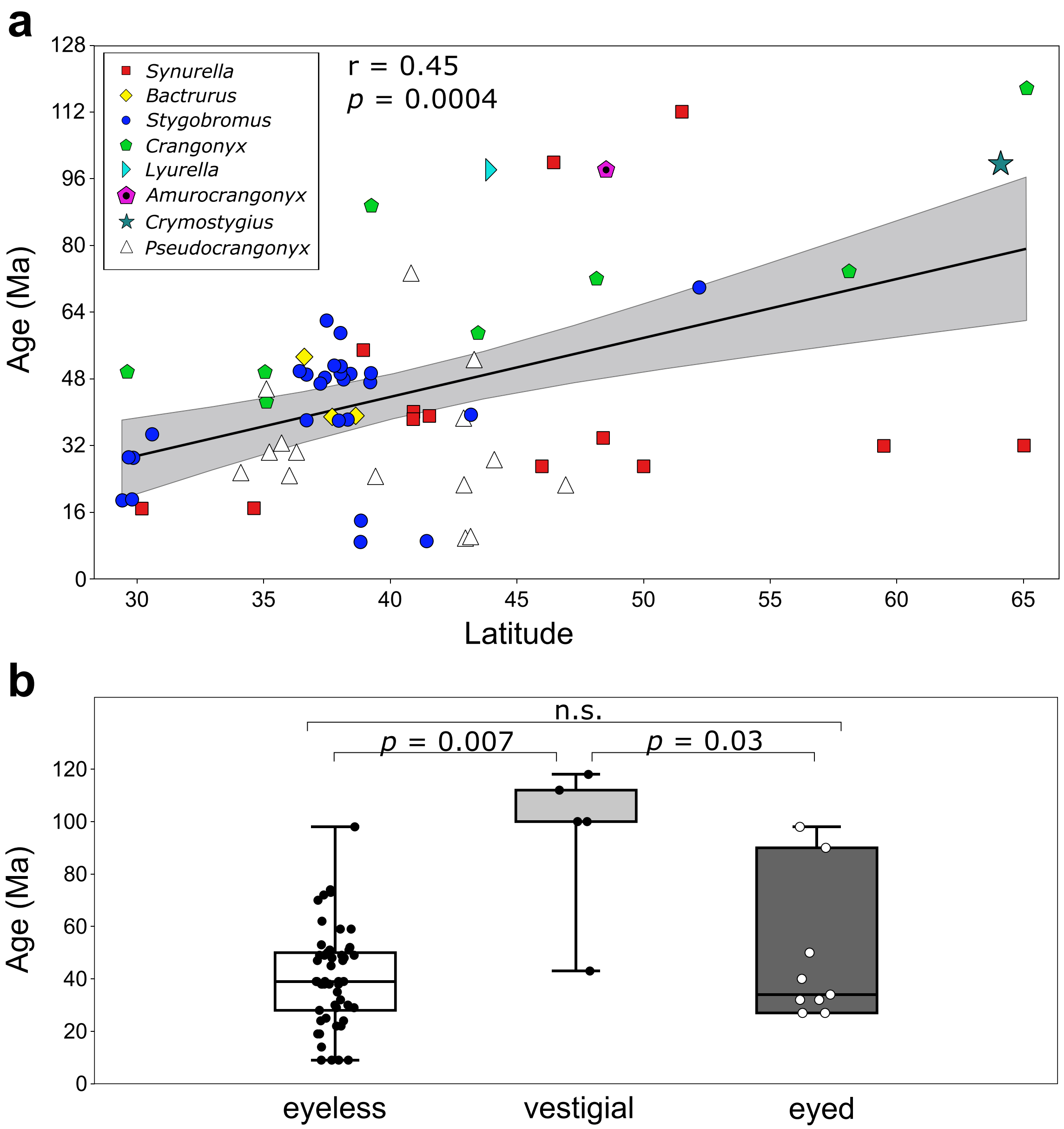

Fig. 4 **a** Relationship between species age and latitude. **b** Box-plots indicating the age variation among eyeless, vestigial eyed, and eyed species in Crangonyctidae, Pseudocrangonyctidae, and Crymostygidae

Petrusek 2017; Mamos et al. 2016; Nahavandi et al. 2013). However, it is noticeably faster than the 1.15% Ma^{-1} arthropod mitochondrial rate of Brower (1994), which has been extensively used in dating divergences in freshwater amphipods (e.g., Copilaş-Ciocianu and Petrusek 2015; Hupało et al. 2019; Liu et al. 2018; Yang et al. 2013). Literature reports on the substitution rates of the 28S and 18S markers on amphipods are almost non-existent. Only Mamos et al. (2016) reported a substitution rate of 0.3% Ma^{-1} for 28S, which is almost twice faster than the one reported herein. Such a difference could be explained by the different approaches to calibrating divergence times (fossils vs. biogeography), different marker datasets, a large discrepancy in the evolutionary timeframe (Ho et al. 2011), or even life history (Saclier et al. 2018). A potential caveat of our study is that the substitution rates and divergence times may have been impacted by the lack of nuclear markers in several of the major crangonyctid clades (clades III, IV, and genus *Lyurella*). However, missing data seem to have only a minor impact on divergence dating with BEAST where the most critical parameters are the calibration points (Zheng and Wiens 2015).

Another issue is that amphipod fossils older than Eocene are ambiguous or misidentified and unfortunately could not be used for calibration. McMenamin et al. (2013) reported a Late Triassic (Norian) giant amphipod that turned out to be the fossilized tail fan of a decapod (Starr et al. 2016). Similarly, Alonso et al. (2000) reported amphipods in Cretaceous amber which, under closer inspection, proved to be misidentified Tanaidacea (Vonk and Schram 2007). Probably one of the oldest putative amphipod fossils is reported by Heggemann et al. (1990) from Middle Jurassic sandstones in northern Thailand. However, the fossil is too poorly preserved to be unambiguously assigned to the Amphipoda. The lack of pre-Eocene amphipod fossils is puzzling and needs further attention. This is probably due to the fact that the amphipod exoskeleton is weakly calcified and is poorly, if at all, preserved in sediments. For example, in sandy shale of the Eilar-Ougi Range in north-western Azerbaijan, Derzhavin (1941) reports that out of the 68 imprints of smooth "gammarids" *Gammarus praecyrius* Derzhavin, 1941, none revealed the detailed structure of uropod 3, which is a taxonomically important feature. Besides the problem of fossilization, a second important aspect is the fact that amphipod taxonomy is based on the analysis of minute morphological details which are difficult to observe even in amber preserved specimens. Additionally, the lack of secondary sex features (indicating maturity) significantly reduces the value of fossilized specimens. Thus, minute individuals can either be juveniles or different taxa altogether which preserved in the same thanatocoenosis. Fortunately, the known Eocene fossils of *Synurella* and *Niphargus* from Baltic amber as well as the gammarid Sarmatian fossils from the Caspian basin do not seem to differ much from the modern forms, making them appropriate for calibrating molecular clocks. We therefore propose for the first time fossil-derived COI, 28S, and 18S rates (Table 3) that can be used to date divergences throughout the Amphipoda.

Historical biogeography

The ancestral range reconstruction reveals that the crangonyctid ancestor occupied a region that comprises nowadays North America, Europe, and Iceland (Fig. 3). However, Iceland can be excluded as it did not exist during the Mesozoic. Coupled with the estimated Early Cretaceous age of the family, this strongly indicates that Crangonyctidae originated on the Laurasian supercontinent. Moreover, given that extant crangonyctids are exclusively found in temperate Holarctic latitudes and that epigean freshwater amphipods are almost absent at equatorial latitudes (Barnard and Barnard 1982, 1983; Serejo and Siqueira 2018; Väinölä et al. 2008), it is very likely that the family originated in a temperate climate. Since such climatic conditions existed mainly above the 45° latitudinal band in Eurasia during the Early Cretaceous (Boucot et al. 2013; Lloyd 1982), we can deduce that the crangonyctid ancestor probably originated northwards of that latitude (Fig. 3).

Even though the Laurasian continental configuration best explains the intercontinental dispersal of crangonyctids throughout the Cretaceous, a number of shallow epicontinental seas that spanned across this landmass likely acted as dispersal barriers. These were the Western Interior Seaway (North America), the Norwegian-Greenland Sea, and the Turgai Straight (Eurasia) (Thiede 1979). However, numerous temporary land bridges likely promoted dispersal by connecting landmasses across the Turgai straight and Norwegian-Greenland Sea throughout the Cretaceous until the Paleocene (Baraboshkin et al. 2003; Scotese 2014; Tiffney 1985; Vakhrameev 1987). The Western Interior Seaway was relatively short lived and vanished by the end of the Cretaceous (Scotese 2014; Tiffney 1985). Likewise, land bridges that connected Greenland and Iceland during the Miocene (McKenna 1981) could explain the presence of *Crangonyx islandicus* Svavarsson & Kristjánsson, 2006 and *Crymostygius thingvallensis* Kristjánsson & Svavarsson, 2004 on the latter.

The uncertainty of the deep nodes in our phylogeny precludes a detailed interpretation of the events that followed the initial diversification of Crangonyctidae. Nevertheless, several pertinent conclusions can be made at the level of the main clades, all of which started their diversification at some point during the Cretaceous. Clade I originated in North America before the Laurasian breakup and is the only one that dispersed to Europe where it is represented by two *Crangonyx* species (*C. chlebnikovi* (Borutzky, 1928) and *C. subterraneus* Spence Bate, 1859). This dispersal occurred at the end of the Cretaceous, when North America and Europe started to break apart (Mosar et al. 2002). Clade II originated and diversified throughout Eurasia since the late Cretaceous, while Eurasian clades III, IV, and V although in an unclear phylogenetic position, seem to be older than most extant crangonyctid lineages. Clade VI is apparently the oldest of the crangonyctid clades and originated in North America and adjacent landmasses. The Iceland endemic species *Crangonyx islandicus* is the earliest branching and oldest lineage in this clade whose age far exceeds that of the island. The same pattern holds for the other Iceland endemic, *Crymostygius thingvallensis*, the sole member of the Crymostygidae (Clade VIII). Moreover, there is also a general tendency towards older evolutionary age with an increase in latitude (Fig. 4). Considering the above points and the Miocene connection of Iceland to Greenland, we hypothesize that Greenland/northern Laurasia was an important crangonyctid evolutionary center and species donor to Iceland and beyond. Long-term survival of freshwater amphipods beneath or in close proximity to glaciers (including Iceland) (Copilaș-Ciocianu et al. 2017; Kornobis et al. 2010; McInerney et al. 2014) provides evidence as to how this relict latitudinal pattern persisted despite the severe climatic oscillations of the Quaternary.

The family Pseudocrangonyctidae (Clade VII), which is distributed across East Asia and Japan, is significantly younger than Crangonyctidae (Fig. 3, Table 2). Nonetheless, its crown age is estimated as Late Cretaceous, indicating that it colonized the Japanese region before it became an archipelago after splitting from the Asian mainland during the Miocene (Iijima and Tada 1990). Its sister relationship with Crymostygidae and their highly disjunct distribution implies that their common ancestor existed throughout the Arctic basin region (Sidorov and Gontcharov 2015), further strengthening our hypothesis that boreal Laurasia was an evolutionary hotspot.

The above biogeographical interpretations are based on the assumption that the crangonyctid ancestor lived in freshwater. Although the phylogenetic analyses strongly support this view, it is also likely that freshwaters were colonized multiple times from marine ancestors, as seems to be the case with other freshwater amphipods (Bauzà-Ribot et al. 2012; Copilaş-Ciocianu and Petrusek 2017; Holsinger 1986; Mamos et al. 2016; McInerney et al. 2014; Stock 1980; Stokkan et al. 2018). Paleontological data indicates that during the Mesozoic numerous lineages of invertebrates evolved in the epicontinental seas of the Arctic basin (Zakharov et al. 2002). Seemingly, this circumstance could also explain the panboreal distribution of the supposedly epigean crangonyctid common ancestor, as well as the younger age of stygobiont *Stygobromus* lineages by independent stranding with marine regressions on different continents/regions (Holsinger 1991); for example, the stranding of *Stygobromus mikhaili* in the Chuy Valley correlates well with these views (Zykin et al. 2008). Furthermore, the overlapping morphology and modern distribution of both the Far Eastern *Amurocrangonyx* and Oregon *Stygonyx* on the Pacific boundaries is correlated with stratigraphic sections of Mesozoic marine fauna (Sey et al. 2004). Considering that the origin of any group is inevitably associated with the evolution of the biotope in which it lives, we conclude that the origin and evolution of crangonyctids is also inextricably linked with the geological evolution of northern Eurasia, the Arctic Ocean basins, and adjacent seas.

Systematic and evolutionary implications

The systematics of the family Crangonyctidae is rather indecisive (Holsinger 1977). Although it is well defined morphologically, the phylogenetic relationships among the genera are not well understood, and even the monophyly of its widespread genera *Crangonyx*, *Stygobromus*, and *Synurella* has not been supported by either morphological or molecular data (Koenemann and Holsinger 2001; Kornobis et al. 2011, 2012). Our extensive taxonomic sampling refines and further strengthens the previous patterns of polyphyly observed in these three genera (Fig. 2).

It appears that the recovered molecular phylogenetic relationships often reflect geographical proximity rather than the morphology-based taxonomy. This means that molecular characters reflect evolutionary relationships that have been shaped by historical biogeographical factors and that molecular and morphological evolution seems to be decoupled, leading to taxonomic incongruence. For example, North American species of *Synurella* are more closely related to North American *Crangonyx* than to Eurasian *Synurella,* while Eurasian *Stygobromus* is more closely related to Eurasian *Synurella* than North American *Stygobromus* (Fig. 2). Similarly, the Caucasian *Lyurella* is closely related to Eurasian species of *Synurella* and *Stygobromus.* However, these patterns do not always hold because the European *Crangonyx chelbnikovi* and *C. subterraneus* are strongly supported as nested within the North American *Stygobromus* (possibly close to *Bactrurus* in the ML analysis), a relationship which is also supported by morphological features such as the presence of the inferior antennal sinus (Sidorov et al. 2012). There are also several cases when the phylogenetic positions of several clades are uncertain within the family, such as clades III and V and *Amurocrangonyx* clade IV (Fig. 2). The phylogenetic position and morphological distinctness of the Icelandic endemic

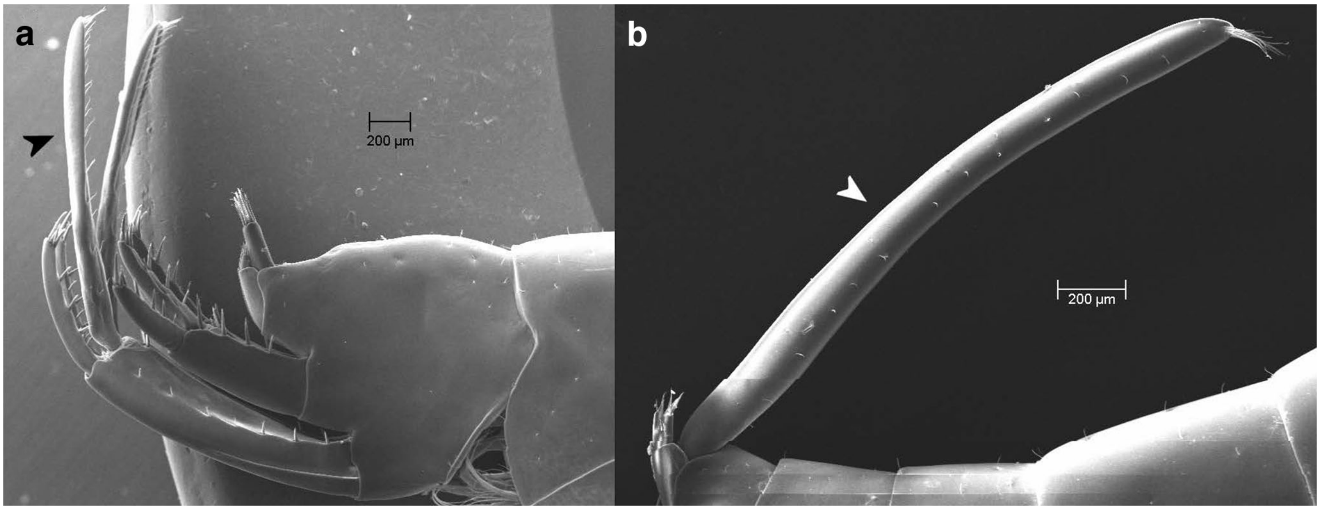

Fig. 5 Scanning electron micrographs. **a** Uropod I of *Synurella derzhavini*, male, Saratov (Russia). **b** Telson of *Bactrurus mucronatus*, male, Indiana (United States of America). Arrows indicate greatly enlarged appendages

Crangonyx islandicus might warrant its elevation to a new genus (Kornobis et al. 2011; Svavarsson and Kristjánsson 2006). Altogether, these results emphasize the complex morphological evolution of Crangonyctidae and that it requires a comprehensive systematic revision that should also take biogeography into account.

A firm conclusion about the evolution of eye regression throughout the family cannot be made at the moment due to the basal polytomy of our phylogeny (Fig. 2). However, we can reject a previous hypothesis that exclusively subterranean and troglomorphic genera such as *Stygobromus* have older origins than the less troglomorphic and often epigean *Crangonyx* and *Synurella* (Holsinger 1994). In fact, the opposite seems to be true because the oldest lineages usually possess relatively developed or vestigial eyes, while the more derived ones (e.g. Clade I) are eyeless (Fig. 2 and 4b). We therefore presume that the ancestral form was an epigean species, possessing eyes and pigmentation and would have lived in the coastal regions of the continents. Hence, distant crangonyctid extant relatives should be sought not in inland karstic caves, but in coastal continental springs, brackish water biotopes of river mouths, and even in the shallow seas. Furthermore, given the general basal placement of *Synurella* lineages (Fig. 2), the presence of this morphotype across the phylogenetic tree (clades II–VI), as well as its wide geographical distribution and significant morphological and genetic diversity, we postulate that the hypothetical crangonyctid ancestor had *Synurella*-like features and a boreal distribution. We also consider that it is necessary to pay attention to the unique morphology of uropod I and telson in *Synurella derzhavini* Behning, 1928 and *Bactrurus mucronatus* (Forbes, 1876), which probably represents the ancestral state of sexual dimorphism in males (Fig. 5). The study of sexual dimorphism in crangonyctids could further help in the elucidation of the evolutionary patterns in this group (cf. *Lyurella* spp.).

Conclusion

The results of our study strongly support the hypothesis that Crangonyctidae originated on the Laurasian supercontinent before its break-up during the Late Cretaceous. Therefore, this family is an excellent model system for testing biogeographical hypotheses of continental-wide plate tectonics vicariance. We hope our study will raise more interest for this group. Further research which includes a broader taxonomic sampling and a phylogenomic approach is needed to examine in more detail the impact of plate tectonics on the vicariance biogeography of Crangonyctidae, narrow down its temporal origins, and resolve its problematic taxonomy.

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