Zurich Open Repository and Archive
University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2023

Phylogenetic structure and molecular species delimitation hint a complex evolutionary history in an Alpine endemic Niphargus clade (Crustacea, Amphipoda)

Knüsel, Mara ; Borko, Špela ; Alther, Roman ; Salussolia, Alice ; Flot, Jean-François ; Altermatt, Florian ; Fišer, Cene ; Stoch, Fabio

Abstract: Subterranean fauna is an important contributor to the global fauna, but it is still understudied and a large part of its taxonomy is not yet resolved. One species complex with unresolved taxonomy is the groundwater amphipod Niphargus ruffoi, endemic to the Alpine chain. Here, we used new samples from across the Alpine arc to review the taxonomic status of the entire clade, including the species N. ruffoi and Niphargus arolaensis. We sequenced four genetic markers from the collected specimens, assessed the phylogenetic position of N. ruffoi within the genus, and studied the structure of this species complex using four molecular species delimitation methods. We tested for recombination using the alignments of the concatenated nuclear rDNA genes. The phylogenetic analyses revealed high support for the monophyly of the studied species complex, defining two lineages (i.e., N. arolaensis and N. ruffoi) within the clade. Molecular species delimitation methods suggested that N. arolaensis is a single species, while N. ruffoi should be considered as a species complex of three (using ITS) to eight (using COI) putative species. Moreover, we found a discrepancy between the different nuclear ribosomal DNA markers, indicating a possible recombination with fragments of 28S DNA of N. ruffoi s. lat. present in the genome of N. arolaensis. For the above-mentioned reasons, the internal phylogenetic structure of N. ruffoi s. lat. could not be fully resolved. Moreover, no clear morphological evidence supported the molecular species delimitation. Consequently, no taxonomic changes were proposed. We postulate that this complex scenario was influenced by Pleistocene climate oscillations with subsequent fragmentation events and secondary contacts, making this an interesting study system to investigate the evolution and biogeography of Alpine clades.

DOI: https://doi.org/10.1016/j.jcz.2023.07.001

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-252770
Journal Article
Published Version

The following work is licensed under a Creative Commons: Attribution 4.0 International (CC BY 4.0) License.

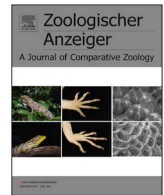
Originally published at:
Knüsel, Mara; Borko, Špela; Alther, Roman; Salussolia, Alice; Flot, Jean-François; Altermatt, Florian; Fišer, Cene; Stoch, Fabio (2023). Phylogenetic structure and molecular species delimitation hint a complex evolutionary history in an Alpine endemic Niphargus clade (Crustacea, Amphipoda). Zoologischer Anzeiger, 306:27-36.
DOI: https://doi.org/10.1016/j.jcz.2023.07.001

JCZ: Zoologischer Anzeiger - A Journal of Comparative Zoology 306 (2023) 27–36

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz



Phylogenetic structure and molecular species delimitation hint a complex evolutionary history in an Alpine endemic *Niphargus* clade (Crustacea, Amphipoda)

Mara Knüsel [a,b,*], Špela Borko [c], Roman Alther [a,b], Alice Salussolia [d], Jean-François Flot [d,e], Florian Altermatt [a,b], Cene Fišer [c], Fabio Stoch [d]

[a] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057, Zurich, Switzerland
[b] Department of Aquatic Ecology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600, Dübendorf, Switzerland
[c] Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia
[d] Evolutionary Biology & Ecology, Université libre de Bruxelles (ULB), Brussels, Belgium
[e] Interuniversity Institute of Bioinformatics in Brussels – (IB)[2], Brussels, Belgium

ARTICLE INFO

Handling Editor: Martin Schwentner

Keywords:
Amphipoda
Niphargus
Alps
Groundwater
Taxonomy
Molecular phylogeny

ABSTRACT

Subterranean fauna is an important contributor to the global fauna, but it is still understudied and a large part of its taxonomy is not yet resolved. One species complex with unresolved taxonomy is the groundwater amphipod *Niphargus ruffoi*, endemic to the Alpine chain. Here, we used new samples from across the Alpine arc to review the taxonomic status of the entire clade, including the species *N. ruffoi* and *Niphargus arolaensis*. We sequenced four genetic markers from the collected specimens, assessed the phylogenetic position of *N. ruffoi* within the genus, and studied the structure of this species complex using four molecular species delimitation methods. We tested for recombination using the alignments of the concatenated nuclear rDNA genes. The phylogenetic analyses revealed high support for the monophyly of the studied species complex, defining two lineages (i.e., *N. arolaensis* and *N. ruffoi*) within the clade. Molecular species delimitation methods suggested that *N. arolaensis* is a single species, while *N. ruffoi* should be considered as a species complex of three (using ITS) to eight (using COI) putative species. Moreover, we found a discrepancy between the different nuclear ribosomal DNA markers, indicating a possible recombination with fragments of 28S DNA of *N. ruffoi* s. lat. present in the genome of *N. arolaensis*. For the above-mentioned reasons, the internal phylogenetic structure of *N. ruffoi* s. lat. could not be fully resolved. Moreover, no clear morphological evidence supported the molecular species delimitation. Consequently, no taxonomic changes were proposed. We postulate that this complex scenario was influenced by Pleistocene climate oscillations with subsequent fragmentation events and secondary contacts, making this an interesting study system to investigate the evolution and biogeography of Alpine clades.

1. Introduction

The subterranean fauna is an important contributor to the global fauna (Gibert and Culver, 2009; Bardgett and van der Putten, 2014), and it is under anthropogenic pressure (Mammola et al., 2019). The high subterranean biodiversity is characterized by restricted distribution ranges of species and a high rate of endemism (Trontelj et al., 2009; Bregović et al., 2019). Despite its rich and unique biodiversity, the subterranean fauna is still understudied (Mammola et al., 2020). First, many subterranean ecosystems remain poorly explored due to a lack in accessibility, the so-called 'Racovitzan impediment' (Ficetola et al., 2019). This impediment results in limited data availability and small sample sizes. Second, a large part of the taxonomy on the subterranean fauna is still unresolved, enforced by morphological convergence, parallel evolution, and the presence of cryptic species (Lefébure et al., 2007; Delić et al., 2017; Eme et al., 2018). The need for increased taxonomic and biogeographic knowledge is of paramount importance, particularly for the protection of subterranean crustaceans, which are typically the most abundant and diverse metazoan group within subterranean ecosystems (Sket, 1999b). This need for knowledge is especially crucial in areas that are most affected by climate change.

Amphipod crustaceans are a key component of the groundwater

* Corresponding author. Eawag, Überlandstrasse 133, 8600, Dübendorf, Switzerland.
E-mail address: mara.knuesel@eawag.ch (M. Knüsel).

https://doi.org/10.1016/j.jcz.2023.07.001
Received 16 May 2023; Received in revised form 30 June 2023; Accepted 6 July 2023
Available online 7 July 2023
0044-5231/© 2023 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

fauna (Sket, 1999a; Väinölä et al., 2008; Zagmajster et al., 2018; Borko et al., 2021). The genus *Niphargus* (Schiödte, 1849) is the most common and species rich subterranean amphipod genus of the West Palearctic. From an ecological point of view, subterranean amphipods (mostly gatherers and predators) contribute substantially to the functioning of groundwater ecosystems. Not only the number of species and their abundances, but also their ability to inhabit nearly all types of groundwater and to hold several trophic positions (Premate et al., 2021), make them an essential part of subterranean species communities (Sket, 1999a). Cryptic species are common in the mega-diverse genus *Niphargus* (more than 400 described species: Horton et al., 2023) and intraspecific variability can be large in comparison to interspecific differences (Fišer et al., 2008; Fišer et al., 2009; Fišer, 2019). Such cryptic species have been studied in various parts of the range of *Niphargus*, including the Balkans, Italy, as well as Central Europe (Lefébure et al., 2007; Trontelj et al., 2009; McInerney et al., 2014; Delić et al., 2017; Eme et al., 2018; Stoch et al., 2022).

Across the whole distribution range of *Niphargus*, the Alps are of particular interest. The succession of glacial periods during the Pleistocene likely prompted the isolation of populations and subsequent recolonization during interglacial periods from local refugia or areas along the glacier borders of the Alpine arc, resulting in a complex history of lineage divergence and secondary contact (Stoch et al., 2020; Delić et al., 2021). A recent study showed that in species occurring along the last maximal extents of glacier borders, the imprint of glaciations might have been stronger than in strictly Alpine *Niphargus* species (Jardim de Queiroz et al., 2022), which include many of the earliest records of *Niphargus* (Godet, 1877; Forel, 1904; Schellenberg, 1934; Strinati, 1966). Recent work has contributed to a better understanding of multiple *Niphargus* species and clades from the Alps (Lefébure et al., 2007; Trontelj et al., 2009; Fišer et al., 2010; Fišer et al., 2017; Fišer et al., 2018; Altermatt et al., 2019; Stoch et al., 2020; Alther et al., 2021; Stoch et al., 2022). These studies highlight the complex biogeography and phylogeographic structure of Alpine *Niphargus* species. Unfortunately, for most of these clades the taxonomy is not yet resolved.

One morphospecies with unresolved taxonomy occurring in the Alpine range is *Niphargus ruffoi* Karaman, 1976. It was first described from a cave in Italy (Gortani abyss, Friuli Venezia Giulia region), near the Slovenian border. By that time, its relationship with *Niphargus thienemanni* Schellenberg, 1934 was not clear due to the inaccurate description of the latter that did not include any drawing. In the original description, three specimens of *N. ruffoi* were reported, two females of 3.3 mm and one male of 3 mm body length (Karaman, 1976). Related specimens that could not be assigned to either *N. ruffoi* or *N. thienemanni* were later mentioned in multiple papers from Switzerland as *Niphargus* cf. *thienemanni* (Fišer et al., 2017; Fišer et al., 2018; Alther et al., 2021). For example, Fišer et al. (2017) noted one juvenile from the Alps in southern Switzerland and Alther et al. (2021) suggested a lineage of at least two species, provisionally labelled *N.* cf. *thienemanni* 1 and *N.* cf. *thienemanni* 2. Since then, the taxonomy of these lineages has remained unresolved due to the limited data availability and uncertain phylogeny, and as a result, *N. ruffoi* had not been considered as a member of the Swiss fauna (Altermatt et al., 2019). A close relative to *N. ruffoi* is *Niphargus arolaensis* Alther, Bongni, Borko, Fišer, and Altermatt, 2021, which has been suggested sister species to *N.* cf. *thienemanni* by Alther et al. (2021). It was recently discovered along the Aare catchment in Switzerland when it came up as a monophyletic lineage in a multilocus phylogeny (Alther et al., 2021). Reported body lengths clearly differ from those of *N. ruffoi* type specimens (7.7 mm for a male and 7.8–9.5 mm for females) (Alther et al., 2021). In addition, the reported distribution and molecular data justified the distinction to previous samples labelled as *N.* cf. *thienemanni*. Recently, there were additional specimens of *N. arolaensis* reported from the Töss catchment in North-Eastern Switzerland, expanding the known distribution of *N. arolaensis* eastwards to another catchment area (Studer et al., 2022). To summarize, the taxonomic position of the previously reported lineages of *N.* cf. *thienemanni* among the closely related *N. ruffoi*, *N. arolaensis*, and *N. thienemanni* remains unclear.

Here, we use new samples from Italy, Austria, Germany, and Switzerland to review the taxonomic status of the entire clade, covering *N. ruffoi*, *N. arolaensis* and specimens previously labelled as *N.* cf. *thienemanni*. We give an overview of the current taxonomic status of *N. ruffoi*, with the goal of advancing one step further in resolving the taxonomy of *Niphargus* in the Alpine arc.

2. Material and methods

2.1. Sampling and origin of specimens

Switzerland. The Swiss samples (eight sites) were collected as part of a countrywide sampling campaign (except NC107, NC171 and ND462). They were obtained at spring catchment boxes (hereafter referred to as spring boxes), which are small facilities used by drinking water providers to source groundwater passively through horizontal perforated pipes. The data collection was conducted by local drinking water providers, with instructions and sampling material provided by the authors of this study (similar as in Alther et al., 2021 and Studer et al., 2022). Specimens were collected using two different methods. First, we asked the water providers to attach a filter net (mesh size 0.8 mm, Sefiltec AG, Höri, Switzerland) to the inlet of the drainage pipe, to filter organisms from the passively flowing spring water. The filter net was attached for approximately seven days, before being checked for organisms. Second, the water providers were instructed to sample the sedimentation/overflow basin of the spring box with a small hand net (mesh size 0.35 mm, JBL GmbH & Co. KG, Neuhofen, Germany). Samples from Waldkirch (voucher id CH22236, 22242, 22851, and 22865) were collected from a filter net that was attached for multiple months. We pre-sorted all organisms in the lab using a stereomicroscope (Leica M205 C), and we stored groundwater amphipod specimens separately, preserving them in 80% ethanol at 4 °C. Samples NC107 and NC171 were collected from streams in 2013 and 2014, as part of the Biodiversity Monitoring Program of Switzerland (Koordinationsstelle BDM, 2014) using kicknet sampling. Sample ND462 was collected from a natural spring in 2019.

Italy. Samples (three sites) were collected during a survey to resolve phylogeny and taxonomy of Italian amphipods (Stoch and Flot, 2017). Topotypes of *N. ruffoi* were collected in the cave (Fontanon di Goriuda) that drains the waters of the Gortani Abyss using a hand net. The other two sites (a cave and a spring) are the only findings of species in this complex from an extensive survey that covered more than 3000 caves and springs in the Southern Alps (from the French to Slovenian borders). Both sites are not located in carbonate rocks but in shale, and specimens were collected using a net with handle for macrobenthic surveys.

Germany. Two sites hosting *N. ruffoi* (one of them included in our analyses) were identified during a multi-year sampling survey conducted by Reinhard Gerecke in the National Park of Berchtesgaden (Bavaria) and the methodology is described in Gerecke and Franz (2006). The specimens were initially identified as *Niphargus forelii* Humbert, 1876 in the interstitial of a spring (Stoch, 2006), but in the present paper assigned to *N. ruffoi*. Out of about 700 springs sampled in the National Park using several methods (hand nets, drift nets and interstitial sampling), the species was found in two springs only.

Austria. A single site of *N. ruffoi* was discovered during an extensive survey of the Austrian amphipod fauna carried out by Erhard Christian (University of Vienna) with the collaboration of local speleological groups. Samples were collected with a hand net.

The list of studied specimens and the origin of samples are available in Supplementary Table S1.

2.2. Molecular analysis

Sequences were obtained in three different laboratories using different protocols that are fully reported in the Supplementary

Material. Overall, we sequenced 23 individuals of *N. ruffoi* from 13 sites. We amplified Folmer's fragment of the mtDNA COI gene (Folmer et al., 1994), and three nuclear markers, namely the complete ITS region (28S flank, ITS1, 5.8S, ITS2 and 28S flank; Flot et al., 2010b) and Verovnik's fragment of the 28S gene (named herein 28S-22, 761 bp; Verovnik et al., 2005) in 22 individuals. Furthermore, a second fragment of 28S (named 28S-66, 530 bp, not overlapping with 28S-22; Ntakis et al., 2015) was sequenced in 17 individuals. For *N. arolaensis,* we used 6 specimens from 3 locations, all sequenced for the same four fragments and one specimen additionally on the histone 3 gene (H3, 331 bp; Colgan et al., 1998).

Chromatograms were inspected, assembled, and cleaned using the programs Sequencher 5.4.6 (Gene Codes) and Geneious 11.0.3 (Dotmatics). Some 28S and ITS chromatograms contained double peaks, as expected in the case of length-variant heterozygotes (Flot et al., 2006); these individuals were phased using the web tool Champuru (Flot, 2007, available online at https://eeg-ebe.github.io/Champuru).

Information on sequenced specimens and GenBank accession codes are available in Supplementary Table S1.

2.3. Phylogenetic inference

To assess the phylogenetic position of *N. ruffoi* within the genus, we assembled the dataset comprising 23 specimens of *N. ruffoi*, 6 specimens of the sister species *N. arolaensis*, and 163 *Niphargus* taxa from different phylogenetic lineages with emphasis on potentially closely related species, each represented by one specimen. We used the family Pseudoniphargidae, represented by *Microniphargus leruthi* Schellenberg, 1934 and two species from genus *Pseudoniphargus* Chevreux, 1901, as an outgroup since it is the sister clade to Niphargidae (Weber et al., 2021). We included available sequences of COI, 28S, and H3 from previous studies (Borko et al., 2022 and references therein) as well as 93 newly obtained sequences (Supplementary Table S1).

For phylogenetic inference analysis we aligned the sequences of COI, H3 and 28S markers using MAFFT 7.3.88 (Katoh and Standley, 2013), using the E–INS–I algorithm with scoring matrix 1PAM/k = 2 and the highest gap penalty. We eliminated poorly aligned positions from both 28S fragments using Gblocks (Talavera and Castresana, 2007). The alignments were concatenated and partitioned by codon position for H3 and COI and with one partition for each part of 28S.

We reconstructed the phylogenetic relationships with maximum likelihood (ML) in IQ-TREE 2.2.0 (Minh et al., 2020) and Bayesian inference (BA) in MrBayes v3.2.6 (Ronquist et al., 2012). For the IQTREE ML analysis, the best-fit substitution model was determined using ModelFinder (implemented in IQTREE; Kalyaanamoorthy et al., 2017). The subsequent phylogenetic analysis included ultrafast bootstrap approximation (UFBoot) and SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010; Hoang et al., 2018). For BA, the optimal substitution model was chosen using Partition Finder 2 (Lanfear et al., 2017) under the corrected Akaike information criterion (AICc). We ran two simultaneous independent runs with four chains each for 20 million generations, sampled every 2000th generation. Convergence was assessed through average standard deviation of split frequencies, LnL trace plots and PSRF (potential scale reduction factor), and the effective sample size. We analysed the results in Tracer 1.7 (Rambaut et al., 2018), discarded the first 25% of trees as burn in and calculated the 50% majority rule consensus tree. This BA analysis was run on the CIPRES Science Gateway (Miller et al., 2010).

2.4. Molecular species delimitation methods and recombination tests

First, we calculated the average uncorrected pairwise genetic differences (i.e., p-distances) for the COI and ITS fragments between *N. ruffoi* and *N. arolaensis* using Geneious 11.0.3.

ASAP (Assemble Species by Automatic Partitioning; Puillandre et al., 2021) was run on the COI sequences of *N. ruffoi* and *N. arolaensis*, using the Kimura two-parameter substitution model on the ASAP web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html).

Species delimitation using the PTP (Poisson Tree Processes) model was also performed using the most recent version (Kapli et al., 2017): after removing duplicates, ML phylogenetic trees were obtained for COI and ITS sequences of *N. ruffoi* and *N. arolaensis* using IQ-TREE 2 and then the PTP analysis was run on the species delimitation server https://mptp.h-its.org/with a P-value threshold of 0.001.

Relationships among haplotypes were explored for each of the four markers (COI, ITS, 28S-22 and 28S-66) using the program HaplowebMaker (Spöri and Flot, 2020) applying the median-joining algorithm. In case of very long indels present in ITS, we preserved only the bases which were different in the different individuals, deleting the highly repetitive parts using AliView 1.25 (Larsson, 2014). In the case of rDNA markers, haplotype networks were turned into haplowebs by adding connections between haplotypes found co-occurring in heterozygous individuals, allowing to delineate FFRs (Fields For Recombination; Doyle, 1995) (Flot et al., 2010a). Given the very low number of heterozygous individuals found in the dataset, results of PTP analysis were superimposed on haplotype networks to improve the delimitation of putative species.

Finally, we tested for recombination using the alignments of the concatenated rDNA genes (ITS, 28S-22 and 28S-66); recombination detection methods were implemented in the RDP4 package (Martin et al., 2015). Default settings were used. Only recombination events detected with a P-value <0.05 after Bonferroni correction were considered.

3. Results

All phylogenetic analyses revealed high support for the monophyly of the studied species complex, regardless of the method of phylogenetic inference used (Fig. 1 and Supplementary Fig. S1). The clade comprised two lineages, corresponding respectively to *N. arolaensis* and *N. ruffoi. Niphargus arolaensis* is a species hitherto confined to Switzerland and showed little genetic divergence in the studied markers. Contrastingly, *N. ruffoi* has a wider distribution and comprised several sub-lineages distributed across Switzerland (here for the first time formally reported from Switzerland), the Western and South-Eastern Alps in Italy, and the North-Eastern Alps in Austria (Fig. 2). Three of these sub-lineages were well supported; however, the hierarchy between them was not resolved. This species complex and its only partially recovered phylogenetic structure was also reflected in the presumed species composition, which could not be delimited satisfactorily. Species delimitation methods suggested that the focal monophylum comprised a single species, *N. arolaensis*, and a species complex, *N. ruffoi* s. lat., encompassing between three and eight putative species (see below).

The species status of *N. arolaensis* in relation to *N. ruffoi* s. lat. was well justified by molecular methods. The mitochondrial marker COI supported the distinction between the two species regardless of the method applied, i.e., ASAP (all of the ten best partitions support the species separation, Supplementary Table S2), PTP ($p < 0.001$, Fig. 3), haplotype network (separated by more than 30 substitutions, Fig. 4) and high genetic distance (uncorrected p distance = 5.5–8.2%, Supplementary Table S3). The results obtained using the ITS nuclear marker were concordant with those obtained with the mitochondrial marker, again distinguishing *N. arolaensis* from *N. ruffoi* using PTP, haploweb (separated by 14 substitutions), and genetic distances (uncorrected p-distance = 5.5–9.3%) (Figs. 3 and 4 and Supplementary Table S4). The fragment 28S-22 confirmed the separate position of *N. arolaensis*, although separated by a single substitution (Fig. 5). By contrast, according to the fragment 28S-66 *N. arolaensis* was joined in the haploweb with two specimens (voucher id CH20114, 20115) that were in all other analyses recognized as members of *N. ruffoi* s. lat (Fig. 5). A recombinant analysis indicated that the fragment 28S-66 in *N. arolaensis* (all vouchers) contains an 84–1457 bp long insertion presumably derived from some individuals of *N. ruffoi* s. lat. (voucher id CH20114, 20115,

Fig. 1. IQ-Tree: Phylogenetic hypothesis based on Maximum Likelihood analysis. Nodes are labelled with ultrafast bootstrap support (UFBoot)/approximate likelihood ratio test (SH-aLRT).

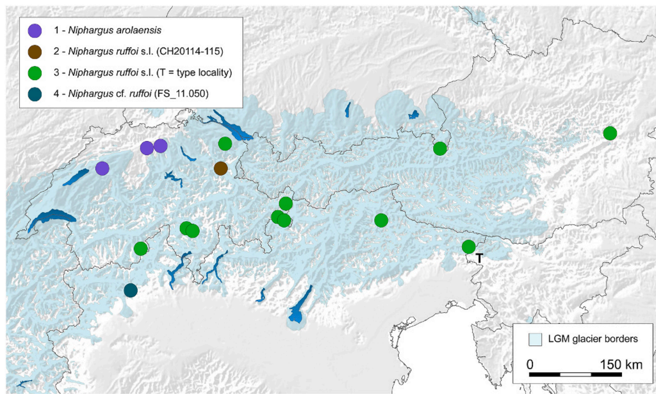


Fig. 2. Map of the Alpine chain showing the distribution of the sampling sites; site colors represent the results of PTP species delimitation reported in Figs. 3 and 4 applied to ITS; the extent of the glaciers during the Last Glacial Maximum (LGM) is reported as well. (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

22865) (Fig. 6).

The possible existence of several species within *N. ruffoi* s. lat. remained unclear. The mitochondrial marker COI suggested that the complex *N. ruffoi* s. lat. may comprise up to eight putative species (ASAP, PTP and haplotype network) that differ by up to 6% in uncorrected p-distances (Figs. 3 and 4 and Supplementary Tabs. S2–3). Analyses of the nuclear marker ITS implied a more conservative solution with three putative species (supported both by PTP and haploweb, Figs. 3 and 4), and, importantly, its phylogenetic tree indicated a slightly different phylogenetic history of the complex than COI did, albeit branch support was not high enough to confirm a marked mitonuclear discordance. Moreover, two individuals (voucher id CH20114, 20115) had a long indel that made them very distinct from the rest of the samples. The distinctness of these two specimens remained even when we treated this indel as a single mutational event (Fig. 4). The structure of the ITS haploweb, however, differed from that of the haplowebs obtained from both 28S fragments. For example, the haploweb of 28S-22 separated individuals of *N. ruffoi* s. str. (i.e., specimens from the type locality) from all other individuals of *N. ruffoi* s. lat. but lumped all three putative species proposed by ITS (Fig. 5). As we could not successfully sequence the fragment 28S-66 in all individuals, we could not use it for further comparisons, but within Swiss specimens, its haploweb was different as well (see above). In brief, within *N. ruffoi* s. lat. we detected a putative mismatch in the hierarchical structure of nuclear (ITS) and mitochondrial (COI) phylogeny, as well as mismatched differentiation between the three nuclear ribosomal fragments (28S-22, 28S-66 and ITS).

We also checked the morphology of a few well-preserved *N. ruffoi* s. lat. individuals from Switzerland. We observed that the distalmost segment of the mandibular palp in Swiss populations was longer than in specimens from the type locality (Karaman, 1976) but the low number of individuals was insufficient to perform a robust and well-supported morphometric analysis.

4. Discussion

Our study revealed a puzzling discrepancy between mitochondrial and nuclear ribosomal DNA signals: although the COI haplotype network was compatible with morphology (separating nicely *N. arolaensis* from *N. ruffoi*), two *N. ruffoi* individuals grouped with *N. arolaensis* in one of the rDNA haplowebs. To understand the causes of this problem, we looked closer at the rDNA data and realized that it displayed a signal suggestive of possible recombination (which might indicate interspecific hybridization), with the breakpoint detected somewhere between the 28S-22 and 28S-66 fragments. Based on the congruence between species delimitation methods based on morphology, COI, and ITS, our study clearly supports the separation between *N. arolaensis,* and a putative species complex we name *N. ruffoi* s. lat. The relatively unambiguous status of *N. arolaensis* has been expected, as these populations distinctly differ from the rest of the entire clade in three genetic markers, with clear morphological diagnostic traits (Alther et al., 2021) and a spatially well-defined distributional range.

The finding of possible evidence for recombination between linked markers within ribosomal DNA was unexpected as such tightly linked markers are usually assumed to evolve in a similar way. The recent finding of recombination between mitochondrial markers in corals (Banguera-Hinestroza et al., 2019), together with our results, suggest that caution should be exerted when using supposedly linked markers for phylogenetic and species delimitation analyses, and that one should check for recombination whenever confronted to discrepancies between markers. Molecular evidence suggests that the recombination event occurred from at least one lineage of *N. ruffoi* s. lat. into *N. arolaensis*, resulting in 28S–66 sequences of *N. arolaensis* being identical to some *N. ruffoi* s. lat. Indeed, this species complex evolved in the Alpine region, which was strongly influenced by late Pliocene and Pleistocene climatic oscillations. It has been suggested that in such multiple fragmentation events mitochondrial DNA responds more sensitively to genetic drift and

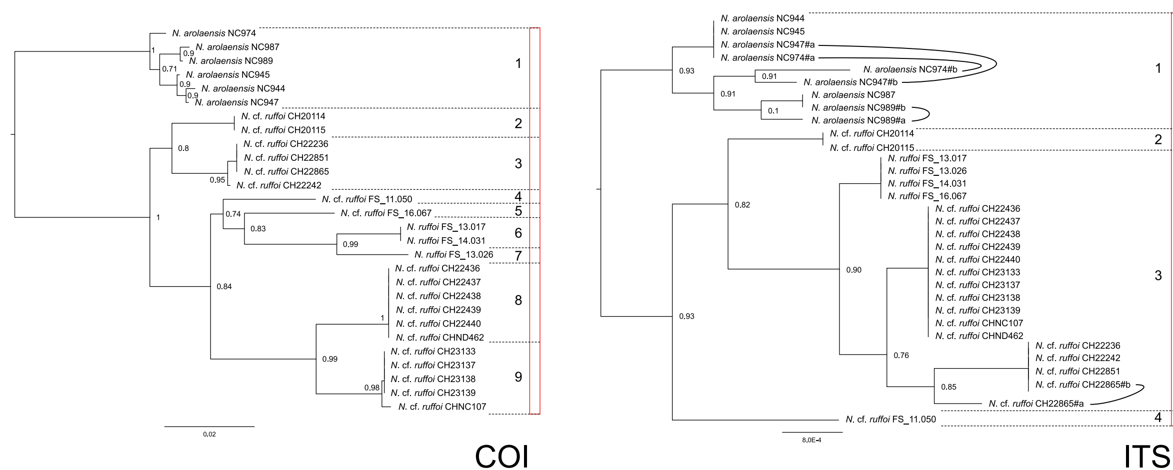


Fig. 3. Maximum Likelihood phylogenetic trees of the *N. ruffoi-N. arolaensis* clade based on COI (left) and ITS (right) markers (outgroups omitted for clarity). Numbers refer to the species delimited using PTP ($P < 0.001$) based on the same trees. For COI, they are also in accordance with the best ASAP partitioning scheme (not shown, ASAP-score 2.5). Arcs reported in the ITS tree connect the two alleles of the same heterozygous individual.

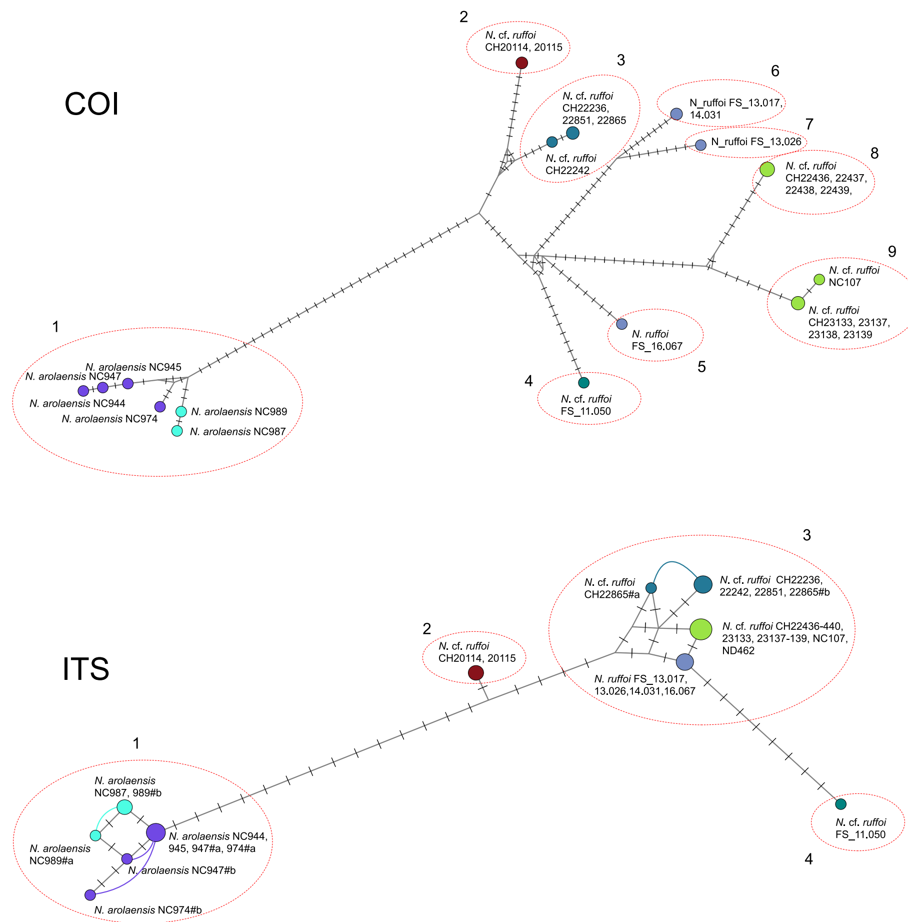


Fig. 4. Haplowebs based on the median joining network algorithm of the COI and ITS sequences; species delimitations are based on the results of PTP analysis reported in Fig. 3. Each color in the ITS haploweb represents distinct FFRs (Fields For Recombination). Arcs reported in the ITS haploweb connect the two alleles of the same heterozygous individuals. (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

evolves much faster than nuclear DNA, as it has smaller effective size. This results in a more homogenous genetic structure in nuclear DNA than in mitochondrial DNA (Després, 2019). The fragmentation followed by secondary contact could also explain recombination events between *N. ruffoi* s. lat. and *N. arolaensis*, and the shared recombinant insert of 28S–66.

While molecular support for *N. ruffoi* s. lat. is unambiguous, its internal phylogenetic structure does not allow more detailed taxonomic conclusions. The relatively large intraspecific distances within *N. ruffoi* s. lat. (for some of them, larger even than the distances between *N. ruffoi* s lat. and *N. arolaensis*) suggest that it is most likely composed of more than one species. But the discrepancies displayed by the different markers and the absence of clear-cut morphological differences make it impossible to ascertain at this stage the actual number of species within *N. ruffoi* s. lat. Overall, while there are strong hints that the entire species complex comprises minimally three species, the evidence is not sufficient to reject a hypothesis of a single pan-Alpine species with mismatched genetic structure. This might be explained by multiple fragmentations and secondary contacts during the past 2 Myr, roughly resembling the evolutionary history of other Alpine species complexes, such as *Niphargus tatrensis* (Stoch et al., 2020) and *Niphargus stygius* (Delić et al., 2021; Stoch et al., 2022). Noteworthy, given the presumed longevity of subterranean animals (Lunghi and Bilandžija, 2022), the generation time might be an order of magnitude longer than in surface species, making the Plio-Pleistocene history even more "recent". For this reason, we propose no taxonomic changes within *N. ruffoi* s. lat. until the complex is analyzed using more sensitive analyses already applied in other amphipod families, using RADseq (Hupało et al., 2023 for Gammaridae), genome skimming (Zapelloni et al., 2021 for Crangonyctidae), and transcriptomes (Liu et al., 2023 for Talitridae) and including additional individuals.

The phylogenetic structure hints to an interesting biogeographic hypothesis proposing that the complex originated in the Western Alps and spread eastward. One of the most basal lineages (*N. arolaensis*) is endemic to Switzerland. Further basal splits of the complex were found in either Switzerland or Western Italy, whereas *N. ruffoi* from the eastern part of the Alps split-off relatively recently. The most parsimonious explanation of such phylogeographic structure implies an origin of this species complex in the Western Alps. This view is even strengthened by the broad phylogenetic structure, where the studied species complex is nested within the clade of species from Switzerland and France. This pattern is analogous with a broader albeit roughly 30–20 Myr older spread of species from west to east (McInerney et al., 2014; Borko et al., 2021). It is possible that the dispersal across the Alps predated Plio-Pleistocene glaciations, but fragmentation events started in the west and proceeded eastward. This hypothesis would concur with the relatively clear differentiation of *N. arolaensis* from the rest of the complex, and a more blurred structure in the more eastern parts of the Alpine arc.

While the present status of the whole species complex is only partially resolved and the taxonomic challenges remain, we identified an interesting study system that could help address many evolutionary and biogeographical enigmas. For example, how can such small species spread that far? Some studies indicate large-bodied species of the North American amphipod *Stygobromus* have larger ranges than small species (Culver and Pipan, 2014), which is in agreement with the notion that

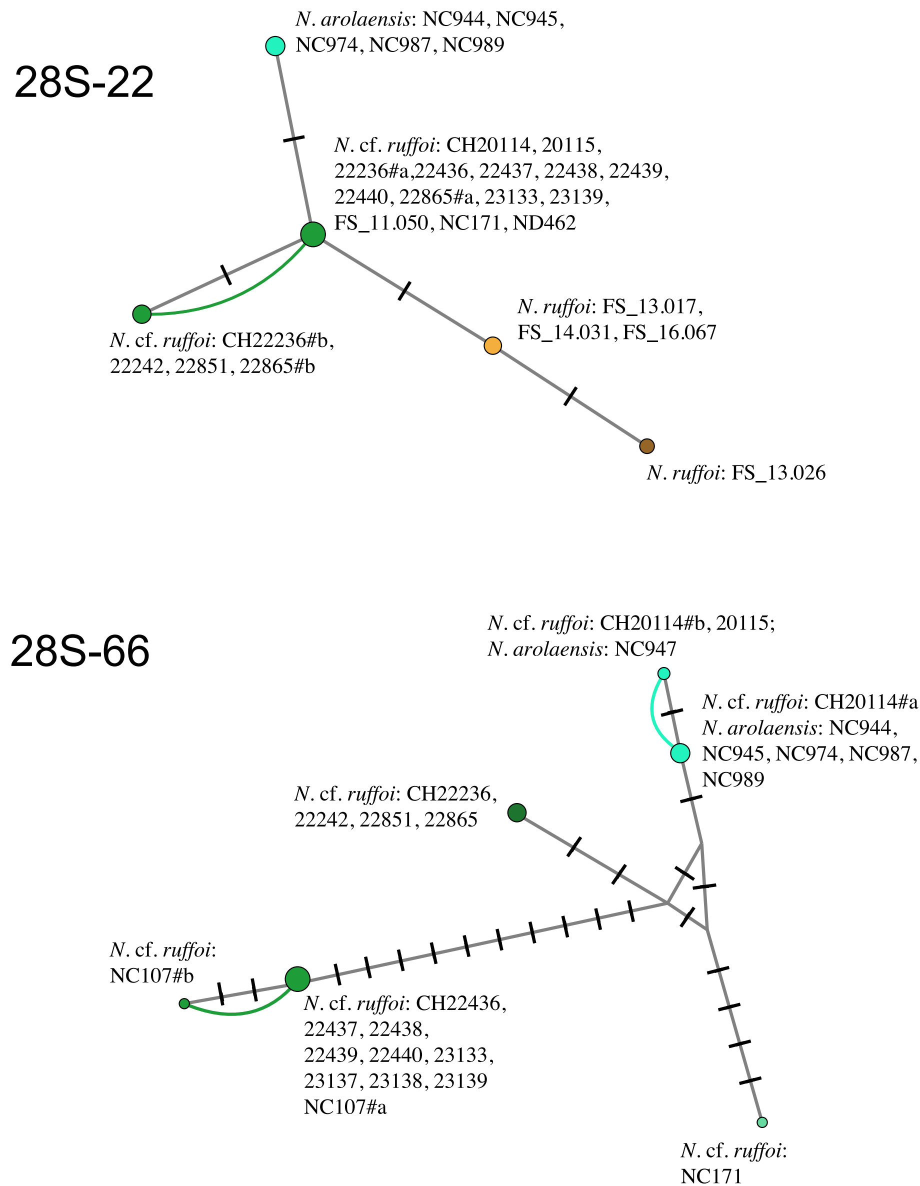


Fig. 5. Haplowebs based on the median joining network algorithm of the 28S-22 and 28S-66 sequences. Each color in the haplowebs represents distinct FFRs (Fields For Recombination). Arcs reported in the haplowebs connect the two alleles of the same heterozygous individuals. (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

larger species are able to move faster or further (Kralj-Fišer et al., 2020). Consequently, all else being equal, larger species can easier maintain gene flow over larger distributional ranges. Second, a detailed analysis of nuclear variation could yield insights into dispersal-fragmentation dynamics, and possibly provide hypotheses on how these species react to climatic fluctuations. Such insights from the past might help us to evaluate how endangered these species might be in the next decades, when the loss of glaciers might lead to a drop of the water table, a phenomenon that apparently happened during glaciations, when water was entrapped in ice cover (Gibbard et al., 2010). Third and most importantly, such complex history may yield new insights into the process of speciation: why do some populations hybridize and others not (Després, 2019), and what are the consequences of hybridization for maintenance of biodiversity (Marques et al., 2019)? We recognize that the collection of additional samples may be challenging due to the Racovitzan impediment (Ficetola et al., 2019); however, new molecular methods, such as eDNA, might improve detection of range boundaries, whereas genome-wide analyses might overcome limitations of sample size.

Funding

CF and ŠB were supported by the Slovenian Research Agency through project J1-2464, program P1-0184 and PhD grant to ŠB. MK, RA, and FA were supported by the Swiss Federal Office for the Environment FOEN/BAFU (project “AmphiWell” to FA and RA) and the Swiss National Science Foundation (grant nr. PP00P3_150698 to FA), as well as the University of Zurich Research Priority Programme on Global Change and Biodiversity (URPP GCB). AS was supported by a DarCo (BIODIV21_0006) PhD grant. FS and JFF were supported by the Belgian Fond de la Recherche Scientifique - FNRS via “Chargé de recherches” fellowship n° FC43267 to FS and “Projet de Recherches” grant n° T.00078.23 to JFF. Part of the equipment used in this study was purchased for the project “Development of research infrastructure for the international competitiveness of the Slovenian RRI space –RI–SI–LifeWatch”. The operation is co-financed by the Republic of Slovenia, Ministry of Education, Science and Sport and the European Union from the European Regional Development Fund. This research was further supported by the Biodiversa + project DarCo

Fig. 6. Recombinant analysis using the alignments of the concatenated rDNA genes (ITS, 28S-22 and 28S-66). Only recombination events detected with $p < 0.05$, after Bonferroni correction, were considered.

(BIODIV21_0006).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appear to have influenced the work reported in this paper.

Data availability

Information on the studied specimens and the origin of samples are provided in the Supplementary Material. Sequences will be uploaded to GenBank.

Acknowledgments

We would like to thank all water providers who contributed samples for this study and Nadine Locher, who did the Swiss laboratory work. Special thanks to the collectors (reported in Table S1) of *Niphargus ruffoi* s. l. specimens from Italy, Germany, and Austria, as well as to Erhard Christian, who provided us with the Austrian material for study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2023.07.001.

References

Altermatt, F., Alther, R., Fišer, C., Švara, V., 2019. Amphipoda (Flohkrebse) der Schweiz: Checkliste, Bestimmung und Atlas. info fauna, Centre suisse de cartographie de la faune, Neuchâtel, p. 389.

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). Subterr. Biol. 39, 1–31. https://doi.org/10.3897/subtbiol.39.66755.

Banguera-Hinestroza, E., Sawall, Y., Al-Sofyani, A., Mardulyn, P., Fuertes-Aguilar, J., Cárdenas-Henao, H., Jimenez-Infante, F., Voolstra, C.R., Flot, J.-F., 2019. mtDNA recombination indicative of hybridization suggests a role of the mitogenome in the adaptation of reef-building corals to extreme environments. bioRxiv. https://doi.org/10.1101/462069.

Bardgett, R.D., van der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. Nature 515, 505–511. https://doi.org/10.1038/nature13855.

Borko, Š., Altermatt, F., Zagmajster, M., Fišer, C., 2022. A hotspot of groundwater amphipod diversity on a crossroad of evolutionary radiations. Divers. Distrib. 28, 2765–2777. https://doi.org/10.1111/ddi.13500.

Borko, Š., Trontelj, P., Seehausen, O., Moškrič, A., Fišer, C., 2021. A subterranean adaptive radiation of amphipods in Europe. Nat. Commun. 12, 3688. https://doi.org/10.1038/s41467-021-24023-w.

Bregović, P., Fišer, C., Zagmajster, M., 2019. Contribution of rare and common species to subterranean species richness patterns. Ecol. Evol. 9, 11606–11618. https://doi.org/10.1002/ece3.5604.

Chevreux, É., 1901. Amphipodes des eaux souterraines de France et d'Algérie. Bulletin de la Société Zoologique de France.

Colgan, D.J., McLauchlan, A., Wilson, G.D.F., Livingston, S.P., Edgecombe, G.D., Macaranas, J., Cassis, G., Gray, M.R., 1998. Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. Aust. J. Zool. 46, 419. https://doi.org/10.1071/ZO98048.

Culver, D.C., Pipan, T., 2014. Shallow Subterranean Habitats: Ecology, Evolution, and Conservation. Oxford University Press, p. 288.

Delić, T., Trontelj, P., Rendoš, M., Fišer, C., 2017. The importance of naming cryptic species and the conservation of endemic subterranean amphipods. Sci. Rep. 7, 3391. https://doi.org/10.1038/s41598-017-02938-z.

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. J. Biogeogr. https://doi.org/10.1111/jbi.14275.

Després, L., 2019. One, two or more species? Mitonuclear discordance and species delimitation. Mol. Ecol. 28, 3845–3847. https://doi.org/10.1111/mec.15211.

Doyle, J.J., 1995. The irrelevance of allele tree topologies for species delimitation, and a non-topological alternative. Syst. Bot. 20, 574. https://doi.org/10.2307/2419811.

Eme, D., Zagmajster, M., Delić, T., Fišer, C., Flot, J.-F., Konecny-Dupré, L., Pálsson, S., Stoch, F., Zakšek, V., Douady, C.J., Malard, F., 2018. Do cryptic species matter in macroecology? Sequencing European groundwater crustaceans yields smaller ranges but does not challenge biodiversity determinants. Ecography 41, 424–436. https://doi.org/10.1111/ecog.02683.

Ficetola, G.F., Canedoli, C., Stoch, F., 2019. The Racovitzan impediment and the hidden biodiversity of unexplored environments. Conserv. Biol. 33, 214–216. https://doi.org/10.1111/cobi.13179.

Fišer, C., 2019. *Niphargus*—a model system for evolution and ecology. In: Encyclopedia of Caves. Elsevier, pp. 746–755.

Fišer, C., Alther, R., Zakšek, V., Borko, Š., Fuchs, A., Altermatt, F., 2018. Translating *Niphargus* barcodes from Switzerland into taxonomy with a description of two new species (Amphipoda, Niphargidae). ZooKeys 113–141. https://doi.org/10.3897/zookeys.760.24978.

Fišer, C., Coleman, C.O., Zagmajster, M., Zwittnig, B., Gerecke, R., Sket, B., 2010. Old museum samples and recent taxonomy: a taxonomic, biogeographic and conservation perspective of the *Niphargus tatrensis* species complex (Crustacea: Amphipoda). Org. Divers. Evol. 10, 5–22. https://doi.org/10.1007/s13127-010-0006-2.

Fišer, C., Konec, M., Alther, R., Švara, V., Altermatt, F., 2017. Taxonomic, phylogenetic and ecological diversity of *Niphargus* (Amphipoda: Crustacea) in the Hölloch cave system (Switzerland). Syst. Biodivers. 15, 218–237. https://doi.org/10.1080/14772000.2016.1249112.

Fišer, C., Sket, B., Trontelj, P., 2008. A phylogenetic perspective on 160 years of troubled taxonomy of *Niphargus* (Crustacea: Amphipoda). Zool. Scripta 37, 665–680. https://doi.org/10.1111/j.1463-6409.2008.00347.x.

Fišer, C., Trontelj, P., Luštrik, R., Sket, B., 2009. Toward a unified taxonomy of *Niphargus* (Crustacea: Amphipoda): a review of morphological variability. Zootaxa 2061, 1–22. https://doi.org/10.11646/zootaxa.2061.1.1.

Flot, J.-F., 2007. Champuru 1.0: a computer software for unraveling mixtures of two DNA sequences of unequal lengths. Mol. Ecol. Notes 7, 974–977. https://doi.org/10.1111/j.1471-8286.2007.01857.x.

Flot, J.-F., Couloux, A., Tillier, S., 2010a. Haplowebs as a graphical tool for delimiting species: a revival of Doyle's "field for recombination" approach and its application to the coral genus *Pocillopora* in Clipperton. BMC Evol. Biol. 10, 372. https://doi.org/10.1186/1471-2148-10-372.

Flot, J.-F., Tillier, A., Samadi, S., Tillier, S., 2006. Phase determination from direct sequencing of length-variable DNA regions. Mol. Ecol. Notes 6, 627–630. https://doi.org/10.1111/j.1471-8286.2006.01355.x.

Flot, J.-F., Wörheide, G., Dattagupta, S., 2010b. Unsuspected diversity of *Niphargus* amphipods in the chemoautotrophic cave ecosystem of Frasassi, central Italy. BMC Evol. Biol. 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. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Forel, F.A., 1904. Le Léman. Librairie de l'Université, Lausanne.

Gerecke, R., Franz, H. (Eds.), 2006. Quellen im Nationalpark Berchtesgaden. Nationalpark Berchtesgaden, Forschungsbericht, p. 272.

Gibbard, P.L., Head, M.J., Walker, M.J.C., 2010. Formal ratification of the Quaternary system/period and the Pleistocene series/epoch with a base at 2.58 Ma. J. Quat. Sci. 25, 96–102. https://doi.org/10.1002/JQS.1338.

Gibert, J., Culver, D.C., 2009. Assessing and conserving groundwater biodiversity: an introduction. Freshw. Biol. 54, 639–648. https://doi.org/10.1111/j.1365-2427.2009.02202.x.

Godet, P., 1877. Sur le *Gammarus puteanus* Koch var. Bulletin de la Société des Sciences Naturelles de Neuchâtel 284–286.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321. https://doi.org/10.1093/sysbio/syq010.

Hoang, D.T., Chernomor, O., Haeseler, A. von, Minh, B.Q., Le Vinh, S., 2018. UFBoot2: improving the ultrafast bootstrap approximation. Mol. Biol. Evol. 35, 518–522. https://doi.org/10.1093/molbev/msx281.

Horton, T., Lowry, J., Broyer, C. de, Bellan-Santini, D., Copilaş-Ciocianu, D., Corbari, L., Costello, M.J., Daneliya, M., Dauvin, J.-C., Fišer, C., Gasca, R., Grabowski, M., Guerra-García, J.M., 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.R., Serejo, C., Souza-Filho, J.F., Tandberg, A.H., Thomas, J.D., Thurston, M., Vader, W., Väinölä, R., Vonk, R., White, K., Zeidler, W., 2023. World Amphipoda database. https://www.marinespecies.org/amphipoda. https://doi.org/10.14284/368.

Humbert, A., 1876. Description du *Niphargus puteanus* var. *Forelii*. Bulletin de la Société vaudoise des Sciences Naturelles 14, 313–392.

Hupało, K., Copilaş-Ciocianu, D., Leese, F., Weiss, M., 2023. Morphology, nuclear SNPs and mate selection reveal that COI barcoding overestimates species diversity in a Mediterranean freshwater amphipod by an order of magnitude. Cladistics 39, 129–143. https://doi.org/10.1111/cla.12520.

Jardim de Queiroz, L., Doenz, C.J., Altermatt, F., Alther, R., Borko, Š., Brodersen, J., Gossner, M.M., Graham, C., Matthews, B., McFadden, I.R., Pellissier, L., Schmitt, T., Selz, O.M., Villalba, S., Rüber, L., Zimmermann, N.E., Seehausen, O., 2022. Climate, immigration and speciation shape terrestrial and aquatic biodiversity in the European Alps. Proc. Biol. Sci. 289 https://doi.org/10.1098/rspb.2022.1020.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., Haeseler, A. von, Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285.

Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A., Flouri, T., 2017. Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. Bioinformatics 33, 1630–1638. https://doi.org/10.1093/bioinformatics/btx025.

Karaman, G.S., 1976. Contribution to the knowledge of the Amphipoda. 72 - four new *Niphargus* species from Italy, *N. duplus, N. stygocharisitalicus, N. ruffoi* and *N. canui* (Gammaridae). HAL 21–50.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.

Koordinationsstelle, BDM, 2014. Biodiversitätsmonitoring Schweiz BDM. Beschreibung der Methoden und Indikatoren. Bundesamt für Umwelt BAFU, Bern, p. 104.

Kralj-Fišer, S., Premate, E., Copilaş-Ciocianu, D., Volk, T., Fišer, Ž., Balázs, G., Herczeg, G., Delić, T., Fišer, C., 2020. The interplay between habitat use, morphology and locomotion in subterranean crustaceans of the genus *Niphargus*. Zoology 139, 125742. https://doi.org/10.1016/j.zool.2020.125742.

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. Mol. Biol. Evol. 772–773. https://doi.org/10.1093/molbev/msw260.

Larsson, A., 2014. AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics 30, 3276–3278. https://doi.org/10.1093/bioinformatics/btu531.

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

Liu, H., Zheng, Y., Zhu, B., Tong, Y., Xin, W., Yang, H., Jin, P., Hu, Y., Huang, M., Chang, W., Ballarin, F., Li, S., Hou, Z., 2023. Marine-montane transitions coupled with gill and genetic convergence in extant crustacean. Sci. Adv. 9, eadg4011 https://doi.org/10.1126/sciadv.adg4011.

Lunghi, E., Bilandžija, H., 2022. Longevity in cave animals. Frontiers in Ecology and Evolution 10. https://doi.org/10.3389/fevo.2022.874123.

Mammola, S., Amorim, I.R., Bichuette, M.E., Borges, P.A.V., Cheeptham, N., Cooper, S.J. B., Culver, D.C., Deharveng, L., Eme, D., Ferreira, R.L., Fišer, C., Fišer, Ž., Fong, D. W., Griebler, C., Jeffery, W.R., Jugovic, J., Kowalko, J.E., Lilley, T.M., Malard, F., Manenti, R., Martínez, A., Meierhofer, M.B., Niemiller, M.L., Northup, D.E., Pellegrini, T.G., Pipan, T., Protas, M., Reboleira, A.S.P.S., Venarsky, M.P., Wynne, J. J., Zagmajster, M., Cardoso, P., 2020. Fundamental research questions in subterranean biology. Biol. Rev. https://doi.org/10.1111/brv.12642.

Mammola, S., Cardoso, P., Culver, D.C., Deharveng, L., Ferreira, R.L., Fišer, C., Galassi, D.M.P., Griebler, C., Halse, S., Humphreys, W.F., Isaia, M., Malard, F., Martinez, A., Moldovan, O.T., Niemiller, M.L., Pavlek, M., Reboleira, A.S.P.S., Souza-Silva, M., Teeling, E.C., Wynne, J.J., Zagmajster, M., 2019. Scientists' warning on the conservation of subterranean ecosystems. Bioscience 69, 641–650. https://doi.org/10.1093/biosci/biz064.

Marques, D.A., Meier, J.I., Seehausen, O., 2019. A combinatorial view on speciation and adaptive radiation. Trends Ecol. Evol. 34, 531–544. https://doi.org/10.1016/j.tree.2019.02.008.

Martin, D.P., Murrell, B., Golden, M., Khoosal, A., Muhire, B., 2015. RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evolution 1, vev003. https://doi.org/10.1093/ve/vev003.

McInerney, C.E., Maurice, L., Robertson, A.L., Knight, L.R.F.D., Arnscheidt, J., Venditti, C., Dooley, J.S.G., Mathers, T., Matthijs, S., Eriksson, K., Proudlove, G.S., Hänfling, B., 2014. The ancient Britons: groundwater fauna survived extreme climate change over tens of millions of years across NW Europe. Mol. Ecol. 23, 1153–1166. https://doi.org/10.1111/mec.12664.

Miller, M.A., Pfeiffer, W., Schwartz, T. (Eds.), 2010. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. IEEE.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., Haeseler, A. von, Lanfear, R., 2020. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37, 1530–1534. https://doi.org/10.1093/molbev/msaa015.

Ntakis, A., Anastasiadou, C., Zakšek, V., Fišer, C., 2015. Phylogeny and biogeography of three new species of *Niphargus* (Crustacea: Amphipoda) from Greece. Zool. Anz. 255, 32–46. https://doi.org/10.1016/j.jcz.2015.02.002.

Premate, E., Borko, Š., Delić, T., Malard, F., Simon, L., Fišer, C., 2021. Cave amphipods reveal co-variation between morphology and trophic niche in a low-productivity environment. Freshw. Biol. https://doi.org/10.1111/fwb.13797.

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

Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior summarization in Bayesian phylogenetics using tracer 1.7. Syst. Biol. 67, 901–904. https://doi.org/10.1093/sysbio/syy032.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. https://doi.org/10.1093/sysbio/sys029.

Schellenberg, A., 1934. Amphipoden aus Quellen, Seen und Höhlen. Zool. Anz. 106, 200–209.

Sket, B., 1999a. High biodiversity in hypogean waters and its endangerment – the situation in Slovenia, the Dinaric carst, and Europe. Crustaceana 72, 767–779. https://doi.org/10.1163/156854099503951.

Sket, B., 1999b. The nature of biodiversity in hypogean waters and how it is endangered. Biodivers. Conserv. 8, 1319–1338. https://doi.org/10.1023/A:1008916601121.

Spöri, Y., Flot, J.-F., 2020. HaplowebMaker and CoMa: two web tools to delimit species using haplowebs and conspecificity matrices. Methods Ecol. Evol. 11, 1434–1438. https://doi.org/10.1111/2041-210X.13454.

Stoch, F., 2006. Asseln und Flohkrebse (Peracarida: Isopoda, Amphipoda). In: Gerecke, R., Franz, H. (Eds.), Quellen im Nationalpark Berchtesgaden, vol. 51. Nationalpark Berchtesgaden, Forschungsbericht, pp. 156–157.

Stoch, F., Christian, E., Flot, J.-F., 2020. Molecular taxonomy, phylogeny and biogeography of the *Niphargus tatrensis* species complex (Amphipoda, Niphargidae) in Austria. Org. Divers. Evol. 20, 701–722. https://doi.org/10.1007/s13127-020-00462-z.

Stoch, F., Flot, J.-F., 2017. Molecular phylogeny and biogeography of freshwater amphipods in Italy: state of the art. Biodiversity Journal 8 (2), 551–552.

Stoch, F., Salussolia, A., Flot, J.-F., 2022. Polyphyly of the *Niphargus stygius* species group (Crustacea, Amphipoda, Niphargidae) in the southern Limestone Alps. bioRxiv. https://doi.org/10.1101/2022.04.28.489871.

Strinati, P., 1966. Faune cavernicole de la Suisse. Éditions du Centre national de la recherche scientifique, Paris.

Studer, A., Knüsel, M., Alther, R., Hürlemann, S., Altermatt, F., 2022. Erfassung der Grundwasserflohkrebse. Studie zur Artenvielfalt und Verbreitung im Einzugsgebiet der Töss. Aqua Gas 102, 14–19.

Talavera, G., Castresana, J., 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. 56, 564–577. https://doi.org/10.1080/10635150701472164.

Trontelj, P., Douady, C.J., Fišer, C., Gibert, J., Gorčki, 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? Freshw. Biol. 54, 727–744. https://doi.org/10.1111/j.1365-2427.2007.01877.x.

Väinölä, R., Witt, J.D.S., Grabowski, M., Bradbury, J.H., Jazdzewski, K., Sket, B., 2008. Global diversity of amphipods (Amphipoda; Crustacea) in freshwater. Hydrobiologia 595, 241–255. https://doi.org/10.1007/s10750-007-9020-6.

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. Mol. Ecol. 14, 4355–4369. https://doi.org/10.1111/j.1365-294X.2005.02745.x.

Weber, D., Stoch, F., Knight, L.R., Chauveau, C., Flot, J.-F., 2021. The genus *Microniphargus* (Crustacea, Amphipoda): evidence for three lineages distributed across northwestern Europe and transfer from Niphargidae to Pseudoniphargidae. Belg. J. Zool. 151 https://doi.org/10.26496/bjz.2021.92.

Zagmajster, M., Malard, F., Eme, D., Culver, D.C., 2018. Subterranean biodiversity patterns from global to regional scales. In: Moldovan, O.T., Kováč, Ľ., Halse, S. (Eds.), Cave Ecology, vol. 235. Springer International Publishing, Cham, pp. 195–227.

Zapelloni, F., Pons, J., Jurado-Rivera, J.A., Jaume, D., Juan, C., 2021. Phylogenomics of the *Hyalella* amphipod species-flock of the Andean Altiplano. Sci. Rep. 11, 366. https://doi.org/10.1038/s41598-020-79620-4.