Shedding light on the Ophel biome: The trans-Tethyan phylogeography of the sulfide shrimp *Tethysbaena* (Peracarida: Thermosbaenacea) in the Levant (#89293)

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Shedding light on the Ophel biome: The trans-Tethyan phylogeography of the sulûde shrimp *Tethysbaena* (Peracarida: Thermosbaenacea) in the Levant

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Background. *Tethysbaena* are small peracarid crustaceans found in extreme environments such as subterranean lakes and thermal springs, represented by endemic species found around the ancient Tethys, including the Mediterranean, Arabian Sea, Mid-East Atlantic, and the Caribbean Sea. Two *Tethysbaena* species are known from the Levant: *T. relicta*, inhabiting the Dead Sea-Jordan Rift Valley, and *T. ophelicola*, found in the Ayyalon cave complex in the Israeli coastal plain, both belonging to the same species-group based on morphological cladistics. Along the biospeleological research of the Levantine subterranean fauna, Tthree biogeographic hypotheses determining their origins were have been proposed: (1) Plioceneeic transgression, (2) Mid-late Mioceneenic transgression, and (3) The Ophel Paradigm, according to which these are inhabitants of a chemosynthetic biome as old as the Cambrian. Methods. *Tethysbaena* specimens of the two Levantine species were collected from subterranean groundwaters. We used the mitochondrial gene cytochrome *c* oxidase subunit I (COI) and a molecular clock approach to establish the phylogeny and assess the divergence times of the Levantine *Tethysbaena*. Results. Contrary to the morphological cladistics-based classifiûcation, we found that *T. relicta* share an ancestor with *Tethysbaena* species from Oman and Dominican Republic, whereas the circum- Mediterranean species (including *T. ophelicola*) share another ancestor. The mean age of the node linking *T. relicta* from the Dead Sea-Jordan Rift Valley and *Tethysbaena* from Oman was 20.13 MYA. The mean estimate for the divergence of *T. ophelicola* from the Mediterranean *Tethysbaena* clade dated to 9.46 MYA. Conclusions. Our results indicate a two-stage colonization of *Tethysbaena* in the Levant: a late Oligocene transgression, through a marine gulf extending from the Arabian Sea, leading to the colonization of *T. relicta* in the Dead Sea-Jordan Rift Valley, whereas *T.*

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3 **(Peracarida: Thermosbaenacea) in the Levant**
4

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Conclusions. Our results indicate a two-stage colonization of *Tethysbaena* in the Levant: a late Oligocene transgression, through a marine gulf extending from the Arabian Sea, leading to the colonization of *T. relicta* in the Dead Sea-Jordan Rift Valley, whereas *T. ophelicola*, originating from the Mesogean ancestor, inhabited anchialine caves in the coastal plain of Israel during the Mid-Miocene.

Introduction

Groundwater fauna (stygofauna) is characterized by short-range endemism and high species crypticity. The unique suite of troglomorphic traits (e.g., loss of pigment, reduced eyes) characterizing stygobionts often hinders distributional studies due to the highly convergent characteristics that can obscure taxonomic relationships (Juan et al. 2010; Porter 2007). As a result, molecular phylogenetic tools have been extensively used over the last two decades to infer stygofauna biogeographies and the underlying processes shaping them (e.g., Abrams et al. 2019; Asmyr et al. 2014; Bauza-Ribot et al. 2012; Bradford et al. 2010; Canovas et al. 2016; Cooper et al. 2023; Finston et al. 2004; Guy-Haim et al. 2018; Jaume 2008; Jurado-Rivera et al. 2017; Marin et al. 2021; Matthews et al. 2020).

Thermosbaenacea is a small order of peracarid crustaceans comprising unique and highly specialized species adapted to extreme aquatic environments, including spring-fed subterranean lakes and thermal springs, with their core populations found deep underground in the inaccessible phreatic waters. Anoxic, sulfide-rich environments are favorable to Thermosbaenacea—often feeding on bacterial mats formed by sulfide-oxidizing bacteria—thus termed "sulfide shrimp" by Por (2014). Based on their distribution, it was assumed that the ancestral habitat of the thermosbaenaceans is the ancient Tethys Sea, and they are represented by relic fauna found around the Mediterranean, the Arabian Sea, Mid-East Atlantic, and the Caribbean Sea (Hou & Li 2018; Wagner 1994). Among thermosbaenaceans, *Tethysbaena* (family: Monodellidae) is the most speciose and widespread genus, comprising 27 species in seven species-groups (Wagner 1994; Wagner & Bou 2021). Only a few of the *Tethysbaena* species-groups ~~were~~ have been analyzed and supported by molecular phylogenetic tools (Canovas et al. 2016; Wagner & Chevaldonne 2020).

> **Commented [RK3]:** Halosbaena and Tulumella are found in more marine anchialine/freshwater cave conditions in Europe, Australia and SE Asia

Two species of *Tethysbaena* are known from Israel: *T. relicta* Por, 1962 (formerly *Monodella relicta*) and *T. ophelicola* Wagner, 2012. Initially, fragments of *T. relicta* were found in the hot spring Hamei Zohar by the Dead Sea in Israel (Por 1962). Later, scattered specimens of the same species were collected from the thermohaline spring En-Nur, on Lake Kinneret shore, a few hundred kilometers to the north (Dimentman & Por 1991), thus inferring that *T. relicta* inhabits the whole groundwater system of the Dead Sea-Jordan Rift Valley aquifer. *T. ophelicola* was

found in the karstic underground basin near Ramla, named Ayyalon-Nesher-Ramla complex (Por 2014; Por et al. 2013; Wagner 2012), 60 km west of the Dead Sea-Jordan Rift Valley, beyond the water divide of Israel.

Based on synapomorphies of the antennular inner flagellum and maxilliped macrosetae (Wagner 1994), it was hypothesized that together with other closely allied species—one species from Somalia (Chelazzi & Messana 1982), four species from Oman (Wagner 2020), one species from Yemen (Wagner & Van Damme 2021)—*T. relicta* and *T. ophelicola* form the "*T. relicta*-group" (Wagner 2012), suggesting a recent common ancestor. An alternate hypothesis can be drawn from the phylogenetic analysis of the prawn *Typhlocaris* (Guy-Haim et al. 2018), which preys on *Tethysbaena* in Ayyalon and En-Nur (Tsurnamal 1978; Tsurnamal 2008; Tsurnamal & Por 1971; Wagner 2012). Four *Typhlocaris* species are known, two of which co-occur with *Tethysbaena*: *Ty. galilea* inhabiting En-Nur spring (Calman 1909; Tsurnamal 1978) and *Ty. ayyaloni* from the Ayyalon cave (Tsurnamal 2008). The two additional *Typhlocaris* species are *Ty. salentina* from Apulia region in Southeastern Italy (Caroli 1923; Froglia & Ungaro 2001) and *Ty. lethaea* from Libya near Benghazi (Parisi, 1921). The molecular phylogeny of *Typhlocaris* species showed that *Ty. ayyaloni* (Israel) and *Ty. salentina* (Italy) are more closely related to each other than either of them is to *Ty. galilea* (Israel) (Guy-Haim et al. 2018). Accordingly, we can hypothesize a similar phylogeographic pattern of the Levantine *Tethysbaena*, where *T. ophelicola* would be more closely related to the Mediterranean species ("*T. argentarii*-group") than to *T. relicta*.

Along the biospeleological research of the Thermosbaenacea and other phyla of subterranean crustaceans represented in the Dead Sea Rift Valley (Syncarida, and the families Bogidiellidae and Typhlocarididae), three paradigms have been proposed to explain their origins: (1) Pliocenic marine transgression (Por 1963), (2) Miocenic Tethys transgression (Dimentman & Por 1991; Por 1987), and (3) The Ophel Paradigm that offered a conceptual framework, within which these styobionts are inhabitants of the ancient chemosynthetic Ophel biome, dating back at least to the Cambrian (Por 2011). Using a molecular clock approach, Guy-Haim et al. (2018) estimated the divergence time of the *Typhlocaris* species. They based their analysis on a calibration node inferred from a regional geological event—the end of the marine connection between the Mediterranean Sea and the Dead Sea-Jordan Rift Valley, marked by the top of Bira formation, dated to 7 MYA (Rozenbaum et al. 2016), separating *Ty. galilea* and the *Typhlocaris* ancestor. The inferred divergence time of *Ty. ayyaloni* and *Ty. salentina* was 5.7 (4.4-6.9) MYA, at the time of the Messinian Salinity Crisis (5.96-5.33 MYA), when the Mediterranean Sea desiccated and lost almost all its Miocene tropical fauna (Por 1987; Por 1989). It is therefore an open question as to whether the same vicariant events have shaped the biogeographies of both predator (*Typhlocaris*) and prey (*Tethysbaena*) subterranean crustaceans.

The main objectives of our study were to (1) reveal the phylogenetic relatedness of the Levantine *Tethysbaena* species, and (2) use these patterns to infer the geological and evolutionary processes that have shaped their divergence patterns.

Materials & Methods

Sampling sites, specimen collection and identification

Specimens of *T. ophelicola* were collected by a hand pump from the inner pool of the Levana cave (31.9223°N, 34.8942°E), part of the Ayyalon-Nesher-Ramla complex (Fig. 1).

Specimens of *T. relicta* were collected by a hand pump from an artificial tunnel near the Dead Sea Shore penetrating the Judea Group aquifer, 6.5 km north of Hamei-Zohar (31.2232°N, 35.3547°E) (Fig. 1). The *locus typicus* of *T. relicta*, the thermal spring of Hamei-Zohar (Por 1962), is no longer accessible since the 1970s, as hotels were built on the spring area.

> **Commented [RK4]:** I would like a comment about the loss of the type locality. How sure are the authors that this spot they have collected in is well connected to the Hamei-Zohar thermal spring and that their specimens have been confirmed (morphology and barcoding?) as the same as the type material. Was type material able to be examined?

Part of the collected specimens was preserved in 70% ethanol and the other in absolute ethanol for morphological and molecular analyses, respectively. Species identification of *T. ophelicola* and *T. relicta* was performed using a stereomicroscope (SZX16, Olympus, Japan) following the identification keys in Por (1962), Wagner (1994) and Wagner (2012).

DNA extraction, amplification and sequencing

Canovas et al. (2016) used both mitochondrial cytochrome *c* oxidase subunit I (COI) and nuclear 28S rRNA genes to assess the genetic population structure of the anchialine *T. scabra* in the Balearic Islands, and found that the 28S rDNA gene showed low genetic variation resulting in a poorly resolved phylogenetic tree, and they, therefore, based their phylogenetic reconstruction and divergence time estimations on the COI gene only. Following their finding, we have used the COI gene in our analysis.

> **Commented [RK5]:** Page et al. used CO1 plus two nuclear genes. There is evidence more broadly that nuclear genes can give a lot of value to the phylogeny.

Total genomic DNA was extracted from each individual using the DNEasy Blood and Tissue kit (QIAGEN, Germany) according to the manufacturer's specifications. Following the DNA extraction, the COI gene was amplified using PCR with universal primers LCO1490 and HCO2198 (Folmer et al. 1994). Reaction conditions were as follows: 94 °C for 2 min, followed by 5 cycles of 94 °C for 40 s, 45 °C for 40 s, and 72 °C for 1 min, and followed by 30 cycles of 94 °C for 40 s, 51°C for 40 s, and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. Obtained PCR products were purified and sequenced by Hylabs (Rehovot, Israel).

Phylogenetic analysis

A total of 22 COI sequences of *Tethysbaena* were analyzed, including *T. ophelicola* (n=3) and *T. relicta* (n=3) obtained in this study. Additional sequences of *T. scabra* (Balearic Islands, n=5), *T. argentarii* (Italy, n=2), *T. ledoyeri* (France, n=2), *T. atlantomaroccana* (Morocco, n=1), and further sequences of *Tethysbaena* sp., unidentified to the species level, from Oman (n=2), Morocco (n=3) and the Dominican Republic (n=1), were obtained from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home). The thermosbaenacean *Halosbaena tulki* was chosen as an outgroup following Page et al. (2016) and used as a root node in the phylogenetic analysis.

> **Commented [RK6]:** I would like to see a comment about how the outgroup was chosen - did the authors look outside of thermosbaenacea as well and run some trees? I assume that divergences would be too great to produce an informative in-group tree but I'd like to see that discussed.

150149   All specimens, collection sites, accession numbers, and related references are summarized in
151150   Table 1.

152151   Sequence alignment was conducted using ClustalW embedded in MEGA v11.0 (Tamura et al.
153152   2021). The best-fitting substitution model was selected according to the Bayesian Information
154153   Criterion using Maximum-likelihood (ML) model selection in MEGA. GBlocks v0.91.1
155154   (Castresana 2000) was used for trimming the ambiguous blocks in the sequence alignment. ML
156155   analysis was performed using the T92+G+I model (BIC= 6112.5) with 1000 bootstrapping
157156   replicates. Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMC) analyses were
158157   conducted with MrBayes v3.2.7a (Ronquist et al. 2012) on XSEDE in the CIPRES v3.3 Science
159158   Gateway portal (https://www.phylo.org/portal2) with nst=2, rates=gamma, and
160159   statefreqpr=fixed(fixedest=equal). Two independent runs of 10,000,000 generations each
161160   performed, sampling every 1000 generations. A burn-in at 25% of the sampled trees was set for
162161   final tree production. Convergence and effective sampling of runs was assessed using Tracer v.
163162   1.6 (Drummond & Rambaut 2007), and the post-burnin tree samples were summarized using the
164163   sumt.

165164   Estimation of divergence times
166165   Molecular clock calculations for cave-dwelling species are often contentious (Page et al. 2008).
167166   Stygobionts often exhibit unique evolutionary characteristics and experiences, including
168167   isolation, reduced gene flow, small population sizes, and distinct selective pressures. These
169168   factors can lead to deviations from a constant rate of molecular evolution among lineages,
170169   rendering a strict molecular clock assumption less realistic. Therefore, we used a relaxed
171170   molecular clock approach (Drummond et al. 2006). Canovas et al. (2016) assessed the
172171   divergence time of the Western Mediterranean *Tethysbaena*, *T. scabra* from the Balearic Islands,
173172   and *T. argentarii* from Italy using the COI gene. They based the substitution rates on the mean
174173   rate estimated for a co-occurring anchialine stygobiont amphipod *Metacrangonyx longipes*,
175174   1.32% per lineage and million years (0.89-1.95, 95% CI) (Bauza-Ribot et al. 2012). Following
176175   Canovas et al. (2016), we implemented this substitution rate in our dataset.

177176   A relaxed-clock MCMC (Markov Chain Monte Carlo) approach using the uncorrelated log-
178177   normal model was implemented in BEAST v2.4 (Drummond & Rambaut 2007; Suchard et al.
179178   2018; Suchard & Rambaut 2009). The Yule process was chosen as speciation process. Three
180179   independent runs, each of 50,000,000 generations, were performed, with sampling every 5000
181180   generations. The three separate runs were then combined (following the removal of 10% burn-in)
182181   using LogCombiner v2.5.2. Log files were analyzed with Tracer v1.6 (Drummond & Rambaut
183182   2007), to assess convergence, confirm the combined effective sample sizes for all parameters,
184183   and ensure that the MCMC chain had run long enough to get a valid estimate of the parameters
185184   (Drummond & Rambaut 2007). Maximum clade credibility (MCC, hereafter) tree was then
186185   produced using TreeAnnotator v2.4.7 (Rambaut & Drummond 2017). FigTree v.1.4.4 (Rambaut

187186   2018) was used to visualize the MCC tree and the highest posterior density (HPD, hereafter)
188187   ranges.

189188   Results

190189   Morphological identification

191190   Specimens of *T. relicta* collected from the Dead Sea tunnel were similar to the specimens from
192191   Hamei-Zohar thermal spring described by Por (1962), and included males, with no ovigerous or
193192   brooding females (Fig. 2A). The average length (excluding antennae) was 2104±181 µm (n=5,
194193   ±SD, hereafter). The following morphological features characterized the specimens as belonging
195194   to *T. relicta*: 8 segments in the main flagellum (endopodite) of antenna 1; 7 terminal
196195   plumidenticulate macrosetae on the maxilliped; the uropod included 5 medial plumose
197196   macrosetae, 11-13 plumose macrosetae in the endopodite, and 16-19 macrosetae in the second
198197   segment of the exopodite. The mean width:length ratio of the telson was 1.15.

199198   Specimens of *T. ophelicola* from Levana cave were similar to the specimens from Ayyalon cave
200199   described by Wagner (2012), and included males, brooding females and postmarsupial juveniles
201200   (Fig. 2B-D). The average length (excluding antennae) was 2276±380 µm in males (n=5) and
202201   2620±139 µm in females (n=5). The following morphological features were found: 7 segments
203202   in the main flagellum (endopodite) of antenna 1; 7 terminal plumidenticulate macrosetae on the
204203   maxilliped; uropod included 4 medial plumose macrosetae and 18-22 plumose macrosetae in the
205204   endopodite and the second segment of the exopodite. The mean width:length ratio of the telson
206   was 1.10.

207   Molecular phylogenetic analysis

208   The DNA barcode consisting of a fragment of 708 bp of the COI gene was sequenced from 6
209   specimens of *T. ayyaloni* and *T. relicta*. Sequences were deposited in NCBI GenBank under
210   accession numbers OR189199-OR189204. The phylogenetic analysis included 16 additional
211   *Tethysbaena* sequences and one *Halosbaena tulki* sequence as an outgroup (Table 1). The overall
212   alignment was 691 bp long, with 227 parsimonious informative sites.

213   ML and Bayesian phylogenetic analyses showed similar tree topologies (Fig. 3). The Levantine
214   *Tethysbaena* species from Israel present polyphyly, where *T. ayyaloni* lies within a
215   Mediterranean clade (including *T. scabra* from the Balearic Islands, *T. ledoyeri* from Southern
216   France and *T. argentarii* from Italy) with 100% bootstrapping support and 0.99 posterior
217   probability, and *T. relicta* clusters with *Tethysbaena* sp. from Oman (100% bootstrapping
218   support and 1.00 posterior probability), and the Dominican Republic (87%/0.83 bootstrapping
219   support/posterior probability), forming the Arabian-Caribbean clade. The Atlantic *Tethysbaena*
220   *T. atlantomaroccana* is a sister taxon to the Mediterranean clade species, although with a lower
221   support/probability. The other Moroccan *Tethysbaena* species from Tasla and Lamkedmya were
222   in a more basal position but showed lower bootstrapping support (<50%).

Commented [RK7]: I am looking for a better confirmation that they belong to T. relicta than "similar to".

Divergence time estimation

Effective sample size (ESS) values were at least 436 and 356 for the posterior and prior statistics, respectively, 1738 for the likelihood statistic, and greater than 1400 for all MRCA times estimates, suggesting good mixing and an effective MCMC sampling of the posterior distribution.

We estimated the ages for eight nodes (Table 2). The youngest node was the most recent common ancestor of *T. leyoderi* from Southern France and *T. scabra* from the Balearic Islands, which returned a mean estimate at 8.31 MYA with 95 % HPD of 10.15-3.97 MYA. The next mean estimate is the divergence of *T. ophelicola* from the clade of *T. leyoderi* and *T. scabra*, dated to 9.46 MYA, with 95% HPD of 14.20-5.71 MYA. The mean age of the most common ancestor of all Mediterranean *Tethysbaena* was 10.71 MYA with 95 % HPD of 16.27-6.04 MYA. The most recent ancestor of the Mediterranean clade and *T. atlantomaroccana* from Morocco was dated to 32.41 MYA with 95 % HPD of 47.53-18.37 MYA. The mean age of the node linking *T. relicta* from the Dead Sea-Jordan Rift Valley and *Tethysbaena* from Oman was 20.13 MYA with 95 % HPD of 41.69-13.25 MYA. The node of the most recent common ancestor of *T. relicta*, *Tethysbaena* from Oman, and the *Tethysbaena* from the Dominican Republic had a mean estimate of 35.84 MYA with 95 % HPD of 51.41-22.16 MYA. The mean age for the node linking the Arabian-Caribbean clade (*T. relicta* + *Tethysbaena* sp. Oman + *Tethysbaena* sp. Dominican Republic) with the Mediterranean-Atlantic clade (*T. scabra* + *T. leyoderi* + *T. ophelicola* + *T. argentarii* + *T. atlantomaroccana*) was 40.42 MYA with 95 % HPD of 56.09-25.72 MYA. The estimate for the root node linking *Tethysbaena* and *Halosbaena* was 79.96 MYA with 95% HPD of 137.8-32.68 MYA.

Discussion

In his monography on Thermosbaenacea, Wagner (1994) divided the Monodellidae family into two genera, the monotypic *Monodella* and the speciose *Tethysbaena*, which he named after the ancient Tethys Sea and the Greek word "³³»¿·»¿" (meaning "to walk"), referring to these animals as "walkers of the Tethys Sea". He noted that although there is a great similarity among the different species, six species-groups can be identified based on morphological characters. With the later finding of *T. exigua* from Southern France, a seventh group was established (Wagner & Bou 2021). Here, we analyzed the phylogenetic relatedness and divergence times of the two Levantine *Tethysbaena* species found in Israel: *T. relicta* from the Dead Sea-Jordan Rift Valley, and *T. ophelicola*, from the Ayyalon-Nesher-Ramla cave complex in central Israel.

According to Wagner (2012) and Wagner & Van Damme (2021), both Levantine species belong to "*T. relicta*-group" (together with four species from Oman, one species from Somalia and one species from Yemen), implying that these are sister taxa sharing a most recent common ancestor. Our results reject the morphology-based cladistics and support the hypothesis suggesting that *T. relicta* shared an ancestor with *Tethysbaena* species from Oman and Dominican Republic,

260   whereas the circum-Mediterranean species (including *T. ophelicola*) share another ancestor.
261   Indeed, discrepancies between morphological cladistics and molecular phylogeny are common in
262   cave fauna and were often attributed to their convergent troglomorphic traits (Bishop & Iliffe
263   2012; Juan et al. 2010; Porter 2007).

264   Three paradigms determining the origin of the Thermosbaenacea and other phyla of subterranean
265   crustaceans represented in the Dead Sea-Jordan Rift Valley (Syncarida, and the families
266   Bogidiellidae and Typhlocarididae) and around the Mediterranean ~~were~~ have been previously defined. The earlier
267   paradigm suggested that the Levantine *Tethysbaena*, among other subterranean salt-water fauna,
268   have resulted from a late Pliocen~~eic~~ pre-glacial (Piacenzian, 3.600-2.588 MYA) marine
269   transgression (Fryer 1964; Hubault 1937; Por 1963). A narrow gulf penetrated into the coastal
270   line near the present-day mount Carmel and then bent southwards along the Dead Sea-Jordan
271   Rift Valley reaching a basin that extended south of the present Dead Sea (Picard 1943).
272   According to this paradigm, the Pliocen~~eic~~ Mediterranean was still inhabited by a very large
273   number of Tethys remnants, including thermosbaenaceans, that were stranded in the Rift Valley
274   and around the Mediterranean.

275   Por (1986) rejected the first paradigm, noting that the Pliocen~~eic~~ Mediterranean no longer
276   contained the tropical fauna that include the *Tethysbaena* ancestor and that the short-lived
277   Pliocen~~eic~~ transgression did not establish viable marine environments. Instead, he posited that
278   these species represent marine fauna colonized by Miocen~~eic~~ transgression (16-10 MYA), the last
279   time that tropical sea penetrated inland in the Levant, and left stranded following a late Miocene
280   regression (6-5.3 MYA) (Dimentman & Por 1991; Por 1987; Por 1989). This second paradigm
281   was supported by Guy-Haim et al. (2018) who used a molecular clock approach to estimate the
282   divergence time of the *Typhlocaris* species, based on a calibration node inferred from the end of
283   the marine connection between the Mediterranean Sea and the Dead Sea-Jordan Rift Valley,
284   marked by the top of Bira formation, dated to 7 MYA (Rozenbaum et al. 2016). They inferred a
285   divergence time of *Typholocaris* from Ayyalon cave and Italy of 5.7 (4.4-6.9) MYA, at the time
286   of the Messinian Salinity Crisis. During this event, the African plate moved towards the Euro-
287   Asian plate, closing the Straits of Gibraltar and temporarily isolating the Mediterranean Sea from
288   the Atlantic Ocean (Krijgsman et al. 1999). As a result, the Mediterranean Sea partly desiccated
289   and transformed into small hypersaline basins, losing almost all its Miocen~~eic~~ tropical fauna,
290   including those able to colonize subterranean waters (Por 1975; Por 1986; Por 1987; Por 1989).

291   With the discovery of the Ayyalon cave system and its endemic stygofauna in 2006, a third
292   paradigm known as "the Ophel Paradigm" was developed by Por (2007). He identified the
293   "Ophel" as a continental subterranean biome, subsisting on chemoautotrophic bacterial food,
294   independently of the exclusive allochthonous epigean food of photoautotrophic origin. Within
295   this biome, *Tethysbaena* are primary consumers, presenting a typical feeding behavior of upside-
296   down swimming-gathering of sulfur bacteria or bacterial mats (Por 2011; Wagner 2012).
297   Following the development of the new chemosynthetic-based biome paradigm, Por presented an

298 alternative to the Tethys stranding paradigm, stating that the "*Ophel paradigm falsified first of*
299 *all my own, previously held views*" on the diversification of the subterranean fauna in the Levant
300 (Por 2011). He noted that the pre-Messinian fauna of the fossiliferous taxa of the foraminiferans,
301 the mollusks and the teleost fishes was similar to the recent Red Sea fauna or different only at the
302 species level, and there is no indication for extinction of crustaceans during the Tertiary, thus
303 inferring that the origin of the subterranean Levantine fauna is of earlier origin (Por 2010). Por
304 suggested that the Ophelic biome is possibly at least as old as the Cambrian, which had a diverse
305 aquatic crustacean and arthropodan palaeofauna, including Thermosbaenacea (Por 2011).

306 Canovas et al. (2016) assessed the divergence time of the Western Mediterranean *Tethysbaena*,
307 *T. scabra* from the Balearic Islands and *T. argentarii* from Italy using the COI gene. They based
308 the substitution rates on the mean rate estimated for a co-occurring anchialine stygobiont
309 amphipod *Metacrangonyx longipes*, 1.32% per lineage and million years (0.89-1.95, 95% CI)
310 (Bauza-Ribot et al. 2012) and estimated the divergence time of *T. scabra* and *T. argentarii* to the
311 early Tortonian, 10.7 MYA. Following Canovas et al. (2016), we have used the COI gene to
312 assess the divergence times of the Levantine *Tethysbaena*, *T. relicta* and *T. opehlicola*, and
313 additional *Tethysbaena* species from around the Mediterranean, Arabian, and Caribbean Sea,
314 using the Australian *Halosbaena* as an outgroup.

315 Our analysis shows that the divergence times of *Tethysbaena* species are earlier than those of
316 *Typhlocaris* species, pre-dating the upper-Miocene Messinian Salinity Crisis. Most divergence
317 events occurred in the Miocene and Oligocene. The Dead Sea-Jordan Rift Valley *T. relicta*
318 shares a most recent common ancestor with *Tethysbaena* from the Arabian Sea (Oman), dated to
319 the early Miocene, 20.13 MYA (with 95% HPD of 41.69 - 13.25), corresponding with the Oligo-
320 Miocene rift-flank uplift of the Arabian plate during the formation of the Red Sea and Gulf of
321 Aden (34-20 MYA) (Omar & Steckler 1995; Stern & Johnson 2010). Both *T. relicta* and the
322 *Tethysbaena* from Oman separated from the Caribbean *Tethysbaena* during the Eocene-
323 Oligocene transition (38-30 MYA), when global cooling and tectonic uplift caused sea level
324 decline and led to the establishment of the modern Caribbean Seaway (Iturralde-Vinent &
325 MacPhee 1999; Iturralde-Vinent 2006; Weaver et al. 2016).

326 The most recent common ancestor of the Mediterranean *Tethysbaena* species—*T. ophelicola*
327 from the coastal plain of Israel, *T. scabra* from the Balearic Islands, *T. ledoyeri* from Southern
328 France, and *T. argentarii* from Italy—dated to the Tortonian in the Mid Miocene, 10.71 MYA
329 (with 95% HPD of 6.27 - 6.04) as was previously found by Canovas et al. (2016). The Ayyalon
330 cave *Tethysbaena*, *T. ophelicola*, separated from other Mesogean (emerging Mediterranean)
331 species around that time, 9.46 MYA (with 95% HPD of 14.20-5.71). The thermal water of the
332 Ayyalon cave complex is part of the Yarkon-Taninim aquifer (Weinberger et al. 1994). During
333 Oligocene-Miocene regressions (28-6 MYA), canyons were entrenched along the Mediterranean
334 Sea shoreline, serving as major outlets of the Yarkon-Taninim aquifer, potentially forming
335 anchialine karst caves (Frumkin et al. 2022; Laskow et al. 2011). Page et al. (2016) hypothesized

336   that the ancestral habitats of Thermosbaenacea are Tethyan anchialine caves. Accordingly, we
337   can assume that the ancestor of *T. ophelicola* inhabited coastal anchialine caves in the Mioceneic
338   ~~Tethys~~.

339   The most recent common ancestor of the Mediterranean and the Arabian-Caribbean clades of
340   *Tethysbaena* is dated to the upper Eocene (40.42 MYA). During that period, the collision
341   between the Arabian Plate and the Eurasian Plate resulted in the uplift of the Zagros Mountains
342   in Iran (Mouthereau et al. 2012). These mountain ranges acted as barriers, further isolating the
343   Arabian Sea from the Mediterranean region (Sanmartin 2003). The oldest, root node
344   (*Tethysbaena-Halosbaena*) dated to 79.96 MYA (with the caveat of a low posterior probability
345   and a large 95% HPD interval, 137.8 - 32.68 MYA). Page et al. (2016) established the
346   phylogeny and divergence dates of the thermosbaeancean *Halosbaena*. They used the
347   *Tethysbaena-Halosbaena* divergence as a calibration node, based on the presence of a
348   continuous band of ocean crust through the length of the North Atlantic, indicating the maximum
349   extent of the Tethys and the final opening of the Atlantic, dated to 107.5 MYA (with 95% HPD
350   of 125-90). Thus, *Tethysbaena* ancestor in both our analysis and in Page et al. (2016) dates to the
351   Cretaceous. The validity of the Paleozoic Ophel-driven hypothesis is also undermined by the
352   deep phylogeny of peracaridean orders based on the small-subunit (SSU) rRNA gene, which
353   showed that the thermosbaenacean lineage does not occupy a basal position relative to other
354   peracarids (Spears et al. 2005).

355   Overall, the molecular clock-based divergence patterns presented here do not support the
356   previously proposed hypotheses regarding the origins of the Levantine *Tethysbaena*. Instead, we
357   infer a complex, two-stage colonization pattern of the *Tethysbaena* species in the Levant: (1) a
358   late Oligocene transgression event, through a marine gulf extending from the Arabian Sea in the
359   East to the Sea of Galilea in the west, leading to the colonization of *T. relicta* in the Dead Sea-
360   Jordan Rift Valley, and (2) *T. ophelicola*, originating from the Mesogean Sea ancestor, inhabited
361   anchialine caves in the coastal plain of Israel during the Mid-Miocene. Our results also show that
362   the Cretaceous *Tethysbaena* ancestor first established in present-day Morocco, and then diverged
363   into two groups. The first is a Tethyan group including Oman, the Dead Sea-Jordan Rift Valley
364   and the Caribbean Sea. The second group formed around the emerging Mediterranean Sea, in its
365   marginal aquifers, including Ayyalon, Southern France, Italy and the Balearic Islands.

366   Conclusions

367   Our results reject the morphology-based cladistics and suggest that *T. relicta* shared a most
368   recent common ancestor with *Tethysbaena* species from Oman and Dominican Republic,
369   whereas the circum-Mediterranean species, including *T. ophelicola*, shared another ancestor. The
370   molecular dating analysis suggest a two-stage colonization of the *Tethysbaena* species in the
371   Levant, explaining their distant origins: a late Oligocene transgression leading to the colonization
372   of *T. relicta* in the Dead Sea-Jordan Rift Valley, and a Miocene transgression in the

373 Mediterranean region followed by a marine regression, stranding *T. ophelicola* in the coastal
374 plain of Israel. The speciose *Tethysbaena* provides an ~~exquisite~~ excellent opportunity for testing
375 paleogeographic paradigms. Here we analyzed the phylogenetic relationships and divergence of
376 nine out of twenty-seven known *Tethysbaena* species using the mitochondrial barcode gene.
377 Future studies should examine additional species utilizing more genes or complete genomes to
378 further unveil the phylogeny and biogeography of this unique group of ancient subterranean
379 crustaceans.

380 The study of these subterranean species is not only an opportunity to broaden our understanding
381 of paleogeography. It is also paramount for the protection of the hidden biodiversity found in
382 these largely inaccessible habitats, which ~~is nonetheless~~are increasingly affected by human activity.
383 Extraction of groundwater for irrigation and other uses, pollution, as well as quarrying, mining,
384 and above-ground development may put these underground ecosystems at severe risk. The
385 unique and often endemic nature of stygobiont species makes them even more prone to
386 extinction, and extensive exploration of this under-explored biome, worldwide, is necessary in
387 order to gain understanding and appreciation of the hidden biodiversity underground – an
388 understanding that may pave the way for conservation of these species and their ecosystems.

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Data Availability Statement

396 The data underlying this article are available in the GenBank Nucleotide Database at
397 https://www.ncbi.nlm.nih.gov/genbank/, and can be accessed with accession numbers OR189199-
398 OR189204.

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576

Figure 1

Tethysbaena distribution and habitats

A. Global *Tethysbaena* distribution. The species included in the phylogenetic analysis are presented in colored circles. Other *Tethysbaena* species are presented in black. Based on documented records in Wagner (1994); Wagner (2012); Wagner (2020) , Cánovas, Jurado0 Rivera, Cerro0Gálvez, Juan, Jaume and Pons (2016); Wagner and Chevaldonné (2020) and Wagner and Bou (2021) . **B.** Levantine distribution of *T. ophelicola* and *T. relicta*. JRV 3 Jordan Rift Valley. **C-D**. *Tethysbaena* Levantine habitats. **C**. An artiûcial tunnel near the Dead Sea, Israel. **D**. Levana (Ayyalon) cave, Israel.
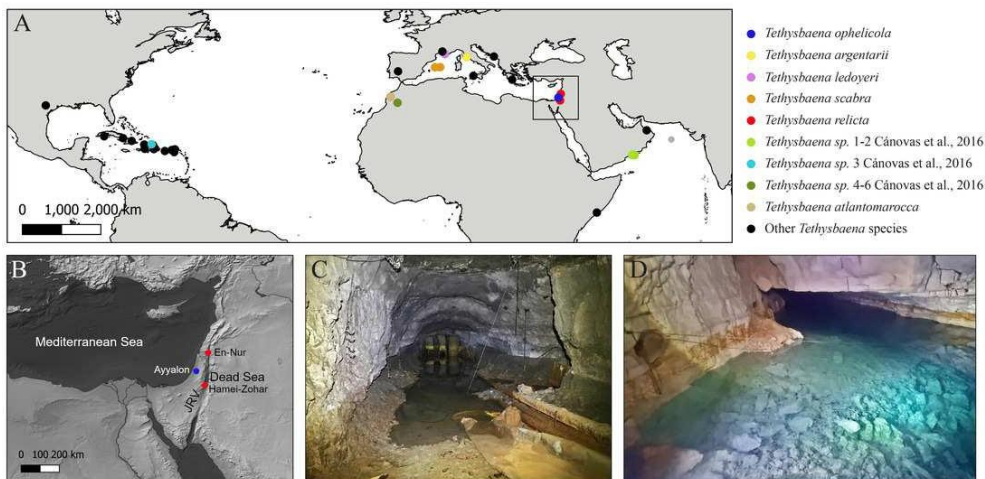
Figure 2

Levantine *Tethysbaena.*

A. *Tethysbaena relicta* Por, 1962 male. **B-D**. *Tethysbaena ophelicola* Wagner, 2012 male (B),
brooding female (C), and postmarsupial juvenile (D). The arrowhead points to the orange
coloration of the gut (B), indicating the presence of sulûde-oxidizing bacteria. The scale bar
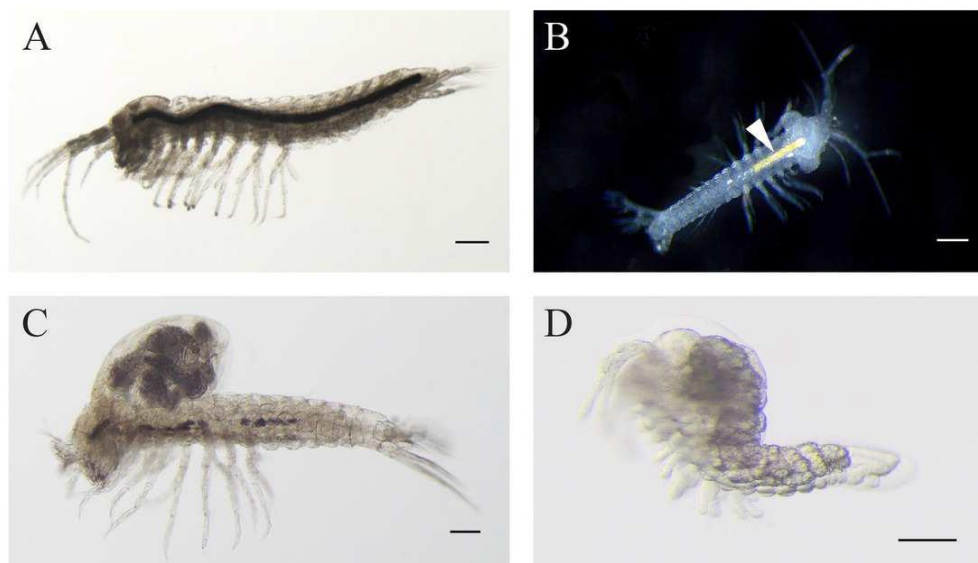denotes 200 μm in A-C and 100 μm in D.

Figure 3

Tethysbaena COI-based phylogenetic tree.

Maximum-Likelihood phylogenetic tree of *Tethysbaena* based on the COI gene, using the T92+G+I substitution model. *Halosbaena tulki* was used as a root node. At each node, the number on the left side of the slash indicates the percentage of ML bootstrap support (1000 replicates), and the right number indicates the Bayesian posterior probability expressed as a decimal fraction, for nodes that received at least 50% support. The scale bar denotes the estimated number of nucleotide substitutions per site.
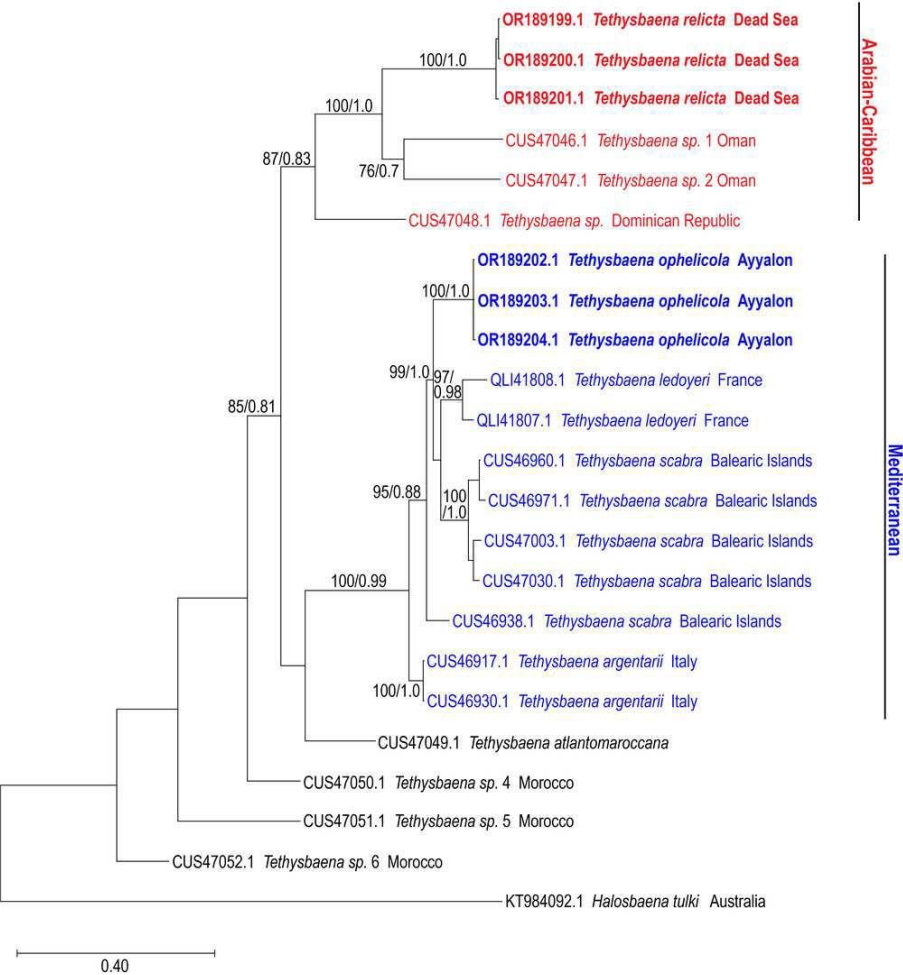
Figure 4

Tethysbaena time tree using the COI gene.

A relaxed MCMC clock using the uncorrelated log-normal model and substitution rates based on Cánovas et al. (2016) were implemented in BEAST v2.4. Mean ages are presented on the nodes, and the 95% HPD (highest posterior density) is presented by the blue bars.
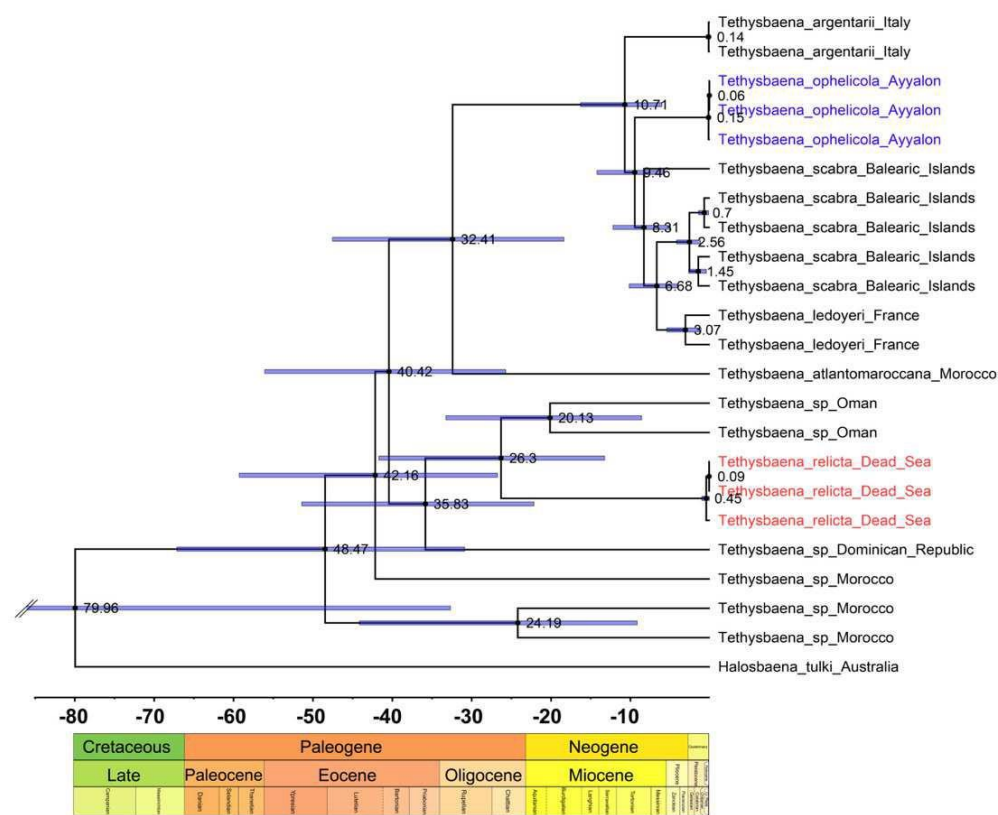
Table 1(on next page)

Tethysbaena species and outgroup included in the phylogenetic analysis.

	Species	Accession number	Locality	Reference
1	*Tethysbaena relicta*	OR189199.1	Dead Sea tunnel, Israel	This study
2	*Tethysbaena relicta*	OR189200.1	Dead Sea tunnel, Israel	This study
3	*Tethysbaena relicta*	OR189201.1	Dead Sea tunnel, Israel	This study
4	*Tethysbaena ophelicola*	OR189202.1	Levana cave, Israel	This study
5	*Tethysbaena ophelicola*	OR189203.1	Levana cave, Israel	This study
6	*Tethysbaena ophelicola*	OR189204.1	Levana cave, Israel	This study
7	*Tethysbaena ledoyeri*	QLI41807.1	Southern France	Wagner and Chevaldonne (2020)
8	*Tethysbaena ledoyeri*	QLI41808.1	Southern France	Wagner and Chevaldonne (2020)
9	*Tethysbaena argentarii*	CUS46917.1	Monte Argentario, Italy	Canovas, Jurado0Rivera, Cerro0Galvez, Juan, Jaume and Pons (2016)
10	*Tethysbaena argentarii*	CUS46930.1	Monte Argentario, Italy	Canovas et al. (2016)
11	*Tethysbaena scabra*	CUS46960.1	Balearic Islands	Canovas et al. (2016)
12	*Tethysbaena scabra*	CUS46971.1	Balearic Islands	Canovas et al. (2016)
13	*Tethysbaena scabra*	CUS47003.1	Balearic Islands	Canovas et al. (2016)
14	*Tethysbaena scabra*	CUS47030.1	Balearic Islands	Canovas et al. (2016)
15	*Tethysbaena scabra*	CUS46938.1	Balearic Islands	Canovas et al. (2016)
16	*Tethysbaena atlantomaroccana*	CUS47049.1	Marrakech, Morocco	Canovas et al. (2016)
17	*Tethysbaena* sp. 1	CUS47046.1	Dhofar coast, Oman	Canovas et al. (2016)
18	*Tethysbaena* sp. 2	CUS47047.1	Dhofar coast, Oman	Canovas et al. (2016)
19	*Tethysbaena* sp. 3	CUS47048.1	Southwest Dominican Republic	Canovas et al. (2016)
20	*Tethysbaena* sp. 4	CUS47050.1	Tasla, Morocco	Canovas et al. (2016)
21	*Tethysbaena* sp. 5	CUS47051.1	Tasla, Morocco	Canovas et al. (2016)
22	*Tethysbaena* sp. 6	CUS47052.1	Lamkedmya, Morocco	Canovas et al. (2016)
23	*Halosbaena tulki* (outgroup)	KT984092.1	Australia	Page, Hughes, Real, Stevens, King and Humphreys (2016)

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Table 2(on next page)

Divergence times for*Tethybaena* species.

Divergence times for *Tethybaena* species as estimated by the Bayesian evolutionary analysis method calculated using the COI gene molecular evolution based on Cánovas et al. (2016) and Bauzà-Ribot, Juan, Nardi, Oromí, Pons and Jaume (2012). Node ages and highest posterior density (±95% HPD) ranges are given in million years round.

	Clade divergence (nodes)	Node age (MYA) (95% HPD range)	Geological period
1	*T scabra* ─ *T leyoderi*	8.31 (10.15 - 3.97)	Miocene
2	*T scabra T leyoderi* ─ *T ophelicola*	9.46 (14.20 - 5.71)	Miocene
3	*T scabra T leyoderi + T ophelicola* ─ *T argentarii*	10.71 (16.27 - 6.04)	Miocene
4	*T scabra T leyoderi + T ophelicola T argentarii* ─ *T atlantomaroccana*	32.41 (47.53 - 18.37)	Oligocene
5	*T relicta* ─ *Tethysbaena* sp. (Oman)	20.13 (41.69 - 13.25)	Miocene
6	*T relicta + Tethysbaena* sp. (Oman) ─ *Tethysbaena* sp. (Dominican Republic)	35.83 (51.41 - 22.16)	Eocene
7	*T scabra T leyoderi + T ophelicola T argentarii + T atlantomaroccana - T relicta + Tethysbaena* sp. (Oman) + *Tethysbaena* sp. (Dominican Republic)	40.42 (56.09 - 25.72)	Eocene
8	*Tethysbaena* ─ *Halosbaena*	79.96 (137.8 - 32.68)	Cretaceous

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