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The genus *Microniphargus* (Crustacea, Amphipoda): evidence for three lineages distributed across northwestern Europe and transfer from Niphargidae to Pseudoniphargidae

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Abstract. *Microniphargus leruthi* Schellenberg, 1934 (Amphipoda: Niphargidae) was first described based on samples collected in Belgium and placed in a monotypic genus within the family Niphargidae. However, some details of its morphology as well as recent phylogenetic studies suggest that *Microniphargus* may be more closely related to *Pseudoniphargus* (Amphipoda: Pseudoniphargidae) than to *Niphargus*. Moreover, *M. leruthi* ranges over 1,469 km from Ireland to Germany, which is striking since only a few niphargids have confirmed ranges in excess of 200 km. To find out the phylogenetic position of *M. leruthi* and check whether it may be a complex of cryptic species, we collected material from Ireland, England and Belgium then sequenced fragments of the mitochondrial cytochrome *c* oxidase subunit 1 gene as well as of the nuclear 28S ribosomal gene. Phylogenetic analyses of both markers confirm that *Microniphargus* is closer to *Pseudoniphargus* than to *Niphargus*, leading us to reallocate *Microniphargus* to Pseudoniphargidae. We also identify three congruent mito-nuclear lineages present respectively in Ireland, in both Belgium and England, and in England only (with the latter found in sympatry at one location), suggesting that *M. leruthi* is a complex of at least three species with a putative centre of origin in England.

Keywords. Species delimitation, haploweb, K/θ, DNA barcoding, cryptic species, *Microniphargus leruthi*.

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Introduction

Microniphargus leruthi Schellenberg, 1934 (family Niphargidae) was first described from Engihoul Cave in Wallonia (Belgium) and placed into a new, monotypic genus considered as closely related

to the genus *Niphargus* Schiödte, 1849 described 85 years before (Schiödte 1849; Schellenberg 1934). *Microniphargus leruthi* is characterised by its small body size (1.2-1.5 mm in length), the scant setulation of its mandibular palps, an evident protrusion on the carpus of its gnathopods (particularly pronounced on the first pair of gnathopods: Knight & Gledhill 2010) and its telson widely incised with an angle of around 80° in its indentation, all of which were used to justify the erection of a new genus (Schellenberg 1934). However, several other genera similarly erected on the basis of distinctive morphological features of unknown variability have been synonymized with *Niphargus* in light of molecular data (see Borko *et al.* 2019).

Although *M. leruthi* is presently placed in the family Niphargidae, the shape of its telson is quite similar to that of the genus *Pseudoniphargus* Chevreux, 1901, which is placed in a different family (Pseudoniphargidae) together with the monotypic *Parapseudoniphargus* Notenboom, 1988. The taxonomic position of the family Pseudoniphargidae, defined on vague morphological characters, has long been controversial, having been included in the superfamilies Hadzioidea, Niphargoidea, Crangonyctoidea, Gammaroidea or included in the families Gammaridae and Melitidae (see Notenboom 1988 for a detailed analysis). Notenboom (1988) in his cladistic analysis placed the family within the families Eriopisidae and Melitidae, whereas Allocrangonyctidae (comprising two stygobitic species from North America) were later considered as the most closely related family. In fact, in their recent revisions of amphipod taxonomy, Lowry & Myers (2013, 2017) included the family Pseudoniphargidae within the superfamily Allocrangonyctoidea, while Niphargidae were allocated to Crangonyctoidea. Recent molecular studies have rejected this hypothesis, suggesting that Pseudoniphargidae are the sister group of Niphargidae (Jurado-Rivera *et al.* 2017; Moškrič & Verovnik 2019; Copilaş-Ciocianu *et al*. 2020). Although numerous mitochondrial sequences of *Pseudoniphargus* are available, there are only three partial 28S sequences for this genus and no genetic data at all for the family Allocrangonyctidae and for the genus *Parapseudoniphargus*, hindering a definitive taxonomic assessment of this clade.

Existing molecular data regarding *M. leruthi* are also scarce, with only 10 sequences available in GenBank so far (Fišer *et al.* 2017; Moškrič & Verovnik 2019), all of which from nuclear markers. No mitochondrial sequence for specimens of *Microniphargus* has been published so far. Moškrič & Verovnik (2019) recovered a (*Microniphargus* + *Pseudoniphargus*) clade as a sister group to *Niphargus* using one protein-coding nuclear gene; however, another protein-coding nuclear marker in the same study yielded a discordant position of *Microniphargus* within *Niphargus.* More recently Copilaş-Ciocianu *et al.* (2020), in a large-scale phylogeny of amphipods based on fragments of the mitochondrial cytochrome *c* oxidase subunit I and of the nuclear histone 3 (H3), 18S and 28S genes, one *Microniphargus*, two *Pseudoniphargus* and two *Niphargus* species, also recovered *Microniphargus* as more closely related to *Pseudoniphargus* than to *Niphargus.*

Pseudoniphargus comprises 71 stygobitic species (Stokkan *et al*. 2018), all strict endemics present in North Africa and Benin, the Mediterranean region, the Iberian Peninsula, the archipelagos of Canaries, Madeira and Azores, and two species in Bermuda, whereas *Parapseudoniphargus* comprises a single, stygobitic species from southern Spain. By contrast, *Microniphargus leruthi* is found in north-western Europe: Belgium (Leruth 1939; Spangenberg 1973; Karaman & Ruffo 1986; Delhez *et al.* 1999), Germany (Spangenberg 1973; Karaman & Ruffo 1986; Fuchs 2007; Matzke *et al.* 2009; Stein *et al.* 2012), Luxembourg (Hoffmann 1963) as well as Ireland (Arnscheidt *et al.* 2008; Knight & Penk 2010; Knight & Gledhill 2010) and Great Britain (Knight & Gledhill 2010). The very large range of *M. leruthi* (over 1,469 km) is unusual as only a few niphargids have ranges exceeding 200 km (Trontelj *et al.* 2009): some species previously considered to be wide-ranging, such as *Niphargus aquilex* Schiödte, 1855 and *Niphargus virei* Chevreux, 1896, have been found to be complexes of cryptic species (Lefébure *et al*. 2006; McInerney *et al*. 2014). The only species with confirmed ranges more extended than *M. leruthi* are *Niphargus hrabei* S. Karaman, 1932 (> 1,300 km) and *Niphargus*

valachicus Dobreanu & Manolache, 1933 (> 3,200 km), two epigean species with enhanced dispersal via surface water (COPILAŞ-CIOCIANU *et al.* 2017). The wide range of *M. leruthi* could therefore be due to the presence of undetected species boundaries.

To resolve these uncertainties, we conducted a molecular study on *M. leruthi* collected in Ireland, England and Belgium using both 28S (nuclear) and COI (mitochondrial) markers. Our aims were (i) to confirm the phylogenetic position of *Microniphargus* relative to the genera *Niphargus* and *Pseudoniphargus* and (ii) to test for the possible existence of cryptic lineages within *M. leruthi*.

Material and methods

Sampling and sequencing

Although we carried out intensive and targeted sampling for *M. leruthi*, especially in caves around the type locality, we were only able to collect it at a single location on the European continent: the Grotte de Comblain (Wallonia, Belgium), which is 20 km away from the type locality. We also collected *M. leruthi* from one site in Ireland (Polldubh, Clare) and two sites in England (Sweetwater Pot, South Devon and Swildon's Hole, Somerset; Fig. 1). All the material (Table 1) was determined morphologically by one of us (L.K.). Specimens were collected by sweeping a long-handle net fitted with a 250 µm mesh collecting bag along the bottom and sides of cave pools, making sure to disturb the substrate to suspend both

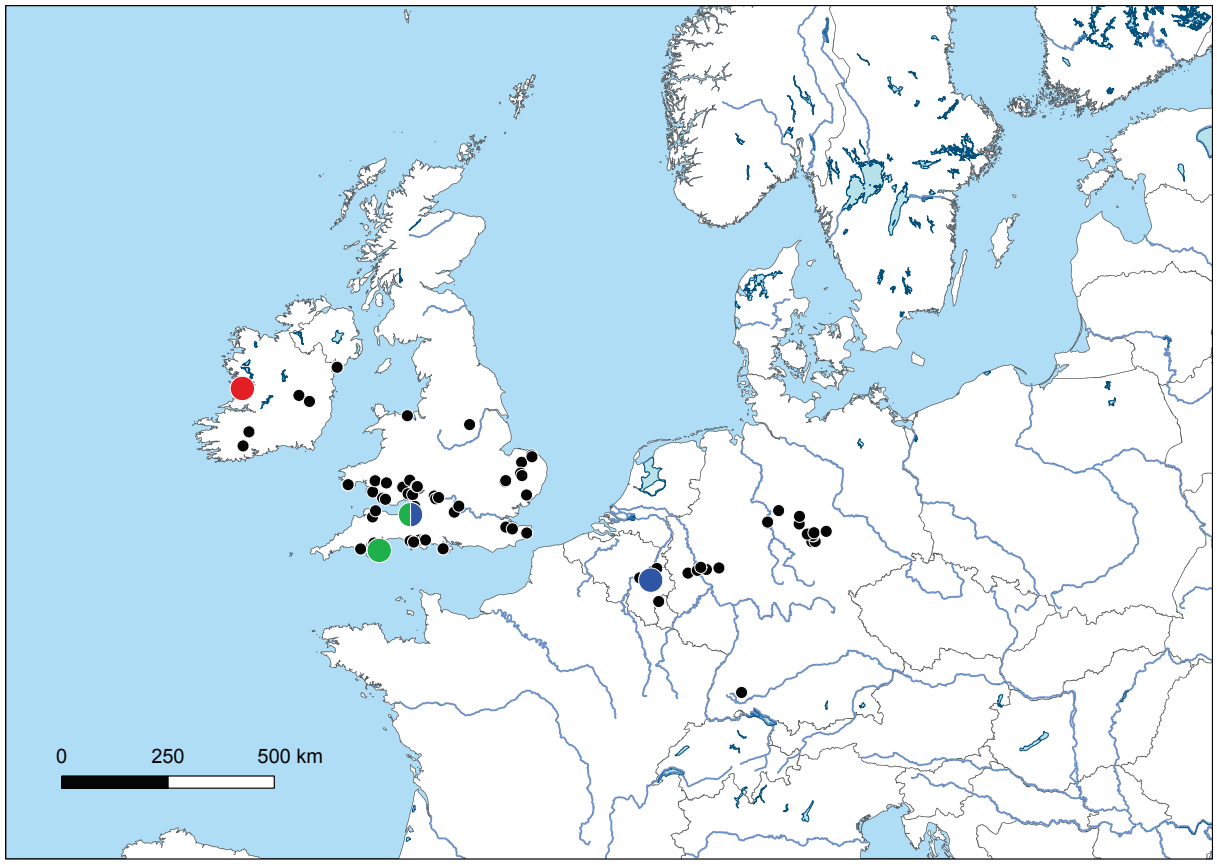

Figure 1 – Map showing the distribution of *Microniphargus leruthi*. Black dots: literature data based on morphological determination (including https://hcrs.brc.ac.uk/hcrs-database, accessed 27th May 2020). Orange: lineage A; blue: lineage B; yellow: lineage C.

TABLE 1

Material newly sequenced for the present study, including GenBank accession numbers; WGS84 coordinates are expressed in decimal degrees.

Isolate code	Species	Sampling site	Country	Latitude	Longitude	Collectors	Date	COI	28S
DW170428-002	*Crangonyx subterraneus*	Interstitial Mümling	Germany	49.843	9.094	Weber D.	28/04/2017	MT993546	MT994446
FS_11.023	*Synurella ambulans*	Lake of Sablici	Italy	45.806	13.576	Stoch F., Fior G.	27/02/2011	MT993547	MT994448
FC_14.1	*Gammarus pulex*	Spring Moulin de Grisendal	France	50.763	1.661	Caroulle F.	26/10/2014	-	MT994447
FS_14.036	*Pseudoniphargus italicus*	Spring near Marineo	Italy	37.946	13.406	Marrone F.	19/04/2013	-	MT994449
FS_18.073	*Pseudoniphargus spiniferus*	Grotte d'Istaurdy	France	43.111	-1.038	Brustel H.	10/05/2018	-	MT994450
DW191250-001	*Microniphargus leruthi*	Sweetwater Pot	England	50.399	-3.491	Knight L.	24/11/2019	MT993556	-
DW191250-002	*Microniphargus leruthi*	Sweetwater Pot	England	50.399	-3.491	Knight L.	24/11/2019	MT993557	MT994443
DW191250-004	*Microniphargus leruthi*	Swildon's Hole	England	51.259	-2.673	Knight L.	2/11/2019	MT993558 MT993559	MT994444
DW191250-011	*Microniphargus leruthi*	Swildon's Hole	England	51.259	-2.673	Knight L.	2/11/2019	MT993560	MT994445
DW171021-001	*Microniphargus leruthi*	Polldubh Cave	Ireland	53.078	-9.287	Knight L., Boulton J., Weber D.	21/10/2017	MT993548	MT994436
DW171021-002	*Microniphargus leruthi*	Polldubh Cave	Ireland	53.078	-9.287	Knight L., Boulton J., Weber D.	21/10/2017	-	MT994437
DW171021-003	*Microniphargus leruthi*	Polldubh Cave	Ireland	53.078	-9.287	Knight L., Boulton J., Weber D.	21/10/2017	MT993549	MT994438
DW190413-007	*Microniphargus leruthi*	Grotte de Comblain	Belgium	50.476	5.566	Knight L., Boulton J., Weber D.	13/04/2019	MT993550 MT993551	MT994439 MT994440
DW190413-008	*Microniphargus leruthi*	Grotte de Comblain	Belgium	50.476	5.566	Knight L., Boulton J., Weber D.	13/04/2019	MT993552 MT993553	MT994441
DW190413-009	*Microniphargus leruthi*	Grotte de Comblain	Belgium	50.476	5.566	Knight L., Boulton J., Weber D.	13/04/2019	MT993554 MT993555	MT994442

TABLE 2

Primers used in the present study.

Primer	Bases	Marker	PCR	Sequencing	Reference
LCO1490-JJ	5‘-CHA CW AAY CAT AAA GAT ATY GG-3‘	COI	x	x	Folmer *et al.* 1994
HCO2198-JJ	5‘-AWA CTT CVG GRT GVC CAA ARA ATC A-3‘	COI	x	x	Folmer *et al.* 1994
Niph15	5‘-CAA GTA CCG TGA GGG AAA GTT-3‘	28S	x		Verovnik *et al.* 2005
Niph15i	5‘-AGA GTC AAA AGA CCG TGA AAC C-3‘	28S		x	present publication
Niph16	5‘-AGG GAA ACT TCG GAG GGA ACC-3‘	28S	x		Verovnik *et al.* 2005
Niph16i	5’-GAT TGG TCT TTC GCC CCT AT-3’	28S		x	present publication
Niph20	5‘-AAA CAC GGG CCA AGG AGT AT-3‘	28S		x	Flot *et al*. 2010b
Niph21	5‘-TAT ACT CCT TGG CCC GTG TT-3‘	28S		x	Flot *et al*. 2010b

sediment and specimens into the water column. The collected specimens were immediately preserved in 96% ethanol and kept at -20°C until DNA was isolated.

Due to the small size of *M. leruthi*, we used one entire specimen for each DNA isolation. DNA was extracted following the standard protocol of the NucleoSpin® Tissue Kit (Macherey-Nagel) except that we performed two elution steps, the first one with 60 µL and the second with 40 µL (instead of a single elution step with 100 µL) to achieve a higher concentration of DNA. The resulting DNA isolates are stored at -20°C in the collections of the Evolutionary Biology & Ecology research unit at the Université libre de Bruxelles (ULB).

The Folmer fragment of the cytochrome *c* oxidase subunit 1 (COI) gene was amplified via polymerase chain reaction (PCR) (Folmer *et al.* 1994) using the primers HCO2198-JJ and LCO1490-JJ (Astrin & Stüben 2008; see Table 2). The PCR mix contained 1µL DNA template (variable concentration), 0.8 µL of each primer (10 pmol/µL), 5µL of DreamTaq DNA Polymerase (Thermo Scientific) and 2.4µL ultrapure water. PCR cycling conditions were an initial 3-min 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 2min elongation step at 65°C.

We also sequenced Verovnik’s fragment of the nuclear 28S ribosomal gene. The primers Niph15 and Niph16 (see Table 2) were used for amplification (Verovnik *et al.* 2005). The PCR mix for 28S contained 2µL of DNA template (variable concentration), 1µl of each primer (10pmol/µL), 0.2µL of REDTaq DNA Polymerase (Sigma-Aldrich), 5µL REDTaq reaction buffer and 15.8µL ultrapure water. PCR cycling conditions for 28S were an initial 3min denaturation step at 95°C; followed by 56 cycles of 30s denaturation at 94°C, 60 s annealing at 45°C, and 90s extension at 72°C.

The amplification success of each PCR reaction was verified using agarose gel electrophoresis, then PCR products were sequenced at Genoscreen (Lille, France). For COI the primer used for sequencing were the same as for PCR amplification, whereas for 28S we used the primers Niph20 and Niph21 (Flot *et al.* 2010b) as well as one or both of two new internal primers located slightly inward of the primers used for initial amplification (Niph15i and Niph16i; see Table 2).

The resulting chromatograms were assembled and cleaned using Sequencher version 4.1.4 (Gene Codes, USA). Whenever double peaks were observed in both the forward and reverse chromatograms of an

individual, we considered this individual as polymorphic and called its two haplotypes (determined using the approach summarized in FONTANETO *et al*. 2015) "a" and "b" in downstream analyses.

Phylogenetic and species delimitation analyses

We compiled comprehensive sets of COI and 28S including all sequences available in GenBank to date, then curated them manually to remove duplicates. The resulting set of 1384 COI sequences was aligned manually, whereas for the 255 sequences of 28S (including two gammarids *Gammarus fossarum* and *Gammarus pulex* and two crangonyctids *Crangonyx subterraneus* and *Synurella ambulans* as outgroups) we used MAFFT 7's E-INS-i mode (KATOH *et al.* 2019).

The comprehensive 28S alignment was used to reconstruct a global phylogeny of niphargid and pseudoniphargid amphipods. The best-fit substitution model, selected using ModelFinder (KALYAANAMOORTHY *et al.* 2017) according to the Bayesian Information Criterion (SCHWARZ 1978), was GTR+F+I+G4 (codes follow the IQTREE manual). Phylogenetic relationships were reconstructed using maximum likelihood with 1,000 ultrafast bootstrap replicates (HOANG *et al*. 2018) in IQ-TREE 2 (MINH *et al*. 2020); 253 out of 255 sequences (including all *Microniphargus* sequences) passed the gap/ambiguity test in IQTree 2 and were used in the analysis.

The comprehensive COI alignment was analysed using ABGD (Automatic Barcode Gap Discovery, available online at https://bioinfo.mnhn.fr/abi/public/abgd/), a distance-based species delimitation tool (PUILLANDRE *et al*. 2012) that first attempts to infer the most likely position of a barcode gap ('initial partitioning') before conducting a second round of splitting by recursively applying the same procedure on the groups defined during the first step ('recursive partitioning'). ABGD was run on the public webserver with default parameters.

A subset of the COI sequences (comprising all new *Microniphargus* sequences, all high-quality, complete *Pseudoniphargus* COI sequences inferred from complete mitochondrial genome sequences from BAUZÀ-RIBOT *et al*. (2012) and STOKKAN *et al.* (2016, 2018) plus two sequences of *Niphargus* and sequences of the Crangonyctidae *Crangonyx subterraneus* and *Synurella ambulans* (as outgroups) was used to build a ML tree using IQ-TREE 2 with the same modalities illustrated for 28S; the best-fit substitution model, selected using ModelFinder (according to the Bayesian Information Criterion was TIM+F+I+G4 (codes follow the IQTREE manual).

Phylogenetic networks were built for the COI and 28S sequences obtained from *Microniphargus* using HaplowebMaker (SPÖRI & FLOT 2020, available online from https://eeg-ebe.github.io/HaplowebMaker/). Average genetic distances between *Microniphargus* sequences identified as belonging to different lineages were computed in MEGA X (KUMAR *et al*. 2018) using uncorrected p-distances. A K/θ species delimitation analysis (BIRKY *et al*. 2010; SCHÖN *et al*. 2012; BIRKY 2013) was performed on the COI sequences of *Microniphargus* using the online program KoT with a K/θ threshold value of 6 (corresponding to a p-value < 0.01; SPÖRI *et al*. 2021, available online from https://eeg-ebe.github.io/KoT).

Results

For both COI and 28S, we successfully sequenced nine *Microniphargus leruthi* specimens. For COI, four individuals (one from Belgium and two from England) displayed one double peak each and were therefore represented by two sequences 'a' and 'b' (with a single base difference between them) in all downstream analyses. One *M. leruthi* individual (from Belgium) displayed a double peak in its 28S chromatograms and was therefore represented by two sequences (with a single base difference between them) in all downstream analyses, whereas all other individuals were homozygous for the 28S marker (Fig. 2).

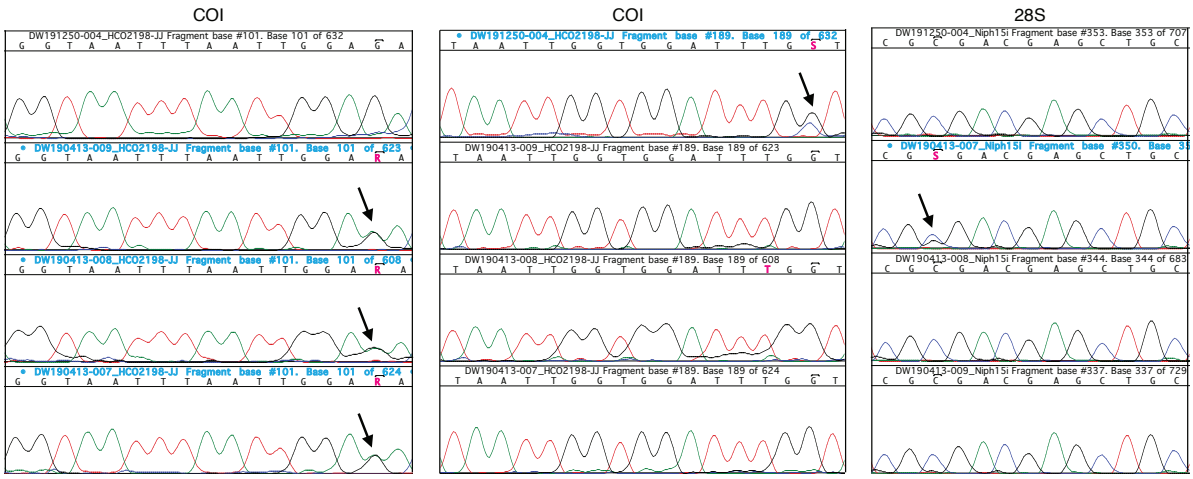

Figure 2 – Screenshots of the Sequencher program showing the double peaks identified in the COI (left and middle panel) and 28S (right panel) chromatograms.

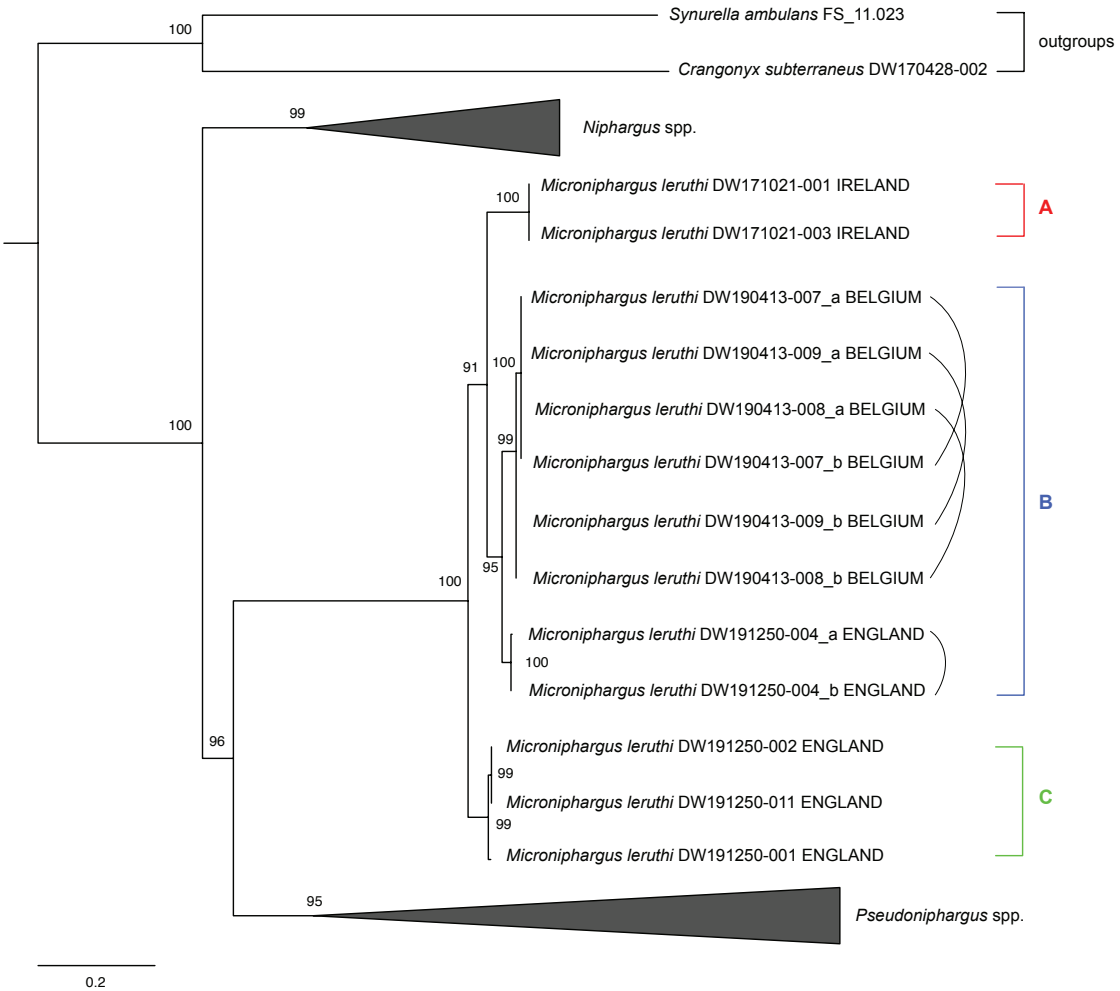

Figure 3 – COI maximum-likelihood phylogeny of *Microniphargus* and *Pseudoniphargus* (with two *Niphargus* and two crangonyctids as outgroups). The tree was turned into a haploweb by adding connections between haplotypes found co-occurring in the same individual.

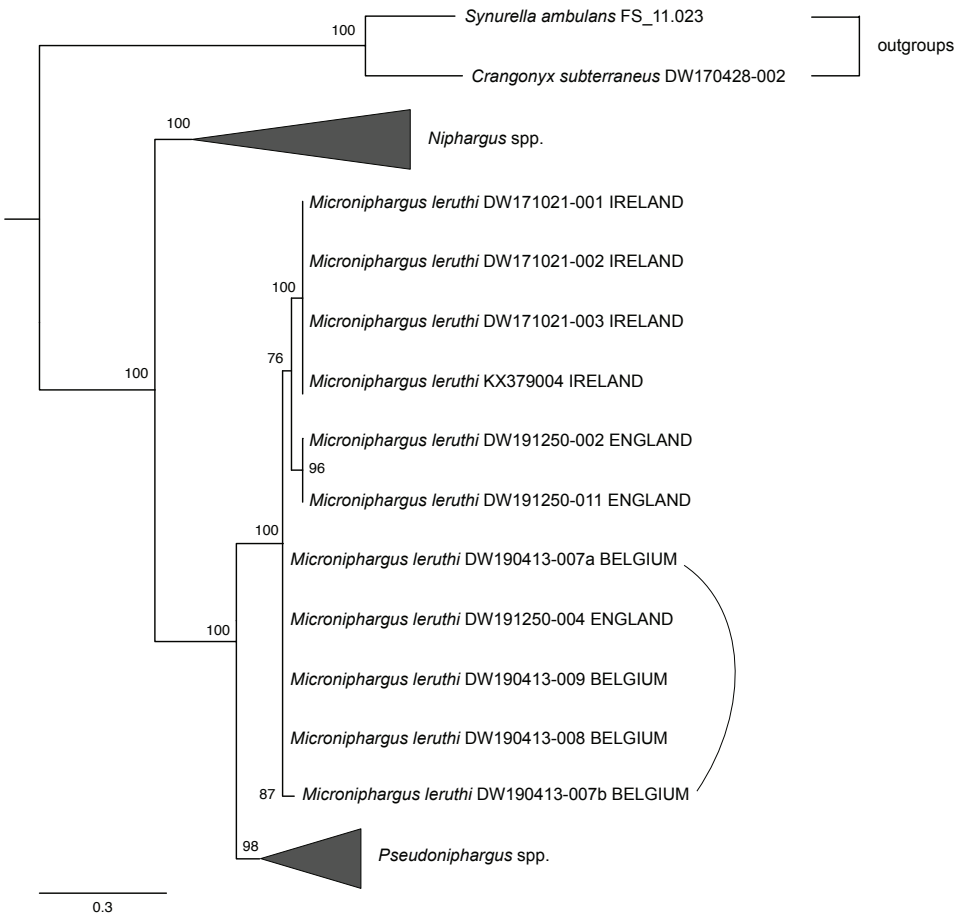

Figure 4 – 28S maximum-likelihood phylogeny of *Microniphargus*, *Niphargus* and *Pseudoniphargus* (with two crangonyctids as outgroups). The tree was turned into a haploweb by adding connections between haplotypes found co-occurring in the same individual.

The COI phylogeny supported a (*Microniphargus* + *Pseudoniphargus*) clade with 96% of ultrafast bootstrap replicates (Fig. 3 and Fig. S1) and revealed *Microniphargus* to be composed of three main clades A (found only in Ireland), B (found both in Belgium and in England) and C (found only in England), with > 99% ultrafast bootstrap support for each of them. Clade B contained two subclades comprising respectively Belgian and English sequences, also with > 99% ultrafast bootstrap support. Clade B and C co-occurred at one sampling site (Fig. 1). The sister-clade relationship between *Pseudoniphargus* and *Microniphargus* was supported with 100% of ultrafast bootstrap replicates in the comprehensive 28S phylogeny, which supported also the monophyly of lineages A and C (with 100% and 96% bootstrap replicates, respectively) but not of B, which was paraphyletic using this marker (Fig. 4 and Fig. S2).

The COI lineages A, B and C were separated by average p-distances of 0.073–0.081 between A and B, 0.072 between A and C, and 0.066 between B and C; whereas the p-distance between the two sub-lineages of B was 0.029. ABGD's initial partitioning of our comprehensive COI dataset supported a three-species hypothesis for *Microniphargus leruthi*, whereas the recursive partitioning favoured a four-species hypothesis separating the Belgian and English sub-lineage of lineage C. The KoT analysis of

Figure 5 – Output of the program KoT applying the K/θ method for species delimitation to the *Microniphargus* COI dataset. At each node are shown the average (Jukes-Cantor corrected) distance K between the corresponding sister clades, Watterson's estimator of genetic diversity θ of each of the two clades, and the ratio of K divided by the largest of the two θ values (for details of the method see Spöri *et al.* 2021). Species were delimited using a threshold K/θ value of 6, i.e. sister clades exhibiting K/θ ratios greater than 6 were considered as putative distinct species.

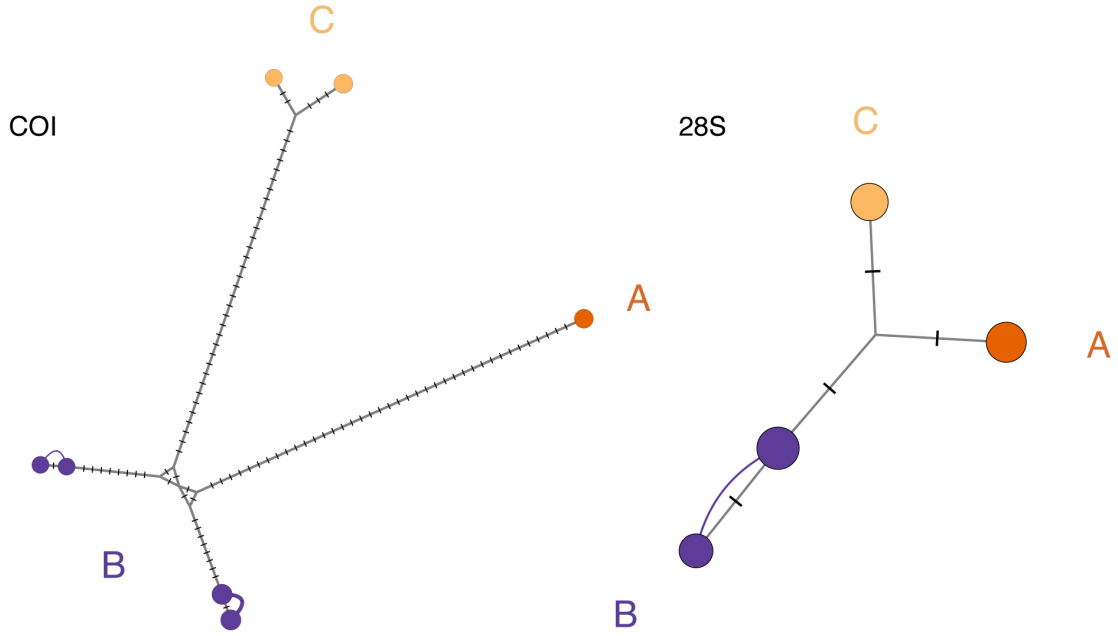

Figure 6 – Median-joining networks of the *Microniphargus* COI and 28S sequences obtained in the present study. The networks were turned into haplowebs by adding connections between haplotypes found co-occurring in the same individual.

the Microniphargus COI sequences supported a four-species scenario as well, with a K/θ ratio of 19.3 between the two subclades within lineage B, itself separated by a gap with a K/θ of 14.0 from lineage C, and finally separated by a gap with a K/θ ratio of 16.8 from lineage A (Fig. 5). By contrast, the 28S haploweb revealed three fields for recombination (FFRs *sensu* DOYLE 1995, i.e., putative species following the criterion of mutual allelic exclusivity; FLOT *et al*. 2010a), corresponding to clades A, B and C (Fig. 6).

Discussion

Key novel, high-quality sequences were acquired

Our newly collected sequences include the first COI sequences of *Microniphargus leruthi* (and of *Crangonyx subterraneus*) made available to date, as well as new 28S sequences that significantly improve the currently available sequences for these two species: the single Verovnik 28S fragment sequence available till now for *C. subterraneus* (EU693288; FIŠER *et al.* 2008) is 100% identical to ours (except for one obvious error at position 25), but its last 140 bp are lacking; the single 28S sequence of *M. leruthi* previously published (KX379004.1; FIŠER *et al.* 2017) is 100% identical to our complete sequences from Ireland, but with the first 59 bp and last 156 bp lacking; whereas the three *Pseudoniphargus* sequences available till now were also highly incomplete. The high-quality 28S and COI sequences we obtained from representative individuals of *C. subterraneus* from Germany*, Pseudoniphargus italicus* from Sicily and *P. spiniferus* from Basses Pyrénées in France, as well as from each of the three lineages of *M. leruthi* identified in our study, will make it easier to include these species as outgroups in future studies of *Niphargus*, *Pseudoniphargus* and other related genera.

Both COI and 28S sequences of *Microniphargus* were found to contain double peaks

Out of the nine *M. leruthi* individuals whose COI marker was sequenced, four (three from Belgium and one from England) presented a double peak in their COI chromatograms, resulting in an intraindividual polymorphism level of 0.15% in these individuals. For the three Belgian specimens the double peak was an R = A or G transition in position 101, whereas for the English specimen it was an S = C or G transversion in position 189 (Fig. 4). These mutations were not synonymous but corresponded to N (asparagine) ↔ D (aspartate) and A (alanine) ↔ G (glycine) mutations in the translation amino acid sequences. Such mitochondrial double peaks are rare in niphargids: for instance, no double peak was observed in the COI chromatograms of the 67 Romanian specimens sequenced in FLOT *et al*. (2014) nor reported for any of the hundreds of niphargids sequenced in EME *et al.* (2018). The presence of two distinct COI sequences in *M. leruthi* individuals may be the result of heteroplasmy, i.e., the presence of two distinct mitochondrial lineages in the cells of an organism, or of a recent numt, i.e., a nuclear pseudogene of a mitochondrial sequence following the transfer and integration of a copy of this sequence in a nuclear chromosome (DIERCKXSENS *et al.* 2020). Determining which one of these two hypotheses is correct in the present case will require whole-genome sequencing, which is beyond the scope of the present study, but in any case, the very limited divergence between the COI sequences found co-occurring in some individuals (with a single double peak per individual) did not hinder downstream phylogenetic analyses.

The genus *Microniphargus* is more closely related to *Pseudoniphargus* than to *Niphargus*

Our COI and 28S phylogenetic trees confirm that all collected specimens assigned to the morphospecies *Microniphargus leruthi* form a monophyletic group that is clearly distinct from *Niphargus* and *Pseudoniphargus*, thereby confirming its status as a separate genus previously established on the sole basis of morphological characters (SCHELLENBERG 1934). The results of our analysis confirm the

conclusions reached by MOŠKRIČ & VEROVNIK (2019) and COPILAŞ-CIOCIANU *et al*. (2020) on the close affinity between *Microniphargus* and *Pseudoniphargus*, suggesting the inclusion of the genus *Microniphargus* within the family Pseudoniphargidae to avoid paraphyly of Niphargidae. Consequently, superfamilies Allocrangonyctoidea and Crangonyctoidea as proposed by LOWRY & MYERS (2013, 2017) turn out to be paraphyletic.

As mentioned in the introduction, a similarity between the two genera can be found in the shape of the telson (which is widely incised and carries one spine on each lobe), and also partly the shape of gnathopod 1. This shape of telson as well as the protrusion on the carpus of gnathopod 1 are found also in Bogidiellidae (another family placed in recent phylogenetic trees not far away from the clade Niphargidae+Pseudoniphargidae: COPILAŞ-CIOCIANU *et al*. 2020) and may be simply symplesiomorphic, in which case the deeply incised, bilobated telson of *Niphargus* would represent an apomorphic character of this genus. However, the small size of *Microniphargus*, the reduced setation of mandibular palp and gnathopods, the lack of elongation of the third uropod in males, and the 1-articulated accessory flagellum of antennulae suggest a major role of paedomorphosis, making it difficult or impossible to correctly allocate this genus within current amphipod taxonomy and phylogeny based on morphological characters alone.

The inclusion of *Microniphargus* within Pseudoniphargidae requires an adjustment in the diagnosis of the family, recently revised by LOWRY & MYERS (2013), with minor changes as follows:

Body depigmented, eyes absent. Antenna 1 longer than antenna 2; accessory flagellum short, or minute, 1–2 articulated. Gnathopod 1 smaller (or weaker) than gnathopod 2; propodus with multiple groups of simple or bifid setae along palmar margin. Urosomites 1 to 3 free, without robust dorsal setae. Urosomite 1 without distoventral robust seta. Uropod 3 biramous; inner ramus minute; outer ramus article 2 absent. Telson notched, distal margin emarginate or nearly straight, with 1–3 robust spines on each lobe.

Microniphargus leruthi comprises at least three cryptic lineages

Our COI phylogeny, ABGD's initial partitioning of our comprehensive COI dataset and our haploweb analysis of 28S sequences of *Microniphargus* support the hypothesis that *Microniphargus leruthi* is composed of three distinct, putatively species-level lineages: clade A found in Ireland, clade B found both in England and in Belgium (with two COI sub-clades consistent with the geographic distance between these two locations), and clade C found so far only in England. By contrast, ABGD's recursive partitioning supports a four-species hypothesis, and so does the KoT analysis. However, the p-distances between the three main lineages A, B and C are all well above the 3% species-level threshold traditionally considered in barcoding studies (HEBERT *et al.* 2003), whereas the average p-distance between the two COI sub-clades of B falls below this symbolic threshold. These arguments, together with the fact that all individuals of lineage B (and only these individuals) display double peaks in their COI chromatograms, lead us to consider tentatively the two sub-clades of lineage B as conspecific and therefore to distinguish at present only three putative species-level lineages within *M. leruthi*.

Although lineage A (found only in Ireland to date) appears geographically separated from the other two, lineages B and C occur in sympatry in at least one location (Swildon's Hole in Somerset), bringing further support to the hypothesis that these two lineages are distinct species. The phylogenetic analysis based on COI could point to an origin of the genus *Microniphargus* in England with subsequent dispersals to Ireland and to Belgium; however, more samples and analyses will be required to test this hypothesis. The fact that lineage B still occurs on both sides of the English Channel is not overly surprising since the land connection between England and continental Europe was only severed about 8,000 years ago (WALLER & LONG 2003).

The hypothesis that the three *Microniphargus leruthi* lineages identified here represent distinct cryptic (or pseudo-cryptic) species will need to be tested further. Doing so will require further collecting and sequencing, as well as detailed morphological analyses using microscopy techniques appropriate for such small specimens.

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References

Arnscheidt J., Hahn H.J. & Fuchs A. (2008). Aquatic subterranean Crustacea in Ireland: results and new records from a pilot study. *Cave and Karst Science* 35 (1): 53–58.

Astrin J.J. & Stüben P.E. (2008). Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). *Invertebrate Systematics* 22 (5): 503–522. https://doi.org/10.1071/IS07057

Bauzà -Ribot M.M., Juan C., Nardi F., Oromi P., Pons J. & Jaume D. (2012). Mitogenomic phylogenetic analysis supports continental-scale vicariance in subterranean thalassoid crustaceans. *Current Biology* 22: 2069–2074. https://doi.org/10.1016/j.cub.2012.09.012

Birky C.W. (2013). Species detection and identification in sexual organisms using population genetic theory and DNA sequences. *PLoS ONE* 8: e52544. https://doi.org/10.1371/journal.pone.0052544

Birky C.W., Adams J., Gemmel M. & Perry J. (2010). Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. *PLoS ONE* 5: e10609. https://doi.org/10.1371/journal.pone.0010609

Borko Š., Collette M., Brad T., Zakšek V., Flot J.-F., Vaxevanopoulos M., Sarbu S.M. & Fišer C. (2019). Amphipods in a Greek cave with sulphidic and non-sulphidic water: phylogenetically clustered and ecologically divergent. *Systematics and Biodiversity* 17 (6): 558–572. https://doi.org/10.1080/14772000.2019.1670273

Copilaş-Ciocianu D., Fišer C., Borza P., Balázs G., Angyal D. & Petrusek A. (2017). Low intraspecific genetic divergence and weak niche differentiation despite wide ranges and extensive sympatry in two epigean *Niphargus* species (Crustacea: Amphipoda). *Zoological Journal of the Linnean Society* 181 (3): 485–499. https://doi.org/10.1093/zoolinnean/zlw031

Copilaş-Ciocianu D., Borko Š. & Fišer C. (2020). The late blooming amphipods: global change promoted post-Jurassic ecological radiation despite Palaeozoic origin. *Molecular Phylogenetics and Evolution* 143: 106664. https://doi.org/10.1016/j.ympev.2019.106664

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

Dierckxsens N., Mardulyn P. & Smits G. (2020). Unraveling heteroplasmy patterns with NOVOPlasty. *NAR Genomics and Bioinformatics* 2 (1): lqz011. https://doi.org/10.1093/nargab/lqz011

Dobreanu E. & Manolache C. 1933. Zur Kenntnis der Amphipodenfauna Rumäniens. *Notationes Biologicae* 1 (3): 102–108.

DOYLE J.J. (1995). The irrelevance of allele tree topologies for species delimitation, and a non-topological alternative. *Systematic Botany* 20 (4): 574–588. https://doi.org/10.2307/2419811

EME D., ZAGMAJSTER M., DELIĆ T., FIŠER C., FLOT J.-F., KONECNY-DUPRÉ L., PÁLSSON S., STOCH F., ZAKŠEK V., DOUADY C.J. & MALARD F. (2018). Do cryptic species matter in macroecology? Sequencing European groundwater crustaceans yields smaller ranges but does not challenge biodiversity determinants. *Ecography* 41 (2): 424–436. https://doi.org/10.1111/ecog.02683

FIŠER C., KONEC M., ALTHER R., ŠVARA V. & ALTERMATT F. (2017). Taxonomic, phylogenetic and ecological diversity of *Niphargus* (Amphipoda: Crustacea) in the Hölloch cave system (Switzerland). *Systematics and Biodiversity* 15 (3): 218–237. https://doi.org/10.1080/14772000.2016.1249112

FIŠER C., SKET B. & TRONTELJ P. (2008). A phylogenetic perspective on 160 years of troubled taxonomy of *Niphargus* (Crustacea: Amphipoda). *Zoologica Scripta* 37 (6): 665–680. https://doi.org/10.1111/j.1463-6409.2008.00347.x

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

FLOT J.-F., WÖRHEIDE G. & DATTAGUPTA S. (2010b). 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., BAUERMEISTER J., BRAD T., HILLEBRAND-VOICULESCU A., SARBU S.M., DATTAGUPTA S. (2014). *Niphargus–Thiothrix* associations may be widespread in sulphidic groundwater ecosystems: evidence from southeastern Romania. *Molecular Ecology* 23 (6): 1405–1417. https://doi.org/10.1111/mec.12461

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 (5): 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

FUCHS A. (2007). *Erhebung und Beschreibung der Grundwasserfauna in Baden-Württemberg*. Thesis, Koblenz-Landau.

HEBERT P.D.N., CYWINSKA A., BALL S.L. & DE WAARD J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society. Biological Sciences* 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

HOANG D.T., CHERNOMOR O., VON HAESELER A., MINH B.Q. & VINH L.S. (2018). UFBoot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution* 35: 518–522. https://doi.org/10.1093/molbev/msx281

HOFFMANN J. (1963). Faune des Amphipodes du Grand-Duché de Luxembourg. *Archives de la Section des Sciences de l'Institut Grand-Ducal* Nouvelle Série 29: 77–128.

JURADO-RIVERA J.A., ÁLVAREZ G., CARO J.A., JUAN C., PONS J. & JAUME D. (2017). Molecular systematics of *Haploginglymus*, a genus of subterranean amphipods endemic to the Iberian Peninsula (Amphipoda: Niphargidae). *Contributions to Zoology* 86 (3): 239–260. https://doi.org/10.1163/18759866-08603004

KALYAANAMOORTHY S., MINH B., WONG T., VON HAESELER A. & JERMIIN L.S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods* 14: 587–589. https://doi.org/10.1038/nmeth.4285

KARAMAN G.S. & RUFFO S. (1986). Amphipoda: *Niphargus*-group (Niphargidae sensu Bousfield, 1982). *In*: BOTOSEANU L. (ed.) *Stygofauna mundi. A Faunistic, Distributional, and Ecological Synthesis of the World Fauna inhabiting Subterranean Waters (including the Marine Interstitial)*: 514–534. Brill, Leiden.

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

KNIGHT L.R.F.D. & GLEDHILL T. (2010). The discovery of *Microniphargus leruthi* Schellenberg, 1934 (Crustacea: Amphipoda: Niphargidae) in Britain and its distribution in the British Isles. *Zootaxa* 2655 (1): 52–56. https://doi.org/10.11646/zootaxa.2655.1.3

KNIGHT L.R.F.D. & PENK M. (2010). Groundwater Crustacea of Ireland: a survey of the stygobitic Malacostraca in caves and springs. *Biology & Environment Proceedings of the Royal Irish Academy* 110 (3): 211–235. https://doi.org/10.3318/BIOE.2010.110.3.211

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 (6): 1547–1549. https://doi.org/10.1093/molbev/msy096

LEFÉBURE T., DOUADY C.J., GOUY M., TRONTELJ P., BRIOLAY J. & GIBERT J. (2006). Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. *Molecular Ecology* 15: 1797–1806. https://doi.org/10.1111/j.1365-294X.2006.02888.x

LERUTH R. (1939). La biologie du domaine souterrain et la faune cavernicole de la Belgique. *Mémoire du Musée royal d'Histoire naturelle de Belgique* 87: 1–506.

LOWRY J.K. & MYERS A.A. (2013). A phylogeny and classification of the Senticaudata subord. nov. (Crustacea: Amphipoda). *Zootaxa* 3610 (1): 1–80. https://doi.org/10.11646/zootaxa.3610.1.1

LOWRY J.K. & MYERS A.A. (2017). A phylogeny and classification of the Amphipoda with the establishment of the new order Ingolfiellida (Crustacea: Peracarida). *Zootaxa* 4265 (1): 1–89. https://doi.org/10.11646/zootaxa.4265.1.1

MATZKE D., FUCHS A., BERKHOFF S., BORK J. & HAHN H.-J. (2009). *Erhebung und Bewertung der Grundwasserfauna Sachen-Anhalts*. Institut für Grundwasserökologie GbR, Im Niederfeld 15, 76829 Landau, Landau.

MCINERNEY C.E., MAURICE L., ROBERTSON A.L., KNIGHT L.R.F.D., ARNSCHEIDT J., VENDITTI C., DOOLEY J.S.G., MATHERS T., MATTHIJS S., ERIKKSON K., PROUDLOVE G.S. & HÄNFLING B. (2014). The Ancient Britons: groundwater fauna survived extreme climate changes over tens of millions of years across NW Europe. *Molecular Ecology* 23: 1153–1166. https://doi.org/10.1111/mec.12664

MINH B.Q., SCHMIDT H.A., CHERNOMOR O., SCHREMPF D., WOODHAMS M.D., VON HAESELER A., LANFEAR R. (2020). IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution* 37: 1530–1534. https://doi.org/10.1093/molbev/msaa015

MOŠKRIČ A. & VEROVNIK R. (2019). Five nuclear protein-coding markers for establishing a robust phylogenetic framework of niphargid crustaceans (Niphargidae: Amphipoda) and new molecular sequence data. *Data in Brief* 25: 104134. https://doi.org/10.1016/j.dib.2019.104134

NOTENBOOM J. (1988). *Parapseudoniphargus baetis*, new genus, new species, a stygobiont amphipod crustacean from the Guadalquivir river basin (southern Spain), with phylogenetic implications. *Journal of Crustacean Biology* 8: 110–121. https://doi.org/10.1163/193724088X00134

PUILLANDRE N., LAMBERT A., BROUILLET S. & ACHAZ G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21 (8): 1864–1877. https://doi.org/10.1111/j.1365-294X.2011.05239.x

SCHIÖDTE J.C. (1849). *Specimen faunæ subterraneae: bidrag til den underjordiske Fauna*. Bianco Luno, Kjöbenhavn.

SCHELLENBERG A. (1934). Eine neue Amphipoden-Gattung aus einer belgischen Höhle, nebst Bemerkungen über die Gattung *Crangonyx*. *Zoologischer Anzeiger* 106 (9): 215–218.

SCHÖN I., PINTO R.L., HALSE S., SMITH A.J., MARTENS K. & BIRKY C.W. (2012). Cryptic species in putative ancient asexual darwinulids (Crustacea, Ostracoda). *PLoS ONE* 7: e39844. https://doi.org/10.1371/journal.pone.0039844

SCHWARZ G. (1978). Estimating the dimension of a model. *The Annals of Statistics* 6 (2): 461–464. https://doi.org/10.1214/aos/1176344136

SPANGENBERG H.-J. (1973). Beitrag zur Faunistik von Höhlengewässern im Zechstein des Südharzes und Kyffhäusers. *Hercynia* Neue Folge, 10 (2): 143–160.

SPÖRI Y. & FLOT J.-F. (2020). HaplowebMaker and CoMa: two web tools to delimit species using haplowebs and conspecificity matrices. *Methods in Ecology and Evolution* 11 (11): 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*. https://doi.org/10.1101/2021.08.17.454531

STEIN H., GRIEBLER C., BERKHOFF S., MATZKE D., FUCHS A. & HAHN H.J. (2012). Stygoregions – a promising approach to a bioregional classification of groundwater systems. *Scientific Reports* 2: 673. https://doi.org/10.1038/srep00673

STOKKAN M., JURADO-RIVERA J.A., JUAN C., JAUME D. & PONS J. (2016). Mitochondrial genome rearrangements at low taxonomic levels: three distinct mitogenome gene orders in the genus *Pseudoniphargus* (Crustacea: Amphipoda). *Mitochondrial DNA Part A* 27: 3579–3589. https://doi.org/10.3109/19401736.2015.1079821

STOKKAN M., JURADO-RIVERA J.A., OROMÍ P., JUAN C., JAUME D. & PONS J. (2018). Species delimitation and mitogenome phylogenetics in the subterranean genus *Pseudoniphargus* (Crustacea: Amphipoda). *Molecular Phylogenetics and Evolution* 127: 988–999. https://doi.org/10.1016/j.ympev.2018.07.002

TRONTELJ P., DOUADY C.J., FIŠER C., GIBERT J., GORIČKI Š., LEFÉBURE T., SKET B. & ZAKŠEK V. (2009). A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? *Freshwater Biology* 54 (4): 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 (14): 4355–4369. https://doi.org/10.1111/j.1365-294X.2005.02745.x

WALLER M.P. & LONG A.J. (2003). Holocene coastal evolution and sea-level change on the southern coast of England: a review. *Journal of Quaternary Science* 18 (3–4): 351–359. https://doi.org/10.1002/jqs.754

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Appendix

Supplementary figures and tables

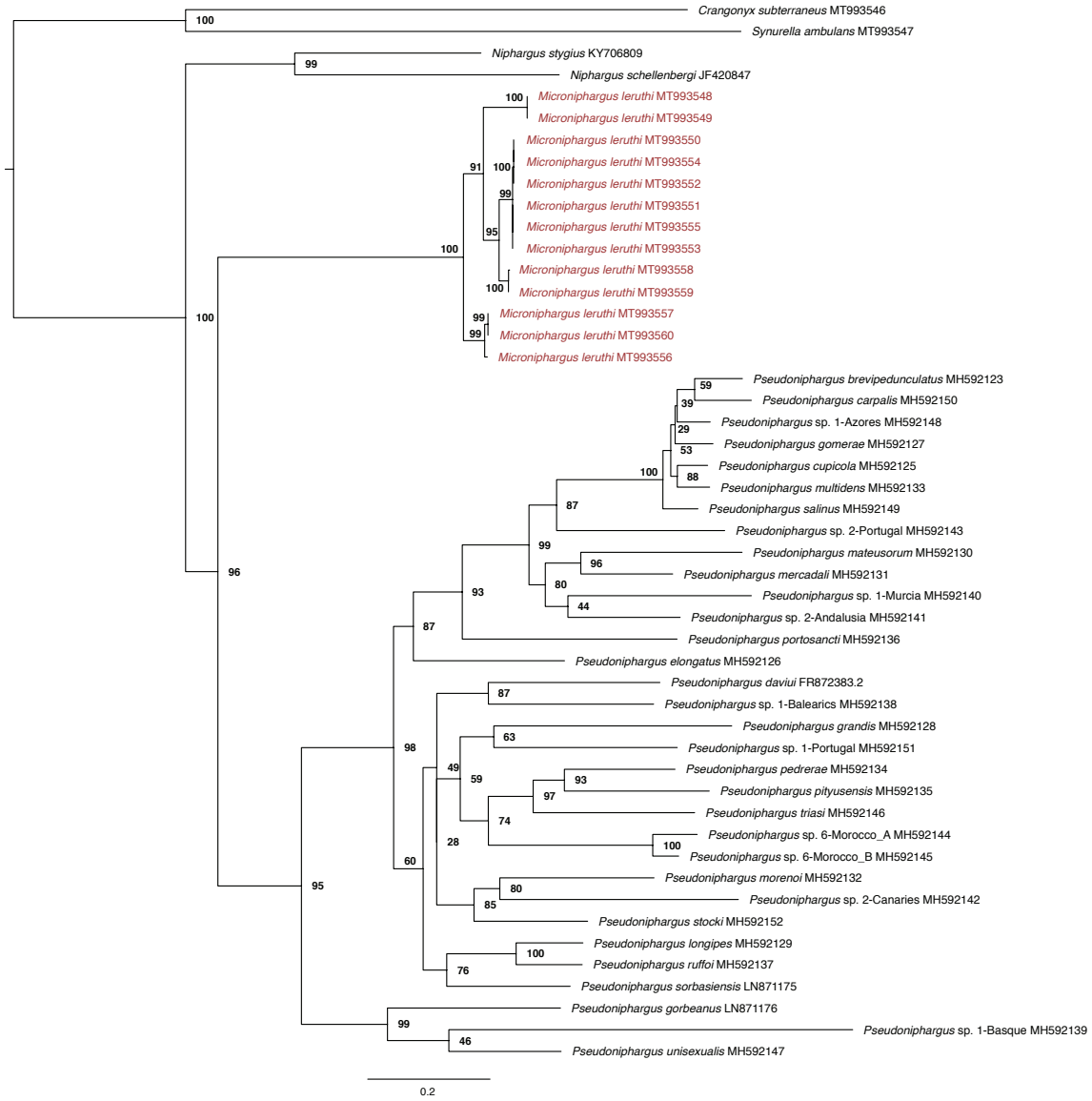

Figure S1 – Detailed version of the COI maximum-likelihood phylogeny.

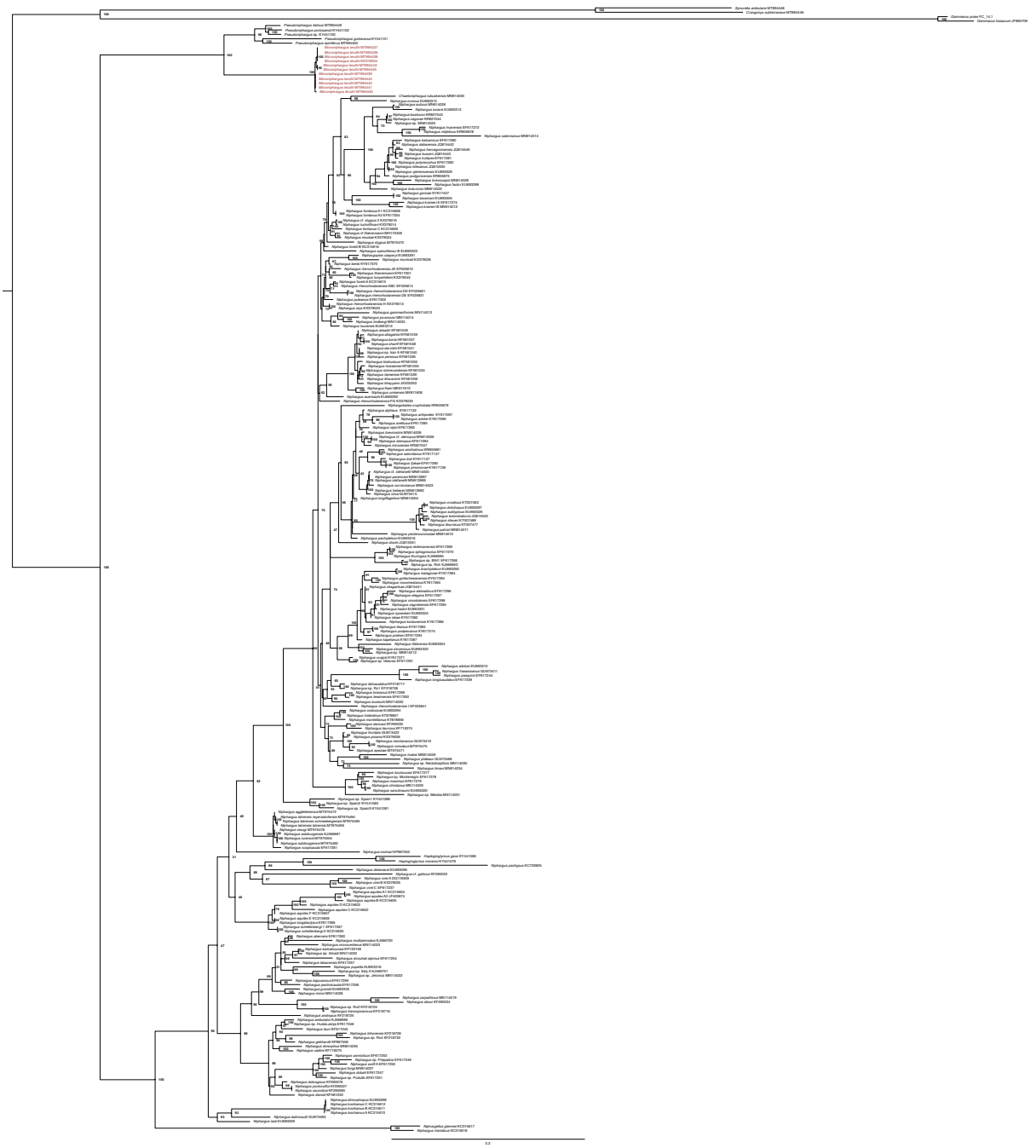

Figure S2 – Detailed version of the 28S maximum-likelihood phylogeny.

TABLE S1

List of all the sequences included in the COI phylogeny, including species names and GenBank accession numbers.

ID	Species	GB accession number
1	*Crangonyx subterraneus*	MT993546
2	*Synurella ambulans*	MT993547
3	*Niphargus stygius*	KY706809
4	*Niphargus schellenbergi*	JF420847
5	*Microniphargus leruthi* A	MT993548
6	*Microniphargus leruthi* A	MT993549
7	*Microniphargus leruthi* B	MT993550
8	*Microniphargus leruthi* B	MT993551
9	*Microniphargus leruthi* B	MT993552
10	*Microniphargus leruthi* B	MT993553
11	*Microniphargus leruthi* B	MT993554
12	*Microniphargus leruthi* B	MT993555
13	*Microniphargus leruthi* C	MT993557
14	*Microniphargus leruthi* A	MT993558
15	*Microniphargus leruthi* A	MT993559
16	*Microniphargus leruthi* C	MT993560
17	*Microniphargus leruthi* C	MT993556
18	*Pseudoniphargus brevipedunculatus*	MH592123
19	*Pseudoniphargus carpalis*	MH592150
20	*Pseudoniphargus cupicola*	MH592125
21	*Pseudoniphargus daviui*	FR872383.2
22	*Pseudoniphargus elongatus*	MH592126
23	*Pseudoniphargus gomerae*	MH592127
24	*Pseudoniphargus gorbeanus*	LN871176
25	*Pseudoniphargus grandis*	MH592128
26	*Pseudoniphargus longipes*	MH592129
27	*Pseudoniphargus mateusorum*	MH592130
28	*Pseudoniphargus mercadali*	MH592131
29	*Pseudoniphargus morenoi*	MH592132
30	*Pseudoniphargus multidens*	MH592133
31	*Pseudoniphargus pedrerae*	MH592134
32	*Pseudoniphargus pityusensis*	MH592135
33	*Pseudoniphargus portosancti*	MH592136
34	*Pseudoniphargus ruffoi*	MH592137
35	*Pseudoniphargus salinus*	MH592149
36	*Pseudoniphargus sorbasiensis*	LN871175
37	*Pseudoniphargus* sp. 1-Azores	MH592148
38	*Pseudoniphargus* sp. 1-Balearics	MH592138
39	*Pseudoniphargus* sp. 1-Basque	MH592139
40	*Pseudoniphargus* sp. 1-Murcia	MH592140
41	*Pseudoniphargus* sp. 1-Portugal	MH592151
42	*Pseudoniphargus* sp. 2-Andalusia	MH592141
43	*Pseudoniphargus* sp. 2-Canaries	MH592142
44	*Pseudoniphargus* sp. 2-Portugal	MH592143
45	*Pseudoniphargus* sp. 6-Morocco A	MH592144
46	*Pseudoniphargus* sp. 6-Morocco B	MH592145
47	*Pseudoniphargus stocki*	MH592152
48	*Pseudoniphargus triasi*	MH592146
49	*Pseudoniphargus unisexualis*	MH592147

TABLE S2 (continued on next four pages)

List of all the sequences included in the 28S phylogeny, including species names and GenBank accession numbers.

ID	Species	Accessionnumber
1	*Gammarus pulex*	MT994447
2	*Gammarus fossarum*	JF965709
3	*Synurella ambulans*	MT994448
4	*Crangonyx subterraneus*	MT994446
5	*Pseudoniphargus italicus*	MT994449
6	*Pseudoniphargus gorbeanus*	KY441101
7	*Pseudoniphargus portosancti*	KY441102
8	*Pseudoniphargus spiniferus*	MT994450
9	*Pseudoniphargus* sp.	KY441100
10	*Micronipharg*us *leruthi*	MT994437
11	*Micronipharg*us *leruthi*	MT994438
12	*Micronipharg*us *leruthi*	MT994439
13	*Micronipharg*us *leruthi*	MT994440
14	*Micronipharg*us *leruthi*	MT994441
15	*Micronipharg*us *leruthi*	MT994442
16	*Micronipharg*us *leruthi*	MT994443
17	*Micronipharg*us *leruthi*	MT994444
18	*Micronipharg*us *leruthi*	MT994445
19	*Micronipharg*us *leruthi*	MT994436
20	*Micronipharg*us *leruthi*	KX379004
21	*Chaetoniphargus lubuskensis*	MN914030
22	*Haploginglymus geos*	KY441086
23	*Haploginglymus morenoi*	KY441079
24	*Niphargellus glenniei*	KC315617
25	*Niphargobates orophobata*	KR905879
26	*Niphargopsis casparyi*	EU693291
27	*Niphargus aberrans*	EF617260
28	*Niphargus aggtelekiensis*	MT975472
29	*Niphargus aitolosi*	EU693310
30	*Niphargus alisadri*	KF581049
31	*Niphargus alpheus*	KY617132
32	*Niphargus altagahizi*	KF581059
33	*Niphargus ambulator*	KJ566699
34	*Niphargus anchialinus*	KR905881
35	*Niphargus andropus*	KF218725
36	*Niphargus antipodes*	KY617097
37	*Niphargus aquilex* A1	KC315604
38	*Niphargus aquilex* A2	JF420874
39	*Niphargus aquilex* B	KC315605
40	*Niphargus aquilex* C	KC315602
41	*Niphargus aquilex* D	KC315603
42	*Niphargus aquilex* E	KC315606
43	*Niphargus aquilex* F	KC315607
44	*Niphargus arbiter*	KY617099
45	*Niphargus arethusa*	EF617285
46	*Niphargus auerbachi*	EU693292
47	*Niphargus aulicus*	MN914026
48	*Niphargus bajuvaricus*	EF617259
49	*Niphargus balcanicus*	EF617280
50	*Niphargus bihorensis*	KF218726
51	*Niphargus bilecanus*	JQ815550
52	*Niphargus bisitunicus*	KF581050

TABLE S2 (continued)

ID	Species	Accessionnumber
53	*Niphargus borisi*	KF581037
54	*Niphargus boskovici*	KR827043
55	*Niphargus brachytelson*	EU693293
56	*Niphargus brevicuspis*	MN914028
57	*Niphargus brevirostris*	MN914008
58	*Niphargus brixianus*	EF617299
59	*Niphargus bureschi*	MN114020
60	*Niphargus buturovici*	MN914022
61	*Niphargus carniolicus*	EF617252
62	*Niphargus carpathicus*	MN114019
63	*Niphargus* cf. *gallicus*	KF290033
64	*Niphargus* cf. *stefanellii*	MN914000
65	*Niphargus* cf. *stenopus*	MN914009
66	*Niphargus* cf. *stygius* 2	KX379016
67	*Niphargus* cf. *thienemanni*	MH172406
68	*Niphargus chagankae*	JQ815441
69	*Niphargus cornicolanus*	MN914003
70	*Niphargus costozzae*	EU693294
71	*Niphargus croaticus*	KT007482
72	*Niphargus cvajcki*	KY617371
73	*Niphargus dabarensis*	JQ815442
74	*Niphargus dalmatinus*	EF617296
75	*Niphargus dancaui*	KF290030
76	*Niphargus daniali*	KF581033
77	*Niphargus darvishi*	KF581041
78	*Niphargus decui*	KF290034
79	*Niphargus delamarei*	EU693295
80	*Niphargus dimorphopus*	EU693296
81	*Niphargus dimorphus*	MN914035
82	*Niphargus dobati*	EF617247
83	*Niphargus dobrogicus*	KF290076
84	*Niphargus dolenianensis*	EF617269
85	*Niphargus doli*	KY617137
86	*Niphargus dolichopus*	EU693297
87	*Niphargus elegans*	EF617297
88	*Niphargus factor*	EU693298
89	*Niphargus fiseri*	MK911610
90	*Niphargus fjakae*	EF617290
91	*Niphargus fongi*	MN914037
92	*Niphargus fontanus* A1	KC315608
93	*Niphargus fontanus* A2	EF617304
94	*Niphargus fontanus* C	KC315609
95	*Niphargus forelii* A	KC315615
96	*Niphargus forelii* B	KC315616
97	*Niphargus frasassianus*	GU973411
98	*Niphargus frontalis*	GU973423
99	*Niphargus gammariformis*	MN114013
100	*Niphargus gebhardti*	KP967556
101	*Niphargus goricae*	KY617447
102	*Niphargus gottscheeanensis*	KY617394
103	*Niphargus grandii*	EU693300
104	*Niphargus hadzii*	EU693301
105	*Niphargus hebereri*	MN913992
106	*Niphargus hercegovinensis*	JQ815549

TABLE S2 (continued)

ID	Species	Accessionnumber
107	*Niphargus hosseiniei*	KF581055
108	*Niphargus hrabei*	MN914029
109	*Niphargus hvarensis*	EF617272
110	*Niphargus ictus*	GU973415
111	*Niphargus ilamensis*	KF581039
112	*Niphargus illidzensis*	EU693304
113	*Niphargus irlandicus*	KC315618
114	*Niphargus iskae*	KY617382
115	*Niphargus jovanovici*	MN114014
116	*Niphargus kapelanus*	KY617387
117	*Niphargus karamani*	EU693305
118	*Niphargus karkabounasi*	KP133156
119	*Niphargus kenki*	KY617370
120	*Niphargus khayyami*	JX535353
121	*Niphargus khwarizmi*	KF581056
122	*Niphargus kochianus* A	KC315610
123	*Niphargus kochianus* B	KC315611
124	*Niphargus kochianus* C	KC315612
125	*Niphargus kolombatovici*	JQ815553
126	*Niphargus kordunensis*	KY617386
127	*Niphargus koukourasi*	EF617277
128	*Niphargus krameri* A	EF617274
129	*Niphargus krameri* B	MN914013
130	*Niphargus kusceri*	JQ815443
131	*Niphargus labacensis*	EF617257
132	*Niphargus ladmiraulti*	GU973463
133	*Niphargus laisi*	EU693309
134	*Niphargus laticaudatus*	KF218717
135	*Niphargus lessiniensis*	EF617300
136	*Niphargus liburnicus*	KT007477
137	*Niphargus likanus*	KY617383
138	*Niphargus lindbergi*	MN114033
139	*Niphargus longicaudatus*	EF617239
140	*Niphargus longidactylus*	EF617266
141	*Niphargus longiflagellum*	MN914004
142	*Niphargus lourensis*	EU693312
143	*Niphargus luchoffmani*	KX379014
144	*Niphargus lunaris*	EU693313
145	*Niphargus malagorae*	KY617384
146	*Niphargus maximus*	EF617279
147	*Niphargus microcerberus*	MN114023
148	*Niphargus miljeticus*	KR905878
149	*Niphargus minor*	MN114028
150	*Niphargus mirocensis*	KR827047
151	*Niphargus molnari*	KP967555
152	*Niphargus montanarius*	GU973419
153	*Niphargus montellianus*	KT878856
154	*Niphargus moogi*	MT975478
155	*Niphargus multipennatus*	KJ566700
156	*Niphargus muotae*	KX379024
157	*Niphargus murimali*	KX379026
158	*Niphargus novomestanus*	KY617364
159	*Niphargus ohridanus*	MN114029
160	*Niphargus orcinus*	EU693315

TABLE S2 (continued)

ID	Species	Accessionnumber
161	*Niphargus pachypus*	KC733825
162	*Niphargus pachytelson*	EU693316
163	*Niphargus parenzani*	MN913997
164	*Niphargus pasquinii*	EF617244
165	*Niphargus patrizii*	MN914011
166	*Niphargus pectencoronatae*	MN914010
167	*Niphargus pectinicauda*	EF617258
168	*Niphargus persicus*	KF581036
169	*Niphargus pincinovae*	KY617139
170	*Niphargus plateaui*	GU973468
171	*Niphargus podgoricensis*	KR905875
172	*Niphargus podpecanus*	KY617374
173	*Niphargus poianoi*	KX379006
174	*Niphargus polymorphus*	EF617282
175	*Niphargus pontoruffoi*	KF290027
176	*Niphargus pretneri*	EF617294
177	*Niphargus pupetta*	EU693318
178	*Niphargus puteanus*	EF617302
179	*Niphargus racovitzai*	KF290065
180	*Niphargus rejici*	EF617283
181	*Niphargus rhenorhodanensis* ABC	EF025814
182	*Niphargus rhenorhodanensis* DE	EF025801
183	*Niphargus rhenorhodanensis* DE	EF025831
184	*Niphargus rhenorhodanensis* FG	KX379033
185	*Niphargus rhenorhodanensis* H	KX379013
186	*Niphargus rhenorhodanensis* I	EF025841
187	*Niphargus rhenorhodanensis* JK	EF025810
188	*Niphargus romuleus*	MT975475
189	*Niphargus salernianus*	MN914014
190	*Niphargus salonitanus*	KY617147
191	*Niphargus salzburgensis*	KJ566697
192	*Niphargus sanctinaumi*	EU693320
193	*Niphargus schellenbergi* 1	EF617267
194	*Niphargus schellenbergi* 2	KC315620
195	*Niphargus scopicauda*	EF617261
196	*Niphargus sharifi*	KF581048
197	*Niphargus slovenicus*	EU693322
198	*Niphargus sohrevardensis*	KF581034
199	*Niphargus* sp. Arkadi	MN114032
200	*Niphargus* sp. BIH1	EF617268
201	*Niphargus* sp. HudaLuknja	EF617246
202	*Niphargus* sp. Iran 9	KF581040
203	*Niphargus* sp. Iskavas	EF617291
204	*Niphargus* sp. Italy A	KJ566701
205	*Niphargus* sp. Jelovica	MN114022
206	*Niphargus* sp. MN914012	MN914012
207	*Niphargus* sp. MN914023	MN914023
208	*Niphargus* sp. Meskla	MN114031
209	*Niphargus* sp. Montenegro	EF617278
210	*Niphargus* sp. Neraidosplilios	MN114030
211	*Niphargus* sp. Podutik	EF617251
212	*Niphargus* sp. Prepadna	EF617249
213	*Niphargus* sp. Ro1	KF218728
214	*Niphargus* sp. Ro2	KF218724

TABLE S2 (continued)

ID	Species	Accession number
215	*Niphargus* sp. Ro4	KF218732
216	*Niphargus* sp. Ro5	KJ566693
217	*Niphargus* sp. Spain1	KY441088
218	*Niphargus* sp. Spain2	KY441083
219	*Niphargus* sp. Spain3	KY441081
220	*Niphargus speziae*	MT975471
221	*Niphargus sphagnicolus*	EF617270
222	*Niphargus spinulifemur* B	EU693323
223	*Niphargus spoeckeri*	EU693324
224	*Niphargus stefanellii*	MN913999
225	*Niphargus stenopus*	EF617284
226	*Niphargus steueri*	KT007489
227	*Niphargus stochi*	JQ815551
228	*Niphargus strouhali alpinus*	EF617254
229	*Niphargus stygius*	MT975470
230	*Niphargus styx*	KX379023
231	*Niphargus subtypicus*	EU693326
232	*Niphargus lurensis*	MT975504
233	*Niphargus tatrensis reyesdorfensis*	MT975494
234	*Niphargus salzburgensis*	MT975492
235	*Niphargus tatrensis schneebergensis*	MT975495
236	*Niphargus tatrensis tatrensis*	MT975496
237	*Niphargus tauri*	EF617245
238	*Niphargus tauricus*	KF719274
239	*Niphargus thienemanni*	EF617301
240	*Niphargus thuringius*	KJ566695
241	*Niphargus timavi*	MN914034
242	*Niphargus tonywhitteni*	KX379045
243	*Niphargus transsylvanicus*	KF218716
244	*Niphargus tridentinus*	KT878857
245	*Niphargus trullipes*	EF617281
246	*Niphargus urmiensis*	MK911608
247	*Niphargus vadimi*	KF719275
248	*Niphargus vinodolensis*	EF617298
249	*Niphargus virei* A	DQ119309
250	*Niphargus virei* B	KX379035
251	*Niphargus virei* C	EF617237
252	*Niphargus vjetrenicensis*	EU693329
253	*Niphargus wolfi* A	EF617250
254	*Niphargus zagorae*	KR827044
255	*Niphargus zagrebensis*	EF617295