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

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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 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*.

TITLE:

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

Short title: Mediterranean stygobiont phylogeography

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

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS & METHODS

Species sampling, genes and outgroup selection

Specimens of *T. galilea* were collected by us, in the covered pool collecting the water of Tabgha spring (32°52′20″N 35°33′00″E) on Lake Kinneret shore (NPA permit 37920). *T. ayyaloni* was collected from the underground groundwater pond in Ayyalon cave (31°54′37″N 34°55′39″E), two specimens of *T. salentina* were provided by Dr. G. Messana Firenze – Italy from two caves in the vicinity of Bari, Italy, Lu Bissu cave (39º59'42"N 15º57'58"E) and Mola di Bari cave (41º03'36"N 17º05'24"E). All samples were fixed and stored in 95% ethanol at -20°C until DNA extraction. The locality of the fourth species, *T. lethaea*, is restricted to Lete Cave, near Benghazi, Libya, and is not accessible. The two specimens of *T. lethaea*, collected by Parisi a century ago (1921), and stored in the Museum National d'Histoire Naturelle, Paris, did not yield DNA.

DNA extraction, amplification and sequencing

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

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

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

Phylogenetic analyses

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

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

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

Divergence time analysis

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

RESULTS

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

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

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

DISCUSSION

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

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

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

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

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

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

The evolutionary rates, even of the same gene, differ in different genera within the same order – indicating that evolutionary rates are not related only to the taxonomic position but also, or mainly, to ecological conditions. We therefore did not use the previously reported substitution rate but the known geological data of the area where *Typhlocaris* occurs. The lower divergence rates found for *Typhlocaris* compared with other crustaceans lead us to the suggestion that the low rates are related to the ecological conditions of the *Thyplocaris* habitat. *Typhlocaris* and other stygobionts are found in isolated subterranean enclosures where species diversity is very low, relative to the regional diversity (Gibert et al. 2009), potentially reducing interspecific competition. The environmental factors in these enclosures are stable, lacking fluctuations. Predators are typically missing in subterranean habitats, resulting in truncated food webs (Gibert & Deharveng 2002). Additionally, evolution rates were correlated with metabolic rates (Martin & 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 the18 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,

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

Table 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).

SUPPLEMETAL INFORMATION - TABLE LEGENDS

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

Table S2. GenBank accession numbers of *Typhlocaris*.

DATA ACCESSIBILITY STATEMENT

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

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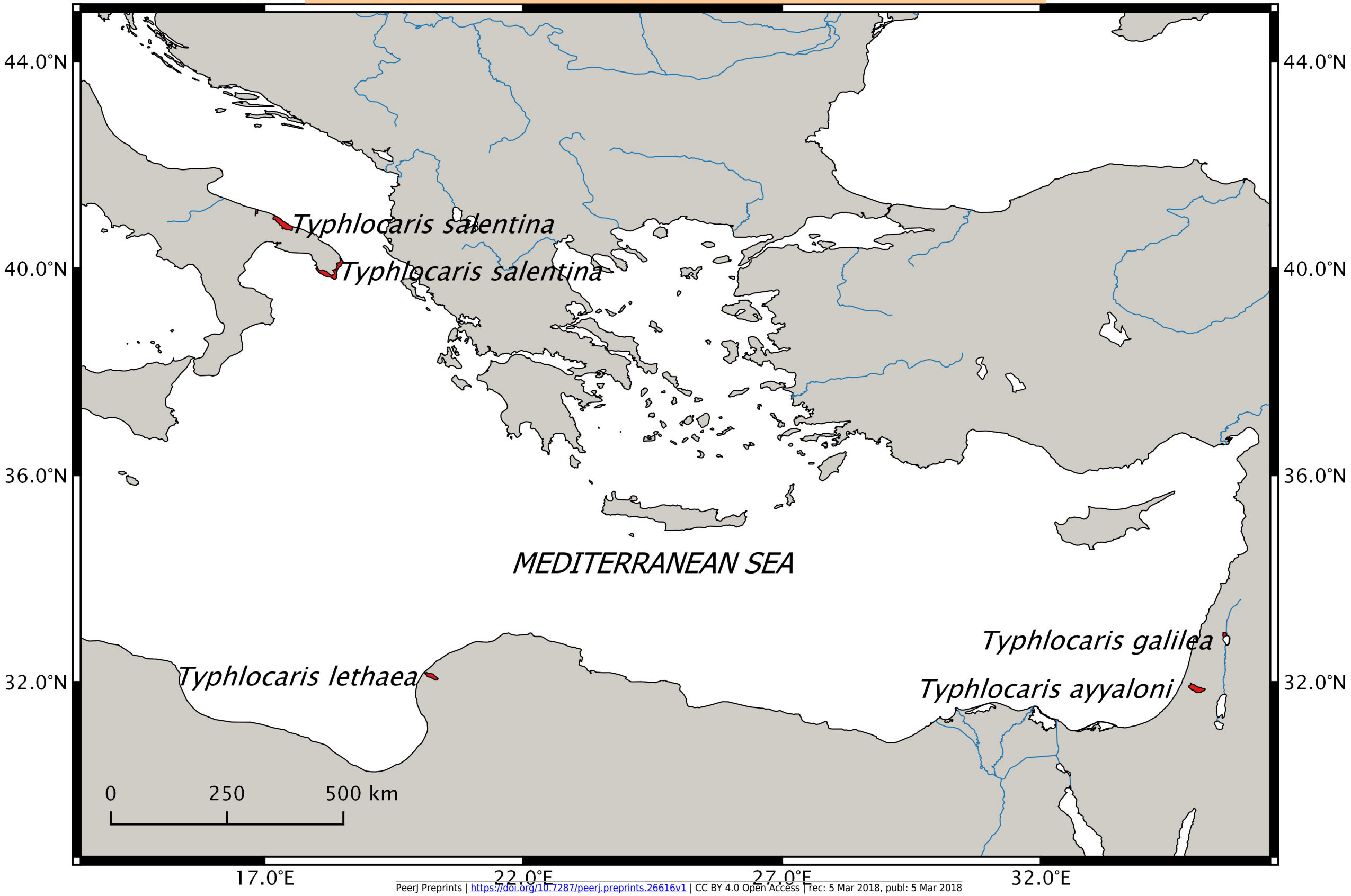

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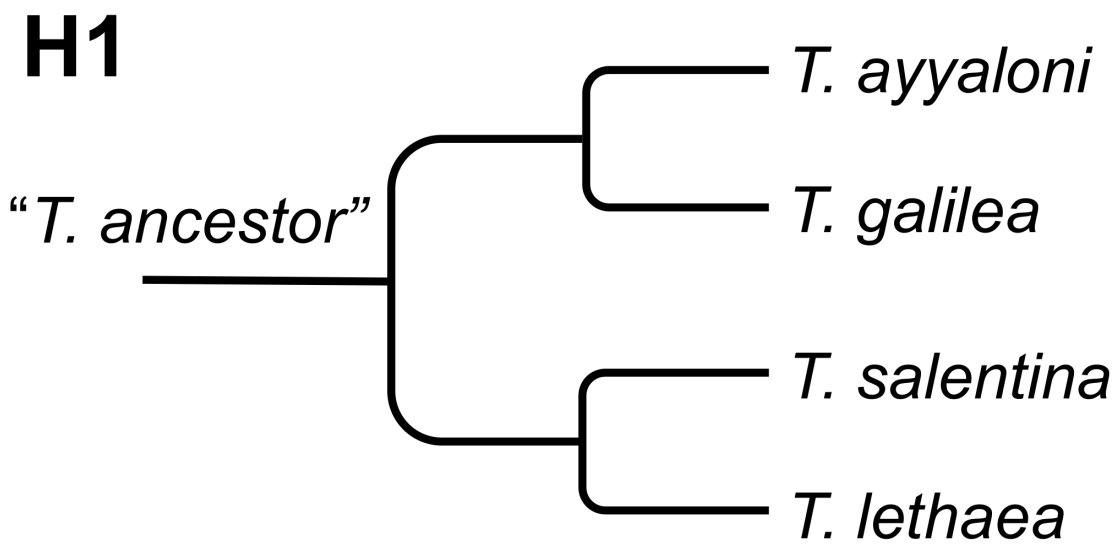
Figure 2(on next page)

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.



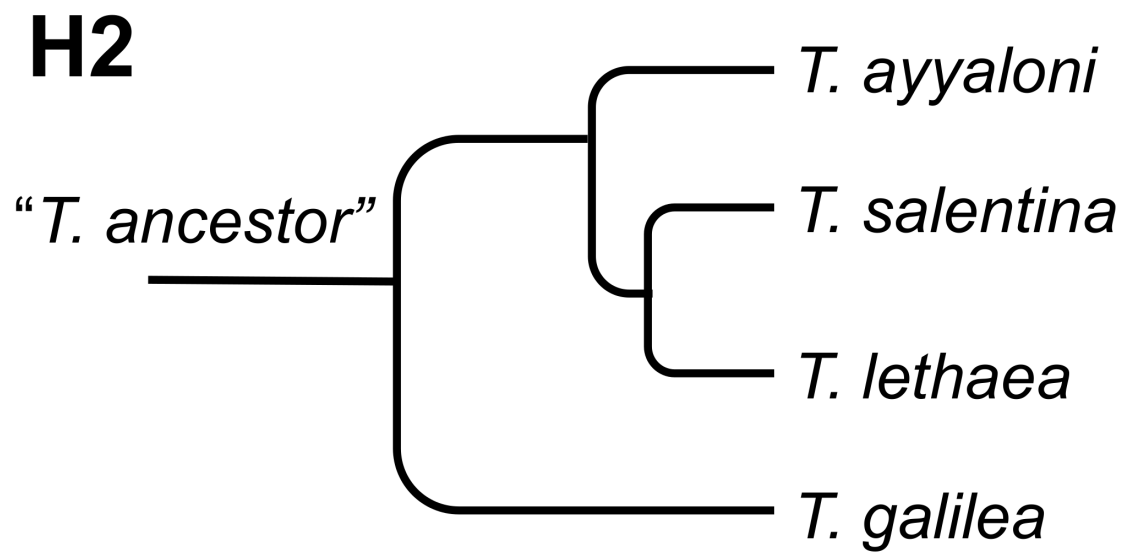


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Figure 3(on next page)

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.

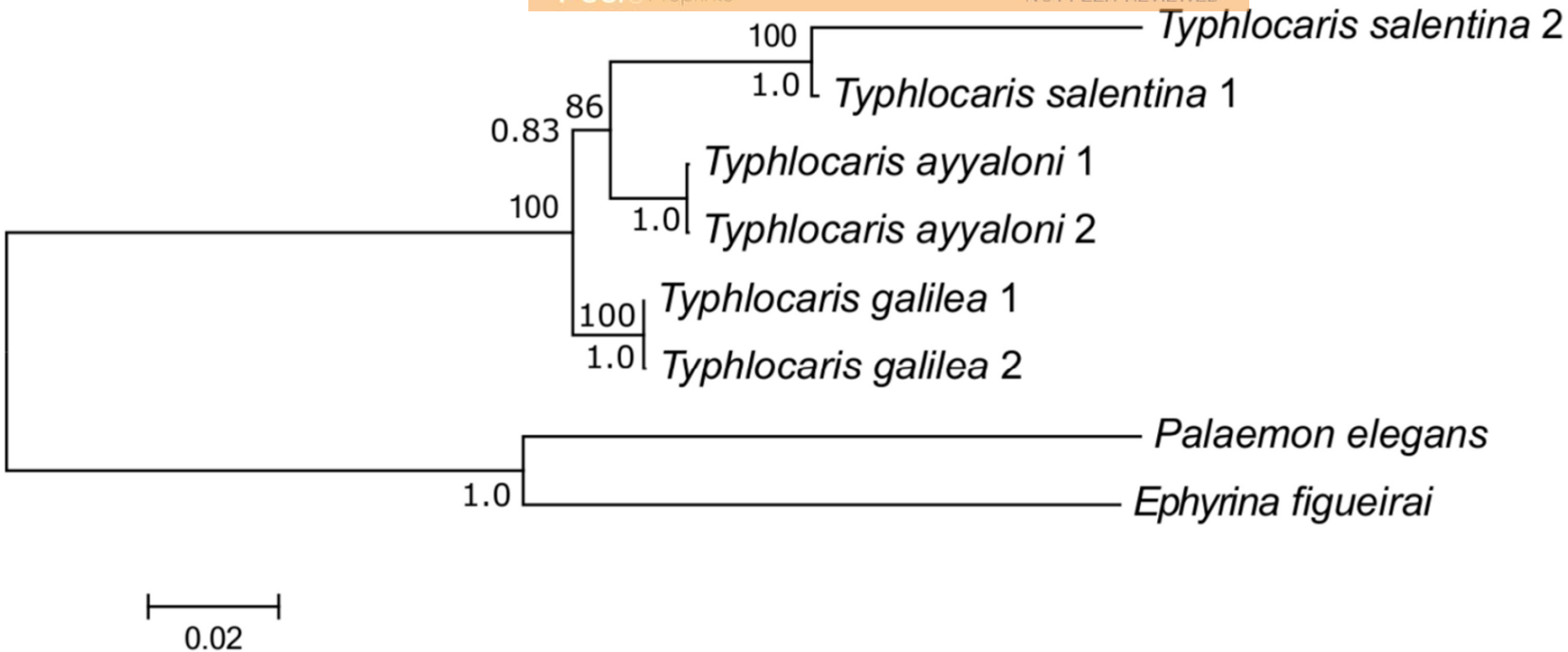

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Table 1(on next page)

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
Typhlocaris	-	COI	25.3 [20.1-26.4]	0.48
	-	16S	40.9 [35.3-47.5]	1.00
(*T. ayyaloni* + *T. salentina*) - *T. galilea*	7.0 (Bira)	COI	7.0 [5.7-8.5]	1.00
		16S	7.0 [4.9-9.2]	1.00
T. ayyaloni - *T. salentina*	-	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	*Typhlocaris* spp. [1]	0.0074	*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]	0.0041	*Sesarma* spp. [4]	0.0065
	Stygiocaris spp. [3]	0.0055-0.0103	*Uca* spp. [5]	0.0090

2

3