

Niphargus tonywhitteni Fišer et al., 2018 recorded for the first time in Bavaria

(Malacostraca, Amphipoda, Niphargidae)

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From 2017 to 2019, 162 sites in Bavaria, including 15 caves and artificial cavities, were surveyed for the presence of Niphargidae. The groundwater amphipod *Niphargus tonywhitteni* Fišer et al., 2018 (Malacostraca, Amphipoda, Niphargidae) was recorded at 6 sites, four of which were caves and artificial cavities, and is new to the fauna of Bavaria. The species affiliation was confirmed by barcoding based on the fragment of the cytochrome *c* oxidase subunit 1 gene (COI). Six clades of this species are largely separated geographically. The clade of Bavarian individuals ranges from north-eastern Switzerland via Tyrol to Baden-Württemberg and Bavaria. It is the clade with by far the widest distribution. Verovnik's fragment of the nuclear 28S ribosomal RNA gene was almost identical in all specimens. Evidence presented suggests that older morphological determinations from Bavaria of *Niphargus fontanus*, perhaps also *Niphargus foreli* and *Niphargus thienemanni*, actually pertain to *N. tonywhitteni*.

Von 2017 bis 2019 wurden in Bayern 162 Standorte, darunter 15 Höhlen und künstliche Hohlräume, auf Besatz von Niphargidae untersucht. Der Grundwasserkrebs *Niphargus tonywhitteni* Fišer et al., 2018 (Malacostraca, Amphipoda, Niphargidae) wurde in Bayern in 6 Fundorten, davon vier Höhlen und künstlichen Hohlräumen, gefunden. Er ist neu für die Fauna Bayerns. Die Artzugehörigkeit wurde mittels Barcodings basierend auf dem Fragment des Gens der Cytochrom-*c*-Oxidase-Untereinheit 1 (COI) bestätigt. Sechs Kladen dieser Art sind geographisch weitgehend getrennt. Die Klade der bayrischen Individuen reicht von der Nordost-Schweiz über Tirol bis Baden-Württemberg und Bayern und hat damit die bei weitem ausgedehnteste Verbreitung. Verovniks Fragment des nuklearen 28S-Ribosomen-RNA-Gens war bei allen Exemplaren weitgehend identisch. Möglicherweise beziehen sich alle älteren morphologischen Bestimmungen aus Bayern von *Niphargus fontanus*, vielleicht auch von *Niphargus foreli* und *Niphargus thienemanni*, tatsächlich auf *N. tonywhitteni*.

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Introduction

Groundwater represents the largest proportion of the Earth's unbound freshwater (Molden 2007). As a primary source of drinking water, it is of paramount

importance for human well-being. It is little known that groundwater is also home to thousands of specialised animal species (Deharveng et al. 2012). These so-called stygobionts (= species living exclusively in groundwater) have adapted to the subterranean way



Fig. 1. *Niphargus tonywhitteni* from the Angerlloch in Bavaria. The photo illustrates well the large gnathopods, especially gnathopod II, which are similar to *Niphargus fontanus* but different to most other *Niphargus* species. Foto: Hansbauer.

of life; they have a so-called cavernicolous morphology, the most striking characteristics of which are the absence of eyes and body pigmentation (Weber 2013).

Within the order Amphipoda, of which the layman may be familiar with the stream amphipod (*Gammarus fossarum*), found in the thousands in many boreal streams, there are several families that have adapted to subterranean life. Members of the family Niphargidae are among the most common representatives of groundwater communities in Europe. *Niphargus* is the by far most species-rich genus with 459 described species (Horton et al. 2022), the vast majority of which are pure stygobionts inhabiting various groundwater habitats. They are often found

in caves (Dobat 1975, Pust 1990, Weber 1991, 2001, Weber et al. 2021a, Zaenker 2008), as caves provide easy access to subterranean water, but actually occur everywhere else in groundwater, including boreholes and wells (Fuchs 2007), the hyporheic interstitial (interstitial = porous gravel/sand aquifers, which can be collected mainly near rivers or in river islands) (Weber & Weigand 2023) and springs as effluents of groundwater (Weber et al. 2020, 2022, Weber 2022). The north-eastern range limit of niphargids runs through Germany roughly following the glaciation boundary of the last ice age from northwest to southeast (Weber 2022). Niphargids are almost completely absent from the northern and north-eastern federal states of Schleswig-Holstein, Mecklenburg-Western Pomerania, Berlin and Brandenburg (Viets 1919). 30 species are listed from Germany, of which about 17 species are plausible. The others are either the result of misidentifications or species that have later been re-classified as synonyms (Weber et al. 2020, Weber 2022).

Bavaria is the federal state with the most frequent type localities for niphargids in Germany: *Niphargellus nollii* in a well at Schönbusch near Aschaffenburg (Schellenberg 1938), *Niphargus caspary* near Munich (Pratz 1866), *Niphargus puteanus* at the Weichselmühle near Regensburg (Stock 1974), *Niphargus schellenbergi* near Lohr am Main (Karaman 1932), and *Niphargus thienemanni* with several type localities close to Garmisch-Partenkirchen (Schellenberg 1934). Nevertheless, *Niphargus* seems to be rather rare in Bavaria (Dobat 1978, Weber 2022). Aside from Saarland (Weber & Flot 2020), Bavaria is the only other German federal state with a Red List of niphargids (Burmeister 2003). Formerly iden-

Table 1. New sequences generated in the present study; WGS84 coordinates are given in decimal degrees and rounded to two decimal places for reasons of cave protection; Natural Areas follows Ssymank (1994) (Biogeografische Regionen und naturräumliche Haupteinheiten Deutschlands/BfN) and are published in German as they do not translate well into English. x = sequenced, but not submitted to Genbank as sequence is incomplete.

| Number | Site | Natural Area | WGS 84 | | Genbank No. | |
|-----------|-----------------------------------------------|-----------------------------|--------|-------|-------------|----------|
| | | | Lat | Lon | COI | 28S |
| 980219-01 | Angerlloch | Schwäb.-Oberbay. Voralpen | 47.56 | 11.29 | OQ456628 | |
| 170824-02 | Höhle ohne Namen | Nördliche Frankenalb | 49.72 | 11.60 | | x |
| 170824-03 | Höhle ohne Namen | Nördliche Frankenalb | 49.72 | 11.60 | OQ456622 | OQ473192 |
| 170824-13 | Höhle ohne Namen | Nördliche Frankenalb | 49.72 | 11.60 | | OQ473190 |
| 170824-14 | Höhle ohne Namen | Nördliche Frankenalb | 49.72 | 11.60 | OQ456623 | OQ473189 |
| 180125-01 | Appelshöhle | Nördliche Frankenalb | 49.57 | 11.65 | OQ456624 | OQ473188 |
| 180617-01 | Quelle ohne Namen | Voralp. Hügel- und Moorland | 47.74 | 11.56 | OQ456625 | |
| 180505-42 | Felsenkeller Egloffstein | Nördliche Frankenalb | 49.70 | 11.25 | | OQ473191 |
| 180705-01 | Quelle zwischen Abfluss Obersee und Königssee | Nördliche Kalkalpen | 47.51 | 12.98 | OQ456626 | OQ473187 |
| 201217-01 | Angerlloch | Schwäb.-Oberbay. Voralpen | 47.56 | 11.29 | OQ456627 | |

tification of niphargids was based exclusively on their morphology, the only possible approach until about 10 years ago (Hartke et al. 2011, McInerney et al. 2014). Burmeister (2003) had already indicated that niphargids were not sufficiently described to be able to identify all specimens morphologically with certainty.

A large-scale project at the Université libre de Bruxelles is concerned with the genetic study of niphargids throughout Europe. One of the aims is to first define the species on the basis of DNA, the genetic material that underlies all animal life, to correct any errors discovered in the morphological description and, if necessary, to describe new species. Some expeditions took the research team to Bavaria where for example they were able to synonymise the Bavarian *Niphargus stadleri* with *N. puteanus* (Weber et al. 2020).

Niphargus tonywhitteni is a species of groundwater amphipod from the family Niphargidae that was described as new to science only five years ago (Fišer et al. 2018). As usual with groundwater amphipods, the species is eyeless and the body is unpigmented, with specimens completely white (Fig. 1). It has been recorded several times in the northern half of Switzerland and rarely in Austria (Fišer et al. 2018, Alther & Altermatt 2021). Three records are known from Germany, all from Baden-Württemberg (Fišer et al. 2018). The type locality is the gravel bed of the river Töss near Winterthur in Switzerland (Fišer et al. 2018). So far, the species has been classified as interstitial (Fišer et al. 2018, Alther & Altermatt 2021).

Materials and methods

In Bavaria, 141 springs, 15 caves and artificial caverns and 6 interstitial sites were investigated from 2017 to 2019. Niphargids were recorded at 40 springs (mainly in the north-western part of Bavaria), 11 caves and artificial cavities and one interstitial site. Springs were sampled by collecting and sieving mud, foliage or moss using a sieve set, with four sieves of mesh sizes 5000 µm, 1000 µm, 500 µm, and 200 µm (Weber et al. 2022). Specimens in caves and artificial caverns were collected opportunistically by manual searching. The interstitial was sampled using the Karaman-Chappuis-method (Chappuis 1942). Captured specimens were immediately preserved in 96% ethanol and kept at -20°C. Further undetermined niphargids, collected from the Angerlloch in 1998 and 2020 by Günter Hansbauer were also included in the study.

A single leg was used for DNA isolation. DNA was extracted using a NucleoSpin Tissue Kit (Macherey-Nagel) following the manufacturers' protocols. DNA isolates are stored at -20°C in the collections of the Evolutionary Biology and Ecology research unit of the Université libre de Bruxelles (ULB), at the Department

of Biology, Biotechnical Faculty, University of Ljubljana and at the Senckenberg Deutsches Entomologisches Institut, Müncheberg.

The standard barcoding fragment of the cytochrome *c* oxidase subunit 1 (COI) gene (Folmer et al. 1994) was used for amplification via polymerase chain reaction (PCR). The primer pair HCO2198-JJ and LCO1490-JJ (Astrin & Stüben 2008) was used. The PCR mix contained 1 µl DNA extract (DNA concentration not measured), 0.8 µl of each primer (10 pmol/µl), 5 µl of DreamTaq DNA Polymerase Master Mix (5 units/µl, Thermo Scientific) and 2.4 µl of ultrapure water. PCR cycling conditions were an initial denaturation step 180 s at 94°C, followed by 36 cycles of denaturation 20 s at 94°C, annealing 45 s at 50°C, and extension 60 s at 65°C. A final elongation step 120 s at 65°C followed.

In addition, Verovnik's fragment of the nuclear 28S ribosomal RNA gene (28S) was investigated. The primers Niph15 and Niph16 (Verovnik et al. 2005) were used for amplification. The PCR mix contained 1 µl of

Table 2. Sequences obtained from the literature, coordinates where only a city name was published were defined as the city centre and rounded to two decimal places.

| Genbank number | WGS84 Lat | WGS84 Lon |
|----------------------------------------|-------------------------------------|-------------|
| KX379080 | 47.46825 | 11.712933 |
| KX379081 | 47.50 | 8.70 |
| MW721678 | 47.361198 | 8.908069 |
| MW721679 | 47.385628 | 8.8513364 |
| MW721680 | 47.385628 | 8.8513364 |
| MW721682 | 47.385628 | 8.8513364 |
| MW721697 | 47.302229 | 8.8752087 |
| MW721700 | 47.430421 | 8.7462744 |
| MW721702 | 47.430421 | 8.7462744 |
| MW721757 | 47.377821 | 8.8658166 |
| OK156970 | 48.3333 | 8.03193 |
| KX379090 | 46.92 | 9.66 |
| KX379099 | 46.87 | 8.89 |
| KX379131 | 48.9946 | 10.0195 |
| MW721721 | 47.3925 | 7.75339 |
| MW721714 | 47.164021 | 7.7936546 |
| MW721694 | 47.483067 | 8.0091128 |
| MW721724 | 47.3925 | 7.75339 |
| MW721730 | 47.248021 | 7.8198516 |
| MW721733 | 47.394277 | 8.4484071 |
| MW721736 | 47.394277 | 8.4484071 |
| MW721741 | 47.215577 | 7.4552177 |
| MW721754 | 47.433808 | 7.7983422 |
| Literature data, incomplete sequences: | | |
| KX379131 | 48.9946 | 10.0195 |
| KY643621 | coordinates could not be identified | |
| MH172432 | 48.10 | 9.79 |
| MW721771 | 47.43703891 | 7.800484964 |
| MW721774 | 47.283935 | 8.6904566 |

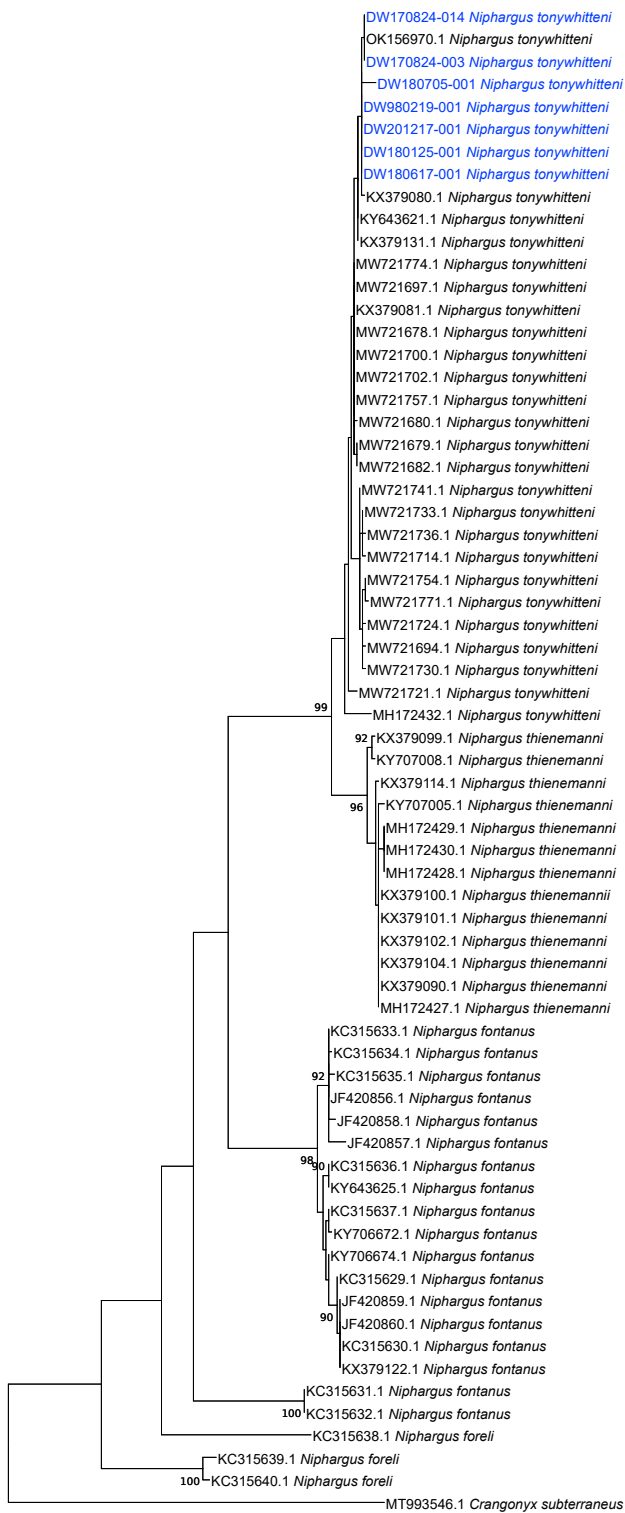


Fig. 2. Phylogenetic tree of *Niphargus tonywhitteni* and a few other *Niphargus* species based on COI sequences, estimated by Maximum likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The newly generated sequences from Bavaria are shown in blue.

DNA extract (DNA concentration not measured), 0.6 µl of each primer (10 pmol/µl), 5 µl of DreamTaq DNA Polymerase Master Mix (5 units/µl, Thermo Scientific) and 2.8 µl ultrapure water. PCR cycling conditions for 28S were an initial denaturation step 180 s at 94°C, followed by 36 cycles of denaturation 20 s at 94°C, annealing 45 s at 45°C, and extension at 60 s 65°C. A final elongation step 120 s at 65°C followed.

PCR products were bi-directionally Sanger-sequenced at Genoscreen, France. The COI marker was sequenced using the same primer pair as during PCR amplification, whereas the 28S marker was sequenced using three primers Niph15 (Verovnik et al. 2005), Niph20, and Niph21 (Flot et al. 2010). Chromatograms were edited and assembled into contigs using Sequencher version 4.1.4 (Gene Codes Corporation, USA).

25 sequences of *N. tonywhitteni* were downloaded from Genbank (Sayers et al. 2021): 1 from Borko et al. (2022), 1 from Eme et al. (2018), 3 from Fišer et al. (2017), 19 from Alther et al. (2021), and 1 from Fišer et al. (2018) (Table 2). For comparison, 13 COI sequences of *N. thienemanni*, 18 of *Niphargus fontanus* and three of *Niphargus foreli* where downloaded (Sayers et al. 2021). One *Cranogonyx subterraneus* sequence was used as an outgroup (Weber et al. 2021b). All sequences longer than the standard number of 658 base pairs were shortened to 658.

Sequences were aligned using Muscle (Edgar 2004) as implemented in MEGA-X (Kumar et al. 2018) using default values. MEGA-X was also used to compute a phylogenetic tree by the Maximum Likelihood method (ML) for the COI marker (Tamura et al. 2004) using default values (Fig. 2).

For the species delimitation procedure, the sequences JF420856, JF420857, JF420858, KC315629, KC315630, KX379131, KY643621, MH172432, MW721771, and MW721774 were deleted from the data set because they were incomplete. In the sequences MW721754, MW721730, MW721724, MW721714, MW721694, MW721741, MW721736, MW721733, MW721721, MW721757, MW721682, MW721697, MW721702, MW721700, MW721680, MW721679, MW721678 and OK156970, an obviously missing T was added at position 1.

Species delimitation was done using tree species delimitation methods: ABGD (Puillandre et al. 2012) was performed at <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html> using default settings, with the exception that the number of steps was set to 100 to achieve more stable results. The Jukes-Cantor (JC69) distance, Kimura (K80) distance with TS/TV of 2.0, as well as Simple distance were selected alternatively. PTP (Zhang et al. 2013) and mPTP (Kapli et al. 2017) were performed at <http://mptp.h-its.org> using default settings.

HaplowebMaker (Spöri & Flot 2020) was used at <https://eeg-ebe.github.io/HaplowebMaker/> to create a haplotype network. As it requires unique bases, three "N" in KX379090 were replaced by bases identical to all other *N. tonywhitteni* sequences.

Results

Between 2017 and 2019, *Niphargus tonywhitteni* was recorded at five sites in the Federal State of Bavaria. The sequences of one of the specimens from the Angerlloch, collected in 1998 and included in the analysis also resulted in *N. tonywhitteni*, although due to preservation for a long time at room temperature, the chromatograms were weak. One further specimen from the Angerlloch were therefore re-collected and confirmed as *N. tonywhitteni*. Three of these sites are located in the Alps, the other three close to each other on the borders of the administrative districts of Upper Palatinate and Upper Franconia. Three are natural caves, one is an artificial cavity and two are springs. All collecting sites are located in karstic limestone. Although the sites were intensively searched, only one to four individuals were present per site.

Seven COI sequences of *N. tonywhitteni*, all from Bavaria, were obtained and uploaded to Genbank (Table 1). They build a monophyletic clade of *N. tonywhitteni* with *N. thienemanni* being another monophyletic clade and closest neighbour (Fig. 2). The species delimitation methods ABGD (Puillandre et al. 2012), PTP (Zhang et al. 2013) and mPTP (Kapli et al. 2017) split *N. foreli* and *N. fontanus* into two species each. In all methods and with all tested distances, both, *N. tonywhitteni* and *N. thienemanni* are each a single and distinct species.

28S was sequenced from seven individuals from four sites. Of these, one locality is not represented in the COI dataset. With the exception of a few unresolved base pairs, all 28S sequences of *N. tonywhitteni* are identical. The 28S sequences confirm the results obtained using the COI sequences: *N. tonywhitteni* and *N. thienemanni* are closely related, but clearly separate and monophyletic. *N. fontanus* and *N. foreli* each yield two clearly separate clades. Thus, two separate species are stored in Genbank under the name of *N. fontanus* as well as another two under *N. foreli*. Additional knowledge cannot be gained from the 28S, so a presentation of the phylogenetic tree is omitted. For the first time, full Verovnik's fragments (Verovnik et al. 2005) of *N. tonywhitteni* were obtained from 7 individuals and uploaded to Genbank.

A haplotype network based on COI sequences of *N. tonywhitteni* (Fig. 3) recognises six distinct clades, each defined by more than three divergent base pairs. The two southern clades, each represented by one individual only, are closely related to each other (8 base pairs difference) as are the four northern clades (4,5,5,7 base pairs). However, the two southern clades differ significantly from the four northern ones (23 base pairs). Four of the six clades are geographically separated (Fig. 4), two overlap.

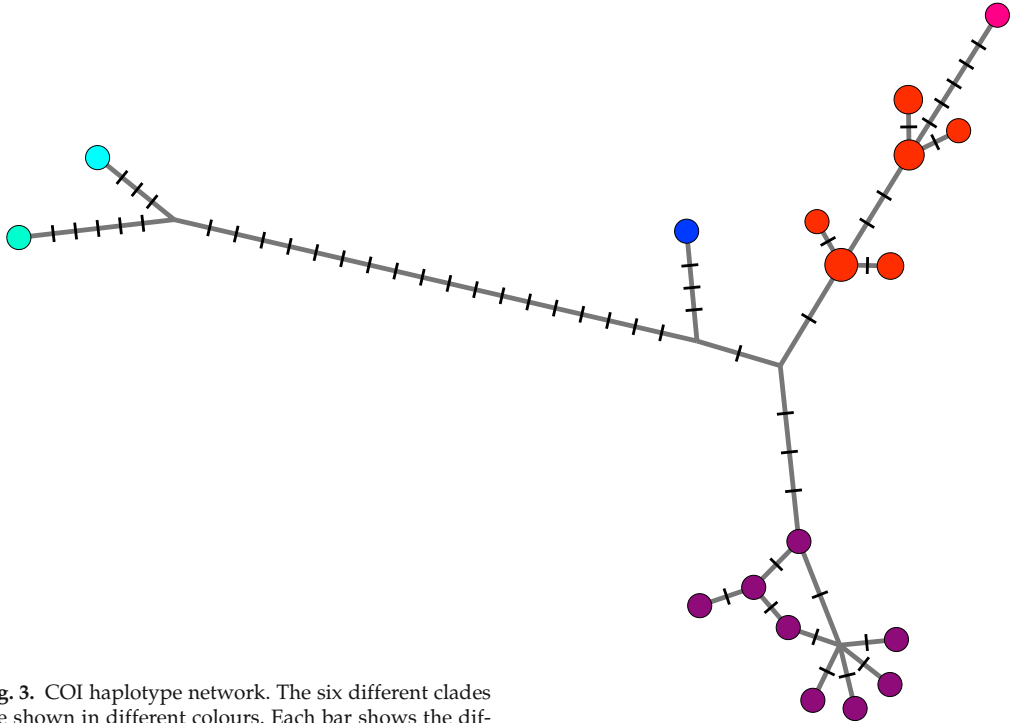


Fig. 3. COI haplotype network. The six different clades are shown in different colours. Each bar shows the difference of one base pair.

Discussion

Comparison to an older species delimitation

When *N. tonywhitteni* was first described (Fišer et al. 2018), *N. thienemanni* and *N. tonywhitteni* were identified as genetically similar, as confirmed in this study. The COI divided them into two separate species according to PTP and bPTP, while ABGD clustered *N. thienemanni* and *N. tonywhitteni* into one species in the default settings. Only by reducing the gap width from 1.5 (default) to 0.5 did two separate species emerge (Fišer et al. 2018). The comprehensive data set now available separates *N. thienemanni* and *N. tonywhitteni* into two species in all tested species delimitation methods, indicating a comprehensive data set is required for stable species delimitations (Weber 2022).

Distribution of *N. tonywhitteni*

While the new sites in the Bavarian Alps are not surprising, as they are close to an already known site in Austria, three further sites, two caves and one artificial cavern, have now been identified in the

Franconian Alb, far north of the previously known range. This is even more surprising as the Franconian Alb, in contrast to the geologically identical Swabian Alb (Dobat 1975, Weber et al. 2021a, 2020), is very poor in niphargids (Dobat 1978).

The strong geographical separation of the individual clades is astonishing. Such a separation of clades within a species, at least within the main distribution area, has not previously been documented for other niphargids from Central Europe, including both *N. puteanus* and *N. schellenbergi*. The various clades of *N. puteanus* overlap in the main distribution area of the South German Scarplands and have then formed satellite populations along the large rivers Rhine and Danube, each consisting of a single clade (Weber et al. 2020). In the case of *N. schellenbergi*, the clades also overlap in the area to the west of the Rhine, while only one clade has managed to colonise east of the Rhine without following a river course (Weber 2022). Both are species of low mountain ranges. The strong geographical separation in *N. tonywhitteni* could therefore be a consequence of the geomorphology of the high Alps, presenting a barrier. This may also explain the presence of a divergent clade in the Berchtesgaden Alps. *Niphargus*

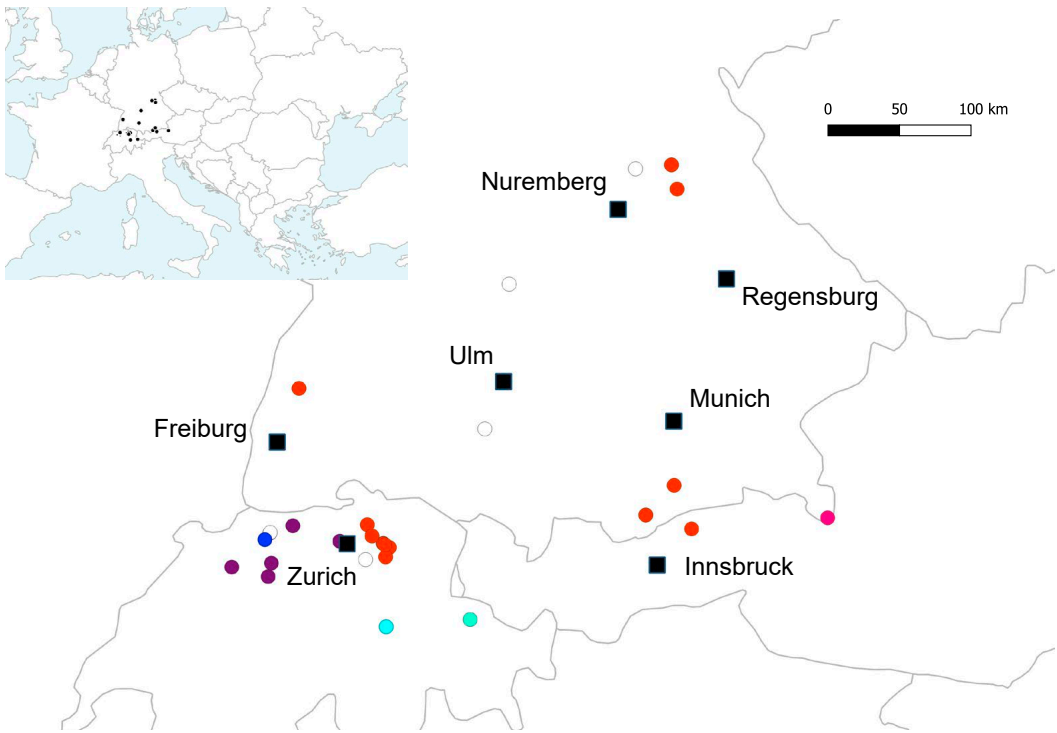


Fig. 4. Sites where *Niphargus tonywhitteni* was recorded. The colours of the six clades correspond to those in Fig. 3. Colourless = incomplete sequence, cannot be allocated to one of the clades.

puteanus is rarely found underground and is more a species of springs (Fuchs 2007, Weber et al. 2020), thus it is better adapted to epigeal life, which has thus enabled it to colonise faster along river courses. Conversely *N. schellenbergi* and *N. tonywhitteni* are true subterranean species that are often found in the interstitial (Fišer et al. 2018, Alther & Altermatt 2021), thus whilst the colonisation of these species might have been slower, their ranges are less limited to river courses as they may spread to other catchments, so long as there is connectivity via the interstitial habitat or conduits at depth in the strata. North of the Alps, high mountain ranges are absent, suggesting that *N. tonywhitteni* has spread relatively easily, whilst to the south, *N. tonywhitteni* has not succeeded in breaking through the Main Alpine Ridge.

Geological restriction

Niphargus tonywhitteni was mainly found in limestone. All other sites were either lime containing sandstone (Zaenker, pers. comm.), the molasse of the Swiss Central Plateau, or in the Swiss Jura, but all in lime containing rocks or conglomerate (Alther, pers. comm.), although intensive collecting was also

performed in Bavaria in non-calcareous areas, where several other *Niphargus* species were recorded. Numerous niphargid species occur in various regions regardless of rock type, such as *N. schellenbergi*, *N. fontanus*, the species of the *Niphargus aquilex* complex, and *Niphargellus arndti* (Weber 2022). Only a few species are restricted to karst in Central Europe, including *Niphargus enslini* (Weber et al. 2021a), *N. puteanus* (Weber et al. 2020) and *Niphargus virei* (Stoch 2004). These are large species and their occurrence almost exclusively in karst areas may be explained by the fact that only well-developed karst has subterranean cavities, large enough to provide sufficient void space in which they can live, been present for thousands of years. However, with a body length of 7.5 to 9.1 mm, *N. tonywhitteni* is a medium-sized species (Fišer et al. 2018) that was also found quite frequently in the interstitial. Thus, this affinity for karst cannot be explained by the presence of large cavities alone. It could be the case that non-karstic groundwaters might not contain sufficient calcium ions for the development and maintenance of the carapaces of some *Niphargus* species, as documented by Rukke (2002) for other Crustacea.

Confusion with *Niphargus fontanus*

Niphargus tonywhitteni is difficult to distinguish morphologically from *N. fontanus*. Moreover, little well-preserved morphological material of *N. tonywhitteni* is available to thoroughly investigate morphological variability within the species (Fišer et al. 2018). The shape of the propodus of gnathopod I tends to be more rectangular in *N. tonywhitteni* compared to *N. fontanus* (Fišer et al. 2018). Only by using sequences, was it possible to establish it as an independent species that is not even particularly closely related to *N. fontanus* (Fišer et al. 2018). The species was actually sequenced for the first time from Bavaria in 2017, i. e. before the date of the first description, but was not recognised as a new species at the time. Genbank (Sayers et al. 2021) also contained errors: as late as 2020, three COI sequences (KX379031, as *N. cf. fontanus*, KX379080, KX379080) were listed on Genbank as *N. fontanus*. Here we have an example that Genbank entries still require checking and that one needs to pay attention to the age of an upload and taxonomic advances since. The errors have since been corrected to *N. tonywhitteni*. With older records of *N. fontanus*, it is no longer possible to determine whether they are actually *N. fontanus* or *N. tonywhitteni*. Even if the specimens still exist in scientific collections, the DNA has deteriorated to such an extent that it can no longer be sequenced. *Niphargus fontanus* has never been confirmed in Bavaria by sequencing and the assumption is that the species may be absent from the region.

Confusion with *Niphargus foreli* or *Niphargus thienemanni*

Due to the large propodus of the gnathopods, *N. tonywhitteni* can be easily differentiated from *N. foreli* and *N. thienemanni*, although an exact comparison with *N. thienemanni*, which was first described as a subspecies of *N. foreli*, is difficult, as Schellenberg's original drawings of *N. thienemanni* lack detail and require updating. Gad (2007) includes two identification keys to *Niphargus* species known from Germany at that time but comparing the original drawings in the first description of *N. tonywhitteni* (Fišer et al. 2018) and following Gad's keys tends to result in determinations of either *N. thienemanni* or *N. foreli* rather than the expected *N. fontanus*, thus they are of limited value. It is not helped by the fact that in the original drawing of the *N. tonywhitteni* type specimen the dactylus of pereopod VII is not drawn as it had obviously been broken off and lost.

Niphargus foreli is now believed to be extinct at the original type locality (Karaman & Ruffo 1990), as well as the neo type locality (Dietmar Straile

Limnological Institute of the University of Konstanz, pers. comm.), and was also not found at the bottom of Lake Starnberg, where it has also been recorded in the past, despite an intensive search by the author in 2022.

All type localities for *N. thienemanni* were sampled without success. Postulating that *N. thienemanni* has withdrawn to higher altitudes with the retreat of the glaciers, numerous springs were sampled at higher altitudes on the Zugspitze (Bavarian Alps), but no niphargids were present. It must therefore be assumed that the species is now extinct within the area where the collections for the first descriptions took place. However, it is noticeable that *N. tonywhitteni* is now known from areas close by (although not specifically at the sites mentioned above). Since it can hardly be expected that *N. tonywhitteni* has displaced the other species in such a short time, it is possible that the earlier records of *N. thienemanni* in fact represent misidentified *N. tonywhitteni*. It is possible that *N. thienemanni* is also a slightly different morphotype of *N. tonywhitteni*.

Two sequences of *N. foreli* are available, both from the Source de la Fouige in the Mercantour National Park, French Alps (McInerney et al. 2014). This spring is far from its core range of Switzerland and southern Germany. The habitat also differs, as the species is usually reported from deep lakes. Both these observations suggest that these sequences are in fact not *N. foreli*. Obtaining new specimens of *N. foreli* from the type locality or fresh specimens from the French spring for morphological examination to see if they actually correspond to *N. foreli* is required to resolve this.

Is the species endangered in Bavaria?

Due to its recent description, *Niphargus tonywhitteni* is not listed on the Bavarian Red List (Burmeister et al. 2003) nor can short or long-term trends be determined from such a newly described species. Thus, its conservation status can only be surmised by considering its ecology and abundance. If it is assumed to be an interstitial species, as supported by observations in Switzerland (Alther et al. 2021), then the species is not endangered in Bavaria, as many interstitial zones, e.g. in the Alpine valleys, remain relatively pristine. However, in Bavaria, based on the results of the current study, we must assume that it is a cavernicolous species. It is certainly not limited to those parts of caves accessible to humans, but also lives within the fissures of the surrounding rock. Nevertheless, this could imply that its habitat might be more limited than that of an interstitial species. Cave visitors should have a responsible attitude when visiting the environment and not collect

any animals, damage any biotopes nor leave any rubbish behind. It goes without saying that the use of carbide lamps is prohibited.

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