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Diversity of *Niphargus* (Crustacea, Amphipoda) in France, as revealed by DNA barcoding approach

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Abstract

Niphargus is the most speciose amphipod genus inhabiting groundwater of the Western Palearctic. The genus is renowned for its notorious taxonomy, associated with large within- but often negligible between-species morphological variation. Results of molecular taxonomy suggested that morphological variation captured less than half of its species diversity, calling for a molecular revision of past taxonomic and faunistic works. Molecular assessment of species diversity within *Niphargus* has been conducted since the 2000s, but it remains spatially incomplete given the genus' extensive geographic range. Here, we provide the first comprehensive assessment of the diversity of *Niphargus* in France using molecular markers. We sequenced the COI mitochondrial marker from 436 individuals collected from 234 locations. Using Assemble Species by Automated Partitioning, we identified 99 molecular operational taxonomic units (MOTU), among which 74 could represent potential new species. In turn, we selected one individual per MOTU, amplified nuclear markers 28S and Histone 3, aligned them with an additional 142 MOTUs from across the genus range, and reconstructed their phylogenetic relationships using the Maximum Likelihood method. We found that several nominal species occurring in France are polyphyletic. Furthermore, *Niphargus* from France comprised lineages derived from deep and terminal splits, some of which showed signs of evolutionary radiation. The phylogenetic relationships are consistent with the hypothesis that *Niphargus* originated in the western part of its range. This study addressed a knowledge gap in the molecular and spatial coverage of *Niphargus*, provided new barcodes for monitoring the diversity of the genus, and identified future directions for research.

Key Words

Amphipoda, cryptic species, groundwater, phylogeny, species delimitation

Introduction

With over 450 described species, the amphipod genus *Niphargus* represents an important part of groundwater species diversity in the Western Palearctic (Zagmajster et al. 2014; Horton et al. 2021). The genus presumably derived from marine ancestors in landmasses that nowadays build Western Europe (McInerney et al. 2014) and subsequently dispersed towards the southeast, where it massively diversified in several adaptive radiations (Borko et al. 2021). Its

frequent occurrence in various types of groundwater (Sket 1977, 1999; Sket and Velkovrh 1981; Karaman and Sarbu 1996) makes it an interesting model system for ecological (Mathieu and Turquin 1992; Premate et al. 2021b; Knüsel et al. 2024), evolutionary (Premate et al. 2021a, 2024a), and conservation studies (Couton et al. 2023a; Premate et al. 2024b). Finally, it holds the potential to integrate science into a broader, non-scientific community through citizen science (Alther et al. 2021; Couton et al. 2023b). Despite the immense potential of the genus to advance the exploration of groundwater biodiversity, its use in routine research protocols is difficult owing to notoriously complex taxonomy.

The taxonomy of *Niphargus* has traditionally been based on analyses of morphological variation (hereafter: morphospecies). Inaccessibility of groundwater and sampling limitations (Ficetola et al. 2019), along with strong, directional environmental selection (Culver and Pipan 2019), have caused major challenges for solving the taxonomy of *Niphargus*. Samples of *Niphargus* often contain few individuals, which do not allow appropriate assessment of morphological variation. Furthermore, selection has repeatedly driven the evolution of phenotypes towards similar optima. Consequently, differences among species are often small compared to within-species variation. Within-species variation is remarkable in many species and is associated with age (Fišer et al. 2008b) and sex (Biró et al. 2024; Premate et al. 2024a). An early study of allozyme variation suggested deep genetic divergence among morphologically similar species (Berettoni et al. 1998). Routine use of molecular approaches strengthened the suggestion of this pioneering study: many morphospecies turned out to be genetically heterogeneous (Lefébure et al. 2006, 2007; Trontelj et al. 2009; McInerney et al. 2014; Delić et al. 2017; Stoch et al. 2022). We estimated that every morphospecies, on average, comprised 2 to 3 genetically distinct putative species, often referred to as molecular operational taxonomic units (MOTUs) (Eme et al. 2017). Molecular methods hold promise to pave the road towards a more stable *Niphargus* taxonomy, allowing relatively reliable species identification using DNA barcodes (Tautz et al. 2003). However, the present coverage of the genetic diversity of *Niphargus* with barcodes is still insufficient, and we are far from a complete species inventory.

Genetic divergence within *Niphargus* morphospecies has been scrutinized in several regions, including Great Britain (McInerney et al. 2014), the Pyrenees (Jurado-Rivera et al. 2017), parts of the Alpine Arc (Altermatt et al. 2014; Stoch et al. 2022; Knüsel et al. 2023, 2024), Germany and Benelux (Weber et al. 2020, 2022), the Dinaric Karst (Borko et al. 2022), Pannonian lowlands (Copilaș-Ciocianu et al. 2017), the Carpathian Arc (Meleg et al. 2013), and Iran (Esmaeili-Rineh et al. 2015). Although France counts more than 30 morphospecies of *Niphargus* (Ginet 1996, Stoch et al. 2024), the assessment of genetic divergence within morphospecies has been restricted to only a few species complexes (Berettoni et al. 1998; Lefébure et al. 2006, 2007; Stoch et al. 2024; Delić et al. 2025; Weber et al. 2025).

In this study, we attempted a broader molecular scrutinization of *Niphargus* diversity in France. We gathered recent samples collected in France by several laboratories and performed the first comprehensive molecular analysis of species diversity using the mitochondrial gene cytochrome c oxidase subunit I (COI). Specifically, we assembled 436 individuals from 234 localities, subjected these data to unilocus COI species delimitation, and defined MOTUs. Then, we assessed differences in the number of species obtained using morphology and COI-based species delimitation. To infer the phylogenetic origin of *Niphargus* from France, we additionally sequenced one individual per MOTU for two nuclear markers and aligned these data with another 142 phylogenetically representative MOTUs to reconstruct evolutionary relationships. Using this phylogeny, we verbally evaluated whether phenotypically similar species diversity evolved through processes of evolutionary radiation or convergent evolution.

Materials and methods

Data acquisition

We gathered a total of 234 samples from caves, mines, springs, and groundwater in unconsolidated sediments (Fig. 1; Suppl. material 1: table S1). Samples were sorted, *Niphargus* individuals examined under a stereomicroscope (Leica M165C), and – whenever possible – identified to species level with the help of morphology. From each sample, we selected 1 to 9 individuals for sequencing. We removed 1 to 3 pereopods for DNA extraction, whereas the rest of the animal was preserved for potential further analyses and additional morphological examination. Small individuals were used whole. The samples were retained in the zoological collections of the Biotechnical Faculty, University of Ljubljana (Slovenia), Université Claude Bernard Lyon 1 (France), and Université libre de Bruxelles (Belgium).

In addition, we harvested data from previous publications and GenBank (date: 31.08.2024) to complement the newly acquired information with the existing data and to harmonize analyses across all studies. All the data are freely available at Zenodo (https://doi.org/10.5281/zenodo.16652237).

Datasets

We prepared two datasets. The first dataset, the species delimitation dataset, was used to infer the MOTU structure of the acquired samples. It comprised 470 individuals sequenced for COI, including 436 individuals from France and 34 individuals from outside France. The latter individuals belonged to morphospecies that have also been reported from France. This dataset was analyzed using Assemble Species by Automated Partitioning (ASAP) (Puillandre et al. 2021).

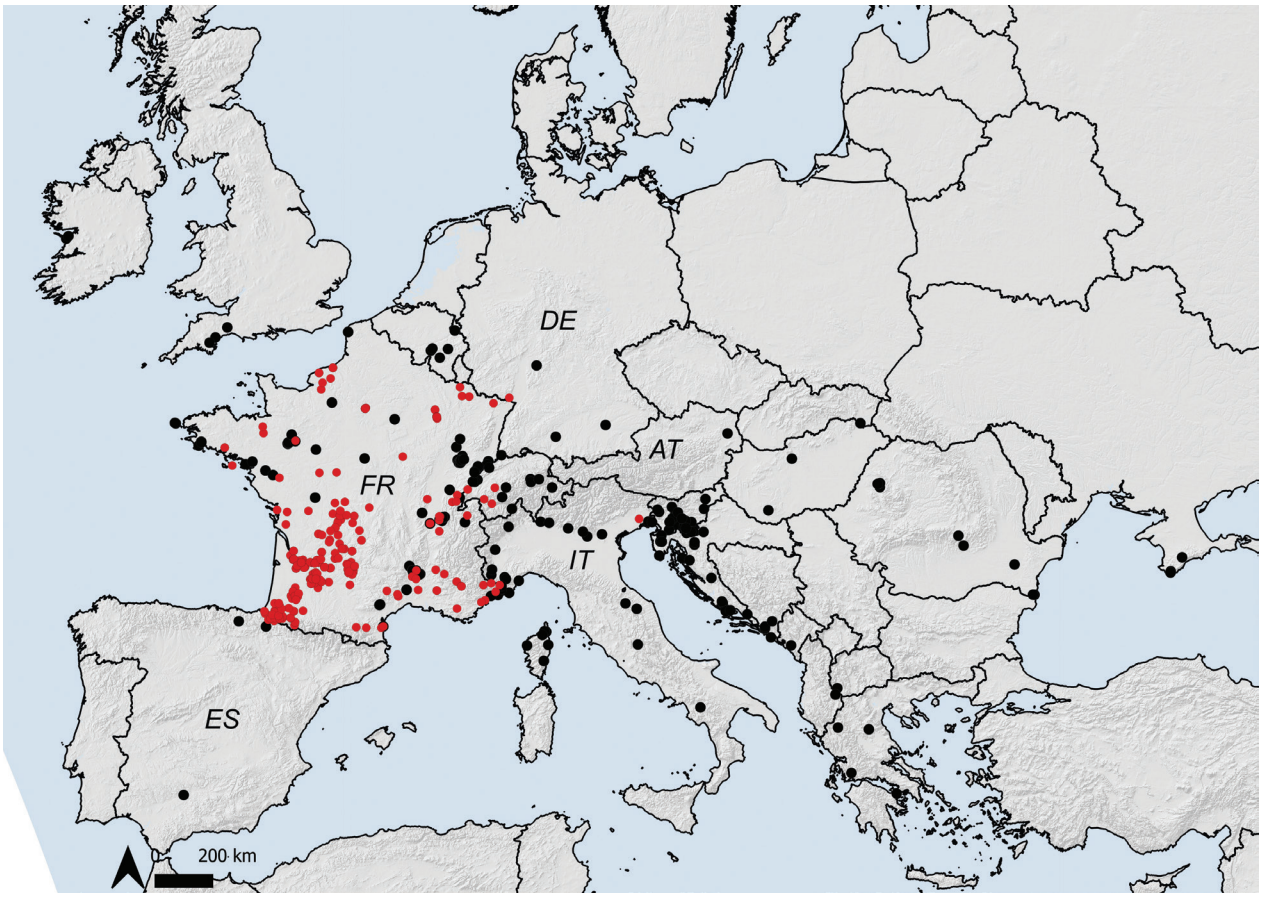

Figure 1. Origin of samples used in this study. Details of the individual MOTU distributions are presented in Suppl. material 1. New records are colored red.

The second dataset, the phylogeny reconstruction dataset, was used to assess the phylogenetic position of MOTUs from France within the genus. The dataset comprised 270 terminals. Its structure was as follows: the target dataset included 107 MOTUs acquired from the ASAP analysis of France, sequenced for COI, 28S, and H3 gene fragments. To adequately present the phylogenetic origin of the MOTUs of interest, we selected another 142 MOTUs belonging to clades from across the genus range. These MOTUs were sequenced for six gene fragments (COI, ARGK, hsp70, EPRS, 18S, and three fragments of 28S) and provided a phylogenetic backbone, tested in previous studies (Borko et al. 2021; Delić et al. 2025). Finally, we added another 21 individuals from France, sequenced only for the 28S gene, to estimate their approximate conspecifics.

Laboratory work, sequencing, and alignment

We extracted genomic DNA using the MagMAX DNA Multi-Sample Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Oligonucleotide primers and amplification protocols were the same as in previous studies (Borko et al. 2021, 2022; Delić et al. 2025) and are detailed in Suppl. material 2. All nucleotide sequences were obtained by the Macrogen Europe laboratory (Amsterdam, Netherlands) using amplification primers and bidirectional Sanger sequencing. Chromatogram assembly and sequence editing were performed in Geneious 11.0.3 (Biomatters Ltd., New Zealand). Edited sequences were aligned using the Geneious MAFFT v7.388 plugin (Katoh and Standley 2013) with the E-INS-i algorithm, scoring matrix 1PAM/k=2, and the highest gap penalty.

Species delimitations and phylogeny reconstruction

To explore genetic differentiation and potential species diversity of the newly sequenced individuals, we subjected the species delimitation dataset (see above; Suppl. material 1: table S1; alignment in Suppl. material 3) to unilocus species delimitation ASAP. ASAP is a hierarchical clustering algorithm that uses pairwise genetic distances. The best partitioning scheme is selected using a composite score based on the probabilities of groups being panmictic species and barcode gap widths (Puillandre et al. 2021). The ASAP delimitation was executed under the Kimura two-parameter substitution model (Kimura-K80) on an online server available at https://bioinfo.mnhn.fr/abi/public/asap/. We also ran a web-available Poisson-tree

process within the Bayesian framework using the same dataset, which – as in previous studies (Borko et al. 2022) – additionally split morphospecies into 158 MOTUs; therefore, we discarded the results of this analysis.

Phylogenetic analyses were inferred using the phylogeny reconstruction dataset. Alignments were concatenated in Geneious. Gap-rich regions from the alignments of non-coding markers were removed using Gblocks 0.91.1 (Talavera and Castresana 2007) with a less stringent selection set, as in previous studies (Borko et al. 2021; Delić et al. 2025). We inferred phylogenetic relationships with a maximum likelihood approach in IQ-TREE 2.3.6 (Minh et al. 2020), using an automatic best-fit substitution model search with ModelFinder (Kalyaanamoorthy et al. 2017) and ultrafast bootstrap to evaluate node support. Additionally, we ran Bayesian inference in MrBayes 3.2.6 (Ronquist et al. 2012). The Markov chain Monte Carlo (MCMC) tree was inferred after two independent runs with four chains for 20 million generations each, sampled every 250 generations. The first 25% of trees were discarded as burn-in, and the remaining trees were used to calculate a 50% majority-rule consensus tree.

Results

We sequenced 23 of the 30 morphospecies known to date from France. Of these, 15 are endemic to France, while the remaining 8 were described in neighboring countries, and their ranges allegedly extend into France. We amplified the barcoding COI fragment for 22 morphospecies, whereas in another species (*N. forelii*), we could only sequence the 28S marker. The geographic distribution of the morphospecies, number of MOTUs per morphospecies, and taxonomic status of the species are summarized in Table 1. The main conclusion of the species delimitation analysis is that each morphospecies comprises between 1 and 24 MOTUs, totaling 99 MOTUs from France. These should be treated as "candidate species" and should be subject to further taxonomic scrutinization (Table 1).

The phylogenetic analysis recovered all previously known major clades, although some of the internal nodes were not supported and some parts of the phylogeny remained ambiguous. Overall, maximum likelihood analysis produced more well-supported nodes than Bayesian analysis (Fig. 2). The 99 studied MOTUs from France belong to 28 phylogenetic lineages. Among the morphospecies comprising more than one MOTU, we detected two noteworthy branching patterns. First, three lineages diversified into species-rich clades (>5 MOTUs) (Fig. 3). These lineages can be attributed to the following morphospecies: *N. virei*, *N. kochianus*, and *N. ladmiraulti*. Additionally, we detected a clade comprising at least two morphologically different species (i.e., ecomorphs). Most of the species within this clade morphologically correspond to *N. ciliatus* (labeled with letters A–G), whereas one species morphologically corresponds to an entirely different species, resembling *N. jovanovici* (Fig. 3). The second emerging pattern is polyphyletic morphospecies complexes – that is, unrelated species that presumably acquired their morphological similarity independently. These include the morphospecies *N. aquilex*, *N. rhenorhodanensis*, and *N. fontanus* (Fig. 4). Notably, the two patterns are not exclusive; the latter three species complexes show a combination of presumed convergent origins followed by diversification.

Discussion

Overall, we find four key results of broad interest. First, the present species diversity of *Niphargus* from France is underestimated. The average number of MOTUs per morphospecies (1:4) slightly exceeds the MOTU–morphospecies ratio estimated for European groundwater peracarids (1:2–3) (Eme et al. 2017). We suggest that this may reflect a decline in classical taxonomy over the past decades and should be perceived as a call for future taxonomic studies. Noteworthy, the number of MOTUs needs to be considered with reservation, as unilocus methods may overestimate the number of species (Hupalo et al. 2022). Conversely, phenomena such as historical mitochondrial introgression may lump species and thus lead to underestimation of this number (Yuan et al. 2023). The results presented herein provide a basis for future taxonomic studies that will in detail explore species complexes using multilocus species delimitations (Yang 2015; Hupalo et al. 2022) within the integrative framework (Padial et al. 2010).

Second, several species-rich lineages indicate that in some parts of France, *Niphargus* underwent substantial diversification (Fig. 3). These lineages are potential candidates for evolutionary radiation. The nature of these putative evolutionary radiations and the mechanisms driving diversification processes in these radiations are not known (Rundell and Price 2009; Simões et al. 2016) and should be addressed in a dedicated study. Such multiple radiations are an interesting comparative and replicated model system for the study of diversification mechanisms on a macroevolutionary scale.

Third, several morphospecies appeared to be polyphyletic. It is unclear whether their morphological similarity has been inherited (Recknagel et al. 2024) or whether it evolved through convergent evolution (Fišer et al. 2018; Struck et al. 2018). We are increasingly aware that much of the biodiversity does not manifest in morphological variation (Fišer and Koselj 2022), yet the mechanisms underlying decoupled genetic and morphological variation are only poorly known (Fišer et al. 2018; Struck et al. 2018). Multiple clades of morphologically similar species, as presented herein, are an interesting model system that may help elucidate this issue.

Fourth, despite the fact that numerous parts of the phylogeny remain unresolved – a well-known problem in *Niphargus* phylogenetics (Fišer et al. 2008a; McInerney et al. 2014) – and despite not calibrating our phylogeny, we

Table 1. Checklist of *Niphargus* from France. The first column refers to the morphospecies checklist derived from the literature (Ginet 1996; Stoch et al. 2024). The second column shows whether the species was sequenced. "Equivalent MOTU(s)" represents the number of taxonomic units determined by ASAP and matching the morphology of the species. Taxonomic status is commented on in the Notes column. We cannot rule out the possibility that some of the species found in France are not conspecific with those described from other countries. The asterisk denotes sequences for species that were not acquired from the samples from France. MOTU – molecular operational taxonomic unit; ASAP – Assemble Species by Automated Partitioning; COI – cytochrome c oxidase subunit I.

Morpho species	Sequence available	Equivalent MOTU(s)	Notes
N. angelieri Ruffo, 1954	yes	1	The species is broadly distributed; its potential differentiation has not been studied. The sequence from the type locality is not available; further explorations are needed.
N. aquilex Schiödte, 1855	yes	17	The species comprises a polyphyletic complex of species. Type *N. aquilex* was described from Great Britain, whereas populations from France require further taxonomic revision. We detected 17 MOTUs from seven phylogenetic lineages. The identity with MOTUs discovered by Weber could not have been established (Weber et al. 2023).
N. balazuci Schellenberg, 1951	yes	1	A species reported only from France. We detected only one MOTU.
N. boulangei Wichers, 1964	yes	1	A species reported only from France. We detected only one MOTU.
N. burgundus Graf & Straškraba, 1967	no	NA	A species reported only from France. The species was not recollected within this study.
N. caspary (Pratz, 1866)	yes*	unclear	A species complex, distributed along the entire Danube and in France; preliminary analyses show some genetic differentiation, yet the data is insufficient to draw any taxonomic hypotheses (unpublished). Populations from France were first described as *N. legeri* but subsequently synonymized with *N. caspary* (Karaman 1982). Type populations of *N. caspary* were described from Germany; populations from France may belong to a different species and require further taxonomic examination. The available sequences were obtained from samples from Austria and Germany.
N. ciliatus Chevreux, 1906	yes	5	A paraphyletic species complex endemic to France (Ginet 1988). All MOTUs are distributed in SW France.
N. corsicanus Schellenberg, 1950	yes	3	A monophyletic species complex (Hovenkamp et al. 1984), part of a clade *N. longicaudatus* with a center of species richness in the Apennines.
N. delamarei Ruffo, 1954	yes	1	The species is broadly distributed; its range extends to Spain (Ruffo 1954; Karaman 1986). Its potential within-species genetic differentiation has not been studied.
N. dimorphopus Stock & Gledhill, 1977	yes*	1	The species was described as a subspecies of *N. kochianus*, and it is nested within this complex (Stock and Gledhill 1977). *N. dimorphopus* has been described from the Netherlands. No sequence from France was acquired, and the status of this species needs to be revised.
N. fontanus Spence Bate, 1859	yes	6	Polyphyletic species complex broadly distributed in W Europe. Type populations were described from Great Britain, and the nominal species range extends to France. The taxonomic status of other MOTUs sharing similar morphology needs to be described.
N. forelii Humbert, 1876	28S marker only	NA	The species was redescribed from Alpine lakes of Switzerland (Karaman and Ruffo 1993). So far, we were not able to genetically characterize populations from Lake Geneva or Lake Constance, the neotype locality. We collected several individuals with corresponding morphology in France; however, their taxonomic status cannot be confirmed without neotype locality. We could not amplify the COI fragment; hence, we cannot evaluate its genetic variation in a similar way as we evaluated the variation of other species complexes.
N. gallicus Schellenberg, 1935	no	NA	The species was reported from France and Romania; while we acquired fresh material from Romania, we could not recollect this species in France. It seems unlikely that a species could maintain a gene flow over such a long distance; hence, we treat this species as unexplored.

Morpho species	Sequence available	Equivalent MOTU(s)	Notes
N. gineti Bou, 1965	no	NA	Species endemic to France and not recollected in our sampling campaigns.
N. gr. *jovanovici*	yes	1	The incompletely identified species was listed in Ginet's catalogue on a level of morpho-group (Ginet 1996). Ginet referred to the monograph of Sket (Sket 1972), who studied species from the River Sava, the tributary of the Danube River, and it seems unlikely that any of these species lives in France. We identified a species that could be considered as a member of morpho-group jovanovici. It is distinct from all known species by the exopodite being longer than the endopodite in uropod I. Finally, we cannot rule out other similar species living in France.
N. kochianus Spence Bate, 1859	yes	8 (excl. *N. dimorphopus*)	The species comprises a complex of species. *N. kochianus* was described from Great Britain, whereas the complex as a monophyletic clade comprises populations from Belgium, the Netherlands, and France (Stock and Gledhill 1977; McInerney et al. 2014). The members of this clade from France need to be taxonomically revisited.
N. ladmiraulti Chevreux, 1901	yes	5	A monophyletic species complex endemic to France.
N. laisi Schellenberg, 1936	yes*	unknown	An interstitial species, distributed in France, Germany, and France (Fišer et al. 2019). The sequences available derive from Germany and Switzerland; thus, conspecific status with individuals from France needs to be established. Potential genetic differentiation within this species is not known.
N. nicaeensis Isnard, 1916	no	NA	A species, described from Nice; we could not recollect it after several attempts.
N. pachypus Schellenberg, 1933	yes	unknown	The species comprises a complex of species. *N. pachypus* has been described from the Netherlands; populations from France require further taxonomic revision.
N. plateaui Chevreux, 1901	yes	3	Polyphyletic species complex endemic to France.
N. quimperensis Citoleux, Flot & Stoch, 2025	yes	1	Endemic species, related to species from Ireland and Great Britain (Stoch et al. 2024).
N. renei G. Karaman, 1986	no	NA	Species endemic to France; we could not recollect it.
N. rhenorhodanensis Schellenberg, 1937	yes	24	A polyphyletic species complex, distributed in France, Germany, Switzerland, and Italy. It comprises over 20 MOTUs in at least 8 clades; in urgent need of taxonomic revisions (Lefébure et al. 2006; Delić et al. 2025).
N. robustus Chevreux, 1901	yes	4	Polyphyletic species complex endemic to France.
N. schellenbergi S. Karaman, 1932	yes	4	Broadly distributed species complex in Switzerland, France, Germany, the Netherlands, and Belgium. In our analyses, we identified 4 MOTUs, a number that is higher than previously assessed (Weber et al. 2023).
N. setiferus Schellenberg, 1937	no	NA	A small species, reported from Jura and not recollected in our analyses.
N. thienemanni Schellenberg, 1934	yes*	1	A species distributed in the Alps, between Switzerland and Germany. Its distribution in Switzerland is well established (Knüsel et al. 2024); however, conspecificity with individuals from France needs to be established. Type material from Germany was never recollected, despite numerous attempts.
N. vandeli Barbé, 1961	no	NA	A species, endemic to France, not recollected in our sampling campaigns.
N. virei Chevreux, 1896	yes	5	A monophyletic species complex, distributed in France, Switzerland, and Belgium. Our study suggests more species than a previous analysis (Lefébure et al. 2006).
Niphargus sp.	yes		Unidentified for different reasons.
Barcode availability	**23 out of 30 morphospecies**	**99 MOTU**	

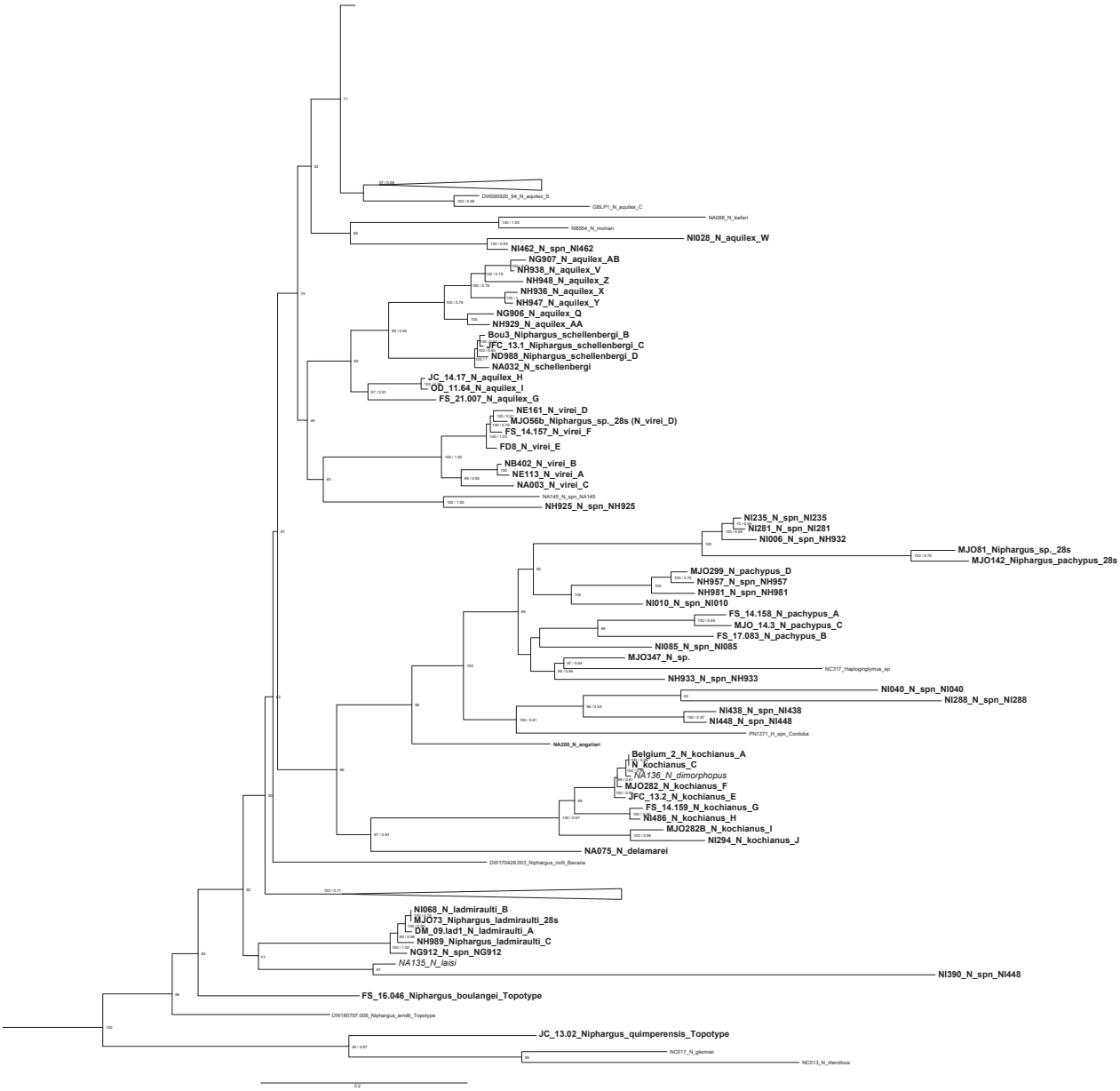

Figure 2. Multilocus phylogenetic tree inferred with the maximum likelihood in IQ-TREE. The first value at the nodes indicates ultrafast bootstrap values; values below 90% are not shown, and these nodes should be considered unreliable. The second value (if present) at the node shows posterior probability. We show posterior probabilities at all nodes that were recovered through Bayesian inference and maximum likelihood (<90%). Some nodes with high bootstrap values were not recovered in Bayesian analysis. Names of terminals in boldface indicate MOTUs from France (molecularly confirmed), whereas names in italics are species listed for France but not sequenced. To enhance readability of the phylogeny, we collapsed the clades not relevant for this study. The phylogeny is divided into two; here, we show the early branching events. The full phylogeny is available in Suppl. material 5: fig. S1. Note: the term "N_spn" is how MOTUs are labeled in an internal database (SubBioDB) and should not be equated with the expression "n. sp.," which indicates a new species and can be used only for species descriptions.

tentatively verbalize some biogeographic implications to be tested in a future dedicated study. Some clades of species from France were derived from basal splits within the *Niphargus* diversification. This corroborates the hypothesis of an ancient (100–70 Mya) origin of *Niphargus* from the territories that nowadays belong to Great Britain, Ireland, and Brittany (McInerney et al. 2014; Borko et al. 2021; Stoch et al. 2024). In contrast, some other lineages were derived from more recent splits, suggesting that they evolved much later and spread westward, such as *N. rhenorhodanensis* (Delić et al. 2025).

The results presented above, however, deserve several comments and cautionary notes. First, exhaustive groundwater sampling was limited to a few regions (Fig. 1). It is reasonable to expect that additional samples will bring up new, yet undescribed, species. Although we cannot predict

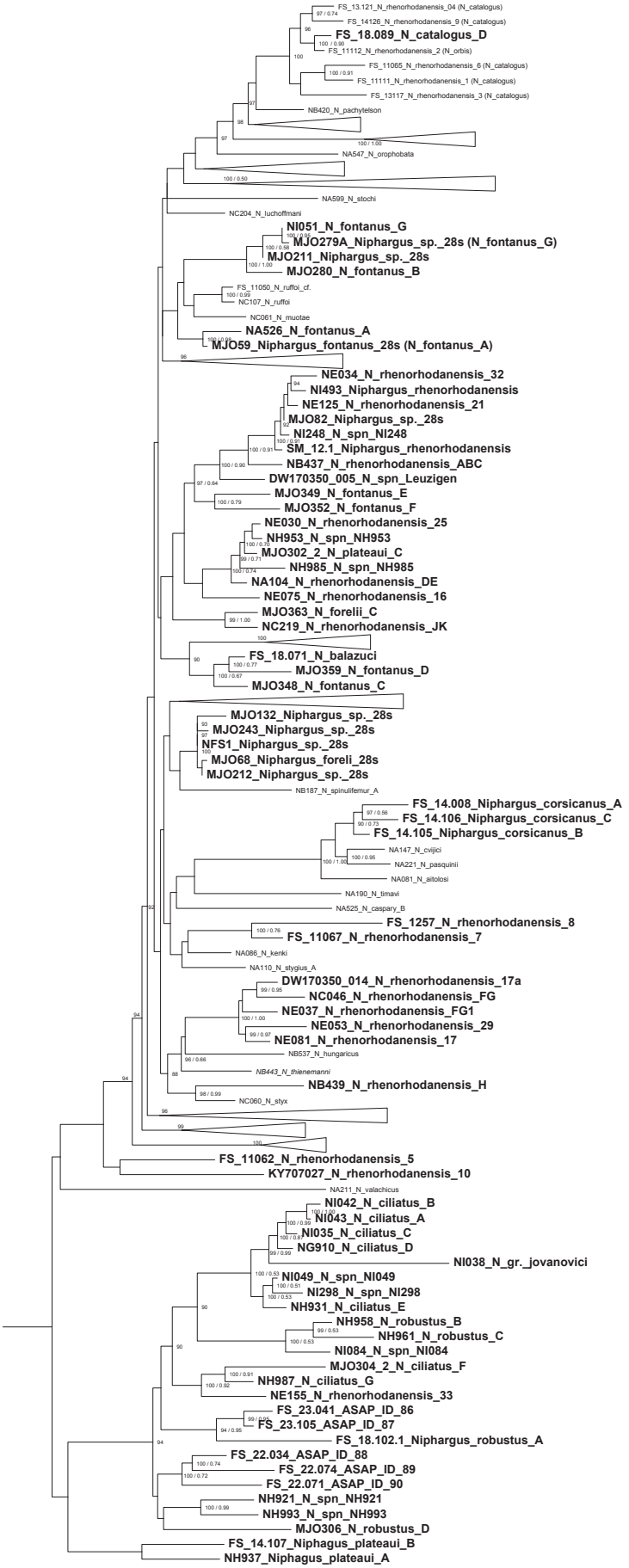

Figure 2. Continued.

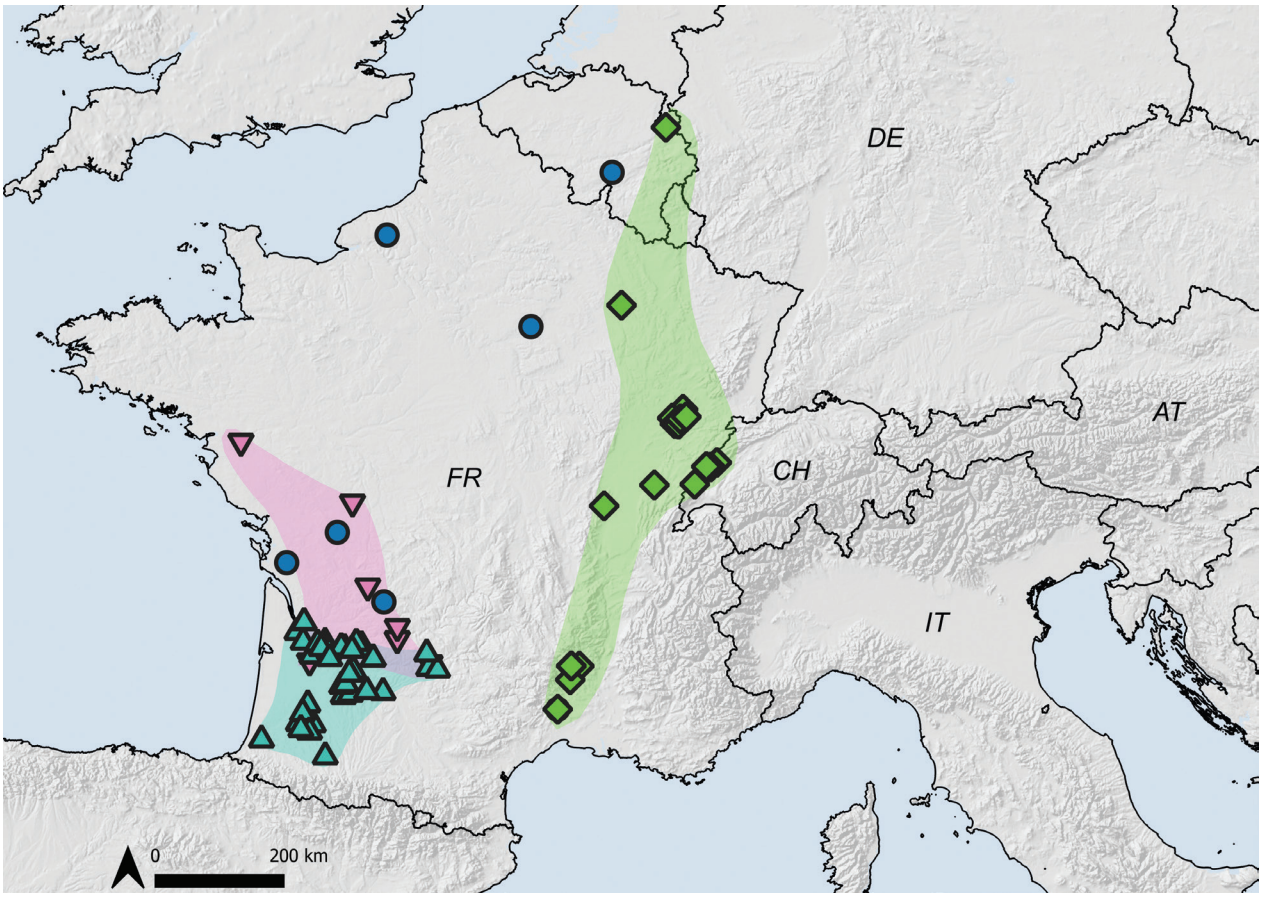

Figure 3. Distribution of three clades with >5 MOTUs that possibly represent evolutionary radiations (blue circles: *N. kochianus*; pink upside-down triangles: *N. ladmiraulti*; green diamonds: *N. virei*) and two sister lineages of *N. ciliatus* (turquoise triangles).

how many more MOTUs may be expected for France, we suggest that the presented data collection represents a solid basis for routine barcoding and further exploration of the genetic diversity of *Niphargus*.

Additionally, morphologically defined species can be divided into two groups: those described from France and those with the type locality abroad. The first group's species epithet (i.e., species name) will remain unchanged (for example, *N. ciliatus* and *N. angelieri*), regardless of taxonomic revisions. These species represent the core of the species in the national checklist for *Niphargus* in France. In contrast, species that were described in other countries but extend their range into France, where they comprise distinct MOTUs (e.g., *N. aquilex*, *N. schellenbergi*, *N. forelii*), may be renamed accordingly once subjected to taxonomic revisions (International Commission on the Zoological Nomenclature 2018). We suggest that such records should not only include the species but also MOTU identities so that they can be easily traced and potentially renamed after taxonomic revisions (see Notes in Table 1). This approach would ease the future administration of species inventories and allow for comparative monitoring results across years.

Conclusion

Our study improved understanding of *Niphargus* diversity in France, identified potentially interesting targets for future macroevolutionary research, and provided a solid base of knowledge for future taxonomy and species barcoding. However, we opened a Pandora's box of a high number of undescribed species, awaiting future generations of taxonomists. The main directions of taxonomic research in France should include 1) sequencing of the remaining morphologically described species, ideally from their type localities (Table 1); 2) resolving the taxonomic status of species that were described outside France (International Commission on the Zoological Nomenclature 2018); 3) describing and naming newly recognized species (Pante et al. 2015); and 4) integrating the undescribed species into nature conservation actions. If appropriately curated, the data assembled in our study represent a solid foundation of the barcoding system that may help monitor the status of the most threatened populations even in the absence of a completed taxonomy (for example, Malard et al. 2020, Saclier et al. 2024).

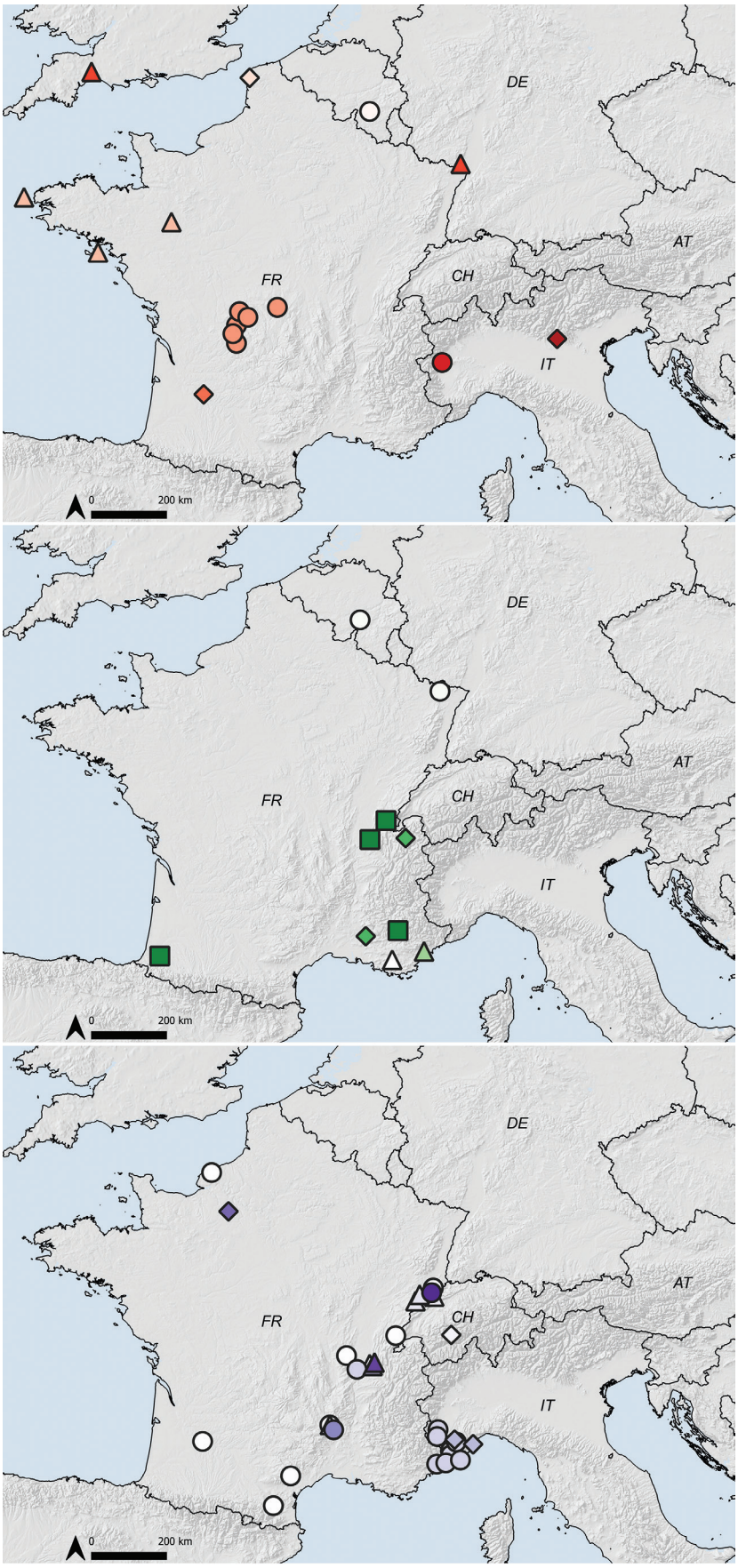

Figure 4. Distribution of polyphyletic species complexes, from top to bottom: *N. aquilex*, *N. fontanus*, and *N. rhenorhodanensis*. Lineages within each species complex are coded with colors and symbols. At present, it is not clear whether similar morphologies were inherited from shared ancestors or whether their similarity was acquired several times independently through convergent evolution.

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Supplementary material 1

Supplementary tables

Authors: Behare Rexhepi, Teo Delić, Gaj Kušar, François Lefebvre, Thierry Alezine, Xavier Chevillot, Marie-José Olivier, Florian Malard, Christophe J. Douady, Fabio Stoch, Jacques Chauvin, Aurélie Husté, Matthieu Fournier, Maja Zagmajster, Cene Fišer

Data type: xlsx

Explanation note: **table S1.** Individuals, localities, and sequences used in the study; **table S2.** Individuals used in the phylogenetic analysis with accession numbers.

Link: https://doi.org/10.3897/zse.101.153239.suppl1

Supplementary material 2

Laboratory protocols

Authors: Behare Rexhepi, Teo Delić, Gaj Kušar, François Lefebvre, Thierry Alezine, Xavier Chevillot, Marie-José Olivier, Florian Malard, Christophe J. Douady, Fabio Stoch, Jacques Chauvin, Aurélie Husté, Matthieu Fournier, Maja Zagmajster, Cene Fišer

Data type: docx

Link: https://doi.org/10.3897/zse.101.153239.suppl2

Supplementary material 3

Alignment used for species delimitation

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Data type: fasta

Link: https://doi.org/10.3897/zse.101.153239.suppl3

Supplementary material 4

Alignment used for phylogenetic reconstruction

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Data type: nex

Link: https://doi.org/10.3897/zse.101.153239.suppl4

Supplementary material 5

Entire phylogeny

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Data type: pdf

Link: https://doi.org/10.3897/zse.101.153239.suppl5