Zootaxa 5222 (6): 501–533
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5222.6.1
http://zoobank.org/urn:lsid:zoobank.org:pub:C9D6A84D-9961-4062-ACF0-CAF3CDE2AA7F

Molecular analyses of groundwater amphipods (Crustacea: Niphargidae) from Luxembourg: new species reveal limitations of morphology-based checklists

DIETER WEBER[1,2,5], JEAN-FRANÇOIS FLOT[1,3], ALAIN C. FRANTZ[2,6] & ALEXANDER M. WEIGAND[2,4]
[1]*Evolutionary Biology & Ecology, C.P. 160/12, Université libre de Bruxelles (ULB), Avenue F.D. Roosevelt 50, B-1050 Brussels, Belgium*
[2]*Musée National d'Histoire Naturelle de Luxembourg, 25 rue Munster, L-2160 Luxembourg, Luxembourg*
[3]*Interuniversity Institute of Bioinformatics in Brussels—(IB)², Brussels, Belgium*
jean-francois.flot@ulb.be; https://orcid.org/0000-0003-4091-7916
[4]*University of Duisburg-Essen, Universitätsstr. 5, D-45141 Essen, Germany*
alexander.weigand@mnhn.lu; https://orcid.org/0000-0001-7587-6531
[5] *dieter.weber124@gmx.de; https://orcid.org/0000-0001-7813-842X*
[6] *alain.frantz@mnhn.lu; https://orcid.org/0000-0002-5481-7142*

Abstract

Niphargus amphipods were collected from 2007 to 2018 at 98 sites comprising artificial caverns, springs and interstitial waters in the Grand Duchy of Luxembourg. Opportunistic sampling was combined with passive trapping. Specimen identification was achieved using morphological keys and molecular data. Initial morphological determination and literature data suggested five species, whereas sequencing of fragments of the mitochondrial cytochrome *c* oxidase subunit 1 gene and nuclear 28S rDNA marker supported the presence of seven species: *Niphargus schellenbergi, Niphargus puteanus, Niphargus fontanus,* one species of the *Niphargus kochianus* complex, and three species of the *Niphargus aquilex* complex. *Niphargus schellenbergi* was by far the most abundant and widespread species. Limited overlap was observed between literature-based records, our initial morphological determinations based on classical taxonomic characters, and genetic sequence data. In general, the combination of phenotypically variable taxa, such as *N. schellenbergi*, and cryptic or near-cryptic species, as in the *N. aquilex* complex, renders morphological identification of niphargids from Luxembourg a challenging or even impossible task. DNA taxonomy will therefore have to be used in future studies of the fauna of this region.

résumé

Plusieurs amphipodes du genre *Niphargus* ont été prélevés de 2007 à 2018 sur 98 sites différents (cavernes artificielles, sources et eaux interstitielles) au sein du Grand-Duché de Luxembourg. Cet échantillonnage a été effectué de manière opportuniste et par piégeage passif. L'identification des échantillons a été réalisée à la fois à l'aide de clés morphologiques et aussi en utilisant des données moléculaires. La détermination morphologique initiale suggérait la présence de cinq espèces, tandis que le séquençage de fragments de la sous-unité 1 de la cytochrome *c* oxidase mitochondriale et du marqueur ADNr nucléaire 28S indique la présence de sept espèces : *Niphargus schellenbergi*, *Niphargus puteanus*, *Niphargus fontanus*, une espèce du complexe *Niphargus kochianus* et trois espèces du complexe *Niphargus aquilex*. *Niphargus schellenbergi* semble de loin l'espèce la plus abondante et la plus répandue. Le recouvrement entre les données basées sur la littérature existante et nos déterminations morphologiques initiales fondées sur les caractères taxonomiques classiques d'un part et les groupements opérés sur la base de séquences génétiques de l'autre apparaît relativement limité. De manière générale, la combinaison de taxons phénotypiquement variables, tels que *N. schellenbergi*, et d'espèces cryptiques ou quasi-cryptiques, comme dans le complexe de *N. aquilex* rend l'identification morphologique des niphargidés du Luxembourg une tâche difficile voire impossible. La taxonomie ADN devra donc être utilisée dans les études futures de la faune de cette region.

Zusammenfassung

Von 2007 bis 2018 wurden im Großherzogtum Luxemburg an 98 Standorten (künstliche Hohlräume, Quellen, Interstitial)

Accepted by K. Tomikawa: 7 Sept. 2022; published: 23 Dec. 2022

Amphipoden der Gattung *Niphargus* gesammelt. Gezieltes Sammeln wurde mit dem Aufstellen von Fallen kombiniert. Die Bestimmung der Tiere erfolgte anhand morphologischer Bestimmungsschlüssel und molekularer Daten. Anfängliche auf Morphologie basierende Bestimmungen in Kombination mit Daten aus der Literatur legten die Existenz von fünf Arten nahe, während die Analyse von Fragmenten des mitochondrialen Cytochrom-*c*-Oxidase-Untereinheit-1-Gens und des nuklearen 28S rDNA Markers sieben Arten belegen konnte: *Niphargus schellenbergi, Niphargus puteanus, Niphargus fontanus*, eine Art des *Niphargus kochianus* Komplexes und drei Arten des *Niphargus aquilex* Komplexes. *Niphargus schellenbergi* war bei weitem die am häufigsten vorkommende und am weitesten verbreitete Art. Die Überlappung zwischen älteren Literaturangaben und unseren anfänglichen morphologischen Bestimmungen mit den genetischen Daten war gering. Die morphologische Identifizierung von Niphargen aus Luxemburg ist bei phänotypisch variablen Taxa wie *N. schellenbergi* und kryptischen oder fast kryptischen Arten wie im *N. aquilex*-Komplex eine schwierige oder sogar unmögliche Aufgabe. Die DNA-Taxonomie wird daher in zukünftigen Studien der Fauna dieser Region verwendet werden müssen.

Key words: Niphargids, alpha diversity, species richness, COI, 28S

Introduction

Niphargidae is a family of amphipod crustaceans that comprises more than 400 formally described species (Horton *et al.* 2021). The family occupies a wide geographical area ranging from Spain (Karaman 2017) to Iran (Esmaeili-Rineh *et al.* 2017) and harbours numerous cryptic species complexes (Fišer & Zagmajster 2009, Meleg *et al.* 2013, Esmaeili-Rineh *et al.* 2019). Mediterranean Europe is the region with the highest known species richness. Almost all *Niphargus* species are obligate groundwater taxa (also called stygobionts) living in groundwater aquifers and groundwater-dependent ecosystems such as springs and riverine interstitial sediments (Fišer 2012). They also demonstrate typical ecophysiological adaptations to a subterranean lifestyle, such as lack of eyes (Schellenberg 1932a, Kureck 1964, Borowsky 2011), depigmentation and increased longevity (Bellan-Santini 2015).

Until the end of the last century, species delimitation and specimen identification of niphargids relied on morphotaxonomy. Yet, already during these times, taxonomic decisions were driven by permanent disputes and frequent revisions. Species were often downgraded to subspecies rank and vice versa: for instance, *Niphargus schellenbergi* became *Niphargus aquilex schellenbergi* (Schellenberg 1932b) then reverted to *N. schellenbergi* (Straškraba 1972). Similarly, new genera such as *Niphargellus* were erected (Schellenberg 1934). The taxonomy and systematics of niphargids are particularly challenging because of the difficulty of disentangling intraspecific and interspecific variation, and thus of defining reliable diagnostic morphological features (Fišer 2012). Although this is a common taxonomic problem, the situation within *Niphargus* is complicated by the fact that some species demonstrate quite distinct phenotypes, whereas others are part of so-called cryptic species complexes displaying nearly conserved morphologies. In such situations, DNA barcoding and molecular taxonomy can often be used to adequately disentangle species identities (Fontaneto *et al.* 2015). For instance, McInerney *et al.* (2014) integrated molecular data to investigate the niphargid fauna from Northern, Western and Central Europe. The authors revealed that several of the known morphospecies (*Niphargus aquilex*, *N. fontanus, N. kochianus, N. schellenbergi*) actually comprise complexes of genetically divergent lineages, which they called Operational Taxonomic Units (OTUs): six for *N. aquilex*, three for *N. fontanus*, four for *N. kochianus* and two for *N. schellenbergi*. As these OTUs were inferred from molecular data, we will refer to them as MOTUs (short for "Molecular Operational Taxonomic Units").

In the Grand Duchy of Luxembourg, the first inventory of niphargids dates back to more than 50 years ago when Hoffmann (1963) reported *Microniphargus leruthi* and four *Niphargus* taxa (*N. aquilex aquilex, N. aquilex schellenbergi, N. virei, N. fontanus*) for the country. Later, Gerecke *et al.* (2005) performed a national survey spanning 41 springs and 30 hyporheic interstitial sites, detecting two *Niphargus* species (*N. aquilex* and *N. schellenbergi*). More recently, Weber (2011), Flot & Weber (2013) and Weigand *et al.* (2016) collected *N. schellenbergi* from several mines and abundant railway tunnels. In this study, we apply for the first time a molecular taxonomic approach to provide a comprehensive inventory of the diversity and distribution of groundwater amphipods in Luxembourg. Our results are discussed in light of previous findings in the country as well as in adjacent regions.

Material and methods

Study region and sampling

Luxembourg is characterised by a rather complex geology, with schist in the North, sandstone and sandy limestone in the centre, limestone or dolomite in the East and Minette iron ore in the very South (Weber 2013). Between 2007–2018, niphargids were collected during various nationwide field campaigns (**Fig. 1A**). Specimens were collected mainly by hand and in an opportunistic way. Sieving was conducted in four mines (Schiefergrouf vu Schläif, Kofferminn Stolzebuerg Hauptsystem, Galerie Merkholtz, Antimonminn Goesdorf) and in one railway tunnel (Tunnel Huldange) with mesh sizes of 5000 µm, 1000 µm, 600 µm and 200 µm. Meat-baited tin cans were placed in five mines (Antimonminn Goesdorf, and the iron ore mines Minière Doihl, Minière Weltschegrond II, Minière Langegronn, Minière Prince Henry) and emptied after 2–3 days. In a parallel study, hundreds of Barber traps (with ethane-1,2-diol, without bait; Barber 1931) were installed to collect soil fauna. Springs were sampled by collecting and sieving mud, foliage or moss (same sieve mesh sizes as above). Interstitial sites were sampled using the Karaman-Chappuis-method (Chappuis 1942; Malard *et al.* 2002).

Captured specimens were immediately preserved in 96% ethanol and kept at -20°C. Whenever possible, at least one male and one female were preserved in 70% ethanol at room temperature for morphological investigation. Specimens collected before 2001 were first preserved in 70% isopropanol, then transferred to 70% ethanol and stored at -20°C. Specimens are stored in the collection of the National Museum of Natural History Luxembourg (MNHNL) **(Supplementary Table 1**).

Morphological identification and literature consulted for past records

All specimens were morphologically identified using available regionally relevant taxonomic keys and primary literature (Ginet 1991-1995; Schellenberg 1932b, 1933, 1935) using a Wang Biomedical stereo microscope with magnification of 7 to 40 times. Further, the following literature sources were consulted for data or status reports of *Niphargus* amphipods in Luxembourg: Sunnen (1957), Hoffmann (1963), Gerecke *et al.* (2005), Weber (2011), Flot & Weber (2013), Meisch & Massard (2015), Weigand *et al.* (2016).

DNA extraction

Since morphological determination of specimens was not always possible (e.g. for juveniles or intermediate morphs) and the aim was to integrate molecular data, a subset of specimens was analysed with two genetic markers. The subset included one specimen of each morphospecies from each site. In case of doubtful morphological identification, two or more specimens were sequenced. Additional specimens of *N. schellenbergi* from Luxembourg were integrated as part of an ongoing phylogeographic study (**Supplementary Table 1**). A single leg of specimens larger than 4 mm, two legs of specimens between 3–4 mm, and an entire specimen for smaller individuals were used for DNA isolation. DNA was extracted using either the DNeasy Blood & Tissue Kit (Qiagen) or the NucleoSpin Tissue Kit (Macherey-Nagel) following the manufacturers' protocols. 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), and in the collection of the National Museum of Natural History Luxembourg (MNHNL). In order to assess whether *N. aquilex sensu stricto* is present in Luxembourg and given that the neotype of *N. aquilex sensu stricto* was not suitable for molecular analyses, we analysed one newly collected *N. aquilex* specimens from a spring in Crowborough (sample UK1 in **Supplementary Table 1**), the neotype locality (Karaman 1980).

PCR and sequencing

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 degenerate primer pair HCO2198-JJ and LCO1490-JJ (Astrin & Stüben 2008; Tab. 2). The PCR mix contained 1 µl DNA extract (of variable concentration), 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 3 minutes denaturation step at 94°C followed by 36 cycles of 20 s denaturation at 94°C, 45 s annealing at 50°C, and 60 s extension at 65°C; then a final elongation step of 2 minutes at 65°C.

Furthermore, a fragment of the nuclear 28S ribosomal RNA gene was investigated using primers Niph15 and Niph16 (Tab. 2 in Verovnik *et al*. 2005). The PCR mix for the 28S marker contained 2 µl of DNA extract (of vari-

able concentration), 1 µl of each primer (10 pmol/µl), 0.2 µl of REDTaq Polymerase (1 unit /ul in 20 mM Tris-HCl, Sigma-Aldrich), 5 µl REDTaq reaction buffer and 15.8 µl ultrapure water. PCR cycling conditions for 28S were an initial 3 minutes denaturation step at 95°C followed by 56 cycles of 30s denaturation at 94°C, 60 s annealing at 45°C, and 90 s extension at 72°C. PCR amplification results were visualised on a 1.2% agarose gel. PCR products were bi-directionally Sanger-sequenced at Genoscreen (Lille, France). The COI marker was sequenced using the same primer pair as during PCR amplification, whereas the 28S marker was sequenced using three primers (**Table 1**).

TABLE 1. PCR and sequencing primers. X letters indicate for which steps a given primer was used.

Primer	Nucleotide sequence	PCR	Sequencing	Reference
LCO1490-JJ	5'-CHA CWA AYC ATA AAG ATA TYG G-3'	X	X	Astrin & Stüben (2008)
HCO2198-JJ	5'-AWA CTT CVG GRT GVC CAA ARA ATC A-3'	X	X	Astrin & Stüben (2008)
Niph15	5'-CAA GTA CCG TGA GGG AAA GTT-3'	X	X	Verovnik *et al.* (2005)
Niph16	5'-AGG GAA ACT TCG GAG GGA ACC-3'	X		Verovnik *et al.* (2005)
Niph20	5'-AAA CAC GGG CCA AGG AGT AT-3'		X	Flot *et al.* (2010a)
Niph21	5'-TAT ACT CCT TGG CCC GTG TT-3'		X	Flot *et al.* (2010a)

Analysis of molecular data

Chromatograms were edited and assembled into contigs using Sequencher version 4.1.4 (Gene Codes Corporation, USA); the resulting consensus sequences were deposited in GenBank (accessions OK378185–OK378243 for 28S and OK380764-OK380901 for COI; Supplementary Table 1). For connecting our specimens to previously delimited species and MOTUs, the COI and 28S sequences from three *N. schellenbergi* individuals from Germany (Flot 2010a) as well as 16 COI sequences and 16 28S sequences used to delimit MOTUs in McInerney *et al*. (2014) were added to the datasets. In the case of COI, a *Crangonyx subterraneus* sequence (accession number MT993546.1) from Weber *et al.* (2020b) was also included as an outgroup for phylogenetic analyses. The resulting datasets were aligned by hands in the case of COI and using MAFFT webserver's E-INS-i algorithm (Katoh *et al*. 2019) in the case of 28S. For the COI marker, MEGAX (Kumar *et al.* 2018) was used to compute a neighbour-joining (NJ) tree of the 139 sequences (the *Crangonyx* outgroup + 136 *Niphargus* specimens, two of which represented by two COI sequences each) using p-distances with 1,000 bootstrap replicates (Felsenstein 1985). In the case of 28S, a haploweb (Flot *et al.* 2010b) based on a minimum spanning network (with additional curves connecting sequences found co-occurring in heterozygous individuals) was produced using HaplowebMaker (Spöri & Flot 2021) by masking all positions with indels in the alignment, then redrawn using Inskscape (Bah 2011). Molecular delimitation of species was also performed using Assemble Species by Automatic Partitioning (ASAP; Puillandre *et al.* 2021) with default parameters and using K over Theta (KoT; Spöri *et al*. 2021) with pairwise deletion, transitivity and a threshold K/theta ration of 6 (corresponding to a 0.99 minimal probability of monophyly).

Results

Taxonomic records from literature

The literature sources named five niphargid species for Luxembourg: *N. schellenbergi*, *N. fontanus*, *N. aquilex*, *N. virei* and *Microniphargus leruthi* (**Table 2**). Additional literature sources were consulted for niphargid records in adjacent regions, which we will refer to in the species-specific discussions.

Taxonomic records from field survey and morphology

A total of 241 sites were visited between 2007 and 2018 (**Fig. 1**). Most of these sites were springs (119; 43%) followed by mines and tunnels (58; 24%), natural caves (34; 14%) and interstitial waters (30; 12%) (**Table 2**). Among all sites, 175 (73%) contained water and from a subset of 98 sites (41% of the total, 56% of water-containing sites) *Niphargus* specimens were successfully sampled—primarily from springs (70% of all sites with water). In total, 1,763 specimens were collected and investigated morphologically. Out of this material, we recognized six *Niphargus* morphotypes: *N. schellenbergi*, *N. fontanus* (sometimes difficult to distinguish clearly from *N. schellenbergi*), *N. puteanus*, *N. kochianus*, *N. aquilex*, and another distinct *Niphargus* morphotype that was later genetically assigned to *N. schellenbergi*.

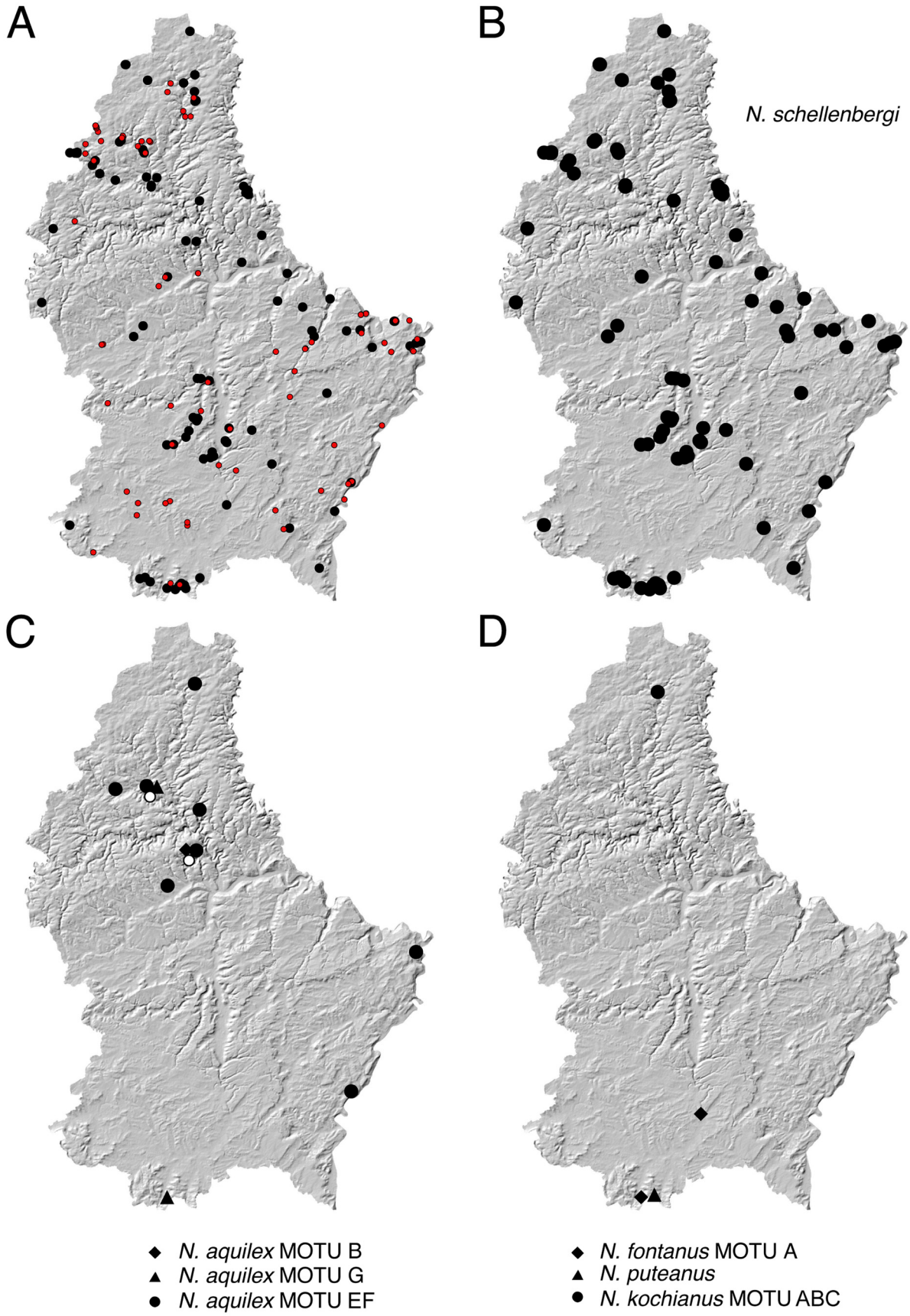

FIGURE 1. Sampling sites. A: All sampling sites with aquatic habitats. Red circles indicate locations where no *Niphargus* specimens were observed; B: Distribution of *N. schellenbergi*; C: Distribution of MOTUs within the *N. aquilex* complex. White dots mark localities where two taxa co-occur; D: Distribution of remaining *Niphargus* taxa.

TABLE 2. Collection sites in Luxembourg from 2007–2018. Barber trap collection sites are not included as they targeted soil species and niphargids in these traps were only found by chance.

	Number of sites visited	Number of sites containing water	Number of sites with *Niphargus*
Springs	119	119	69
Natural caves	34	0	0
Mines and tunnels	58	26	20
Interstitial waters	30	30	9
Total	241	175	98

Taxonomic records from molecular species delimitation

169 specimens were sequenced for the COI marker alone (99 specimens), for the 28S marker alone (16 specimens) or for both markers (54 specimens) (**Supplementary Table 1**). Among those, 135 specimens were newly sequenced for this study (**identifiers for COI sequences MNHNL006-20 to MNHNL140-20**), whereas 18 COI and 17 28S sequences were added from previous studies (Flot & Weber 2013, Weigand *et al.* 2016). COI sequences were obtained for a total of 93 sites and 28S sequences for 50 sites. We found in Luxembourg three of the seven *N. aquilex* MOTUs identified by McInerney *et al.* (2014)—namely, MOTU B, MOTU E and MOTU F—and delineated one additional *N. aquilex* MOTU that had not been previously observed, which we will refer to as MOTU G. Specimen UK1 from the neotype locality of *N. aquilex* in Crowborough (UK) turned out to belong to *N. aquilex* MOTU B. All specimens of *N. fontanus* collected in Luxembourg were assigned to *N. fontanus* (without suffix) following Trontelj *et al*. (2009), which corresponds to *N. fontanus* MOTU A following McInerney *et al*. (2014).

The COI chromatograms of two individuals comprised double peaks (three in BR1 and one in KF2), either as a result of heteroplasmy or because of the presence of numts, i.e. nuclear pseudogenes. The COI haplotypes of these specimens were phased using Clark's method (Clark, 1990) and then included in downstream analyses.

ASAP applied to the COI dataset (**Tab. 3**) detected seven *Niphargus* MOTUs among the Luxembourg individuals sequenced for this marker: *N. aquilex* MOTU E (10 specimens), *N. aquilex* MOTU F (6 specimens), *N. aquilex* MOTU G (one specimen), *N. fontanus* MOTU A (three specimens), *N. kochianus* (one specimen), *N. puteanus* (one specimen), *N. schellenbergi* (113 specimens). In contrast to the results of McInerney *et al*. (2014), this analysis lumped together the two *N. aquilex* MOTUs A1 and A2, the two *N. fontanus* MOTUs A1 and A2 as well as the three *N. kochianus* MOTUs A, B and C.

TABLE 3. Results of ASAP species delimitation based on COI sequences (individuals from Luxembourg are underlined)

Niphargus aquilex MOTU A	KC315623(A1) JF420841(A2)
Niphargus aquilex MOTU B	UK1
Niphargus aquilex MOTU E	GK2 GK3 GK9 GK13 GK14 GK15 GK16 MK2 GO2 GO3 KC315621(E)
Niphargus aquilex MOTU F	II1 IL3 IL4 IS1 WP1 MH2 KC315622(F)
Niphargus aquilex MOTU G	MK3
Niphargus fontanus MOTU A	KE1 KE2 LG2 KC315633(A1) KC315629(A2) KC315630(A2)
Niphargus fontanus MOTU C	KC315632
Niphargus kochianus MOTU ABC	KC315682(A) KC315687(A) KC315688(A) MU5 KC315661(B) KC315662(B) KC315667(B) KC315659(C) KC315660(C)
Niphargus puteanus	QL1
Niphargus schellenbergi	AK1 AS1 AS2 AW1 AW2 BA1 BA2 BA3 BB1 BC1 BR1 BR2 DF1 DI1 DK1 DK2 DL1 DL2 DL3 DL4 DL5 DL6 DL7 DU1 DU2 DU3 EC1 EH1 EH2 FN1 GD1 GD2 GK1 GK10 GK11 GK12 GK4 GK5 GK6 GK7 GK8 GO1 HN1 HR1 HS1 IA1 IL1 IL2 IW1 KC1 KF1 KF2 KF3 KK1 KO1 KR1 KS1 KT1 KZ1 LB1 LB2 LE1 LE2 LG1 LI1 MF1 MH1 MK1 ML1 MU1 MU2 MU3 MU4 NE1 NR1 PA1 QA1 QB1 QG1 QL2 QN1 QS1 QT1 RM1 RP1 SB1 SB2 SC1 SF1 SG1 SI1 SL1 SM1 SW1 SW2 SW3 TF1 TI1 TN1 TO1 TS1 UE1 UR1 VM1 WD1 WE1 WH1 WI1 WN1 WR1 WS1 WT1 WT2 WT3 WU1 WY1

KoT applied to the same COI dataset (**Tab. 4**) detected eight *Niphargus* MOTUs among the Luxembourg individuals sequenced for this marker, as the 10 specimens attributed by ASAP to *N. aquilex* MOTU E were split by KoT into two MOTUs both considered distinct from *N. aquilex* MOTU E. The result of KoT was more similar to the conclusions of McInerney *et al.* (2014) in separating the two *N. aquilex* MOTUs A1 and A2 as well as the three *N. kochianus* MOTUs A, B and C, but the two *N. fontanus* MOTUs A1 and A2 were still lumped by KoT.

TABLE 4. Results of KoT species delimitation based on COI sequences (individuals from Luxembourg are underlined)

Niphargus aquilex MOTU A1	KC315623
Niphargus aquilex MOTU A2	JF420841
Niphargus aquilex MOTU B	UK1
Niphargus aquilex MOTU E	KC315621(E)
	GK2 GK3 GK9 GK13 GK14 GK15 GK16 MK2
	GO2 GO3
Niphargus aquilex MOTU F	II1 IL3 IL4 IS1 WP1 MH2 KC315622(F)
Niphargus aquilex MOTU G	MK3
Niphargus fontanus MOTU A	KE1 KE2 LG2 KC315633(A1) KC315629(A2) KC315630(A2)
Niphargus fontanus MOTU C	KC315632
Niphargus kochianus MOTU A	KC315682 KC315687 KC315688
Niphargus kochianus MOTU B	MU5 KC315661 KC315662 KC315667
Niphargus kochianus MOTU C	KC315659 KC315660
Niphargus puteanus	QL1
Niphargus schellenbergi	AK1 AS1 AS2 AW1 AW2 BA1 BA2 BA3 BB1 BC1 BR1 BR2 DF1 DI1 DK1 DK2 DL1 DL2 DL3 DL4 DL5 DL6 DL7 DU1 DU2 DU3 EC1 EH1 EH2 FN1 GD1 GD2 GK1 GK10 GK11 GK12 GK4 GK5 GK6 GK7 GK8 GO1 HN1 HR1 HS1 IA1 IL1 IL2 IW1 KC1 KF1 KF2 KF3 KK1 KO1 KR1 KS1 KT1 KZ1 LB1 LB2 LE1 LE2 LG1 LI1 MF1 MH1 MK1 ML1 MU1 MU2 MU3 MU4 NE1 NR1 PA1 QA1 QB1 QG1 QL2 QN1 QS1 QT1 RM1 RP1 SB1 SB2 SC1 SF1 SG1 SI1 SL1 SM1 SW1 SW2 SW3 TF1 TI1 TN1 TO1 TS1 UE1 UR1 VM1 WD1 WE1 WH1 WI1 WN1 WR1 WS1 WT1 WT2 WT3 WU1 WY1

The 28S chromatograms of four individuals comprised double peaks and those specimens were treated as heterozygous in downstream analyses: *Niphargus aquilex* MOTU F (GK16 with 2 double peaks, GK3 with 2 double peaks, MK2 with 3 double peaks) and *N. aquilex* MOTU G (LG3 with 1 double peak).

The ASAP analysis for 28S (**Table 5**) detected six *Niphargus* MOTUs among the Luxembourg individuals sequenced for this marker: *N. aquilex* MOTUs A&B (one specimen), *N. aquilex* MOTUs E&F (12 specimens), *N. aquilex* MOTU G (two specimens), *N. fontanus* MOTUs A&B (two specimens), *N. puteanus* (one specimen) and *N. schellenbergi* (35 specimens). It also lumped the three *N. kochianus* MOTUs A, B and C from McInerney *et al*. (2014) into a single putative species.

The KoT analysis for 28S (**Table 6**) detected seven *Niphargus* MOTUs among the Luxembourg individuals sequenced for this marker: the difference was that *N. aquilex* MOTU E and *N. aquilex* MOTU F, which were lumped by ASAP, were separated by KoT. However, one heterozygous individual (MK2) was represented in both MOTUs, as one of his haplotypes grouped with *N. aquilex* MOTU E and the other haplotype grouped with *N. aquilex* MOTU F. This suggests that these two MOTUs are actually a single species, and the final result of KoT can therefore be considered to be six MOTUs, just like ASAP. The KoT analysis of the 28S data also lumped *N. kochianus* MOTUs A, B and C. Compared to ASAP, however, KoT separated *N. aquilex* MOTU A from *N. aquilex* MOTU B, thereby making it possible to attribute individual IS3 to *N. aquilex* MOTU B.

TABLE 5. Results of the ASAP species delimitation based on 28S sequences (individuals from Luxembourg are underlined)

Niphargus aquilex MOTU AB	IS3 UK1 KC315604(A1) JF420874(A2) KC315605(B)
Niphargus aquilex MOTU C	KC315602(C)
Niphargus aquilex MOTU D	KC315603(D)
Niphargus aquilex MOTU EF	GK2 GK3 GK16 GO2 II1 IL3 IS1 IS2 MH2 MH3 MK2 WP1 KC315606(E) KC315607(F)
Niphargus aquilex MOTU G	LG3 MK3
Niphargus fontanus MOTU AB	KE1 LG4 KC315608(A1) KC315614(A2) EF025852(B)
Niphargus fontanus MOTU C	KC315609(C)
Niphargus kochianus ABC	KC315610(A) KC315611(B) KC315612(C)
Niphargus kochianus D	KC315613(D)
Niphargus puteanus	QL1 EF617302
Niphargus schellenbergi	DF1 DL1 DL2 DL8 DU1 DU2 DU3 FN2 FO1 GF1 GK1 HN1 HS1 IL1 IL2 IL5 IW1 KO1 KS1 KT1 LB1 LG1 LI1 MH1 MU6 NE1 NR1 NW1 PA1 PH1 QH1 RP2 SC1 TH1 TI1 UE1 WI1 AS1

TABLE 6. Results of the KoT species delimitation based on 28S sequences (individuals from Luxembourg are underlined, and the asterisk indicates one heterozygous individual whose two haplotypes were in two different MOTUs)

Niphargus aquilex MOTU A	KC315604(A1) JF420874(A2)
Niphargus aquilex MOTU B	IS3 UK1 KC315605(B)
Niphargus aquilex MOTU C	KC315602(C)
Niphargus aquilex MOTU D	KC315603(D)
Niphargus aquilex MOTU E	GK2 GK3 GK16 GO2 MK2* KC315606(E)
Niphargus aquilex MOTU F	II1 IL3 IS1 IS2 MH2 MH3 MK2* WP1 KC315607(F)
Niphargus aquilex MOTU G	LG3 MK3
Niphargus fontanus MOTU AB	KE1 LG4 KC315608(A1) KC315614(A2) EF025852(B)
Niphargus fontanus MOTU C	KC315609(C)
Niphargus kochianus ABC	KC315610(A) KC315611(B) KC315612(C)
Niphargus kochianus D	KC315613(D)
Niphargus puteanus	QL1 EF617302
Niphargus schellenbergi	DF1 DL1 DL2 DL8 DU1 DU2 DU3 FN2 FO1 GF1 GK1 HN1 HS1 IL1 IL2 IL5 IW1 KO1 KS1 KT1 LB1 LG1 LI1 MH1 MU6 NE1 NR1 NW1 PA1 PH1 QH1 RP2 SC1 TH1 TI1 UE1 WI1 AS1

Finally, the haploweb analysis of 28S was congruent with both the ASAP and KoT results in delimiting six species among the Luxembourg individuals sequenced for this marker, suggesting the presence of a total of seven *Niphargus* species in Luxembourg (since the single *N. kochianus* sampled in Luxembourg had no 28S sequence available) (**Fig. 2**). Compared to COI, this consensus of the various approaches applied to 28S differs only by the number of MOTUs delimited for *N. aquilex*: whereas COI results suggest the presence of four *N. aquilex* MOTUs in Luxembourg (MOTUs B, E, F and the new MOTU G), the 28S results indicate that *N. aquilex* MOTUs E and F may be a single species. In the remaining part of our paper, we chose the most conservative approach and considered *N. aquilex* MOTUs E and F as conspecific (as well as *N. kochianus* MOTUs A, B and C), yielding the following tentative list of seven molecularly defined *Niphargus* species for Luxembourg: *N. aquilex* MOTU B, *N. aquilex* MOTU EF, *N. aquilex* MOTU G, *N. fontanus* MOTU A, *N. kochianus* MOTU ABC, *N. puteanus*, and *N. schellenbergi* (**Fig. 3** and **Table 7**).

TABLE 7. Recorded presence of niphargids in Luxembourg. Past literature records compared to the findings of the present study (morphology and DNA-based).

Species	Known from past literature findings	Initial morphological findings (this study)	Molecular findings (this study)
N. schellenbergi	Yes	Yes, two morphotypes	Yes, a single lineage
N. fontanus	Yes	(Yes)	Yes, MOTU A
N. aquilex	Yes	Yes	Yes, MOTUs B, EF and G
N. kochianus	No	Yes	Yes, MOTU ABC
N. puteanus	No	Yes	Yes
N. virei	Yes	No	No
Microniphargus leruthi	Yes	No	No

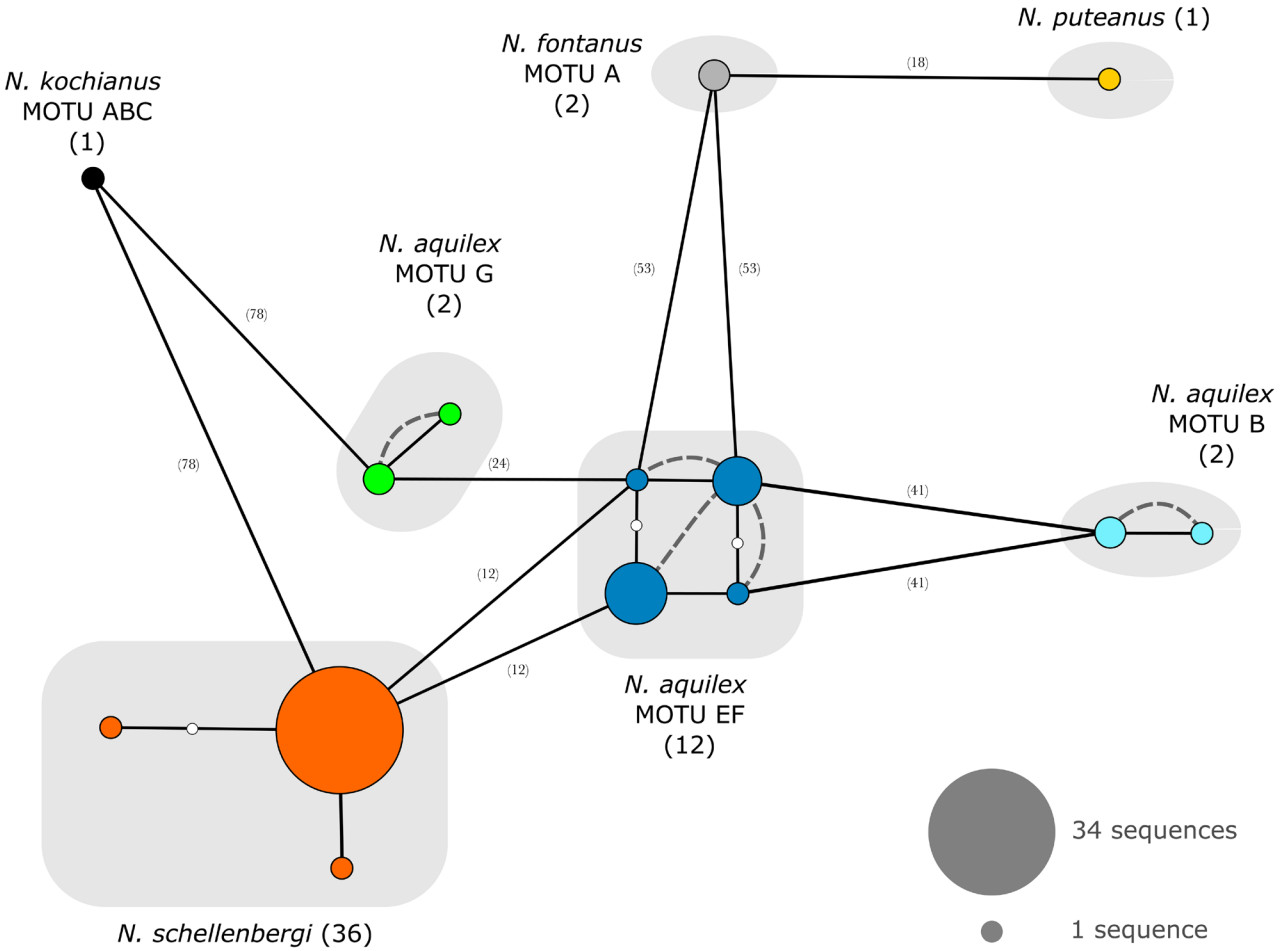

FIGURE 2. Haploweb for *Niphargus* 28S sequences obtained from Luxembourg. The size of the circles is proportional to the number of sequences bearing a specific 28S allele. The 28S alleles of heterozygous specimens are connected by dotted curves. Hypothetical, unsampled haplotypes are shown as white dots. *Niphargus kochianus* MOTU ABC is represented by a consensus of the three sequences from McInerney *et al.* (2014), and the 28S sequence of specimen UK1 from the neotype locality of *Niphargus aquilex sensu stricto* is included in the analysis (in *Niphargus aquilex* MOTU B). The species delineation shown as boxes is based on the consensus of the ASAP and KoT outcomes, stating the number of sequences for each species.

Distribution and abundance

Niphargus schellenbergi was by far the most abundant and widespread species in Luxembourg. It was collected in springs, interstitial sites, mines and abandoned railway tunnels (**Fig. 1**, **Table 8**). Records originated from all types of rocks, in a temperature regime from 5.9 to 14.9°C (n = number of measured sites = 42, x = arithmetic average = 9.8, s = standard deviation = 2.0), in German hardness ranging from 1° to 46° (n = 23, x = 14.5, s = 13.1), pH values

from 4.2 to 7.9 (n = 13, x = 6.6, s = 2.1) and in total dissolved solid values ranging from 39 to 451 ppm (n = 53, x = 246, s = 165). All the other species were much rarer (*N. fontanus* MOTU A and *N. aquilex* MOTUs EF and G), with single-specimen records for *N. puteanus*, *N. kochianus* MOTU ABC and *N. aquilex* MOTU B).

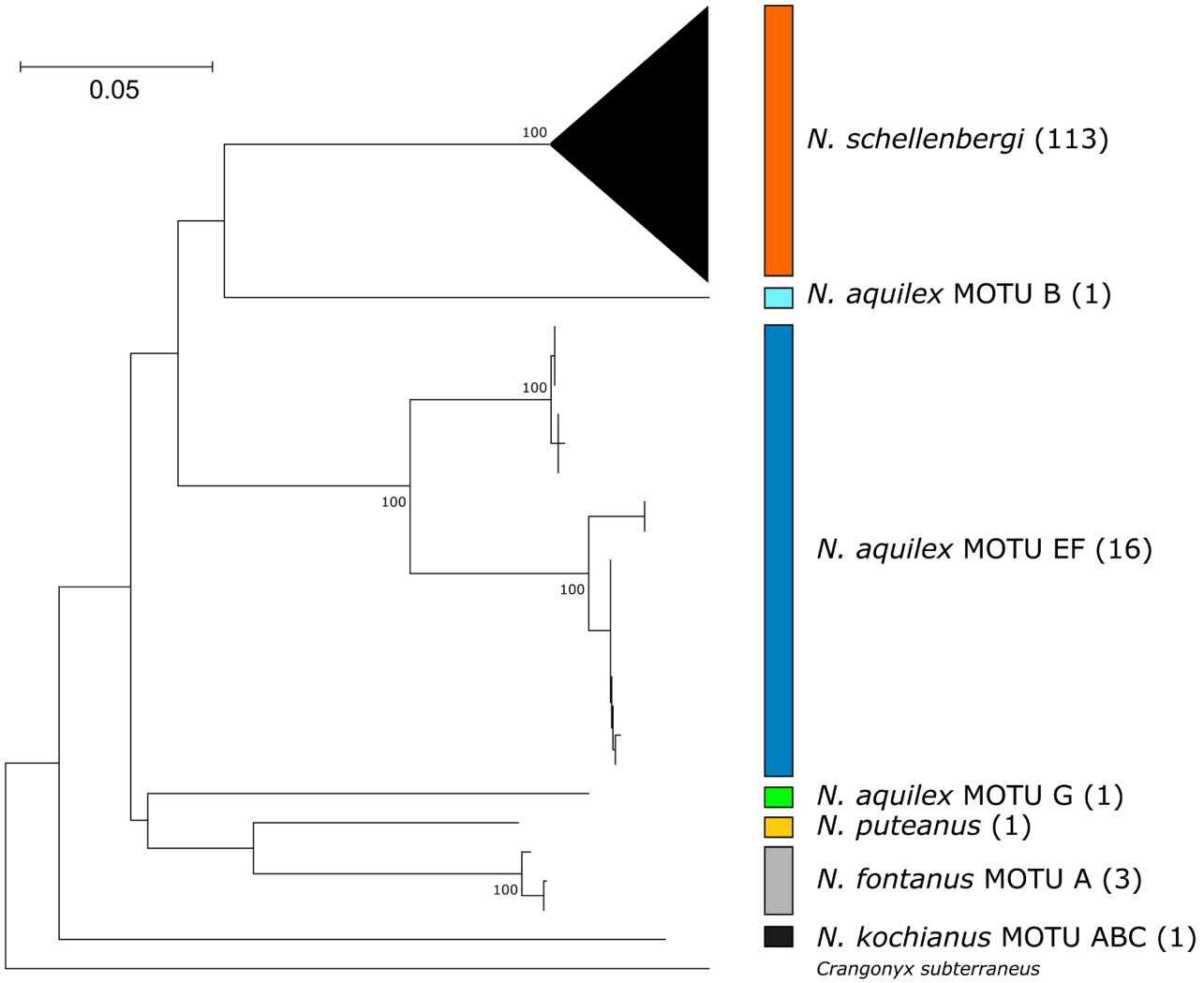

FIGURE. 3. Neighbor-joining tree of *Niphargus* COI sequences from Luxembourg. This tree was generated using p-distances in MEGA X. Coloured boxes indicate the different species delineated by ASAP and correspond with the 28S haploweb. *Niphargus aquilex* MOTU B from Luxembourg had no COI sequence data, hence the COI sequence of individual UK1 from the neotype locality of *Niphargus aquilex sensu stricto* was included.

TABLE 8. Luxembourg's *Niphargus* species and their main habitats [from Weber & Flot (2013), Weigand *et al.* (2016) and the present study] * = including artificial cavities and tunnels.

Species	springs	caves*	interstitial	main habitat
Niphargus aquilex MOTU B	–	–	**1**	interstitial
Niphargus aquilex MOTU EF	**1**	**1**	**6**	interstitial, spring, mine
Niphargus aquilex MOTU G	–	**2**	–	mine
Niphargus fontanus MOTU A	**1**	**1**	–	spring, mine
Niphargus kochianus MOTU ABC	**1**	–	–	interstitial
Niphargus puteanus	**1**	–	–	spring
Niphargus schellenbergi	**68**	**20**	**6**	spring, mine, interstitial

Discussion

Niphargid diversity in Luxembourg with notes on morphological and ecological peculiarities

Microniphargus leruthi Schellenberg, 1934 has been found in Luxembourg only once (Hoffmann 1963) in a galena mine in the North of Luxembourg. This mine is not accessible anymore. Our collections in springs around this mine only yielded *N. schellenbergi*. *Microniphargus leruthi* was also reported from adjacent areas along the Meuse River in Belgium (e.g. Fišer *et al.* 2018) and in Germany (Spangenberg 1973; Karaman & Ruffo 1986). The presence of *M. leruthi* in Luxembourg seems therefore possible, although unconfirmed by our present study.

Niphargus aquilex Schiödte, 1855 extends over an area ranging from South East England to Western Germany. MOTU B had only been reported so far in England, and our study is the first published report of this species from continental Europe. MOTU EF is frequently observed from Devon (UK) to Saxony (Germany). It comprises two genetically well-separated COI clades with no apparent morphological differences and that were lumped in our 28S molecular analyses. MOTU G is new to science, and can be also found in the Czech Republic and in several sites in Germany (unpublished data). In Luxembourg, we collected it in an ancient schist mine in the Oesling (53 m past the entrance) and in a Minette mine in the South of Luxembourg (850 m from the entrance). The description of this new species is in preparation.

Hoffmann (1963; as *N. aquilex aquilex*) stated that the *N. aquilex* morphospecies was less common in Luxembourg than *N. schellenbergi*, but could be found in all regions as well as in different habitats such as mines, water catchments, wells, and springs, although never in streamlets or rivers. Gerecke *et al.* (2005) did not report any *N. aquilex* in springs, but with 146 specimens from ten sites, the morphospecies seemed common in interstitial water. Gerecke *et al.* (2005) already assumed that *N. aquilex* comprises several species, even though specimens they examined were described as morphologically extremely close to the original English material. In our study, MOTU B and MOTU EF co-occurred in the interstitial of the Sauer River. Within MOTU EF, both clades were detected in Luxembourg, with one exclusively inhabiting interstitial sites in the Oesling region in the North of Luxembourg, which also seems to be the main habitat for this lineage. The other clade was found in springs, mines and interstitial sites. Both clades showed overlapping distributions in Luxembourg.

Niphargus fontanus Spence Bate, 1859 is regularly found in Belgium (Leruth 1939; Delhez *et al.* 1999; Stoch *et al.* 2004; Fišer *et al.* 2018) and in the western part of Germany (Weber 2001, 2012). Population densities seem always far lower than for *N. schellenbergi* (Leruth 1939; Delhez *et al.* 1999; Stoch *et al.* 2004; Fišer *et al.* 2018; Weber 2001, 2012). This morphospecies nowadays is recognised as a species complex comprising three main clades (Trontelj *et al.* 2009; as *N. fontanus*, *N.* cf. *fontanus* 1, and *N.* cf. *fontanus* 2). One clade is reported from Germany, Switzerland, and Austria (Trontelj *et al*. 2009; Fišer *et al*. 2016), one clade is known from France (Trontelj *et al.* 2009, McInerney *et al.* 2014), and the third clade is present in UK, France and Belgium (Hardtke *et al.* 2011, Lefébure *et al.* 2007, Trontelj *et al.* 2009, Altermatt *et al.* 2014, McInerney *et al.* 2014). In Luxembourg, only *N. fontanus* MOTU A (sensu McInerney *et al.* 2014) was detected, and only at two sites: in the Minière Langegronn occurring in syntopy with *N. schellenbergi* and in the spring of Klengelbuer (genetically confirmed by two specimens). The type locality for *N. fontanus* is located in Great Britain (Spence Bate 1859) with currently only one clade found in this country (Trontelj *et al.* 2009; Hartke *et al.* 2011; McInerney *et al.* 2014). Once the three clades will be formally described, specimens from Luxembourg most probably belong to *N. fontanus sensu stricto* since they cluster with the British *N. fontanus* MOTU A.

N. schellenbergi and *N. fontanus* morphologically much resemble each other. The ovoid gnathopods for *N. fontanus* versus the trapezoidal gnathopods of *N. schellenbergi* on Luxembourg's material are by far not as easy distinguishable, as shown in many drawings (e.g. Gledhill *et al.* 1976; Hartke *et al.* 2011). Because the drawings of Hoffmann (1963) do not clearly indicate the gnathopods of *N. fontanus*, it is likely that Hoffmann confused these two species.

Although literature records exist from the Minière Laangebierg for this species (being not identical to Minière Langegronn) and from springs near Echternach (Hoffmann 1963), and the species is frequently mentioned to be a typical member of the Minette mines in the South of Luxembourg, our investigations of sites where Hoffmann (1963) reported *N. fontanus* only yielded specimens of *N. schellenbergi*.

Niphargus kochianus Spence Bate, 1859 is a species complex currently under revision. McInerney *et al.* (2014) distinguished four MOTUs, but the authors did not investigate specimens from the whole distribution area. For Luxembourg, a single specimen at the Sickerquelle Maulusmühle was provisionally assigned by genetics as

N. kochianus MOTU ABC (lumping three MOTUs from McInerney *et al.* 2014, including MOTU B reported as most widespread MOTU in the Benelux area). At this spring, we found eight niphargid specimens in total, but none was an adult suitable for morphological determination. The five additional specimens that were sequenced from this site were identified as *N. schellenbergi*.

Niphargus puteanus (C.L. Koch in Panzer, 1836) is widely distributed in Baden-Württemberg (Dobat 1975; Weber *et al.* 2020a) and parts of Bavaria (Fišer *et al.* 2016), but rarely found in North Rhine-Westphalia (Weber 1991) and Switzerland (Chappuis 1924; Altermatt *et al.* 2014). Occurrences from West of the Rhine River are generally sparse (Weber *et al.* 2020a). It was previously reported from a single site in Belgium [Leloup & Jacquemart 1963; but this was not confirmed in a later study by Fišer *et al.* (2018)], as well as from four sites in Alsace (Hertzog 1930; and our own investigation), all very close to the Rhine River. For a general overview of the distribution and habitat preferences of the species see Weber *et al.* (2020a).

Only one specimen of *N. puteanus* was found in a spring in the Minette region in the South of Luxembourg, where it occurred in sympatry with *N. schellenbergi*. This single-specimen record was confirmed by conducting a second DNA isolation then by performing separate PCRs and separate sequencing reactions for both markers. *Niphargus puteanus* and *N. schellenbergi* are two spring-inhabiting species that seem to exclude each other (Weber, pers. obs.).

Niphargus schellenbergi S. Karaman, 1932 is the most abundant and widespread niphargid species in Belgium (Leruth 1939; Delhez & Houssa 1969; Notenboom 1982; Stoch *et al.* 2004; Fišer *et al.* 2018), the Netherlands (Stock 1961; Cuppem 1978; Weber 2018) and the Western part of Germany (Weber 2001, 2012). It is therefore not surprising that this is the case for Luxembourg as well. Hoffmann (1963; as *Niphargus aquilex schellenbergi*) considered this species to be common in springs in the Oesling and the Gutland. Notably, Hoffmann (1963) reported this species also from nine streamlets and rivers. Gerecke *et al.* (2005) observed *N. schellenbergi* in 26 springs and at two interstitial sites. This indicates that *N. schellenbergi* is a common species in springs of Luxembourg, but rarer in the interstitial. Our records of *N. schellenbergi* confirm the wide habitat range of this species. The majority of findings originated from springs, but we also recovered the species in subterranean and interstitial habitats. Notably, in five mines (Minn vun Asselbuer, two specimens; Ardoisière de Merkholtz, three specimens; Schiefergrouf vu Schläif II, one specimen, Schiefergrouf vu Pärel, three specimens; Minière Laange Gronn XII, 33 specimens) and in one railway tunnel (Eisebunns Tunnel Fouhren, nine specimens), *N. schellenbergi* specimens were found in Barber traps outside the water and up to 3 m from the edges of the mine lakes. Flooding of the traps before they were collected seems unlikely as the traps were still filled with ethane-1,2-diol. We therefore assume that specimens crawled on the damp ground from the subterranean river into the traps. Such a behaviour was reported by Sket (2004) for the amphipod *Typhlogammarus mrazeki* (Schäferna, 1907), but to the best of our knowledge never for niphargids.

Comparison of COI as well as of 28S to *N. schellenbergi* molecular data from the type locality (data not shown) indicates that all Luxembourgish specimens belong to *N. schellenbergi sensu stricto*. Furthermore, the morphology of the specimens collected in Luxembourg generally matched the morphological variability found at the type locality (Karaman 1932; re-collected by the first author in 2018). However, a few specimens displayed strong deviations from this morphology. Some, but by far not all adult specimens from the Minière Doihl exhibited a slightly to strongly rounded epimeral plate III. This is indicative of the *N. aquilex* complex. The rami of uropod II of these *N. schellenbergi* are about 60% of the length of their peduncle, while in *N. schellenbergi* normally it is close to 100%. All other characters, such as 3–4 spines in the dactylus of the gnathopods, indicate them as belonging to *N. schellenbergi*. One adult male (individual DL1 in **Supplementary Table 1**) very clearly possessed this rounded epimeral plate but sequencing revealed it as a *N. schellenbergi* instead (as was the case for all sequenced specimens from Minière Doihl). In addition, some specimens had relatively short spines (of about 40% of the telson length) at the end of the telson, whereas all the specimens of our collections had spines >60% of the length of the telson (thereby differing from the literature, e.g. the first description by Karaman in 1932). All other deviations from the first description (e.g. antennae I 40% of body length vs. 33% in the original description) seemed to be within the variability of this species (according to our own investigations, data not shown).

All adult males from Sept-Fontaines deviated strongly from all known *N. schellenbergi* morphs. They had 5 spines at the dactyli of the gnathopods whereas all other adult specimens of our material showed 3–4 spines. Their 3rd epimeral plate was slightly rounded. The end-spines of their telsons had a length only 40–50% of the telson length. The specimens bear 26–28 segments on the flagellum of antennae I. *Niphargus schellenbergi* normally possesses 22–25 segments on this part. It seems thus likely that *N. schellenbergi* harbors a much greater intraspecific morphological variability than currently recognized.

Niphargus virei Chevreux, 1896 was reported by Hoffmann (1963) from two sites: Minière Langegronn and Sept-Fontaines (=Sieweboure) in the city of Luxembourg. It was stated to co-occur with *N. schellenbergi*. We were not able to re-collect *N. virei* in Luxembourg, although we re-visited both sites. Our collection in Sept-Fontaines resulted in 42 individuals (9 males, 11 supposed females and 22 juveniles). Their morphology deviated strongly from the typical *N. virei* as well as from *N. schellenbergi*, to the point they could have been considered a different, undescribed species. Surprisingly, however, both genetic markers allocated the specimens clearly to *N. schellenbergi*. Field sampling at the other potential *N. virei* site (Minière Langegronn) only yielded *N. schellenbergi* (confirmed by morphology, COI and 28S). In springs above the mines, where predominantly *N. schellenbergi* was detected, one specimen was assigned to *N. puteanus*.

The presence of *N. virei* in Luxembourg remains doubtful. For the moment, we assume that Hoffmann (1963) was misled by the peculiar morphotype of *N. schellenbergi* in Sept-Fontaines. It might explain why Hoffmann (1963) did not attribute them to *N. schellenbergi*, but it does not explain why he identified them as belonging to *N. virei*. Given the morphological characters stated by Hoffmann (1963) for his material from Minière Langegronn (**Table 9**), we can exclude the possibility that he had collected *N. puteanus* (based on overall size, number of spines of the dactylus, length of end spines of telson, length coxal plate IV, end spines:telson) or *N. virei* (based on number of spines on the dactylus, telson length:width, end spines:telson). Currently, no known morphospecies fits his investigations and we tentatively interpret his results as misidentifications.

TABLE 9. Relevant morphological characters of *Niphargus puteanus* and *Niphargus virei* in comparison to the specimen drawn by Hoffmann (1963) determined as *N. virei.*

	N. puteanus	*N. virei*	Hoffmann (1963)
Size in mm	10–22	about 35	30–33
Coxal plate IV	not longer than V	longer than V	longer than V
Spines on the dactylus of the gnathopods	5–8	2–10	1
Epimeral plate 3, angle at posterior edge	80°–120°	90°	90°
Uropod I internal branch:external branch	1.2–2	1.2–1.35	1.25
Telson length:width	0.86–1.05	1.2–1.25	1
End spines of telson	3	4–5	5
End spines:telson	0.33–0.53	0.25–0.44	0.22

Combining past and present findings

Our integrative taxonomic survey for groundwater amphipods of the family Niphargidae in Luxembourg detected *N. schellenbergi* as the predominant species. All other taxa were less frequent (*N. fontanus* MOTU A, *N. aquilex* MOTUs EF and G), and for three species only a single-specimen record was retrieved (*N. aquilex* MOTU B, *N. kochianus* MOTU ABC, *N. puteanus*). When compared, species overlap between literature reports (**Fig. 4**), morphology-based identifications from our study and DNA-based species identifications was limited to three (morpho)species: *N. aquilex, N. fontanus* and *N. schellenbergi*. Neither *M. leruthi* nor *N. virei* were re-detected.

As stated above, we faced several difficulties when identifying specimens based on their morphology, partly leading to questionable or imprecise determination results attributable to the following causes:

- mistakes or ambiguities in determination keys (e.g. in Ginet, 1991-1995, where *N. schellenbergi* is described with the external ramus of uropod 1 being longer than internal, whereas at least in Luxembourg and further Central European material both rami have similar lengths);
- lack of taxonomic keys to distinguish species within species complexes (*N. aquilex* complex*, N. kochianus* complex), which so far can only be addressed using genetic data;
- mismatches between the historical drawings of diagnostic morphological characters and their features in the specimens at hand (e.g. the supposedly ovoid gnathopods in *N. fontanus* compared to the square-shaped gnathopods of *N. schellenbergi*);
- high intraspecific morphological variability, as e.g. observed in *N. schellenbergi* for which two distinct morphotypes were observed. Without molecular data (COI and 28S), we would have wrongly described the distinct phenotype from Sept-Fontaines as a species new to science;

- abundance of juveniles among our collected individuals, which are always difficult to determine with confidence.

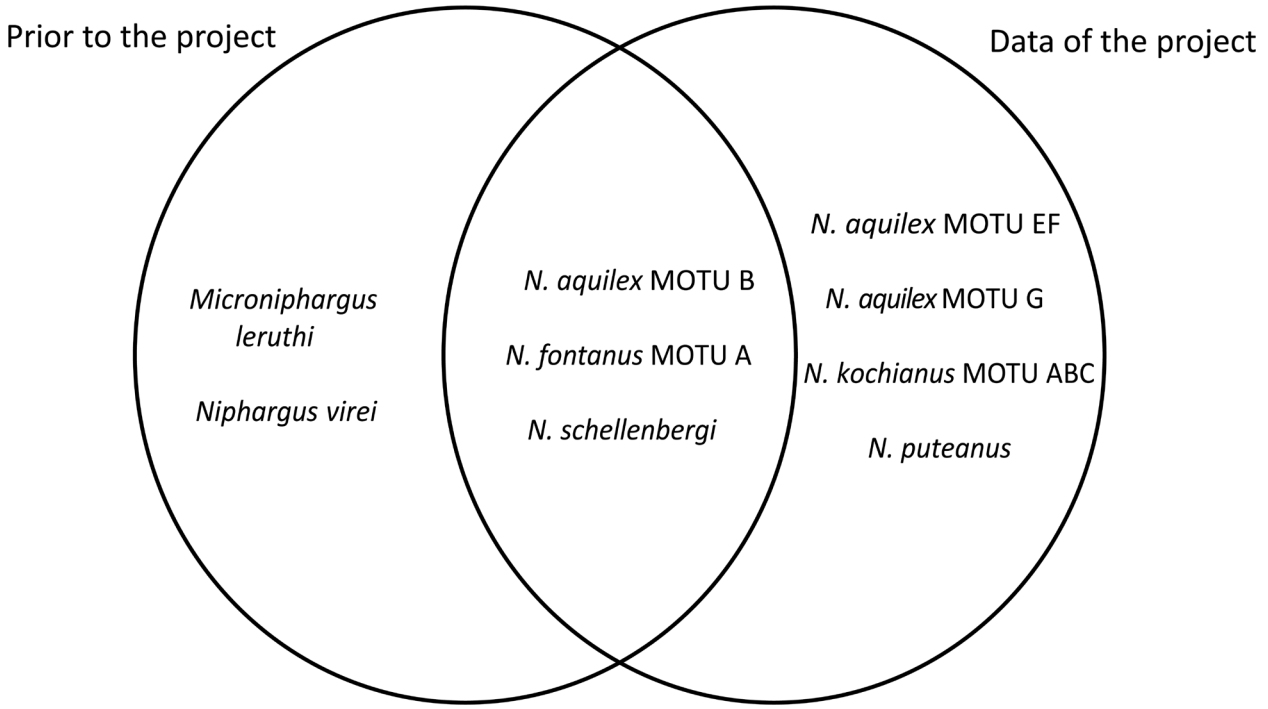

FIGURE 4. Venn diagram of niphargids from Luxembourg, comparing historical literature findings and genetic findings of this study. The presence of *Microniphargus leruthi* seems likely, but the species was not recollected.

The outcomes of our integrative taxonomic survey hence strongly corroborate previous statements that morphology-based identifications of the niphargid fauna of Northern France, the Benelux countries and Germany is error-prone, or, for some species complexes, even impossible (McInerney *et al.* 2014; Weber *et al*. 2020b). Currently, only adult *N. puteanus* can be reliably identified using their morphology (Weber *et al*. 2020a). For some part also, an accurate morphological determination of *N. schellenbergi* seems possible, i.e. when referring to i) the form of gnathopods, ii) the setae on the outer ramus of the dactylus of gnathopods and iii) the rounded epimeral plate; the length of the two rami of uropod I, on the other hand, does not appear informative. In contrast, several individuals not matching the set of diagnostic characters mentioned in the original description of *N. schellenbergi* by Karaman (1932) turned out to belong to this species after integration of mitochondrial and nuclear sequence data.

In conclusion, a safe determination of niphargids from Luxembourg—and the Greater Region of Saarland, Lorraine, Luxembourg, Wallonia and Rhineland-Palatinate—appears only possible using molecular data. The commonly applied COI barcode marker seems highly suitable here as species delineations and assignments were consistent with nuclear 28S data. A large-scale molecular inventory of niphargids in the Greater Region would also allow shedding further light on the distribution and ecological preferences of the rare species detected in our study.

Acknowledgements

We thank Robert Dondelinger, Christine Harbusch, Thomas Sebastian Lechner, Christa Locke, Ann-Cathrin Thierry and Verena Weber for their assistance in collecting specimens in Luxembourg. We also acknowledge Jessica Durkota and Tim Johns for their help in collecting material of *Niphargus aquilex* from the type locality in England. Fabio Stoch confirmed the morphological determination of some unusual morphotypes of the *N. aquilex* group and of *N. schellenbergi*, and uploaded sequence data to GenBank. Tim Johns determined the *N. aquilex* material from England. The German Science Foundation (DFG, WE 6055/1-1), the National Museum of Natural History Luxembourg and the Fonds de la Recherche Scientifique (FNRS, CDR J.0272.17) financially supported this project.

References

Astrin, J.J. & Stüben, P.E. (2009) Molecular phylogeny in "nano-weevils" description of a new subgenus *Nanoacalles* and two new species of *Calacalles* from the Macaronesian Islands (Curculionidae: Cryptorhynchidae). *Zootaxa*, 2300, 51–67.

Bah, T. (2011) *Inkscape. Guide to a vector drawing program. 4th Edition.* Prentice Hall, Boston, 504 pp.

Barber, H.S. (1931) Traps for cave-inhabiting insects. *Journal of the Elisha Mitchell Scientific Society*, 46, 259–266.

Bellan-Santini, D. (2015) Order Amphipoda Latreille, 1816. *In*: von Vaupel Klein, J.C., Charmantier-Daures, M. & Schram, F.R. (Eds.), Treatise on zoology—anatomy, taxonomy, biology. *The Crustacea*, 5, pp. 93–248. https://doi.org/10.1163/9789004232518_006

Borowsky, B. (2011) Response to light in two eyeless cave dwelling amphipods (*Niphargus ictus* and *Niphargus frasassianus*). *Journal of Crustacean Biology*, 31 (4), 613–616. https://doi.org/10.1651/10-3450.1

Chappuis, P.-A. (1924) Die Fauna der unterirdischen Gewässer der Umgebung von Basel. *Archiv für Hydrobiologie*, 14, 1–88.

Chappuis, P.-A. (1942) Eine neue Methode zur Untersuchung der Grundwasserfauna. *Acta scientiarum mathematicarum et naturalium / Universitas Francisco Joseephina, Kolozsvar*, 6, 3–7.

Chevreux, M.E. (1896) Sur un Amphipode d'eau douce, *Niphargus Virei* nov. sp., provenant des grottes du Jura. *Bulletin du muséum d'histoire naturelle*, 2, 136–137.

Clark, A.G. (1990) Inference of haplotypes from PCR-amplified samples of diploid populations. *Molecular Biology and Evolution*, 7 (2), 111–122. https://doi.org/10.1093/oxfordjournals.molbev.a040591

Delhez, F. & Houssa, M. (1969) L'Araine de Richeronfontaine à Liège. Etude écologique de la faune cavernicole d'un réseau souterrain artificiel. *Les Naturalistes Belges*, 50 (4), 194–212.

Delhez, F., Dethier, M. & Hubart, J.-M. (1999) Contribution à la connaissance de la faune des grottes de Wallonie. *Bulletin des Chercheurs de la Wallonie*, 39, 27–54.

Dobat, K. (1975) Die Höhlenfauna der Schwäbischen Alb mit Einschluss des Dinkelberges, des Schwarzwaldes und des Wutachgebietes. *Abhandlungen zur Karst- und Höhlenkunde, Reihe D, Paläontologie*, Zoologie, 2, 260–381.

Esmaeili-Rineh, S., Mamaghani-Shishvan, M., Fišer, C., Akmali, V. & Najafi, N. (2019) Range sizes of groundwater amphipods (Crustacea) are not smaller than range sizes of surface amphipods: a case study from Iran. *Contributions to Zoology*, 1, 1–13. https://doi.org/10.1163/18759866-20191418

Esmaeili-Rineh, S., Sari, A., Fišer, C. & Bargrizaneh, Z. (2017) Completion of molecular taxonomy: description of four amphipod species (Crustacea: Amphipoda: Niphargidae) from Iran and release of database for morphological taxonomy. *Zoologischer Anzeiger*, 271, 57–79. https://doi.org/10.1163/18759866-20191418

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39, 783–791. https://doi.org/10.2307/2408678

Fišer, C. (2012) *Niphargus*: A model system for evolution and ecology. *In:* White, W.B. & Culver, D.C. (Eds.), *Encyclopaedia of caves. 2nd Edition*. Academic press, Amsterdam, pp. 555–564. https://doi.org/10.1016/B978-0-12-814124-3.00090-X

Fišer, C. & Zagmaister, M. (2009) Cryptic species from cryptic space: the case of *Niphargus fongi* sp. n. (Amphipoda, Niphargidae). *Crustaceana*, 82 (5), 593–614. https://doi.org/10.1163/156854009X407704

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

Fišer, C., Konec, M., Alther, R., Švara, V. & Altermatt, F. (2016) Taxonomic, phylogenetic and ecological diversity of *Niphargus* (Amphipoda: Crustacea) in the Hölloch cave system (Switzerland). *Systematics and Biodiversity*, 15, 218–237. https://doi.org/10.1080/14772000.2016.1249112

Flot, J.-F. & Weber, D. (2013) Amphipods from caves of the Grand Duchy of Luxembourg. *Ferrantia*, 69, 186–190.

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

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

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

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

Fontaneto, D., Flot, J.-F. & Tang, C.Q. (2015) Guidelines for DNA taxonomy, with a focus on the meiofauna. *Marine Biodiversity*, 45, 433–451.
https://doi.org/10.1007/s12526-015-0319-7

Gerecke, R., Stoch, F., Meisch, C. & Schrankel, I. (2005) Die Fauna der Quellen und des hyporheischen Interstitials in Luxembourg. *Ferrantia*, 41, 1–140.

Ginet, R. (1991-1995) *Bilan systématique du genre Niphargus en France*. s.n., Lyon, 243 pp.

Gledhill, T, Sutcliffe, D.W. & Williams, W.D. (1976) *Key to the British freshwater Crustacea: Malacostraca. Freshwater Biological Association, scientific publications*, 32, 1–72.

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

Hertzog, L. (1930) Notes sur quelques Crustacés nouveaux pour la plaine d'Alsace (Bas-Rhin). *Bulletin de l'Association Philomathique d'Alsace et de Lorraine*, 7 (5), 355–364.

Hoffmann, J. (1963) Faune des Amphipodes du Grand-Duché de Luxembourg. Crustacea, Malacostraca, Amphipoda. Musée d'Histoire Naturelle Luxembourg. *Extrait des Archives de la Section des Sciences de l'Institut Grand-Ducal, Nouvelle Série*, 29, 77–128.

Horton, T., Lowry, J., De Broyer, C., Bellan-Santini, D., Coleman, C.O., Corbari, L., Costello, M.J., Daneliya, M., Dauvin, J.-C., Fišer, C., Gasca, R., Grabowski, M., Guerra-García, J.M., Hendrycks, E., Hughes, L., Jaume, D., Jazdzewski, K., Kim, Y.-H., King, R., Krapp-Schickel, T., LeCroy, S., Lörz, A.-N., Mamos, T., Senna, A.R., Serejo, C., Sket, B., Souza-Filho, J.F., Tandberg, A.H., Thomas, J.D., Thurston, M., Vader, W., Väinölä, R., Vonk, R., White, K. & Zeidler, W. (2021) World Amphipoda Database. Available from: http://www.marinespecies.org/amphipoda (accessed 20 September 2021)
https://doi.org/10.14284/368

Karaman, G.S. (1980) Contribution to the knowledge of the Amphipoda 113. Redescription of *Niphargus aquilex* Schiödte and its distribution in Great Britain. *Biosistematika*, 6 (2), 175–185.

Karaman, G. (2017) New data on two subterranean species of the family Niphargidae from Spain, *Niphargus gallicus* Schell., 1935 and *N. delamarei* Ruffo, 1954 (Contribution to the Knowledge of the Amphipoda 282). *Contributions, Section of Natural, Mathematical and Biotechnical Sciences*, 36 (2), 105–120.
https://doi.org/10.20903/csnmbs.masa.2015.36.2.72

Karaman, G.S. & Ruffo, S. (1986) Amphipoda: *Niphargus*-group (Niphargidae sensu Bousfield, 1982) *In*: Botosaneanu L. *Stygofauna Mundi*, 514–535.

Karaman, S. (1932) 5. Beitrag zur Kenntnis der Süsswasser-Amphipoden (Amphipoden unterirdischer Gewässer). *Prirodoslovne Razprave*, 2, 179–232.

Katoh, K., Rozewicki, J. & Yamada, K.D. (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, 20 (4), 1160–1166.
https://doi.org/10.1093/bib/bbx108

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.
https://doi.org/10.1093/molbev/msy096

Koch, C.L. (1836) *Gammarus puteanus*. *In*: Panzer, G.W.F. (Ed.), *Fauna Insectorum Germanica Initia*, 138, pp. 1–2.

Kureck, A. (1964) Light sensitivity in the amphipode, *Niphargus aquilex schellenbergi* Karaman. *Experientia*, 20 (9), 523–524.

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

Leloup, E. & Jacquemart, S. (1963) Ecologie d'une tourbière bombée (Haute-Ardenne, La Fange aux Mochettes). *Mémoires de l'Institut royal des Sciences naturelles de Belgique*, 149, 1–159.

Leruth, R. (1939) Biologie du domaine souterrain et faune cavernicole de la Belgique. *Memoires du Musee royal d'Histoire naturelle de Belgique, Bruxelles*, 87, 1–506.

Malard, F., Dole-Olivier, M.-J., Mathieu, J. & Stoch, F. (2002) *Sampling manual for the assessment of regional groundwater biodiversity*. European Project PASCALIS, Villeurbanne, 74 pp.

McInerney, C.E., Maurice, L, Robertson, A.L., Knight, L.R.F.D., Arnscheidt, J., Venditti, C., Doodley, J.S.G., Mathers, T., Matthijs, S., Eriksson, K., Proudlove, G.S. & Hänfling, B. (2014) The ancient Britons: groundwater fauna survived extreme climate change over tens of millions of years across NW Europe. *Molecular Ecology*, 23 (5), 1153–1166.
https://doi.org/10.1111/mec.12664

Meisch, C. & Massard, J.A. (2015) Les recherches sur les crustacés (Crustacea) du Luxembourg: aperçu historique. *Bulletin de la Société des naturalistes luxembourgeois*, 116, 381–390.

Meleg, J.N., Zakšek, V., Fišer, C., Kelemen, B.S. & Moldovan, O.T. (2013) Can environment predict cryptic diversity? The case of *Niphargus* inhabiting Western Carpathian groundwater. *PLoS ONE*, 8, 1–13.
https://doi.org/10.1371/journal.pone.0076760

Notenboom, J. (1982) Een inventarisatie van de fauna in een aantal Zuid-Limburgse waterputten. *Natuurhistorisch Maandblad*,

71 (2), 27–32.
Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, 21, 1864–1877.
https://doi.org/10.1111/j.1365-294X.2011.05239.x
Schellenberg, A. (1932a) Vier blinde Amphipodenarten aus einem Brunnen Oberbayerns. *Zoologischer Anzeiger*, 98 (5/6), 131–139.
Schellenberg, A. (1932b) Deutsche subterrane Amphipoden. *Zoologischer Anzeiger*, 99 (11/12), 311–323.
Schellenberg, A. (1933) *Niphargus puteanus* am alten Fundort neu entdeckt, Revision der deutschen *Niphargus*-Arten. *Zoologischer Anzeiger*, 102 (9/10), 255–257.
Schellenberg, A. (1934): Eine neue Amphipoden-Gattung aus einer belgischen Höhle, nebst Bemerkungen über die Gattung *Crangonyx*. *Zoologischer Anzeiger*, 106 (9), 125–128.
Schellenberg, A. (1935) Schlüssel der Amphipodengattung *Niphargus* mit Fundortangaben und mehreren neuen Formen. *Zoologischer Anzeiger*, 111 (7/8), 204–211.
Schiödte, J.C. (1855) Om den i England opdagede Art af Hulekrebe-Slaegten *Niphargus*. Oversigt over det Kgl. danske Vidensk. Selsk. Forhandl. *Medlemmers Arb. Kjobenhavn*, 1855, 349–351.
Sket, B. (2004) The cave hygropetric—a little known habitat and its inhabitants. *Archiv für Hydrobiologie*, 160 (3), 413–425.
https://doi.org/10.1127/0003-9136/2004/0160-0413
Spangenberg, H.-J. (1973) Beitrag zur Faunistik von Höhlengewässern im Zechstein des Südharzes und Kyffhausers. *Hercynia*, Neue Folge, 10 (2), 143–160.
Spence Bate, C.S. (1859) On the genus *Niphargus* (Schiödte). *Proceedings of the Dublin university zoological and botanical association*, 1, 237–240.
Spöri, Y. & Flot, J. (2020) HaplowebMaker and CoMa: two web tools to delimit species using haplowebs and conspecificity matrices. *Methods in Ecology and Evolution*, 1, 1434–1438.
https://doi.org/10.1111/2041-210X.13454
Spöri, Y., Stoch, F., Dellicour, S., Birky, C.W. & Flot, J.-F. (2021) KoT: an automatic implementation of the K/θ method for species delimitation. *BioRxiv*, 2021.08.17.454531. [published online]
https://doi.org/10.1101/2021.08.17.454531
Straškraba, M. (1972) Les groupement des espèces du genre *Niphargus* (sensu lato). *In*: Ruffo, S. (Ed.), *Actes du Ier Colloque International sur le Genre Niphargus, Verona*, 1972, 85–90.
Stoch, F., Malard, F., Castellarini, F., Dole-Olivier, M.-J. & Gibert, J. (2004) *Statistical analyses and identification of indicators.* European Project Report WP7 D8 (PASCALIS No EVK-CT-2001-00121), Villeurbanne, 156 pp.
Stock, J.H. (1961) Ondergrondse waterdieren in Zuid-Limburg. *Overdruk uit het Natuurhistorich Maandblad,* 50 (7-8), 77–85.
Sunnen, T. (1957) Les *Niphargus* des chambres de captage du Grès de Luxembourg. *Archives de l'Institut grand-ducal, Section des sciences naturelles, physiques et mathématiques*, New Series, 24, 57–60.
Trontelj, P., Douady, C.J., Fišer, C., Gibert, J., Gorički, Š., Lefébure, T., Sket, B. & Zakšek, V. (2009) Molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? *Freshwater Biology*, 54, 727–744.
https://doi.org/10.1111/j.1365-2427.2007.01877.x
Verovnik, R., Sket, B. & Trontelj, P. (2005) The colonization of Europe by the freshwater crustacean *Asellus aquaticus* (Crustacea: Isopoda) proceeded from ancient refugia and was directed by habitat connectivity. *Molecular Ecology*, 14, 4355–4369.
https://doi.org/10.1111/j.1365-294X.2005.02745.x
Weber, D. (1991) *Die Evertebratenfauna der Höhlen und künstlichen Hohlräume des Katastergebietes Westfalen einschließlich der Quellen- und Grundwasserfauna. Abhandlungen zur Karst- und Höhlenkunde, München*, 25, 1–701.
Weber, D. (2001) Die Höhlenfauna und -flora des Höhlenkatastergebietes Rheinland-Pfalz/Saarland. 4. Teil. *Abhandlungen zur Karst- und Höhlenkunde, München*, 33, 1–1088.
Weber, D. (2011) Höhlenfaunenerfassung in Luxemburg. *Mitteilungen des Verbandes der deutschen Höhlen- und Karstforscher e.V., München*, Jg. 2011 (3), 99–100.
Weber, D. (2012) *Die Höhlenfauna und -flora des Höhlenkatastergebietes Rheinland-Pfalz/Saarland. 5. Teil. Abhandlungen zur Karst- und Höhlenkunde, München*, 36, 1–2367.
Weber, D. (2013) *Die Höhlenfauna Luxemburgs. Ferrantia*, 69, 5–95.
Weber, D. (2018) 'Grot garnelen' in Limburg. *SOK Medelingen*, 69, 18–23.
Weber, D., Flot, J.-F., Weigand, H. & Weigand, A.M. (2020) Demographic history, range size and habitat preferences of the groundwater amphipod *Niphargus puteanus* (C.L. Koch in Panzer, 1836). *Limnologica*, 8, 125765.
https://doi.org/10.1016/j.limno.2020.125765
Weber, D., Stoch, F., Knight, L.R.F.D., Chauveau, C.A. & Flot, J.-F. (2021) The genus *Microniphargus* (Crustacea, Amphipoda): evidence for three lineages distributed across northwestern Europe and transfer from Niphargidae to Pseudoniphargidae. *Belgian Journal of Zoology*, 161, 169–191.
https://doi.org/10.1101/2020.08.25.266817
Weigand, A.M., Kremers, J. & Grabner, D.S. (2016) Shared microsporidian profiles between an obligate (*Niphargus*) and facultative subterranean amphipod population (*Gammarus*) at sympatry provide indications for underground transmission pathways. *Limnologica*, 58, 7–10.
https://doi.org/10.1016/j.limno.2016.01.005

SUPPLEMENTARY TABLE 1. Overview of sequenced specimens used in the present article. Habitat: mine = artificial cavern mined for minerals; tunnel = artificial cavern with two opposite entrances on same level; interstitial = hyporheic porous sediment zone along and under the river bed; rheocrene = flowing spring where water emerges into a defined downstream channel; helocrene = spring where water emerges diffusely over a wider area; limnocrene = flowing spring where water emerges upwards into a lake or lakelet; basin = spring where water flows mostly via a rheocrene or a man-made pipe into a basin. Lat. = Latitude, Long. = Longitude. 28S and COI refer to molecular sequences available, either new from this study (with Genbank number) or from the literature (with x). To protect subterranean sites, we have removed the 3rd decimal place of the WGS84 coordinates for all artificial caverns.

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
AK1	Quelle am aale Koepchen	rheocrene	20.05.2018	*Niphargus schellenbergi*	Luxembourg	50.073	6.047		OK380764	
AS1	Schiefergrouf Asselborn	mine	14.03.2017	*Niphargus schellenbergi*	Luxembourg	50.10	5. 96	OK378185	OK380765	
AS2	Schiefergrouf Asselborn	mine	14.03.2017	*Niphargus schellenbergi*	Luxembourg	50.10	5. 96		OK380766	
AW1	Sickerquelle auf Weg	helocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	50.005	5.954		OK380767	
AW2	Sickerquelle auf Weg	helocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	50.005	5.954		OK380768	
BA1	Source 1 Bambesch	rheocrene	25.08.2018	*Niphargus schellenbergi*	Luxembourg	49.636	6.116		OK380769	
BA2	Source 1 Bambesch	rheocrene	25.08.2018	*Niphargus schellenbergi*	Luxembourg	49.636	6.116		OK380770	
BA3	Source 1 Bambesch	rheocrene	25.08.2018	*Niphargus schellenbergi*	Luxembourg	49.636	6.116		OK380771	
BB1	Source 2 Bambesch	rheocrene	25.08.2018	*Niphargus schellenbergi*	Luxembourg	49.636	6.116		OK380772	
BC1	Lavoir Brachtenbach	basin	27.02.2017	*Niphargus schellenbergi*	Luxembourg	50.019	5.908		OK380773	
BR1	Source rue de Bridel	rheocrene	26.08.2018	*Niphargus schellenbergi*	Luxembourg	49.629	6.113		OK380774, OK380775	
BR2	Source rue de Bridel	rheocrene	26.08.2018	*Niphargus schellenbergi*	Luxembourg	49.629	6.113		OK380776	
DF1	Quelle 1 Decken Fiels	rheocrene	03.01.2017	*Niphargus schellenbergi*	Luxembourg	49.643	6.041	OK378186	OK380777	
DI1	Quelle 2 Decken Fiels	rheocrene	03.01.2017	*Niphargus schellenbergi*	Luxembourg	49.642	6.028		OK380778	
DK1	Quelle Diekirch	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	49.875	6.154		OK380779	
DK2	Quelle Diekirch	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	49.875	6.154		OK380780	
DL1	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85	OK378187	OK380781	
DL2	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85	OK378188	OK380782	
DL3	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85		OK380783	
DL4	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85		OK380784	
DL5	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85		OK380785	
DL6	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85		OK380786	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
DL7	Minière Doihl	mine	26.07.2016	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85		OK380787	
DL8	Minière Doihl	mine	04.10.2014	*Niphargus schellenbergi*	Luxembourg	49.54	5. 85	OK378189		
DU1	Jettenhöhle	cave	02.05.2019	*Niphargus schellenbergi*	Germany	51.69	10.27	x	x	Flot 2010
DU2	Jettenhöhle	cave	02.05.2019	*Niphargus schellenbergi*	Germany	51.69	10.27	x	x	Flot 2010
DU3	Jettenhöhle	cave	02.05.2019	*Niphargus schellenbergi*	Germany	51.69	10.27	x	x	Flot 2010
EC1	Quelle südlich Echternach	rheocrene	31.01.2018	*Niphargus schellenbergi*	Luxembourg	49.811	6.455		OK380788	
EH1	Quelle 2 südlich Echter-nach	rheocrene	31.01.2018	*Niphargus schellenbergi*	Luxembourg	49.811	6.454		OK380789	
EH2	Quelle 2 südlich Echter-nach	rheocrene	31.01.2018	*Niphargus schellenbergi*	Luxembourg	49.811	6.454		OK380790	
FN1	Sept-Fontaines	rheocrene	11.08.2018	*Niphargus schellenbergi*	Luxembourg	49.627	6.100		OK380791	
FN2	Sept-Fontaines	rheocrene	11.08.2018	*Niphargus schellenbergi*	Luxembourg	49.627	6.100	OK378190		
FO1	Eisebunns Tunnel Fouhren	tunnel	21.03.2015	*Niphargus schellenbergi*	Luxembourg	49.91	6.19	OK378191		
GD1	Grünelscheid-Quelle	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	49.976	5. 87		OK380792	
GD2	Grünelscheid-Quelle	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	49.976	5. 87		OK380793	
GF1	Quelle bei Grundhaff	rheocrene	13.07.2016	*Niphargus schellenbergi*	Luxembourg	49.835	6.328	OK378192		
GK1	Source de Girsterklaus	basin	04.09.2016	*Niphargus schellenbergi*	Luxembourg	49.785	6.498	OK378193	OK380794	
GK10	Source de Girsterklaus	basin	17.02.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380795	
GK11	Source de Girsterklaus	basin	17.02.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380796	
GK12	Source de Girsterklaus	basin	17.02.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380797	
GK13	Source de Girsterklaus	basin	17.02.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498		OK380798	
GK14	Source de Girsterklaus	basin	17.02.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498		OK380799	
GK15	Source de Girsterklaus	basin	17.02.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498		OK380800	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
GK16	Source de Girsterklaus	basin	30.01.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498	OK378194, OK378195	OK380801	
GK2	Source de Girsterklaus	basin	10.07.2016	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498	OK378196	OK380802	
GK3	Source de Girsterklaus	basin	04.09.2016	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498	OK378197, OK378198	OK380803	
GK4	Source de Girsterklaus	basin	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380804	
GK5	Source de Girsterklaus	basin	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380805	
GK6	Source de Girsterklaus	basin	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380806	
GK7	Source de Girsterklaus	basin	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380807	
GK8	Source de Girsterklaus	basin	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.785	6.498		OK380808	
GK9	Source de Girsterklaus	basin	30.01.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.785	6.498		OK380809	
GO1	Interstitial Gouschtenger-bach 1	interstitial	12.10.2018	*Niphargus schellenbergi*	Luxembourg	49.607	6.386		OK380810	
GO2	Interstitial Gouschtenger-bach 1	interstitial	12.10.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.607	6.386	OK378199	OK380811	
GO3	Interstitial Gouschtenger-bach 1	interstitial	12.10.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.607	6.386		OK380812	
HN1	Hunnebuer 4	rheocrene	04.02.2017	*Niphargus schellenbergi*	Luxembourg	49.727	6.087	OK378200	OK380813	
HR1	Hunnebur	rheocrene	01.05.2018	*Niphargus schellenbergi*	Luxembourg	49.727	6.08		OK380814	
HS1	Quelle 4 Helmsange	rheocrene	04.01.2017	*Niphargus schellenbergi*	Luxembourg	49.649	6.143	OK378201	OK380815	
IA1	Interstitial Attert bei Evelingen	interstitial	12.10.2017	*Niphargus schellenbergi*	Luxembourg	49.775	5.953		OK380816	
II1	Interstitial Wiltz	interstitial	02.09.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.969	5.901	OK378202	OK380817	
IL1	Interstitial Schlënner	interstitial	01.08.2017	*Niphargus schellenbergi*	Luxembourg	49.949	6.066	OK378203	OK380818	
IL2	Interstitial Schlënner	interstitial	01.08.2017	*Niphargus schellenbergi*	Luxembourg	49.949	6.066	OK378204	OK380819	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
IL3	Interstitial Schlënner	interstitial	01.08.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.949	6.066	OK378205	OK380820	
IL4	Interstitial Schlënner	interstitial	01.08.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.949	6.066		OK380821	
IL5	Interstitial Schlënner	interstitial	01.08.2017	*Niphargus schellenbergi*	Luxembourg	49.949	6.066	OK378206		
IS1	Interstitial Sauer	interstitial	02.08.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.888	6.094	OK378207	OK380822	
IS2	Interstitial Sauer	interstitial	02.08.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.888	6.094	OK378208		
IS3	Interstitial Sauer	interstitial	02.08.2017	*Niphargus aquilex* MOTU B	Luxembourg	49.888	6.094	OK378209		
IW1	Interstitial Wiltz 2	interstitial	02.09.2017	*Niphargus schellenbergi*	Luxembourg	49.963	5.971	OK378210	OK380823	
KC1	Quelle beim Kalkesbach	basin	14.05.2017	*Niphargus schellenbergi*	Luxembourg	49.796	6.363		OK380824	
KE1	Klengelbuer	rheocrene	28.01.2018	*Niphargus fontanus* MOTU A	Luxembourg	49.571	6.149	OK378211	OK380825	
KE2	Klengelbuer	rheocrene	28.01.2018	*Niphargus fontanus* MOTU A	Luxembourg	49.571	6.149		OK380826	
KF1	Kofferminn Stolzebuerg	mine	23.02.2018	*Niphargus schellenbergi*	Luxembourg	49.97	6. 15		OK380827	
KF2	Kofferminn Stolzebuerg	mine	23.02.2018	*Niphargus schellenbergi*	Luxembourg	49.97	6. 15		OK380828, OK380829	
KF3	Kofferminn Stolzebuerg	mine	23.02.2018	*Niphargus schellenbergi*	Luxembourg	49.97	6. 15		OK380830	
KK1	Kuhtränke	basin	11.05.2017	*Niphargus schellenbergi*	Luxembourg	49.794	6.296		OK380831	
KO1	Source Kopstal K8	rheocrene	06.01.2017	*Niphargus schellenbergi*	Luxembourg	49.677	6.085	OK378212	OK380832	
KR1	Katzebour 1	rheocrene	13.12.2016	*Niphargus schellenbergi*	Luxembourg	49.631	6.119		OK380833	
KS1	Source Kopstal non Capté	rheocrene	06.01.2017	*Niphargus schellenbergi*	Luxembourg	49.674	6.083	OK378213	OK380834	
KT1	Katzebour 2	rheocrene	13.12.2016	*Niphargus schellenbergi*	Luxembourg	49.631	6.119	OK378214	OK380835	
KZ1	Quelle an der Kreuzung	limnocre-ne	11.02.2018	*Niphargus schellenbergi*	Luxembourg	50.009	5.951		OK380836	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
LB1	Lédeleschbur	rheocrene	07.01.2017	*Niphargus schellenbergi*	Luxembourg	49.678	6.076	OK378215	OK380837	
LB2	Lédeleschbur	rheocrene	07.01.2017	*Niphargus schellenbergi*	Luxembourg	49.678	6.076		OK380838	
LE1	Strassenböschung L'Écluse	rheocrene	01.02.2018	*Niphargus schellenbergi*	Luxembourg	49.570	6.357		OK380839	
LE2	Strassenböschung L'Écluse	rheocrene	01.02.2018	*Niphargus schellenbergi*	Luxembourg	49.570	6.357		OK380840	
LG1	Minière Langegronn	mine	24.07.2016	*Niphargus schellenbergi*	Luxembourg	49.46	6.04	OK378216	OK380841	
LG2	Minière Langegronn	mine	01.01.2016	*Niphargus fontanus* MOTU A	Luxembourg	49.46	6.04		OK380842	
LG3	Minière Langegronn	mine	01.01.2016	*Niphargus aquilex* MOTU G	Luxembourg	49.46	6.04	OK378217, OK378218		
LG4	Minière Langegronn	mine	24.01.2016	*Niphargus fontanus* MOTU A	Luxembourg	49.46	6.04	OK378219		
LI1	Quelle Laangebierg Italien	rheocrene	27.04.2016	*Niphargus schellenbergi*	Luxembourg	49.468	6.076	OK378220	OK380843	
MF1	Source à Moestroff	rheocrene	20.05.2018	*Niphargus schellenbergi*	Luxembourg	49.864	6.243		OK380844	
MH1	Mëchelbaach	interstitial	30.06.2017	*Niphargus schellenbergi*	Luxembourg	49.852	6.012	OK378221	OK380845	
MH2	Mëchelbaach	interstitial	30.06.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.852	6.012	OK378222	OK380846	
MH3	Mëchelbaach	interstitial	30.06.2017	*Niphargus aquilex* MOTU EF	Luxembourg	49.852	6.012	OK378223		
MK1	Galerie Merkholtz	mine	23.02.2018	*Niphargus schellenbergi*	Luxembourg	49.97	5. 97		OK380847	
MK2	Galerie Merkholtz	mine	23.02.2018	*Niphargus aquilex* MOTU EF	Luxembourg	49.97	5. 97	OK378224, OK378225	OK380848	
MK3	Galerie Merkholtz	mine	23.02.2018	*Niphargus aquilex* MOTU G	Luxembourg	49.97	5. 97	OK378226	OK380849	
ML1	Source non captée Muellerthal	rheocrene	11.05.2017	*Niphargus schellenbergi*	Luxembourg	49.787	6.301		OK380850	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
MU1	Sickerquelle Maulusmühle	helocrene	16.05.2017	*Niphargus schellenbergi*	Luxembourg	50.095	6.023		OK380851	
MU2	Sickerquelle Maulusmühle	helocrene	16.05.2017	*Niphargus schellenbergi*	Luxembourg	50.095	6.023		OK380852	
MU3	Sickerquelle Maulusmühle	helocrene	16.05.2017	*Niphargus schellenbergi*	Luxembourg	50.095	6.023		OK380853	
MU4	Sickerquelle Maulusmühle	helocrene	16.05.2017	*Niphargus schellenbergi*	Luxembourg	50.095	6.023		OK380854	
MU5	Sickerquelle Maulusmühle	helocrene	16.05.2017	*Niphargus kochianus* MOTU ABC	Luxembourg	50.095	6.023		OK380855	
MU6	Sickerquelle Maulusmühle	helocrene	16.05.2017	*Niphargus schellenbergi*	Luxembourg	50.095	6.023	OK378227		
NE1	Quelle 1 Neuhäuschen	rheocrene	02.06.2017	*Niphargus schellenbergi*	Luxembourg	49.625	6.232	OK378228	OK380856	
NR1	Quelle Nr. 1	rheocrene	07.01.2017	*Niphargus schellenbergi*	Luxembourg	49.674	6.083	OK378229	OK380857	
NW1	Interstitial Nieder-wampach	interstitial	02.09.2017	*Niphargus schellenbergi*	Luxembourg	50.001	5.824	OK378230		
PA1	Schiefergrouf vu Pärel	mine	30.01.2016	*Niphargus schellenbergi*	Luxembourg	49.81	5. 77	OK378231	OK380858	
PH1	Minière Prince Henry	mine	02.09.2016	*Niphargus schellenbergi*	Luxembourg	49.47	5. 99	OK378232		
QA1	Quelle 2 Laangebierg Italien	rheocrene	10.02.2018	*Niphargus schellenbergi*	Luxembourg	49.467	6.066		OK380859	
QB1	Quelle 6 Laangebierg Italien	helocrene	10.02.2018	*Niphargus schellenbergi*	Luxembourg	49.468	6.069		OK380860	
QG1	Quelle 3 Laangebierg Italien	rheocrene	10.02.2018	*Niphargus schellenbergi*	Luxembourg	49.467	6.067		OK380861	
QH1	Quelle 1 Helmsange	rheocrene	04.01.2017	*Niphargus schellenbergi*	Luxembourg	49.651	6. 14	OK378233		
QL1	Quelle 4 Laangebierg Italien	limnocre-ne	10.02.2018	*Niphargus puteanus*	Luxembourg	49.467	6.068	OK378234	OK380862	
QL2	Quelle 4 Laangebierg Italien	limnocre-ne	10.02.2018	*Niphargus schellenbergi*	Luxembourg	49.467	6.068		OK380863	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
QN1	Quelle 5 Laangebierg Italien	limnocrene	10.02.2018	*Niphargus schellenbergi*	Luxembourg	49.467	6.068		OK380864	
QS1	Quelle Schiltzhaus	rheocrene	31.01.2018	*Niphargus schellenbergi*	Luxembourg	49.787	6.507		OK380865	
QT1	Quelle am Strassenrand	rheocrene	15.05.2017	*Niphargus schellenbergi*	Luxembourg	49.829	6.227		OK380866	
RM1	Brunnen Rue de Mamer	rheocrene	12.02.2017	*Niphargus schellenbergi*	Luxembourg	49.661	6.069		OK380867	
RP1	Quelle unter den Raschpetzern	rheocrene	04.02.2017	*Niphargus schellenbergi*	Luxembourg	49.667	6.146		OK380868	
RP2	Quelle unter den Raschpetzern	rheocrene	04.02.2017	*Niphargus schellenbergi*	Luxembourg	49.667	6.146	OK378235		
SB1	Strassenbassin	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	50.017	5.905		OK380869	
SB2	Strassenbassin	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	50.017	5.905		OK380870	
SC1	Schenzelbuer	rheocrene	16.01.2017	*Niphargus schellenbergi*	Luxembourg	49.666	6. 19	OK378236	OK380871	
SF1	Quelle Schleif	rheocrene	11.02.2018	*Niphargus schellenbergi*	Luxembourg	49.992	5. 86		OK380872	
SG1	Strassenquelle Girst	basin	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.781	6.487		OK380873	
SI1	Quelle bei Schleif	rheocrene	21.05.2018	*Niphargus schellenbergi*	Luxembourg	49.986	5.855		OK380874	
SL1	Schiefergrouf vu Schläif	mine	28.02.2017	*Niphargus schellenbergi*	Luxembourg	50.00	5. 82		OK380875	
SM1	Quelle über Schendelsemillen	helocrene	01.05.2018	*Niphargus schellenbergi*	Luxembourg	49.725	6.103		OK380876	
SW1	Schwiewelbur	rheocrene	28.01.2018	*Niphargus schellenbergi*	Luxembourg	49.546	6.272		OK380877	
SW2	Schwiewelbur	rheocrene	28.01.2018	*Niphargus schellenbergi*	Luxembourg	49.546	6.272		OK380878	
SW3	Schwiewelbur	rheocrene	28.01.2018	*Niphargus schellenbergi*	Luxembourg	49.546	6.272		OK380879	
TF1	Tümpelquelle Fromburg	limnocrene	31.01.2018	*Niphargus schellenbergi*	Luxembourg	49.777	6.414		OK380880	
TH1	Tunnel Huldange	tunnel	14.12.2013	*Niphargus schellenbergi*	Luxembourg	50.16	6.03	OK378237		
TI1	Quelle Thilsmillen	rheocrene	03.01.2017	*Niphargus schellenbergi*	Luxembourg	49.462	6.059	OK378238	OK380881	
TN1	Saint Thomas Nebenquelle	rheocrene	14.03.2017	*Niphargus schellenbergi*	Luxembourg	50.114	5.908		OK380882	
TO1	Source Saint Thomas	basin	14.03.2017	*Niphargus schellenbergi*	Luxembourg	50.114	5.909		OK380883	

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
TS1	Tussen-Quelle	rheocrene	28.02.2017	*Niphargus schellenbergi*	Luxembourg	50.16	6.03		OK380884	
UE1	Quelle Uechelsbaach	rheocrene	27.04.2016	*Niphargus schellenbergi*	Luxembourg	49.478	6.105	OK378239	OK380885	
UK1	Spring		20.05.2018	*Niphargus aquilex* MOTU B	Luxembourg	51.056	0.182	OK378240, OK378241	OK380886	
UR1	Wiesenquelle Urspelt	basin	20.05.2018	*Niphargus schellenbergi*	Luxembourg	50.085	6.045		OK380887	
VM1	Source au Vieux Moulin	rheocrene	31.01.2018	*Niphargus schellenbergi*	Luxembourg	49.798	6.389		OK380888	
WD1	Quelle 5 Weidendall	rheocrene	12.02.2017	*Niphargus schellenbergi*	Luxembourg	49.653	6.064		OK380889	
WE1	Quelle beim Wemperbach	rheocrene	21.05.2018	*Niphargus schellenbergi*	Luxembourg	50.106	6.044		OK380890	
WH1	Waschhaus Schandel	rheocrene	12.10.2017	*Niphargus schellenbergi*	Luxembourg	49.789	5. 97		OK380891	
WI1	Quelle Winteringen	rheocrene	05.01.2017	*Niphargus schellenbergi*	Luxembourg	49.498	6.333	OK378242	OK380892	
WN1	Wiesenquelle Nidderland	helocrene	16.05.2017	*Niphargus schellenbergi*	Luxembourg	50.074	6.049		OK380893	
WP1	Interstitial Wamperbach	interstitial	21.05.2018	*Niphargus aquilex* MOTU EF	Luxembourg	50.106	6.044	OK378243	OK380894	
WR1	Quelle am Wegrand	rheocrene	15.05.2017	*Niphargus schellenbergi*	Luxembourg	49.829	6.227		OK380895	
WS1	Wegequelle Syr	helocrene	21.05.2018	*Niphargus schellenbergi*	Luxembourg	49.904	5.786		OK380896	
WT1	Waldquelle Girst	rheocrene	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.781	6.487		OK380897	
WT2	Waldquelle Girst	rheocrene	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.781	6.487		OK380898	
WT3	Waldquelle Girst	rheocrene	30.01.2018	*Niphargus schellenbergi*	Luxembourg	49.781	6.487		OK380899	
WU1	Geborstene Wasserleitung	rheocrene	11.05.2017	*Niphargus schellenbergi*	Luxembourg	49.823	6.276		OK380900	
WY1	Lavoir Weydig	basin	28.08.2018	*Niphargus schellenbergi*	Luxembourg	49.717	6.331		OK380901	
EF025852	Lorrain, Vaux, Moselle			*Niphargus fontanus* MOTU AB	France	6	49	x		Lefébure *et al*. 2007
EF617302	Spring at the Gasthof Zur Walba, Regensburg			*Niphargus puteanus*	Germany	12	49	x		Trontelj *et al*. 2009
JF420841	Weser, Hessisch Oldendorf			*Niphargus aquilex* MOTU A	Germany	9	52		x	Hartke *et al*. 2011
JF420874	North German Plain, Weser, Hessisch Oldendorf			*Niphargus aquilex* MOTU AB	Germany	9	52	x		Hartke *et al*. 2011

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315602	Pleine-Fougeres, Brittany			*Niphargus aquilex* MOTU C	France	2	49	x		McInerney *et al*. 2013
KC315603	Pleine-Fougeres, Brittany			*Niphargus aquilex* MOTU D	France	2	49	x		McInerney *et al*. 2013
KC315604	Damage Barton, War-combe, Devon			*Niphargus aquilex* MOTU AB	United Kingdoms	-4	51	x		McInerney *et al*. 2013
KC315604	North Kenwood, Devon			*Niphargus aquilex* MOTU AB	United Kingdoms	-4	51	x		McInerney *et al*. 2013
KC315604	Netton Farm, Christow , Devon			*Niphargus aquilex* MOTU AB	United Kingdoms	-4	51	x		McInerney *et al*. 2013
KC315604	Postlake Farm, Devon			*Niphargus aquilex* MOTU AB	United Kingdoms	-3	51	x		McInerney *et al*. 2013
KC315604	Ashfields well, Half Moon village, Devon			*Niphargus aquilex* MOTU AB	United Kingdoms	-4	51	x		McInerney *et al*. 2013
KC315604	Swallow great limber, North England			*Niphargus aquilex* MOTU AB	United Kingdoms	0	54	x		McInerney *et al*. 2013
KC315604	River Till			*Niphargus aquilex* MOTU AB	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315604	Anglesey, Cae Glan-y-Mor			*Niphargus aquilex* MOTU AB	United Kingdoms	-4	53	x		McInerney *et al*. 2013
KC315604	Welton le wold, North England			*Niphargus aquilex* MOTU AB	United Kingdoms	0	53	x		McInerney *et al*. 2013
KC315604	North elkington, North England			*Niphargus aquilex* MOTU AB	United Kingdoms	0	53	x		McInerney *et al*. 2013
KC315604	Beelsby, North England			*Niphargus aquilex* MOTU AB	United Kingdoms	0	54	x		McInerney *et al*. 2013
KC315604	Hendale wood great limber			*Niphargus aquilex* MOTU AB	United Kingdoms	0	54	x		McInerney *et al*. 2013

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315604	South Ferriby			*Niphargus aquilex* MOTU AB	United Kingdoms	0	54	x		McInerney *et al*. 2013
KC315604	Havant, Hampshire			*Niphargus aquilex* MOTU AB	United Kingdoms	-1	51	x		McInerney *et al*. 2013
KC315605	Little Stour, Kent			*Niphargus aquilex* MOTU AB	United Kingdoms	1	51	x		McInerney *et al*. 2013
KC315606	Fountain on village square of Lesterny			*Niphargus aquilex* MOTU EF	Belgium	5	50	x		McInerney *et al*. 2013
KC315606	Water well nr 3 in Wavreille			*Niphargus aquilex* MOTU EF	Belgium	5	50	x		McInerney *et al*. 2013
KC315607	Stour Park, Dorset			*Niphargus aquilex* MOTU EF	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315607	Pridhamsleigh, Devon			*Niphargus aquilex* MOTU EF	United Kingdoms	-4	50	x		McInerney *et al*. 2013
KC315607	Plymouth garden centre, Devon			*Niphargus aquilex* MOTU EF	United Kingdoms	-4	50	x		McInerney *et al*. 2013
KC315607	Red Hill, Dorset			*Niphargus aquilex* MOTU EF	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315608	Ashwell Springs, Hertfordshire			*Niphargus fontanus* MOTU AB	United Kingdoms	0	52	x		McInerney *et al*. 2013
KC315608	Dunstable, Hertfordshire			*Niphargus fontanus* MOTU AB	United Kingdoms	0	52	x		McInerney *et al*. 2013
KC315608	South Wales, Breconshire, Ogof Ffynnon Ddu			*Niphargus fontanus* MOTU AB	United Kingdoms	-4	52	x		McInerney *et al*. 2013
KC315608	Little Gaddesden, Hertfordshire			*Niphargus fontanus* MOTU AB	United Kingdoms	-1	52	x		McInerney *et al*. 2013
KC315608	Wheathampstead, Hertfordshire			*Niphargus fontanus* MOTU AB	United Kingdoms	0	52	x		McInerney *et al*. 2013

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315608	Amersham, Buckingham-shire			*Niphargus fontanus* MOTU AB	United Kingdoms	-1	52	x		McInerney *et al*. 2013
KC315608	St Cuthberts Swallet, Somerset			*Niphargus fontanus* MOTU AB	United Kingdoms	-3	51	x		McInerney *et al*. 2013
KC315608	Little Stour, Kent			*Niphargus fontanus* MOTU AB	United Kingdoms	1	51	x		Hänfling et. al. 2008
KC315609	39 Pz2-Balmettes			*Niphargus fontanus* MOTU C	France	6	46	x		McInerney *et al*. 2013
KC315609	39 St Rambert			*Niphargus fontanus* MOTU C	France	5	46	x		McInerney *et al*. 2013
KC315610	Amersham, Buckingham-shire			*Niphargus kochianus* MOTU ABC	United Kingdoms	-1	52	x		Hänfling et. al. 2008
KC315610	Waterston Cress Beds, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315610	Ashwell Springs, Hert-fordshire			*Niphargus kochianus* MOTU ABC	United Kingdoms	0	52	x		McInerney *et al*. 2013
KC315610	Luton			*Niphargus kochianus* MOTU ABC	United Kingdoms	0	52	x		McInerney *et al*. 2013
KC315610	Little Gaddesden, Hert-fordshire			*Niphargus kochianus* MOTU ABC	United Kingdoms	-1	52	x		McInerney *et al*. 2013
KC315610	Warmwell, Cressbeds, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315610	Martinstown well, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315610	Hurst Green cottage, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315610	Throop, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315610	West Lodge, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315610	Barcombe farm, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	51	x		McInerney *et al*. 2013
KC315610	Brightwalton Holt, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdoms	-1	51	x		McInerney *et al*. 2013
KC315610	Calversley Farm, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdoms	-1	51	x		McInerney *et al*. 2013
KC315610	Crane Bridge, Cotswolds			*Niphargus kochianus* MOTU ABC	United Kingdoms	-2	52	x		McInerney *et al*. 2013
KC315610	Digswell, Herfordshire			*Niphargus kochianus* MOTU ABC	United Kingdoms	0	52	x		McInerney *et al*. 2013
KC315610	Cowdown, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdoms	-1	52	x		McInerney *et al*. 2013
KC315611	Résurgence at the cave Tridaine			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315611	Water well of the Castle of Houx			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315611	Water well 4 in Champalle			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315611	Stone quarry of Warnant			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315611	Water catchment gallery of Senenne (in Spontin)			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315611	Resurgence in Embiérin			*Niphargus kochianus* MOTU ABC	Belgium	6	50	x		McInerney *et al*. 2013
KC315611	Water well in Prés de l'Abreux			*Niphargus kochianus* MOTU ABC	Belgium	6	51	x		McInerney *et al*. 2013

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315611	Water well in the Reijmer-stokker-Dorpstraat			*Niphargus kochianus* MOTU ABC	Netherlands	6	51	x		McInerney *et al*. 2013
KC315611	Water well in Koeberg			*Niphargus kochianus* MOTU ABC	Netherlands	6	51	x		McInerney *et al*. 2013
KC315612	Water well 4 in Cham-palle			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315612	Water well of the Castle of Houx			*Niphargus kochianus* MOTU ABC	Belgium	5	50	x		McInerney *et al*. 2013
KC315613	39 Boutillon			*Niphargus kochianus* MOTU D	France	3	47	x		McInerney *et al*. 2013
KC315614	Main gallery of the Néblon			*Niphargus fontanus* MOTU AB	Belgium	5	50	x		McInerney *et al*. 2013
KC315614	Water catchment gallery of Senenne (in Spontin)			*Niphargus fontanus* MOTU AB	Belgium	5	50	x		McInerney *et al*. 2013
KC315614	Water catchment in Crupet			*Niphargus fontanus* MOTU AB	Belgium	5	50	x		McInerney *et al*. 2013
KC315621	Water well nr 3 in Wavreille			*Niphargus aquilex* MOTU EF	Belgium	5	50		x	McInerney *et al*. 2013
KC315621	Fountain on village square of Lesterny			*Niphargus aquilex* MOTU EF	Belgium	5	50		x	McInerney *et al*. 2013
KC315622	Pridhamsleigh, Devon			*Niphargus aquilex* MOTU EF	United Kingdom	-4	50		x	McInerney *et al*. 2013
KC315622	Plymouth garden centre, Devon			*Niphargus aquilex* MOTU EF	United Kingdom	-4	50		x	McInerney *et al*. 2013
KC315623	Hendale wood great limber			*Niphargus aquilex* MOTU A	United Kingdom	0	54		x	McInerney *et al*. 2013
KC315623	Welton le wold, North England			*Niphargus aquilex* MOTU A	United Kingdom	0	53		x	McInerney *et al*. 2013

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315623	Beelsby, North England			*Niphargus aquilex* MOTU A	United Kingdom	0	54		x	McInerney *et al*. 2013
KC315623	Swallow great limber, North England			*Niphargus aquilex* MOTU A	United Kingdom	0	54		x	McInerney *et al*. 2013
KC315623	North Elkington, North England			*Niphargus aquilex* MOTU A	United Kingdom	0	53		x	McInerney *et al*. 2013
KC315629	Main gallery of the Néblon			*Niphargus fontanus* MOTU A	Belgium	5	50		x	McInerney *et al*. 2013
KC315630	Water catchment in Crupet			*Niphargus fontanus* MOTU A	Belgium	5	50		x	McInerney *et al*. 2013
KC315632	39 St Rambert			*Niphargus fontanus* MOTU B	France	5	46		x	McInerney *et al*. 2013
KC315633	Little Gaddesden, Hertfordshire			*Niphargus fontanus* MOTU A	United Kingdom	-1	52		x	McInerney *et al*. 2013
KC315633	Wheathampstead, Hertfordshire			*Niphargus fontanus* MOTU A	United Kingdom	0	52		x	McInerney *et al*. 2013
KC315633	St. Cuthberts Swallet, Somerset			*Niphargus fontanus* MOTU A	United Kingdom	-3	51		x	McInerney *et al*. 2013
KC315633	Dunstable, Hertfordshire			*Niphargus fontanus* MOTU A	United Kingdom	0	52		x	McInerney *et al*. 2013
KC315633	South Wales, Breconshire, Ogof Ffynnon Ddu			*Niphargus fontanus* MOTU A	United Kingdom	-4	52		x	McInerney *et al*. 2013
KC315659	Water well of the Castle of Houx			*Niphargus kochianus* MOTU ABC	Belgium	5	50		x	McInerney *et al*. 2013
KC315660	Water well of the Castle of Houx			*Niphargus kochianus* MOTU ABC	Belgium	5	50		x	McInerney *et al*. 2013
KC315661	Water well in Koeberg			*Niphargus kochianus* MOTU ABC	Netherlands	6	51		x	McInerney *et al*. 2013

.....continued on the next page

SUPPLEMENTARY TABLE 1. (Continued)

Code	Object name	Ha-bitat	Collection date	Species	Country	Lat.	Long.	28S	COI	**Literature**
KC315662	Water well in Koeberg			*Niphargus kochianus* MOTU ABC	Netherlands	6	51		x	McInerney *et al*. 2013
KC315662	Water well in the Reijmer-stokker-Dorpstraat			*Niphargus kochianus* MOTU ABC	Netherlands	6	51		x	McInerney *et al*. 2013
KC315667	Water well in Koeberg			*Niphargus kochianus* MOTU ABC	Netherlands	6	51		x	McInerney *et al*. 2013
KC315682	Brightwalton Holt, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdom	-1	51		x	McInerney *et al*. 2013
KC315682	Cowdown, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdom	-1	52		x	McInerney *et al*. 2013
KC315682	Hurst Green cottage, Dorset			*Niphargus kochianus* MOTU ABC	United Kingdom	-2	51		x	McInerney *et al*. 2013
KC315682	Calversley Farm, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdom	-1	51		x	McInerney *et al*. 2013
KC315682	Crane Bridge, Cotswolds			*Niphargus kochianus* MOTU ABC	United Kingdom	-2	52		x	McInerney *et al*. 2013
KC315682	Little Gaddesden, Hertfordshire			*Niphargus kochianus* MOTU ABC	United Kingdom	-1	52		x	McInerney *et al*. 2013
KC315687	Calversley Farm, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdom	-1	51		x	McInerney *et al*. 2013
KC315688	Calversley Farm, Berkshire			*Niphargus kochianus* MOTU ABC	United Kingdom	-1	51		x	McInerney *et al*. 2013

ONLINE SUPPLEMENTARY MATERIAL

Online Supplementary Data 1. Sequencher file containing all the COI chromatograms of the present study (136 chromatogram pairs, two of which displayed double peaks attributable to Numts or heteroplasmy).

Online Supplementary Data 2. Sequencher file containing all the 28S chromatograms of the present study (55 chromatogram pairs, five of which displayed double peaks attributable to heterozygosity).

Online Supplementary Data 3. FASTA alignment of all the COI sequences used in the present study (138 sequences from 136 individuals from the present study plus three sequences from Flot 2010 and 16 sequences from McInerney *et al.* 2014).

Online Supplementary Data 4. FASTA alignment of all the 28S sequences used in the present study (60 sequences from 55 individuals from the present study plus three sequences from Flot 2010 and 16 sequences from McInerney *et al.* 2014).