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

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**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 *Typlocaris* 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 sister species, both sister to *T. galilea* (Israel). The divergence time of *T. ayyaloni* and *T. salentina* from *T. galilea* was 5.7 [4.4-6.9] Ma according to COI, and 5.8 [3.5-7.2] Ma according to 16S, based on Bira calibration. The computed interspecific evolutionary rates were 0.0077 substitutions/Myr for COI, and 0.0046 substitutions/Myr for 16S.

**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 water bodies inhabited by *Typhlocaris*.

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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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21    **ABSTRACT**

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23    of endemism and small range, shaped by vicariance, limited dispersal, and evolutionary rates.

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25    and identified the geological and evolutionary processes that have shaped its divergence pattern.

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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.

32    **Results.** The multi-locus ML/Bayesian trees of the concatenated seven gene sequences showed

33    that *T. salentina* (Italy) and *T. ayyaloni* (Israel) are sister species, both sister to *T. galilea* (Israel).

34    The divergence time of *T. ayyaloni* and *T. salentina* from *T. galilea* was  5.7 [4.4-6.9] Ma

35    according to COI, and 5.8 [3.5-7.2] Ma according to 16S, based on Bira calibration. The

36    computed interspecific evolutionary rates were 0.0077 substitutions/Myr for COI, and 0.0046

37    substitutions/Myr for 16S.

38    **Discussion.** Two consecutive vicariant events have shaped the phylogeographic patterns of

39    *Typhlocaris* species. First, *T. galilea* was tectonically isolated from its siblings in the

40    Mediterranean Sea by the arching uplift of the central mountain range of Israel ca. 7 Ma.

41    Secondly, *T. ayyaloni* and *T. salentina* were stranded and separated by a marine transgression ca.

42    6 Ma, occurring just before the Messinian Salinity Crisis. Our estimated molecular evolution

43    rates were in one order of magnitude lower than the rates of closely related crustaceans, as well

44    as of other stygobiont species. We suggest that this slow evolution reflects the ecological

45    conditions prevailing in the highly isolated subterranean water bodies inhabited by *Typhlocaris*.

46

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 water body, 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 (Por et al.

58    2013). Among them are at least four crustaceans, found in sites located along the Dead Sea Rift

59    valley with congeneric taxa found in the Mediterranean coastal plain and even in brackish

60    groundwater in the south of Israel. These obligate stygobionts are regarded as relicts of extinct

61    marine fauna of ancient Mediterranean transgressions (Por 1963). The most prominent members

62    of this faunal assemblage are the large blind prawns of the genus *Typhlocaris*.  Four species of

63    this genus are known from four localities around the east Mediterranean Sea (*Figure 1*). Each

64    locality is inhabited by a different species with no congenerics in the open sea. Two species are

65    known from Israel: *T. galilea* (Calman 1909) from the Tabgha spring on Lake Kinneret

66    shore, and the recently discovered *T. ayyaloni* (Tsurnamal 2008), found in the karstic

67    underground basin near Ramla, named Ayyalon cave, about 200 km south of Tabgha. The third

68    species - *T. salentina* Caroli, 1923 was described from the Zinzulusa cave in Southern Italy and

69    was recently found in other two caves in southern Italy (Froglia & Ungaro 2001). The fourth

70    species, *T. lethaea* Parisi, 1921 is known from Libya near Benghazi. In the IUCN Red List of

71    Threatened Species, *T. galilea* and *T. ayyaloni* are defined as endangered, and *T. salentina* as

72    vulnerable. No data later than 1960 on *T. lethaea* is available (De Grave 2013).

73    The ancestor of *Typhlocaris* ("T. ancestor") and the other marine taxa survived the regression of

74    the Mediterranean Sea that occurred during the Messinian Salinity Crisis (MSC), 5.96 to 5.33

75    Ma, in caves and groundwater basins. Most probably, they were extirpated from the

76    Mediterranean Sea waters when the Mediterranean desiccated and transformed to small

77    hypersaline basins. During this crisis, the Mediterranean Sea lost almost all its Miocene tropical

78    fauna, including those able to colonize subterranean waters (Por 1975; Por 1986; Por &

79    Dimentman 2006). Therefore, the stranding of the *Typhlocaris* species and the separation from

80    their common ancestor have likely preceded the 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, during the Zanclean (5.3-3.6 Ma). The

84    timing of this scenario contradicts the pre-MSC stranding described above. According to Por

85    (1963), the *Typhlocaris* species expanded their distribution into the Jordan valley when it was

86    submerged for a brief period during the Zanclean marine transgression. The coastal plain was

87    also submerged by this transgression that possibly also covered a part of the south of Israel (Por

88    1963). Those faunal elements were left behind when the shore has retreated during the regression

89    that followed the transgression in the early Pliocene. Similarly, Horowitz (2001) suggested that

90    during the Pliocene, two successive transgressive cycles have occurred in the Zanclean and the

91    Piacenzian, separated by a regression. Thus, according to this scenario, *T. galilea* and *T. ayyaloni*

92    were separated together or at successive events from the Mediterranean fauna, and are thus sister

93    taxa (H1, *Figure 2*).

94    A recent study of the eastern Galilee (Rozenbaum et al. 2016) suggests a second scenario (H2,

95    *Figure 2*). The marine transgression into the Dead Sea valley, bringing along *T. galilea*, was

96    associated with a subsidence of the eastern Galilee. The Dead Sea rift valley, accommodating

97    several water bodies, became tectonically isolated from the Mediterranean by the arching uplift

98    of the central mountain range of Israel. This uplift also divided the groundwater basins of the

99    Dead Sea basin from those associated with the Mediterranean, thus resulting in an earlier

100   divergence of *T. galilea* than the divergence of its sister species. Contrastingly, the other three

101   *Typhlocaris* species were found in coastal to inland aquifers that are not isolated from the

102   Mediterranean by a tectonic barrier. They could be stranded in the coastal aquifers by a

103   regression that was not necessarily associated with a tectonic event. This scenario (H2) is

104   supported by the finding of marine macrofossils within the late Miocene Bira Formation of the

105   SE Galilee-Jordan valley indicating its association with a marine transgression (Shaked-Gelband

106  et al. 2014). Ar-Ar dates of volcanics interbedded within the Bira Formation show that the

107  earliest marine invasion into the SE Galilee-Jordan valley happened between 11 and 10 Ma

108  (Rozenbaum et al. 2016; for earlier dating see Shaliv 1989). Ongoing subsidence of the SE

109  Galilee basin, coupled with rising sea level, resulted in the invasion of the Mediterranean water

110  and establishment of a seaway that connected it to the evolving Dead Sea Rift in the east, as

111  represented by parts of the Bira Formation. Seawater could have flowed to the SE Galilee basin

112  either due to global sea level rise above the low barrier near the coastline or due to tectonic

113  subsidence of the Yizre'el Valley which had already started to develop. The detachment of this

114  region from the Mediterranean occurred ca. 7Ma, when the Mediterranean Sea level started

115  falling during the Messinian, followed by freshwaters gradually replacing the saline waters of the

116  Bira lagoon. Thus, the main marine ingression is constrained to the Tortonian, prior to the MSC.

117  Further to the NE, within the Hula valley, Syria and Lebanon, there is no indication of this

118  marine transgression, demonstrating that the marine water came from the Mediterranean and not

119  from the NE (Rozenbaum et al. 2016). This is consistent with the circum-Mediterranean

120  distribution of the four *Typhlocaris* species.

121  The main objectives of our study were: (1) to reveal the phylogenetic relationships of the

122  *Typhlocaris* species, and to use these patterns to (2) infer the geological and evolutionary

123  processes that have shaped their divergence patterns.

124  **MATERIALS & METHODS**

125  *Species sampling, genes and outgroup selection*

126  Specimens of *T. galilea* were collected by us, in the covered pool collecting the water of Tabgha

127  spring (32°52′20″N 35°33′00″E) on Lake Kinneret shore (NPA permit 37920). *T. ayyaloni* was

128  collected from the underground groundwater pond in Ayyalon cave (31°54′37″N 34°55′39″E),

129  two specimens of *T. salentina* were provided by Dr. G. Messana Firenze – Italy from two caves

130  in the vicinity of Bari, Italy, Lu Bissu cave (39°59'42"N 15°57'58"E) and Mola di Bari cave

131  (41°03'36"N 17°05'24"E). All samples were fixed and stored in 95% ethanol at -20°C until

132  DNA extraction. The locality of the fourth species, *T. lethaea*, is restricted to Lete Cave, near

133  Benghazi, Libya, and is not accessible. The two specimens of *T. lethaea*, collected by Parisi a

134    century ago (1921), and stored in the Museum National d'Histoire Naturelle, Paris, did not yield

135    DNA.

136    *DNA extraction, amplification and sequencing*

137    DNA was extracted using Macherey–Nagel genomic DNA isolation kit (Düren, Germany),

138    following the manufacturer's recommended protocol. The primers used for gene amplification

139    are detailed in the Supplemental Information, including both primers from former studies and

140    newly designed primers for this study (*Table S1*). REDTaq ReadyMix R2523 (Sigma-Aldrich,

141    St. Louis, MO) was used for sequence amplification by PCR (Saiki et al. 1988). Amplification

142    was carried out in a personal combi-thermocycler (Biometra, Germany) according to the profiles

143    listed in *Table S1*. PCR products were purified by centrifugation using a High Pure PCR product

144    purification kit (Roche Diagnostics GmbH, Mannheim, Germany) or by Mclab laboratories (San

145    Francisco, California). PCR products were sequenced on both strands using an ABI PRISM 3100

146    Genetic Analyzer (Applied Biosystems) by McLab laboratories (San Francisco, US).

147    Three mitochondrial genes (12S rRNA; 16S rRNA; Cytochrom oxygnese subunit 1 (*COI*)) and

148    four nuclear genes (18S rRNA; 28S rRNA, Internal transcribed spacer (*ITS*); Histon 3 (*H3*))

149    were chosen for analysis. For phylogenetic inference of all seven gene partitions, we used

150    *Ephyrina figueirai* Crosnier & Forest, 1973 (family: Acanthephyridae), and *Palaemon*

151    *elegans* Rathke, 1837 (family: Palaemonidae), as outgroup species that belong to the Caridea, the

152    same infraorder of *Typhlocaris*, because sequences of the seven genes used in our analysis were

153    available in GenBank. Considering that both *Palaemon* and *Typhlocaris* belong to the same

154    superfamily (Palaemonoidea), and since Palaemonoidea is paraphyletic (Kou et al. 2013), *E.*

155    *figueirai* was chosen as a root node.

156    The *Typhlocaris* sequences were deposited in the GenBank under accession numbers

157    KY593415-KY593454. In addition to the newly generated sequences, two sequences of *T.*

158    *salentina* were obtained from GenBank and included in the molecular analysis. The list of taxa,

159    localities and GenBank accession numbers included in the analysis is detailed in the

160    Supplemental Information (*Table S2*).

161    *Phylogenetic analyses*

162    Sequence alignment was conducted using ClustalX embedded in MEGA v6.0 (Tamura et al.

163    2013).The sequences were concatenated to form a multi-gene matrix using Geneious v7.1 (http://

164    www.geneious.com/), including the three *Typhlocaris* sequences and two outgroups, delimited

165    into seven partitions, one for each gene. MEGA v6.0 (Tamura et al. 2013) was used in order to

166    select the best fitting substitution model for each partition according to the Bayesian Information

167    Criterion (*Table 1*).

168    Maximum likelihood analysis of the aligned partitions was conducted using RAxML v8.2.9

169    (Stamatakis 2014) on XSEDE server in the CIPRES Science Gateway portal (Miller et al. 2010)

170    using a GTRCAT model of evolution with 50 rate categories with 1000 bootstrapping replicates.

171    Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMC) analyses were conducted

172    with MrBayes v3.2 on XSEDE with GTR model (Ronquist et al. 2012). Search was conducted

173    with four chains (three cold, one hot) with trees sampled every 100 generations. Three 100

174    generations analyses were conducted to verify likelihood convergence and burn-in parameter.

175    *Estimation of evolutionary rates*

176    Since the molecular clock calculations for cave-dwelling species are often contentious (Page et

177    al. 2008), we used multiple genes and a relaxed molecular clock approach (Drummond et al.

178    2006). To estimate the divergence time of *Typhlocaris* species, we first performed analyses

179    based on accepted molecular evolution rates of the mitochondrial genes COI and 16S rRNA for

180    crustaceans: 0.0140 nucleotide substitutions per Myr (Knowlton & Weigt 1998), and 0.0090

181    substitutions per Myr (Sturmbauer et al. 1996). As an alternative approach, we used a calibrated

182    tree based on a regional geological event. A similar approach was applied by Bauzà-Ribot et al.

183    (2012) that used two paleogeographic events as a calibration point to establish the divergence

184    pattern of the stygobiont family Metacrangonyctidae (Amphipoda). The top of Bira formation,

185    dated to 7 Ma (Rozenbaum et al. 2016), marks the end of the marine connection between the

186    Mediterranean and the Dead Sea valley. Therefore we assume that this event indicates the

187    isolation of *T. galilea* from its sister taxa, and we used it as a calibration node. Using Bira

188    formation as a calibration node, solely allowed the estimation of the divergence time of the sister

189    species, *T. ayyaloni* and *T. salentina*, and thus infer the geological event that led to this

190    separation.

191    Bayesian evolutionary analysis was used to obtain the evolutionary rates of COI and 16S genes

192    under the favored tree topology, based on the ML analysis. A relaxed-clock MCMC approach

193    using the uncorrelated log-normal model was implemented in BEAST v2.4 (Drummond &

194    Bouckaert 2015) on XSEDE server in the CIPRES Science Gateway portal (Miller et al. 2010),

195    using 10 million generations, and sampling every 1000th generation. Models of sequence

196    evolution for each gene were determined using the corrected Akaike information criterion in

197    JModelTest v2.1 (Darriba & Posada 2014, *Table 2*) on XSEDE server. The Yule process was

198    chosen as speciation process for both genes. Log files were analyzed with Tracer v1.6 (Rambaut

199    et al. 2015), to assess convergence and confirm that the combined effective sample sizes for all

200    parameters were larger than 200, in order to ensure that the MCMC chain had run long enough to

201    get a valid estimate of the parameters (Drummond & Rambaut 2007). All resulting trees were

202    then combined with LogCombiner v1.8.2, with a burn-in of 25%. A maximum credibility tree

203    was then produced using TreeAnnotator v2.1.2 (Rambaut & Drummond 2015).

204    **RESULTS**

205    The concatenated alignment of the seven genes was 7761 bp long, out of which 1645 were

206    parsimonious informative. The substitution models selected for all the genes/partitions with the

207    corrected Akaike Information Criterion and the Bayesian Information Criterion scores is

208    presented in *Table 1*. *Figure 3* presents a maximum likelihood (ML) tree of the concatenated

209    sequences, showing that *T. salentina* and *T. ayyaloni* are more closely related to each other than

210    either of them is to *T. galilea*. Out of the seven genes used for the analysis, five gene sequences

211    (ITS, 28S, COI, 12S, 16S) presented this topology. The remaining gene trees, of 18S and H3,

212    had slightly different topology. However, the bootstrap support of the nodes connecting

213    *Typhlocaris* species in these two trees was less than 50%. The topology of the five gene

214    phylogenetic tree supports the hypothesis suggesting that *T. galilea* was separated from its

215    presumed marine ancestor earlier than the separation of *T. ayyaloni* and *T. salentina* (H2, *Figure*

216    *2*).

217    Using the common evolutionary rates for crustacean COI and 16S genes, 0.0140 and 0.0090

218    substitutions/Myr, respectively (Knowlton & Weigt 1998; Sturmbauer et al. 1996), the

219    divergence time estimations for *T. galilea* and *T. salentina-T.ayyaloni* clade were 3.7 [3.0-4.5]

220 and 3.3 (2.3-4.2) Ma, respectively (means [95% highest probability density intervals]). The

221 divergence time between *T. ayyaloni* and *T. salentina* was estimated as 3.2 (2.4-3.8) Ma

222 according to COI and as 2.6 (1.6-3.4) according to 16S (*Table 2*). These estimations suggest that

223 the divergence of *Typhlocaris* species has happened two million years after the Zanclean

224 reflooding of the Mediterranean Sea, thus under no apparent vicariant conditions.

225 Using 7 Ma as the detachment time that isolated *T. galilea* from the Mediterranean Sea (top Bira

226 formation), the divergence time of *T. ayyaloni* and *T. salentina* was according to COI gene – 6.0

227 [4.5-7.2] Ma and according to the 16S gene – 5.9 [3.6-7.4] Ma (*Table 2*), suggesting that these

228 are relicts of the last high level of the Mediterranean Sea before the MSC. The computed

229 evolutionary rates for COI – 0.0077 substitutions/Myr and for 16S – 0.0046 substitutions/Myr,

230 are notably lower than the molecular clock rates found in previous crustacean studies (*Table 3*).

231 The evolutionary rates of ITS, 28S, and 12S were 0.0104, 0.0184, 0.0115 substitutions/Myr,

232 respectively.

## DISCUSSION

234 Marine regressions are the most significant vicariant events forming physical barriers and

235 structuring stygoboint speciation (Boutin & Coineau 2012; Culver et al. 2009; Notenboom 1991;

236 Porter 2007; Stock 1993). Other influential vicariant events include uplilft of mountain ridges

237 (Bauzà-Ribot et al. 2012; Humphreys & Danielopol 2005; Reid et al. 2002), and events that

238 destroy or close off aquatic dispersal corridors (Barr & Holsinger 1985; Holsinger 2012). Using

239 molecular techniques, we established the phylogeny of *Typhlocaris* species, and showed that *T.*

240 *salentina* (Italy) and *T. ayyaloni* (Israel) are sister species, both sister to *T. galilea* (Israel). These

241 phylogeographic relationships indicated that more than one vicariant event have shaped the

242 speciation pattern of *Typhlocaris*. First, *T. galilea* was tectonically isolated from the

243 Mediterranean Sea by the arching uplift of the central mountain range of Israel, ~7 Ma (Matmon

244 et al. 2003; Wdowinski & Zilberman 1997). Later, *T. ayyaloni* and *T. salentina* were stranded

245 and separated by a marine regression ~6 Ma, as a result of the Messinian Salinity Crisis.

246 The fourth *Typhlocaris* species, *T. lethaea*, was missing from our analysis due to the

247 inaccessibility of Lete Cave, Libya, where it is found. Hypothetically, adding *T. lethaea* to the

248    phylogenetic analysis, could have resulted in a modified tree topology, and potentially, in

249    different scenario of speciation (e.g., finding that *T. galilea* and *T. lethaea* are sister species will

250    compel a modification of the inferred speciation model). The long branch of one of the *T.*

251    *salentina* specimens likely reflects the difference between populations originating in different

252    cave systems in southern Italy, where the samples were collected (Lu Bissu and Mola di Bari

253    caves). Both the effect of *T. lethaea* on the phylogeographic pattern of *Typhlocaris*, and the

254    population genetics of *T. salentina* in the caves and wells of Salento and southern Murge karst

255    systems, warrant each for an independent study.

256    Commonly, the final closure of the Isthmus of Panama that has occurred approximately 3 Ma

257    (Coates et al. 1992; Keigwin 1982; Keigwin 1978; O'Dea et al. 2016) is used for estimation and

258    calibration of divergence time of crustaceans. Knowlton and Weigt (1998) and Williams *et al.*

259    (2001) found that the substitution rate of COI is 0.0140 per Myr. This finding is based on the

260    pairs of transisthmian snapping shrimp *Alpheus* from Panama: *A. estuarensis – A. colombiensis*,

261    and *A. nepenulitimus – A. chacei.* Schubart *et al.* (1998) calibrated the substitution rate of 16S

262    rDNA using trans-isthmian pairs of crabs of the genus *Sesarma* (Grapsidae) and then used this

263    rate to estimate a date for the origin of the Jamaican lineage *Sesarma,* the substitution rate of

264    *Sesarma* was 0.0065 per Myr. Sturmbauer *et al.* (1996) used the same gene from populations of

265    the fiddler crab *Uca vocator,* from either side of the Isthmus of Panama to estimate divergences

266    rates of *Uca*. The sequence divergence rate was 0.0090 per Myr; this rate was used to estimate

267    the time divergence between clades of terrestrial *Uca* from different parts of the globe.

268    Craft *et al.* (2008) and Page *et al.* (2008) that studied the phylogeography of atyids did not use

269    the rates of transisthmian organisms to calibrate the molecular clock but estimated it

270    independently for the studied taxa. Craft *et al.* (2008) studied *Halocaridina* from the Hawaiian

271    Archipelago. To calibrate the molecular clock, they used the age of the earliest eruption

272    of Kilauea volcano in Hawaii, 50–100 Ka, and the genetic data of the groups of

273    *Halocaridina* that occur along the flank of this volcano. They found an exceptionally

274    high divergence rate of 0.2 per Myr in COI gene of *Halocaridina*. They noted that this rate is

275    in sharp contrast to the commonly utilized evolution rates for arthropods 0.0140-0.0170 per Myr

276    (Williams et al. 2001). Page *et al.* (2008) studied the cave atyids *Stygiocaris* from Cape Range

277    area in Western Australia. It is accepted that the emergence of the Cape Range Anticline in the

278  Miocene isolated *Stygiocaris lancifera* and *S. stylifera*, leading to their speciation, therefore,

279  Page *et al.* (2008) used this event, 7–10 Ma, as a calibration point to estimate rates of molecular

280  divergence. This yielded a wide range of evolutionary rates for the *S. lancifera / stylifera* node:

281  0.0133-0.0516 substitutions/Myr in COI and 0.0055-0.0103 substitutions/Myr in 16S, relatively

282  lower than other atyid studies, but still higher than the rate we found for *Typhlocaris*.

283  Our estimated low evolutionary rates in *Typhlocaris* correspond with the analysis of Zakšek et al.

284  (2009) that studied the phylogeography the cave shrimp *Troglocaris anophthalmus*. To estimate

285  the divergence time they referred to the divergence rate of COI used for transisthmian species of

286  *Alpheus* across the Isthmus of Panama (Knowlton and Weigt, 1998). Zakšek *et al.* (2009),

287  therefore, stated that for *Troglocaris*, the rate calculated by Knowlton and Weigt (1998) can be

288  used only for estimation of the order of magnitude of divergence time because it is the most

289  commonly used rate for decapods. Nonetheless, they found COI patristic distances between

290  phylogroups that are much lower (0.05-0.08) than the accepted patristic COI distance of 0.16

291  substitutions per nucleotide position found to optimally separate intra-from interspecies

292  divergence in other crustaceans (Lefébure et al. 2007).

293  The rates found by us are in one order of magnitude lower than those found for *Alpheus,* the

294  common crustacean used for calibration of divergence time (Knowlton & Weigt 1998), and

295  lower or similar to the rates of other stygobionts (*Table 3*). An exception is the case of the

296  stygobiont amphipod family Metacrangonyctidae, which was shown to undergo rapid evolution

297  using mitochondrial protein-coding genes (Bauzà-Ribot et al. 2012). The average rate estimated

298  by Bauzà-Ribot et al. was 0.1090 substitutions/Myr, one order of magnitude higher than the rates

299  acceptable for other crustaceans. They suggested that this high rate might result from frequent

300  population bottlenecks. Evolutionary rates, even of the same gene, may vary between different

301  genera within the same order –indicating that evolutionary rates are not related only to the

302  taxonomic position but also, or mainly, to ecological conditions. We therefore did not use the

303  previously reported substitution rate but the known geological data of the area where *Typhlocaris*

304  occurs to infer its divergence rate and time. The lower divergence rates found for *Typhlocaris*

305  compared with other crustaceans lead us to the suggestion that the low rates are related to the

306  ecological conditions of the *Typhlocaris* habitat. *Typhlocaris* and other stygobionts are found in

307  isolated subterranean basins where species diversity is very low, relative to the regional diversity

308   (Gibert et al. 2009), reducing interspecific competition. The environmental factors in these

309   habitats are stable, lacking fluctuations. Predators are typically missing in subterranean habitats,

310   resulting in truncated food webs (Gibert & Deharveng 2002). Additionally, evolution rates were

311   correlated with metabolic rates (Martin & Palumbi 1993). Species with low metabolic rates (e.g.,

312   deep-sea fauna) are generally characterized by reduced nucleotide substitution rates. It was

313   hypothesized that limited light reduces visual predation pressure and selects for reduced

314   locomotory ability and metabolic capacity (da Silva et al. 2011). This may be just as well the

315   case of stygobiont evolution. Thus, the combined unique ecological and biological conditions

316   (dark habitat, environmental stability, low richness, lack of interspecific competition) may lead

317   to stability and low rate of gene divergence. This is in agreement with the statement of Mayr

318   (1963) that competition and allopatry are important elements of speciation and evolutionary

319   divergence.

320   Culver (1976) noted that the most striking feature of the organization of Appalachian cave-

321   stream communities is the reduction in intensity of competition. One of the suggested

322   explanations is that, with increasing time in caves, species evolve a life-history strategy of high

323   metabolic efficiency and low reproductive rate, a strategy that may itself reduce interspecific

324   competition. We thus may assume that the higher divergence rates found in non-stygobiont

325   crustaceans are related to competition. The classical taxa used for calibration of molecular dating

326   are the 18 species of *Alpheus* at both sides of the Isthmus of Panama (Knowlton and Weigt,

327   1998). Knowlton (1993) observed aggressive behavior among species including individuals that

328   belong to a nominal species from both sides of the Isthmus of Panama, supporting our

329   assumption on the role of competition in delimiting evolutionary rates.

330   Using evolutionary biology, we can identify processes that promote or maintain phenotypic and

331   genetic diversity in natural populations. This is of a great importance particularly when the

332   studied organisms are under high risk of becoming extinct. While many studies confirmed that

333   interspecific competition and environmental variation drive genetic diversification, there is little

334   phylogeographic evidence linking environmental stability with low genetic variation. Further

335   molecular investigations of stygobionts and other organisms of stable environments will shed

336   light on universality of their temporal mode of speciation.

337

338 **CONCLUSIONS**

339 Our results indicated that two separate vicariant event shaped the distribution patterns of the

340 blind cave-dwelling shrimp *Typhlocaris*. During the late Miocene, *T. galilea* was tectonically

341 isolated from the Mediterranean Sea by the arching uplift of the central mountain range of Israel,

342 ca. 7 Ma. During the Messinian Salinity Crisis, *T. ayyaloni*, geographically adjacent to *T. galilea*,

343 and *T. salentina* were stranded and separated by a marine transgression. A future investigation of

344 the divergence time of *T. lethaea* may shed more light on the transgression events leading to the

345 disjunct phylogeographic pattern of *Typhlocaris*. Furthermore, the evolutionary rates of

346 *Typhlocaris* estimated in this study (0.0077 substitutions/Myr in Cytochrome Oxidase Subunit 1

347 (COI) and 0.0046 substitutions/Myr in 16S rRNA) were in one order of magnitude lower than

348 the rates of closely related crustaceans, and lower than other stygobiont species. These low rates

349 may result from the low predation stress and the low diversity, leading to low interspecific

350 competition, which characterizes the highly isolated subterranean habitats inhabited by

351 *Typhlocaris*.

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506  **FIGURE LEGENDS**

507  **Figure 1.** Distribution map of *Typhlocaris* species (colored in red) based on spatial data from

508  NatureServe and IUCN (International Union for Conservation of Nature). *The IUCN Red List of*

509  *Threatened Species. Version 2014.1.* (http://www.iucnredlist.org). Downloaded on 28 January

510  2018. Map made using Natural Earth data (http://www.naturalearthdata.com).

511  **Figure 2.** Schemes describing the two hypotheses of development of the disjunct distribution of

512  *Typhlocaris*. H1: the peri-Mediterranean transgression scenario. H2: tectonic isolation of the

513  eastern Galilee from the Mediterranean followed by stranding to the coastal aquifers by

514  ingressions.

515  **Figure 3.** Multi-locus Maximum Likelihood tree of the genus Typhlocaris, based on combined

516  12S rRNA + 16S rRNA + COI + 18S rRNA + 28S rRNA + ITS + H3 genes (total 7761 bp). At

517  each node, the number above the branch indicates the percentage of ML bootstrap support (1000

518  replicates) from RAxML analysis with the GTRCAT model of evolution. The number below the

519  branch at each node indicates the Bayesian posterior probability expressed as a decimal fraction

520  for nodes that received at least 50% support in at least one analysis. The scale bar denotes the

521  estimated number of nucleotide substitutions per site.

522  **TABLE LEGENDS**

523  **Table 1.** Nucleotide analysis and substitution models selected (out of 24 candidate models) for

524  all the genes/partitions.

525  **Table 2.** Divergence times (and 95% CI) for Typhlocaris species as estimated using Bayesian

526  evolutionary analysis method calculated using COI and 16S gene molecular evolution rates and

527  using calibration based on Bira formation.

528   **Table 3.** Comparison between the COI and 16S molecular evolution rates estimated in this and

529   previous crustacean studies: [1] this study, [2] Knowlton & Weigt (1998), [3] Page et al. (2008), [4]

530   Schubart et al. (1998), [5] Sturmbauer et al. (1996), [6] Ketmaier et al. (2003), [7] Craft et al.

531   (2008).

532   **SUPPLEMETAL INFORMATION - TABLE LEGENDS**

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

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

535   **DATA ACCESSIBILITY STATEMENT**

536   The authors confirm that all data underlying the findings are fully available without restriction.

537   All DNA sequences generated in this research were deposited in the GenBank. The list of

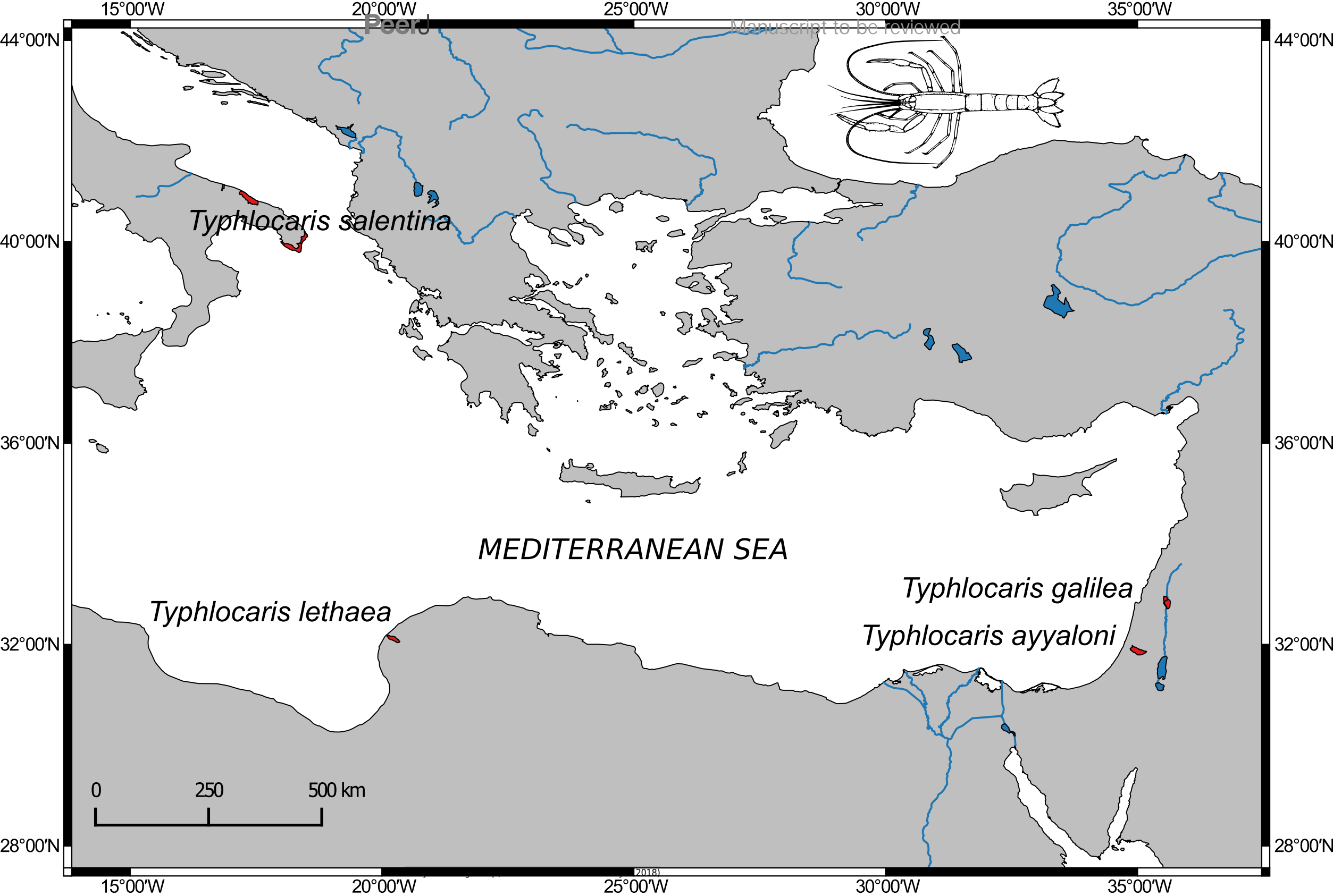
538   primers used and designed for this study and the list of taxa, localities and GenBank accession

539   numbers are detailed in the Supplemental Information (Table S1 and S2, respectively) and will

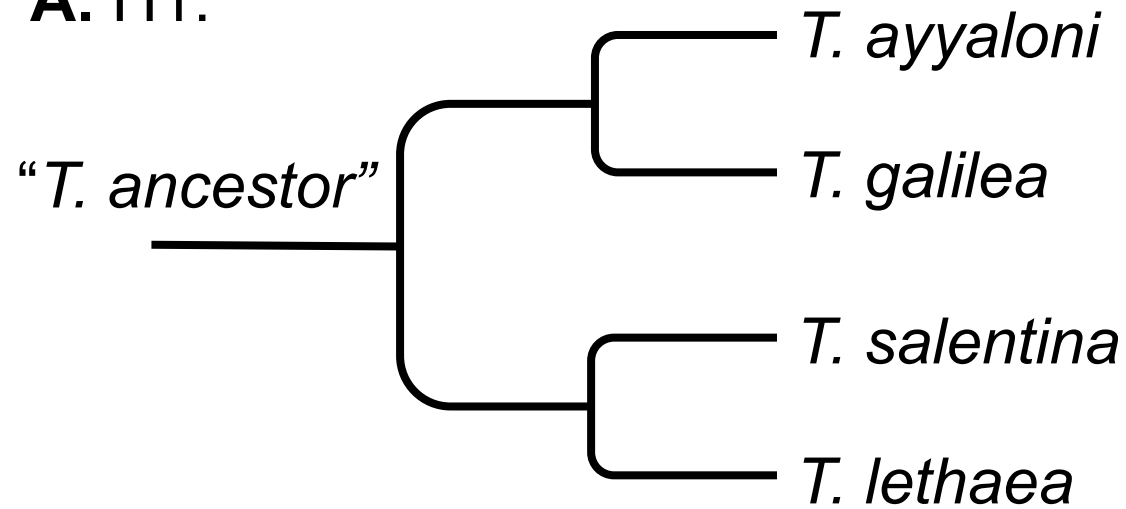
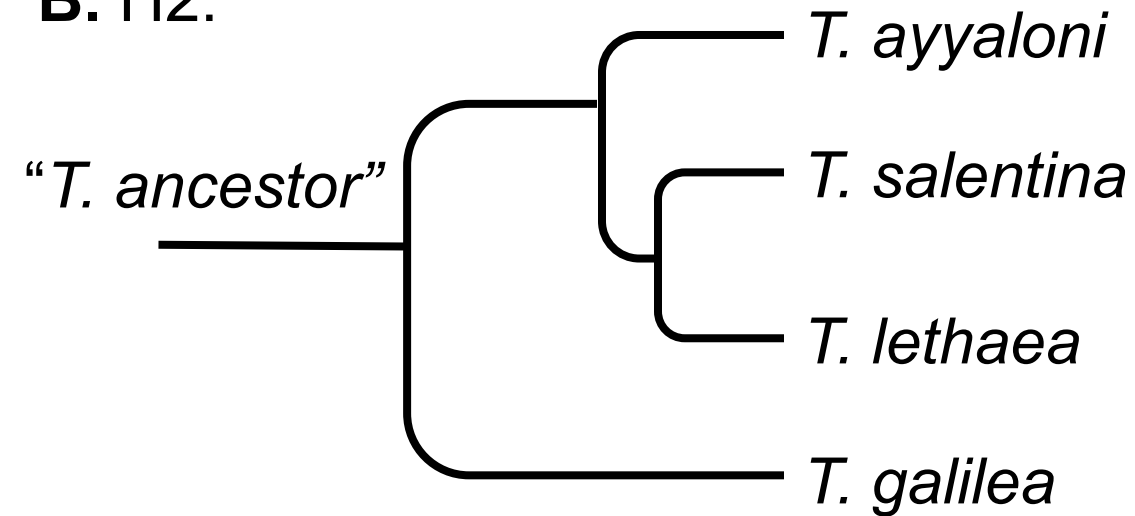
540   be made available in the data repository PANGAEA.

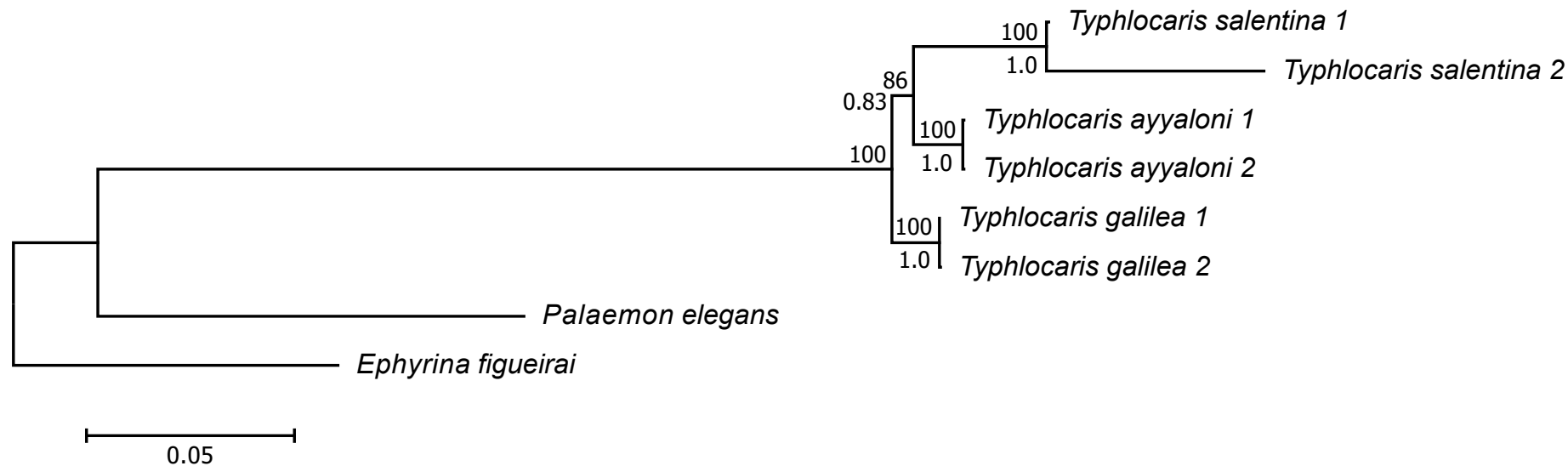
Distribution map of *Typhlocaris* species (colored in red) based on spatial data from NatureServe and IUCN (International Union for Conservation of Nature).

*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).

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

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

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.

Nucleotide analysis and substitution models selected (out of 24 candidate models) for all the genes/partitions.

| Partition | Length (bp) | Informative Positions | Variable Positions | Model | Nst-rates |
|---|---|---|---|---|---|
| 12S | 394 | 161 | 236 | T92+G | 6 - Gamma |
| 16S | 972 | 160 | 221 | HKY+G | 2 - Gamma |
| COI | 663 | 254 | 286 | GTR+G+I | 6 - Gamma |
| 18S | 1914 | 263 | 342 | K2+G | 2 - Gamma |
| 28S | 2059 | 306 | 659 | T92+G | 6 - Gamma |
| ITS | 1795 | 612 | 1523 | T92+G | 6 - Gamma |
| H3 | 358 | 50 | 97 | K2+G | 2 - Gamma |

Divergence times (and 95% CI) for Typhlocaris species as estimated using Bayesian evolutionary analysis method calculated using COI and 16S gene molecular evolution rates and using calibration based on Bira formation.

| Clade divergence | Gene | Node age (Myr) [range] non-calibrated | Calibration node | Node age (Myr) [range] calibrated | Posterior probability |
|---|---|---|---|---|---|
| *Typhlocaris* | COI | 13.4 (10.6-14.0) | – | 19.9 [17.3-22.5] | 0.48 |
| | 16S | 19.1 (16.5-22.2) | | 41.5 [35.8-48.5] | 1.00 |
| (*T. ayyaloni + T. salentina*) - *T. galilea* | COI | 3.7 (3.0-4.5) | 7.0 (Bira) | | 1.00 |
| | 16S | 3.3 (2.3-4.2) | | | 1.00 |
| *T. ayyaloni - T. salentina* | COI | 3.2 (2.4-3.8) | – | 5.7 [4.4-6.9] | 0.76 |
| | 16S | 2.6 (1.6-3.4) | | 5.8 [3.5-7.2] | 0.76 |

3

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).

| Gene | Stygofauna | | Non-Stygofauna | |
|---|---|---|---|---|
| | Species | Substitutions /Myr | Species | Substitutions /Myr |
| COI mtRNA | *Typhlocaris* spp. [1] | 0.0077 | *Alpheus* spp. [2] | 0.0140 |
| | *Stygiocaris* spp. [3] | 0.0133-0.0516 | *Halocaridina* spp. [7] | 0.2000 |
| | *Stenasellus* spp. [6] | 0.0125 | | |
| 16S rRNA | *Typhlocaris* spp. [1] | 0.0046 | *Sesarma* spp. [4] | 0.0065 |
| | *Stygiocaris* spp. [3] | 0.0055-0.0103 | *Uca* spp. [5] | 0.0090 |