**Demographic history, range size and habitat preferences of the groundwater amphipod
Niphargus puteanus (C.L. Koch in Panzer, 1836)**

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Abstract

Niphargus puteanus is the oldest described species of its genus and, in the past, was used as a taxonomic annotation for any subterranean amphipod record. For that reason, no clear knowledge exists about its actual range size and habitat preferences. We here applied a molecular taxonomic and phylogeographical approach based on mitochondrial and nuclear DNA to shed light on its distribution and to infer its demographic history. Furthermore, we analysed aquifer types and water flow regimes to provide a clearer picture of the species' ecological requirements.

Our results indicate that *N. puteanus* is widely distributed north of the Alps, having its core range in the geomorphological natural region of the 'South German Scarplands' (SGS). Additionally, isolated satellite populations exist in the Taunus and the Sauerland, and two single individuals were collected in Luxembourg and in Austria, respectively. The species' maximal distribution range reaches 756 km between the two single-specimen records and 371 km within the SGS. A very high haplotype diversity was observed, revealing the presence of seven haplotype groups. All the haplogroups were present in the SGS and exhibited distinct spatial-genetic patterns. We thus inferred a high degree of population isolation, with the SGS being a potential long-term refugium for *N. puteanus*. Historical drift of specimens along major riverine networks (Rhine, Danube) may provide an evolutionary dispersal mechanism explaining the formation of satellite populations.

From an ecological perspective, populations of *N. puteanus* were found to be closely associated with fast-flowing spring environments within fissured aquifers. This species should therefore be classified as both crenophile and stygophile, effectively exploiting resources of surface as well as subsurface spring waters.

keywords: distribution; genetic diversity; phylogeography; ecological classification; DNA taxonomy; *Niphargus stadleri*; *Niphargus puteanus*

Introduction

Groundwater comprises the largest fraction of unbound freshwater on Earth and represents the primary drinking water source for half of the global human population (Tushaar, 2007). Despite its paramount importance for human well-being, the recognition of groundwater as an aquatic ecosystem and home for often enigmatic species is still at an early stage. Nevertheless, some 2,000 obligate groundwater metazoans are known from Europe (Gibert and Culver, 2004). These so-called stygobionts are typically characterised by a lack of eye and body pigmentation and a considerably prolonged lifespan compared to their surface-water counterparts (Bellan-Santini, 2015). Stygobionts often have small distribution ranges or are even sometimes known from a single locality only (Trontelj et al., 2009; Fišer et al., 2016). Because of limited resource availability, the subterranean environment presumably supports small local communities (Gibert and Deharveng, 2002). Furthermore, geographically separated communities are often quite distinct in their species pools, suggesting that distribution patterns result from past vicariance events rather than from active migration (Stock, 1993; Trontelj et al., 2009; Culver et al., 2009). The detection of cryptic diversity – in particular for morphospecies assumed to be geographically widespread – is a frequent phenomenon in molecular studies dealing with stygobionts (Zakšek et al., 2009; Camacho et al., 2011; Niemiller et al., 2012; Délic et al., 2017; Eme et al., 2018), where morphospecies are often revealed to harbour more than one distinct molecularly defined species. As a result, estimations about the global number of stygobiont species are in the range of 50,000 – 100,000 (Culver and Holsinger, 1992).

Members of the amphipod genus *Niphargus* are among the most common representatives of groundwater communities of Europe. More than 425 species have been described for the West Palearctic (Horton et al., 2018), with the large majority being strictly stygobiont and inhabiting diverse groundwater habitats. Prominent exceptions comprise *Niphargus hrabei* and *Niphargus valachicus*, which have re-colonised surface freshwaters and occur over a wide geographical area (Copilaş-Ciocianu et al., 2018). Subterranean amphipods are frequently used as models in ecology and biogeography (Fišer, 2019). Over the last decade, it has become more and more obvious that our knowledge on the ecology and distribution of many niphargid species is heavily impacted by erroneous or ambiguous past taxonomic assignments (Fišer et al., 2016, 2018a, b). Species delimitation as well species identification based on potentially diagnostic morphological characters seems insufficient as intraspecific and interspecific variability ranges can largely overlap (Fišer et al., 2018b). Adding to this, several *Niphargus* morphospecies have been revealed to be complexes of cryptic or pseudocryptic species (e.g. McInerney et al., 2014). The latter refers to a population initially considered part of a morphospecies, but identified by molecular data as a separate entity and found out to be distinguishable by morphological characters upon re-investigation. It is thus not surprising that recent studies make use of integrative research designs applying a combination of morphological and molecular characteristics for *Niphargus* species identification. Molecular species identification using DNA taxonomy (Fontaneto et al., 2015) has the advantage to be applicable to developmental stages (e.g. juveniles) or sexes, which – in case they are not dealt with in taxonomic keys – are regularly omitted. An increase of high-quality occurrence data leads in turn to more robust datasets, which is one the major obstacles for current groundwater ecological and biodiversity research (Zagmajster et al., 2019; Mokany et al., 2019).

Probably due to its relatively large body size of up to 2.2 cm in adult males, the first ever described niphargid species was *Niphargus puteanus* (C.L. Koch in Panzer, 1836) – at that time referred to as *Gammarus puteanus*. This species is commonly observed in aquatic subterranean biotopes (caves, mining galleries and interstitial) and groundwater discharges (springs and wells) in South-Western Germany (Baden-Württemberg), where it is considered to have its main distribution. Some isolated populations have been described from North and North-Western Germany (Sauerland, Pust, 1990; the Harz Mountains, Hartmann, 1978/79, 2004), Southern Poland (Arndt, 1923), Bulgaria (Bureš, 1929; Beron et al., 2004, referring to Fage, 1926), Switzerland (Moeschler, 1989), Belgium (Leloup and Jacquemart, 1963), France, Italy, Croatia and Slovenia (Wolf, 1934-38). However, morphology-based species records of the past can be questionable. For example, very early records of *N. puteanus* (or *G.*

puteanus) may actually refer to any groundwater niphargid or even gammarid, as this taxonomic annotation was frequently used as a lumping bin for amphipod populations found in and adapted to the dark (see Brehm, 1955). Likewise, distribution data provided by Schellenberg (1932) remain unclear as he synonymised the species with *Niphargus fontanus*. Finally, repeated intensive sampling campaigns in the Hölloch cave system (Switzerland) did not re-detect the five previously reported *Niphargus* species, including *N. puteanus* (Fišer, et al. 2016): instead, the authors described three new niphargids from this location and emphasised that all past species records from there were probably misidentifications. The species was also historically reported from Belgium, but in their recent survey Fišer et al. (2018a) were not able to re-detect it.

In the original description by Koch (1836), the type locality of *N. puteanus* comprises two wells near Regensburg (Germany, Bavaria) and a well in Zweibrücken (Germany, Rhineland-Palatinate). However, as carefully noted by previous authors (e.g. Wrzesniowski, 1890; Schellenberg, 1933), the two illustrated and investigated specimens were quite different from each other. Moreover, the original material of Koch (1836) seems to be lost (Goedmakers, 1972). For these reasons, Stock (1974) established a neotype and defined a neotype locality referring to the spring Weichselmühle (=Weichselmühle) near Regensburg (South-Western Germany).

The present work aimed to provide robust and updated insights into the ecology, genetic diversity, distribution and demographic history of the groundwater amphipod *N. puteanus*. First, we performed molecular species delimitation to provide high-quality presence records for reconstructing the actual distribution range of the species. Second, the obtained dataset was used to generate insights into the genetic diversity of the species, and to investigate the connectivity (or lack thereof) of the populations sampled. The combined spatio-genetic data were then used to infer the plausibility and potential origin of isolated satellite populations, and to help reconstructing the demographic history of *N. puteanus*. Finally, we investigated the available information on aquifer types and water-flow regimes to provide a more sophisticated picture of the species' ecological requirements and its ecological classification.

Material and Methods

Datasets and presence records of N. puteanus

From 2016 to 2018, more than 1,400 sites were visited and investigated for the presence of *Niphargus puteanus* in the target region covering Austria, Belgium, the Czech Republic, Germany, Luxembourg, Poland and Switzerland (**Supplementary Figure S1**). We also included the data available in two major databases – the biospeleological register of the Federal State of Hesse in Germany (Reiss et al., 2009, updated 2018) and a private register for subterranean taxa of Baden-Württemberg in Germany (Hannes Köble). Further published records from Stock (1974), Pust (1990), Bernasconi (1994), Trontelj et al. (2007), Altermatt et al. (2014), Esmaeili-Rineh et al. (2015), Fišer et al. (2017, 2018), Eme et al. (2018), the German Barcode of Life project (GBOL), the Amphipod database of Switzerland (<https://www.amphipod.ch>) and the private collection of Fabio Stoch were integrated. Overall, our dataset comprised 3,333 presence records of the genus *Niphargus* in the study area (dataset 'observed all') including 154 potential presence records of *Niphargus puteanus* (dataset 'observed Np', **Supplementary Table S1**). Of the latter, 58 localities were (re-)visited and successfully sampled for living *N. puteanus* specimens, which were used for subsequent molecular cross-validation and analysis of genetic diversity. We also intensively collected around Zweibrücken (i.e. one of the two sampling regions stated in the original description by Koch in 1836) and found many niphargids, but were not able to re-sample *N. puteanus* there.

Laboratory work

DNA was extracted using either the DNeasy Blood & Tissue Kit (Qiagen) or the NucleoSpin Tissue Kit (Macherey-Nagel), following the manufacturers' protocols. A single leg was dissected and dried for 10 min at 60°C before use. DNA isolates were stored at -20°C in the collections of the Evolutionary Biology and Ecology research unit of the Université libre de Bruxelles (ULB, Solbosch campus). The standard animal barcoding fragment of the cytochrome c oxidase subunit 1 (COI) gene (Folmer et al., 1994) was

amplified via polymerase chain reaction (PCR) using the primer pair HCO2198 and LCO1490 (Folmer et al., 1994) at the beginning of the lab trials. Later, the degenerate primers HCO2198-JJ and LCO1490-JJ (Astin and Stüben, 2008; **Supplementary Table S2**) were used, as they produced slightly clearer chromatograms. PCR mixes contained 1 µL of DNA extract (of variable concentration), 0.8 µL of each primer (10 pmol/µL), 5 µL of DreamTaq DNA Polymerase Master Mix (Thermo Scientific) and 2.4 µL of ultrapure water. PCR cycling conditions were an initial 3 min denaturation step at 94°C, 36 cycles of 20 s denaturation at 94°C, 45 s annealing at 50°C, and 60 s extension at 65°C; and a final elongation step of 2 min at 65°C. The amplification results of the PCR reactions were visualised using gel electrophoresis on a 1.2% agarose gel. Bi-directional Sanger-sequencing was performed at Genoscreen (Lille, France) using the same primers as for PCR amplification.

A fragment of the nuclear 28S ribosomal RNA gene was investigated using the PCR primer pair of Verovnik et al. (2005). The PCR mix for the 28S marker contained 2 µL of DNA extract (of variable concentration), 1 µL of each primer (10 pmol/µL), 0.2 µL of REDTaq Polymerase (Sigma-Aldrich), 5 µL of REDTaq reaction buffer and 15.8 µL of ultrapure water. PCR cycling conditions for 28S were an initial 3 min denaturation at 95°C, 56 cycles of 30 s denaturation at 94°C, 60 s annealing at 45°C, and 90 s extension at 72°C. The amplification results of PCR reactions were visualised on a 1.5% agarose gel and Sanger-sequenced using three primers (see **Supplementary Table S2**) at Genoscreen (Lille, France).

Molecular analyses

The newly generated chromatograms were edited and assembled into contigs using Sequencher version 4.1.4 (Gene Codes Corporation, USA). Sequences were aligned using MUSCLE (Edgar, 2004) as implemented in MEGA 7.0.21 (Kumar et al., 2016). Additional public COI sequences annotated as *Niphargus puteanus* were retrieved from the Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007), the German Barcode of Life project (GBOL, Geiger et al., 2016) and NCBI GenBank as of 13.01.2019. Of the 43 downloaded sequence records we did not include in the final COI dataset those that a) represented double entries of the same specimen in different databases, b) contained any stop codon potentially reflecting pseudogenes, c) contained more than three ambiguities or d) were less than 600 bp in length.

Species delimitation

The Automatic Barcode Gap Discovery (ABGD) approach (Puillandre et al., 2012) was performed on a dataset comprising the COI alignment of Eme et al. (2017, 2018; this dataset included 1,380 niphargid sequences, 16 of which were annotated as *N. puteanus*), the downloaded COI sequences and our newly generated sequences. The COI alignment was trimmed to 510 bp. ABGD was run twice under default settings, using the Kimura-K80 as well as the Jukes-Cantor-JC69 genetic distance models. Likewise, the nuclear 28S dataset was enlarged by seven sequences of *N. puteanus* from NCBI GenBank (**Supplementary Table S4**).

Network analyses

All COI sequences were aligned using the MUSCLE plugin (Edgar, 2004) with three iterations, as implemented in the Geneious 5.4.7 software (Kearse et al., 2012). The number of haplotypes (N), haplotype diversity (Hd), nucleotide diversity (π), Tajima's D (Tajima, 1989), Fu's Fs (Fu, 1997), Fu and Li's D* and F* (Fu and Li, 1993), haplotype mismatch distribution, the Harpending's raggedness index hri (Harpending, 1994) and the R2 statistic (Ramos-Onsins and Rozas, 2002) were all calculated using the DnaSP v5 software (Librado and Rozas, 2009). The latter two statistics are goodness-of-fit estimates for comparing the observed mismatch distribution against an expected distribution for historically stable populations (Rogers and Harpending, 1992). The statistical significance of the parameters was tested by using 1,000 simulated coalescent replicates. 28S sequences were aligned by hand. For each marker, a median-joining haplotype network (Bandelt et al., 1999) was generated using the software Network 5 (Fluxus Technology Ltd.). In the case of COI, haplotype clades were defined using a nested clade analysis (Templeton, 1998) and represented as boxes on the median-joining network. In the case of 28S, the median-joining network was turned into a haploweb by adding curves

connecting haplotypes found co-occurring in heterozygous individuals (Flot et al., 2010). All final graphics were generated using Inkscape 0.92 (Bah, 2011).

Analyses of range size, aquifer types and microhabitat conditions

The 154 plausible presence records of *N. puteanus* largely followed the delineation of geomorphological natural regions, clustering geographically into the South German Scarplands (SGS), Taunus (Hesse, Germany), Sauerland (North Rhine-Westphalia, Germany), Luxembourg and Austria. These geographical regions were used for inferring the species range size. Point-to-point distance measurements were calculated in QGIS 3.4.1 (QGIS Development Team, 2019). We calculated both the maximal Euclidean distance between two records within the same pre-defined geographical region as well as all minimal Euclidean distances between two points in different geographical regions.

The precise location of each 154 presence records in a specific aquifer type category was analysed using the georeferenced Spatial Data of the International Hydrogeological Map of Europe 1 : 1,500,000 (Version IHME1500 v1.1, Düscher et al., 2015) of the Federal Institute for Geosciences and Natural Resources (BGR, Hannover, Germany). This dataset records six generalised aquifer type categories delineated according to their geological porosity and productivity in terms of general groundwater yield: 1) porous and highly productive aquifers, 2) porous and low to moderately productive aquifers, 3) fissured/karstified and highly productive aquifers, 4) fissured/karstified and low to moderately productive aquifers, 5) locally aquiferous rocks, either porous or fissured, and 6) practically non-aquiferous rocks, either porous or fissured. A distinct category ("200") refers to inland waters such as rivers and lakes (**Supplementary Figure S1**).

To investigate whether the distribution of *Niphargus* and *N. puteanus* presence records in the six aquifer type categories deviated from expected aquifer type category distributions, two simulation datasets with random sampling points were produced in QGIS. First, the maximal and minimal latitudinal and longitudinal WGS84 coordinates (xmin, xmax, ymin and ymax) of the *N. puteanus* occurrence dataset were used to define a square element (dataset 'exp_{square}'). This element was created as the geographic area for the generation of 1,000 random sampling points building null models for the different aquifer types. Subsequently, these models were used to infer the expected (simulated) frequency of each aquifer type category. Second, a polygon was used as the boundary element instead of a square, as defined by all outermost records of the 'observed all' dataset (dataset 'exp_{polygon}'). Similarly, 1,000 random sampling points were generated to infer the frequency of each aquifer type category within the polygon, which then was used as a second null model distribution. The two different boundaries were used (i.e. square and polygon) to account for uncertainties when setting an artificial margin for the simulated datasets. But even then it has to be highlighted that the null models produced can be to some extent biased by reflecting dispersal histories rather than ecological preferences (i.e. random sampling points outside the dispersal-accessible zone represent unavailable rather than unfavourable sites).

Pearson's χ^2 (Pearson, 1900) implemented in the R software environment was applied to test whether the aquifer type category frequencies from both observed datasets ('observed all' and 'observed Np') differed significantly from the randomly sampled point datasets (exp_{square} and exp_{polygon}). Further, we tested whether the aquifer type category distribution in the observed presence record dataset of *N. puteanus* differed from the distribution of aquifer type categories in the 'all *Niphargus*' dataset.

When available (i.e. stated in the literature or collected during time of sampling), microhabitat characteristics were investigated for all 154 presence records. Habitat types and flow conditions were recorded. We distinguished between the habitat types 'spring', 'cave', 'well', 'interstitial' and 'mining gallery'. Springs were further subdivided into 'spring (rheocrene)', 'spring (helocrene)', 'spring (limnocrene)' and 'spring (basin)' following Springer and Stevens (2008). Flow conditions ranged from 'low' to 'extreme'.

Results

Genetic diversity and phylogeographic analyses

The results of the ABGD analyses of the COI marker were consistent with Eme et al. (2018) in delimiting five MOTUs (**Supplementary Table S3**) among the sequences annotated as *N. puteanus* in Eme et al. (2017) fell into five MOTUs. One of these MOTUs included twelve public sequences as well as all of our 42 newly generated COI sequences; as one of our sequences was of a specimen from the neotype locality, we equated this MOTU (ABGD cluster 3) with *Niphargus puteanus* sensu stricto, which was labelled by Eme et al. (2018) as MOTU T259 (based on 16% COI threshold), M71 (based on PTP) and B72 (based on bPTP), respectively (**Supplementary Table S3**). All specimens of the other four MOTUs originated from Italy and were omitted for all downstream analyses.

Three *N. puteanus* COI sequences (KX379079, KX379118, KX379139) had insufficient lengths and were therefore only used as geographical presence records of haplotype groups but not for analyses of haplotype and nucleotide diversity or demography estimates. The final dataset of 51 COI sequences revealed a total of 36 haplotypes with 66 polymorphic sites (**Supplementary Table S4**). Among these haplotypes, 32 were singletons whereas the four others were shared by up to nine specimens: H1 was shared by BA1, BA2, DS1, GQ1, OS1, QF1, QL1, SW1 and WS1; H2 was shared by DH1, GH1, IM1, SH1 and SP1; H3 was shared by DE1, OZ1 and QD1; and H4 was shared by KW2 and QH2. The overall haplotype diversity H_d was 0.961 ± 0.00032 whereas the overall nucleotide diversity π was 0.01727.

The nested clade analysis assembled these 36 haplotypes into seven haplotype groups (**Fig. 1**, HG3-1 to 3-7). The most frequently sampled haplotype groups were HG3-4 and HG3-6. HG3-1 was mainly distributed along the Neckar River in the South German Scarplands (SGS) and comprised also the two specimens sampled from the Sauerland satellite population. HG3-2 comprised three specimens from the Upper Neckar, the Austrian sample as well as the specimen from the neotype locality. HG3-3 was restricted to the very south-west of the SGS. HG3-4 was distributed in the SGS between Upper Danube and Upper Neckar (Swabian Jura), at the Lower Main (Odenwald-Spessart-Rhön) and in the East and West Neckar and Tauber Gäu Plateaus. HG3-5 comprised a single specimen from the Eastern Swabian Keuper-Lias Plains, whereas HG3-6 harboured most of the investigated specimens and spanned across the Swabian Jura, Lower Neckar, Eastern Neckar and Tauber Gäu Plateaus, in addition to the two specimens from the Taunus satellite population and from Luxembourg (**Fig. 2B**). Finally, HG3-7 harbours a single specimen from the Kraichgau close to the Salzach River, which is a headwater of the Saalbach River and part of the Rhine catchment.

Fig. 1. Median-joining haplotype network of the COI marker. The seven haplotype groups (3-1 to 3-7) delineated by the nested clade analysis are represented with different colours (haplotypes in white are the median vectors inferred by the median-joining algorithms). The size of each circle is proportional to the frequency of the corresponding haplotype in the overall dataset. Lines represent the mutational pathway between individual haplotypes, with small black circles representing hypothetical but not sampled haplotypes.

Fig. 2. Distribution area of *Niphargus puteanus* and haplotype groups. A) Geomorphological regions in which *N. puteanus* was detected. Single specimens were collected in Austria and Luxembourg. The inset places these presence records in a broader geographical context. B) Distribution of the haplotype groups HG3-1 to HG3-7. Small white circles indicate localities where specimens were identified morphologically as *N. puteanus* but not sequenced, whereas small grey circles indicate the locations of specimens that were validated genetically using 28S or a smaller COI fragment but could not be attributed to one of the seven haplogroups for that reason. The background of the map shows the different aquifer type categories with blue colours referring to fissured aquifers, and orange colours to porous aquifers (for details see **Supplementary Figure S1**).

Fu's F_s test for neutral molecular evolution was significantly negative, suggesting an excess of rare haplotypes ($F_s = -14.954$, $p = 0.001$), which is often taken as evidence for past population expansion. The three remaining tests for neutral molecular evolution (D , D^* and F^*) were also negative but not significantly (**Tab. 1**).

A multimodal haplotype mismatch distribution was revealed (**Fig. 3**). When compared with the distribution expected in case of a historical stable population, both goodness-of-fit parameters hri (0.0162 , $p = 0.268$) and R_2 (0.0793 , $p = 0.209$) did not significantly deviate from zero (**Tab. 1**). Given that the R_2 statistic is supposedly suitable for small sample sizes such as ours (Ramos-Onsins and Rozas, 2002), these results are compatible with the hypothesis of a long-term stable population.

Tab. 1. Test statistics for neutral molecular evolution and goodness-of-fit estimates. Significant values are marked in bold.

parameter	value	p -value
D	-0.97983	0.180
F_s	-14.954	0.001
D^*	-0.95332	0.162
F^*	-1.11734	0.133
hri	0.0162	0.268
R_2	0.0793	0.209

Fig. 3. COI haplotype mismatch distribution. Comparison of the observed haplotype mismatch distribution (solid line) and a modelled distribution expected from a historically stable population (dashed line).

The analysis of a fragment of the nuclear 28S rDNA for 47 specimens (27 of which had been also sequenced for COI) yielded nine alleles, one of which (allele 1) was shared by 42 specimens (89%; **Tab. S1, Fig. 4**). Individual BL2 from the literature (Fišer et al. 2017) was initially published as homozygous for this marker, but careful re-examination of its chromatogram revealed a minor peak that had been overlooked during automatic base-calling (**Supplementary Figure S2**). Overall, six heterozygous individuals were detected (BL2, EZ1, HA1, QG1, SH1 and WQ1). Other alleles were found in two specimens (allele 2 in GW1 and TU1; allele 3 in BL2 and SQ1) or detected in the heterozygote QG1. The nine alleles (A1-A9) were separated from each other by a maximum of five mutational steps. The resulting haploweb (Flot et al., 2010) contained three fields for recombination (*sensu* Doyle, 1995): 44

of the 47 specimens sequenced were interconnected via shared haplotypes, whereas the three remaining specimens (TU1, GW1 and QG1) formed two distinct groups.

Fig. 4. Haplotype network of 28S haplotypes. The 28S haplotypes of each individual are coloured according to the COI haplogroup to which it belongs, with grey indicating missing COI data. Next to each haplotype are listed the names of the specimens harboring it, with -a and -b indicating the two haplotypes of heterozygous specimens (connected by black curves in the haplotype network). Black circles represent hypothetical (unsampled) 28S haplotypes. Sequences retrieved from NCBI GenBank are in italics and marked with asterisks.

Range size

In total, 61 (40%) of the 154 presence localities integrated in our study were checked using molecular data (COI and/or 28S), further validating the presence of *N. puteanus* at those localities. The two most distant records were separated by an Euclidean geographical distance of 756 km (Tab. 2, Fig. 2A), corresponding to the single specimens found in Austria and Luxembourg. A more or less continuous distribution spanned the South German Scarplands (SGS), which approximately encompasses 300 x 340 km (max. 371 km between two records) – or 300 x 240 km (max. 272 km) when excluding the isolated Regensburg population in the East. Both satellite populations occupied a small range with a maximal point-to-point Euclidean distance of 50 km in the Sauerland and 41 km in the Taunus.

Tab. 2. Euclidean distances for records of *N. puteanus* within and between geographic regions. Estimates between regions refer to minimal point-to-point calculations, whereas estimates within a geographical region represent the maximal Euclidean distance. SGS = South German Scarplands.

SGS	371				
Taunus	87	41			
Sauerland	183	115	50		
Luxembourg	175	143	214	0	
Austria	317	640	696	756	0
	SGS	Taunus	Sauerland	Luxembourg	Austria

Aquifer types

The distributions of expected and observed presence records according to specific aquifer type categories are given in **Tab. 3**. All Pearson's Chi²-tests were significant, indicating a deviation of the observed aquifer type distributions from the null distributions in all pairwise comparisons (**Tab. 4**). Aquifer type category '200' was excluded from the test statistics because there were no value in this category in the simulated datasets. The majority of presence records for *Niphargus puteanus* was from fissured, highly productive aquifers (82 observations, 53.2%); this aquifer type category only made up 22.5 and 24% in the simulated datasets (exp_{square} and exp_{polygon}), respectively, vs. 29.4% of all *Niphargus* presence records. Furthermore, fissured and low to moderately productive aquifers (type category 4) comprised 28.6% of all *Niphargus* records and 25.3% of *N. puteanus* records, but no more than 11.1 and 13.0% in the simulated datasets. Compared to the simulated expectations, the proportion of presence records for *N. puteanus* was higher in fissured aquifers (categories 3 and 4) and lower in locally aquiferous or practically non-aquiferous rocks as well as in porous, low to moderately productive aquifers (categories 2, 5 and 6). There was only a slight deviation from the observed and expected values for the aquifer type category 1 (porous, highly productive). However, when compared to all *Niphargus* records (3.8% in category 1), the 16 presence records of *N. puteanus* (10.4%) were overrepresented in this aquifer type category.

Microhabitat characteristics

The majority of the 154 localities harbouring populations of *N. puteanus* belonged to the habitat type 'spring' (77%; **Tab. 5**). Of those, rheocene springs – i.e. springs where an aquifer discharges into a defined surface channel – constituted more than half (53%). Fewer records originated from the habitat types 'cave' (12%) and 'mining gallery' (9%). Since flow regimes were assessed during our own field surveys, but were not always reported in the literature, statements on flow conditions at the time of sampling could be made for only 58% of all presence sites. The large majority of flow conditions under which *N. puteanus* specimens were collected could be classified as 'medium' (27/89, 30%) and 'high' (38/89, 43%).

Tab. 3. Aquifer type categories. Seven aquifer type categories were distinguished and their observed and expected distributions indicated. Observed all = all *Niphargus* presence records included; observed NP = only presence records of *N. puteanus* included; exp_{square} and exp_{polygon} refer to expected distributions following two simulated datasets.

		observed all		observed Np		exp _{square}		exp _{polygon}	
aquifer description	type	N	%	N	%	N	%	N	%
porous, highly productive	1	127	3.8	16	10.4	109	10.9	130	13.0
porous, low to moderately productive	2	53	1.6	0	0	85	8.5	125	12.5
fissured, highly productive	3	979	29.4	82	53.2	225	22.5	240	24.0
fissured, low to moderately productive	4	954	28.6	39	25.3	111	11.1	130	13.0
locally aquiferous rocks, porous or fissured	5	419	12.6	2	1.3	147	14.7	194	19.4
practically non-aquiferous rocks, porous or fissured	6	797	23.9	14	9.1	323	32.3	181	18.1
inland waters	200	4	0.1	1	0.7	0	0	0	0

Tab. 4. Results of Chi²-tests between expected and observed aquifer type distributions for *N. puteanus*. Observed all = all *Niphargus* presence records included; observed Np = only presence records of *N. puteanus* included; exp_{square} and exp_{polygon} refer to expected distributions following two simulated datasets.

Chi²-test

Expected	Observed	Chi ²	df	p-value
exp _{square}	observed all	1417,4	5	< 1E ⁻¹⁵
exp _{polygon}	observed all	1343,7	5	< 1E ⁻¹⁵
exp _{square}	observed Np	151,4	5	< 1E ⁻¹⁵
exp _{polygon}	observed Np	126,7	5	< 1E ⁻¹⁵
observed all	observed Np	80,5	5	< 1E ⁻¹⁵

Tab. 5. Habitat type and water flow conditions during time of sampling of *N. puteanus* populations. Subcategories are indicated for the habitat type 'spring'. N.a. = not available. Rheocrene = flowing spring, emerging into one or more confined stream channels; limnocrene = emergence of confined or unconfined aquifers in pool(s); helocrene = often indistinct or multiple springs seeping from shallow, unconfined aquifers. Definitions following Springer and Stevens (2008).

habitat type	N	%	flow condition				n.a.
			low	medium	high	extreme	
spring	118	77	13	26	34	8	37
spring (rheocrene)	63	41	5	22	26	8	2
spring (limnocrene)	3	2	2		1		
spring (helocrene)	6	4	2				4
spring (basin)	17	11	4	4	7		2
not specified	29	19					29
cave	18	12	1		4	2	11
mining gallery	14	9					14
interstitial	3	2		1			2
well	1	<1					1

Discussion

This study expands our knowledge on the distribution, genetic diversity, demographic history and ecology of the groundwater amphipod *Niphargus puteanus*. Molecular data were used to cross-validate past presence records and to infer the actual distribution range. This was particularly important for satellite populations and two single-specimen findings, which were strongly disconnected geographically from the assumed core range in South-Western Germany. Subsequently, the same geographic and genetic datasets were exploited to reveal spatio-genetic patterns, infer the genetic connectivity of populations and disentangle the potential origin of satellite populations. Finally, the aquifer types and microhabitat conditions (habitat type and water flow condition) where *N. puteanus* populations occurred were investigated, providing a clearer picture of the species' ecological requirements.

Species delimitation and identification

DNA taxonomy allowed us to delimit *N. puteanus*, first using COI with Automatic Barcode Gap Discovery then confirming the resulting species hypothesis with a haplotype analysis of 28S. Among the five COI MOTUs delimited by ABGD, we equated one with *N. puteanus* whereas the four other MOTUs from Italy (as published in Eme et al., 2018 and morphologically resembling *N. puteanus*) must be considered distinct species. Those specimens are separated by at least 14.9% COI p-distance from its closest *N. puteanus* member in our dataset. Most likely, they could refer to *Niphargus rotundus* and/or *Niphargus sestaputeanus* instead, which are morphologically very close to *N. puteanus* (Karaman, 2016). Likewise, findings from the Central Alps are potential results of former misidentifications, as highlighted e.g. by Fišer et al. (2018) for the Hölloch cave system. The same might be true for early literature records from Bulgaria (Fage, 1926), Poland (Arndt, 1923) and the Balkan Peninsula (Wolf, 1934-38). To circumvent future errors related to geographical and genetic data for *N. puteanus*, data repositories for geographic (e.g. GBIF) and genetic information (e.g. NCBI GenBank) should be updated accordingly.

28S sequences supported the distinctiveness of *N. puteanus* as defined here compared with the species from Italy (data not shown). The 28S haploweb of *N. puteanus* contained three distinct fields for recombinations (FFRs), i.e., putative species according to this marker. In the case of individuals TU1 and GW1 from the literature, examination of the chromatograms might have revealed uncalled double peaks connecting them to the main haplotype shared by nearly all *N. puteanus* individuals examined (as turned out to be the case for individual BL2) but we could not test this hypothesis. In the case of QG1, sequencing of additional individuals from the same locality will be required to find out whether the disconnection of this specimen from the rest of the network is merely an undersampling artefact or points to an actual species boundary undetected by COI.

Morphological analysis of our molecularly checked specimens enabled us to confirm that adult *N. puteanus* males can be reliably identified using a combination of morphotaxonomic characters, at least in our study region: i) average size of 10-16 mm (exceptionally up to 22 mm), ii) coxal plate IV not significantly longer than coxal plate V, iii) elongated uropod III (much longer than that of females), iv) length of inner ramus of uropod I 20%-100% longer than that of outer ramus, v) epimeral plate III with an angle of 80°-120° but not rounded at the postero-ventral corner, and vi) square-shaped propodi of the gnathopods with their dactyli bearing 5-8 seta along the outer margin. Adult females have the same size, look similar, but uropod III is not elongated and the inner ramus of uropod I is only 10-30 % longer than outer ramus, which makes the differentiation from *N. schellenbergi* difficult: the only diagnostic trait appears to be the number of setae on the dactyli of the gnathopods (higher than 4 in *N. puteanus* whereas *N. schellenbergi* has 3-4).

DNA taxonomy allowed us to identify unambiguously a number of juvenile *N. puteanus* specimens, providing robust geographical records and new insights via genetic data. At the same time, we must stress the fact that a relatively high intraspecific genetic diversity was observed. In the case of COI, the two most divergent sequences of *N. puteanus* were separated by 23 mutations corresponding to a genetic p-distance of 3.5%, which is higher than the 3% threshold-value commonly applied in DNA barcoding studies (Hebert et al., 2003), but much less than commonly observed mean genetic distances for amphipods (Costa et al., 2006, Trontelj et al., 2009, Délic et al., 2017). Still, such level of intraspecific

genetic diversity may cause problems when sampling frequency and/or the distribution area covered are low (Bergsten et al., 2012) – a situation frequently observed for groundwater animals. Hence, although DNA taxonomy appears very promising for the future characterisation of stygobionts, their identification using DNA barcoding will require well developed and curated DNA barcode reference libraries, in which each target species is represented by several DNA barcodes originating from throughout its distribution range (Weigand et al., 2019). In the optimal case, type material or newly collected specimens from the type locality should be targeted. Here, for instance, we were able to integrate a DNA barcode from the neotype locality of *N. puteanus* near Regensburg (Germany). Also, group-specific distance thresholds must be applied to account for taxonomically variable patterns of intra- vs. interspecific genetic distances.

We also briefly want to address the taxonomic status of *Niphargus stadleri* S. Karaman, 1932, a species described from Southern Germany (Bavaria). One year after its original description, the species had been already synonymised with *N. puteanus* (Schellenberg, 1933) – a view later supported by Stock (1974) during its neotypification of *N. puteanus*. However, the World Register of Marine Species (WoRMS, Horton et al., 2019) still treats this taxon as accepted. In his first description, Karaman (1932) states five collecting sites between Aschaffenburg and Würzburg: the Jakobsbrunn close to Altenbuch as the type locality, the Nähler Brunnen, the Springbrunn, the Wildscheuerbrunn and the Schulzekanzebrunn, all close to Bischbrunn. We identified two springs close to Altenbuch: the Frickenbrunn and another spring 600 m to the South (referred to as Quelle im Frickengrund). Since re-naming of springs in this region frequently took place during the last century, we assume that the Jakobsbrunn by Karaman (1932) is the spring now called Frickenbrunn. This spring contained no *Niphargus* specimens, but the Quelle im Frickengrund was successfully sampled. The Nähler Brunnen (Karaman 1932), now called Nählerbrunn, contained *Niphargus*. In another spring 1 km to the East, the Schleifhorquelle, we also collected niphargids. The Springbrunn, now Springbrunnen, was located but not visited. The Jakobsbrunn, the Wildscheuerbrunn and the Schulzekanzebrunn could not be located. All collected specimens clustered within the morphological variability observed for *N. puteanus*. Two of them (NA1 from the Nählerbrunn and SH1 from the Schleifhorquelle) were sequenced for COI, yielding nucleotide sequences typical for *N. puteanus*. In addition, SH1 was sequenced for 28S and turned out to harbour the most frequent *N. puteanus* haplotype. We take this result as further genetic support for the synonymisation of *N. stadleri* with *N. puteanus*.

Distribution range

A more or less continuous distribution range of 300 x 340 km was inferred for *N. puteanus* in the South German Scarplands (SGS). Additionally, genetic data supported the existence of two disconnected satellite populations in Western Germany (Sauerland, Taunus) and revealed single-specimens records for Luxembourg and Austria. Our integrative dataset thus cross-validated the presence of *N. puteanus* in five geographic regions, with the easternmost and westernmost findings (respectively in Austria and in Luxembourg) separated by a Euclidean distance of 756 km. Yet, we must again highlight that the Luxembourgish as well as the Austrian record are based on a single specimen each and that no further localities or specimens are known from both regions. We almost certainly can exclude i) laboratory mistakes (as morphological identifications and genetic results from multiple DNA extractions, PCRs and sequencing runs always produced consistent results and a unique Austrian haplotype), ii) mislabelling of samples / specimens (as potentially transposed digits in sampling dates and specimen labelling would not lead to localities with *N. puteanus* occurrences), and iii) field trip artefacts from passive transportation (as equipment was always inspected and cleaned, and the chronologically closest field trips did not yield specimens of *N. puteanus*). A more conservative geographical estimate excluding these two single-specimen reports would lead to a maximal Euclidean distance of approximately 430 km (between the Sauerland populations and localities near Regensburg). Consequently, the distribution range of *N. puteanus* seems to be restricted to the north of the Alps (see also the discussion in the previous paragraph).

When compared to the distribution ranges of other macro-stygobionts, the observed range size for *N. puteanus* is relatively large. In their meta-analysis including two European *Niphargus* morphospecies

complexes, Trontelj et al. (2009) only very rarely detected geographical ranges spanning more than 200 km. Based on the integration of molecular data, they revealed that traditionally recognised macrostygobionts with large ranges are often formed by cryptic lineages with much more narrow distribution ranges. Such pattern is also common for other taxonomic groups of terrestrial subsurface habitats (Weigand et al., 2013; Zhang and Li, 2014). However, one cryptic lineage of the *Niphargus virei* complex in the study of Trontelj et al. (2009) had populations separated by 700 km. In another geographical setting, Esmaeili-Rineh et al. (2019) revealed almost similar ranges for groundwater and surface amphipods in the mountain ranges of Iran, again averaging less than 200 km Euclidean distances but with maximal linear distance of 644 km for one *Niphargus* MOTU. Our study suggests that *N. puteanus* is another such exceptional case. The most obvious factor responsible for the large geographic distribution of max. 756 km, or 430 km when excluding single records from Luxembourg and Austria, are its isolated satellite populations. Still, when one considers its core range in the SGS only, *N. puteanus* occupies a continuous distribution area of 300 x 340 km. One explanation for this rather large continuous range size may be that local aquifers span a wide geographical area (e.g. the Swabian Jura) and/or that the dynamic (paleo-)hydrogeology of the SGS has remodelled frequently the regional underground discharge system during the Pleistocene (Hötzl, 1996; Baumann and Romanov, 2008). A more detailed discussion follows below.

Demographic history

In total, the COI of 51 specimens from populations within all five pre-defined geographic regions was sequenced. Those sequenced individuals formed seven haplotype groups, which were often confined to distinct geographic regions. Since most of the molecular records originated from the South German Scarplands (SGS), a closer look into this region seems reasonable. In the SGS, haplotype groups demonstrate distinct spatio-genetic patterns, with particularly the upper catchments of the Neckar and Danube acting as small-scale hotspots harbouring six haplotype groups – only HG3-7 was found in the nearby Salzach (Rhine catchment). Following the assumption that older genetic variants are more centrally located (Excoffier and Langaney, 1989; Golding, 1987; Pfenninger and Posada, 2002), the oldest (or most central) haplotype group seems to be HG3-4, which also spans the largest geographical area within the SGS. From this more ancient group, other haplotype groups might have been formed during the colonisation of adjacent aquifers and catchments (e.g. HG3-3, 5 and 7). Finally, repeated regional bottleneck events could have led to the genetic diversification of those groups. The observation of a more or less conserved spatio-genetic pattern of haplotype groups also points to a rather infrequent genetic intermixture of populations situated in different aquifers and catchments. This is further corroborated by the unusually high haplotype diversity observed ($H_d = 0.961$), meaning that a random sample of two sequences from our dataset will yield in 96% of the cases two distinct haplotypes. Since observed nucleotide and haplotype diversities are particularly high in the SGS, it seems likely that this region acted as a refugium for *N. puteanus* during Pleistocene glaciations. Tests for the neutrality of molecular evolution as well as the mismatch distribution are in agreement with a long-term favourable environment in the SGS, as they favour a scenario of historically stable populations. The deviating result of Fu's FS test can be attributed to the high degree of low-frequency haplotypes observed in our dataset, which the test relies on for detecting past population expansion. However, the absence of more central, high-frequency and assumedly older haplotypes (the “star-like pattern”) further supports a long-term stable but geographically scattered population. On the contrary, haplotypes revealed for the satellite populations in Western Germany (Taunus, Sauerland) as well as for Luxembourg and Austria do not form distinct haplotype groups but cluster among specimens and haplotype groups found in the SGS.

One particularly striking feature of the SGS is its high (paleo-)hydrogeological dynamic, which must be set into context with the observation of spatially confined haplotype groups in this region. During the orogenesis of this karstic region, stream capture and the reshaping of unequally permeable strata frequently occurred, leading to a spatial reorientation of catchment and aquifer boundaries. Adding to this, the SGS is one of Central Europe's seismic hotspots (Giardini et al., 2013). Today, the incipient Danube Sinkhole hydrogeological anomaly might best illustrate this geomorphological dynamic: water from the Upper Danube suddenly disappears near Immendingen and Möhringen as well as next to

Fridingen, reaching the surface in the Aachtopf spring located 11.8 km to the south and 180 metres lower. As such, part of the water from the Upper Danube drains into the Rhine catchment (Hötzl, 1996; Baumann and Romanov, 2008). This phenomenon shows a strong variation between years, with the disappearance of the Upper Danube taking place during 10 - 300 days per year. The Danube-Aach underground drainage system started to develop in the mid-Pleistocene and the repeated formation and retraction of ice sheets temporarily blocked or formed subsurface flow paths and groundwater outlets. The Wutach River provides another example of a river capture, as it directly drained into the Danube until the last glacial maximum, but is nowadays a tributary of the Rhine River (Hebestreit, 1995). It seems highly plausible that this general (paleo-)hydrogeological dynamic allowed survival of distinct *N. puteanus* populations and fostered the diversification of haplotype groups in the western SGS during the Pleistocene. The absence of *N. puteanus* populations in the adjacent 'Alpine foreland' geomorphological natural region (to the south) might be attributable to the effect of the Alpine glaciers, which extended far north during the last glacial period (Gaar et al., 2019). However, the question remains of how individuals formed satellite populations despite sometimes large geographical distances and potential environmental barriers.

Potential origins of satellite populations

For inferring the origins of *N. puteanus* satellite populations (Sauerland, Taunus) and single-specimen records (Luxembourg, Austria) we can consider three non-exclusive scenarios. The first hypothesises a "riverine trajectory" scenario. Occasional flooding events generally release large quantities of groundwater and thereby can wash out stygobionts that drift along river channels (here primarily affecting the rivers Danube, Neckar and Rhine). Indeed, specimens of the cave salamander *Proteus* are commonly found in remote epigean environments after events of seasonal flooding (Aljančič et al., 2016). These flooding events are interpreted as rare opportunities for downstream river migration, allowing the expansion of distribution areas. A similar hypothesis with the Rhine River acting as an evolutionary long-term trajectory was proposed for an amphibic microgastropod (Weigand et al., 2012) and seems likely for *Bythiospeum* groundwater gastropods, in particular for explaining the differentiation between clades 1 and 2 (Richling et al., 2016).

Second, we can consider an "anthropogenic transportation" scenario, in which human-mediated passive transportation via mining, caving or cave diving equipment allowed *N. puteanus* to colonise new regions. Traditional mining sites are located in three of the four areas outside the SGS (in the Sauerland, Taunus and Luxembourg) and mining in the Swabian Jura dates back to medieval times. Today, the Swabian Jura is likewise a prominent European caving and cave diving hotspot.

As a third alternative, an "aquifer hopping" scenario seems plausible, which conceptually links to the "interstitial highway" hypothesis of Ward and Palmer (1994). Adjacent aquifers with suitable environmental conditions could have been colonised in a stepwise process, as seems likely for the diversification of the haplotype groups within the SGS. This repeated colonisation of aquifers overcoming environmental barriers might be particularly likely at times of seasonal flooding, and in areas close to aquifer / catchment boundaries. If both circumstances are combined, distances between aquifers / catchments might be low and the number of migrating individuals periodically increased. In addition, it has to be noted that favourable habitat patches might be quite ephemeral and that the network of interconnected habitat patches can frequently change through time.

The likeliness of the aforementioned scenarios has to be set into individual geographic context. Indeed, both *N. puteanus* satellite populations and the Austrian record are located in proximity to the rivers Danube and Rhine, which directly originate in or have confluences (i.e. the Neckar River) in the SGS. Furthermore, they do not harbour distinct haplotype groups, but all genetically analysed specimens cluster within haplotype groups also revealed for the SGS. As such, a riverine trajectory seems generally plausible for the foundation of the Taunus and Sauerland populations (with the Rhine River as vector), as well as for the Regensburg populations within the SGS and potentially the Austrian record (with the Danube as vector). The "interstitial highway" scenario seems less likely for long-distance colonisations, as the geology and porosity of aquifers in-between the satellite populations and the SGS do not particularly favour a stepwise colonisation process of this rather large amphipod species (see also

Castellarini et al., 2007). However, this colonisation process might play a more crucial role for the spread of a local population upon its arrival.

One exceptional case refers to the geographically rather isolated Luxembourgish single-specimen record collected from a limnocene spring near the old iron ore mine 'Laangebierg'. The specimen bears haplotype H1, which is shared with the SGS (e.g. at the Lower Neckar) and the Taunus population. A stepwise colonisation is less plausible, as aquifers in-between this locality and its nearest population in the Taunus were well sampled but did not yield a single additional record for *N. puteanus*. Furthermore, the south-west to north-east flow direction of the Moselle would hinder a passive drifting of specimens along riverine surface trajectories. When excluding labelling and sampling mistakes (as discussed in the previous section), the "anthropogenic transportation" scenario might be the best explanation. Cave divers frequently visit both, the Swabian Jura as well as the nowadays-flooded mining galleries in Southern Luxembourg, so that transport of specimens by wet diving material appears possible. Furthermore, a large migration wave of German mining workers arrived in Southern Luxembourg at the end of the 19th and in the early 20th century (Musée National des Mines de Fer Luxembourgeoises, pers. comm.). Thus, another possibility would be that mining workers were responsible for this potential translocation.

Ecological requirements and classification

Our final objective was to infer the ecological requirements of *N. puteanus*, such as habitat preferences and potential environmental barriers. The first observation we can draw from the species' presence records is the accumulation of findings in spring habitats (77%), most of them rheocrenes (Bornhauser, 1913). Rheocrenes, however, were also the most common habitat type sampled. The majority of springs for which precise ecological data were available showed medium to high flow regimes (73%). Since the formation of springs is more likely in regions where a permeable stratum encounters a less permeable layer, it is not surprising that almost all presence records originate from regions close to aquifer boundaries. Likewise, an increased water flow velocity is best maintained if the underlying aquifer allows a fast transportation of larger water quantities. This is documented by our finding of a statistically more likely occurrence of *N. puteanus* populations in springs situated in fissured aquifers, further distinguishable by high, medium to low productivity. The few presence records from caves and mining galleries can be attributed to findings near the entrance zone (e.g. Mordloch cave near Eybach) – as such mimicking fast-flowing spring habitats – or simply might be the result of washed-in specimens. The latter is particularly likely for findings in the Donauhöhle (the cave of the Aachtopf spring) and the Blautopfhöhle (cave of the Blautopf spring), which are passed by enormous quantities of water each day. Discharge conditions strongly vary during the year but are in the range of 1.300 – 24,000 L/s for the Aachtopf, and 300 – 30,000 L/s for the Blautopf (Kaufmann and Romanov, 2008). As such, *N. puteanus* still seems to be able to establish permanent subterranean populations but its classification as a stygobiont species must be questioned. Available faunistic data from borehole samplings could help address this topic. A study by Hahn & Fuchs (2009) investigated 304 boreholes distributed across the entire area of Baden-Württemberg (including the Swabian Jura), sampled twice during the years 2001 and 2002. Although most of the boreholes were located in porous aquifers (67%), a considerable number also comprised karstic (17%) and fractured aquifers (11%). In total, twelve nipharigid species were sampled but not a single *N. puteanus* specimen was detected. A bias introduced by screen widths (0.5 mm in sandstone, 1.5 for the rest) negatively affecting in particular the immigration of adult *N. puteanus* specimens (which can be up to 2.2 cm long) into the boreholes cannot be excluded. However, the more stagnant flow conditions in boreholes are in sharp contrast with the aforementioned fast-flow conditions preferred by *N. puteanus* and could explain its absence. Accordingly, *N. puteanus* was not found in slow flowing subterranean waters in the Swabian Jura (pers. obs.). Given our results and data from the literature, we propose to consider *N. puteanus* as a crenophile and stygophile species, particularly inhabiting surface and subsurface waters of fast-flowing springs.

Conclusions and outlook

The incorporation of genetic data allowed us to draw a precise picture of the actual distribution range of the groundwater amphipod *Niphargus puteanus*. After omitting questionable presence records of the species southwards the Alps, the distribution range of *N. puteanus* remains rather large for a *Niphargus* species. Even if our dataset was limited and biased towards few specimens from multiple locations, the spatio-genetic data further enabled us to infer i) a generally high level of population isolation (indicated by a high haplotype diversity and distinct spatio-genetic patterns), ii) a long-term stable metapopulation in the South German Scarplands, and iii) possible scenarios for origins of the geographically more separated satellite populations. Historical drift of specimens along the Danube and Rhine is discussed as a likely long-term dispersal mechanism. Analysis of the geological setting of presence records allowed to describe *N. puteanus* as a crenophile and stygophile species instead of being strictly stygobiont. The species seems to inhabit preferably the interconnected surface and subsurface microhabitats of spring environments with high discharge rates, which are mainly found in fissured aquifers. The large body size reached by this species might be in fact attributable to its particular ecology in actively exploring epigean spring environments and exploiting their richer resources. In the future, the connectivity of populations in the Swabian Jura should be accessed using genomic approaches such as ddRAD sequencing (Peterson et al., 2012) to investigate potential environmental barriers and drivers of population diversification in this hydrologically highly dynamic region in closer detail.

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Appendices

Supplementary Figures

Figure S1. Map indicating sampling effort and presence records in aquifer type categories

Figure S2. Comparison of 28S chromatograms of individuals SQ1 (top), BL2 (middle) and SN1 (bottom)

Supplementary Tables

Table S1. Presence records of *N. puteanus* used for the inference of the distribution in different aquifer type categories

Table S2. Primer sequences for PCR and sequencing

Table S3. Results of the Automatic Barcoding Gap Discovery approach

Table S4. Metadata of the genetically analysed specimens. Due to insufficient length, GenBank accession numbers provided in brackets were used for presence records only

Supplementary Data

Data S1. FASTA alignment of the COI sequences used in the ABGD analysis

Data S2. FASTA alignment of the COI sequences used for all other analyses

Data S3. FASTA alignment of the 28S sequences used in the haploweb analysis

Data S4. COI chromatograms generated in the present study

Data S5. 28S chromatograms generated in the present study

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A

B

SQ1_DW160731-03_Niph21 Fragment base #204. Base 204 of 573

BL2_NC142_28Srtest2 Fragment base #204. Base 204 of 930

SN1_DW161219-69_Niph21 Fragment base #204. Base 204 of 559

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Dieter Weber: Conceptualization, Validation, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing. **Jean-François Flot:** Conceptualization, Methodology, Validation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition. **Hannah Weigand:** Conceptualization, Methodology, Software, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing. **Alexander Weigand:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.