



RESEARCH ARTICLE

Speciation of a subterranean amphipod on the glacier margins in South Eastern Alps, Europe

Teo Delić¹ | Peter Trontelj¹ | Valerija Zakšek¹ | Anton Brancelj^{2,3} |
Tatjana Simčič² | Fabio Stoch⁴ | Cene Fišer¹

¹SubBio Lab, Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

²Department of Organisms and Ecosystems Research, National Institute of Biology Ljubljana, Ljubljana, Slovenia

³Faculty of Environmental Sciences, University of Nova Gorica, Nova Gorica, Slovenia

⁴Evolutionary Biology & Ecology, Université libre de Bruxelles (ULB), Bruxelles, Belgium

Correspondence

Teo Delić, SubBio Lab, Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.
Email: teo.delic@bf.uni-lj.si

Funding information

Javna Agencija za Raziskovalno Dejavnost RS

Handling Editor: Hans-Peter Comes

Abstract

Aim: Climatic oscillations altered distributions through migration, extinction, adaptation or speciation. Their joint effect has been rarely studied. Here, we tested how Pleistocene climatic oscillations shaped the evolutionary history and distribution of a subterranean amphipod. We tested four predictions for extant populations: (i) populations of good dispersers moving along the ice-sheet margin should have homogenous genetic structure and broad tolerance to temperatures; (ii) adaptation to local temperatures evolved in poor dispersers with spatially structured populations; (iii) Pleistocene extinctions in the range of historical glaciers should reflect founder effects and high sensitivity to low temperatures; (iv) para- or allopatric speciation could unfold through diverse scenarios as a response to local adaptation and constrained dispersal.

Location: North-Western part of the Balkan Peninsula, Europe.

Taxon: *Niphargus stygius*, Amphipoda, Crustacea.

Methods: We used time-calibrated multilocus phylogenies and multilocus delimitations to test independently evolving lineages. For each lineage, separately we assessed historical population dynamics and dispersal potential, modelled climatic niches and mapped them onto palaeoclimatic maps. Additionally, we conducted enzymatic and respiratory tests of thermal tolerance, and reconstructed historical distribution range using phylogeographical diffusion analyses.

Results: We identified four independently evolving tentative species, dated to the Pleistocene, with no evidence of extinction events. Three main lineages are parapatric with few pairwise syntopies and no traces of hybridization. They evolved in allopatry and possibly maintained constant effective population sizes. The lineages are phylogeographically substructured, indicating low recent dispersal. They differ in their climatic niche envelopes and physiological responses to unfavourable temperatures.

Main conclusions: We infer that periodic drops of groundwater in the Pleistocene caused two vicariant events and the onset of two allopatric speciation events, followed by phases of dispersal leading to secondary contact and occasional overlap of ranges. Speciation was seemingly fostered by eco-physiological differentiation due to adaptation to local climates.

KEYWORDS

adaptation, Amphipoda, climate change, eco-physiology, *Niphargus*, phylogeography, Pleistocene

1 | INTRODUCTION

Climates importantly shape species distributions, community structure and species richness patterns (Chen et al., 2011; Francis & Currie, 2003; Hawkins et al., 2003). The premise that climate conditions can explain past and predict future biodiversity patterns, makes them attractive for macroecology and macroevolution (Hewitt, 2000; Ivory et al., 2016; Quintero & Wiens, 2013; Saupe et al., 2019). The accuracy of future predictions, however, depends on our understanding of how past climate oscillations tailored species distributions and evolution (Burbrink et al., 2016; Mammola et al., 2018; Quintero & Wiens, 2013; Sánchez-Fernández et al., 2016).

The climate oscillations can be translated into key drivers of biodiversity patterns, including dispersal, extinction, speciation and adaptation. During the Pleistocene, biodiversity patterns were largely reshaped. Cooling, ice-cover, drought and permafrost conditions drove species into refugia where they managed to survive (Copilaş-Ciocianu et al., 2017; Eme et al., 2014; Marzahn et al., 2016). Non-migratory species that could not escape the harsh abiotic conditions likely went extinct (Eme et al., 2015; Zgajmajster et al., 2014). Shifts in distributions and temporary isolation in allopatric refugia reshaped genetic structure of populations (Knowles & Massatti, 2017; Lanier et al., 2015; Verovnik et al., 2005; Wachter et al., 2016) and, on some occasions, triggered allopatric speciation (Carstens & Knowles, 2007; Huang et al., 2020; Knowles, 2001; Knowles & Richards, 2005). The fate of marginal populations depended on their capacity to either accommodate to local environmental conditions through phenotypic plasticity or to adapt via natural selection (Keller & Seehausen, 2012). Differential adaptation between peripheral and central populations could attenuate geneflow and trigger parapatric ecological speciation (Castellanos-Morales et al., 2016) by multiple mechanisms (Keller & Seehausen, 2012).

Subterranean invertebrates are excellent model organisms to study the translation of climatic oscillations into biodiversity drivers due to two important characteristics of subterranean environments. First, the temperature in subterranean environments approximates the mean annual temperature at the surface (Culver & Pipan, 2019). The effects of short-term and catastrophic climate fluctuations are buffered (Dominguez-Villar et al., 2015). Therefore, aggregated measures of climatic variables that are used in reconstructions of past climates (Waldock et al., 2018) fit subterranean ecosystems better than surface ecosystems and the links between climate changes and biological processes are clearer. Second, subterranean species are generally poor dispersers (Bregović et al., 2019; Trontelj et al., 2009; but see Delić et al., 2020). Theoretically, climate changes in climatically buffered environments can yield two opposing outcomes onto weak dispersers. In the absence of temperature variation, thermal tolerance of subterranean organisms presumably narrows, making them more vulnerable to long-term climate changes and, if dispersal is limited, more vulnerable to extinction (Mammola et al., 2018; Mermillod-Blondin et al., 2013). Yet, climate changes in such buffered environment are slowed, and temporally delayed (Dominguez-Villar et al., 2015), leaving organisms sufficient time to adapt. Local

adaptation is not swamped by gene-flow. Besides, within deep carbonate massifs the temperature can greatly vary at the local scale, favouring thermal generalists over specialists (Trontelj et al., 2019). Thus, subterranean populations might have even a higher chance of survival than surface populations. Indirect evidence supports both theoretical predictions. On the organismal level, subterranean species are thermally either extremely sensitive or tolerant (Colson-Proch et al., 2009; Issartel, Hervant, et al., 2005; Issartel, Renault, et al., 2005; Issartel et al., 2006; Lencioni et al., 2010; Mammola et al., 2018, 2019; Sánchez-Fernández et al., 2016). On the species and community levels, species ranges and species richness patterns follow the boundaries of glaciers unveiling local extinctions (Eme et al., 2014, 2015, 2017; Foulquier et al., 2008; Zgajmajster et al., 2014). Nevertheless, a few species apparently survived under glaciated areas, possibly indicating local adaptation (Bjarni et al., 2007; Holsinger, 1980; Holsinger & Shaw, 1987; Lefébure et al., 2007). Explicit studies of multiple effects of climate changes onto subterranean organisms are rare.

In this study, we explored whether and how dispersal, extinction, speciation and adaptation during the Pleistocene affected biogeography of the subterranean amphipod crustacean *Niphargus stygius* (Schiödte, 1847). An early analysis, limited in sampling, detected substantial genetic variation between populations of this species (Delić et al., 2017). *Niphargus stygius* is an omnivore distributed in the north-west Balkan Peninsula (Delić et al., 2017; Sket, 1974), extending from the Adriatic coast up to 1900 m a.s.l. in the South-Eastern Alps (Brancelj, 2002). The area in the South-Eastern Alps was covered by glaciers during the Pleistocene. The current distribution pattern implies that the studied species had the opportunity to undergo local adaptation and possibly speciation in north-south direction. Moreover, the species is distributed across the watershed between the Soča River in the west, which drains into the Adriatic Sea, and the Sava River in the east, draining into the Black Sea. Hence, the hydrological situation might have facilitated allopatric speciation between the two basins, as it is already known from other subterranean crustaceans (Konec et al., 2016).

We tested the role of dispersal, extinction, adaptation and speciation using species delimitations, population demographic history, ancestral range reconstruction, ecological niche modelling and eco-physiological tests. The tested predictions were as follows.

1. *Dispersal*: If a species was a good disperser, populations responded to climate oscillations by dispersal. If so, the contemporary populations show little geographic structure, with many shared haplotypes. Moreover, populations do not differ in their thermal preferences given that the effect of local adaptation is swamped by immigrants. Ecological niche models between phylogroups are similar to each other.
2. *Adaptation*: In the absence of dispersal, populations became spatially structured and could adapt to local conditions. In this case, we should expect differences among geographically remote populations in climatic niche envelopes and in their physiological response to temperatures.

3. **Extinction:** In the absence of dispersal and ability to adapt, populations in glaciated areas went extinct. Hence, we predict spatially structured lineages, showing no signs of eco-physiological differentiation, but being sensitive to cold. If the ice-covered areas were re-colonized recently, these populations should show signs of founder effect and reduced genetic diversity.
4. **Speciation:** Speciation could unfold in different scenarios. We explored possibilities of speciation (i) between the two drainage basins and (ii) along the altitudinal gradient. The first scenario predicts allopatric speciation without signs of population contraction (Knowles & Richards, 2005), possibly followed by secondary sympatry. Differences in eco-physiology are possible, but not a prerequisite in this case. In contrast, the second scenario predicts that eco-physiological differentiation of marginal, possibly genetically less diverse populations yielded parapatric ecological speciation (Castellanos-Morales et al., 2016). In both scenarios, splitting events should be dated to the Pleistocene.

2 | MATERIALS AND METHODS

2.1 | General outline

We first used a multilocus species delimitation approach to disentangle the structure of *N. stygius* species complex. In turn, we analysed the population structure of each lineage to quantify their dispersal potential and past demography. For each putative species, we

constructed climatic niche envelopes to explore the ecological differences between lineages. In a complementary experimental analysis, we assessed the temperature sensitivity of the putative species exposed to suboptimal temperatures by measuring respiratory rates and biochemical markers. Finally, we reconstructed past dispersal to infer allo-parapatry and colonization of the current range. To test for parapatric ecological speciation, we projected climatic niche envelopes onto palaeoclimatic maps.

2.2 | Sampling

For molecular analyses and ecological niche modelling, 56 localities were sampled from the entire range of the *N. stygius* species complex (Figure 1), in total 167 specimens. For physiological and biochemical measurements, we sampled specimens from five localities with roughly similar environmental temperature (9°C). We gathered 30 individuals from each locality. A list of the samples and specimens used in the analyses is available in Material S1. The samples are deposited in the collection of the Department of Biology (Biotechnical Faculty, University of Ljubljana, Slovenia).

2.3 | Molecular analyses

Genomic DNA was extracted from one of the pereopods, or in case of small individuals, from the entire specimens, using Sigma

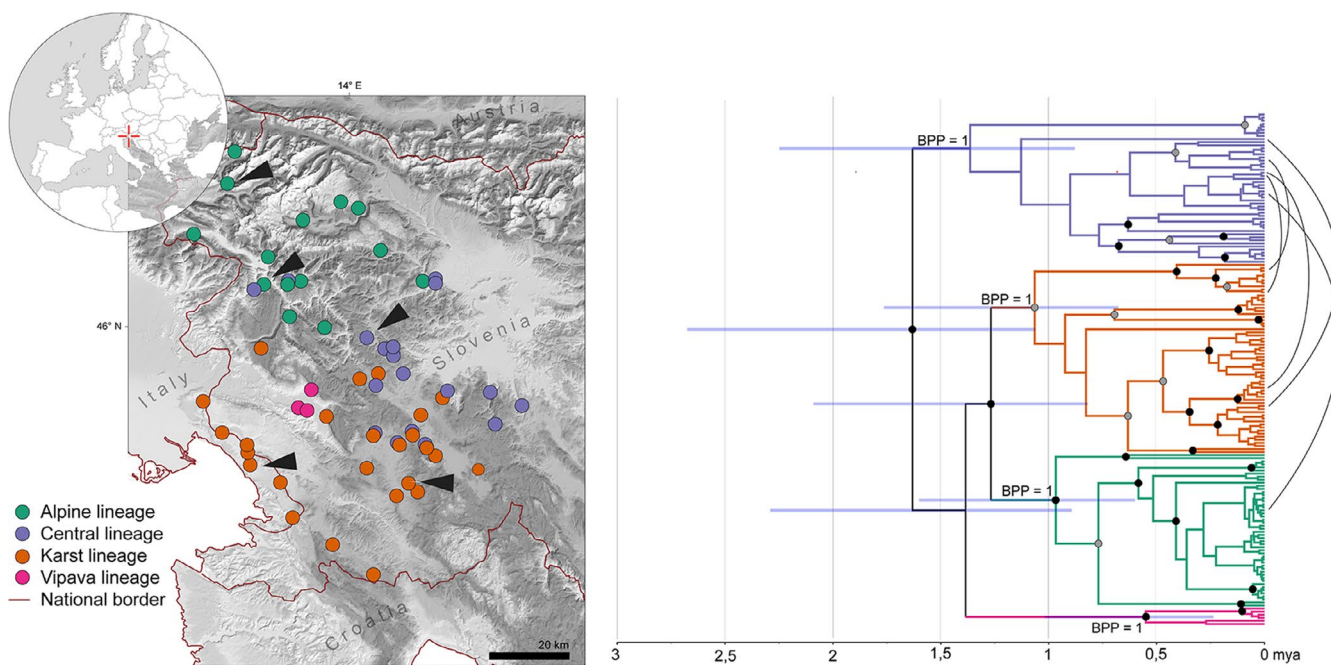


FIGURE 1 Distribution of the *Niphargus stygius* species complex, with the main four lineages. Black arrows indicate the populations used for the biochemical analyses (left). The time-calibrated maximum clade credibility tree (right) of the species complex was derived from a BEAST analysis using COI, 28S rDNA I and II, ITS and EF1 gene fragments. Posterior probabilities of major splits are denoted as grey (>0.95) and black (>0.99) dots. The posterior probabilities for the putative species, as inferred from the multilocus species delimitation with BPP, are shown on the nodes. Branches representing syntopically occurring individuals are connected with arched lines

Aldrich GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). One mitochondrial (Cytochrome Oxidase I—COI) and four nuclear gene partitions (two parts of 28S rRNA, Internal Transcribed Spacer—ITS and Elongation Factor—EF1) were amplified in polymerase chain reaction (PCR) with standard primers and protocols (details in Material S2). PCR products were purified using Exonuclease I and FastAP (Thermo Scientific) and sequenced in both directions with the same amplification primers by Macrogen Europe (the Netherlands). Sequence chromatograms were visually checked, assembled and edited in GENEIOUS 8.0.4 (Biomatters), aligned using MAFFT 7 (Kato & Standley, 2013), and partitions were concatenated in GENEIOUS. The most probable substitution model and partitioning scheme were chosen in PARTITIONFINDER 2.1.1 (Lanfear et al., 2017). A concatenated dataset (details in Material S2) from 167 specimens was used to infer the phylogenetic structure and delineate putative species.

2.4 | Phylogenetic analyses and species delimitation

The phylogenetic structure of the species complex was inferred using the concatenated alignment within the multispecies coalescence framework and Bayesian inference as implemented in BEAST 1.8.1 (Drummond et al., 2012). All the analyses were run using the strict clock prior for mitochondrial COI to a mean value of 0.018, estimated for amphipods (Nahavandi et al., 2013), and an interval between 0.0095 and 0.0265 substitutions per site in million years. The molecular clock for other gene partitions, treated as independent loci, was estimated relative to the COI clock, and the priors were set to default values for uniform clock priors. We tested for alternative historical population sizes (constant, expansion, exponential and logistic). To account for possible mitonuclear discordance, we compared the topologies of COI tree versus nuclear markers tree in R package 'dendextend' (Galili, 2015). All the analyses were run for 50 million generations, sampled every 1000th generation, and repeated twice. Convergence of the resulting MCMC chains and effective samples size was checked in TRACER 1.6. Independent runs were combined in LOGCOMBINER 1.8.2 after discarding the first 20% trees as burn-in. Simulation analysis producing results comparable to Akaike information criterion (AIC) in Bayesian Monte Carlo context, AICM (Baele et al., 2012), was used to compare different population growth scenarios. The maximum clade credibility tree was assembled using Tree ANNOTATOR 1.8.2 (Drummond et al., 2012).

Species were delimited using Bayesian Phylogenetics & Phylogeography 3.1 (BPP; Yang & Rannala, 2014). The BPP analysis was used to assess Bayesian posterior probabilities for alternative species delimitation scenarios via reversible-jump Markov chain Monte Carlo (rjMCMC) and nearest neighbour interchange on the multilocus molecular dataset. rjMCMC was run for 50,000 generations, sampled every fifth generation and the first 2000 samples were discarded as burn-in. Fine-tuning parameters, heredity and locus rates were estimated, while the ancestral population size θ and the root age τ were tested against three alternative scenarios (large

population, deep divergence; small population, shallow divergence and large population, shallow divergence) following Leaché and Fujita (2010). Each run was repeated twice to confirm the robustness of the results.

2.5 | Temporal changes in effective population size

Demographical responses to palaeogeographic changes, including the Pleistocene glaciations, were estimated using a partial, lineage-specific multilocus dataset. The lineage-specific datasets counted 50, 62 and 49 specimens for the Alpine, Karst and Central lineage (see Figure 1) respectively. Four specimens from the Vipava lineage were excluded from the analysis (see Section 3). Changes in effective population sizes were assessed using Extended Bayesian Skyline Plot as implemented in software BEAST 1.8.1, which allows simultaneous analysis of data from unlinked loci, taking into account specific mode of inheritance and improving the reliability of changes in demography. However, it should be kept in mind that the accuracy of estimation of effective size population is limited when relying on a small number of markers. The main part of the analysis was set the same way as described in Section 2.4. In addition, we used two tests to assess whether the genetic variation of the delimited lineages deviated from neutral expectations due to either a recent population expansion or selection: Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997), both implemented in DNASP 6.12.01 (Rozas et al., 2017).

2.6 | Climatic niche envelope modelling and niche overlap

To test for ecological differences among the three main lineages of the *N. stygius* species complex, and to estimate whether these differences could emerge during speciation under historical climatic conditions, we reconstructed climatic niche envelopes for all lineages. We acknowledge that the results of these analyses need to be treated with caution. Realized distributions, which are the basis for the calculation of climatic niche envelopes, may be an outcome of biotic and abiotic factors. Thus inferences of environmental niche envelopes from distributional data may underestimate true climatic niche boundaries. Nevertheless, some studies suggest that such models return reasonable good niche estimates (Kearney et al., 2010; Lee-Yaw et al., 2016; Warren et al., 2014). Moreover, if underestimated (e.g. too small) and projected onto the past layers, niche envelope represents a conservative estimate of the suitability of past habitats.

Climatic niche envelopes were modelled using bioclimatic data obtained from WorldClim 2.1 (Fick & Hijmans, 2017; <http://www.worldclim.org>). The original dataset offers climatic data at the scale of 30 s. The environmental layers were delimited with a buffered rectangle surrounding the full extent of known occurrence points (45.3–46.7°N and 13.2–14.8°E) using ArcGIS 10.1 (ESRI). For construction of the final models, we selected two climatic variables that best present environmental conditions within the subterranean



realm. As the temperature within subterranean habitats reflects the mean annual temperature on the surface, we used the mean annual temperature (Bio 1). In addition, we used annual precipitation (Bio 12), as a proxy of surface production and potential connectivity between patches of shallow subterranean habitats (aphotic habitats close to the surface), often exploited by *N. stygius*.

To account for spatially uneven sampling and spatial autocorrelation that may bias the model estimation (Bahn & McGill, 2012; Hijmans, 2012), we superimpose two grids of different cell sizes (1 × 1 km, 5 × 5 km) on the data. From each cell, only one site was randomly selected for the subsequent analyses. Climatic niche envelopes were estimated using a machine learning algorithm based on the principle of maximum entropy, MAXENT 3.4.1 (Phillips et al., 2006). This method requires presence-only data, it performs well compared to other modelling techniques, and has already been tested on subterranean animals (Elith & Leathwick, 2009; Mammola et al., 2018). The dataset was randomly divided into a training set (75% of the occurrences) and a validation set (25% occurrence sites). Models were evaluated by running 100 replications with cross-validation and using the area under the receiver operating characteristic curve (ROC).

The similarity in climatic niche envelopes among lineages was estimated using Schoener's *D* distances (Warren et al., 2008), ranging from 0 (no overlap) to 1 (complete overlap), and occurrence probability maps (R package 'phyloclim'; Heibl & Calenge, 2013). To assess whether niche envelopes between lineage-pairs differ significantly, we used niche equivalency test that compares the overlap of the focal lineage pair against overlaps expected from random draws from the same underlying distribution (R package 'dismo'; Hijmans et al., 2015). To assess the suitability of habitats during the Last Maximum Glacial (LGM) environmental conditions (21,000 years ago), we projected ecological niche models onto the paleoclimate layer for Pleistocene, LGM (Brown et al., 2018), with a spatial resolution of 5 × 5 km.

2.7 | Ancestral range reconstruction

In order to estimate whether the three main lineages evolved in close proximity or allopatry, we reconstructed ancestral ranges of the *N. stygius* lineages using Bayesian phylogeographical diffusion analysis (Lemey et al., 2010). The analysis was performed in BEAST using COI sequences and the same settings as described in 'Phylogenetic analyses and species delimitation' section. Spatial origins and the historical dispersal routes were visualized in the software SPREAD 1.0.7 (Bielejec et al., 2011).

2.8 | Thermal sensitivity

To test whether different lineages of the studied species complex differ in thermal sensitivity, we measured physiological and biochemical response of five populations at three temperatures. The

populations belonged to three lineages (one Central, two Karst and two Alpine populations, see Section 3). The water temperature at all five sampling sites was approximately the same (9°C), thus the differences among populations can be attributable to phylogenetic contingency alone. Animals were brought to the laboratory and acclimated for 21 days in a dark acclimatized chamber at 9°C. Thermal sensitivity was assessed at three temperatures (5.5, 9, 14°C) by measuring stress response. Stress response was estimated by respiratory rate (*R*), the respiratory capacity of electronic transport system (ETS, a biochemical measure of the potential metabolic activity, or theoretical maximum respiratory rate), and specific activity of the catalase (CAT), one of the crucial enzymes responsible for the cellular defence system against oxidative stress (Cho et al., 2000). In order to normalize the measurements, we measured also wet body mass. Laboratory protocols are described in Material S3. Respiration, CAT activity and wet body mass data were log transformed to meet assumptions of the normal distribution.

The data were analysed using linear mixed effect models (LMM). We performed two analyses. In either analysis pointers of stress, that is the respiratory rate and catalase activity, were used as response variables. Wet body mass, phylogenetic lineage affiliation and experimental temperature were used as predictive variables, while different populations within a lineage were treated as a random variable. Additionally, in the model analysing respiration, we included also ETS, given it can affect the respiratory rate. Statistical analyses were run in R statistical environment (R Core Team, 2016) using the 'nlme' package (Pinheiro et al., 2016).

We first tested both models with all possible interactions, then simplified them using the top-down protocol for LMMs, and compared them using the AIC value (Zuur et al., 2009). Comparisons between the groups were made using Tukey's post hoc test as implemented in a 'multcomp' package (Hothorn et al., 2016). To quantify how much variance is explained by the model itself (R^2), we used a summary statistics proposed by Nakagawa and Schielzeth (2013) implemented in the R package 'MuMIn' (Barton, 2015). The method estimates goodness-of-fit of a model using marginal and conditional R^2 which describe how much variance is explained by the predictors alone or by the predictors together with random variables (Nakagawa & Schielzeth, 2013).

3 | RESULTS

3.1 | Phylogenetic analyses, demographic models and species delimitation

Multilocus phylogenetic analysis suggested the subdivision of *N. stygius* into four parapatric to sympatric phyletic lineages, referred to as Central, Alpine, Karst and Vipava lineage (Figure 1). With the exception of the hydrologically limited Vipava lineage, the remaining lineages were distributed in both, Adriatic and Black-Sea basins. All splitting events dated to the Pleistocene, including the large 95% credible intervals. The first split separating the Central lineage

from the rest of the complex dates to 1.6 Myr (95% credible interval 2.7–1.1 Myr). The next two splits followed at 1.4 Myr (95% credible interval 2.3–0.9 Myr) and 1.27 Myr (95% credible interval 2.09–0.82 Myr), with respective separations of the Vipava lineage from the Karst+Alpine lineage, and Karst from Alpine lineage.

Mitochondrial and nuclear data largely agree in the four-lineage structure, but show signs of possible mitonuclear discordance from two different source (Material S4). The first one comes from the weak support of the basal relationships between lineages and can be attributed to short branches between lineages. The second one is limited to some populations of the Alpine and Central lineage and shows a pattern characteristic of sporadic hybridization events over a wide zone of contact between the ranges of the two lineages.

Nevertheless, multilocus species delimitation suggested that the four lineages can be viewed as putative species. The four-species hypothesis received the highest posterior probabilities, regardless the population size and the root age priors used (Table 1). The species status of the Karst lineage is indirectly supported also by the absence of any signs of hybridization and mitonuclear discordance despite its overlapping distribution and co-occurrence with other lineages. All known localities of the Vipava lineage fall within the range of the Karst lineage, while the Central and Karst lineage are syntopic in four caves.

All further analyses were performed only on the three large, exhaustively sampled lineages, named Central, Karst and Alpine. The Vipava lineage was excluded from all subsequent analyses given that we found only few individuals in three geographically close localities.

The deep splits among clades within each of the three lineages roughly correspond to sub-catchments and indicate low dispersal between populations. All lineages showed relatively stable effective population sizes during the past 1 Myr. With the exception of F_u 's F_s in the Karst lineage, Tajima's D and F_u 's F_s were not significant, implying neutral evolution (Table 2). Likewise, the effective population sizes were stable, with negligible drop and subsequent Holocene growth in the Alpine and Central lineage (Figure 2).

3.2 | Modelling of past and present climatic niche envelopes

The models of climatic niche envelopes can be considered plausible for all three lineages, given that the area under ROC curve was

above the threshold value of 0.7, a value suggesting relative confidence of the results (Hosmer et al., 2013). As the grid size did not affect our conclusions, we report the results from 5×5 km cell size. Species pairwise climatic niche envelope overlap was moderate to high, ranging from 0.51 to 0.68 (Rödder & Engler, 2011). The largest niche overlap was found between the Central and Karst lineage (Schoener's $D = 0.75$), followed by the Central and Alpine lineage (Schoener's $D = 0.65$) and the Karst and Alpine lineage pairs (Schoener's $D = 0.51$) (Table 2, Figure 3). The niche equivalency test showed that climatic niche envelopes were significantly different from each other, that is the probability that their pairwise differences was $p = 1$ (Table 3).

Projections of climatic niche envelopes onto palaeoclimatic layers suggested substantial displacement of the suitable conditions during the past 21,000 years for all three lineages. The most suitable conditions for Karst lineage were met at similar territory as present distribution, with notable extensions to the south and east of its present distribution. The Central lineage met the most suitable conditions in the central part of its present distribution, while the Alpine lineage met suitable climatic conditions within approximately the same territory, with an extension to the southeast of its present distribution.

3.3 | Tests of thermal sensitivity and ancestral range reconstructions

Individuals from all three lineages differed in their physiological and biochemical responses to temperature. Respiratory rates were best explained using a complex model that included lineage identity, body mass, ETS, temperature and four interactions (lineage:logETS, lineage:T, logETS:T, lineage:logETS:T) as predicting variables, and sampling site as a random term. Alternative models received lower AIC support and were discarded as less likely. Tukey's post hoc test suggested that Karst lineage had significantly higher respiratory rates than Alpine lineage ($p = 0.015$) and Central lineage ($p = 0.0177$). The two latter did not differ significantly from each other ($p = 0.685$) (Figure 4, Table 4). The interaction between temperature and lineage identity that would quantify differential response to temperature gradient, was not significant.

The analysis of specific activity of catalase (CAT) yielded partially congruent results to the analysis of respiratory rates. The best

| | Prior θ | Prior τ | Algorithm 0 | | Algorithm 1 | |
|-----------------------------|----------------|--------------|------------------|------------------|------------------|------------------|
| | | | Run 1 | Run 2 | Run 1 | Run 2 |
| large pop., deep div. | G (1, 10) | G (1, 10) | 1 (4 sp.) | 1 (4 sp.) | 1 (4 sp.) | 1 (4 sp.) |
| small pop., shallow div. | G (2, 2000) | G (2, 2000) | 1 (4 sp.) | 1 (4 sp.) | 1 (4 sp.) | 1 (4 sp.) |
| large pop., shallow div. | G (1, 10) | G (2, 2000) | 1 (4 sp.) | 1 (4 sp.) | 1 (4 sp.) | 1 (4 sp.) |

TABLE 1 Bayesian support for the four independent lineages (printed in bold), acquired from multilocus species delimitation as implemented in BPP (Yang & Rannala, 2014) under different population size, node divergence time and algorithm priors

Note: The four species scenario was the only supported in all runs.



TABLE 2 Results of estimation of possible past demographic expansions, for the Central, Alpine and Karst lineage by Tajima's *D* and Fu's *F*. Values printed in bold indicate statistical significance.

| | Tajima's <i>D</i> | Fu's <i>F</i> |
|-----------------|--------------------------------|---------------------------------|
| | Mean (95% CI)/ <i>p</i> -value | Mean (95% CI)/ <i>p</i> -value |
| Central lineage | -0.10 (-1.55, 1.79)/0.62 | -0.58 (-7.95, 6.82)/0.79 |
| Karst lineage | -0.11 (-1.64, 1.81)/0.67 | -0.60 (-9.08, 7.5)/ 0.04 |
| Alpine lineage | -0.11 (-1.65, 1.82)/0.96 | 0.45 (-7.28, 6.37)/0.88 |

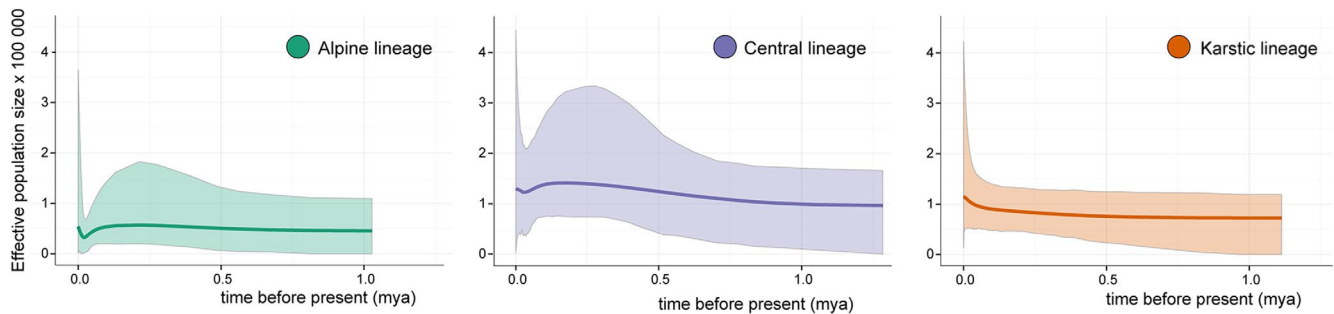


FIGURE 2 Extended Bayesian skyline plots showing the changes in effective population size over time for the three *Niphargus stygius* lineages: Alpine, Central and Karst. The central line of each plot represents the median value of population size and the shaded area indicates the 95% confidence interval

model that predicted CAT activity included lineage identity, body mass and their interaction as predicting factors, and population as a random factor. Pairwise comparisons indicated that CAT was significantly lower in Alpine lineage, when compared to Karst lineage ($p = 0.0037$) and Central lineage ($p = 0.0367$). No difference in CAT activity was detected between Karst and Central lineage ($p = 0.9780$). In addition, we found no evidence suggesting that response to modified temperature differed between lineages (the interaction between lineage and temperature was not significant).

Ancestral range reconstruction implies that these three lineages apparently emerged in allopatry. Successive dispersal led to secondary contact, parapatry and even some syntopic populations (Figure 5).

4 | DISCUSSION

The *N. stygius* species complex experienced a complex evolutionary history. We detected three possible speciation events. The origin of Vipava lineage, represented by only six individuals, presently cannot be clarified. By contrast, distribution, population structure and ecophysiological characteristics of the remaining three lineages provide limited insight into the two remaining putative speciation events.

The oldest split, between the Central and (Alpine+Karst) lineages, roughly follows an East-West division but does not correspond to the current watershed between Adriatic and Black Sea Basin. Range reconstructions and the stable population size of the Central lineage indeed imply allopatric speciation, followed by secondary contact with Alpine and Karst lineages. The climatic niche envelope of the Central lineage differs from climatic

niche envelopes of the remaining two lineages and imply that local adaptation could additionally accelerate speciation in allopatry (Keller & Seehausen, 2012). This hypothesis is, to some extent, supported by projections of the present niche envelope onto past climatic conditions. Projected niche envelopes indicate historical spatial segregation of the area with suitable conditions that could lead to competitive exclusions between Central and (Alpine+Karst) lineages. Interestingly, the assumed suitable conditions, and the presumed spatial origin of the lineage, inferred from the range reconstruction coincide relatively well (Figures 3 and 5). The ecophysiological measurements support weak differentiation among lineages, as Central lineage differs from Alpine and Karst lineage in CAT and respiratory rates respectively. The speciation likely dates back to the first half of the Pleistocene, when cooling of the atmosphere, and the establishment of glaciers across Europe, already induced climate aridification and affected the water level (Gibbard et al., 2010). There is no accurate information of the level of groundwater, but considering the marine regression of the Adriatic (Correggiari et al., 1996), a substantial lowering of groundwater table seems a reasonable hypothesis. In addition, stepwise aridification of central and eastern European landmasses (Bugge et al., 2013) might have lowered the accessibility of subsurface subterranean habitats which are often exploited by *N. stygius*, that is the hypotelmic and epikarstic waters (Culver & Pipan, 2014). If so, temporary groundwater connections between the Danubian and the Adriatic watershed were disrupted and, thus, might grounded allopatric speciation.

The recent split between the Alpine and Karst lineage may correspond to the hypothesized process of parapatric ecological speciation. In ecological speciation, species evolve as a by-product

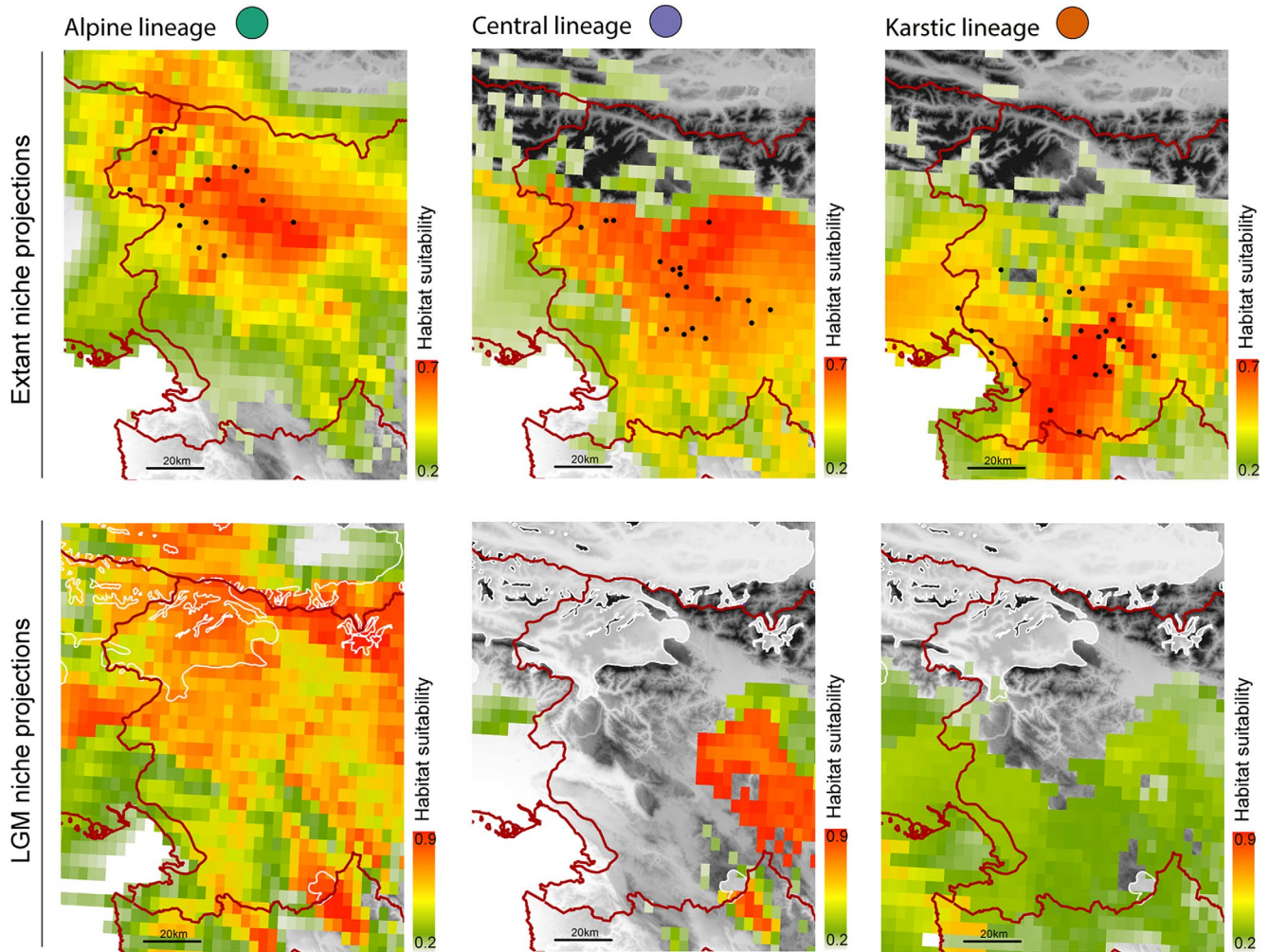


FIGURE 3 Habitat suitability of the three *Niphargus stygius* lineages; Alpine, Karst, Central, based on the occurrence data and selected bioclimatic variables (bio1, bio12; Fick & Hijmans, 2017). The upper three images present recent habitat suitability projections, while the lower three depicts the habitat suitability during LGM (Last Glacial Maximum, 21,000 years ago) for each lineage. White outlines in the lower row present the maximum extent of the Alpine and Dinaric glaciers during LGM

of adaptation to local environment (Nosil, 2012). A possible mechanism behind reproductive isolation along climatic gradients could be the mortality of maladapted migrants, or environment-induced poorer performance due to physiological stress (e.g. Tobler et al., 2011). In the studied case, the two lineages differ in both, climatic niche envelope and physiological response to temperature, suggesting some ecological differentiation. However, some details refute this hypothesis. If environmental conditions affected fitness of migrants, physiological response to unfavourable temperature should integrate stress response and a shift to suboptimal temperature, as expected for ectotherms. The former should be reflected in a significant interaction between temperature and phylogenetic lineage, which is not the case in our study. Moreover, if stress really affected fitness, the Karst lineage, occupying the warmest parts of the range of the species complex (Figures 1 and 3), should show traces of extinction and range retraction during last glacial maximum, which is again not the case (Figures 2 and 3). Finally, the ancestral range reconstruction implies allopatric

speciation. Therefore, we tentatively conclude that this speciation event began in allopatry, with both emerging species adapting to local conditions. The observed eco-physiological differences possibly prevented lineages to hybridize and enforced the completion of speciation when both evolving species came into contact (Keller & Seehausen, 2012; Reeve et al., 2016).

Overall, we hypothesize that the *Niphargus stygius* species complex should be treated as a set of populations bound to areas with different ecological parameters that speciated due to a complex interplay of geomorphological complexity, historical climatic shifts and limited dispersal potential. During the Pleistocene environmental perturbations, a combination of extrinsic factors (drop of water level, climate oscillations, elevation gradient), and intrinsic factors (low dispersal, capacity to accommodate to local conditions) prolonged periods of isolation and prompted speciation to the point of no return. The prolonged periods in allopatry allowed eco-physiological differentiation on the basis of local adaptation. Hereafter, dispersion enabled the establishment of secondary contacts and sympatry zones

TABLE 3 Comparison of the predicted environmental niche models (ENMs) for three *Niphargus stygius* lineages: Alpine, Central and Karst geographical niche overlap calculated in *phyloclim* and test of niche equivalency in *dismo*, both expressed as Schoener's *D* distances, ranging from 0 (no overlap) to 1 (complete overlap)

| Niche overlap (phyloclim) | | Niche equivalency (dismo) | |
|---------------------------------|------|---------------------------------|----------------------|
| Schoener's <i>D</i> , 1 km grid | | Schoener's <i>D</i> , 1 km grid | |
| Central-Alpine | 0.70 | Central-Alpine | 0.67 (<i>p</i> = 1) |
| Central-Karst | 0.73 | Central-Karst | 0.78 (<i>p</i> = 1) |
| Alpine-Karst | 0.55 | Alpine-Karst | 0.54 (<i>p</i> = 1) |
| Schoener's <i>D</i> , 5 km grid | | Schoener's <i>D</i> , 5 km grid | |
| Central-Alpine | 0.65 | Central-Alpine | 0.61 (<i>p</i> = 1) |
| Central-Karst | 0.75 | Central-Karst | 0.80 (<i>p</i> = 1) |
| Alpine-Karst | 0.51 | Alpine-Karst | 0.50 (<i>p</i> = 1) |

Note: *p* value in niche equivalency test show support for the null hypothesis, i.e. that niches are different.

TABLE 4 Results of the Tukey's post hoc test for respiratory rates and specific activity of CAT, below and above the diagonal respectively

| | Central lineage | Karst lineage | Alpine lineage |
|-----------------|-----------------|---------------|----------------|
| Central lineage | | 0.978 | 0.0367 |
| Karst lineage | 0.0177 | | 0.0037 |
| Alpine lineage | 0.6851 | 0.0149 | |

Significant values are bolded.

between the lineages. In this phase of speciation, the processes of eco-physiological differentiation prevented lineages to hybridize, at least in the case of the Karst and the Alpine lineage. For example, with different thermal optima, co-occurring individuals will show different mating motivation and activity depending on the local water temperature. Whenever this is shifted strongly towards the optimum of either lineage, intra-lineage mating will dominate over crosses. Finally, the Bayesian skyline plots show no hard evidence of extinction (Figure 2).

Our study case is intriguing in two aspects. First, our conclusions emerged only when we combined multiple and diverse sources of information (genetics, distribution, ecology, physiology). Without any of these views, the conclusions would change. This illustrates how challenging the reconstructions of speciation are. We recognize that several aspects of our study remained correlative. For example, understanding fitness consequences of the presumed adaptation to local temperatures would require breeding experiments and transplant experiments; these data might further inform about the origin of these lineages. Second, our study illustrates that various factors contribute differently to the process of speciation (Butlin et al., 2012). While allopatry was important in both speciation events, we

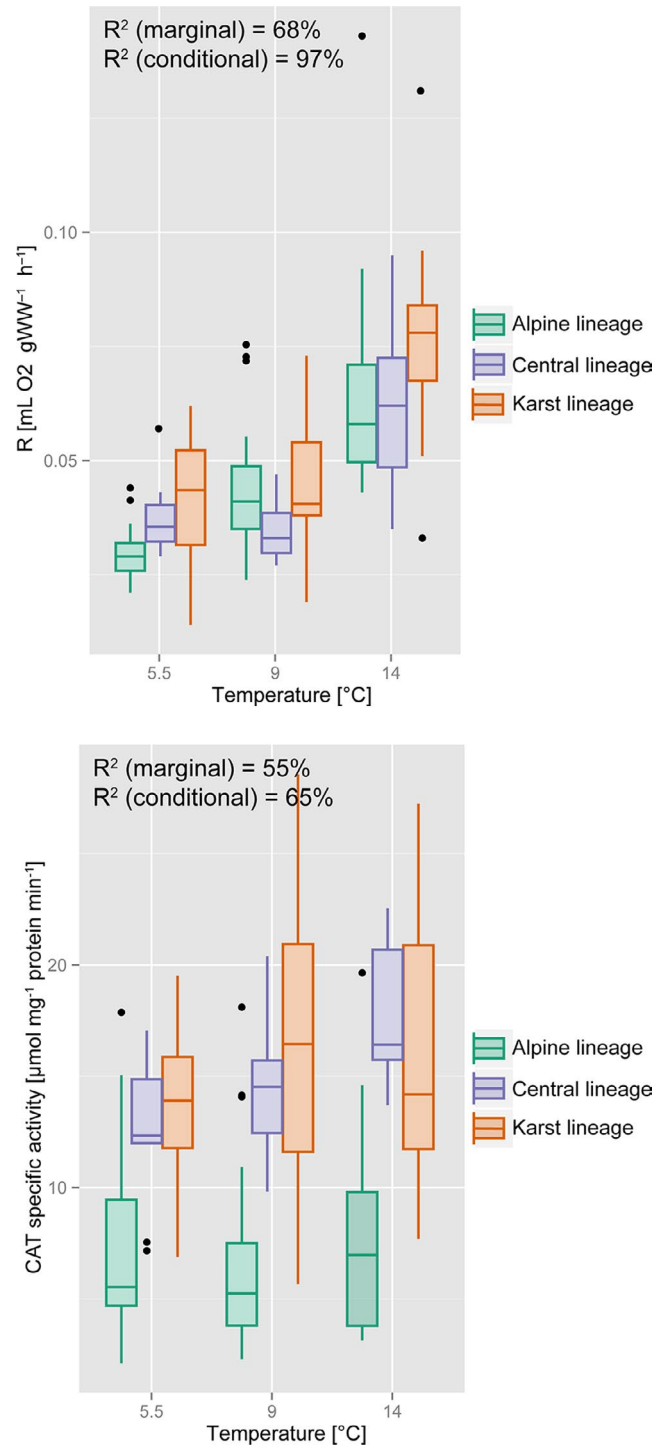


FIGURE 4 Boxplots showing the differences in respiratory rate (*R*) and specific activity of catalase (CAT) between the three lineages exposed to three different temperatures (5.5, 9 and 14°C). Sample outliers are shown as black dots

tentatively hypothesize ecological differentiation was important only for the second speciation to unfold. This conclusion is in line with a previous meta-analysis that found only limited generality in how Pleistocene glaciations affected evolutionary changes in diverse taxa (Burbrink et al., 2016).

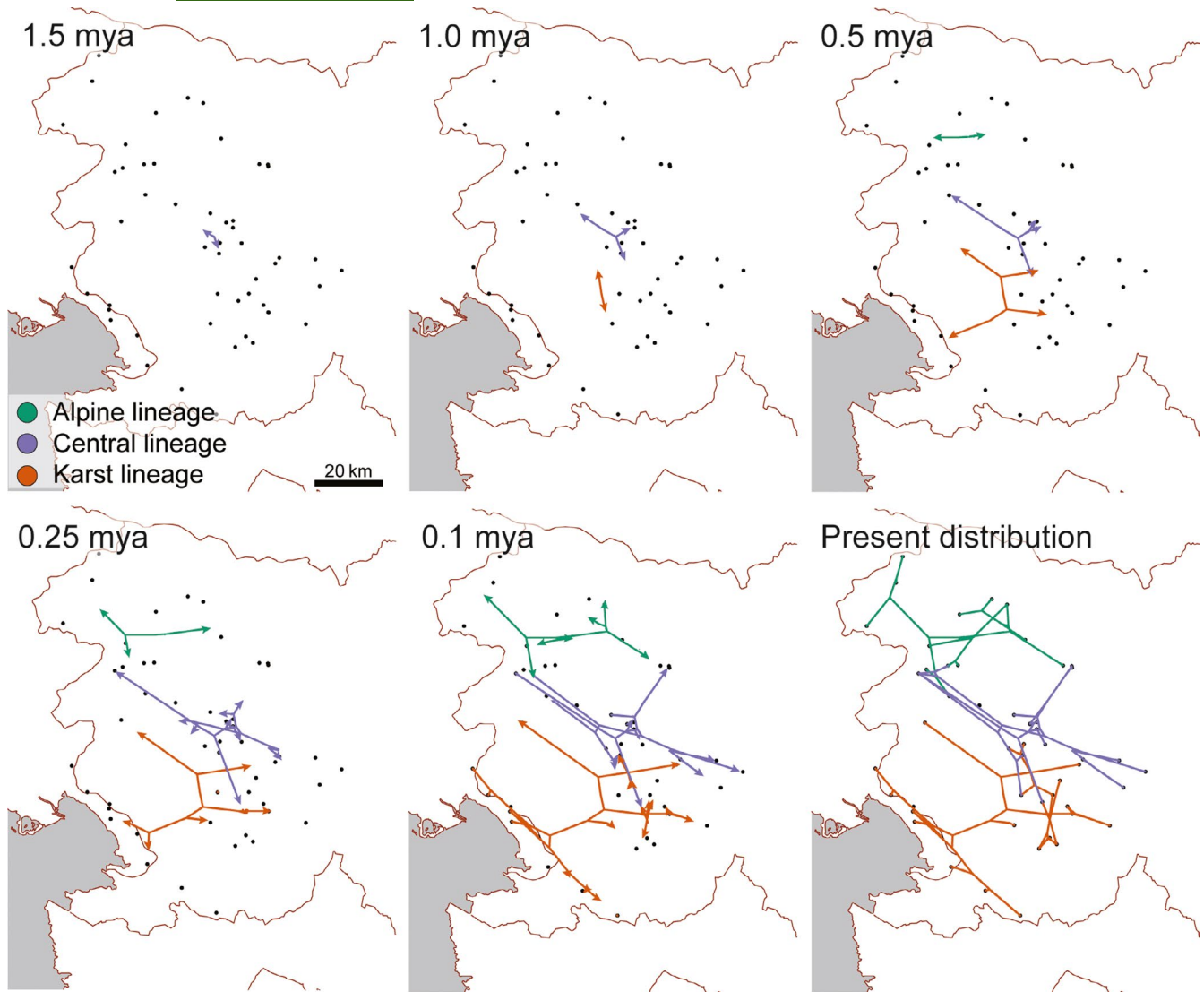


FIGURE 5 Geographical origin and dispersal patterns of the three lineages of *Niphargus stygius*, as inferred by Bayesian diffusion models from COI gene sequences. The timeframes were estimated by postulating a global COI molecular clock rate for gammaridean amphipods (0.018 substitutions per site per Mya; Nahavandi et al., 2013)

We conclude that studies aiming at predicting the impact of future climate oscillations need to thoroughly consider not only the climatic niche envelopes estimated from current species distribution, but also species propensity to evolve and disperse, in order to formulate sound hypotheses. Even then, the results should be considered with a sufficient amount of scepticism.

ACKNOWLEDGEMENTS

The study was funded by the Slovenian Research Agency (programs P1-0184 and P1-0255, projects N1-0069, N1-0096, J1-2464 and Z1-9164). No permits were required to conduct the research. The authors thank all the collectors or cavers accompanying them during the field work; Maja Zagmajster, Slavko Polak, Boris Sket, Primož Presetnik, Janja Matičič, Klara Hercog, Matija Perne, Andrej Drevenšek and all the co-workers.

CONFLICT OF INTEREST

The authors declare they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

All sequences are deposited in GenBank; accession numbers are available in the data files. All the data are available on Zenodo Digital Repository, <https://doi.org/10.5281/zenodo.5148050>.

ORCID

Teo Delić <https://orcid.org/0000-0003-4378-5269>

Peter Trontelj <https://orcid.org/0000-0003-4057-6912>

Valerija Zakšek <https://orcid.org/0000-0002-6503-7424>

Anton Brancelj <https://orcid.org/0000-0002-8767-3894>

Tatjana Simčič <https://orcid.org/0000-0001-6540-926X>

Fabio Stoch <https://orcid.org/0000-0003-4535-3769>

Cene Fišer <https://orcid.org/0000-0003-1982-8724>

REFERENCES

- Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M. A., & Alekseyenko, A. V. (2012). Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology and Evolution*, 29(9), 2157–2167. <https://doi.org/10.1093/molbev/mss084>
- Bahn, V., & McGill, B. J. (2013). Testing the predictive performance of distribution models. *Oikos*, 122(3), 321–331. <https://doi.org/10.1111/j.1600-0706.2012.00299.x>
- Barton, K. (2015). MuMIn: Multi-model inference. R package version 1.7.11. <https://cran.r-project.org/web/packages/MuMIn/index.html>
- Bielejec, F., Rambaut, A., Suchard, M. A., & Lemey, P. (2011). SPREAD: Spatial phylogenetic reconstruction of evolutionary dynamics. *Bioinformatics*, 27, 2910–2912. <https://doi.org/10.1093/bioinformatics/btr481>
- Bjarni, K., Kristjánsson, B. K., & Svavarsson, J. (2007). Subglacial refugia in Iceland enabled groundwater amphipods to survive glaciations. *The American Naturalist*, 170, 292–296. <https://doi.org/10.1086/518951>
- Brancelj, A. (2002). Fauna: Zooplankton, benthos and fish. In A. Brancelj (Ed.), *High-mountain lakes in the Eastern part of the Julian Alps* (pp. 137–157). ZRC Publishing and National institute of Biology.
- Bregović, P., Fišer, C., & Zagamajster, M. (2019). Contribution of rare and common species to subterranean species richness patterns. *Ecology and Evolution*, 9(20), 11606–11618. <https://doi.org/10.1002/ece3.5604>
- Brown, J. L., Hill, D. J., Dolan, A. M., Carnaval, A. C., & Haywood, A. M. (2018). PaleoClim, high spatial resolution paleoclimate surfaces for global land areas. *Nature – Scientific Data*, 5, 180254.
- Buggle, B., Hambach, U., Kehl, M., Marković, S. B., Zöller, L., & Glaser, B. (2013). The progressive evolution of a continental climate in SE-Central European lowlands during the Middle Pleistocene recorded in loess paleosol sequences. *Geology*, 41, 771–774.
- Burbrink, F. T., Chan, Y. L., Myers, E. A., Ruane, S., Smith, B. T., & Hickerson, M. J. (2016). Asynchronous demographic responses to Pleistocene climate change in Eastern Nearctic vertebrates. *Ecology Letters*, 12(12), 1457–1467. <https://doi.org/10.1111/ele.12695>
- Butlin, R., Debelle, A., Kerth, C., Snook, R. R., Beukeboom, L. W., Castillo Cajas, R. F., & Schilthuizen, M. (2012). What do we need to know about speciation? *Trends in Ecology and Evolution*, 27(1), 27–39.
- Carstens, B. C., & Knowles, L. L. (2007). Shifting distributions and speciation: Species divergence during rapid climate change. *Molecular Ecology*, 16, 619–627. <https://doi.org/10.1111/j.1365-294X.2006.03167.x>
- Castellanos-Morales, G., Gámez, N., Castillo-Gámez, R. A., & Eguarte, L. E. (2016). Peripatric speciation of an endemic species driven by Pleistocene climate change: The case of the Mexican prairie dog (*Cynomys mexicanus*). *Molecular Phylogenetics and Evolution*, 94, 171–181. <https://doi.org/10.1016/j.ympev.2015.08.027>
- Chen, I.-C., Hill, J. K., Ohlemüller, R., Roy, D. B., & Thomas, C. D. (2011). Rapid range shifts of species associated with high levels of climate warming. *Science*, 333(6045), 1024–1026.
- Cho, Y., Park, E., & Lim, C. (2000). Catalase, glutathione S-transferase and thioltransferase respond differently to oxidative stress in *Schizosaccharomyces pombe*. *Journal of Biochemistry and Molecular Biology*, 33(4), 344–348.
- Colson-Proch, C., Renault, D., Gravot, A., Douady, C. J., & Hervant, F. (2009). Do current environmental conditions explain physiological and metabolic responses of subterranean crustaceans to cold? *Journal of Experimental Biology*, 212(12), 1859–1868. <https://doi.org/10.1242/jeb.027987>
- Copilaş-Ciocianu, D., Rutová, T., Pařil, P., & Petrusek, A. (2017). Epigean gammarids survived millions of years of severe climatic fluctuations in high latitude refugia throughout the Western Carpathians. *Molecular Phylogenetics and Evolution*, 112, 218–229. <https://doi.org/10.1016/j.ympev.2017.04.027>
- Correggiari, A., Roveri, M., & Trincardi, F. (1996). Late Pleistocene and Holocene evolution of the North Adriatic Sea. II Quaternario. *Italian Journal of Quaternary Sciences*, 9(2), 697–704.
- Culver, D. C., & Pipan, T. (2014). *Shallow subterranean habitats: Ecology, evolution, and conservation*, 1st ed. Oxford University Press.
- Culver, D. C., & Pipan, T. (2019). *The Biology of caves and other Subterranean habitats* (2nd ed.). Oxford University Press.
- Delić, T., Stoch, F., Borko, Š., Flot, J. F., & Fišer, C. (2020). How did subterranean amphipods cross the Adriatic Sea? Phylogenetic evidence for dispersal–vicariance interplay mediated by marine regression–transgression cycles. *Journal of Biogeography*, 47, 1875–1887. <https://doi.org/10.1111/jbi.13875>
- Delić, T., Trontelj, P., Rendoš, M., & Fišer, C. (2017). The importance of naming cryptic species and the conservation of endemic subterranean amphipods. *Scientific Reports*, 7(1), 3391. <https://doi.org/10.1038/s41598-017-02938-z>
- Domínguez-Villar, D., Lojen, S., Krklec, K., Baker, A., & Fairchild, I. J. (2015). Is global warming affecting cave temperatures? Experimental and model data from a paradigmatic case study. *Climate Dynamics*, 45(3–4), 569–581. <https://doi.org/10.1007/s00382-014-2226-1>
- Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29(8), 1969–1973. <https://doi.org/10.1093/molbev/mss075>
- Elith, J., & Leathwick, J. R. (2009). Species distribution models: Ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution, and Systematics*, 40(1), 677–697. <https://doi.org/10.1146/annurev.ecolsys.110308.120159>
- Eme, D., Malard, F., Colson-Proch, C., Jean, P., Calvignac, S., Konecny-Dupré, L., Hervant, F., & Douady, C. J. (2014). Integrating phylogeography, physiology and habitat modelling to explore species range determinants. *Journal of Biogeography*, 41(4), 687–699. <https://doi.org/10.1111/jbi.12237>
- Eme, D., Zagamajster, M., Delić, T., Fišer, C., Flot, J.-F., Konecny-Dupré, L., Pálsson, S., Stoch, F., Zakšek, V., Douady, C. J., & Malard, F. (2017). Do cryptic species matter in macroecology? Sequencing European groundwater crustaceans yields smaller ranges but does not challenge biodiversity determinants. *Ecography*, 41(2), 424–436. <https://doi.org/10.1111/ecog.02683>
- Eme, D., Zagamajster, M., Fišer, C., Galassi, D., Marmonier, P., Stoch, F., Cornu, J.-F., Oberdorff, T., & Malard, F. (2015). Multi-causality and spatial non-stationarity in the determinants of groundwater crustacean diversity in Europe. *Ecography*, 38(5), 531–540. <https://doi.org/10.1111/ecog.01092>
- Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: New 1km spatial resolution climate surfaces for global land areas. *International Journal of Climatology*, 37(12), 4302–4315. <https://doi.org/10.1002/joc.5086>
- Foulquier, A., Malard, F., Lefebure, T., Douady, C. J., & Gibert, J. (2008). The imprint of Quaternary glaciers on the present-day distribution of the obligate groundwater amphipod *Niphargus virei* (Niphargidae). *Journal of Biogeography*, 35(3), 552–564. <https://doi.org/10.1111/j.1365-2699.2007.01795.x>
- Francis, A. P., & Currie, D. J. (2003). A globally consistent richness–climate relationship for angiosperms. *American Naturalist*, 161(4), 523–536. <https://doi.org/10.1086/368223>
- Fu, Y.-X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147, 915–925. <https://doi.org/10.1093/genetics/147.2.915>
- Galili, T. (2015). Dendextend: An R package for visualizing, adjusting, and comparing trees of hierarchical clustering. *Bioinformatics*, 31(22), 3718–3720. <https://doi.org/10.1093/bioinformatics/btv428>
- Gibbard, P. L., Head, M. J., & Walker, M. J. & Subcommission on Quaternary Stratigraphy (2010). Formal ratification of the Quaternary System/Period and the Pleistocene Series/Epoch with a base at 2.58 Ma. *Journal of Quaternary Science*, 25(2), 96–102.

- Hawkins, B. A., Field, R., Cornell, H. V., Currie, D. J., Guégan, J.-F., Kaufman, D. M., Kerr, J. T., Mittelbach, G. G., Oberdorff, T., O'Brien, E. M., Porter, E. E., & Turner, J. R. G. (2003). Energy, water and broad-scale geographic patterns of species richness. *Ecology*, 84(12), 3105–3117. <https://doi.org/10.1890/03-8006>
- Heibl, C., & Calenge, C. (2013). Phyloclim: Integrating phylogenetics and climatic niche modeling. R package version 0.9.5. <https://cran.r-project.org/web/packages/phyloclim/index.html>
- Hewitt, G. (2000). The genetic legacy of the Quaternary ice ages. *Nature*, 405, 907–913. <https://doi.org/10.1038/35016000>
- Hijmans, R. J. (2012). Cross-validation of species distribution models: removing spatial sorting bias and calibration with a null model. *Ecology*, 93, 679–688.
- Hijmans, R. J., Phillips, S., Leathwick, J., & Elith, J. (2015). Dismo: Species distribution modelling. R package version 1.1-4. <https://cran.r-project.org/web/packages/dismo/index.html>
- Holsinger, J. R. (1980). *Stygobromus canadensis*, a new subterranean amphipod crustacean (Crangonyctidae) from Canada, with remarks on Wisconsin refugia. *Canadian Journal of Zoology*, 58(2), 290–297.
- Holsinger, J. R., & Shaw, D. P. (1987). *Stygobromus quatsinensis*, a new amphipod crustacean (Crangonyctidae) from caves on Vancouver Island, British Columbia, with remarks on zoogeographic relationships. *Canadian Journal of Zoology*, 65(9), 2202–2209.
- Hosmer, D. W., Lemeshow, J. S., & Sturdivant, R. X. (2013). *Applied logistic regression* (3rd ed.). John Wiley & Sons Inc.
- Hothorn, T., Bretz, F., Westfall, P., Heiberger, R. M., Schuetzenmeister, A., Scheibe, S., & Hothorn, M. T. (2016). Multcomp: Simultaneous inference in general parametric models. R package version 1.4-16. <https://cran.r-project.org/web/packages/multcomp/index.html>
- Huang, J.-P., Hill, J. G., Ortego, J., & Knowles, L. L. (2020). Paraphyletic species no more – Genomic data resolve a Pleistocene radiation and validate morphological species of the *Melanoplus scudderii* complex (Insecta: Orthoptera). *Systematic Entomology*, 45(3), 594–605.
- Issartel, J., Hervant, F., Voituron, Y., Renault, D., & Vernon, P. (2005). Behavioural, ventilatory and respiratory responses of epigeal and hypogean crustaceans to different temperatures. *Comparative Biochemistry and Physiology – A Molecular and Integrative Physiology*, 141(1), 1–7. <https://doi.org/10.1016/j.cbpb.2005.02.013>
- Issartel, J., Renault, D., Voituron, Y., Bouchereau, A., Vernon, P., & Hervant, F. (2005). Metabolic responses to cold in subterranean crustaceans. *The Journal of Experimental Biology*, 208(15), 2923–2929. <https://doi.org/10.1242/jeb.01737>
- Issartel, J., Voituron, Y., Odagescu, V., Baudot, A., Guillot, Geneviève, Ruaud, J.-P., Renault, D., Vernon, P., & Hervant, Frédéric (2006). Freezing or supercooling: How does an aquatic subterranean crustacean survive exposures at subzero temperatures? *The Journal of Experimental Biology*, 209, 3469–3475. <https://doi.org/10.1242/jeb.02387>
- Ivory, S. J., Blome, M. W., King, J. W., McGlue, M. M., Cole, J. E., & Cohen, A. S. (2016). Environmental change explains cichlid adaptive radiation at Lake Malawi over the past 1.2 million years. *Proceedings of the National Academy of Sciences*, 113(42), 11895–11900. <https://doi.org/10.1073/pnas.1611028113>
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), 772–780. <https://doi.org/10.1093/molbev/mst010>
- Kearney, M. R., Wintle, B. A., & Porter, W. P. (2010). Correlative and mechanistic models of species distribution provide congruent forecasts under climate change. *Conservation Letters*, 3(3), 203–213. <https://doi.org/10.1111/j.1755-263X.2010.00097.x>
- Keller, I., & Seehausen, O. (2012). Thermal adaptation and ecological speciation. *Molecular Ecology*, 21(4), 782–799. <https://doi.org/10.1111/j.1365-294X.2011.05397.x>
- Knowles, L. L. (2001). Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Molecular Ecology*, 10, 691–701. <https://doi.org/10.1046/j.1365-294x.2001.01206.x>
- Knowles, L. L., & Massatti, R. (2017). Distributional shifts-not geographic isolation – As a probable driver of montane species divergence. *Ecography*, 40(12), 1475–1485. <https://doi.org/10.1111/ecog.02893>
- Knowles, L. L., & Richards, C. L. (2005). Importance of genetic drift during Pleistocene divergence as revealed by analyses of genomic variation. *Molecular Ecology*, 14, 4023–4032. <https://doi.org/10.1111/j.1365-294X.2005.02711.x>
- Konec, M., Delic, T., & Trontelj, P. (2016). DNA barcoding sheds light on hidden subterranean boundary between Adriatic and Danubian drainage basins. *Ecohydrology*, 9(7), 1304–1312. <https://doi.org/10.1002/eco.1727>
- Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2017). Partitionfinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 34, 772–773.
- Lanier, H. C., Massatti, R., He, Q., Olson, L. E., & Knowles, L. L. (2015). Colonization from divergent ancestors: Glaciation signatures on contemporary patterns of genomic variation in Collared Pikas (*Ochotona collaris*). *Molecular Ecology*, 24, 3688–3705.
- Leaché, A. D., & Fujita, M. K. (2010). Bayesian species delimitation in West African forest geckos (*Hemidactylus fasciatus*). *Proceedings of the Royal Society B*, 277, 3071–3077.
- Lee-Yaw, J. A., Kharouba, H. M., Bontrager, M., Mahony, C., Csörgő, A. M., Noreen, A. M. E., Li, Q., Schuster, R., & Angert, A. L. (2016). A synthesis of transplant experiments and ecological niche models suggests that range limits are often niche limits. *Ecology Letters*, 19, 710–722. <https://doi.org/10.1111/ele.12604>
- Lefebvre, T., Douady, C. J., Malard, F., & Gibert, J. (2007). Testing dispersal and cryptic diversity in a widely distributed groundwater amphipod (*Niphargus rhenorhodanensis*). *Molecular Phylogenetics and Evolution*, 42, 676–686. <https://doi.org/10.1016/j.ympev.2006.08.020>
- Lemey, P., Rambaut, A., Welch, J. J., & Suchard, M. A. (2010). Phylogeography takes a relaxed random walk in continuous space and time. *Molecular Biology and Evolution*, 27, 1877–1885. <https://doi.org/10.1093/molbev/msq067>
- Lencioni, V., Bernabò, P., & Latella, L. (2010). Cold resistance in two species of cave-dwelling beetles (Coleoptera: Cholevidae). *Journal of Thermal Biology*, 35(7), 354–359. <https://doi.org/10.1016/j.jtherbio.2010.07.004>
- Mammola, S., Goodacre, S. L., & Isaia, M. (2018). Climate change may drive cave spiders to extinction. *Ecography*, 41(1), 233–243. <https://doi.org/10.1111/ecog.02902>
- Mammola, S., Piano, E., Malard, F., Vernon, P., & Isaia, M. (2019). Extending Janzen's hypothesis to temperate regions: A test using subterranean ecosystems. *Functional Ecology*, 33(9), 1638–1650. <https://doi.org/10.1111/1365-2435.13382>
- Marzahn, E., Mayer, W., Joger, U., Ilgaz, Ç., Jablonski, D., Kindler, C., & Fritz, U. (2016). Phylogeography of the *Lacerta viridis* complex: Mitochondrial and nuclear markers provide taxonomic insights. *Journal of Zoological Systematics and Evolutionary Research*, 54(2), 85–105.
- Mermillod-Blondin, F., Lefour, C., Lalouette, L., Renault, D., Malard, F., Simon, L., & Douady, C. J. (2013). Thermal tolerance breadths among groundwater crustaceans living in a thermally constant environment. *The Journal of Experimental Biology*, 216, 1683–1694. <https://doi.org/10.1242/jeb.081232>
- Nahavandi, N., Ketmaier, V., Plath, M., & Tiedemann, R. (2013). Diversification of Ponto-Caspian aquatic fauna: Morphology and molecules retrieve congruent evolutionary relationships in *Pontogammarus maeoticus* (Amphipoda: Pontogammaridae). *Molecular Phylogenetics and Evolution*, 69(3), 1063–1076. <https://doi.org/10.1016/j.ympev.2013.05.021>
- Nakagawa, S., & Schielzeth, H. (2013). A general and simple method for obtaining R² from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, 4(2), 133–142.

- Nosil, P. (2012). *Ecological speciation*. Oxford University Press.
- Phillips, S. J., Anderson, R. P., & Schapire, R. E. (2006). Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, 190(3–4), 231–259. <https://doi.org/10.1016/j.ecolm.odel.2005.03.026>
- Pinheiro, J., Bates, D., DebRoy, S., Heisterkamp, S., & Van Willigen, B. (2016). nlme: Linear and nonlinear mixed effects models. R package version 3.1-149. <https://cran.r-project.org/web/packages/nlme/index.html>
- Quintero, I., & Wiens, J. J. (2013). Rates of projected climate change dramatically exceed past rates of climatic niche evolution among vertebrate species. *Ecology Letters*, 16(8), 1095–1103. <https://doi.org/10.1111/ele.12144>
- R Core Team. (2016). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.
- Reeve, J., Ortiz-barrientos, D., Engelsta, J., & Engelsta, J. (2016). The evolution of recombination rates in finite populations during ecological speciation. *Proceedings of the Royal Society B: Biological Sciences*, 238, 1841. <https://doi.org/10.1098/rspb.2016.1243>.
- Rödger, D., & Engler, J. O. (2011). Quantitative metrics of overlaps in Grinnellian niches: Advances and possible drawbacks. *Global Ecology and Biogeography*, 20(6), 915–927. <https://doi.org/10.1111/j.1466-8238.2011.00659.x>
- Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 34, 3299–3302. <https://doi.org/10.1093/molbev/msx248>.
- Sánchez-Fernández, D., Rizzo, V., Cieslak, A., Faille, A., Fresneda, J., & Ribera, I. (2016). Thermal niche estimators and the capability of poor dispersal species to cope with climate change. *Scientific Reports*, 6, 23381. <https://doi.org/10.1038/srep23381>
- Saupe, E. E., Myers, C. E., Peterson, A. T., Soberón, J., Singarayer, J., Valdes, P., & Qiao, H. (2019). Spatio-temporal climate change contributes to latitudinal diversity gradients. *Nature Ecology & Evolution*, 3, 1419–1429. <https://doi.org/10.1038/s41559-019-0962-7>.
- Sket, B. (1974). *Niphargus stygius* (Schiodte) (Amphipoda, Gammaridae) – die Neubeschreibung des Generotypus, Variabilität, Verbreitung und Biologie der Art, I. *Biološki Vestnik*, 22, 91–103.
- Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585–595. <https://doi.org/10.1093/genetics/123.3.585>.
- Tobler, M., Palacios, M., Chapman, L. J., Mitrofanov, I., Bierbach, D., Plath, M., Arias-Rodriguez, L., Garcia de León, F. J., & Mateos, M. (2011). Evolution in extreme environments: Replicated phenotypic differentiation in livebearing fish inhabiting sulfidic springs. *Evolution*, 65(8), 2213–2228. <https://doi.org/10.1111/j.1558-5646.2011.01298.x>
- Trontelj, P., Borko, Š., & Delić, T. (2019). Testing the uniqueness of deep terrestrial life. *Scientific Reports*, 9(1), 1–9. <https://doi.org/10.1038/s41598-019-51610-1>
- Trontelj, P., Douady, C. J., Fišer, C., Gibert, J., Gorički, Š., Lefebure, T., Sket, B., & Zakšek, V. (2009). A molecular test for cryptic diversity in ground water: How large are the ranges of macrostygobionts? *Freshwater Biology*, 54(4), 727–744. <https://doi.org/10.1111/j.1365-2427.2007.01877.x>
- Verovnik, R., Sket, B., & Trontelj, P. (2005). The colonization of Europe by the freshwater crustacean *Asellus aquaticus* (Crustacea: Isopoda) proceeded from ancient refugia and was directed by habitat connectivity. *Molecular Ecology*, 14, 4355–4369. <https://doi.org/10.1111/j.1365-294X.2005.02745.x>
- Wachter, G. A., Papadopoulou, A., Muster, C., Arthofer, W., Knowles, L. L., Steiner, F. M., & Schlick-Steiner, B. C. (2016). Glacial refugia, recolonization patterns and diversification forces in Alpine-endemic *Megabunus* harvestmen. *Molecular Ecology*, 25, 2904–2919.
- Waldock, C., Dornelas, M., & Bates, A. E. (2018). Temperature-driven biodiversity change: Disentangling space and time. *BioScience*, 68(11), 873–884.
- Warren, D. L., Cardillo, M., Rosauer, D. F., & Bolnick, D. I. (2014). Mistaking geography for biology: Inferring processes from species distributions. *Trends in Ecology and Evolution*, 29(10), 572–580. <https://doi.org/10.1016/j.tree.2014.08.003>
- Warren, D. L., Glor, R. E., & Turelli, M. (2008). Environmental niche equivalency versus conservatism: Quantitative approaches to niche evolution. *Evolution*, 62(11), 2868–2883. <https://doi.org/10.1111/j.1558-5646.2008.00482.x>
- Yang, Z., & Rannala, B. (2014). Unguided species delimitation using DNA sequence data from multiple loci. *Molecular Biology and Evolution*, 31(12), 3125–3135. <https://doi.org/10.1093/molbev/msu279>
- Zagmajster, M., Eme, D., Fišer, C., Galassi, D., Marmonier, P., Stoch, F., Cornu, J.-F., & Malard, F. (2014). Geographic variation in range size and beta diversity of groundwater crustaceans: Insights from habitats with low thermal seasonality. *Global Ecology and Biogeography*, 23(10), 1135–1145. <https://doi.org/10.1111/geb.12200>
- Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. Springer.

BIOSKETCHES

The authors are connected by their interests in natural history of specialized, cave adapted taxa from the Dinaric Karst and evolutionary mechanisms underlying their speciation and diversification patterns. They collaborate on questions of subterranean amphipod biodiversity based at SubBio Lab, University of Ljubljana, Slovenia (<http://subbio.net/>), Evolutionary Biology & Ecology, Université libre Bruxelles (<http://ebe.ulb.ac.be/>), National Institute of Biology (<http://www.nib.si/>) and University of Nova Gorica (<http://www.ung.si/>).

Author contributions: C.F., P.T. and T.D. conceived the ideas, T.D., F.S. and V.Z. collected the data, T.D. analysed the data, T.S. and A.B. executed the laboratory experiments, and T.D., C.F. and P.T. led the writing.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Delić, T., Trontelj, P., Zakšek, V., Brancelj, A., Simčič, T., Stoch, F., & Fišer, C. (2021). Speciation of a subterranean amphipod on the glacier margins in South Eastern Alps, Europe. *Journal of Biogeography*, 00, 1–13. <https://doi.org/10.1111/jbi.14275>