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Phylogeny of cave-dwelling atyid shrimp *Troglocaris* in the Dinaric Karst based on sequences of three mitochondrial genes

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Abstract

Background and Purpose: *The main purposes of this study was to revise the current taxonomy of the genus Troglocaris in the Dinarids in the light of molecular phylogenetic results from three major areas of disjunct distribution (Southern France, West Dinarids, and West Caucasus), and additionally to test the subfamily relationships between Paratyinae and Typhlatyinae in the Dinarids.*

Materials and Methods: *This study was performed on populations of the cave-dwelling shrimp Troglocaris from three disjunct areas of distribution: Southern France, Dinaric Karst and Western Caucasus using mitochondrial genes for 16S rRNA, cytochrome oxidase I and cytochrome oxidase II subunits. We combined mitochondrial data from shrimp populations to clarify the evolutionary relationship inside (within) the genus Troglocaris.*

Results: *Our results, based on phylogenetic analysis of three mitochondrial genes from 14 populations of the closely related atyid taxa, do not support the monophyly of the genus Troglocaris. Moreover, new insights were introduced in the Atyidae subfamily status. At subfamily level, a difference in current taxonomy was observed which excluded the genus Spelaeocaris from Typhlatyinae and placed it inside Paratyinae. Additionally the closest relative to the French species Troglocaris inermis appears to be the surface-dwelling shrimp Atyaephyra desmaresti.*

Conclusions: *The separation of the oldest Western Troglocaris lineage from the Dinaro-Caucasian lineages is estimated to have occurred in the Late Miocene. Also DNA sequence data suggest that Troglocaris hercegovinensis from South Herzegovina and Troglocaris kutaissiana complex from Western Caucasus are sister species.*

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INTRODUCTION

The genus *Troglocaris* is the most widespread cave-dwelling decapod in Europe with a disjunct range extended to West Caucasus. In the Dinaric Karst, its distribution encompasses a number of major drainage basins and biogeographical regions characterized by the presence of *Troglocaris* main predator, the European cave salamander *Proteus anguinus anguinus* Laurenti, 1768 *(1)*. Originally, four species were described *(2, 3)*: *Troglocaris anophthalmus* (Kollar 1848), *T. hercegovinensis* (Babić 1922), *T. inermis* Fage, 1937, and *T. kutaissiana* (Sadovsky

1930). The nominal species *T. anophthalmus* from the Dinaric Karst was later treated as three distinct subspecies (*T. anophthalmus anophthalmus* (Kollar 1848), *T. anophthalmus intermedia* Babić, 1922, and *T. anophthalmus planinensis* Birstein, 1948) while *T. kutaissiana* from the West Transcaucasus was listed as five distinct subspecies (*T. kutaissiana kutaissiana, T. kutaissiana ablaskiri* Birstein, 1939, *T. kutaissiana fagei* Birstein, 1939*, T. kutaissiana jusbaschjani* Birstein, 1948*, T. kutaissiana osterloffi* Jusbaschjan, 1940) *(2)*. Mugue *et al. (4)* have added an additional species for West Transcaucasus, *Troglocaris birsteini*. Their short summary contains no information of diagnostic value, and we thus consider the species as *nomen nudum*. *T. hercegovinensis* was originally described as *Troglocaridella hercegovinensis* by Babić *(5)* who originally created that separate genus.

The remarkable discovery of a cave dwelling atyid shrimp was the monotypic *Spelaeocaris pretneri* Matjašič 1956 from south-eastern Herzegovina, which was later transferred to the genus *Typhlatya* by Sanz and Platvoet *(6)*. On the basis of all the data mentioned above it is obvious that the current status of species inside *Troglocaris* genera is not satisfactorily resolved.

Allozyme studies on the diversity of *Troglocaris* populations from Italy suggest that allopatric speciation was followed by secondary sympatric speciation which has promoted the high genetic diversity of the nominal species in the Western Dinaric Karst *(7)*.

In a recent phylogenetic study Zakšek *et al. (8)* showed that the currently recognized taxonomic diversity of *Troglocaris*, which was predicted by Sket *(1)* as the most species-rich cave-dwelling shrimp, is probably lower than the number of distinct lineages on the molecular phylogenetic tree. After all previous studies the current status and the patterns of speciation of the genus *Troglocaris* and *Spelaeocaris* remain to be completely elucidated.

The main purpose of this study was to revise the current taxonomy of the genus *Troglocaris* in the Dinarids in the light of molecular phylogenetic results from three major areas of disjunct distribution (Southern France, West Dinarids, and West Caucasus), and additionally to test the subfamily relationships between Paratyinae and Typhlatyinae in the Dinarids. To gain such results a comprehensive molecular phylogenetic analysis, using different methods of phylogenetic inference, was applied on parts of three mitochondrial genes (16S rRNA, cytochrome oxidase I and cytochrome oxidase II).

MATERIALS AND METHODS

Taxon sampling

Our sampling includes one or more individuals of each of the nominate taxa [*Troglocaris anophthalmus* (Kollar 1848), *T. inermis* Fage 1937, *T. intermedia* Babić, 1922, *T. kutaissiana* (Sadovsky 1930), *T. planinensis* (Birštejn 1950)], originating from different localities throughout their area of distribution (Figure 1). A total of 12 *Troglocaris* populations were analyzed from 11 localities (Table 1). We chose two additional sister taxa, a cave-dwelling shrimp *Spelaeocaris*, currently member of Typhlatyinae *(2, 3)* and an epigean freshwater shrimp *Atyaephyra desmaresti* (Millet 1848), currently a member of Paratyinae *(2, 3)* to verify relationships between Paratyinae and Typhlatyinae. To root our phylogenetic trees two additional taxa were chosen as outgroups in analyses: epigean decapod *Astacus astacus* (Linnaeus 1758) and epigean amphipod *Gammarus balcanicus* (Schäferna 1922).

The specimens were preserved in 96% ethanol and stored at –20°C until extraction of DNA. From the majority of samples all three genes were analyzed. However, some samples failed to amplify for all three genes, possibly due to degraded genomic DNA. Sequences are stored

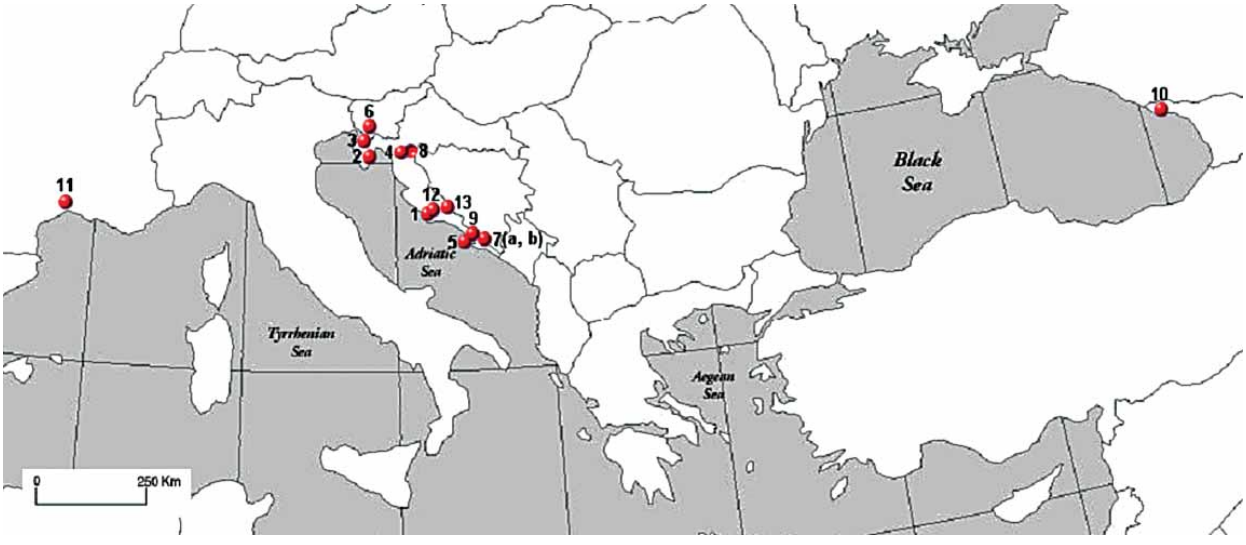

Figure 1. Distribution of analyzed haplotypes. The numbers correspond to the haplotype names presented in Table 1.

TABLE 1

List of taxa with indicated haplotype group, geographical origin and collection numbers (specimen tissue voucher abbreviations) of all taxa sequenced and analyzed in this study along with GenBank accession numbers of sequences used in phylogenetic inference. The numbers in parentheses represent collection localities referred according to the Fig. 1.

Subfamily (current taxonomy)	Lineages	Taxon (current taxonomy)	Geographical origin	Haplotype	Voucher abbreviation	Name of sequence used in phylogenetic inference	GenBank Accesion Number
Paratyinae	»Anophthalmus«	*T. anophthalmus (Kollar, 1948)*	Mandalina špilja, anchihaline cave, Šibenik, HR **(1)**	*Troglocaris n. sp. 1*	A1	A1 16S	DQ320019
Paratyinae	»Anophthalmus«	*T. anophthalmus* (Kollar, 1948)	Tunnel Plomin, spring in tunnel, HR **(2)**	*T. anophthalmus* A	A2	A2 16S A2 COI A2 COII	DQ320020 DQ320036 DQ320052
Paratyinae	»Anophthalmus«	*T. anophthalmus (Kollar, 1948)*	Jama pod Krogom, cave, Mlini, HR-Sl **(3)**	*T. anophthalmus* AN	AN1	AN1 16S AN1 COI AN1 COII	DQ320021 DQ320037 DQ320053
Paratyinae	»Dinaro-Caucasian«	*T. hercegovinensis* (Babić, 1922)	Spring Rupečica, Ivanci, Ogulin, HR **(4)**	*Troglocaris* n. sp. 2	L1	L1 16S L1 COI	DQ320022 DQ320038
Paratyinae	»Anophthalmus«	*T. anophthalmus* (Kollar, 1948)	Cave near Jurjevići, Pelješac peninsula, HR **(5)**	*Troglocaris* n. sp. 3	P1	P1 16S P1 COI P1 COII	DQ320023 DQ320039 DQ320054
Paratyinae	»Anophthalmus«	*T. planinensis* (Birštejn, 1948)	Planinska jama, cave, Planina, Sl **(6)**	*T. a. planinensis*	PL1	PL1 6S PL1 COI PL1 COII	DQ320024 DQ320040 DQ320055
Paratyinae	»Anophthalmus«	*T. anophthalmus (Kollar, 1948)*	Vjetrenica, cave, Popovo polje, BA **(7a)**	*Troglocaris* n. sp. 4	T21	T21 16S T21 COI T21 COII	DQ320025 DQ320041 DQ320056
Paratyinae	»Anophthalmus«	*T. intermedia* (Babić, 1922)	Kukuruzovića špilja, cave, Vaganac, Rakovica, HR **(8)**	*T. a. intermedia*	T24	T24 16S T24 COI T24 COII	DQ320026 DQ320042 DQ320057
Paratyinae	»Anophthalmus«	*T. anophthalmus* (Kollar, 1948)	Jama u Predolcu, cave, Metković, HR **(9)**	*Troglocaris* n. sp. 4	T26	T26 16S T26 COII	DQ320027 DQ320058
Paratyinae	»Dinaro-Caucasian«	*T. hercegovinensis (Babić, 1922)*	Vjetrenica, cave, Popovo polje, BA **(7b)**	*T. hercegovinensis*	TH1	TH1 16S TH1 COI	DQ320028 DQ320044

Paratyinae	»Dinaro-Caucasian«	*Troglocaris* aggr. *kutaissiana* (Sadovsky, 1930)	Novo Aphonskaya, cave, Novyy Aphon, Abkhazia, **(10)** GE	*Troglocaris kutaissiana*	T-A	T-A 16S T-A COI	DQ320029 DQ320045
Paratyinae	»Western«	*Troglocaris inermis* (Fage, 1937)	Gouffre des Cent Fons cave, Causse de la Selle Hérault, (11)	*Troglocaris inermis*	T-I	T-I 16S T-I COI T-I COII	DQ320030 DQ320046 DQ320059
Paratyinae	»Western«	*Atyaephyra desmaresti* (Millet, 1831)	Krka River, Skradin, HR **(12)**	*Atyaephyra desmaresti*	OG1	OG1 16S OG1 COI	DQ320031 DQ320047
Typhlatyinae	»Dinaro-Caucasian«	*Spelaeocaris pretneri* (Matjašič, 1956)	Tunnel Orlovac I,Kotlić, Trilj, HR **(13)**	*Spelaeocaris pretneri*	OG2	OG2 16S OG2 COI	DQ320032 DQ320048
Astacidae		*Astacus astacus* (Linnaeus, 1758)	Slugovina, stream, Varaždinske Toplice, HR	*Astacus astacus*	OG3	OG3 16S OG3 COII	DQ320033 DQ320061
Gammaridae		*Gammarus balcanicus* Schäferna, 1922	Spring Plitvice, Plitvice Lakes, HR	*Gammarus balcanicus*	OG4	OG4 16S	DQ320034

in GenBank under accession numbers from DQ320019 to DQ320061.

DNA extraction, gene amplification, and sequencing

DNA extraction – Total genomic DNA was extracted from alcohol preserved whole single individuals or parts of organism e.g. pleon or carapax using the Qiagen DNeasy® Tissue Kit following the Qiagen DNeasy Protocol for Animal Tissues (Qiagen, Germany).

Gene amplification – PCR products were amplified via Mastercycler Personal (Eppendorf, Germany) using HotMasterMix (Eppendorf, Germany) in 50 μL reactions containing: 25 μL HotMasterMix-a, 22 μL mQ H_2O, 1 μL of DNA and 1 μL of each primer. For the 16S rRNA, primers 16Sar (5' – CGC CTG TTT ATC AAA AAC AT – 3') and 16Sbr (5' – CCG GTC TGA ACT CAG ATC ACG T – 3') from Simon *et al. (9)* were used. The COI gene fragment was amplified using primers LCO 1490 (5' – GGT CAA CAA ATC ATA AAG ATA TTG G – 3') and HCO 2198 (5' – TAA ACT TCA GGG TGA CCA AAA AAT CA – 3') from Folmer *et al. (10)* and for the amplification of COII gene primers C2-J-3138 (5' – AGA GCT TCA CCC TTA ATA GAG CAA – 3') and C2--N-3661 (5' – CCA CAA ATT TCT GAA CAT TGA CCA – 3') from Morrison *et al. (11)* were used.

For all amplifications an initial denaturation step at 94°C was applied for 2 min. The amplifications were followed by: (1) for 16S rDNA – 35 cycles of 20s at 94°C, 20s at 55°C, and 40s at 65°C, and a final extension for 7min at 65°C; (2) for COI – 35 cycles of 20s at 94°C, 20s at 50°C, and 40s at 65°C, and a final extension for 7min at 65°C and (3) for COII – 35 cycles of 20s at 94°C, 20s at 48°C, and 40s at 65°C, and a final extension for 7min at 65°C were used. The PCR products were electrophoresed on a 1 % agarose gel, soaked in ethidium bromide for 15 minutes, and visualized by ultraviolet light. PCR products were purified with Qiagen PCR Quick Purification KIT (Qiagen, Germany).

Sequencing

Mitochondrial genes for 16S rRNA, COI and COII were sequenced for this study. For the sequencing purposes we used services of MWG-BIOTECH AG (Ebersberg, Germany) and »DNA Service« in the Institute Ruđer Bošković (Zagreb, Croatia) executed on Applied Biosystems 3730xl DNA Analyzer. In order to improve accuracy each sample was sequenced for both heavy and light strand. Sequence chromatograms were viewed and edited manually using CHROMAS LITE 2.0 (Technelysium Pty., Queensland, Australia). Forward and reverse sequences were checked for base ambiguity in BIOEDIT 7.0.5.2 *(12)* before consensus sequences were compiled and aligned with CLUSTALX *(13)* using default parameters. The mean total nucleotide composition of different mtDNA genes were analyzed with MEGA 3.1 *(14)*. Base compositions are characteristic for invertebrate mt DNA and are 67% for A+T and 33 % for

G+C. The lengths of the alignments used for further analyses were: 416 bases of 16S, 573 bases of COI, and 408 bases of COII gene.

Phylogenetic analyses

Pairwise sequence distances were calculated using the HKY85 + Γ of nucleotide sequence evolution and a neighbor joining tree was generated using PHYLIP 3.65 *(15)*. We used published rates for decapod crustaceans and relied on a global molecular clock for COI in decapods according to Knowlton and Weigt *(16)*, Schubart *et al. (17)* and Wares and Cunningham *(18)* to estimate the time of the main phylogenetic events. Nevertheless, we simultaneously used the 16S rRNA rates *(17, 19)* as a double checking measure for timing of divergence.

Maximum parsimony analyses were performed using PAUP* 4.0b10 *(20)*. Parsimony analysis included unweighted and weighted parsimony with heuristic search using random sequence addition with 100 replicates and tree--bisection-reconnection (TBR) branch swapping. Support for individual clades was evaluated using nonparametric bootstrapping *(21)* obtained from 1000 bootstrap replicates found by heuristic or exact search in PAUP* using the same options as the individual searches.

Maximum likelihood analyses were performed using PAUP* 4.0b10 *(20)*. MODELTEST 3.7 *(22)* and MT GUI *(23)* were used to select the best-fit model of nucleotide substitution for the data sets. The model of substitution was evaluated using Likelihood Ratio Tests (LRT) and the Akaike Information Criterion (AIC). The Hasegawa Kishino Yano model of nucleotide substitution with gamma distribution (HKY85 + Γ) *(24)* under both criteria was chosen as best for estimation of an ML tree.

Bayesian analysis was performed using *MR.BAYES* 3.1.1. *(25)*. We specified Hasegawa Kishino Yano model of nucleotide substitution with gamma distribution (HKY85 + Γ) *(24)* for given data based on prior modeltest analyses. MR. BAYES uses Markov Chain Monte Carlo to approximate the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations (data). Priors were set according to the suggested model. No initial values were assigned to the model parameters, and empirical nucleotide frequencies were used. Four Markov chains were run for 1 000 000 generations and trees were sampled every 100 generations to yield a posterior probability distribution of 10,000 trees. After eliminating the first 1000 trees as »burn-in«, we constructed a 50% majority-rule consensus tree, with nodal values representing the probability (posterior probability) that the recovered clades exist, given the aligned sequence data

RESULTS

The phylogenetic analyses based on four different methods of phylogenetic reconstruction (neighbor joining, maximum parsimony, maximum likelihood and Bayesian analysis) resulted in the production of trees that supported same main branching. Results of these analyses are presented only as the most parsimonious consensus phylogenetic tree based on 16S rRNA sequences (Figure 2) to avoid unnecessary duplication of identical phylograms. There is general agreement between these

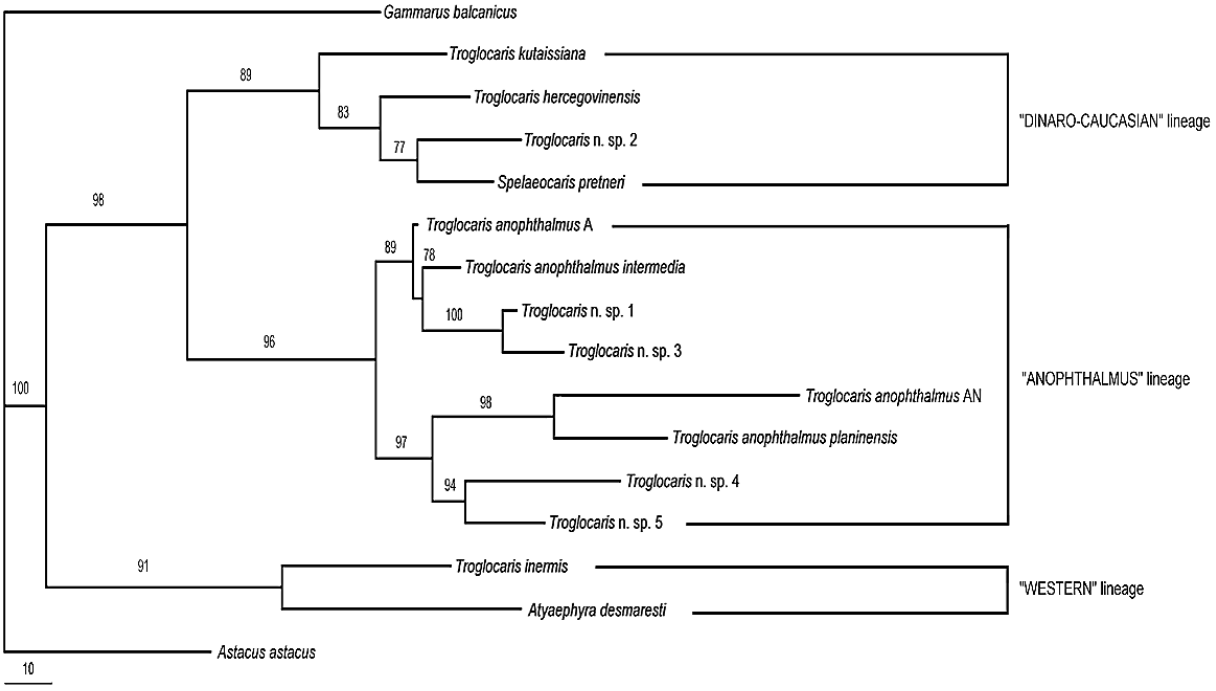

Figure 2. *Consensus tree derived from maximum parsimony analysis based on mitochondrial 16S rRNA sequence data rooted using* Astacus astacus *outgropup. Numbers at nodes indicate bootstrap support (1000 replicates). Labels indicate major clades of the* Troglocaris.

phylogenies indicating that overall topology is robust and not affected by the optimality criterion or gene marker utilized. Three main clades were apparent within the genus *Troglocaris*: (1) a clade containing species from »Dinaro-Caucasian« lineage (2) a clade containing species from »Anophthalmus« lineage and (3) a clade containing species from »Western« lineage. The »Dinaro--Caucasian« lineage was paraphyletic due to nesting of *Spelaeocaris pretneri* within the traditional *Troglocaris* species. Together with *Troglocaris hercegovinensis* from the Vjetrenica cave and *Troglocaris* n. sp. 1 from the Lika region, *S. pretneri* from Dalmatia was identified as sister taxa. As such, the prominent feature of this phylogeny is that *Troglocaris* does not form a monophyletic clade. The »Anophthalmus« lineage contained taxa distributed from the Istra region to south-eastern parts of Bosnia and Herzegovina. Finally the »Western« lineage include disjunct population of *T. inermis* from the south of France, clearly separated from Dinaric and Caucasian species, but in sister-relationship with surface-dwelling freshwater shrimp *Atyaephyra desmaresti* from the southern part of Dalmatia (Croatia).

Overall high bootstrap values supporting major branching in the neighbor joining and parsimonious trees, as well as high posterior probabilities in Bayesian trees, demonstrated the robustness of the phylogenetic analyses. To summarize, our phylogeny is well resolved.

The pairwise sequence divergences, under HKY85 + Γ model, among samples ranged from 2.6 to 23.2% for COI and 4.4 to 18.3% for 16S. The COI clock calibration of 1.4% to 2.6% sequence divergence per million years for snapping shrimp *(16)* was used. Mean rate of 2% sequence difference per million years (MYA) for the COI gene were applied. According to this rate, when applied to sequence distances, the split between Caucasian and Western European lineage might have appeared around more than 15 MYA. The second oldest split separated the Caucasian populations from »Anophthalmus« ancestors 8 MYA ago and the last split between »Anophthalmus« populations is around 5 MYA (Figure 3). Applied sequence difference of approximately 1% per million years (MYA) for 16S rRNA showed congruent results as that gained with COI rates.

DISCUSSION

The phylogeny within genus *Troglocaris*, as well as relationship to *Spelaeocaris* and *Atyaephyra,* genera is estimated. The data, when evaluated by different methods of phylogenetic inference, yielded phylogenetic trees, none of which supported the monophyly of the genus *Troglocaris*. Therefore, the genus *Troglocaris* cannot be considered monophyletic due to the positioning of *Troglocaris* and *Spelaeocaris* on all phylogenetic trees. The inter-relationship and intra-relationships of the atyid subfamilies are also different from the hypotheses suggested by Holthuis *(2, 3)*. Representatives of Holthuis's subfamily Paratyinae (*Troglocaris* and *Atyaephyra*) were found in distinct clades, while *Spelaeocaris* which, according to Holthuis's belongs to Typhlatyinae, was nested inside Paratyinae. However, Sanz and Platvoet hypothesized in 1995 that the genus *Spelaeocaris* together with the genus *Typhlatya* belongs to the subfamily Typhlatyinae. Later, d'Udekem d'Acoz *(26)* also transferred Eurasiatic *Troglocaris schmidti jusbaschjani* Birstein, 1948 to *Typhlatya* as *T. jusbaschjani*. Furthermore, Jaume and Bréhier *(27)*, who described the new *Typhlatya* species from southern France (*T. arfeae* Jaume & Bréhier, 2005), supported the revisions according to Sanz & Platvoet from 1995 and d'Udekem d'Acoz from 1999. The arguments that *Spelaeocaris* and *Typhlatya* might be the same morphs *(4, 26)* are unsupported by the recent molecular phylogenetic analysis. Moreover, according to Zakšek *et al.* in 2007 the genus *Typhlatya* was separated from *Troglocaris* and *Spelaeocaris*, which is in concordance with our data. Further-

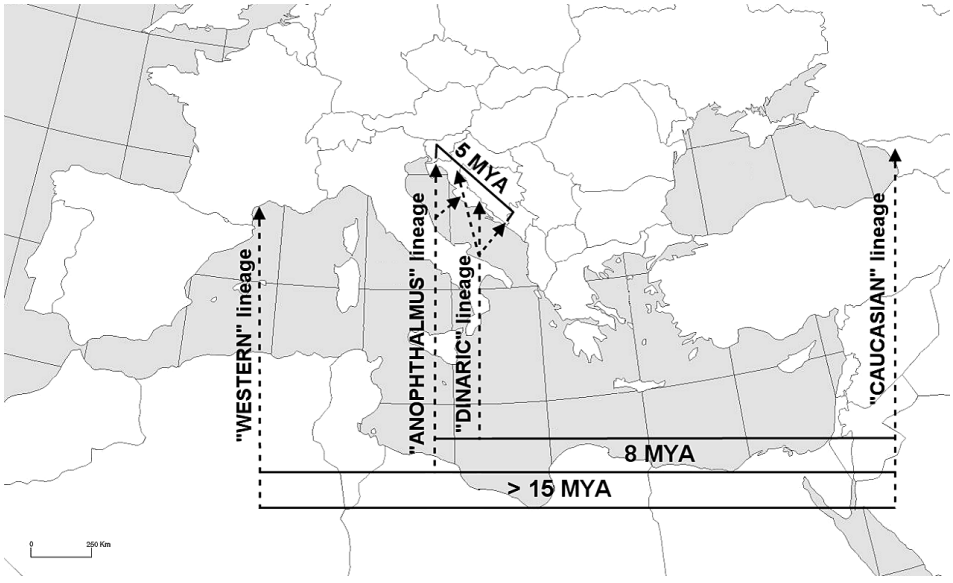

Figure 3. *Estimates of divergence times of recorded* Troglocaris *lineages*.

more, although our field data suggest that *Troglocaris hercegovinensis* and *T. anophthalmus* are isolated, nevertheless their ranges of distribution overlap. We found evidence that these two species are isolated to different hydrological systems in the Vjetrenica cave in Bosnia and Herzegovina. *T. hercegovinensis* is found in the upper part of the cave and *T. anophthalmus* inhabits the deepest part of the cave. Variation in the local distribution of these two species may reflect therefore some ecological differences, such as habitat preferences and water temperature amplitude.

Phylogeographical analysis of known distribution range and newly established localities, together with results of the taxonomic *(28, 29)* and phylogenetic studies *(8)*, indicate that distribution patterns of the cave dwelling shrimp *Troglocaris* from the Dinaric Karst and Western Caucasus were related to geological and hydro-geological events in the Mediterranean region during the Late Miocene *(30, 31, 32)*. More to the point, we propose that the last contact between the southern France species *Troglocaris inermis* and Dinaro-Caucasian species was probably achieved early in the Middle Miocene, when in northern Italy and north-western part of Dinarides was opened seaway *(30)* between the Mediterranean and the Paratethys. In particular, the complete separation of the »Western« lineage of the genus *Troglocaris* took place some 15 million years ago during the Miocene. According to the molecular clock applied, the historical events leading to the main splits in the genus took place during the second half of the Miocene. At that time the land mass of the Adriatic microplate separated the Paratethys from the paleo-Mediterranean Sea *(33, 34)*. The resulting two major drainages might have formed a basis for the split of the ancestral *Troglocaris* into the Caucasian and Dinaric lineages. Additionally, the evolutionary separation of Caucasian populations occurred before the Paratethys dried out 5.5 MYA ago during the Messinian salinity crisis *(30)*. The geological events of that period had major impacts on the European freshwater fauna *(35)*. According to Prezmann *(36)*, the cave-dwelling decapods started their subterranean life prior to the Pleistocene, which is in concordance with the karstification in the Mediterranean countries that was initiated by active groundwater movements during the Late Miocene *(30)*. During that time, *Troglocaris* ancestors may have started speciation in the groundwaters. Moreover, recently Zakšek *et al. (8)* support the freshwater origin of a *Troglocaris* ancestor in the Dinaric region and the Caucasus after the Pannonian part of the Paratethys dried out during the Late Miocene.

However, a historic analysis of *Troglocaris* lineage distribution patterns should not take global geomorphological evidence exclusively, but also include local ecological facts . At present four new species are recognized, all of which occur in the Dinaric Karst where some populations remained trapped in geo-hydrologically isolated regions *(37)*.

For some Caucasian populations, there is no consensus regarding their affiliations to *Troglocaris kutaissiana (4)*. The status of these populations remains to be investigated as well as topotypic material of all nominal *Troglocaris* species (including the genus *Spelaeocaris*) in order to elucidate the taxonomic differences between them from the morphological point of view.

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