PENSOFT
SENCKENBERG world of biodiversity
Leibniz Association

Extra-Mediterranean glacial refugia and range dynamics in a groundwater amphipod species, *Niphargus fontanus* Spence Bate, 1859, in western Central Europe

Dieter Weber[1], Martin Wendt[2], Thomas Schmitt[1,3]

1 *Senckenberg Deutsches Entomologisches Institut, Eberswalder Straße 90, 15374 Müncheberg, Germany*
2 *Leibniz-Zentrum für Agrarlandschaftsforschung (ZALF) e. V., Eberswalder Straße 84, 15374 Müncheberg, Germany*
3 *Entomology and Biogeography, Institute of Biochemistry and Biology, Faculty of Science, University of Potsdam, 14476 Potsdam, Germany*

https://zoobank.org/7503EC5A-29A3-40D3-9335-21B6545A3703

Corresponding author: Dieter Weber (dieter.weber124@gmx.de)

Received 23 November 2023
Accepted 04 November 2024
Published 19 February 2025

Academic Editors Martin Schwendtner, Anna Hundsdörfer

Citation: Weber D, Wendt M, Schmitt T (2025) Extra-Mediterranean glacial refugia and range dynamics in a groundwater amphipod species, *Niphargus fontanus* Spence Bate, 1859, in western Central Europe. Arthropod Systematics & Phylogeny 83: 15–29. https://doi.org/10.3897/asp.83.e116160

Abstract

The biogeography, taxonomy and systematics of ground water organisms is still poorly understood. This is partly due to the difficult accessibility of the habitats and the expert knowledge required for identification. Nevertheless, due to the large distribution range and limited dispersal possibilities of amphipods such as the genus *Niphargus*, important insights can be gained into biogeographical patterns and evolutionary processes in subterranean ecosystems. *Niphargus* is the most species-rich genus of freshwater amphipods worldwide and holds great potential for cryptic species whose identification is important for the reconstruction of biogeographic patterns and events. Therefore, we assessed the genetic patterns of *Niphargus fontanus* Spence Bate, 1859 (Amphipoda: Niphargidae). We sampled the species all over its current distribution and sequenced one mitochondrial and three nuclear gene fragments. We discovered that most records from France were probably misidentifications, and that the species does not occur in central and southern France. Nevertheless, the distribution area extends from Wales to Bavaria, which could make it one of the largest distribution areas within the genus. Compared with other *Niphargus*, the genetic diversity and differentiation of *N. fontanus* is low and most likely evolved since the mid-Pleistocene Transition, but reflects a clear phylogeographic pattern with about 13 genetic lineages. These apparently stand for a number of extra-Mediterranean glacial refugia from which postglacial expansion was low to moderate. However, few cases of disjunction within these genetic lineages exist, most likely resulting from rapid expansions along river Rhine which otherwise mostly acted as a dispersal barrier.

Key words

Biogeography, dispersal barriers, mid-Pleistocene Transition, cryptic glacial refugia, groundwater organisms, subterranean ecosystems

1. Introduction

The distribution patterns of animals and plants has been fascinating scientists since long (Darwin 1838, Wallace 1876). In particular, the effects of geological dynamics and climatic fluctuations on distribution patterns has been intensively discussed (Avise 2000). In Europe, the glacial cycles of the Pleistocene had particularly severe influences on range changes (Hewitt 1996), and the retreat of warm-adapted species to so-called Mediterranean refugia located in specific areas of the Mediterranean Basin has been postulated many decades ago (De Lattin

1949). However, it is getting increasingly clear that Mediterranean refugia were not the only refugia for temperate species in Europe but that survival was also possible further north in small, climatically favoured pockets in so-called extra-Mediterranean refugia (Schmitt and Varga 2012).

While the European phylogeographic patterns of terrestrial and epigean (i.e. surface) fresh water organisms are already well understood in their dynamics throughout time (Hewitt 2004), groundwater organisms are much more hidden and hence enigmatic, even in Europe. Although groundwater forms by far the largest habitats on the continents (Molden 2007), we are far away from a comprehensive understanding of its species, and there is still much need for single case studies. Despite its lack of nutrients, some groups, especially crustaceans, have managed to colonise groundwater. In this aspect, amphipods of the family Niphargidae are of particular interest. They live almost exclusively in groundwater, and as much as 447 species have already been described for its most species-rich genus *Niphargus* (Horton et al. 2022) (accessed 26 Oct 2024).

Representatives of the genus *Niphargus* occur from Spain in the west (Karaman 2017a) to Iran in the east (Esmaeili-Rineh et al. 2017). In southern France, Italy and the Balkan Peninsula, the genus is particularly rich in species, but the number of species decreases towards the north and north-east (Zagmajster et al. 2014). Thus, seven or eight species are present in Belgium (Fišer et al. 2018), six in the Netherlands (Notenboom 1994), about seven in England, the Channel Islands and Wales (McInerney et al. 2014, Knight et al. 2015), two in Ireland (McInerney et al. 2014, Weber et al. 2021b), and none in Scotland. 30 species are mentioned for Germany, of which only about 17 are plausible (Weber 2022), while Poland hosts five (Skalski 1994). Niphargid species usually colonise particularly small geographical areas in the southern parts of the genus' distribution (Fišer et al. 2006, Karaman 2017b) but much larger areas in the northern parts (McInerney et al. 2014, Weber 2022). However, only a few reliable data based on genetic analyses exist that address their distributions in more northern areas, i.e. *Niphargus puteanus* (C.L. Koch in Panzer, 1836): 340 km core range, 756 km if satellite populations are included (Weber et al. 2020), *Niphargus schellenbergi* S. Karaman, 1932: 500 km (Weber 2022), *Niphargus tonywhitteni* Fiser, Alther, Zaksek, Borko, Fuchs & Altermatt, 2018: 400 km (Weber 2023), and *Niphargus enslini* S. Karaman, 1932: 95 km (Weber et al. 2021a).

Generally, the older publications on *Niphargus* deal with their identification and distribution and are based on morphology (Karaman 1932, Schellenberg 1935b, 1942). More recent studies included genetic analyses, hereby unravelling numerous cryptic species (e.g. Stoch 1998, Hartke et al. 2011, McInerney et al. 2014, Eme et al. 2018, Horton et al. 2022, Weber 2022b). Further aspects, such as their ecology (Weber and Weigand 2023) or biogeographical dynamics, are still mostly unknown. Although a publication of Schellenberg (1935b) almost 100 years ago already had glacial relics in its title, it is mostly restricted to the delivery of a list of sampling sites. As an exception, the more than 50-years-old publication by Ginet (1971) deals with the biogeography of *Niphargus virei* Chevreux, 1896 and *Niphargus rhenorhodanensis* Schellenberg, 1937. As discovered afterwards, both taxa represent a species complex (Lefébure et al. 2006, 2007, Delić et al. 2023), strongly reducing the value of the concepts expressed in Ginet's publication due to a mixing of taxa. In addition, only some few more biogeographic studies on *Niphargus* from its northern distribution area based on genetic analyses have been added since then (McInerney et al. 2014, Weber et al. 2020).

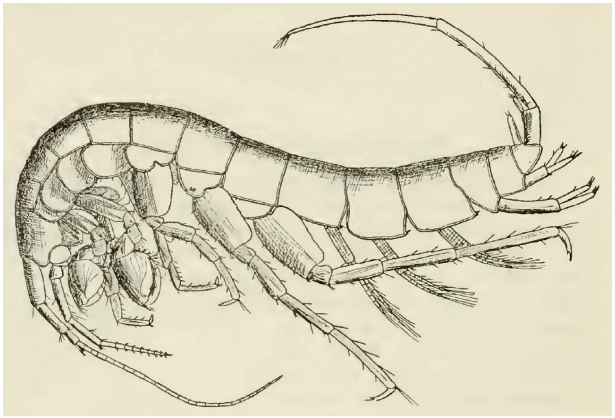

Figure 1. Drawing of *Niphargus fontanus* from Chevreux 1901.

This paper deals with the biogeographic history of *Niphargus fontanus* (Fig. 1), a widespread but rare species in the northern parts of the distribution of this genus. This species was first described by Spence Bate (1859) more than 160 years ago. Unfortunately, the type locality was not published, and Spence Bate apparently had specimens from two sites available. One of these was Corsham (20 km east of Bristol, SW England). For the description, however, Spence Bate used specimens from another not named site because he noted that the specimens described differed morphologically from those of Corsham. A tube with two specimens in the British Museum of Natural History collection is labelled: "Ringwood, Hampshire, England Hants. coll. Rev. A. R. Hogan. 1952: 5:7: 123–124", where 1952 is not the date of collection (Gledhill 1980). These two specimens were considered as syntypes from which one was selected as lectotype with the registration no. 1978: 190 (Gledhill 1980). Therefore, Ringwood (10 km north of Bornemouth, SW England) is the type locality. Since its description in the UK, *N. fontanus* has been reported from the Channel Islands (Knight et al. 2015), France (Chevreux 1901), Belgium (Wolf 1934), Germany (Wolf 1934), and Switzerland (Alther et al. 2021).

Thus, the published records based on morphological determinations revealed a wide distribution of *N. fontanus* of 1150 km as the crow flies. This is rather large, even for the northern distribution range of the genus *Niphargus*, as a distribution from 100 to a maximum of 750 km, more likely 100 to 500 km, should be assumed (see above). We therefore have to question whether this wide distribution based on morphological determination might

be due to failure to recognise cryptic or pseudo-cryptic species, as it was the case of *Niphargus aquilex* Schioedte, 1855 (Weber et al. 2023).

Doubtlessly, *Niphargus fontanus* s. str. (for more information on the delimitation of this species see material and methods) is distributed from England to Germany and Switzerland (McInerney et al. 2014). However, its numerous mentions from France (many records from the Grand Est to Provence, rarely in Auvergne and Pyrenees), all determined only morphologically, remain questionable (Gibert et al. 2005). If these records are correct, they would stand for glacial survival of this species, at least partly, in Mediterranean refugia. If not, *N. fontanus* would represent a species with exclusive extra-Mediterranean refugia north of the Mediterranean Basin (Schmitt 2009, Schmitt and Varga 2012). We therefore hypothesise: The populations so far considered as *N. fontanus* in central and southern France belong to different species. Also, we consequently assume survival of *N. fontanus* in permafrost regions or even under the glacial shield.

So far, all investigated representatives of the genus *Niphargus* in the northern part of the distribution range of the genus have a high level of intra- and interspecific diversity (Weber et al. 2020, 2021a, 2022b, 2023). As only its larger distribution range is remarkably distinguishing *N. fontanus* from these species, we assume that it has a similar genetic make-up to these other *Niphargus* species. Based on this, our second hypothesis is that *N. fontanus* has a genetic diversity and differentiation comparable with other species of this genus in the northern part of its distribution.

Following Ward and Palmer (1994), the distribution of the interstitial freshwater meiofauna is influenced by a variety of different ecological factors at different geographical scales. Since *N. fontanus* is not abundant but widespread, and since it has been proven that not only small species but also juveniles of medium-sized *Niphargus* species occur in the interstitial (Weber and Weigand 2023), it has to be assumed that *N. fontanus* spreads in the free space of the interstitial. Therefore, our third hypothesis is that large river systems should not hamper but might even foster the dispersal and hence the range size of *N. fontanus*.

2. Material and methods

2.1. Study species

McInerney et al. (2014) reported three strongly distinct genetic groups of *N. fontanus* from the UK, Belgium and France (A–C). However, sequences of *N. fontanus* B, only reported from Vaux (close to Metz, Dept. Moselle, France), were not deposited in openly accessible databases (McInerney et al. 2014) and therefore cannot be considered as they are unknown. *N. fontanus* C was collected only in the French Jura, but was not further encountered, although we intensively searched for it. However, the existing sequence from Genbank is not clustering within other *N. fontanus* individuals, hence most likely resembling a different species. As a consequence, *N. fontanus* A is considered as *N. fontanus* s. str. and studied in this publication.

2.2. Collecting and preservation of material

From 2016 to 2020, individuals of *N. fontanus* were collected (and confirmed as such by sequencing) as follows: five specimens from three sites in the United Kingdom, three specimens from two sites in Belgium, six specimens from five sites in France, seven specimens from four sites in Luxembourg, and 57 specimens from 28 sites in Germany (Table S1). They were collected from hyporheic interstitial (three specimens), springs (27 specimens), cave waters (including artificial cavities; puddles, lakes, and rivers; 44 specimens), and boreholes (one specimen).

Interstitial was sampled using the Karaman-Chappuis-method (Chappuis 1942). In caves and artificial cavities, specimens were collected mainly in an opportunistic way by hand with the assistance of a small tea sieve. A sieve set with mesh sizes of 5 mm, 1 mm, 0.5 mm, and 0.2 mm was mainly used for springs and partly in caves and artificial cavities. Mud, foliage and moss was rinsed with water and niphargids were collected in the three lower sieves with a spring steel forceps. Meat-baited tin cans with chicken liver as bait were used in shaft wells and partly in caves and artificial cavities. They were emptied after 2–3 days.

Specimens were sorted, and individuals identified as *N. fontanus* by sequencing were used for further analyses in this study. Each specimen was separately labelled (in case of > 20 specimens from the same site, 5–10, preferably juveniles, were kept in one tube), preserved in 96 % non-denaturated ethanol and kept at -20°C. Whenever possible, at least one male and one female were preserved in 70 % ethanol at room temperature for morphological investigation.

2.3. DNA Extraction, PCR and sequencing

One pereiopod of each specimen was used for DNA isolation. DNA was extracted using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek) following the "DNA Extraction and Purification from Tissue" protocol. Samples were lysed using Proteinase K (Omega Bio-Tek).

The Folmer's fragment of the cytochrome *c* oxidase subunit 1 (COI) gene was amplified via polymerase chain reaction (PCR) (Folmer et al. 1994). The primers (see Table S2 for primers of all sequenced markers) HCO2198-JJ and LCO1490-JJ (Astrin and Stüben 2008) were used for COI amplification. The PCR mix contained 1.5 µl DNA extract (of variable concentration), 1 µl primer mix (10 pmol/µl, corresponding to 0.5 µl of each primer), 7.5 µl of 2x Multiplex PCR Master Mix (Qiagen) con-

taining HotStarTaq Plus DNA Polymerase, and 5 µl of ultrapure water. The mix was heated in a thermocycler to 95°C, kept for 5 min, and then treated 38 cycles with 30 s at 95°C, 90 s at 49°C and 60 s at 72°C, followed by a final elongation step of 30 min at 68°C. The COI marker was sequenced with the same primer pair as used for PCR amplification.

In addition, a fragment of the nuclear 28S ribosomal RNA gene (28S) was sequenced with two primers (Verovnik et al. 2005), named later as Niph15 and Niph16 (Weber et al. 2021b). The PCR mix contained 2 µl DNA extract (with variable concentration), 1 µl of each primer (10 pmol/µl), 0.2 µl REDTaq polymerase (Sigma-Aldrich), 5 µl REDTaq reaction buffer and 15.8 µl ultrapure water. Sequencing conditions were an initial 3 min denaturation step at 95°C, followed by 56 cycles of 30 s denaturation at 94°C, 60 s annealing at 45°C, and 90 s extension at 72°C. Sanger sequencing was performed with the three primers Niph15, Niph20, Niph21 (Flot et al. 2010a).

The ITS region (18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence) (ITS) was also analysed. The PCR mix contained 1 µl DNA extract (of variable concentration), 1 µl ITS primer mix (10 pmol/µl, corresponding to 0.5 µl of each primer), 7.5 µl of 2x Multiplex PCR Master Mix, and 5.5 µl of ultrapure water. The mix was heated in a thermocycler to 95°C, kept for 5 min, and then treated 35 cycles with 30 s at 95°C, 90 s at 52°C and 60 s at 72°C, followed by a final elongation step of 30 min at 68°C.

The histone H3 gene (H3) was amplified using the primers H3aF2 and H3aR2 (Colgan et al. 1998). The PCR mix contained 1 µl DNA extract (of variable concentration), 1 µl H3 primer mix (10 pmol/µl, corresponding to 0.5 µl of each primer), 7.5 µl of 2x Multiplex PCR Master Mix, and 5.5 µl of ultrapure water. The mix was heated in a thermocycler to 95°C, kept for 5 min, and then treated 38 cycles with 30 s at 95°C, 60 s at 50°C and 90 s at 72°C, followed by a final elongation step of 30 min at 68°C. The H3 marker was sequenced with the same primer pair as used for PCR amplification.

Amplification success of PCR reactions in all cases was verified via agarose gel electrophoresis. Bidirectional Sanger sequencing was performed by Genoscreen and Macrogen.

2.4. Datasets analysed

For our publication, we generated 72 COI sequences from 41 sites (Table S1). Additionally, we downloaded all COI sequences which were named as *N. fontanus* from Genbank (40 sequences). Furthermore, we added all COI sequences from Genbank (excluding all incomplete ones) of the *Niphargus krameri* Schellenberg species group with 23 species defined by Stoch et al. (2024) as sister group to *N. fontanus*. Then, we added our *N. fontanus* sequences, collapsed duplicates and obtained 126 haplotypes. We processed a NJ tree (Fig. S1) in Mega X (Kumar et al. 2018) using default settings; we did not correct obvious mistakes as this was not in scope of our paper; we used this dataset for ASAP species delimitation (Puillandre et al. 2021). Specimens named in Genbank as *N. fontanus*, but belonging to other species by ASAP (i.e. representing misidentifications) were omitted for further analysis. Finally, after this purification procedure, we ended up with 38 additional COI sequences from Genbank (Hartke et al. 2011, McInerney et al. 2014, Eme et al. 2018, Alther et al. 2021).

We created a Bayesian tree based on the mtDNA dataset using Beast v. 2.5 (Bouckaert et al. 2014). A sequence of *N. krameri* was used as outgroup to root the tree. We used an HKY model with empirical base frequency settings and a gamma category count of 4. Based on the ESS values, the coalescent exponential tree setting was chosen over the coalescent constant population size tree setting. We used a relaxed clock log normal setting with a clock-rate of 0.0177 (Papadopoulou et al. 2010). We performed the analysis with 30 million generations, sampling every 3000 iterations. The MCMC chain was controlled for convergence in Tracer v. 1.7.1 and a burn-in of 10 % was applied. We used TREEANNONTATOR v. 2.5 to generate a consensus tree with common ancestor height. We used FigTree v. 1.4.4 (Rambout 2018) for visualisation.

The generated input and consensus tree was used for an ancestral occurrence reconstruction analysis using RASP v. 4.2 (Yu et al. 2020). Based on the BiogeoBears model test, an S-Dec analysis was performed with maxarea settings of 4. Additionally, a Bayesian Binary MCMC (BBM) analysis was performed. The number of maximum areas was kept at 4, the fixed JC+G (Jukes-Cantor + Gamma) was used with 5,000,000 cycles, 10 chains, a temperature of 0.1 and sampling every 100 generations. The individual sequences were assigned to regions coded as consecutive numbers (R1–R7) from south-east to north-west. These areas were R1 (Switzerland), R2 (south-western Germany, mostly Baden-Württemberg), R3 (Alsace in north-eastern France), R4 (western Germany, mostly North Rhine-Westphalia and Hesse), R5 (Moselle + Meuse), R6 (British Channel region) and R7 (England and Wales). Alternative assignments for the French samples were tested and are further described in Figure S2.

For nuclear genes, we generated 18 *N. fontanus* sequences of 28S from 17 different sites, spread all over the distribution area (Table S1). For interspecific comparison of intraspecific genetic diversity and differentiation, 28S sequences of *N. aquilex* (12 individuals) and *N. schellenbergi* (55) were downloaded from Genbank (accessed 5 April 2023). We also added 46 *N. puteanus* 28S sequences from Weber et al. (2020). All other *N. puteanus* sequences in Genbank were excluded as there are obviously numerous unrelated species stored there under this species name. The haplotype network was created using HaplowebMaker (Spöri and Flot 2020).

Ten ITS sequences were generated from ten sites. So far, no ITS sequences of *N. fontanus* were available in Genbank. Ten H3 sequences were generated and supplemented by three H3 sequences from Genbank.

All in all, we sequenced approximately 2,000 niphargids of which 371 were collected in the southern half of France, allowing for a comprehensive understanding of the distribution of *N. fontanus*. Species identity of all of them was identified using Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastp). DnaSP v. 6 (Rozas et al. 2017) was used for calculating haplotype mismatch distribution (Patarnello et al. 2007), Tajima's *D* (Tajima 1989), and Li's *D** and *F** (Fu and Li 1993). Colours and names of colours follow https://www.w3schools.com/colors/colors_names.asp (Fig. S3). Genbank accession numbers of all sequences of *N. fontanus* are given in Table S1.

3. Results

Using all COI sequences of *N. fontanus* and COI sequences from Genbank of the *Niphargus krameri* group with 23 species defined by Stoch et al. (2024) as sister group to *N. fontanus*, the ASAP species delimitation method identified 24 MOTUs (= Molecular Operational Taxonomic Units) in partition 1, and 12 MOTUs in partition 2. In both partitions, it found *N. fontanus* being one single species with 46 haplotypes while one haplotype stored in Genbank as *N. fontanus* from two sites (in Departments Ain and Haut-Savoie, France) clearly does not belong to this species and hence has to be considered as misidentification (Table S3).

We confirmed *N. fontanus* by COI sequences in the UK, Belgium (Wallonia), Luxembourg, Germany (North Rhine-Westphalia, Hesse, Rhineland-Palatinate, Baden-Württemberg, Bavaria) and Switzerland. In France, we confirmed its presence only in the very North and in Alsace. In turn, not a single specimen of the 371 niphargids collected in southern France was identified as *N. fontanus*. The *Niphargus* sequenced from southern France belong to the species or species complexes *Niphargus kochianus* Spence Bate, 1859, *N. rhenorhodanensis*, *N. schellenbergi*, *N. virei*, and to three species whose sequences are not yet stored in Genbank.

While the nuclear 28S fragment (994 bp) of *N. fontanus* (Fig. 2) only shows two haplotypes with only one base pair difference, three sympatric *Niphargus* taxa have considerably higher internal diversity and differentiation. Thus, *N. puteanus* shows eight haplotypes with a maximum of three base pairs difference, *N. schellenbergi* seven haplotypes with a maximum of 24 base pairs difference. The *N. aquilex* complex has eight haplotypes with a maximum of 76 base pairs difference.

The ten *N. fontanus* specimens sequenced for the nuclear ITS from ten different localities split into four haplotypes; two specimens had sequences with 1936 base pairs and with three base pairs difference between them, eight with 1785 base pairs, seven of them identical and one with two base pairs difference. All differences occur exclusively in ITS1; the distribution of these four haplotypes had no geographical pattern. The ten self-generated H3 sequences (330 bp) also differ only slightly from each other (maximal difference: 13 base pairs, double peaks included). They have numerous double peaks, almost exclusively at codon position 3. Of the three sequences downloaded from Genbank, only KJ566724 shows a single double peak, the others at best unresolved "N". The sequences of KJ566710 and KF484715, downloaded from Genbank, are very different from all other H3 sequences and therefore should not be conspecific. No phylogeographic pattern was observed in the distribution of the H3 sequences.

COI of *N. fontanus* has 46 haplotypes that differ in up to 18 base pairs (Figs. 3, 4a, b). Thus, *N. fontanus* is less genetically diverse in the studied mitochondrial and nuclear gene fragments if compared with other widespread *Niphargus* species. By a haplotype network (Fig. 3) and a neighbour joining tree (Fig. 4b), 13 genetic lineages of COI (defined as a minimum of three mutational steps among haplotypes) are distinguished. Some of these genetic lineages show a wide and partly discontinuous distribution, while others are geographically restricted (Fig.

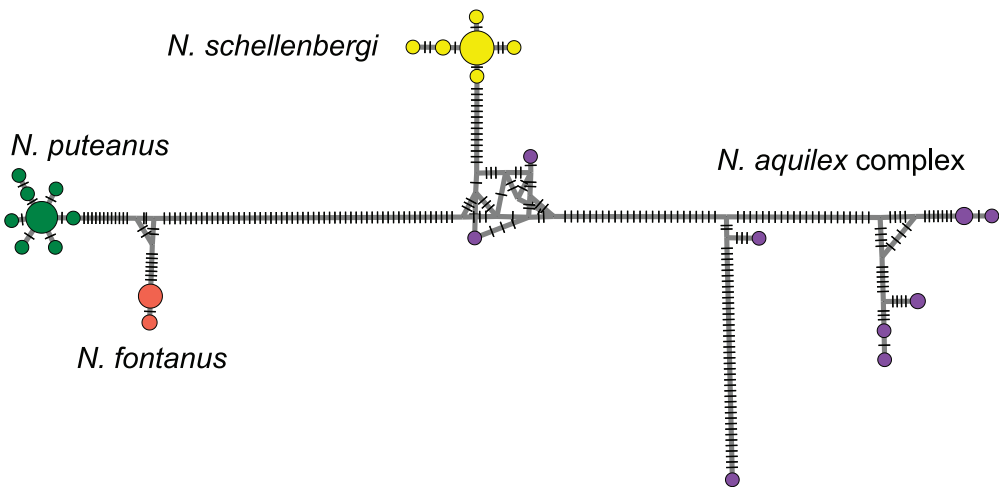

Figure 2. Haplotype network of 28S with *Niphargus fontanus* (tomato), *Niphargus puteanus* (green), *Niphargus schellenbergi* (yellow) and the *Niphargus aquilex* species complex (blueviolet). Colours refer to the code given in Figure S3.

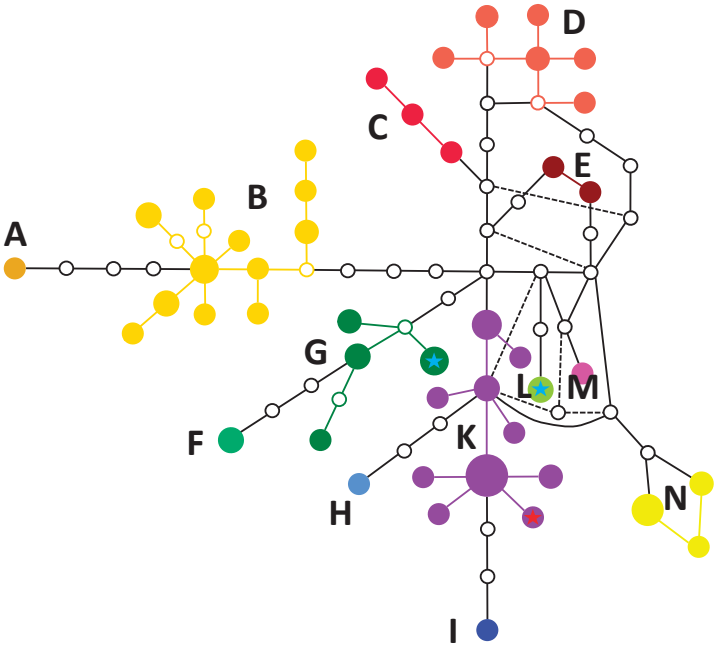

Figure 3. COI haplotype network of the 13 haplogroups (different colours) of *Niphargus fontanus*. Open circles represent haplotypes not present in our sequences. The haplotypes marked with a blue star contain one incomplete sequence each. The haplotype marked with a red star contains a sequence with one undissolved base pair.

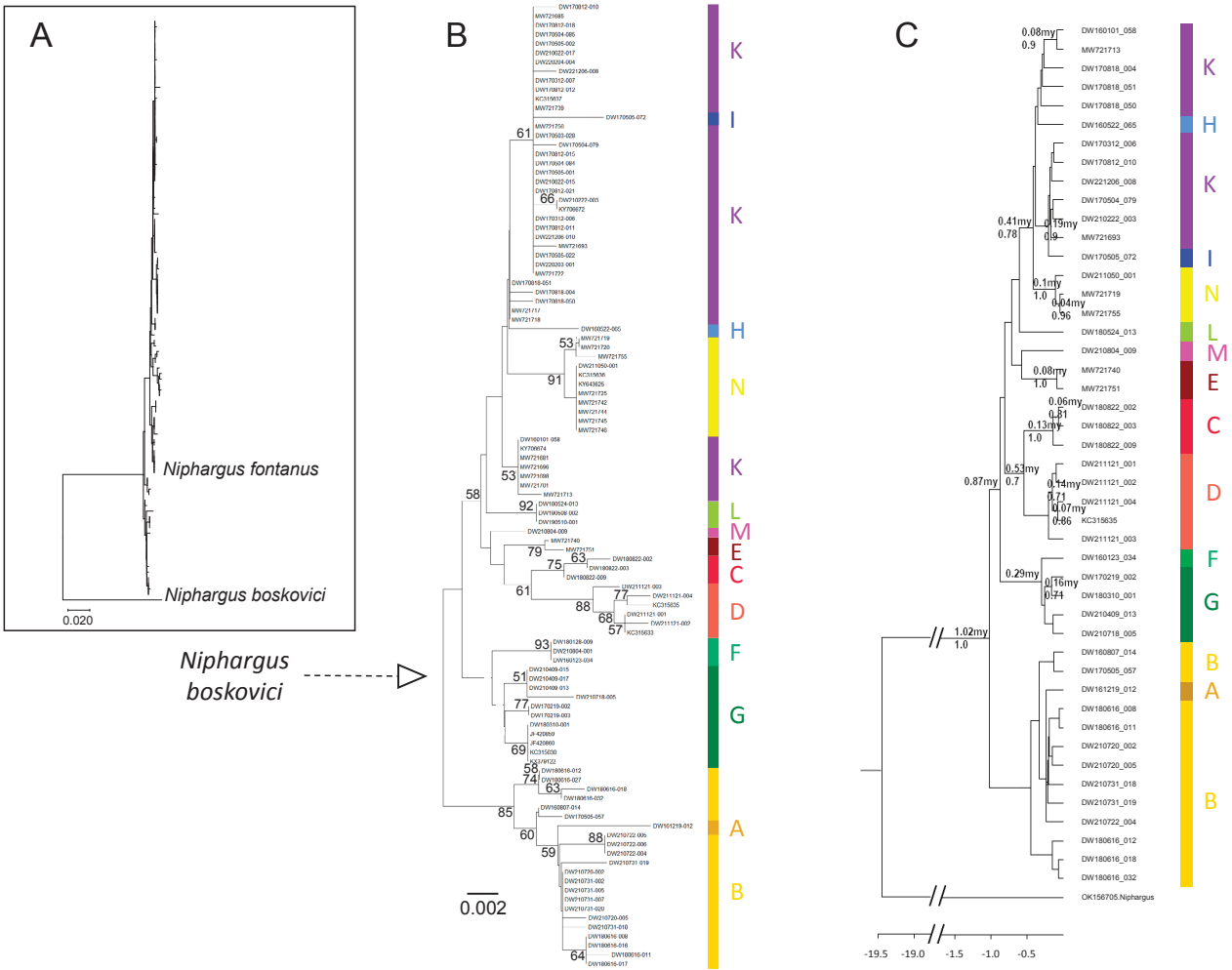

Figure 4. Neighbor joining trees based on COI sequences of *Niphargus fontanus* with *N. boskovici* as outgroup (**A**) and without outgroup (**B**). Bootstrap values (only values >50 %) are based on 3.000 iterations. **C** Bayesian tree based on COI sequences of *Niphargus fontanus* using Beast v. 2.5 (Bouckaert et al. 2014) and a relaxed clock log normal setting with a clock-rate of 0.0177 (Papadopoulou et al. 2010). The 13 genetic lineages defined via the haplotype network are indicated by the bars besides the trees.

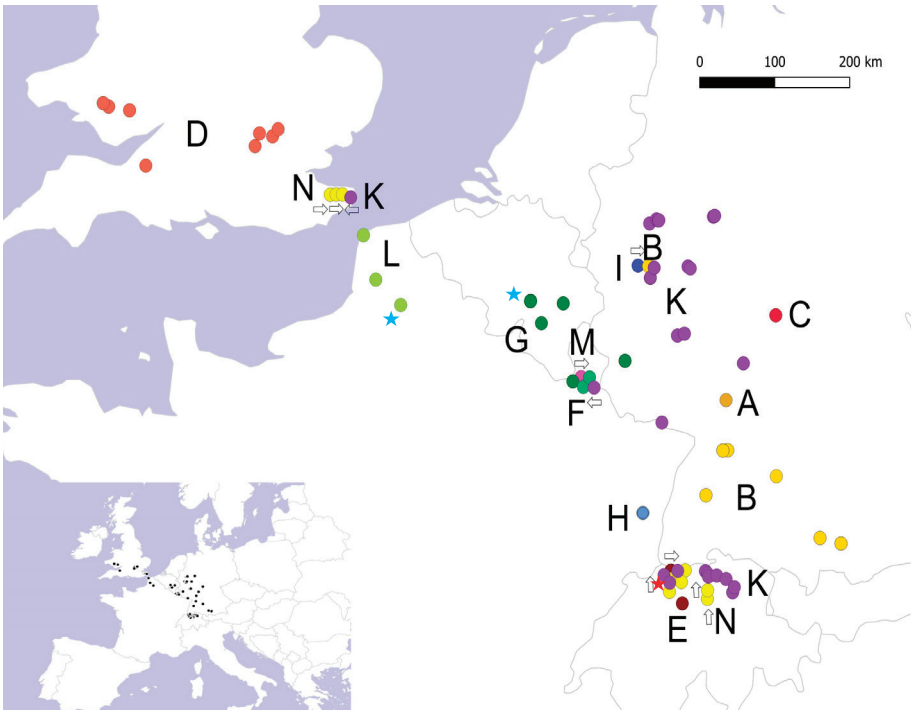

Figure 5. Map of the 13 haplogroups of *Niphargus fontanus*. The colours correspond to those of the COI haplotype network (Fig. 3). Arrows point to the actual location, which has been moved on the map, otherwise multiple haplogroups would overlap. The sites marked with a blue star contain one incomplete sequence each. The sites marked with a red star contains one undissolved base pair.

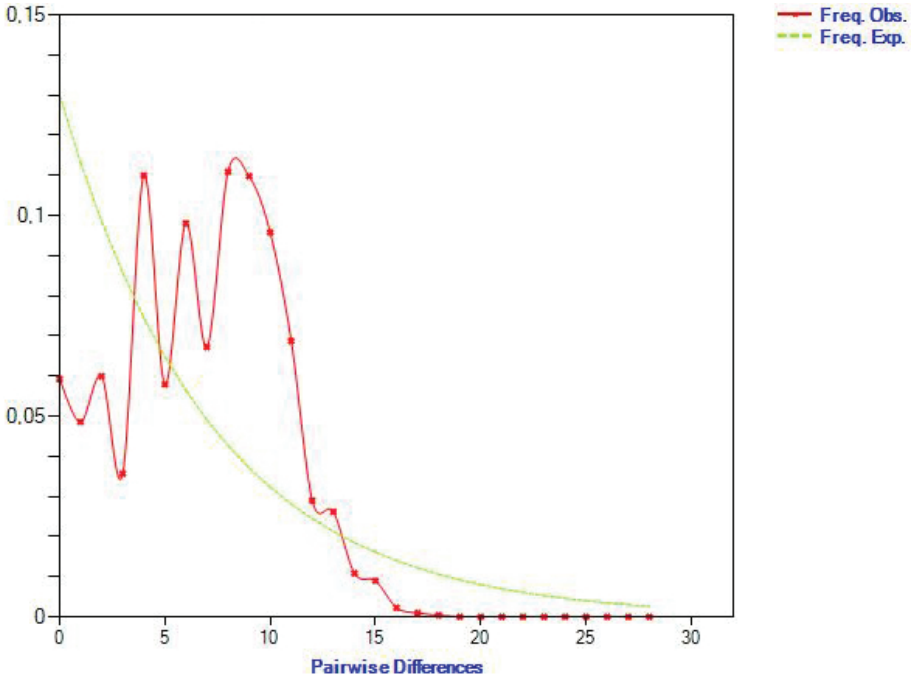

Figure 6. COI mismatch-distribution of *Niphargus fontanus*.

5); six lineages are represented by one single haplotype, i.e. A, F, H, I, L, M (Figs. 3, 4). The COI mismatch distribution (Fig. 6) shows a strong trend towards low pairwise differences. Tajima's *D* (-1,20302), Fu and Li's *D** (-2,29795) and Fu and Li's *F**:(-2,21666) are all negative, but not significant.

The Beast analysis resulted in a topology with high Effective Sample Size (ESS > 500), with an initial, well supported separation of the south-western German region R2 from all others around 1 My ago. Less well supported consecutive splits around 800 ky ago separated the Moselle region as a distinct clade, followed by several internal splits, also within regions, indicating a complex biogeographical history (Fig. 4c).

The DEC analysis postulated 14 dispersal, 8 vicariance, and 2 extinction events. Most of these events involved small steps occurring within haplogroup K. The supported origin of *N. fontanus* were R2-R4-R5 with 17.0 % followed by R1-R2-R5 with 12.8 %. All remaining possible regions of origin scored below 7 % likelihood (Fig. 7). The BBM analysis declared the region R2 (SW Germany) as the most likely origin of the species with 75.8 % and the region R5 (Moselle + Meuse) with 16.7 % as the second. 16 dispersal events and 10 vicari-

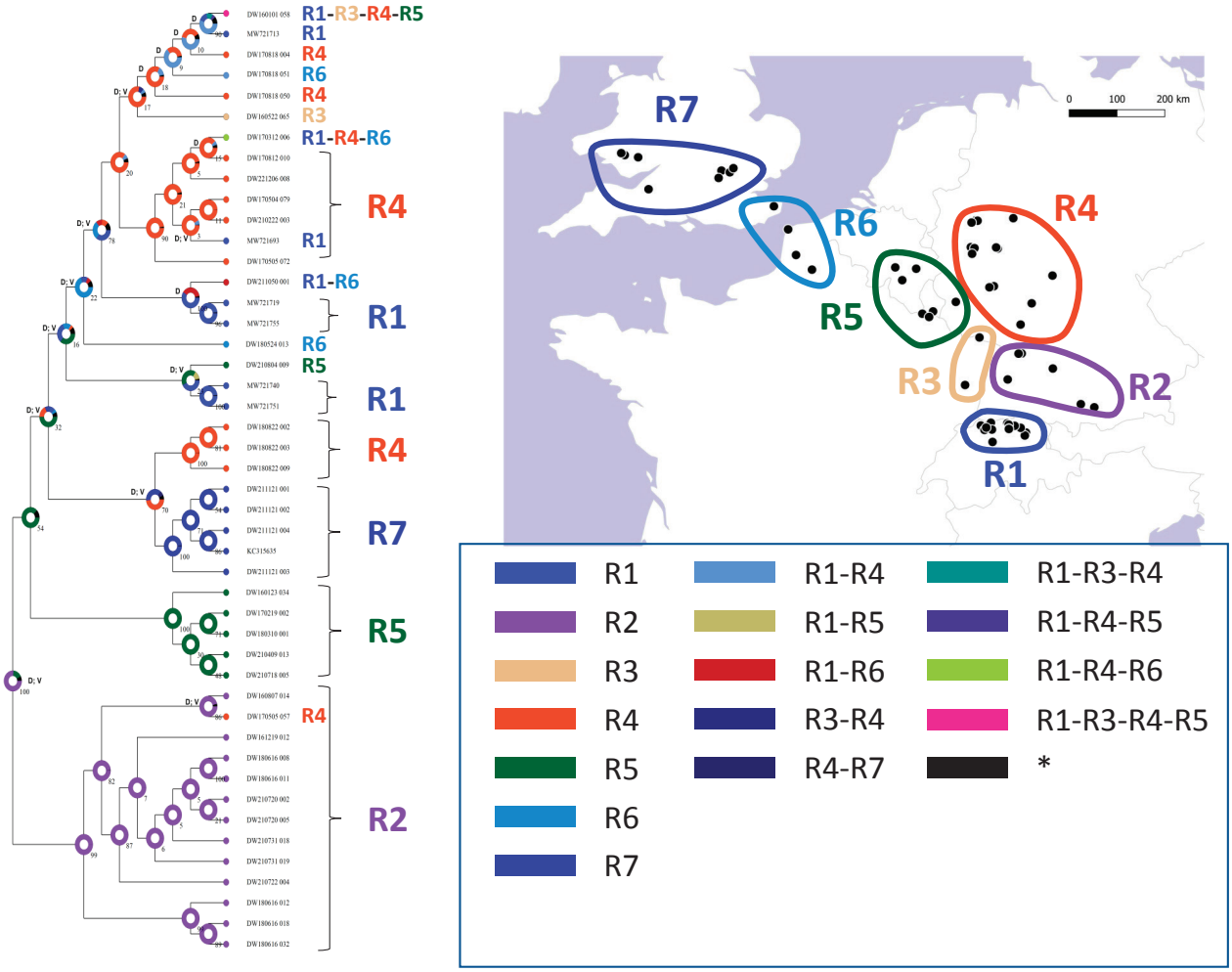

Figure 7. Bayesian Binary MCMC analysis result of *Niphargus fontanus* based on mtDNA (COI) haplotypes. Bayesian posterior probability is giving next to each node. Dispersal events and vicariance events are indicated with the letters "D" and "V" next to each node. The pie-charts and their respective colours indicate the origin of the most recent common ancestor. The regional affiliation of the haplotypes to the regions R1–R7 is given; the classification of these regions is illustrated in the map. Colour codes are explained in the legend.

ance events were postulated by the BBM analysis. The alternative BBM and DEC settings proposed region R2 also the sole or partial origin of *N. fontanus* (Fig. S2).

4. Discussion

In this study, we confirmed the existence of *N. fontanus* s. str. for Wales and southern England, the northernmost parts of France and Alsace, western and southern Germany as well as northern Switzerland. However, although *N. fontanus*, based on morphological determination (Gibert et al. 2005) was indicated for the central and southern parts of France, none of our numerous samples from that area morphologically resembles this species. The only haplotype in Genbank labelled as *N. fontanus* from southern France published by McInerney et al. (2014), finally turned out not to belong to this species. Additionally, all our sequenced 371 individuals of the genus and all data from Genbank from that region belong to other *Niphargus* species. Thus, only *N. fontanus* A distributed in western and central Europe should be accepted as this species. Records of the species from France south of its northernmost parts and Alsace apparently all are misidentifications. This supports our first hypothesis. However, the wide distribution of *N. fontanus* of 1,150 km as the crow flies (from Wales to Bavaria) remains. *N. fontanus* therefore is the species with the largest distribution in the north-western part of the genus' distribution, as far as known up to now.

Comparing the genetic diversity and differentiation of *N. fontanus* against other members of this genus from West, North-West and Central Europe, we obtained remarkable differences in nuclear and mitochondrial DNA. Thus, the nuclear 28S locus of *N. fontanus* had only two haplotypes differing only at one position (Fig. 2). In contrast, the 28S of *N. puteanus* had a star-like structure with a maximum of three mutational steps, thus having a considerably higher diversity and also assumed age (Weber et al. 2020). The star-like structure of *N. schellenbergi* even had up to five mutational steps (Weber 2022), hence calling for an even older structure. The oldest structure

in nuclear genes was obtained for the *N. aquilex* complex with dozens of mutational steps between the single haplotypes and no star-like structures at all (Weber et al. 2023). This supports an old fragmentation in this taxon and the formation of numerous cryptic species (Fig. 2). Therefore, we have to reject hypothesis 2 for the nuclear level, especially as two other nuclear genes sequences for *N. fontanus* (ITS, H3) gave quite similar results. Consequently, the youngest genetic structure should be in *N. fontanus* if compared against all other here included species in this genus.

Considering the mitochondrial COI marker, we obtained a somewhat different picture with a considerably higher diversity and differentiation, although still being lower than in other related species (Weber et al. 2020, Weber 2022), again underlining the more recent evolutionary history of *N. fontanus* if compared with other *Niphargus* species. Hence, hypothesis 2 also has to be rejected for the mitochondrial level, here represented by the COI fragment. However, the difference is not as sharp as at the nuclear level, here represented by the 28S marker, because of the about ten times higher mutation rate of the mitochondrial COI, a typical difference between nuclear and mitochondrial DNA (Haag-Liautard et al. 2008). Consequently, a considerable mitogenomic diversity with pronounced phylogeographic pattern has already evolved again after a relatively recent bottleneck. This bottleneck apparently is still reflected in the low diversity of the analysed nuclear markers due to their generally lower mutation rate.

The complete lack of genetically confirmed records from central and southern France is calling for the absence of a Mediterranean refugium sensu De Lattin (1949) in *N. fontanus*, and no refugium in southern France for whose existence numerous examples have accumulated for other species until today (Vitali and Schmitt 2017, with references therein). Hence, the second part of hypothesis 1 of exclusive glacial survival north of the Mediterranean refugial belt in extra-Mediterranean refugia seems more plausible, and is also supported by our Beast and RASP analysis, which predicted survival along the last glacial-interglacial cycles without major extinction events. Although survival of ice ages in climatically favoured pockets in the permafrost zone got strong support over the last few decades for many terrestrial and limnic organisms (review: Schmitt and Varga 2012), it is still mostly unknown for organisms living in groundwaters, but see e.g. Holsinger (1980). Further supporting survival of groundwater organisms in permafrost areas during ice ages, some few *Niphargus* species have been found exclusively at the north-western limit of the genus' distribution, e.g. *Niphargus irlandicus* in Ireland (Schellenberg 1932), *Niphargus glenniei* in Cornwall and Devon (England) (Spooner 1952, Knight and Johns 2015) and two apparent endemics, of which only one location is known so far, *Niphargus boulangei* (Wichers 1964) from northern France and *Niphargus carolinensis* (Weber and Brad 2023) from North Rhine-Westphalia. Our data presented here hence indicate that *N. fontanus* is another example of glacial survival in permafrost zones.

Analysing the phylogeographic pattern of the mitochondrial information of *N. fontanus*, we distinguished 13 lineages composed of one (i.e. lineages A, F, H, I, L, M) to 12 haplotypes (i.e. lineage B) (Fig. 3). Based on the average mutation rates of COI, i.e. 0.0177/My (Papadopoulou et al. 2010), the differentiation into these intraspecific lineages most likely started about 1 My ago, with the follow-up splits taking place around 800–900 ky ago (Fig. 4C), simultaneously with the onset of the more rigid glaciations at the beginning of the mid-Pleistocene, i.e. the mid-Pleistocene Transition (Menning 2018). This fully agrees with the almost absent differentiation in the nuclear markers 28S, ITS and H3; however, these nuclear markers have to be interpreted with care due to the small sample size analysed. Thus, the drastic cooling might have produced a strong bottleneck still visible in the poor nuclear diversity, also setting the likely starting point for most of the mitogenome differentiation. However, the afterwards evolution of the phylogeographic structure most likely results from complex range fluctuations reflected in the non-unimodal mismatch distribution pattern (Fig. 6).

Only three mitochondrial lineages of *N. fontanus* have wide geographic distributions including range disjunctions. For example, lineage K has a large distribution (northern Switzerland via Alsace and Palatinate to northern Westphalia) on both sides of river Rhine but also one isolated individual in south-easternmost England. Lineage B restricted to south-western Germany has one additional specimen in North Rhine-Westphalia. Lineage N is restricted to Switzerland, but has an additional occurrence in south-eastern England (Fig. 5). All other ten lineages have much more restricted extant distributions, always confined to one side of river Rhine. This distribution pattern also is apparent when referring to the Rhine of the Pleistocene cold stages flowing from its present mouth via what is now the south-western North Sea and the present English Channel into the Atlantic Ocean, with the Thames as its right tributary (Bridgland and D'Olier 1995, Antoine et al. 2003, Busschers et al. 2007, Patton et al. 2017).

In addition, most mitochondrial lineages seem to be well geographically separated from each other; if changing the separation criterion for a genetic lineage from three to four mutational steps, the one-haplotype-lineages F, H and I would fuse with the geographically extended lineages G and K. In all three cases, these mergers are located within or adjoining to the distributions of the lineages they would merge with (Fig. 3). Hence, the geographic separation among the then resulting ten lineages would be even stronger. Consequently, the centres of origin might be within the extant distributions of the respective genetic lineages. These results are consistent with the results obtained from the analyses of Tajima's *D*, Fu and Li's *D** and Fu and Li's *F**, all of which were negative but nevertheless not significantly different from zero. Hence, *N. fontanus* might show geographic stability over most of the time as its most typical biogeographic feature, only interspersed by some few dispersal events with most

of them happening more than 300 ky ago and lineage K being the most expansive lineage of *N. fontanus*.

Following the RASP analysis of the mitochondrial information (Fig. 7), the centre of origin of *N. fontanus* should be located in south-western Germany (i.e. Baden-Württemberg) with subsequent dispersal across river Rhine to the Moselle-Meuse region, with complex follow-up range dynamics (see below). However, considering the high phylogenetic diversity in northern Switzerland with three geographically overlapping lineages, i.e. a higher phylogenetic diversity than in any other of the regions defined for the RASP analysis, this region might have been the first one colonised out of Baden-Württemberg (and not the Moselle-Meuse region) or even might be the origin of the entire species. In the latter case, Baden-Württemberg would have been the first region colonised out of Switzerland. However, our data do not allow to clearly prefer one of these mutually exclusive scenarios. In any case, no further emigration from or immigration to Baden-Württemberg has taken place later on and the regional genetic lineage evolved independently since then.

The evolution of the strong phylogeographic mitochondrial diversity in northern Switzerland also might have been fostered by the complex glaciation history of the Swiss Midlands, which e.g. during the Würm glaciation were never completely covered by ice (Seguinot et al. 2018), thus offering a patchwork of temporarily fluctuating retreat areas. This pattern even might reflect temporal survival of *N. fontanus* under the ice shield when extending more, e.g. during the preceding Riss glaciation, hence making the survival of the endemic lineages possible; a similar case of below-glacier survival is known since long for Canada (Holsinger 1980). Interestingly, a quite similar phylogeographic structure was also obtained for *N. tonywhitteni* (Weber 2023) with high genetic diversity in Switzerland but low diversity in the much larger region of Baden-Württemberg-Bavaria-Austria.

The flowing waters of river Rhine downstream Lake Constance apparently represent a strong dispersal barrier for *N. fontanus*. This is supported by the fact that all lineages (apart for lineages N and K) have their continuous distribution areas only on one side of the river. Hence hypothesis 3 that rivers are not impacting the phylogeographic patterns of groundwater organisms has to be rejected in this case. Nevertheless, river Rhine was not a completely unsurmountable barrier as our genetic data support at least three crossings, as discussed in the following.

A first crossing of river Rhine might have taken place early in the intraspecific differentiation during the early mid-Pleistocene when the species either expanded from Baden-Württemberg (region 2) north-westwards to either the Moselle-Meuse region 5 or southwards to northern Switzerland (region 1), or from northern Switzerland northwards to adjoining Baden-Württemberg. Even if it initially seems unlikely that *N. fontanus* dispersed from Baden-Württemberg to Switzerland, it must be considered that the species could have dispersed from the Danube via the Danube seepage through the Aach cave and via the Aach (Schetter 2017, Käss 2021) to the area of Lake Constance, from where the pathway to Switzerland is not far, even if including a crossing of river Rhine. However, neither the Danube nor the Rhine are significant barriers in this region: In dry seasons, the Danube has completely dried up or seeped away and the Alpine Rhine only carries a small amount of water here.

Later, coming either from the Moselle-Meuse region or from northern Switzerland, the ancestors of lineage C must have crossed river Rhine, most likely further north than the first crossing for not colliding with lineage B in Baden-Württemberg, i.e. being hindered by high density blocking (Waters et al. 2013). The resulting lineage C today represents a relict lineage at the actual limit of the species distribution in the German federal state of Hesse, just recorded for a single locality. The most recent Rhine-crossing of lineage K apparently is so recent that still no major differentiation has taken place on both sides of the river. Maybe this lineage even expanded quickly from northern Switzerland, even the early Postglacial is possible, crossing the river somewhere in the Middle Rhine Valley and quickly expanding further north reaching to the northern parts of North Rhine-Westphalia. However, also the opposite direction is possible but less supported by our RASP analysis (Fig. 7).

On the other hand, the entire distribution of *N. fontanus* let us argue that the interstitial along the Rhine river basin, of which river Thames was a right-hand tributary during glacial periods (Patton et al. 2017), is a connecting structure; and not a barrier as the river's floating waters (see above). This is supported by the fact that the vast majority of *N. fontanus* individuals was recorded from the larger Rhenanian system; the rare exceptions are some few populations in areas draining to the Danube in Baden-Württemberg and south-western Bavaria (see below). It therefore is likely that individuals of *N. fontanus* disperse in the interstitial along river Rhine and its tributaries during climatically favourable times. Later, when conditions deteriorated, they apparently were trapped in refugia, starting differentiation in allopatry, even reaching southern Britain via river Thames.

The occurrence of haplogroup B in the catchment area of Rhine and Danube might be explained by the fact that the Danube sinkhole between Immendingen and Möhringen feeds Danube water to river Aach via a karst cave system with its discharge in the Aachtopf and then to river Rhine. Thus, the upper course of Danube can partly be regarded as a tributary of Rhine (Knop 1878). Thus, this pathway even might be used twice for dispersal (see above).

Three mitochondrial lineages with wide geographic distribution (i.e. B, G, K) are closely related (i.e. three or four mutational steps) to one or two, respectively, of the lineages composed of one single haplotype, i.e. A, F, H and I (Fig. 3). What occured for the entire species at a larger geographical and temporal scale, was likely repeated within these three groups of lineages again, i.e. expansion during suitable climatic conditions out of one refugium with later restriction to more scattered distributions in the wake of climatic deterioration. Interest-

ingly, diversifications within these groups of lineages apparently followed the peripatric pattern of differentiation (Mayr 1982, Coyne and Orr 2004) with the most derived haplotypes (i.e. the single haplotype lineages) surviving in small areas at the margin of the respective distribution.

Finally, in some exceptional cases and again rejecting hypothesis 3, the Rhine river system apparently served as a kind of "dispersal highway". This is most likely in the mitochondrial lineage N mostly restricted to northern Switzerland and lineage K found in northern Switzerland and western Germany. However, both lineages were also detected at rather restricted sites near Dover in south-eastern England (Fig. 5), i.e. close to the Würm ice age Rhine river system (see above), crossing the British Chanel from east to west (Patton et al. 2017). Thus, a long-distance dispersal event by rafting down the interstitial of river Rhine many hundreds of km during the Würm ice age is the most likely explanation for these peculiar disjunctions. A similar incident is also likely for the occurrence of lineage B close to the North Rhine several hundreds of km away from its other populations in Baden-Württemberg.

When comparing distribution ranges, the total distribution of *N. fontanus* with more than 1,000 km from Wales to western Bavaria is considerably larger than in most other *Niphargus* species, although the time to reach this distribution (measured by the intraspecific differentiation of the respective species) is even shorter. No other *Niphargus* species in this part of Europe achieved such a large distribution: neither *N. schellenbergi*, which has the same body size and apart from the gnathopods also almost the same body shape (Weber 2022); nor the rather crenophilic *N. puteanus* (Weber et al. 2020); nor small *Niphargus* species (such as *N. boulangei* Wichers, 1964, the *N. kochianus* Spence Bate, 1859 group (Ginet 1996, Gibert et al. 2005) or the *N. aquilex* group (Weber et al. 2023), which due to their small body sizes would be expected to spread more quickly in the narrow spaces of the interstitial. Note that the more eastern species *N. hrabei* S. Karaman, 1932 and *N. valachicus* Dobreanu & Manolache, 1933 might have similarly large or even more extended ranges, if their actual species identification is correct of which some doubt is well justified (https://www.gbif.org/species/4417257).

Consequently, the chance to disperse must be remarkably higher in *N. fontanus* than in the other western European species. As this species possesses no characteristics that might enhance its own dispersal power compared to related species, we disbelieve that it is a particularly dispersive species. Rather, we believe that the conditions in the interstitial accompanying river Rhine and its tributaries has been particularly suitable for some dispersal events of *Niphargus* species and hence could foster the large distribution of *N. fontanus*, even allowing for long distance dispersal in exceptional cases. Nevertheless, it remains somewhat enigmatic why *N. fontanus*, in particular, has dispersed so much more extensively than other species also occurring in the Rhine drainage.

5. Declarations

Authors' contributions. DW: Conceptualisation, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing – Original draft, Writing – Review and Editing, Visualization, Project administration. MW: Formal analysis, Writing - Review and Editing, Visualization. TS: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – Review and Editing, Visualization, Supervision.

Competing interests. The authors declare no competing interests.

Permissions. The authors had all necessary permissions.

6. Acknowledgements

Ulrich Brämer, Jean-François Flot, Tim Jones, Sabine Kaufmann, Lee Knight, Hans Martin Luz, Florian Malard, Stephan Schild, Michael Strassburger, Klaus Timmerberg, Jens Trasberger, Monika Weber, Verena Schuhmacher, and Jörg Zahlmann assisted in collecting the *Niphargus* material.

7. References

Alther R, Bongni N, Borko Š, Fišer C, Altermatt F (2021) Citizen science approach reveals groundwater fauna in Switzerland and a new species of *Niphargus* (Amphipoda, Niphargidae). Subterranean Biology 39: 1–31. https://doi.org/10.3897/subtbiol.39.66755

Antoine P, Coutard J, Gibbard P, Hallegouet B, Lautridou J, Ozouf J (2003) The Pleistocene rivers of the English Channel region. Journal of Quaternary Science 18(3–4): 227–243. https://doi.org/10.1002/jqs.762

Astrin JJ, Stüben PE (2008) Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera:Curculionidae). Invertebrate Systematics 22(5): 503. https://doi.org/10.1071/IS07057

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge, Mass, 447 pp.

Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, Suchard MA, Rambaut A, Drummond AJ (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. Prlic A (Ed.). PLoS Computational Biology 10(4): e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Bridgland DR, D'Olier B (1995) The Pleistocene evolution of the Thames and Rhine drainage systems in the southern North Sea Basin. Geological Society, London, Special Publications 96(1): 27–45. https://doi.org/10.1144/GSL.SP.1995.096.01.04

Busschers FS, Kasse C, Van Balen RT, Vandenberghe J, Cohen KM, Weerts HJT, Wallinga J, Johns C, Cleveringa P, Bunnik FPM (2007) Late Pleistocene evolution of the Rhine-Meuse system in the southern North Sea basin: imprints of climate change, sea-level oscillation and glacio-isostacy. Quaternary Science Reviews 26(25–28): 3216–3248. https://doi.org/10.1016/j.quascirev.2007.07.013

Chappuis PA (1942) Eine neue Methode zur Untersuchung der Grundwasserfauna. Acta scientiarum mathematicarum et naturalium / Universitas Francisco Joseephina (Kolozsvar) 6: 3–7.

Chevreux E (1901) Amphipodes des eaux souterraines de France et d'Algérie. Bulletin de la société zoologique de France 26: 168–174.

Colgan DJ, McLauchlan A, Wilson GDF, Livingston SP, Edgecombe GD, Macaranas J, Cassis G, Gray MR (1998) Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. Australian Journal of Zoology 46(5): 419. https://doi.org/10.1071/ZO98048

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, Mass, 545 pp.

Darwin C (1838) The Zoology of the Voyage of H.M.S. Beagle. Smith, Elder & Co., London.

De Lattin G (1949) Beiträge zur Zoogeographie des Mittelmeergebietes. Verhandlungen der deutschen Zoologischen Gesellschaft 42: 143–151.

Delić T, Borko Š, Premate E, Rexhepi B, Alther R, Knüsel M, Malard F, Weber D, Stoch F, Flot J-F, Fišer C, Altermatt F (2023) Evolutionary origin of morphologically cryptic species imprints co-occurrence and sympatry patterns. Ecology. preprint https://doi.org/10.1101/2023.09.13.557531

Eme D, Zagmajster M, Fišer C, Flot J-F, Koneny-Dupré L, Snaebjörn P, Stoch F, Zakšek V, Douady CJ, Malard F, Delić T (2018) 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

Esmaeili-Rineh S, Sari A, Fišer C, Bargrizaneh Z (2017) Completion of molecular taxonomy: description of four amphipod species (Crustacea: Amphipoda: Niphargidae) from Iran and release of database for morphological taxonomy. Zoologischer Anzeiger 271: 57–79. https://doi.org/10.1016/j.jcz.2017.04.009

Fišer C, Sket B, Stoch F (2006) Distribution of four narrowly endemic *Niphargus* species (Crustacea: Amphipoda) in the western Dinaric region with description of a new species. Zoologischer Anzeiger 245(2): 77–94. https://doi.org/10/djv95w

Fišer C, Zagmajster M, Dethier M (2018) Overview of Niphargidae (Crustacea: Amphipoda) in Belgium: distribution, taxonomic notes and conservation issues. Zootaxa 4387(1): 47–74. https://doi.org/10.11646/zootaxa.4387.1.2

Flot J-F (2010) Vers une taxonomie moléculaire des amphipodes du genre *Niphargus*: exemples d'utilisation de séquences d'ADN pour l'identification des espèces. Bulletin de la Société des Sciences Naturelles de l'Ouest de la France 32(2): 62–68.

Flot J-F, Wörheide G, Dattagupta S (2010) Unsuspected diversity of *Niphargus* amphipods in the chemoautotrophic cave ecosystem of Frasassi, central Italy. BMC Evolutionary Biology 10: 171. https://doi.org/10.1186/1471-2148-10-171

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294–299.

Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. Genetics 133(3): 693–709. https://doi.org/10.1093/genetics/133.3.693

Gibert J, Brancelj A, Camacho A, Castellarini F, Broyer CD, Deharveng L, Marie-José Dole-Olivier, Douady CJJ, Galassi DMP, Malard F, Martin P, Michel G, Sket B, Stoch F, Trontelj P, A. G. Valdecasas (2005) Groundwater biodiversity. protocols for the assessment and conservation of aquatic life in the subsurface (PASCALIS): overview and main results. https://doi.org/10.13140/RG.2.1.2306.1528

Ginet R (1971) Biogéographie de *Niphargus* et *Caecosphaeroma* (Crustacés troglobies) dans les départements francais du Jura et de l'Ain. Origine; influence des glaciations. Actes du 4ème Congrès Suisse de Spéléologie: 186–198.

Ginet R (1996) Bilan systématique du genre *Niphargus* en France. Société linnéenne de Lyon, Lyon, 242 pp.

Gledhill T (1980) Designation and description of the lectotype of *Niphargus fontanus* Bate. Crustaceana. Supplement 6: 27–37.

Haag-Liautard C, Coffey N, Houle D, Lynch M, Charlesworth B, Keightley PD (2008) Direct Estimation of the Mitochondrial DNA Mutation Rate in *Drosophila melanogaster*. Hurst LD (Ed.). PLoS Biology 6(8): e204. https://doi.org/10.1371/journal.pbio.0060204

Hartke TR, Fišer C, Hohagen J, Kleber S, Hartmann R, Koenemann S (2011) Morphological and molecular analyses of closely related species in the stygobiontic genus *Niphargus* (Amphipoda). Journal of Crustacean Biology 31(4): 701–709. https://doi.org/10.1651/10-3434.1

Hewitt G (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society 58(3): 247–276. https://doi.org/10.1006/bijl.1996.0035

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Willis KJ, Bennett KD, Walker D (Eds). Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 359(1442): 183–195. https://doi.org/10.1098/rstb.2003.1388

Holsinger JR (1980) *Stygobromus canadensis*, a new subterranean amphipod crustacean (Crangonyctidae) from Canada, with remarks on Wisconsin refugia. Canadian Journal of Zoology 58(2): 290–297. https://doi.org/10.1139/z80-034

Horton T, Lowry J, De Broyer C, Bellan-Santini D, Coleman CO, Corbari L, Costello M, Daneliya M, Dauvin J-C, Fišer C, Gasca R, Grabowski M, Guerra-García JM, Hendrycks E, Hughes L, Jaume D, Jazdzewski K, Kim Y-H, King R, Krapp-Schickel T, LeCroy S, Lörz A-N, Mamos T, Senna A, Serejo C, Sket B, Souza-Filho JF, Tandberg AH, Thomas JD, Thurston M, Vader W, Väinölä R, Vonk R, White K, Zeidler W (2022) World Amphipoda Database. https://doi.org/10.14284/368

Karaman GS (2017a) New data on two subterranean species of the family Niphargidae from Spain, *Niphargus gallicus* Schell., 1935 and *N. delamarei* Ruffo, 1954 (contribution to the knowledge of the amphipoda 282. Contributions, Section of Natural, Mathematical and Biotechnical Sciences 36(2): 105–120.

Karaman GS (2017b) On the endemic subterranean amphipod *Niphargus versluysi* S. Karaman, 1950 (fam. Niphargidae) In Greece (contribution to the Knowledge of the Amphipoda 297). Biologia Serbica 39(2): 52–67. https://doi.org/10.5281/ZENODO.1068964

Karaman S (1932) 5. Beitrag zur Kenntnis der Süsswasser-Amphipoden. Prirodoslovne razprave 2: 179–232.

Käss W (2021) Das Donau-Aach-System. Schweizerbart Science Publishers, Stuttgart, Germany. Available from: http://www.schweizerbart.de//publications/detail/isbn/9783510968626/Geologisches_Jahrbuch_Reihe_A_Heft_A165.

Knight LRFD, Johns T (2015) Auto-ecological studies on *Niphargus aquilex* (Schiodte, 1855) and *Niphargus glenniei* (Spooner, 1952) (Crustacea: Amphipoda: Niphargidae). Cave and Karst Science 42: 63–77.

Knight LRFD, Brancelj A, Hänfling B, Cheney C (2015) The groundwater invertebrate fauna of the Channel Islands. Subterranean Biology 15: 69–94. https://doi.org/10.3897/subtbiol.15.4792

Knop A (1878) Über die hydrographischen Beziehungen zwischen der Donau und der Aachquelle im badischen Oberlande. Neues Jahrbuch für Mineralogie, Geologie und Palaeontologie Jg. 1878: 350–363.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms.

Battistuzzi FU (Ed.). Molecular Biology and Evolution 35(6): 1547–1549. https://doi.org/10.1093/molbev/msy096

Lefébure T, Douady CJ, Malard F, Gibert J (2007) Testing dispersal and cryptic diversity in a widely distributed groundwater amphipod (*Niphargus rhenorhodanensis*). Molecular Phylogenetics and Evolution 42(3): 676–686. https://doi.org/10.1016/j.ympev.2006.08.020

Lefébure T, Douady CJ, Gouy M, Trontelj P, Briolay J, Gibert J (2006) Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. Molecular Ecology 15(7): 1797–1806. https://doi.org/10.1111/j.1365-294X.2006.02888.x

Mayr E (1982) Processes of speciation in animals. Progress in Clinical and Biological Research 96: 1–19.

McInerney CE, Maurice L, Robertson AL, Knight LRFD, Arnscheidt J, Venditti C, Dooley JSG, Mathers T, Matthijs S, Erikkson K, Proudlove GS, Hänfling B (2014) The Ancient Britons: Groundwater fauna survived extreme climate changes over tens of millions of years across NW Europe. Molecular Ecology 23: 1153–1166. https://doi.org/10.1016/j.jcz.2006.05.003

Menning M (2018) Die Stratigraphische Tabelle von Deutschland 2016 (STD 2016). Zeitschrift der Deutschen Gesellschaft für Geowissenschaften 169(2): 105–128. https://doi.org/10.1127/zdgg/2018/0161

Molden D (2007) Water for Food Water for Life. Routledge. https://doi.org/10.4324/9781849773799

Notenboom J (1994) Netherlands. In: Encyclopaedia Biospeologica, 757–759.

Papadopoulou A, Anastasiou I, Vogler AP (2010) Revisiting the Insect Mitochondrial Molecular Clock: The Mid-Aegean Trench Calibration. Molecular Biology and Evolution 27(7): 1659–1672. https://doi.org/10.1093/molbev/msq051

Patarnello T, Volckaert FAMJ, Castilho R (2007) Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographical break? Molecular Ecology 16(21): 4426–4444. https://doi.org/10.1111/j.1365-294X.2007.03477.x

Patton H, Hubbard A, Andreassen K, Auriac A, Whitehouse PL, Stroeven AP, Shackleton C, Winsborrow M, Heyman J, Hall AM (2017) Deglaciation of the Eurasian ice sheet complex. Quaternary Science Reviews 169: 148–172. https://doi.org/10.1016/j.quascirev.2017.05.019

Puillandre N, Brouillet S, Achaz G (2021) ASAP: assemble species by automatic partitioning. Molecular Ecology Resources 21(2): 609–620. https://doi.org/10.1111/1755-0998.13281

Rambout AD (2018) Figtree version 1.4.4. Institute of Evolutionary Biology.

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. Molecular Biology and Evolution 34(12): 3299–3302. https://doi.org/10.1093/molbev/msx248

Schellenberg A (1932) Bemerkungen über subterrane Amphipoden Großbritanniens. Zoologischer Anzeiger 99: 49–58.

Schellenberg A (1935a) Der *Niphargus* des Thüringer Waldes und die Glazialreliktenfrage. Arch. für Hydrobiologie 29: 274–281.

Schellenberg A (1935b) Schlüssel der Amphipodengattung *Niphargus* mit Fundortangaben und mehreren neuen Formen. Zoologischer Anzeiger 111(7/8): 204–211.

Schellenberg A (1942) Krebstiere oder Crustacea. IV: Flohkrebse oder Amphipoda. Jena, 252 pp.

Schetter H (2017) Die Aachquelle. Impressionen einer Groß-Höhle. 68 pp.

Schmitt T (2009) Biogeographical and evolutionary importance of the European high mountain systems. Frontiers in Zoology 6(1): 9. https://doi.org/10.1186/1742-9994-6-9

Schmitt T, Varga Z (2012) Extra-Mediterranean refugia: The rule and not the exception? Frontiers in Zoology 9(1): 22. https://doi.org/10.1186/1742-9994-9-22

Seguinot J, Ivy-Ochs S, Jouvet G, Huss M, Funk M, Preusser F (2018) Modelling last glacial cycle ice dynamics in the Alps. The Cryosphere 12(10): 3265–3285. https://doi.org/10.5194/tc-12-3265-2018

Skalski AW (1994) Poland. In: Encyclopaedia Biospeologica, 767–770.

Spence Bate C (1859) On the genus *Niphargus* (Schiödte). Natural History Review, and quarterly journal of science 6: 163–166.

Spooner GM (1952) A new subterranean gammarid (Crustacea) from Britain. Proceedings of the Zoological Society of London 121(4): 851–859. https://doi.org/10.1111/j.1096-3642.1952.tb00787.x

Spöri Y, Flot J (2020) HaplowebMaker and CoMa: Two web tools to delimit species using haplowebs and conspecificity matrices. Jarman S (Ed.). Methods in Ecology and Evolution 11(11): 1434–1438. https://doi.org/10.1111/2041-210X.13454

Stoch F (1998) Revision of the *Niphargus stygius* – group in Venetia and Trentino (northeastern Italy) with description of three new species. Bollettino del Museo Civico di Storia Naturale di Verona 22: 229–274.

Stoch F (2004) Pascalis Project: niphargids, unpublished project.

Stoch F, Citoleux R, Weber D, Salussolia A, Flot J-F (2024) New insights into the origin and phylogeny of Niphargidae (Crustacea: Amphipoda), with description of a new species and synonymization of the genus *Niphargellus* with *Niphargus*. Zoological Journal of the Linnean Society 202: zlae154. https://doi.org/10.1093/zoolinnean/zlae154

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

Trontelj P, Douady CJ, Fišer C, Gibert J, Gorički Š, Lefébure T, Sket B, Zakšek V (2009) A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? 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

Vitali F, Schmitt T (2017) Ecological patterns strongly impact the biogeography of western Palaearctic longhorn beetles (Coleoptera: Cerambycoidea). Organisms Diversity & Evolution 17(1): 163–180. https://doi.org/10.1007/s13127-016-0290-6

Wallace AR (1876) The geographical distribution of animals, with a study of the relations of living and extinct faunas as elucidating the past changes of the earth's surface. Macmillan and Co, London. https://doi.org/10.5962/bhl.title.30514

Ward JV, Palmer MA (1994) Distribution patterns of interstitial freshwater meiofauna over a range of spatial scales, with emphasis on alluvial river-aquifer systems. Hydrobiologia 287(1): 147–156. https://doi.org/10.1007/BF00006903

Waters JM, Fraser CI, Hewitt GM (2013) Founder takes all: density-dependent processes structure biodiversity. Trends in Ecology & Evolution 28(2): 78–85. https://doi.org/10.1016/j.tree.2012.08.024

Weber D (2022) Molecular phylogeny and systematics of Central and Western European niphargids (Crustacea: Amphipoda). Université liebre de Bruxelles, Bruxelles, 265 pp.

Weber D (2023) *Niphargus tonywhitteni* Fišer et al. 2018 recorded for the first time in Bavaria (Malacostraca, Amphipoda, Niphargidae). Spixiana 46(1): 9–19.

Weber D, Weigand AM (2023) Groundwater Amphipods of the hyporheic interstitial: a case study from Luxembourg and the Greater Region. Diversity 15(3): 411. https://doi.org/10.3390/d15030411

Weber D, Brad T (2023) *Niphargus carolinensis* sp. nov. (Amphipoda: Niphargidae), an Endemic Species in the Northern Distribution Area of the Genus. Hydrobiology 2(4): 554–574. https://doi.org/10.3390/hydrobiology2040037

Weber D, Flot J-F, Weigand H, Weigand AM (2020) Demographic history, range size and habitat preferences of the groundwater amphipod *Niphargus puteanus* (C.L. Koch in Panzer, 1836). Limnologica 82: 125765. https://doi.org/10.1016/j.limno.2020.125765

Weber D, Brad T, Stoch F, Flot J-F (2021a) Rediscovery and redescription of *Niphargus enslini* Karaman, 1932 (Amphipoda, Niphargidae) in southern Germany. Subterranean Biology 40: 65–89. https://doi.org/10.3897/subtbiol.40.73017

Weber D, Brad T, Weigand A, Flot J-F (2023) Water diviners multiplied: cryptic diversity in the *Niphargus aquilex* species complex in Northern Europe. Zoology. preprint https://doi.org/10.1101/2023.08.13.553147

Weber D, Stoch F, Knight LRFD, Chauveau C, Flot J-F (2021b) The genus *Microniphargus* (Crustacea, Amphipoda): evidence for three lineages distributed across northwestern Europe and transfer from Niphargidae to Pseudoniphargidae. Belgian Journal of Zoology 151: 169–191. https://doi.org/10.26496/bjz.2021.92

Wichers HJ (1964) Sur une nouvelle espèce d'Amphipode, intermédiaire entre *Niphargus* et *Niphargellus*, du Boulonnais. Beaufortia 147(11): 185–198.

Wolf B (1934) Animalium Cavernarum Catalogus. Verlag für Naturwissenschaften, 's-Gravenhage, 918 pp.

Yu Y, Blair C, He X (2020) RASP 4: Ancestral State Reconstruction Tool for Multiple Genes and Characters. Yoder A (Ed.). Molecular Biology and Evolution 37(2): 604–606. https://doi.org/10.1093/molbev/msz257

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: Range size and beta diversity in non-seasonal habitats. Global Ecology and Biogeography 23(10): 1135–1145. https://doi.org/10.1111/geb.12200

Supplementary Material 1

Tables S1–S3

Authors: Weber D, Wendt M, Schmitt T (2025)
Data type: .zip
Explanation notes: Table S1. Collecting sites and Genbank numbers of *Niphargus fontanus*. In order to protect caves and subterranean cavities, their coordinates were only given to two decimal places. — **Table S2.** PCR primers used for DNA amplification. — **Table S3.** Number of haplotypes, species identification by ASAP partition 1 and partition 2. Column A shows the number of the haplotype, columns B and C the results of the species limitation by ASAP partition 1 and partition 2 (partition 1 corresponds to *Niphargus fontanus*), column D indicates which haplotype belongs to *Niphargus fontanus*, and column E shows the inventory number used in our paper and corresponding to Table S1 or the name in Genbank

Link: https://doi.org/10.3897/asp.82.e116160.suppl1

Supplementary Material 2

Figures S1–S3

Authors: Weber D, Wendt M, Schmitt T (2025)
Data type: .pdf
Explanation notes: Figure S1. Neighbor-Joining tree with default settings. The crown group is formed by *Niphargus fontanus*, followed by one haplotype that is saved in Genbank as *N. fontanus*, but belongs to another species (marked as *Niphargus* sp. (1). The numbers reflect haplotypes, see Table S3, column 1. Obvious mistakes in Genbank, like one clade naming with two species names (*Niphargus balcanicus* and *Niphargus kusceri*) or one species name occurring several times (like *Niphargus vietrenicensis*) are not corrected. — **Figure S2.** Modified graphical output from (a, c, e) Dispersal-Extinction-Cladogensis (DEC) analysis and (b, d) Bayesian Binary MCMC (BBM) analysis (exported from RASP) based on the mtDNA dataset. Colour code of each node indicates the most likely state of alternative ancestral ranges of *N. fontanus* with the posterior probability for the node next to it. Tip labels contain the area codes and correspond to the GenBank accessions codes of Table S1. The map provides the defined regions of *N. fontanus* distribution. The legend provides the colour codes with the corresponding region codes. — **Figure S3.** Names of the colours used in Figs. 2, 3, 4 and 5 following https://www.w3schools.com/colors/colors_names.asp.

Link: https://doi.org/10.3897/asp.82.e116160.suppl2