The genus *Microniphargus* (Crustacea, Amphipoda): evidence for three lineages distributed across northwestern Europe and transfer 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 1,469km from Ireland to Germany, which is striking since only a few niphargids have confirmed ranges in excess of 200km. 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, DNA barcoding, cryptic species, *Microniphargus leruthi*, Niphargidae, Pseudoniphargidae

Running title

Microniphargus in northwestern Europe

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* Schiødte, 1849 (Schellenberg 1934). *M. leruthi* is characterised by its small body size (1.2-1.5mm 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* when molecular data became available (see e.g. Borko *et al.* 2020).

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 1988a for a detailed analysis). Notenboom (1988b) 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 (2012, 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, Copilas-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 Copilas-Ciocianu *et al.* (2020), in a large-scale phylogeny of amphipods comprising 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 200km (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 (McInerney et al. 2014; Lefébure et al. 2006). The only species with confirmed ranges more extended than *M. leruthi* are *Niphargus hrabei* S. Karaman, 1932 (>1,300km) and *Niphargus valachicus* Doboreanu & Manolache 1933 (>3,200km), 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 in Galway) and two sites in England (Sweetwater Pot in South Devon and Swildon's Hole in 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 in order to suspend both 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 whole 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 μ L) 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 1 μ L DNA template (variable concentration), 0.8 μ L of each primer (10pmol/ μ L), 5 μ L of DreamTaq DNA Polymerase (Thermo Scientific) and 2.4 μ L ultrapure water. PCR cycling conditions were an initial 3-min denaturation step at 94°C followed by 36 cycles of 20s denaturation at 94°C, 45s annealing at 50°C, and 60s 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 2 μ L of DNA template (variable concentration), 1 μ L of each primer (10pmol/ μ L), 0.2 μ L of REDTaq DNA Polymerase (Sigma-Aldrich), 5 μ L REDTaq reaction buffer and 15.8 μ L 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, Wörheide, *et al.* 2010) 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 “a” and “b” in downstream analyses.

Phylogenetic 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 (Kato *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 at <https://eeg-ebi.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 p-distances.

Results

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

Our comprehensive 28S phylogeny including all published sequences of *Microniphargus*, *Niphargus*, *Pseudoniphargus* supported a (*Microniphargus* + *Pseudoniphargus*) clade with 100% of the ultrafast bootstrap replicates (Fig. 3). Our COI phylogeny confirmed this sister-clade relationship between *Pseudoniphargus* and *Microniphargus* (supported by 96% of the ultrafast bootstrap replicates) and revealed the latter 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 comprised 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 COI lineages A, B and C were separated by averages 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 C 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 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. 2010) corresponding to clades A, B and C (Fig. 5).

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's 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. For the three Belgian specimens it was a R = A or G transition in position 101, whereas for the English specimen it was S = C or G transversion in position 189. 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 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 show that *Microniphargus* is monophyletic and clearly distinct from *Niphargus* and *Pseudoniphargus*, thereby confirming its status as a separate genus established on 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 (2012, 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, bilobed 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 (2012), 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 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. Although ABGD's recursive partitioning supports a four-species hypothesis, the p-distances between these three main lineages 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 C as conspecific and therefore to distinguish 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 marker strongly suggest an origin of the genus *Microniphargus* in England, with subsequent dispersals to Ireland and to Belgium. 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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Figures and tables

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). Red: lineage A; blue: lineage B; green: lineage C.

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: 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.

Figure 4: 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 5: 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.

Table 1: Material newly sequenced for the present study

Table 2: Primers used in the present study

Supplementary material

Figure S1: Complete, uncondensed version of the 28S maximum-likelihood phylogeny in Figure 3

Figure S2: Complete, uncondensed version of the COI maximum-likelihood phylogeny in Figure 4

Table S1: List of all the sequences included in the 28S phylogeny, including species names and GenBank accession numbers

Table S2: List of all the sequences included in the COI phylogeny, including species names and GenBank accession numbers

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). Red: lineage A; blue: lineage B; green: lineage C.

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Table 1: Material newly sequenced for the present study

Voucher code	Species	Sampling site	Country	Latitude (WGS84)	Longitude (WGS84)	Collectors	Date	COI acc numbers	28S acc numbers
DW170428-002	<i>Crangonyx subterraneus</i>	Interstitial Mümling	Germany	49.843	9.094	Weber D.	28/04/17		
FS_11.023	<i>Synurella ambulans</i>	Lake of Sablici	Italy	45.806	13.576	Stoch F., Fior G.	27/02/11		
FC_14.1	<i>Gammarus pulex</i>	Spring Moulin de Grisendal	France	50.763	1.661	Carouille F.	26/10/14	-	
FS_14.036	<i>Pseudoniphargus italicus</i>	Spring near Marineo	Italy	37.946	13.406	Marrone F.	19/04/13	-	
FS_18.073	<i>Pseudoniphargus spiniferus</i>	Grotte d'Istaurdy	France	43.111	-1.038	Brustel H.	10/05/18	-	
DW191250-001	<i>Microniphargus leruthi</i>	Sweetwater Pot	England	50.399	-3.491	Knight L.	24/11/19		-
DW191250-002	<i>Microniphargus leruthi</i>	Sweetwater Pot	England	50.399	-3.491	Knight L.	24/11/19		
DW191250-004	<i>Microniphargus leruthi</i>	Swildon's Hole	England	51.259	-2.673	Knight L.	02/11/19		
DW191250-011	<i>Microniphargus leruthi</i>	Swildon's Hole	England	51.259	-2.673	Knight L.	02/11/19		
DW171021-001	<i>Microniphargus leruthi</i>	Pollidubh Cave	Ireland	53.078	-9.287	Knight L., Boulton J., Weber D.	21/10/17		
DW171021-002	<i>Microniphargus leruthi</i>	Pollidubh Cave	Ireland	53.078	-9.287	Knight L., Boulton J., Weber D.	21/10/17	-	
DW171021-003	<i>Microniphargus leruthi</i>	Pollidubh Cave	Ireland	53.078	-9.287	Knight L., Boulton J., Weber D.	21/10/17		
DW190413-007	<i>Microniphargus leruthi</i>	Grotte de Comblain	Belgium	50.476	5.566	Knight L., Boulton J., Weber D.	13/04/19		
DW190413-008	<i>Microniphargus leruthi</i>	Grotte de Comblain	Belgium	50.476	5.566	Knight L., Boulton J., Weber D.	13/04/19		
DW190413-009	<i>Microniphargus leruthi</i>	Grotte de Comblain	Belgium	50.476	5.566	Knight L., Boulton J., Weber D.	13/04/19		

Note: accession numbers will be added upon acceptance of manuscript

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	Astrin <i>et al.</i> 2008
HCO2198-JJ	5'-AWA CTT CVG GRT GVC CAA ARA ATC A-3'	COI	x	x	Astrin <i>et al.</i> 2008
Niph15	5'-CAA GTA CCG TGA GGG AAA GTT-3'	28S	x		Verovnik <i>et al.</i> 2005
Niph15i	5'-AGA GTC AAA AGA CCG TGA AAC C-3'	28S		x	unpublished
Niph16	5'-AGG GAA ACT TCG GAG GGA ACC-3'	28S	x		Verovnik <i>et al.</i> 2005
Niph16i	5'-GAT TGG TCT TTC GCC CCT AT-3'	28S		x	unpublished
Niph20	5'-AAA CAC GGG CCA AGG AGT AT-3'	28S		x	Flot <i>et al.</i> 2010
Niph21	5'-TAT ACT CCT TGG CCC GTG TT-3'	28S		x	Flot <i>et al.</i> 2010

Figure S2: Complete, uncondensed version of the COI maximum-likelihood phylogeny in Figure 4

Table S1: List of all the sequences included in the 28S phylogeny, including species names and GenBank accession numbers

ID	Species	Accession number	ID	Species	Accession number
1	<i>Gammarus pulex</i>	FC_14.1	129	<i>Niphargus krameri</i> B	MN914013
2	<i>Gammarus fossarum</i>	JF965709	130	<i>Niphargus kusceri</i>	JQ815443
3	<i>Synurella ambulans</i>	FS_11.023	131	<i>Niphargus labacensis</i>	EF617257
4	<i>Crangonyx subterraneus</i>	DW170428-002	132	<i>Niphargus ladmiraulti</i>	GU973463
5	<i>Pseudoniphargus italicus</i>	FS_14.036	133	<i>Niphargus laisi</i>	EU693309
6	<i>Pseudoniphargus gorbeanus</i>	KY441101	134	<i>Niphargus laticaudatus</i>	KF218717
7	<i>Pseudoniphargus portosancti</i>	KY441102	135	<i>Niphargus lessiniensis</i>	EF617300
8	<i>Pseudoniphargus spiniferus</i>	FS_18.073	136	<i>Niphargus liburnicus</i>	KT007477
9	<i>Pseudoniphargus</i> sp.	KY441100	137	<i>Niphargus likanus</i>	KY617383
10	<i>Microniphargus leruthi</i>	DW171021-002	138	<i>Niphargus lindbergi</i>	MN114033
11	<i>Microniphargus leruthi</i>	DW171021-003	139	<i>Niphargus longicaudatus</i>	EF617239
12	<i>Microniphargus leruthi</i>	DW190413-007a	140	<i>Niphargus longidactylus</i>	EF617266
13	<i>Microniphargus leruthi</i>	DW190413-007b	141	<i>Niphargus longiflagellum</i>	MN914004
14	<i>Microniphargus leruthi</i>	DW190413-008	142	<i>Niphargus lourensis</i>	EU693312
15	<i>Microniphargus leruthi</i>	DW190413-009	143	<i>Niphargus luchoffmani</i>	KX379014
16	<i>Microniphargus leruthi</i>	DW191250-002	144	<i>Niphargus lunaris</i>	EU693313
17	<i>Microniphargus leruthi</i>	DW191250-004	145	<i>Niphargus malagorae</i>	KY617384
18	<i>Microniphargus leruthi</i>	DW191250-011	146	<i>Niphargus maximus</i>	EF617279
19	<i>Microniphargus leruthi</i>	DW171021-001	147	<i>Niphargus microcerberus</i>	MN114023
20	<i>Microniphargus leruthi</i>	KX379004	148	<i>Niphargus miljeticus</i>	KR905878
21	<i>Chaetoniphargus lubuskensis</i>	MN914030	149	<i>Niphargus minor</i>	MN114028
22	<i>Haploginglymus geos</i>	KY441086	150	<i>Niphargus mirocensis</i>	KR827047
23	<i>Haploginglymus morenoi</i>	KY441079	151	<i>Niphargus molnari</i>	KP967555
24	<i>Niphargellus glenniei</i>	KC315617	152	<i>Niphargus montanarius</i>	GU973419
25	<i>Niphargobates orophobata</i>	KR905879	153	<i>Niphargus montellianus</i>	KT878856
26	<i>Niphargopsis casparyi</i>	EU693291	154	<i>Niphargus moogi</i>	FS_14.177
27	<i>Niphargus aberrans</i>	EF617260	155	<i>Niphargus multipennatus</i>	KJ566700
28	<i>Niphargus aggtelekiensis</i>	FS_14.015	156	<i>Niphargus muotae</i>	KX379024
29	<i>Niphargus aitolosi</i>	EU693310	157	<i>Niphargus murimali</i>	KX379026
30	<i>Niphargus alisadri</i>	KF581049	158	<i>Niphargus novomestanus</i>	KY617364
31	<i>Niphargus alpheus</i>	KY617132	159	<i>Niphargus ohridanus</i>	MN114029
32	<i>Niphargus altagahizi</i>	KF581059	160	<i>Niphargus orcinus</i>	EU693315
33	<i>Niphargus ambulator</i>	KJ566699	161	<i>Niphargus pachypus</i>	KC733825
34	<i>Niphargus anchialinus</i>	KR905881	162	<i>Niphargus pachytelson</i>	EU693316
35	<i>Niphargus andropus</i>	KF218725	163	<i>Niphargus parenzani</i>	MN913997
36	<i>Niphargus antipodes</i>	KY617097	164	<i>Niphargus pasquinii</i>	EF617244
37	<i>Niphargus aquilex</i> A1	KC315604	165	<i>Niphargus patrizii</i>	MN914011
38	<i>Niphargus aquilex</i> A2	JF420874	166	<i>Niphargus pectencoronatae</i>	MN914010
39	<i>Niphargus aquilex</i> B	KC315605	167	<i>Niphargus pectinicauda</i>	EF617258
40	<i>Niphargus aquilex</i> C	KC315602	168	<i>Niphargus persicus</i>	KF581036
41	<i>Niphargus aquilex</i> D	KC315603	169	<i>Niphargus pincinovae</i>	KY617139
42	<i>Niphargus aquilex</i> E	KC315606	170	<i>Niphargus plateau</i>	GU973468

43	<i>Niphargus aquilex</i> F	KC315607	171	<i>Niphargus podgoricensis</i>	KR905875
44	<i>Niphargus arbiter</i>	KY617099	172	<i>Niphargus podpecanus</i>	KY617374
45	<i>Niphargus arethusa</i>	EF617285	173	<i>Niphargus poianoi</i>	KX379006
46	<i>Niphargus auerbachii</i>	EU693292	174	<i>Niphargus polymorphus</i>	EF617282
47	<i>Niphargus aulicus</i>	MN914026	175	<i>Niphargus pontoruffoi</i>	KF290027
48	<i>Niphargus bajuvaricus</i>	EF617259	176	<i>Niphargus pretneri</i>	EF617294
49	<i>Niphargus balcanicus</i>	EF617280	177	<i>Niphargus pupetta</i>	EU693318
50	<i>Niphargus bihorensis</i>	KF218726	178	<i>Niphargus puteanus</i>	EF617302
51	<i>Niphargus bilecanus</i>	JQ815550	179	<i>Niphargus racovitzae</i>	KF290065
52	<i>Niphargus bisitunicus</i>	KF581050	180	<i>Niphargus rejici</i>	EF617283
53	<i>Niphargus borisi</i>	KF581037	181	<i>Niphargus rhenorhodanensis</i> ABC	EF025814
54	<i>Niphargus boskovici</i>	KR827043	182	<i>Niphargus rhenorhodanensis</i> DE	EF025801
55	<i>Niphargus brachytelson</i>	EU693293	183	<i>Niphargus rhenorhodanensis</i> DE	EF025831
56	<i>Niphargus brevicuspis</i>	MN914028	184	<i>Niphargus rhenorhodanensis</i> FG	KX379033
57	<i>Niphargus brevirostris</i>	MN914008	185	<i>Niphargus rhenorhodanensis</i> H	KX379013
58	<i>Niphargus brixianus</i>	EF617299	186	<i>Niphargus rhenorhodanensis</i> I	EF025841
59	<i>Niphargus bureschi</i>	MN114020	187	<i>Niphargus rhenorhodanensis</i> JK	EF025810
60	<i>Niphargus buturovici</i>	MN914022	188	<i>Niphargus romuleus</i>	FS_14.032
61	<i>Niphargus carniolicus</i>	EF617252	189	<i>Niphargus salernianus</i>	MN914014
62	<i>Niphargus carpathicus</i>	MN114019	190	<i>Niphargus salonitanus</i>	KY617147
63	<i>Niphargus</i> cf. <i>gallicus</i>	KF290033	191	<i>Niphargus salzburgensis</i>	KJ566697
64	<i>Niphargus</i> cf. <i>stefanellii</i>	MN914000	192	<i>Niphargus sanctinaumi</i>	EU693320
65	<i>Niphargus</i> cf. <i>stenopus</i>	MN914009	193	<i>Niphargus schellenbergi</i> 1	EF617267
66	<i>Niphargus</i> cf. <i>stygius</i> 2	KX379016	194	<i>Niphargus schellenbergi</i> 2	KC315620
67	<i>Niphargus</i> cf. <i>thienemanni</i>	MH172406	195	<i>Niphargus scopicauda</i>	EF617261
68	<i>Niphargus chagankae</i>	JQ815441	196	<i>Niphargus sharifi</i>	KF581048
69	<i>Niphargus cornicolanus</i>	MN914003	197	<i>Niphargus slovenicus</i>	EU693322
70	<i>Niphargus costozzae</i>	EU693294	198	<i>Niphargus sohrevardensis</i>	KF581034
71	<i>Niphargus croaticus</i>	KT007482	199	<i>Niphargus</i> sp. Arkadi	MN114032
72	<i>Niphargus cvajcki</i>	KY617371	200	<i>Niphargus</i> sp. BIH1	EF617268
73	<i>Niphargus dabarensis</i>	JQ815442	201	<i>Niphargus</i> sp. HudaLuknja	EF617246
74	<i>Niphargus dalmatinus</i>	EF617296	202	<i>Niphargus</i> sp. Iran 9	KF581040
75	<i>Niphargus dancaui</i>	KF290030	203	<i>Niphargus</i> sp. Iskavas	EF617291
76	<i>Niphargus daniali</i>	KF581033	204	<i>Niphargus</i> sp. Italy A	KJ566701
77	<i>Niphargus darvishi</i>	KF581041	205	<i>Niphargus</i> sp. Jelovica	MN114022
78	<i>Niphargus decui</i>	KF290034	206	<i>Niphargus</i> sp. MN914012	MN914012
79	<i>Niphargus delamarei</i>	EU693295	207	<i>Niphargus</i> sp. MN914023	MN914023
80	<i>Niphargus dimorphopus</i>	EU693296	208	<i>Niphargus</i> sp. Meskla	MN114031
81	<i>Niphargus dimorphus</i>	MN914035	209	<i>Niphargus</i> sp. Montenegro	EF617278
82	<i>Niphargus dohati</i>	EF617247	210	<i>Niphargus</i> sp. Neraidosplilios	MN114030
83	<i>Niphargus dobrogicus</i>	KF290076	211	<i>Niphargus</i> sp. Podutik	EF617251
84	<i>Niphargus dolienianensis</i>	EF617269	212	<i>Niphargus</i> sp. Prepadna	EF617249
85	<i>Niphargus doli</i>	KY617137	213	<i>Niphargus</i> sp. Ro1	KF218728
86	<i>Niphargus dolichopus</i>	EU693297	214	<i>Niphargus</i> sp. Ro2	KF218724
87	<i>Niphargus elegans</i>	EF617297	215	<i>Niphargus</i> sp. Ro4	KF218732
88	<i>Niphargus factor</i>	EU693298	216	<i>Niphargus</i> sp. Ro5	KJ566693
89	<i>Niphargus fiseri</i>	MK911610	217	<i>Niphargus</i> sp. Spain1	KY441088

90	<i>Niphargus fjakae</i>	EF617290	218	<i>Niphargus</i> sp. Spain2	KY441083
91	<i>Niphargus fongi</i>	MN914037	219	<i>Niphargus</i> sp. Spain3	KY441081
92	<i>Niphargus fontanus</i> A1	KC315608	220	<i>Niphargus speziae</i>	FS_11.069
93	<i>Niphargus fontanus</i> A2	EF617304	221	<i>Niphargus sphagnicolus</i>	EF617270
94	<i>Niphargus fontanus</i> C	KC315609	222	<i>Niphargus spinulifemur</i> B	EU693323
95	<i>Niphargus forelii</i> A	KC315615	223	<i>Niphargus spoeckeri</i>	EU693324
96	<i>Niphargus forelii</i> B	KC315616	224	<i>Niphargus stefanellii</i>	MN913999
97	<i>Niphargus frassassianus</i>	GU973411	225	<i>Niphargus stenopus</i>	EF617284
98	<i>Niphargus frontalis</i>	GU973423	226	<i>Niphargus steueri</i>	KT007489
99	<i>Niphargus gammariformis</i>	MN114013	227	<i>Niphargus stochi</i>	JQ815551
100	<i>Niphargus gebhardtii</i>	KP967556	228	<i>Niphargus strouhali alpinus</i>	EF617254
101	<i>Niphargus goricae</i>	KY617447	229	<i>Niphargus stygius</i>	FS_11.006
102	<i>Niphargus gottscheeanensis</i>	KY617394	230	<i>Niphargus styx</i>	KX379023
103	<i>Niphargus grandii</i>	EU693300	231	<i>Niphargus subtypicus</i>	EU693326
104	<i>Niphargus hadzii</i>	EU693301	232	<i>Niphargus lurensis</i>	FS_18.076
105	<i>Niphargus hebereri</i>	MN913992	233	<i>Niphargus tatrensis reyesdorfensis</i>	FS_16.088
106	<i>Niphargus hercegovinensis</i>	JQ815549	234	<i>Niphargus salzburgensis</i>	FS_16.043
107	<i>Niphargus hosseiniei</i>	KF581055	235	<i>Niphargus tatrensis schneebergensis</i>	FS_16.089
108	<i>Niphargus hrabei</i>	MN914029	236	<i>Niphargus tatrensis tatrensis</i>	FS_16.090
109	<i>Niphargus hvarensis</i>	EF617272	237	<i>Niphargus tauri</i>	EF617245
110	<i>Niphargus ictus</i>	GU973415	238	<i>Niphargus tauricus</i>	KF719274
111	<i>Niphargus ilamensis</i>	KF581039	239	<i>Niphargus thienemanni</i>	EF617301
112	<i>Niphargus illidzensis</i>	EU693304	240	<i>Niphargus thuringius</i>	KJ566695
113	<i>Niphargus irlandicus</i>	KC315618	241	<i>Niphargus timavi</i>	MN914034
114	<i>Niphargus iskae</i>	KY617382	242	<i>Niphargus tonywhitteni</i>	KX379045
115	<i>Niphargus jovanovici</i>	MN114014	243	<i>Niphargus transsylvanicus</i>	KF218716
116	<i>Niphargus kapelanus</i>	KY617387	244	<i>Niphargus tridentinus</i>	KT878857
117	<i>Niphargus karamani</i>	EU693305	245	<i>Niphargus trullipes</i>	EF617281
118	<i>Niphargus karkabounasi</i>	KP133156	246	<i>Niphargus urmiensis</i>	MK911608
119	<i>Niphargus kenki</i>	KY617370	247	<i>Niphargus vadimi</i>	KF719275
120	<i>Niphargus khayyami</i>	JX535353	248	<i>Niphargus vinodolensis</i>	EF617298
121	<i>Niphargus khwarizmi</i>	KF581056	249	<i>Niphargus virei</i> A	DQ119309
122	<i>Niphargus kochianus</i> A	KC315610	250	<i>Niphargus virei</i> B	KX379035
123	<i>Niphargus kochianus</i> B	KC315611	251	<i>Niphargus virei</i> C	EF617237
124	<i>Niphargus kochianus</i> C	KC315612	252	<i>Niphargus vjetrenicensis</i>	EU693329
125	<i>Niphargus kolombatovici</i>	JQ815553	253	<i>Niphargus wolfi</i> A	EF617250
126	<i>Niphargus kordunensis</i>	KY617386	254	<i>Niphargus zagorae</i>	KR827044
127	<i>Niphargus koukourasi</i>	EF617277	255	<i>Niphargus zagrebensis</i>	EF617295
128	<i>Niphargus krameri</i> A	EF617274			

Note 1: yellow = GenBank accession numbers to be included after manuscript acceptance; in red: accession numbers to be added as soon as they will be received

Table S2: List of all the sequences included in the COI phylogeny, including species names and GenBank accession numbers

ID	Species	GB accession number
1	<i>Crangonyx subterraneus</i>	DW170428-002
2	<i>Synurella ambulans</i>	FS_11.023
3	<i>Niphargus stygius</i>	KY706809
4	<i>Niphargus schellenbergi</i>	JF420847
5	<i>Microniphargus leruthi</i> A	DW171021-001
6	<i>Microniphargus leruthi</i> A	DW171021-003
7	<i>Microniphargus leruthi</i> B	DW190413-007_a
8	<i>Microniphargus leruthi</i> B	DW190413-007_b
9	<i>Microniphargus leruthi</i> B	DW190413-008_a
10	<i>Microniphargus leruthi</i> B	DW190413-008_b
11	<i>Microniphargus leruthi</i> B	DW190413-009_a
12	<i>Microniphargus leruthi</i> B	DW190413-009_b
13	<i>Microniphargus leruthi</i> C	DW191250-002
14	<i>Microniphargus leruthi</i> A	DW191250-004_a
15	<i>Microniphargus leruthi</i> A	DW191250-004_b
16	<i>Microniphargus leruthi</i> C	DW191250-011
17	<i>Microniphargus leruthi</i> C	DW191250-001
18	<i>Pseudoniphargus brevipedunculatus</i>	MH592123
19	<i>Pseudoniphargus carpalis</i>	MH592150
20	<i>Pseudoniphargus cupicola</i>	MH592125
21	<i>Pseudoniphargus daviui</i>	FR872383.2
22	<i>Pseudoniphargus elongatus</i>	MH592126
23	<i>Pseudoniphargus gomerae</i>	MH592127
24	<i>Pseudoniphargus gorbeanus</i>	LN871176
25	<i>Pseudoniphargus grandis</i>	MH592128
26	<i>Pseudoniphargus longipes</i>	MH592129
27	<i>Pseudoniphargus mateusorum</i>	MH592130
28	<i>Pseudoniphargus mercadali</i>	MH592131
29	<i>Pseudoniphargus morenoi</i>	MH592132
30	<i>Pseudoniphargus multidentis</i>	MH592133
31	<i>Pseudoniphargus pedrerae</i>	MH592134
32	<i>Pseudoniphargus pityusensis</i>	MH592135
33	<i>Pseudoniphargus portosancti</i>	MH592136
34	<i>Pseudoniphargus ruffoi</i>	MH592137
35	<i>Pseudoniphargus salinus</i>	MH592149
36	<i>Pseudoniphargus sorbasiensis</i>	LN871175
37	<i>Pseudoniphargus</i> sp. 1-Azores	MH592148
38	<i>Pseudoniphargus</i> sp. 1-Balearics	MH592138
39	<i>Pseudoniphargus</i> sp. 1-Basque	MH592139
40	<i>Pseudoniphargus</i> sp. 1-Murcia	MH592140
41	<i>Pseudoniphargus</i> sp. 1-Portugal	MH592151
42	<i>Pseudoniphargus</i> sp. 2-Andalusia	MH592141
43	<i>Pseudoniphargus</i> sp. 2-Canaries	MH592142

44	<i>Pseudoniphargus</i> sp. 2-Portugal	MH592143
45	<i>Pseudoniphargus</i> sp. 6-Morocco A	MH592144
46	<i>Pseudoniphargus</i> sp. 6-Morocco B	MH592145
47	<i>Pseudoniphargus stocki</i>	MH592152
48	<i>Pseudoniphargus triasi</i>	MH592146
49	<i>Pseudoniphargus unisexualis</i>	MH592147

Note: GenBank accession numbers (yellow cells) will be added upon manuscript acceptance