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Environmental DNA reveals cryptic diversity within the subterranean amphipod genus *Pseudocrangonyx* Akatsuka & Komai, 1922 (Amphipoda: Crangonyctoidea: Pseudocrangonyctidae) from central Japan

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ABSTRACT

An environmental DNA (eDNA) detection method confirms the presence of the subterranean amphipod genus *Pseudocrangonyx* Akatsuka & Komai, 1922 in the city of Kyoto, Honshu, Japan for the first time in more than 90 years. Phylogenetic trees using partial sequences of the 16S rRNA gene of eDNA samples from Kyoto and specimens collected from various locations in Japan and Korea highlight that two genetically distinct taxonomic units inhabit Kyoto's subterranean environments. Results reveal that the ranges of distinct phylogroups of *Pseudocrangonyx* widely overlap with each other around Lake Biwa in central western Honshu. The eDNA results increase our understanding of the species richness and complicated evolutionary history of these stygobitic amphipods.

Key Words: East Asia, groundwater, molecular phylogeny, *Pseudocrangonyx kyotonis*, *Pseudocrangonyx shikokunis*, stygobitic species

Groundwater environments harbor diverse metazoan groups worldwide (Griebler *et al.*, 2014). The crangonyctoidean family Pseudocrangonyctidae is a species-rich and abundant taxon known from East Asian subterranean habitats (Holsinger, 1993, 1994). The family comprises 30 valid species included in two genera, *Procrangonyx* Schellenberg, 1934 and *Pseudocrangonyx* Akatsuka & Komai, 1922, the former consisting of three species, and the latter 27 species (Nakano *et al.*, 2018; Tomikawa *et al.*, 2019; Lee *et al.*, 2020).

The true species richness of *Pseudocrangonyx* inhabiting Japan was revealed in recent molecular phylogenetic studies (Tomikawa *et al.*, 2016; Tomikawa & Nakano, 2018), and nine species, including several undescribed species, were recognized from the Japanese Archipelago alone (Tomikawa & Nakano, 2018; Tomikawa *et al.*, 2019). The systematic status of the two Japanese species, *P. kyotonis* Akatsuka & Komai, 1922 and *P. shikokunis* Akatsuka & Komai, 1922, nevertheless remained unclear due to the lack of genetic data for topotypic materials from the type localities.

Pseudocrangonyx kyotonis was originally collected from an aqueduct in Kyoto, Honshu, and *P. shikokunis* was obtained from a well in Tomioka (now the urban area of Anan city), Tokushima Prefecture, Shikoku (Akatsuka & Komai, 1922). Although type materials of these two species preserved in ethanol are kept in the National Museum of Nature and Science, Tsukuba, Japan (Tomikawa & Nakano, 2018), no DNA sequences were known. In addition to the type specimens, some topotypic specimens of *P. kyotonis* collected in the 1920s (see Uéno, 1927) have been kept at the Kyoto University Museum, but all of them are dry (based on an unpublished observation by KT and TN, unpublished data). DNA extraction from these old specimens is thus deemed almost impossible, and it was desirable to collect new specimens to reveal the true

phylogenetic position of *P. kyotonis* and *P. shikokunis*. Specimens are unfortunately difficult to collect at the type localities owing to the decreasing number of operational water wells.

Detection of species via environmental DNA (eDNA) is a useful approach for finding cryptic faunal diversity, including that for stygobitic vertebrates and invertebrates (Ficetola *et al*., 2008; Rees *et al.*, 2014; Gorički *et al*., 2017; Niemiller *et al*., 2017; Vörös *et al*., 2017). We used eDNA to find evidence for the presence of *P. kyotonis* and *P. shikokunis* at their respective type localities. Additional specimens of the species were also collected from caves around the type localities. Genetic data obtained from both eDNA detection and traditional field collecting surveys were analyzed along with the previously published DNA sequences, and the phylogenetic positions of the newly obtained samples were determined.

We collected 1 l each of 42 water samples in Kyoto for *P. kyotonis* from July 2017 to October 2018, and 19 water samples in Tokushima Prefecture for *P. shikokunis* in September 2018 (Fig. 1; Supplementary material Table S1). We also collected one negative control sample, which was extracted from deionized water during the October 2018 survey to test for cross-contamination during eDNA extraction. The negative controls were treated as samples throughout the tests. Workplace and tools were respectively sterilized with 70% ethanol and a bleach solution before all laboratory work. Water samples were filtered immediately after collection using 50 ml sterile syringes (Thermo, Tokyo, Japan) and 0.45-μm Sterivex filter cartridges (Millipore, Burlington, MA, USA). The filter cartridges were kept at 4 °C during transport to the laboratory and then stored at −20 °C until the DNA extraction. The eDNA from the filter cartridges was extracted using a DNeasy blood and tissue kit (Qiagen, Hilden,

Germany) following Miya *et al.* (2016).

A two-step polymerase chain reaction (PCR) protocol was applied for the library preparation. One PCR negative control (MilliQ water) was prepared for each procedure. For PCR amplification of the mitochondrial 16S rRNA (16S) region, a specific primer pair, Amphipoda16S_F (5′-AAAGGGACGATAAGACCC-3′) and Amphipoda16S_R (5′-CGCTGTTATCCCTARAGTAAC-3′), were designed at the Primer3 website (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) by modifying the previously published Crust16S_F/R primers (Berry *et al.*, 2017) based on the 16S sequences of *Pseudocrangonyx* amphipods determined by Tomikawa *et al.* (2016). These primers were used with the MiSeq sequencing primer and six random bases described in Ushio *et al.* (2017). First-round PCR was performed with a 10 μl volume containing 1 μl of the extracted eDNA, 6 μl of Multiplex PCR Master Mix (Qiagen), 0.5 μl of each primer (5 μM), and 2 μl of MilliQ water. The first PCR reaction mixtures were heated to 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 57 °C for 1 min 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Although the cycle number of first-round PCR was often set as 35 in previous studies, we increased it to 40 according to Miya & Sado (2019) because PCRs with 35 cycles failed in most of the samples. The first PCR was replicated three times, then pooled. The first PCR products were purified following Kinoshita *et al.* (2019).

Second-round PCR was also performed adding MiSeq adaptor sequences and index sequences to both amplicon ends, and carried out in 24 μl volumes containing 2 μl of the first PCR products, 12 μl of KAPA HiFi HotStart ReadyMix (2×) (KAPA Biosystems, Wilmington, WA, USA), 1.4 μl of each forward/reverse primer (5 μM), and 7.2 μl of MilliQ water. The second PCR thermal cycle profile was as follows: 95 °C for

3 min, followed by 12 cycles of 98 °C for 20 s, 72 °C for 15 s, and 72 °C for 5 min. The second-round PCR products were mixed in equal amounts, and then purified using 1:1 ratio of Agencourt AMPure XP (Beckman Coulter, High Wycombe, UK) to the product. The target size of the DNA (ca. 350 bp, including the barcode, primer, MiSeq sequencing primer, index, and MiSeq adapters) in the purified product was separated using E-Gel SizeSelect (Thermo Fisher, Waltham, MA, USA) and the concentration of the separated amplicon measured by Qubit dsDNA HS assay kit and Qubit 3.0 Fluorometer (Life Technologies, Paisley, UK). The concentration of the target amplicons was then adjusted to 2 nM using MilliQ water, and the DNA solution was applied the MiSeq platform (Illumina, San Diego, CA, USA). MiSeq sequencing was performed with a MiSeq reagent nano kit v2 (300-cycle) following the manufacturer's protocols.

The raw MiSeq sequence data were converted from a bcl file to a FASTQ file using the bcl2fastq v2.18.0.12 (Illumina). All the following analyses were performed using the commands in Claident v0.2.2018.05.29 (Tanabe & Toju, 2013; http://www.claident.org). The FASTQ files were demultiplexed using the "clsplitseq" command. The forward and reverse sequences were merged using the "clconcatpair" command. The low-quality sequences were removed from the merged sequences using the "clfilterseq" command with the "minqual = 30" and "maxplowqual = 0.1" options, then the noisy sequences were removed using the "clcleanseqv" command with the "pnoisycluster = 0.5" option. The molecular operational taxonomic units (MOTUs) were selected by clustering with 97% cutoff similarity using the "clclassseqv" command. *De novo* chimera removal was performed using the "clrunuchime" command with the default option. All the MOTUs were identified as known taxa by the query-centric auto-k-nearest-neighbor (QCauto)

method (Tanabe & Toju, 2013) based on the lowest common ancestor (LCA) algorithm (Huson *et al.*, 2007) using the “clidentseq” and “classingtax” commands with the “animals_mt_genus” database. The 16S rRNA sequences of *Pseudocrangonyx* within this database were provided by the previous systematic study (Tomikawa *et al.*, 2016), and thus their identities were adequately confirmed.

As a result of MiSeq sequencing, 51 water samples, one DNA extraction negative control, and two PCR negative controls finally generated 1,280,738 sequences. These MiSeq sequence data was deposited to INSDC through DNA Data Bank of Japan (DDBJ) under an accession number DRA010003. After data processing, 677,044 sequences were used for BLAST, then 292,363 sequences were assigned to a specific taxon. According to QCauto analysis, 209,354 sequences could be identified as any of the family-, genus-, or species-group taxa. The DNA sequences identified as being derived from pseudocrangonyctids, amphipods belonging to *Pseudocrangonyx* or Pseudocrangonyctidae, were detected from three locations (locality numbers 3, 17, and 18 in Figure 1; Supplementary material Table S1). One sequence identified as location 3 (Fig. 1) was discarded before analysis, because only six reads were detected from the location. The other two sequences were deposited with INSDC through DDBJ (Supplementary material Table S2).

In addition to the eDNA analysis, specimens of *Pseudocrangonyx* were obtained from subterranean streams in four caves in Honshu and Shikoku from September 2018 to March 2019: Kagu-no-iwaya Cave (48 in Figures 1, 2A; cave entrance 33.8968°N, 134.6478°E; 11 specimens, voucher numbers KUZ Z2058–Z2065, Z2432–Z2434), Anan, Tokushima Prefecture, 28 September 2018; Nosaka Mine (xii in Figure 2A; 35.60571°N, 136.01503°E; two specimens, KUZ Z2071, Z2446), Tsuruga city, Fukui

Prefecture, 25 October 2018 and 7 March 2019; Kurotengu-no-ana Cave (xi in Figure 2A; 34.32442°N, 136.40457°E; KUZ Z2145–Z2152, Z2435–Z2443), Taiki town, Mie Prefecture; and Shurei-suiketsu Cave (x in Figure 2A; 34.41793°N, 136.69475°E; eight specimens, KUZ Z2153–Z2160), Ise city, Mie Prefecture, on 6 December 2018. Specimens were collected using a fine-mesh hand-net and fixed in 70% ethanol; the specimens were deposited in the Zoological Collection, Kyoto University (KUZ). No specimens were obtained from the sites where the water samples were collected in Kyoto and Tokushima Prefecture.

Genomic DNA of the four specimens (KUZ Z2065, Z2071, Z2145, and Z2153) was extracted following the method in Nakano & Tomikawa (2018). A primer set, PCR and cycle sequencing reactions, and DNA sequencing for 16S were performed using a method described by Tomikawa *et al.* (2016). The obtained sequences were assembled using DNA BASER (Heracle Biosoft, Piteşti, Romania). Four sequences were obtained and deposited with INSDC through DDBJ (Supplementary material Table S2).

The phylogenetic positions of the present eDNA as well as amphipod samples were estimated based on the dataset analyzed in Lee *et al.* (2020) along with three short eDNA sequences (149 or 152 bp) and four voucher-based sequences (414–420 bp) (Supplementary material Table S2). In total, 27 OTUs were included and their sequences aligned using MAFFT v7.427 L-INS-i (Katoh & Standley, 2013), yielding a 435 bp alignment. Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI). The best fit model was identified as GTR+I+G with the corrected Akaike information criterion using PartitionFinder v2.1.1 (Lanfear *et al.*, 2017) with the “all” algorithm. The ML phylogenetic tree was calculated using IQ-TREE v2.0-rc1 (Minh *et al.*, 2020) with non-parametric bootstrapping (BS) conducted

with 1,000 replicates. BI tree and Bayesian posterior probabilities (PP) were estimated using MrBayes v3.2.7a (Ronquist *et al.*, 2012). Two independent runs for four Markov chains were conducted for 2 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v1.7.1 (Rambaut *et al.*, 2018), and the first 5,001 trees were discarded based on the results.

The obtained BI tree (mean ln-Likelihood [*L*] = −3992.80; Fig. 2B) showed an identical topology to that of the ML tree (ln *L* = −3965.69; not shown). The analyses surprisingly revealed that the eDNA sequences obtained from underground water in Kyoto represented two genetically very distinct clades. The eDNA sequence (RMR-098-17; eDNA Kyoto 1 in Figure 2B) formed a well-supported clade (lineage A in Figure 2B) along with *P. komaii* Tomikawa & Nakano, 2018 from Gifu Prefecture, Japan, and two unidentified specimens (*Pseudocrangonyx* spp. 7, 8) newly collected from Mie Prefecture (BS = 95%, PP = 1.0). The remaining eDNA sequence (RMR-098-14; eDNA Kyoto 2) belonged to the clade (lineage B) consisting of the Korean *P. daejeoensis* Lee, Tomikawa, Nakano & Min, 2018, *P. uenoi* Tomikawa, Abe & Nakano, 2019 from Shiga Prefecture, Japan, and an unidentified *Pseudocrangonyx* sp. 6 collected from Fukui Prefecture (BS = 78%, PP = 0.99).

The results of the eDNA analyses confirmed the presence of *Pseudocrangonyx* in Kyoto for the first time since Uéno (1927). The previous study presumed that *Pseudocrangonyx* sp. 4 and *Pseudocrangonyx* sp. 5 might represent true *P. kyotonis* and *P. shikokunis*, respectively, because their collection sites were close to the respective type localities (Tomikawa *et al.*, 2016). Our results, however, suggest that *Pseudocrangonyx* sp. 4 may be a distinct unidentified species of the genus. The

specimen (KUZ Z2065) from Kagu-no-iwaya Cave, which is located on an upper mountainside behind Anan city (the type locality of *P. shikokunis*) (Fig. 1), belonged to the sp. 5 phylogroup, although we failed to obtain any *Pseudocrangonyx* eDNA sequences from the exact type locality. It is thus highly likely that the sp. 5-phylogroup may contain the true *P. shikokunis*. Because its intra-group genetic diversity is deemed high, this phylogroup may consist of several distinct species, and their taxonomic status should be clarified by a future taxonomic study.

The present phylogenetic trees revealed that at least two distinct units of *Pseudocrangonyx* inhabit groundwater in Kyoto. Uéno (1927) stated that *P. kyotonis* exhibited morphological variation, especially with regard to antenna 1 and uropod 3, with antenna 1 primary flagellum being 15-articulate in the holotype (Akatsuka & Komai, 1922) *versus* around 25-articulate in the four specimens of Uéno (1927). The uuropod 3 peduncle was described as having an outer ramus of approximately 0.15× (Akatsuka & Komai, 1922) in contrast to approximately 0.25−0.28× in Uéno (1927). It is nevertheless difficult to determine whether the present eDNA OTUs, eDNA Kyoto 1 in lineage A and eDNA Kyoto 2 in lineage B (Fig. 2B) correspond to those morphological variants. *Pseudocrangonyx komaii*, which was once identified as *P. kyotonis* (Nunomura, 1975), in lineage A bears 13 or 14 articles in its antenna 1 primary flagellum, which agrees with the *P. kyotonis* holotype. The outer ramus of uropod 3 in *P. komaii*, is clearly obviously shorter than that of the *P. kyotonis* holotype. The uropod 3 peduncle of *P. komaii* is 0.3 times as long as the outer ramus (Tomikawa & Nakano, 2018). *Pseudocrangonyx uenoi* in lineage B also possessed a short antenna 1 primary flagellum, which is 11-articulate, and the uropod 3 of its peduncle is 0.4 times as long as the outer ramus (Tomikawa *et al.*, 2019). The phylogenetic position of *P. kyotonis*

within the genus unfortunately remains unclear, and additional specimens are needed to further evaluate its systematic status, including its morphological variants.

The phylogenies also highlighted that the ranges of lineages A and B widely overlap around Lake Biwa in central western Honshu. Tomikawa & Nakano (2018) shed light on the distribution overlap between *P. akatsukai* and the sp. 5 phylogroup in the Chugoku District of Honshu, Shikoku, and Kyushu. Our study reveals that this Honshu region, especially around Lake Biwa, is also home to *Pseudocrangonyx* amphipods belonging to distinct phylogroups. There are still many unidentified species of this genus in need to be described, and it is essential to clarify their taxonomic status and precise phylogenetic relationships to elucidate the evolution of the morphological diversity and biogeography of this stygobitic group.

Some false negative samples might have been included in our results because we collected groundwater samples only once in each site. Many previous studies repeated the sample collection for at least three times in the same site to prevent false negative results (Fukumoto *et al.*, 2015). Although our results were not based on a quantitative eDNA survey of abundance of groundwater organisms, we were able to elucidate the cryptic species richness of the subterranean *Pseudocrangonyx* of Japan. In addition to the *Pseudocrangonyx* amphipods, 11 MOTUs, such as decapods and hexapods, were detected from the groundwater samples (Supplementary material Table S1). But, all of these crustaceans were not deemed to be subterranean. In the future, repeatable and intensive sampling of groundwater for an eDNA meta-barcoding analysis should be performed to estimate the abundance and richness of subterranean organisms in Kyoto city and other localities in Japan.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Environmental DNA reads obtained via MiSeq sequencing.

S2 Table. Samples used for the molecular phylogenetic analyses.

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REFERENCES

Akatsuka, K. & Komai, T. 1922. *Pseudocrangonyx*, a new genus of subterranean amphipods from Japan. *Annotationes Zoologicae Japonenses*, **10**: 119–126.

Berry, T.E., Osterrieder, S.K., Murray, D.C., Coghlan, M.L., Richardson, A.J., Grealy, A.K., Stat, M., Bejder, L. & Bunce, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecology and Evolution*, **7**: 5435–5453.

Ficetola, G. F., Miaud, C., Pompanon, F. & Taberlet, P. 2008. Species detection using environmental DNA from water samples. *Biology Letters*, **4**: 423–425.

Fukumoto, S., Ushimaru, A. & Minamoto, T. 2015. A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: A case study of giant salamanders in Japan. *Journal of Applied Ecology*, **52**: 358–365.

Gorički, Š., Stanković, D., Snoj, A., Kuntner, M., Jeffery, W.R., Trontelj, P., Pavićević, M., Grizelj, Z., Năpăruş-Aljančič, M. & Aljančič, G. 2017. Environmental DNA in subterranean biology: range extension and taxonomic implications for *Proteus*. *Scientific Reports*, **7**: 45054.

Griebler, C., Malard, F. & Lefébure, T. 2014. Current developments in groundwater ecology—from biodiversity to ecosystem function and services. *Current Opinion in Biotechnology*, **27**: 159–167.

Holsinger, J.R. 1993. Biodiversity of subterranean amphipod crustaceans: global patterns and zoogeographic implications. *Journal of Natural History*, **27**: 821–835.

Holsinger, J.R. 1994. Pattern and process in the biogeography of subterranean amphipods. *Hydrobiologia*, **287**: 131–145.

Huson, D.H., Auch, A.F., Qi, J. & Schuster, S.C. 2007. MEGAN analysis of metagenomic data. *Genome Research*, **17**: 377–386.

Katoh, K. & Standley, D.M. 2013. MAFFT multiple sequence alignment software

version 7: improvements in performance and usability. *Molecular Biology and Evolution*, **30**: 772–780.

Kinoshita, G., Yonezawa, S., Murakami, S. & Isagi, Y. 2019. Environmental DNA collected from snow tracks is useful for identification of mammalian species. *Zoological Science*, **36**: 198–207

Koressaar, T. & Remm, M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics*, **23**: 1289–1291.

Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T. & Calcott, B. 2017. PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, **34**: 772–773.

Lee, C.-W., Tomikawa, K., Nakano, T. & Min, G.-S. 2018. A new species of the genus *Pseudocrangonyx* (Crustacea, Amphipoda, Pseudocrangonyctidae) from Korea. *ZooKeys*, **735**: 27–44.

Lee, C.-W., Tomikawa, K., Nakano, T. & Min, G.-S. 2020. A new species of the genus *Pseudocrangonyx* (Crustacea: Amphipoda: Pseudocrangonyctidae) from Simbok Cave, Korea. *Zootaxa*, **4731**: 321–334.

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.

Miya, M. & Sado, T. 2019. Multiple species detection using MiFish primers. In: *Environmental DNA sampling and experimental manual Version 2.1* (eDNA Methods Standardization Committee, ed.), pp. 55–92. The eDNA Society, Otsu,

Japan.

Miya, M., Minamoto, T., Yamanaka, H., Oka, S.-I., Sato, K., Yamamoto, S., Sado, T. & Doi, H. 2016. Use of a filter cartridge for filtration of water samples and extraction of environmental DNA. *Journal of Visualized Experiments*, **117**: e54741 [doi: 10.3791/54741].

Nakano, T. & Tomikawa, K. 2018. Reassessment of the groundwater amphipod *Paramoera relicta* synonymizes the genus *Relictomoera* with *Paramoera* (Crustacea: Amphipoda: Pontogeneiidae). *Zoological Science*, **35**: 459–467.

Nakano, T., Tomikawa, K. & Grygier, M.J. 2018. Rediscovered syntypes of *Procrangonyx japonicus*, with nomenclatural consideration of some crangonyctoidean subterranean amphipods (Crustacea: Amphipoda: Allocrangonyctidae, Niphargidae, Pseudocrangonyctidae). *Zootaxa*, **4532**: 86–94.

Niemiller, M.L., Porter, M.L., Keany, J., Gilbert, H., Fong, D.W., Culver, D.C., Hobson, C.S., Kendall, K.D., Davis, M.A. & Taylor, S.J. 2017. Evaluation of eDNA for groundwater invertebrate detection and monitoring: a case study with endangered *Stygobromus* (Amphipoda: Crangonyctidae). *Conservation Genetics Resources*, **10**: 247–257.

Nunomura, N. 1975. *Pseudocrangonyx kyotonis* collected from Kumaishi-do Cave (Gifu Prefecture). *Nature Study*, **21**: 11 [in Japanese].

Rambaut, A., Drummond, A.J., Xie, D., Baele, G. & Suchard, M.A. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, **67**: 901–904.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., Gough, K.C. & Crispo, E. 2014. The detection of aquatic animal species using environmental DNA

- a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, **51**: 1450–1459.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, **61**: 539–542.

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

Tanabe, A.S. & Toju, H. 2013. Two new computational methods for universal DNA barcoding: a benchmark using barcode sequences of bacteria, archaea, animals, fungi, and land plants. *PLoS ONE*, **8**: e76910 [doi: org/10.1371/journal.pone.0152242].

Tomikawa, K. & Nakano, T. 2018. Two new subterranean species of *Pseudocrangonyx* Akatsuka & Komai, 1922 (Amphipoda: Crangonyctoidea: Pseudocrangonyctidae), with an insight into groundwater faunal relationships in western Japan. *Journal of Crustacean Biology*, **38**: 460–474.

Tomikawa, K., Abe, Y. & Nakano, T. 2019. A new stygobitic species of the genus *Pseudocrangonyx* (Crustacea: Amphipoda: Pseudocrangonyctidae) from central Honshu, Japan. *Species Diversity*, **24**: 259–266.

Tomikawa, K., Nakano, T., Sato, A., Onodera, Y. & Ohtaka, A. 2016. A molecular phylogeny of *Pseudocrangonyx* from Japan, including a new subterranean species (Crustacea, Amphipoda, Pseudocrangonyctidae). *Zoosystematics and Evolution*, **92**: 187–202.

Uéno, M. 1927. Notes on some subterranean isopods and amphipods of Japan. *Memoirs of the College of Science, Kyoto Imperial University, Series B*, **3**: 355–368.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. & Rozen, S.G. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Research*, **40**: e115 [doi:10.1093/nar/gks596].

Ushio, M., Fukuda, H., Inoue, T., Kobayashi, M., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh, M. & Miya, M. 2017. Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, **17**: e63–e75 [doi: 10.1111/1755-0998.12690].

Vörös, J., Márton, O., Schmidt, B.R., Gál, J.T. & Jelić, D. 2017. Surveying Europe's only cave-dwelling chordate species (*Proteus anguinus*) using environmental DNA. *PLoS ONE*, **12**: e0170945 [doi: org/10.1371/journal.pone.0170945].

FIGURE LEGENDS

Figure 1. Sampling locations for eDNA analyses. Closed circles denote the locations where pseudocrangonyctid eDNA was detected.

Figure 2. Collection locations of the eDNA water samples and specimens used for phylogenetic analyses; location numbers in Arabic numerals correspond to those in Figure 1 (**A**). Bayesian inference tree for 435 bp of mitochondrial 16S rRNA markers; numbers on nodes represent nonparametric bootstrapping (BS) values for maximum likelihood and Bayesian posterior probabilities (PP); BS higher than 50% and PP higher than 0.90 are indicated (**B**).

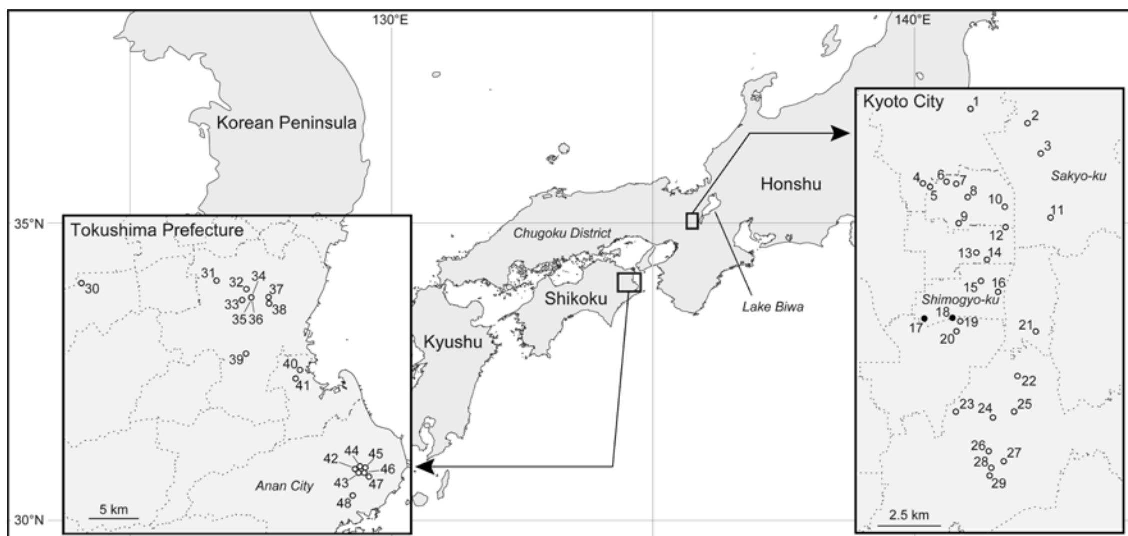

Figure 1. Sampling locations for eDNA analyses. Closed circles denote the locations where pseudocrangonyctid eDNA was detected.

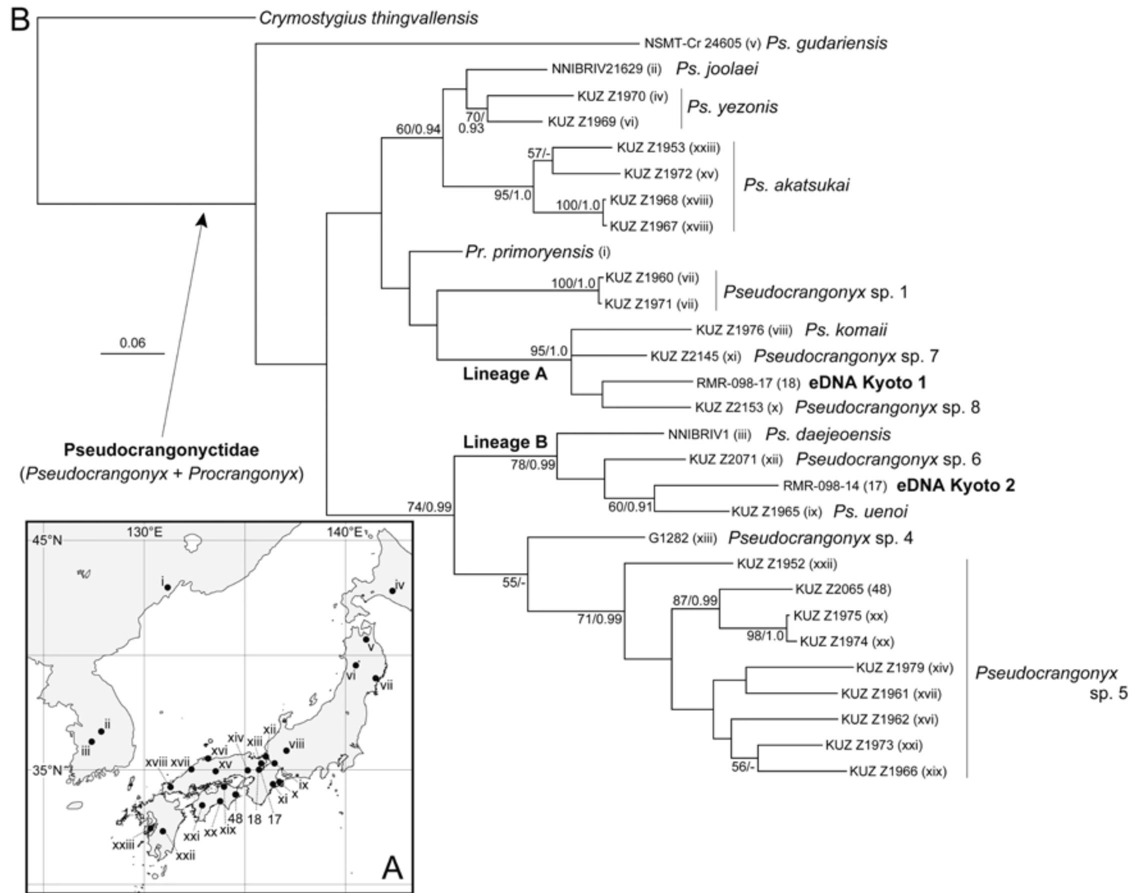

Figure 2. Collection locations of the eDNA water samples and specimens used for phylogenetic analyses; location numbers in Arabic numerals correspond to those in Figure 1 (**A**). Bayesian inference tree for 435 bp of mitochondrial 16S rRNA markers; numbers on nodes represent nonparametric bootstrapping (BS) values for maximum likelihood and Bayesian posterior probabilities (PP); BS higher than 50% and PP higher than 0.90 are indicated (**B**).