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 sympathy 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*.

**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* Schrödte, 1849 described 85 years before (SCHÖDTE 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; MOŠKRIĆ & 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; Moškrić & Verovnik 2019), all of which from nuclear markers. No mitochondrial sequence for specimens of *Microniphargus* has been published so far. Moškrić & 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 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 & RUFOX 1986; DELHEZ et al. 1999), Germany (SPANGENBERG 1973; KARAMAN & RUFOX 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 (TRONTELL et al. 2009); some species previously considered to be wide-ranging, such as Niphargus aquilex Schrödte, 1855 and *Niphargus virei* Chevreux, 1896, have been found to be complexes of cryptic species (LEFEBURE 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 (Copilaș-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
| 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     | Caroule 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'Istaury                  | 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     | 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     | MT994439     |
| DW190413-008 | *Microniphargus leruthi*        | Grotte de Comblain                | Belgium  | 50.476   | 5.566     | Knight L., Boulton J., Weber D. | 13/04/2019 | MT993552     | MT994441     |
| DW190413-009 | *Microniphargus leruthi*        | Grotte de Comblain                | Belgium  | 50.476   | 5.566     | Knight L., Boulton J., Weber D. | 13/04/2019 | MT993554     | MT994442     |
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 & Stübner 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 (10 pmol/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 94°C followed by 56 cycles of 30s 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.

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

| 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 CGG TGA GGG AAA GTT-3’    | 28S    | x   |            | Verovnik et al. 2005 |
| Niph15i  | 5’-AGA GTC AAA AGA CGG 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   |
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).
connections between haplotypes found co-occurring in the same individual. Niphargus

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.
The COI phylogeny supported a \((\text{Microniphargus} + \text{Pseudoniphargus})\) clade with 96% of ultrafast bootstrap replicates (Fig. 3 and Fig. S1) and revealed \text{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 \text{Pseudoniphargus} and \text{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 \text{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 haplotype 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 intradividual 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 (DIERCKXSSENS 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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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 capicola                     | MH592125            |
| 21 | Pseudoniphargus davii                       | 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-Canaries               | 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                      | Accession number |
|----|------------------------------|------------------|
| 1  | Gammarus pulex               | MT994447         |
| 2  | Gammarus fossarum            | JF965709         |
| 3  | Synurella ambulans           | MT994448         |
| 4  | Crangonyx subtierraneus      | MT994446         |
| 5  | Pseudoniphargus italicus     | MT994449         |
| 6  | Pseudoniphargus gorbeanus    | KY441101         |
| 7  | Pseudoniphargus portosancti  | KY441102         |
| 8  | Pseudoniphargus spiniferus   | MT994450         |
| 9  | Pseudoniphargus sp.          | KY441100         |
| 10 | Microniphargus leruthi       | MT994437         |
| 11 | Microniphargus leruthi       | MT994438         |
| 12 | Microniphargus leruthi       | MT994439         |
| 13 | Microniphargus leruthi       | MT994440         |
| 14 | Microniphargus leruthi       | MT994441         |
| 15 | Microniphargus leruthi       | MT994442         |
| 16 | Microniphargus leruthi       | MT994443         |
| 17 | Microniphargus leruthi       | MT994444         |
| 18 | Microniphargus leruthi       | MT994445         |
| 19 | Microniphargus leruthi       | MT994436         |
| 20 | Microniphargus 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 agge telekensis    | 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 antiquipes         | 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 auerbach           | EU693292         |
| 47 | Niphargus alicus             | MN914026         |
| 48 | Niphargus bajauvaricus       | EF617259         |
| 49 | Niphargus balcanicus         | EF617280         |
| 50 | Niphargus biorensis          | KF218726         |
| 51 | Niphargus bilecanus          | JQ815550         |
| 52 | Niphargus bisitunicus        | KF581050         |
| ID  | Species                   | Accession number |
|-----|---------------------------|------------------|
| 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 dantali         | 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 dolianensis     | 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 fontamus A1     | KC315608         |
| 93  | Niphargus fontamus A2     | EF617304         |
| 94  | Niphargus fontamus C      | KC315609         |
| 95  | Niphargus forelli A       | KC315615         |
| 96  | Niphargus forelli B       | KC315616         |
| 97  | Niphargus frasassianus    | GU973411         |
| 98  | Niphargus frontalis       | GU973423         |
| 99  | Niphargus gammariformis   | MN114013         |
| 100 | Niphargus gebhardtii      | 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         |
| ID | Species              | Accession number |
|----|----------------------|------------------|
| 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 khayami    | 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         |
| ID | Species | Accession number |
|----|---------|-----------------|
| 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 poianaio | KX379006 |
| 174 | Niphargus polymorphus | EF617282 |
| 175 | Niphargus pontoruffoi | KF290027 |
| 176 | Niphargus pretneri | EF617294 |
| 177 | Niphargus pumetta | 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 salontanus | KY617147 |
| 191 | Niphargus salzburgensis | KJ566697 |
| 192 | Niphargus sanitnaumi | 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* speeckeri                | 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* tatensis reyesdorfenisis  | MT975494         |
| 234 | *Niphargus* salzburgensis            | MT975492         |
| 235 | *Niphargus* tatensis schneebergensis  | MT975495         |
| 236 | *Niphargus* tatensis tatrensis       | MT975496         |
| 237 | *Niphargus* tauri                     | EF617245         |
| 238 | *Niphargus* tauricus                 | KF719274         |
| 239 | *Niphargus* thiemenmanni             | EF617301         |
| 240 | *Niphargus* thuringius               | KJ566695         |
| 241 | *Niphargus* timavi                   | MN914034         |
| 242 | *Niphargus* tonywhitteni             | KX379045         |
| 243 | *Niphargus* transsylvanicus          | KF218716         |
| 244 | *Niphargus* tridentinus              | KX878857         |
| 245 | *Niphargus* trullipes                | EF617281         |
| 246 | *Niphargus* urmiensis                | MK911608         |
| 247 | *Niphargus* vadiii                   | KF719275         |
| 248 | *Niphargus* vinodiensis              | EF617298         |
| 249 | *Niphargus* virei A                  | DQ119309         |
| 250 | *Niphargus* virei B                  | KX379035         |
| 251 | *Niphargus* virei C                  | EF617237         |
| 252 | *Niphargus* vjetrenicensis           | EU693329         |
| 253 | *Niphargus* wolffi A                 | EF617250         |
| 254 | *Niphargus* zagorae                  | KR827044         |
| 255 | *Niphargus* zagrebensis              | EF617295         |