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Multiple transgressions and slow evolution shape the phylogeographic pattern of the blind cave-dwelling shrimp *Typhlocaris*

Tamar Guy-Haim^{Corresp., 1,2}, Noa Simon-Blecher³, Amos Frumkin⁴, Israel Naaman⁴, Yair Achituv³

¹ Marine Ecology, GEOMAR, Helmholtz Centre for Ocean Research, Kiel, Germany

² Marine Biology, Israel Oceanographic and Limnological Research, Haifa, Israel

³ The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel

⁴ Institute of Earth Science, Hebrew University of Jerusalem, Jerusalem, Israel

Corresponding Author: Tamar Guy-Haim

Email address: tguy-haim@geomar.de

Background. Aquatic subterranean species often exhibit disjunct distributions, with high level of endemism and small range, shaped by vicariance, limited dispersal, and evolutionary rates. We studied the disjunct biogeographic patterns of an endangered blind cave shrimp, *Typhlocaris*, and identified the geological and evolutionary processes that have shaped its divergence pattern.

Methods. We collected *Typhlocaris* specimens of three species (*T. galilea*, *T. ayyaloni*, and *T. salentina*), originating from subterranean groundwater caves by the Mediterranean Sea, and used three mitochondrial genes (12S, 16S, COI) and four nuclear genes (18S, 28S, ITS, H3) to infer their phylogenetic relationships. Using the radiometric dating of a geological formation (Bira) as a calibration node, we estimated the divergence times of the *Typhlocaris* species and the molecular evolution rates.

Results. The multi-locus ML/Bayesian trees of the concatenated seven gene sequences showed that *T. salentina* (Italy) and *T. ayyaloni* (Israel) are more closely related than *T. galilea* (Israel). The divergence time of *T. ayyaloni* and *T. salentina* from *T. galilea* was according to COI - 6.0 [4.5-7.2] Ma and according to 16S - 5.9 [3.6-7.4] Ma. The computed interspecific evolutionary rates for COI - 0.0074 substitutions/Myr and for 16S - 0.0041 substitutions/Myr.

Discussion. Two consecutive vicariant events have shaped the phylogeographic patterns of *Typhlocaris* species. First, *T. galilea* was tectonically isolated from its siblings in the Mediterranean Sea by the arching uplift of the central mountain range of Israel ca. 7 Ma. Secondly, *T. ayyaloni* and *T. salentina* were stranded and separated by a marine transgression ca. 6 Ma, occurring just before the Messinian Salinity Crisis. Our estimated molecular evolution rates were in one order of magnitude lower than the rates of closely related crustaceans, as well as of other stygobiont species. We suggest that this slow evolution reflects the ecological conditions prevailing in the highly isolated subterranean enclosures inhabited by *Typhlocaris*.

1 TITLE:

2 **Multiple transgressions and slow evolution shape the phylogeographic**
3 **pattern of the blind cave-dwelling shrimp *Typhlocaris***

4 Short title: Mediterranean stygobiont phylogeography

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7 Tamar Guy-Haim^{1,2*}, Noa Simon-Blecher³, Amos Frumkin⁴, Israel Naaman⁴, Yair Achituv³

8

9 ¹GEOMAR, Helmholtz Centre of Ocean Research Kiel, Marine Ecology, Düsternbrooker Weg 20, Kiel 24105,
10 Germany.

11 ² Israel Oceanographic and Limnological Research, National Institute of Oceanography. P.O. Box 8030, Haifa
12 31080, Israel.

13 ³The Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat Gan 529002 Israel.

14 ⁴Institute of Earth Science, The Hebrew University of Jerusalem, Jerusalem 9190401 Israel.

15

16 *Corresponding author: Tamar Guy-Haim, GEOMAR, Helmholtz Centre of Ocean Research
17 Kiel, Düsternbrooker Weg 20, Kiel 24105, Germany. tguy-haim@geomar.de. Office: +49 431
18 6004508, Mobile: +49 16 24037340, Fax: +49 431 6001671

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21 **ABSTRACT**

22 **Background.** Aquatic subterranean species often exhibit disjunct distributions, with high level
23 of endemism and small range, shaped by vicariance, limited dispersal, and evolutionary rates.

24 We studied the disjunct biogeographic patterns of an endangered blind cave shrimp, *Typhlocaris*,
25 and identified the geological and evolutionary processes that have shaped its divergence pattern.

26 **Methods.** We collected *Typhlocaris* specimens of three species (*T. galilea*, *T. ayyaloni*, and *T.*
27 *salentina*), originating from subterranean groundwater caves by the Mediterranean Sea, and used
28 three mitochondrial genes (12S, 16S, COI) and four nuclear genes (18S, 28S, ITS, H3) to infer
29 their phylogenetic relationships. Using the radiometric dating of a geological formation (Bira) as
30 a calibration node, we estimated the divergence times of the *Typhlocaris* species and the
31 molecular evolution rates.

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33 that *T. salentina* (Italy) and *T. ayyaloni* (Israel) are more closely related than *T. galilea* (Israel).
34 The divergence time of *T. ayyaloni* and *T. salentina* from *T. galilea* was according to COI – 6.0
35 [4.5-7.2] Ma and according to 16S – 5.9 [3.6-7.4] Ma. The computed interspecific evolutionary
36 rates for COI – 0.0074 substitutions/Myr and for 16S – 0.0041 substitutions/Myr.

37 **Discussion.** Two consecutive vicariant events have shaped the phylogeographic patterns of
38 *Typhlocaris* species. First, *T. galilea* was tectonically isolated from its siblings in the
39 Mediterranean Sea by the arching uplift of the central mountain range of Israel ca. 7 Ma.
40 Secondly, *T. ayyaloni* and *T. salentina* were stranded and separated by a marine transgression ca.
41 6 Ma, occurring just before the Messinian Salinity Crisis. Our estimated molecular evolution
42 rates were in one order of magnitude lower than the rates of closely related crustaceans, as well
43 as of other stygobiont species. We suggest that this slow evolution reflects the ecological
44 conditions prevailing in the highly isolated subterranean enclosures inhabited by *Typhlocaris*.

45 **KEYWORDS:** cave, divergence time, Mediterranean Sea, Messinian Salinity Crisis, stygofauna,
46 subterranean, transgression, *Typhlocaris*.

47 **INTRODUCTION**

48 The biogeographic distribution patterns of populations of aquatic subterranean organisms
49 (stygobionts) are characterized by a small range and high degree of endemism, originating from
50 limited dispersal abilities and vicariant events, isolating the subterranean basins (Christman et al.
51 2005; Culver & Holsinger 1992; Culver et al. 2009; Culver & Sket 2000; Gibert & Deharveng
52 2002; Porter 2007). Sometimes the entire distribution of a stygobiont species is restricted to a
53 single subterranean enclosure, exposing it to a substantial risk of extinction due to natural and
54 anthropogenic pressures such as salt water intrusion, pollution, climate change, and
55 overexploitation of groundwater for drinking and agricultural purposes, resulting in habitat
56 destruction (Culver & Pipan 2009; Danielopol et al. 2003; Gibert et al. 2009).

57 The aquatic subterranean fauna of the Levant is comprised of typical stygofauna. Among them
58 are at least four crustaceans, found in sites located along the Dead Sea Rift valley with
59 congeneric taxa found in the Mediterranean coastal plain and even in brackish groundwater in
60 the south of Israel. These obligate stygobionts are regarded as relicts of extinct marine fauna of
61 ancient Mediterranean transgressions (Por 1963). The most prominent member of this faunal
62 assemblage is the large blind prawn of the genus *Typhlocaris*. Four species of this genus are
63 known from four localities around the east Mediterranean Sea (Figure 1). Each locality is
64 inhabited by a different species with no congeners in the open sea. Two species are known
65 from Israel: *T. galilea* (Calman 1909) from the Tabgha spring on Lake Kinneret shore, and the
66 recently discovered *T. ayyaloni* (Tsumamal 2008), found in the karstic underground basin near
67 Ramla, named Ayyalon cave, about 200 km south of Tabgha. The third species - *T. salentina*
68 Caroli, 1923 was described from the Zinzulusa cave in Southern Italy and was recently found in
69 other two caves in southern Italy (Frogliia & Ungaro 2001). The fourth species, *T. lethaea* Parisi,
70 1921 is known from Libya near Benghazi. In the IUCN Red List of Threatened Species, *T.*
71 *galilea* and *T. ayyaloni* are defined as endangered, and *T. salentina* as vulnerable. No data later
72 than 1960 on *T. lethaea* is available (De Grave 2013).

73 *Typhlocaris* and the other marine taxa survived the regression of the Mediterranean Sea that
74 occurred during the Messinian Salinity Crisis (MSC), 5.96 to 5.33 Ma, in caves and groundwater
75 basins. Most probably, they were extirpated from the Mediterranean Sea waters when the

76 Mediterranean desiccated and transformed to small hypersaline basins. During this crisis, the
77 Mediterranean Sea lost almost all its Miocene tropical fauna, including those able to colonize
78 subterranean waters (Por 1975; Por 1986; Por & Dimentman 2006). Therefore, the stranding of
79 the *Typhlocaris* species and the separation from their common ancestor have likely preceded the
80 MSC.

81 Two scenarios were proposed to explain the disjunct distribution of *Typhlocaris* (H1 and H2,
82 Figure 2). Por (1963; 1975; 2006) suggested that *Typhlocaris* species have been stranded along
83 the shores of a peri-Mediterranean Pliocene transgression. The timing of this scenario contradicts
84 the pre-MSC stranding described above. Accordingly, the *Typhlocaris* species expanded their
85 distribution into the Jordan valley when it was submerged for a brief period during the Zanclean
86 marine transgression. The coastal plain was also submerged by this transgression that possibly
87 also covered a part of the south of Israel (Por 1963). Those faunal elements were left behind
88 when the shore has retreated during the regression that followed the regression in the early
89 Pliocene. Similarly, Horowitz (2001) suggested that during the Pliocene, two successive
90 transgressive cycles have occurred in the Zanclean and the Piacenzian, separated by a regression.
91 Thus, according to this scenario, *T. galilea* and *T. ayyaloni* were separated together or at
92 successive events from the Mediterranean fauna, and are thus sister taxa (H1, Figure 2).

93 A recent study of the eastern Galilee (Rozenbaum et al. 2016) suggests a second scenario (H2,
94 Figure 2). The marine transgression into the Dead Sea valley, bringing along *T. galilea*, was
95 associated with a subsidence of the eastern Galilee. The Dead Sea rift valley, accommodating
96 several water bodies, became tectonically isolated from the Mediterranean by the arching uplift
97 of the central mountain range of Israel. This uplift also divided the groundwater basins of the
98 Dead Sea basin from those associated with the Mediterranean. Contrastingly, the other three
99 *Typhlocaris* species are found in coastal to inland aquifers that are not isolated from the
100 Mediterranean by a tectonic barrier. They could be stranded in the coastal aquifers by an
101 ingression that was not necessarily associated with a tectonic event. This hypothesis is supported
102 by the finding of marine macrofossils within the late Miocene Bira Formation of the SE Galilee-
103 Jordan valley indicating its association with a marine transgression (Shaked-Gelband et al.
104 2014). Ar-Ar dates of volcanics interbedded within the Bira Formation show that the earliest
105 marine invasion into the SE Galilee-Jordan valley happened between 11 and 10 Ma (Rozenbaum

106 et al. 2016; for earlier dating see Shaliv 1989). Ongoing subsidence of the SE Galilee basin,
107 coupled with rising sea level, resulted in the invasion of the Mediterranean water and
108 establishment of a seaway that connected it to the evolving Dead Sea Rift in the east, as
109 represented by parts of the Bira Formation. Seawater could have flowed to the SE Galilee basin
110 either due to global sea level rise above the low barrier near the coastline or due to tectonic
111 subsidence of the Yizre'el Valley which had already started to develop. The detachment of this
112 region from the Mediterranean occurred ca. 7Ma, when the Mediterranean Sea level started
113 falling during the Messinian, followed by freshwaters gradually replacing the saline waters of the
114 Bira lagoon. Thus, the main marine ingression is constrained to the Tortonian, prior to the MSC.
115 Further to the NE, within the Hula valley, Syria and Lebanon, there is no indication of this
116 marine transgression, demonstrating that the marine water came from the Mediterranean and not
117 from the NE (Rozenbaum et al. 2016). This is consistent with the circum-Mediterranean
118 distribution of the four *Typhlocaris* species.

119 The main objectives of our study were: (1) to reveal phylogenetic relationship of the *Typhlocaris*
120 species, and (2) to infer the geological processes that have shaped their divergence pattern.

121 MATERIALS & METHODS

122 *Species sampling, genes and outgroup selection*

123 Specimens of *T. galilea* were collected by us, in the covered pool collecting the water of Tabgha
124 spring (32°52'20"N 35°33'00"E) on Lake Kinneret shore (NPA permit 37920). *T. ayyaloni* was
125 collected from the underground groundwater pond in Ayyalon cave (31°54'37"N 34°55'39"E),
126 two specimens of *T. salentina* were provided by Dr. G. Messina Firenze – Italy from two caves
127 in the vicinity of Bari, Italy, Lu Bissu cave (39°59'42"N 15°57'58"E) and Mola di Bari cave
128 (41°03'36"N 17°05'24"E). All samples were fixed and stored in 95% ethanol at -20°C until
129 DNA extraction. The locality of the fourth species, *T. lethaea*, is restricted to Lete Cave, near
130 Benghazi, Libya, and is not accessible. The two specimens of *T. lethaea*, collected by Parisi a
131 century ago (1921), and stored in the Museum National d'Histoire Naturelle, Paris, did not yield
132 DNA.

133

134 *DNA extraction, amplification and sequencing*

135 DNA was extracted using Macherey–Nagel genomic DNA isolation kit, following the
136 manufacturer’s recommended protocol. The primers used for gene amplification are detailed in
137 the Supplemental Information, including both primers from former studies and newly designed
138 primers for this study (*Table S1*). REDTaq ReadyMix R2523 (Sigma-Aldrich, St. Louis, MO)
139 was used for sequence amplification by PCR (Saiki et al. 1988). Amplification was carried out in
140 a personal combi-thermocycler (Biometra, Germany) according to the profiles listed in *Table S1*.
141 PCR products were purified by centrifugation using a High Pure PCR product purification kit
142 (Roche Diagnostics GmbH, Mannheim, Germany) or by Mclab laboratories (San Francisco,
143 California). PCR products were sequenced on both strands using an ABI PRISM 3100 Genetic
144 Analyzer (Applied Biosystems) by Mclab laboratories (San Francisco, US).

145 Three mitochondrial genes (12S rRNA; 16S rRNA; Cytochrom oxygnese subunit 1 (*COI*)) and
146 four nuclear genes (18S rRNA; 28S rRNA, Internal transcribed spacer (*ITS*); Histon 3 (*H3*))
147 were chosen for analysis. For phylogenetic inference of all seven gene partitions, we used
148 *Ephyrina figueirai* Crosnier & Forest, 1973, and *Palaemon elegans* Rathke, 1837, as outgroup
149 species, belonging to the same infraorder of *Typhlocaris*, Caridae. For divergence time
150 estimation, we used two transisthmian pairs of *Alpheus*: *A. estuarensis* – *A. colombiensis*, and *A.*
151 *anepenulitimus* – *A. chacei* (Knowlton & Weigt 1998; Williams et al. 2001).

152 The sequences were deposited in the GenBank under accession numbers KY593415-KY593454.
153 In addition to the newly generated sequences, two sequences of *T. salentina* were obtained from
154 GenBank and included in the molecular analysis. The list of taxa, localities and GenBank
155 accession numbers included in the analysis is detailed in Supplemental Information (*Table S2*).

156 *Phylogenetic analyses*

157 Sequence alignment was conducted using ClustalX embedded in MEGA v6.0 (Tamura et al.
158 2013). The sequences were concatenated to form a multi-gene matrix using Geneious v7.1 ([http://](http://www.geneious.com/)
159 www.geneious.com/), including the three *Typhlocaris* sequences and two outgroups, delimited
160 into seven partitions, one for each gene. MEGA v6.0 (Tamura et al. 2013) was used in order to

161 select the best fitting substitution model for each partition according to the Bayesian Information
162 Criterion (*Table 1*).

163 Maximum likelihood analysis of the aligned partitions was conducted using RAxML v8.2.9
164 (Stamatakis 2014) on XSEDE server in the CIPRES Science Gateway portal (Miller et al. 2010)
165 using a GTRCAT model of evolution with 50 rate categories with 1000 bootstrapping replicates.
166 Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMC) analyses were conducted
167 with MrBayes v3.2 on XSEDE with GTR model (Ronquist et al. 2012). Search was conducted
168 with four chains (three cold, one hot) with trees sampled every 100 generations. Three 100
169 generations analyses were conducted to verify likelihood convergence and burn-in parameter.

170 *Divergence time analysis*

171 Since the molecular clock calculations for cave-dwelling species are often contentious (Page et
172 al. 2008), we used multiple genes and a relaxed molecular clock approach (Drummond et al.
173 2006). The top of Bira formation, dated to 7 Ma (Rozenbaum et al. 2016), marks the end of the
174 marine connection between the Mediterranean and the Dead Sea valley. Therefore we assume
175 that this event indicates the isolation of *T. galilea* from its sister taxa, and we used it as a
176 calibration node. A relaxed-clock MCMC approach using the uncorrelated log-normal model
177 was implemented in BEAST v2.4 (Drummond & Bouckaert 2015) on XSEDE server in the
178 CIPRES Science Gateway portal (Miller et al. 2010), using 10 million generations, and sampling
179 every 1000th generation. Models of sequence evolution for each gene were determined using the
180 corrected Akaike information criterion in JModelTest v2.1 (Darriba & Posada 2014, *Table 2*) on
181 XSEDE server. The Yule process was chosen as speciation process for both genes. Log files
182 were analyzed with Tracer v1.6 (Rambaut et al. 2015), to assess convergence and confirm that
183 the combined effective sample sizes for all parameters were larger than 200, in order to ensure
184 that the MCMC chain had run long enough to get a valid estimate of the parameters (Drummond
185 & Rambaut 2007). All resulting trees were then combined with LogCombiner v1.8.2, with a
186 burn-in of 25%. A maximum credibility tree was then produced using TreeAnnotator v2.1.2
187 (Rambaut & Drummond 2015).

188

189 RESULTS

190 The concatenated alignment of the seven genes was 7761 bp long, out of which 1645 were
191 parsimonious informative. The substitution models selected for all the genes/partitions with the
192 corrected Akaike Information Criterion and the Bayesian Information Criterion scores is
193 presented in *Table 1*. Figure 3 presents a maximum likelihood (ML) tree of the concatenated
194 sequences, showing that *T. salentina* and *T. ayyaloni* are more closely related than *T. galilea*.
195 Neighbour-Joining multi-gene trees of the three species of *Typhlocaris* showed the same
196 topology. Also, out of the seven genes used for the analysis, five gene sequences (ITS, 28S, COI,
197 12S, 16S) presented this topology. The remaining gene trees, of 18S and H3, had slightly
198 different topology. However, the bootstrap support of the nodes connecting *Typhlocaris* species
199 in these trees was less than 50%.

200 Our analyses support the hypothesis suggesting that *T. galilea* was separated from its presumed
201 marine ancestor earlier than *T. ayyaloni* and *T. salentina* (H2, Figure 2).

202 Using 7 Ma as the detachment time that isolated *T. galilea* from the Mediterranean Sea (top Bira
203 formation), the divergence time of *T. ayyaloni* and *T. salentina* was according to COI gene – 6.0
204 [4.5-7.2] Ma and according to the 16S gene – 5.9 [3.6-7.4] Ma (*Table 2*), suggesting that these
205 are relicts of the last high level of the Mediterranean Sea before the MSC. The computed
206 evolutionary rates for COI – 0.0074 substitutions/Myr and for 16S – 0.0041 substitutions/Myr,
207 are notably lower than the molecular clock rates found in previous crustacean studies (*Table 3*).
208 The evolutionary rates of ITS, 28S, and 12S were 0.0104, 0.0184, 0.0115 substitutions/Myr,
209 respectively.

210 DISCUSSION

211 Marine regressions are the most significant vicariant events structuring stygoboint speciation
212 (Culver et al. 2009; Porter 2007). Using molecular techniques, we showed that two vicariant
213 events have shaped the phylogeographic patterns of *Typhlocaris* species. First, *T. galilea* was
214 tectonically isolated from the Mediterranean Sea by the arching uplift of the central mountain
215 range of Israel, ~7 Ma. Later, *T. ayyaloni* and *T. salentina* were stranded and separated by a
216 marine transgression ~6 Ma, as a result of the Messinian Salinity Crisis.

217 Commonly, the final closure of the Isthmus of Panama that has occurred approximately 3 Ma
218 (Coates et al. 1992; Keigwin 1982; Keigwin 1978; O’Dea et al. 2016) is used for estimation and
219 calibration of divergence time of crustaceans. Knowlton and Weigt (1998) and Williams *et al.*
220 (2001) found that the substitution rate of COI is 0.0140 per Myr. This finding is based on the
221 pairs of transisthmian snapping shrimp *Alpheus* from Panama: *A. estuarensis* – *A. colombiensis*,
222 and *A. nepenulitimus* – *A. chacei*. Schubart *et al.* (1998) calibrated the substitution rate of 16S
223 rDNA using trans-isthmian pairs of crabs of the genus *Sesarma* (Grapsidae) and then used this
224 rate to estimate a date for the origin of the Jamaican lineage *Sesarma*, the substitution rate of
225 *Sesarma* was 0.0065 per Myr. Sturmbauer *et al.* (1996) used the same gene from populations of
226 the fiddler crab *Uca vocator*, from either side of the Isthmus of Panama to estimate divergences
227 rates of *Uca*. The sequence divergence rate was 0.0090 per Myr; this rate was used to estimate
228 the time divergence between clades of terrestrial *Uca* from different parts of the globe.

229 Craft *et al.* (2008) and Page *et al.* (2008) that studied the phylogeography of atyids did not use
230 the rates of transisthmian organisms to calibrate the molecular clock but estimated it
231 independently for the studied taxa. Craft *et al.* (2008) studied *Halocaridina* from the Hawaiian
232 Archipelago. To calibrate the molecular clock, they used the age of the earliest eruption
233 of Kilauea volcano in Hawaii, 50–100 Ka, and the genetic data of the groups of
234 *Halocaridina* that occur along the flank of this volcano. They found an exceptionally
235 high divergence rate of 0.2 per Myr in COI gene of *Halocaridina*. They noted that this rate is
236 in sharp contrast to the commonly utilized evolution rates for arthropods 0.0140-0.0170 per Myr
237 (Williams et al. 2001). Page *et al.* (2008) studied the cave atyids *Stygiocaris* from Cape Range
238 area in Western Australia. It is accepted that the emergence of the Cape Range Anticline in the
239 Miocene isolated *Stygiocaris lancifera* and *S. stylifera*, leading to their speciation, therefore,
240 Page *et al.* (2008) used this event, 7–10 Ma, as a calibration point to estimate rates of molecular
241 divergence. This yielded a wide range of evolutionary rates for the *S. lancifera* / *stylifera* node:
242 0.0133-0.0516 substitutions/Myr in COI and 0.0055-0.0103 substitutions/Myr in 16S, relatively
243 lower than other atyid studies, but still higher than the rate we found for *Typhlocaris*.

244 Zakšek *et al.* (2009) studied the phylogeography the cave shrimp *Troglocaris anophthalmus*. To
245 estimate the divergence time they referred to the divergence rate of COI used for transisthmian
246 species of *Alpheus* across the Isthmus of Panama (Knowlton and Weigt, 1998). Zakšek *et al.*

247 (2009), therefore, stated that for *Troglocaris*, the rate calculated by Knowlton and Weigt (1998)
248 can be used only for estimation of the order of magnitude of divergence time because it is the
249 most commonly used rate for decapods. Nonetheless, they found COI patristic distances between
250 phylogroups that are much lower (0.05-0.08) than the accepted patristic COI distance of 0.16
251 substitutions per nucleotide position found to optimally separate intra- from interspecies
252 divergence in other crustaceans (Lefébure et al. 2007).

253 The rates found by us are in one order of magnitude lower than those found for *Alpheus*, the
254 common crustacean used for calibration of divergence time, as well as the rates of other
255 stygobionts (Page et al. 2008). Corresponding with our analysis, the low COI patristic distance
256 found in several phylogroups of a cave shrimp by Zakšek et al. (2009), may indicate a lower
257 evolution rate. Unlike *Typhlocaris* species that are each restricted to a limited isolated
258 subterranean enclosure, Zakšek et al. (2009) studied 50 isolated populations of the stygobiont
259 shrimp *Troglocaris anophthalmus*, whose range of distribution is more than 500 km. In the
260 Balkan Peninsula, this taxon is composed of four or possibly five monophyletic, geographically
261 defined phylogroups. It is assumed that during periodical floods, the cave dwelling *Troglocaris*
262 are frequently washed out of their subterranean habitat and reach other caves. Eventually each
263 was genetically adapted to the ecological condition of the new subterranean environmental
264 conditions.

265 The evolutionary rates, even of the same gene, differ in different genera within the same order –
266 indicating that evolutionary rates are not related only to the taxonomic position but also, or
267 mainly, to ecological conditions. We therefore did not use the previously reported substitution
268 rate but the known geological data of the area where *Typhlocaris* occurs. The lower divergence
269 rates found for *Typhlocaris* compared with other crustaceans lead us to the suggestion that the
270 low rates are related to the ecological conditions of the *Typhlocaris* habitat. *Typhlocaris* and
271 other stygobionts are found in isolated subterranean enclosures where species diversity is very
272 low, relative to the regional diversity (Gibert et al. 2009), potentially reducing interspecific
273 competition. The environmental factors in these enclosures are stable, lacking fluctuations.
274 Predators are typically missing in subterranean habitats, resulting in truncated food webs (Gibert
275 & Deharveng 2002). Additionally, evolution rates were correlated with metabolic rates (Martin
276 & Palumbi 1993). Species with low metabolic rates (e.g., deep-sea fauna) are generally

277 characterized by reduced nucleotide substitution rates. It was hypothesized that limited light
278 reduces visual predation pressure and selects for reduced locomotory ability and metabolic
279 capacity (da Silva et al. 2011). This may be just as well the case of stygobiont evolution. Thus,
280 the combined unique ecological and biological conditions (dark habitat, environmental stability,
281 low richness, lack of interspecific competition) lead to stability and low rate of gene divergence.
282 This is in agreement with the statement of Mayer (1963) that competition and allopatry are
283 important elements of speciation and evolutionary divergence.

284 Culver (1976) noted that the most striking feature of the organization of Appalachian cave-
285 stream communities is the reduction in intensity of competition. One of the suggested
286 explanations is that, with increasing time in caves, species evolve a life-history strategy of high
287 metabolic efficiency and low reproductive rate, a strategy that may itself reduce interspecific
288 competition. We assume that the higher divergence rate found in other crustacean is related to
289 competition. The classical taxa used for calibration of molecular dating are the 18 species of
290 *Alpheus* at both sides of the Isthmus of Panama (Knowlton and Weigt, 1998). Knowlton (1993)
291 observed aggressive behavior among species including individuals that belong to a nominal
292 species from both sides of the Isthmus of Panama, supporting our assumption on the role of
293 competition in delimiting evolutionary rates.

294 Using evolutionary biology, we can identify processes that promote or maintain phenotypic and
295 genetic diversity in natural populations. This is of a great importance particularly when the
296 studied organisms are under high risk of becoming extinct. While many studies confirmed that
297 interspecific competition and environmental variation drive genetic diversification, there is little
298 phylogeographic evidence linking environmental stability with low genetic variation. Further
299 molecular investigations of stygobionts and other organisms of stable environments will shed
300 light on universality of their temporal mode of speciation.

301 **CONCLUSIONS**

302 Our results indicated that two separate vicariant event shaped the distribution patterns of the
303 blind cave-dwelling shrimp *Typhlocaris*. During the late Miocene, *T. galilea* was tectonically
304 isolated from the Mediterranean Sea by the arching uplift of the central mountain range of Israel,

305 ca. 7 Ma. During the Messinian Salinity Crisis, *T. ayyaloni*, geographically adjacent to *T. galilea*,
306 and *T. salentina* were stranded and separated by a marine transgression. A future investigation of
307 the divergence time of *T. lethaea* may shed more light on the transgression events leading to the
308 disjunct phylogeographic pattern of *Typhlocaris*. Furthermore, the evolutionary rates of
309 *Typhlocaris* estimated in this study (0.0074 substitutions/Myr in Cytochrome Oxidase Subunit 1
310 (COI) and 0.0041 substitutions/Myr in 16S rRNA) were in one order of magnitude lower than
311 the rates of closely related crustaceans, and lower than other stygobiont species. These low rates
312 may result from the low predation stress and the low diversity, leading to low interspecific
313 competition, which characterizes the highly isolated subterranean enclosures inhabited by
314 *Typhlocaris*.

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438 **FIGURE LEGENDS**

439 **Figure 1.** Distribution map of *Typhlocaris* species (colored in red) based on spatial data from
440 NatureServe and IUCN (International Union for Conservation of Nature). *The IUCN Red List of*
441 *Threatened Species. Version 2014.1.* (<http://www.iucnredlist.org>). Downloaded on 28 January
442 2018. Map made using Natural Earth data (<http://www.natureearthdata.com>).

443 **Figure 2.** Schemes describing the two hypotheses of development of the disjunct distribution of
444 *Typhlocaris*. H1: the peri-Mediterranean transgression scenario. H2: tectonic isolation of the
445 eastern Galilee from the Mediterranean followed by stranding to the coastal aquifers by
446 ingressions.

447 **Figure 3.** Multi-locus Maximum Likelihood tree of the genus *Typhlocaris*, based on combined
448 12S rRNA + 16S rRNA + COI + 18S rRNA + 28S rRNA + ITS + H3 genes (total 7761 bp). At
449 each node, the number above the branch indicates the percentage of ML bootstrap support (1000
450 replicates) from RAxML analysis with the GTRCAT model of evolution. The number below the
451 branch at each node indicates the Bayesian posterior probability expressed as a decimal fraction
452 for nodes that received at least 50% support in at least one analysis. The scale bar denotes the
453 estimated number of nucleotide substitutions per site.

454 **TABLE LEGENDS**

455 **Table 1.** Nucleotide analysis and substitution models selected (out of 24 candidate models) for
456 all the genes/partitions with the corrected Akaike Information Criterion and the Bayesian
457 Information Criterion.

458 **Table 2.** Divergence times (and 95% CI) for *Typhlocaris* species as estimated using Bayesian
459 evolutionary analysis method based on COI and 16S genes and calibrated based on Bira
460 formation.

461 **Table 3.** Comparison between the COI and 16S molecular evolution rates estimated in this and
462 previous crustacean studies: ^[1] this study, ^[2] Knowlton & Weigt (1998), ^[3] Page et al. (2008), ^[4]
463 Schubart et al. (1998), ^[5] Sturmbauer et al. (1996), ^[6] Ketmaier et al. (2003), ^[7] Craft et al.
464 (2008).

465 SUPPLEMENTAL INFORMATION - TABLE LEGENDS

466 **Table S1.** List of the primers used for gene amplification in this study and PCR profiles.

467 **Table S2.** GenBank accession numbers of *Typhlocaris*.

468 DATA ACCESSIBILITY STATEMENT

469 The authors confirm that all data underlying the findings are fully available without restriction.
470 All DNA sequences generated in this research were deposited in the GenBank. The list of
471 primers used and designed for this study and the list of taxa, localities and GenBank accession
472 numbers are detailed in the Supplemental Information (Table S1 and S2, respectively) and will
473 be made available in the data repository PANGAEA.

Figure 1(on next page)

Distribution map of *Typhlocaris* species (colored in red) based on spatial data from NatureServe and IUCN (International Union for Conservation of Nature).

The IUCN Red List of Threatened Species. Version 2014.1. (<http://www.iucnredlist.org>).

Downloaded on 28 January 2018. Map made using Natural Earth data

(<http://www.naturalearthdata.com>).

17.0°E

22.0°E

27.0°E

32.0°E

44.0°N

44.0°N

40.0°N

40.0°N

36.0°N

36.0°N

32.0°N

32.0°N

Typhlocaris salentina

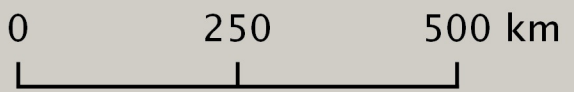
Typhlocaris salentina

MEDITERRANEAN SEA

Typhlocaris lethaea

Typhlocaris galilea

Typhlocaris ayyaloni



17.0°E

22.0°E

27.0°E

32.0°E

Figure 2 (on next page)

Schemes describing the two hypotheses of development of the disjunct distribution of *Typhlocaris*.

H1: the peri-Mediterranean transgression scenario. H2: tectonic isolation of the eastern Galilee from the Mediterranean followed by stranding to the coastal aquifers by ingressions.

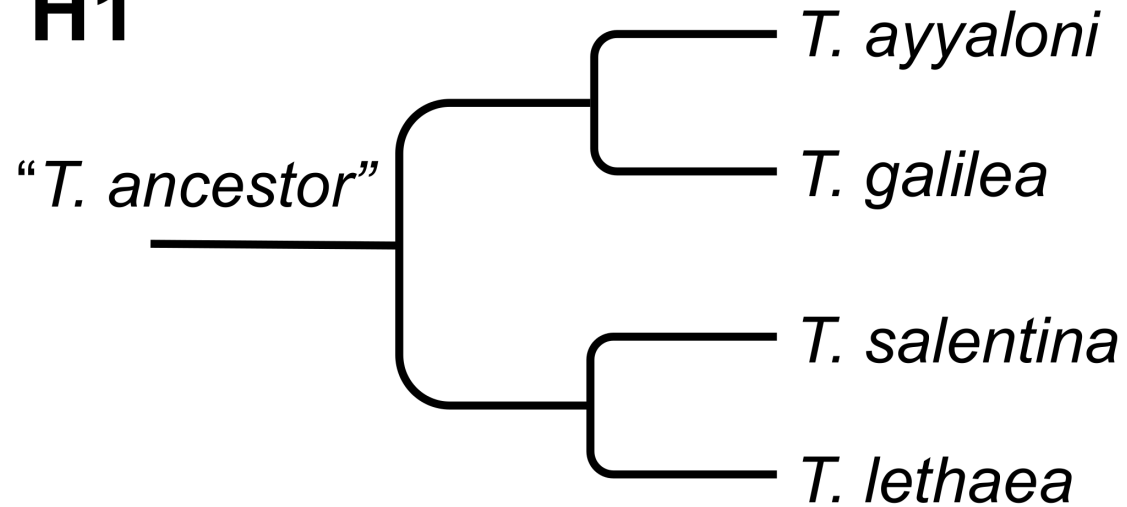
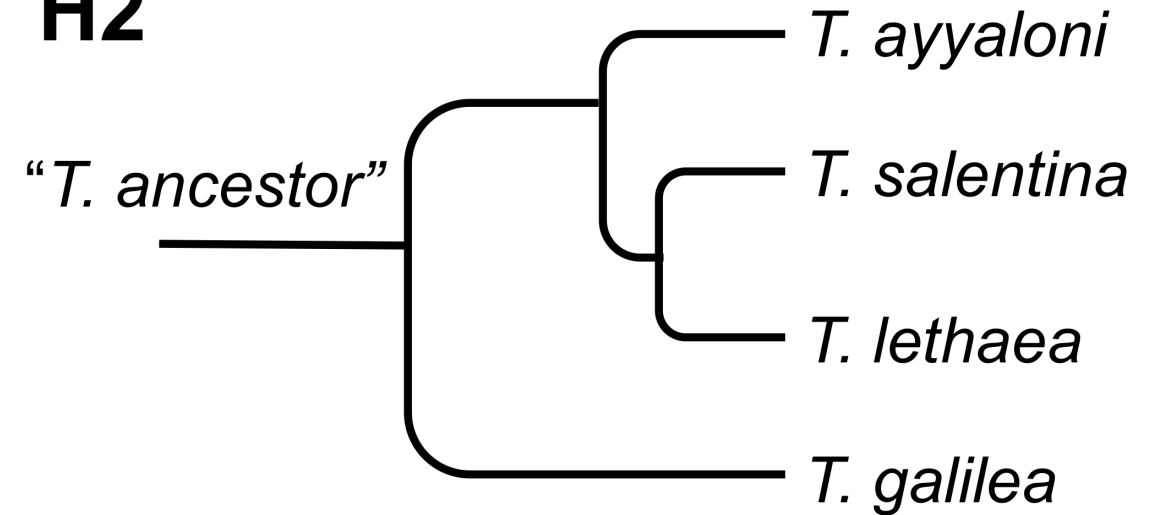
H1**H2**

Figure 3(on next page)

Multi-locus Maximum Likelihood tree of the genus *Typhlocaris*, based on combined 12S rRNA + 16S rRNA + COI + 18S rRNA + 28S rRNA + ITS + H3 genes (total 7761 bp).

At each node, the number above the branch indicates the percentage of ML bootstrap support (1000 replicates) from RAxML analysis with the GTRCAT model of evolution. The number below the branch at each node indicates the Bayesian posterior probability expressed as a decimal fraction for nodes that received at least 50% support in at least one analysis. The scale bar denotes the estimated number of nucleotide substitutions per site.

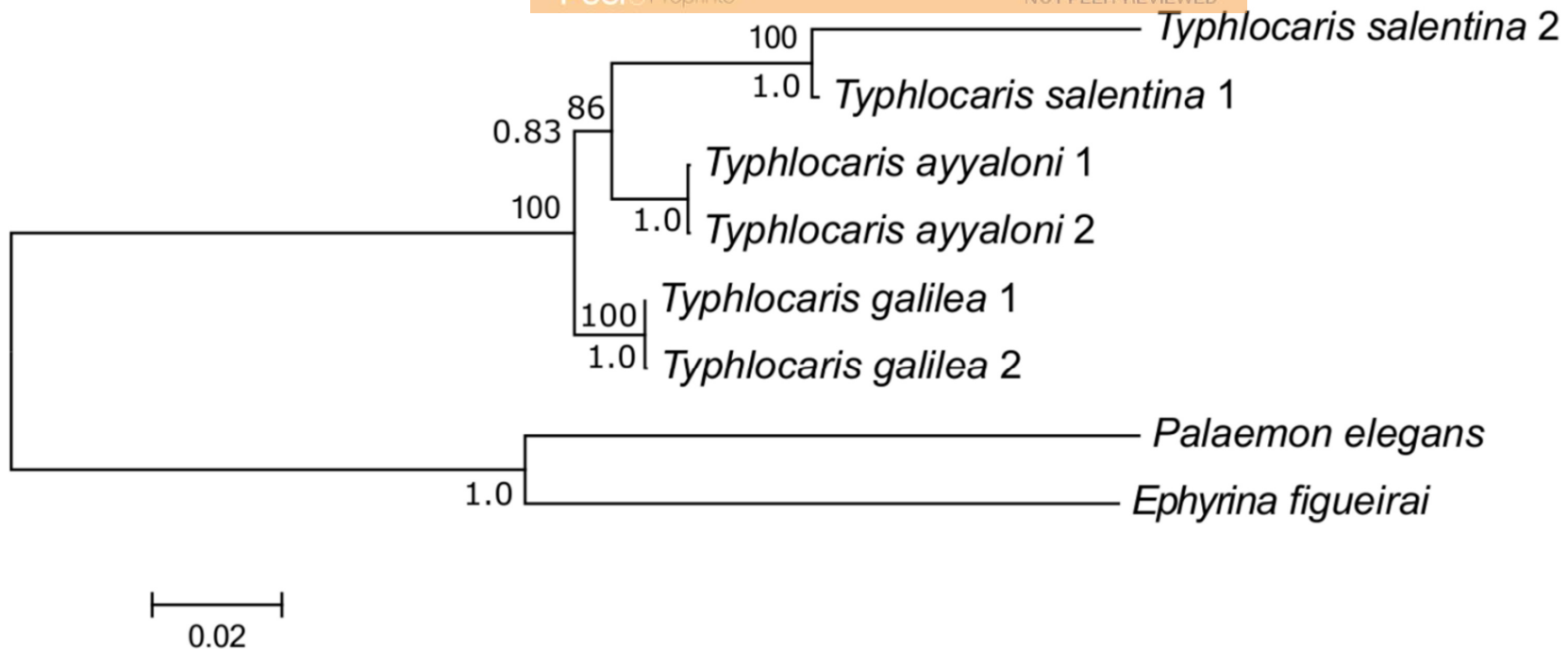


Table 1 (on next page)

Nucleotide analysis and substitution models selected (out of 24 candidate models) for all the genes/partitions with the corrected Akaike Information Criterion and the Bayesian Information Criterion

.

1

Partition	Length (bp)	Informative Positions	Variable Positions	Model	Nst-rates	BIC	AICc
12S	394	161	236	T92+G	6 - Gamma	2572	2465
16S	972	160	221	HKY+G	2 - Gamma	4179	3062
COI	663	254	286	GTR+G+I	6 - Gamma	5366	5008
18S	1914	263	342	K2+G	2 - Gamma	6750	6640
28S	2059	306	659	T92+G	6 - Gamma	5194	5117
ITS	1795	612	1523	T92+G	6 - Gamma	4185	4014
H3	358	50	97	K2+G	2 - Gamma	1736	1572

2

Table 2 (on next page)

Divergence times (and 95% CI) for *Typhlocaris* species as estimated using Bayesian evolutionary analysis method based on COI and 16S genes and calibrated based on Bira formation.

1

Clade divergence	Calibration node	Gene	Node age (Myr) [range]	Posterior probability
<i>Typhlocaris</i>	-	COI	25.3 [20.1-26.4]	0.48
	-	16S	40.9 [35.3-47.5]	1.00
<i>(T. ayyaloni + T. salentina) - T. galilea</i>	7.0 (Bira)	COI	7.0 [5.7-8.5]	1.00
		16S	7.0 [4.9-9.2]	1.00
<i>T. ayyaloni - T. salentina</i>	-	COI	6.0 [4.5-7.2]	0.76
	-	16S	5.6 [3.4-7.3]	0.76

2

Table 3(on next page)

Comparison between the COI and 16S molecular evolution rates estimated in this and previous crustacean studies.

^[1] this study, ^[2] Knowlton & Weigt (1998), ^[3] Page et al. (2008), ^[4] Schubart et al. (1998), ^[5] Sturmbauer *et al.* (1996), ^[6] Ketmaier et al. (2003) , ^[7] Craft et al. (2008).

1

Gene	Stygofauna		Non-Stygofauna	
	Species	Substitutions /Myr	Species	Substitutions /Myr
COI mtRNA	<i>Typhlocaris</i> spp. ^[1]	0.0074	<i>Alpheus</i> spp. ^[2]	0.0140
	<i>Stygiocaris</i> spp. ^[3]	0.0133-0.0516	<i>Halocaridina</i> spp. ^[7]	0.2000
	<i>Stenasellus</i> spp. ^[6]	0.0125		
16S rRNA	<i>Typhlocaris</i> spp. ^[1]	0.0041	<i>Sesarma</i> spp. ^[4]	0.0065
	<i>Stygiocaris</i> spp. ^[3]	0.0055-0.0103	<i>Uca</i> spp. ^[5]	0.0090

2

3