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A collaborative backbone resource for comparative studies of subterranean evolution: the World Asellidae database

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Complete List of Authors:	Saclier, Nathanaelle; Université Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA; Université de Montpellier, ISEM, CNRS, Univ. Montpellier, IRD, EPHE Konecny-Dupré, Lara; Universite Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA Grison, Philippe; Muséum National d'Histoire Naturelle, CNRS, BBEES, Unité Bases de données sur la Biodiversité, Ecologie, Environnement et Sociétés, Duchemin, Louis; Université Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA Eme, David; INRAE, UR-RiverLY Martin, Chloé; Muséum National d'Histoire Naturelle, CNRS, BBEES, Unité Bases de données sur la Biodiversité, Ecologie, Environnement et Sociétés, Callou, Cécile; Muséum National d'Histoire Naturelle, CNRS, BBEES, Unité Bases de données sur la Biodiversité, Ecologie, Environnement et Sociétés, Lefebure, Tristan; Université Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA Francois, Clementine M; Université Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA Issartel, Colin; Université Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA Lewis, Julian; Virginia Museum of Natural History; Lewis and Associates, Cave, Karst and Groundwater Biological Consulting Stoch, Fabio; Université Libre de Bruxelles, ULB, Evolutionary Biology & Ecology Sket, Boris; University of Ljubljana Biotechnical faculty, Department of Biology, SubBio Lab Gottstein, Sanja; University of Zagreb, Department of Biology Delić, Teo; University of Ljubljana Biotechnical faculty, Department of Biology, SubBio Lab Zagmajster, Maja; University of Ljubljana Biotechnical faculty, Department of Biology, SubBio Lab Grabowski, Michal; University of Lodz, Department of Invertebrate Zoology & Hydrobiology, Faculty of Biology & Environmental Protection Weber, Dieter; Musée national d'histoire naturelle; Senckenberg Deutsches Entomologisches Institut Reboleira, Ana Sofia; Universidade de Lisboa Faculdade de Ciências, Departamento de Biologia Animal, and Centre for Ecology, Evolution and

	Environmental Changes & CHANGE – Global Change and Sustainability Institute; Natural History Museum of Denmark, University of Copenhagen Palatov, Dmitry; A N Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences Paragamian, Kaloust; Hellenic Institute of Speleological Research Knight, Lee; Hypogean Crustacea Recording Scheme Michel, Georges; Commission Wallonne d'Etude et de Protection des Sites Souterrains Lefebvre , Francois; SEPANSO Aquitaine Malek Hosseini, Mohammad-Javad; ZRC SAZU, Jovan Hadži Institute of Biology; NIB, Department of Organisms and Ecosystems Research, National Institute of Biology Camacho, Ana; CSIC, Dpto. Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales Gartzia De Bikuña, Begoña; Anbiotek, Investigación científica y técnica del medio ambiente, Limnology Area Directora; Anbiolab, BIC Bizkaia Taleb, Amina; Abou Bekr Belkaid University Tlemcen, Laboratoire d'Écologie et Gestion des Ecosystèmes Naturels, University of Tlemcen Belaidi, Nouria; Abou Bekr Belkaid University Tlemcen, Laboratoire d'Écologie et Gestion des Ecosystèmes Naturels, University of Tlemcen Tuekam Kayo, Raoul; University of Bamenda, Faculty of Science, Department of Zoology Galassi, Diana; University of L'Aquila, Department of Life, Health and Environmental Sciences Moldovan, Oana; Emil Racovita Institute of Speleology; Centro Nacional de Investigación sobre la Evolución Humana Malard, Florian; Université Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA Douady, Christophe; Universite Claude Bernard Lyon 1, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA; Institut Universitaire de France

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Title:

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Authors:

Nathanaelle Saclier [1,2], Lara Konecny-Dupré [1], Philippe Grison [3], Louis Duchemin [1], David Eme [4], Chloé Martin [3], Cécile Callou [3], Tristan Lefébure [1], Clémentine François [1], Colin Issartel [1], Julian J. Lewis [5,6], Fabio Stoch [7], Boris Sket [8], Sanja Gottstein [9], Teo Delić [8], Maja Zagmajster [8], Michal Grabowski [10], Dieter Weber [11,12], Ana Sofia P.S. Reboleira [13,14], Dmitry Palatov [15], Kaloust Paragamian [16], Lee R.F.D. Knight [17], Georges Michel [18], Francois Lefebvre [19], Mohammad-Javad Malek Hosseini [20,21], Ana I. Camacho [22], Begoña Gartzia De Bikuña [23,24], Amina Taleb [25], Nouria Belaidi [25], Raoul P. Tuekam Kayo [26], Diana Maria Paola Galassi [27], Oana Teodora Moldovan [28,29], Christophe J. Douady [1,30]*, Florian Malard [1,]*

* Equal contribution

Correspondence: Florian Malard, Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, F-69622, Villeurbanne, France.

Email: Florian.Malard@univ-lyon1.fr

Authors' affiliations:

[1] Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, Ville-urbanne, France

[2] ISEM, CNRS, Univ. Montpellier, IRD, EPHE, Montpellier, France

[3] BBEES, Unité Bases de données sur la Biodiversité, Ecologie, Environnement et Sociétés, Muséum National d'Histoire Naturelle, CNRS, Paris, France

[4] INRAE, UR-RiverLY, Lyon, France

[5] Virginia Museum of Natural History, Martinsville, Virginia, USA

[6] Lewis and Associates, Cave, Karst and Groundwater Biological Consulting, Borden, Indiana, USA

[7] Evolutionary Biology & Ecology, Université libre de Bruxelles (ULB), Bruxelles, Belgium

[8] University of Ljubljana, Biotechnical Faculty, Department of Biology, SubBio Lab, Jamnikarjeva 101, Ljubljana, Slovenia

[9] University of Zagreb, Faculty of Science, Department of Biology, Horvatovac 102A, 10000 Zagreb, Croatia

[10] Department of Invertebrate Zoology & Hydrobiology, Faculty of Biology & Environmental Protection, University of Lodz, 90-237 Lodz, Poland

[11] Musée National d'Histoire Naturelle de Luxembourg, 25 Rue Münster, L-2160 Luxembourg, Luxembourg

[12] Senckenberg Deutsches Entomologisches Institut, Eberswalder Straße 90, 15374 Müncheberg, Germany

[13] Departamento de Biologia Animal, and Centre for Ecology, Evolution and Environmental Changes & CHANGE – Global Change and Sustainability Institute, Faculdade de Ciências, Universidade de Lisboa, Campo Grande 1749-016, Lisbon, Portugal

[14] Natural History Museum of Denmark, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark

[15] A.N. Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences, Moscow, Russia.

[16] Hellenic Institute of Speleological Research, Megalou Alexandrou 179, 71306 IRAKLEIO, Crete, Greece

[17] Hypogean Crustacea Recording Scheme, No.1, The Linhay, North Kenwood Farm, Oxton, Nr Kenton, Devon, EX6 8EX, United Kingdom
[18] CWEPSS, Commission Wallonne d'Etude et de Protection des Sites Souterrains, avenue G. Gilbert 20, 1050 Bruxelles, Belgium
[19] SEPANSO Aquitaine, 1 rue de Tauzia, 33800 Bordeaux, France
[20] Jovan Hadži Institute of Biology, Research Centre of the Slovenian Academy of Sciences and Arts (ZRC-SAZU), Ljubljana, Slovenia
[21] Department of Organisms and Ecosystems Research, National Institute of Biology (NIB), Ljubljana, Slovenia
[22] Museo Nacional de Ciencias Naturales (CSIC). Dpto. Biodiversidad y Biología Evolutiva. C/ José Gutiérrez Abascal 2., 28006-Madrid, Spain
[23] Anbiotek, Investigación científica y técnica del medio ambiente, Limnology Area Directora, Ribera de Axpe 11. B201, 48950 Erandio, Bizkaia, Spain
[24] Anbiolab, BIC Bizkaia Astondo bidea, edificio 612, Parque Científico y Tecnológico de Bizkaia, 48160 Derio, Spain
[25] Laboratoire d'Écologie et Gestion des Ecosystèmes Naturels, University of Tlemcen, Algeria
[26] University of Bamenda, Faculty of Science, Department of Zoology, POBox 39, Bambili, Cameroon
[27] Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy
[28] Emil Racovita Institute of Speleology, Clinicilor 5, 400006 Cluj-Napoca, Romania
[29] Centro Nacional de Investigación sobre la Evolución Humana, Paseo Sierra de Atapuerca 3, 09002 Burgos, Spain
[30] Institut Universitaire de France, Paris, France

Abstract

Transition to novel environments, such as groundwater colonization by surface organisms, provides an excellent research ground to study phenotypic evolution. However, interspecific comparative studies on evolution to groundwater life are few because of the challenge in assembling large ecological and molecular resources for species-rich taxa comprised of surface and subterranean species. Here, we make available to the scientific community an operational set of working tools and resources for the Asellidae, a family of freshwater isopods containing hundreds of surface and subterranean species. First, we release the World Asellidae database (WAD) and its web application, a sustainable and FAIR solution to producing and sharing data and biological material. WAD provides access to thousands of species occurrences, specimens, DNA extracts and DNA sequences with rich metadata ensuring full scientific traceability. Second, we perform a large-scale dated phylogenetic reconstruction of Asellidae to support phylogenetic comparative analyses. Of 424 terminal branches, we identify 34 pairs of surface and subterranean species representing independent replicates of the transition from surface water to groundwater. Third, we exemplify the usefulness of WAD for documenting phenotypic shifts associated with colonization of subterranean habitats. We provide the first phylogenetically controlled evidence that body size of males decreases relative to that of females upon groundwater colonization, suggesting competition for rare receptive females selects for smaller, more agile males in groundwater. By making these tools and resources widely accessible, we open up new opportunities for exploring how phenotypic traits evolve in response to changes in selective pressures and trade-offs during groundwater colonization.

KEYWORDS collaborative database, phylogeny, comparative analysis, phenotypic evolution, molecular resources, subterranean biodiversity

1 | INTRODUCTION

Homo sapiens has been fascinated by the subterranean world throughout its history (Mammola and Martinez, 2020) and the peculiar features of subterranean organisms have attracted scientists since their first discovery over the 16th to 17th centuries (Malard, 2022). However, it was not until the mid-twentieth century that the idea that the subterranean world provides an excellent research ground for addressing general scientific questions in ecology and evolution gained momentum (Poulson and White, 1969; Mammola et al., 2020). A long-standing perspective of subterranean life evolution is that of convergence whereby phylogenetically distant organisms acquire similar phenotypes because of a convergent selective environment that includes no light, environmental stability and energy limitation (Christiansen, 1961; Pipan and Culver, 2012). Since the 2000's, a broader evolutionary perspective of subterranean life has emerged, one that has also incorporated the role of non-adaptive processes (Lefébure et al., 2017; Wilkens and Strecker, 2017; Policarpo et al., 2021) and divergent selection (Trontelj et al., 2012; Fišer et al., 2023) in shaping the phenotype of organisms.

Phylogenetically controlled and replicated comparisons between closely related surface and subterranean organisms provide ideal models to study evolution during colonization of a novel environment (Protas and Jeffery, 2012; Saclier et al., 2018; Rétaux and Jeffery, 2023). Indeed, surface organisms that colonize subterranean habitats experience dramatic environmental changes (e.g. darkness, food limitation) and evolve characteristic regressive (e.g. reduced eyes and pigment) and constructive (e.g. increased extra-optic sensory structures) traits (Culver and Pipan, 2019; Hose et al. 2022). Subterranean colonization is considered an irreversible habitat transition because it leads to eye degeneration (Niemiller et al., 2013; Langille et al., 2022).

Only in very rare cases, blind and depigmented animals can re-colonize surface habitats that are characterized by low competitive pressure (Copilas-Ciocianu et al., 2018).

The scientific scope of surface-subterranean comparative studies ultimately depends on the acquisition of ecological and molecular resources - from biological trait data to phylogenetic and genomic resources - in model organisms. These resources are increasingly becoming available at intraspecific level in species comprised of surface and subterranean populations such as the teleost *Astyanax mexicanus* (Kowalko et al., 2020; Gross et al., 2023), the isopod *Asellus aquaticus* (Konec et al., 2015; Protas et al., 2023), the amphipod *Gammarus minus* (Fong et al., 2023) and the urodele amphibian *Proteus anguinus* (Kostanjšek et al., 2023). However, comparative studies at the interspecific level remain scarce essentially because of the difficulty in assembling large-scale phylogenetic and species trait data sets in clades comprised of multiple surface and subterranean species (Stern et al., 2017; Lefébure et al., 2017; Saclier et al., 2018; Mammola et al., 2019; Langille et al., 2022). Although intraspecific studies often provide deeper insights into the genetic and developmental basis of phenotypic traits, only interspecific studies can document evolutionary changes taking place over time periods longer than the lifespan of natural populations.

Performing phylogenetic comparative analyses of clades comprised of surface and subterranean species faces several challenges. First, only a few clades of metazoans contain both a high number of surface species and subterranean species because the surface ancestors of many subterranean taxa went extinct (Humphreys, 2000). Candidate clades often have a wide geographic distribution, sometimes spanning several continents, which makes it particularly difficult to obtain biological material (Mammola and Isaia, 2017; Faille, 2019; Fišer, 2019a;

Lukić, 2019). Second, the taxonomic units to be used in comparative analyses are not firmly established. Molecular species delimitation methods often reveal highly divergent operational taxonomic units within subterranean described species that have been historically delimited based on morphological criteria (Fišer et al., 2018; Eme et al., 2018). Third, we lack large dated phylogenies of clades with multiple independent subterranean colonization events (but see Ledford et al., 2011; Morvan et al., 2013; Stern et al., 2017). Last, when phylogenetic inferences are available, biological traits for the taxonomic units of interest are often not available in the literature and voucher specimens for measuring those traits are difficult to locate (but see Mammola et al., 2022).

Here, we address the aforementioned challenges by releasing the World Asellidae database (WAD) and phylogeny, a backbone resource to support comparative studies on life evolution in subterranean habitats. The Asellidae (Isopoda, Pancrustacea) is one of the few families of aquatic metazoans containing both surface and subterranean species, thereby potentially providing multiple independent replicates of the transition from surface water to groundwater. First, we describe the guiding principles and content of WAD, a collaborative database specifically designed to promote the joint production and sharing of primary ecological and molecular data and metadata by multiple research laboratories. Second, we take advantage of new sequence data available in WAD for two mitochondrial genes and two nuclear genes to perform a large-scale dated phylogenetic reconstruction of the Asellidae family that can be used more widely in future comparative studies. Third, we exemplify the usefulness of WAD for documenting phenotypic changes associated with colonization of subterranean habitats. We use the Asellidae phylogeny and body size (BS) data from literature articles and morphological measurements made on specimen lots referenced in WAD to test for differences in male and

female BS between surface and groundwater habitats. We predict no difference in female BS between habitats because fecundity selection probably favors large-bodied females with large brood sizes in both habitats. In contrast, we predict smaller-bodied males in groundwater than in surface water due to a shift in male mating strategy. In surface water, we hypothesize that competition for synchronously receptive females selects for large males that are more likely to win mating contests (Bertin and Cezilly, 2003). In groundwater, competition for rare, highly asynchronous, receptive females potentially favors smaller, more agile males that are more likely to be successful in finding mates (Andersson, 1994; Blanckenhorn, 2000).

2 | MATERIALS AND METHODS

2.1 | The World Asellidae Database (WAD)

We use the free and open-source application GOTIT (https://github.com/gotit-dev/gotit; Malard et al., 2020) to input, manage and share ecological and molecular data and metadata in WAD. The application manages every step of an every-day laboratory workflow process leading to the production of species occurrence data and DNA sequences. A demo version of GOTIT application is available at https://gotit.cnrs.fr. WAD hosts all species occurrence data, sampling and sequencing metadata and biological vouchers (specimens, microscopic slides and DNA extracts) generated over the workflow (Table 1). The database also manages species occurrence data and DNA sequence metadata from the literature and biodiversity facilities, the bibliographic referencing of information and the assignment of DNA sequences to molecular operational taxonomic units (MOTUs). We provide in supplemental figures 1 and 2 (SI Figures 1 and 2) the simplified and full logical models of the database, respectively. User access to WAD, either as a data end-user or contributor, is at https://gotit.univ-lyon1.fr upon request from the corresponding author.

2.2 | Species delimitation and dated phylogeny

2.2.1 | Taxon sampling and molecular data

To build the phylogeny, we extracted from WAD an initial molecular data set representing 299 described and undescribed morphospecies of Aselloidea (278 Asellidae and 21 Stenasellidae used as outgroup). Specimens were collected at 943 localities in Europe, North America, North Africa and Asia (SI Table 1). Localities spanned a wide range of surface and subsurface fresh-water habitats including lotic and lentic surface water bodies, cave streams and pools, the hyporheic zone of surface streams and groundwater in unconsolidated sediments. Throughout this paper, we used the term morphospecies to refer to species, either formally described or undescribed (i.e. waiting a formal description), that were identified based on morphological criteria. Species names of North American asellids follow the latest taxonomic revision to be published by Lewis and coauthors (2023). For morphological identification of specimens to species level, we relied on the shape of male copulatory organs (pleopods 2), plus secondary characteristics including the morphology of the male pereopods 1 and 4, pleopods 3-5, and uropods (Lewis et al., 2023). We dissected copulatory pleopods 1 and 2 of male specimens and mounted them on slide for examination using a compound microscope.

We used the Chelex protocol of Casquet and coauthors (2012) to extract DNA from specimen. We incubated three pereopods of each specimen in a solution of 150 µl of 7% chelex and 10 µl of proteinase K at 15 mg / ml for 90 minutes at 56 °C, and then 15 minutes at 90 °C. We amplified DNA using primers targeting the mitochondrial cytochrome oxidase subunit I (COI) gene, the 16S mitochondrial rDNA gene, the FASTKD4 nuclear gene and the 28S nuclear rDNA gene. We provide in supplemental tables 2 and 3 (SI Tables 2 and 3) the list of all PCR

primers, among which 66 were specifically designed as part of this study. For the two mitochondrial genes, we applied several methods to prevent misleading inclusion of nuclear mitochondrial DNA segments (NUMT) in the data set, including different primer pairs, long-range amplification and pre-PCR dilution of genomic DNA (Calvignac et al., 2011).

We amplified 16S fragments with two independent pairs of primers (SI Tables 2 & 3). PCR settings were as follows: one step of 3 min at 95 °C; 35 cycles of 20 s at 95 °C, 30 s at 53 °C, 30 s at 72 °C; and one step of 5 min at 72 °C. We performed PCRs for COI fragments using a previously optimized protocol (Calvignac et al., 2011), but with a Taq polymerase (Eurobiotaq) amount of 0.05 U instead of 0.15 U and a PCR volume of 25 µl instead of 35 µl. We used the following PCR settings: one step of 3 min at 95 °C, 37 cycles of 20 s at 95 °C, 30 s at 51 °C, 45 s at 72 °C, and one step of 5 min at 72 °C. A semi-nested PCR was performed whenever the first amplification failed. Using the first PCR product as DNA template, we performed a second PCR using one of the two primers used in the first PCR and another, different primer. The second round PCR was run on 1 µl of the first round PCR product, using the same settings as above but 35 cycles. We amplified FASTKD4 fragments using several pairs of primers (SI Tables 2 & 3) with the following PCR settings: one step of 5 min at 95 °C, 38 cycles of 30 s at 95 °C, 45 s at 54 °C, 45 s at 72 °C, and one step of 5 min at 72 °C. As for the COI gene, we performed a semi-nested PCR whenever the first amplification failed. We completed PCRs for 28S fragments with two independent pairs of primers in order to detect divergent copies. We used the following PCR settings: one step of 3 min at 95 °C; 37 cycles of 30 s at 95 °C, 30 s at 62 °C, 30 s at 72 °C; and one step of 5 min at 72 °C.

Microsynth France SAS (Vaulx-en-Velin, France) performed Sanger sequencing for the four genes. Chromatograms were visualized with FinchTV (Geospiza, Seattle, WA, USA). All sequences were aligned with Muscle as implemented in Seaview (Gouy et al., 2010) and checked visually for the presence of anomalies, including stop codons and frameshifts for protein coding genes.

2.2.2 | Molecular operational taxonomic units (MOTUs)

We delimited MOTUs based on a COI alignment of 1385 haplotypes, which were defined from the sequences obtained from 2093 specimens belonging to 299 morphospecies of Aselloidea (SI Tables 1 & 4). We used the following procedure to select specimens for which we obtained COI sequences. Whenever possible, we first obtained 16S sequences from three specimens of each morphospecies present at a site. Second, we obtained a COI sequence for each specimen whose 16S sequence differed by more than 5 nucleotides with any 16S sequence of the two other specimens.

We used the fixed COI threshold method (TH) implemented by Lefébure and coauthors (2006) for crustaceans, and the Poisson tree processes (PTP) proposed by Zhang and coauthors (2013), to delimit MOTUs. The TH method was previously used in several studies for delimiting species of asellids (Morvan et al., 2013; Eme et al., 2013, 2018). It is based on the observation made from hundreds COI sequences of crustaceans that two clades diverging by more than 0.16 substitution per site, as measured by patristic distances, have a strong probability (ca. 0.99%) of belonging to different described morphospecies. It is a conservative method insofar as it identifies both fewer MOTUs and MOTUs that are more divergent than tree-based methods such as the PTP method (Eme et al., 2018). We applied the TH and PTP methods on a COI

haplotype alignment in which the longest sequence with the fewest ambiguities was retained as the best representative sequence for any given haplotype. We constructed a COI haplotype phylogeny in maximum likelihood with PhyML (Guindon et al., 2010) using a GTR + G + I model of evolution and Stenasellidae species as outgroup. We computed patristic distances from this phylogeny with the R package "ape" (Paradis et al., 2004) and delimited MOTUs according to the TH method with the "cluster" package (Maechler et al., 2002). To delimit MOTUs with the PTP method, we ran mPTP v0.2.2 (https://github.com/Pas-Kapli/mptp) using 400 000 MCMC generations, with a thinning of 400 and 0.1 (10%) burn-in.

We performed pairwise taxonomic comparisons between the three different sets of species hypotheses delimited using morphology, the TH method, and the PTP method. For each pairwise comparison, we provided the number of species delimited by each of the two methods as well as the number of matches, splits, lumps and reshuffling (see Eme et al. (2018) for a definition of these four categories).

2.2.3 | Four-gene alignment and phylogeny

We produced alignments of the COI, 16S, FASTKD4 and 28S genes for 424 MOTUs of aselloids delimited with the TH method (SI Tables 5 to 9). Using MOTUs delimited with the TH method rather than the PTP method limited the risk of considering two populations of the same species as belonging to two distinct MOTUs. In each alignment, we retained the longest sequence with the fewest ambiguities to represent each MOTU, using the chimera assembler script (https://github.com/TristanLefebure/chimera_assembler.pl). We aligned the 28S and 16S genes with MAFFT Q-INS-i using default parameters (Katoh and Standley, 2013) and the COI and FASTKD4 genes with PRANK codon (Löytynoja and Goldman, 2008). Sites ambiguously

aligned were removed with Gblocks (Castresana 2000). We used the four genes for 424 MOTUs to build a phylogeny with PhyloBayes (Lartillot et al., 2009) under a CAT-GTR model of evolution. To guarantee the absence of polytomy, a threshold of 10% was set to obtain the majority consensus tree, meaning that each clade must be found in at least 10% of the trees of the Markov process after burn-in. We computed *posterior probabilities* to estimate the support of tree topologies and rooted the tree using species of Stenasellidae as outgroup.

Using the phylogeny, we identified pairs of surface and groundwater asellid species that provided independent replicates of the ecological transition from surface water to groundwater. We ensured independence among pairs by selecting them so that the tree paths from one species to the other within a pair did not contain any branches in common with any other pairs (Felsenstein, 1988). For comparison with intraspecific studies, using independent species pairs is statistically more robust than using replicate pairs of surface and groundwater populations within a single species (Rétaux and. Jeffery, 2023). Indeed, replicate populations pairs within species can be statistically dependent if gene flow still occurs among surface populations.

2.2.4 | Time-scale phylogeny

In the absence of fossil records for the Aselloidea, we used well-identified paleobiogeographic events to constrain the age of 17 nodes in the phylogeny (see SI Table 10 for a description of these events). Paleobiogeographic calibration points spanned a period ranging from 300 to 2 Myr before present. We estimated divergence times among aselloids with PhyloBayes using a CAT-GTR + G + I model, the 17 calibration points as soft bounds, a birth-death prior on divergence time and a log-normal auto-correlation of the substitution rates among branches

(Lepage et al., 2007). The effect of any given calibration point on divergence time estimates was assessed by removing that given calibration point during time tree reconstruction.

2.3 | Comparative phylogenetic analyses of body size

2.3.1 | Body size and sexual body size dimorphism

Here, we provide a case study of body size and sexual body size dimorphism to show how WAD resources and the World Asellidae phylogeny allow exploring how phenotypic traits evolve upon groundwater colonization. We completed literature data with laboratory measurements made on specimen lots contained in WAD to quantify the maximum body size of adult males and females of 162 asellid MOTUs included in the World Asellidae phylogeny (SI Table 11). We defined body size as the distance between the anterior margin of the cephalon and the posterior margin of the pleotelson (Prevorčnik et al. 2004). Maximum body size (subsequently abbreviated to BS) provides an estimator of the size of full-grown specimens in a species: it avoids including immature specimens and is often the only measurement provided in publications. For each MOTU, we provide in SI Table 11 our best estimate of the number of specimens used for quantifying BS, as the exact number is not always reported in the source articles. For measurements made on specimen lots contained in WAD, we took photos of specimens with a DP25 Olympus camera connected to a dissecting microscope (SZX16 Olympus) and measured BS using ImageJ (Schneider et al., 2012).

To quantify sexual body size dimorphism (SBSD), we used the size dimorphism index (SDI) as follows (Lovich and Gibbons, 1992; Fairbairn, 2007):

$$\mathrm{SDI} = \frac{\text{Body size of largest sex}}{\text{Body size of smallest sex}} - 1$$

343 SDI equals zero for monomorphic species in which the two sexes have the same body size and
344 is arbitrarily given a negative sign when males are larger than females.
345
346 2.3.2 | Habitat specialization and habitat size
347 We used presence and absence of eyes and body pigment as evidence of specialization to
348 surface water and groundwater habitats, respectively. Hence, in the ensuing text, groundwater
349 species designate eyeless and depigmented species whereas surface water species designate
350 occulated and/or pigmented species. Of the 162 MOTUs included in the phylogenetic
351 comparative analyses (see below), 61 were surface water species and 101 were groundwater
352 species.
353
354 We assessed the size of habitat or pore volume available to species because it is potentially a
355 major determinant of maximum BS (Pipan and Culver, 2017). We used a fuzzy coding approach
356 (Chevenet et al., 1994; Degen et al., 2018) to assess habitat size because most groundwater
357 ecological studies do not provide any quantitative estimates of pore volume available to species.
358 For the 162 asellid MOTUs incorporated in the comparative analyses, we attributed positive
359 scores (from 0=no affinity to 3=strong affinity) to three categories of habitat size (large,
360 medium and small pore volumes). We attributed habitat size scores independently of the eye
361 and pigmentation status of species. Hence, we assigned a high affinity for large size habitats to
362 species living in the benthic layer of surface streams as well as to those living in the benthic
363 layer of cave streams. Scores were attributed to all species separately by two of us (F.M. and
364 J.J.L.) using species occurrence data per habitat category as guideline data (data extracted from
365 WAD). Then, we corrected for inconsistencies between the two sets of scores to produce a
366 single "habitat trait categories per species" matrix. We provide the species habitat scores and

the scoring procedure in supplemental table 11 (SI Table 11) to ensure data traceability and reproducibility, and potential revision of scores in the event of new habitat data of species. Then, we performed a fuzzy correspondence analysis (COA) of the "habitat trait categories per species" matrix (Chevenet et al., 1994) and used the coordinates of species along the first axis of the COA, representing 85% of total variability, as quantitative surrogates of their habitat size. The COA was performed using the R package "ade4" (Thioulouse et al., 2018).

2.3.3 | Data analysis

We performed phylogenetic generalized least-squares (PGLS) regression models (Martins and Hansen, 1997) to test for the effect of habitat specialization and habitat size and its interaction on BS of females and males and SDI. To account for phylogenetic non-independence among species, we used the Asellidae time-scale phylogeny, pruned to the 162 MOTUs for which BS data were available for the two sexes. We selected the best model of trait evolution and its associated covariance structure - in this study, the Brownian motion model - according to minimum Akaike information criterion. We tested the significance of each predictor (i.e. habitat specialization and habitat size) in the regression by comparing with a likelihood ratio test (LRT) a model without the predictor to a model with the predictor. We assessed the proportion of variance explained by phylogenetic regressions using Cox-Snell pseudo-R2. PGLS were performed in R using the "APE" (Paradis et al., 2004) and "nlme" (Pintero et al., 2022) packages.

3 | RESULTS

3.1 | The World Asellidae Database (WAD)

The database contains 9438 distributional records for 163 surface water species and 285 groundwater species of Asellidae belonging to 23 genera (Tables 1 and 2). Asellids are widely distributed in the Northern Hemisphere with species belonging to four formerly recognized groups of morphospecies, which occupy distinct but partially overlapping distribution ranges (Figure 1). All four groups include both surface and groundwater species, although in different proportions. (Table 2). The first group is the "*Asellus* pattern", so named by Henry and Magniez (1995) in reference to the specific shape of copulatory organs shared by several genera of Asellidae. It has nine genera (61 species); all distributed in Asia and North Western America, except the genus *Asellus,* which is also represented in Europe by the widespread *Asellus aquaticus* species complex (Verovnik et al., 2005). The second group to which we refer as the North American asellids include seven genera (152 described species), all located in North America, except *Gallasellus* and *Baicalasellus*, which are endemic to western France and Lake Baikal (Russia), respectively. The third group containing the two genera *Bragasellus* and *Synasellus* (56 species) is endemic to the Iberian Peninsula. The fourth group corresponding to the genus *Proasellus* (174 species) extends from southern Scandinavia to northern Africa and from Portugal to Iran.

WAD describes the content of 1943 specimen lots, which were sampled by 324 collectors in 38 countries. Lot description comprises the number of male and female mature specimens, juveniles and ovigerous females. Collection material referenced in WAD also includes 4362 specimen DNA extracts and 1584 specimen microscopic slides. Specimen lots and DNA extracts are preserved at -20°C in the zoology collection at University Claude Bernard of Lyon: they are available for subsequent collaborative morphological and molecular analyses upon request from the corresponding author.

WAD provides metadata – from sampling of specimens to PCR and chromatogram settings – for 8914 validated sequences of Asellidae belonging to two mitochondrial and three nuclear genes (Table 1). Of these, 3692 sequences were submitted to NCBI, essentially from the present article authors, as part of previous studies, and 4082 sequences were submitted as part of the present study (SI Table 12). In WAD, COI sequences are assigned to MOTUs using different molecular species delimitation methods. The geographic distribution of MOTUs within morphospecies can be visualized using ready-to-use queries implemented in GOTIT application (SI Figure 3).

3.2 | The Asellidae timetree

3.2.1 | Molecular operational taxonomic units (MOTUs)

The TH and PTP molecular species delimitation methods provided respectively, 1.6 and 1.9 more MOTUs than morphospecies (Figure 2). The two molecular methods essentially split morphospecies into smaller clusters of individuals. Reshuffling cases were rare: of the 466 and 557 MOTUs respectively delimited by TH and PTP, only 10 (2.1%) and 12 (2.2%) fell in that category. PTP split morphospecies into smaller clusters than TH, thereby generating 1.2 more species hypotheses than TH.

3.2.2 | Time-scale phylogeny

The phylogeny included 384 MOTUs of asellids delimited with the TH method. They collectively represented 268 morphospecies, among which 195 were formally described (Figure 3, Table 2). The tree topology recovered the monophyly of the four main species groups described above and that of all asellid genera, with posterior probabilities > 0.9, except the

genus *Conasellus* (*PP*=0.43) (Figure 3, SI Figure 4). The *Asellus* pattern (group 1 in Figures 1 and 3) formed a sister clade to the rest of a larger clade comprised of the North American asellid clade (group 2), the *Bragasellus* + *Synasellus* clade (group 3), and the *Proasellus* clade (group 4). However, relationships among the later three clades were not resolved. The tree topology for *Proasellus* was also consistent with earlier subdivisions of this species-rich genus into four clades (Morvan et al., 2013). Within that genus, the slavus clade was sister to a larger clade comprised of the ibero-aquitanian, anophtalmus-coxalis, and Alpine clades, but the relationships among the later three clades were not resolved (Figure 3).

Divergence time estimates were robust to the removal of any single paleobiogeographic calibration point, except the deepest one that constrained the divergence between Stenasellidae and Asellidae to be more recent than 300 Myr (SI Figure 5). Removing this point yielded older divergence times, notably pushing back the divergence between the Stenasellidae and Asellidae to 300 Myr (95% Credibility Interval [CI]: 415-222 Myr) instead of 139 Myr (CI: 174-106 Myr), when including it (Figure 3, SI Figure 5). The diversification of Asellidae might have started in early Cretaceous (132 Myr, CI: 168-102 Myr) and that of *Proasellus* at the end of Cretaceous or beginning of the Paleogene (72 Myr, CI: 88-58 Myr).

We identified up to 34 independent pairs of surface and groundwater asellid species in the phylogeny (Figure 3, SI Figure 4). Species pairs were present in all four major groups of asellid species, although several species-rich clades were almost exclusively comprised of groundwater species, including the Alpine *Proasellus* clade, *Synasellus* and *Caecidotea*. The uneven distribution of species pairs among the *Proasellus* (21 pairs), North American asellids (10 pairs) and the *Asellus* pattern (one pair) essentially reflected a too low sampling in the latter

two groups (Table 2). Transitions to groundwater have probably occurred throughout the evolutionary history of the Asellidae (Figure 3, SI Figure 4). Using the speciation event leading to a congeneric species pair as a surrogate of the transition time to groundwater (but see discussion), some transitions occurred less than 10 million years ago (6.9, CI: 11.4 -4.2 Myr for the *Asellus aquaticus – A. kosswigi* species pair), whereas others potentially occurred much longer ago (at most 40.4, CI: 50.9 - 30.4 Myr for the *Conasellus burkensis - Conasellus reddelli* species pair) (SI Figure 4).

3.3 | Comparative phylogenetic analyses of body size

Female and male BS ranged from 2.3 to 18 mm (mean=6.3±2.7 mm, n=162 MOTUs) and from 2.1 to 25 mm mean=7.3±4.1 mm, n=162 MOTUs), respectively (SI Table 11, SI Figure 6). BS increased significantly with habitat size, both for females and males (Table 3, Figure 4, SI Table 13). Species colonizing open habitats, both above (e.g. surface lakes and streams) and below ground (e.g. cave streams), had larger BS than species colonizing interstitial habitats (e.g. groundwater in unconsolidated sediment). The effect of habitat specialization on BS was gender dependent (Table 3, Figure 4, SI Table 13). Male BS was significantly smaller in groundwater species than in surface water species, whereas we found no significant differences in female BS between surface water and groundwater species. However, habitat specialization accounted for a smaller proportion of variance in male body size (Cox-Snell $R^2 = 0.074$) than habitat size ($R^2 = 0.161$) (SI Table 13). We found no interactions between the effects of habitat size and habitat specialization on male and female body size, indicating that constraints imposed by the size of habitats on body size applied similarly to eyeless and depigmented species and occulated and/or pigmented species.

Asellids showed substantial variation in sexual body size dimorphism (SBSD) among species. Of the 162 species examined in this study, 94 (58 %) exhibited male-biased dimorphism, 62 (38.3 %) exhibited female-biased dimorphism and 6 (3.7 %) were monomorphic for BS. We found a significant effect of habitat specialization on SBSD: mean SDI was -0.33±0.22 (n=61) and 0.01 ±0.28 (n=101) for surface water species and groundwater species, respectively (Table 3, Figure 4, SI Table 13). Males were larger than females in 57 of 61 (i.e. 93.4 %) surface water species examined in this study, whereas they were larger than females in only 37 of 101 (i.e. 36.6 %) groundwater species. The size dimorphism index (SDI) decreased significantly with increasing habitat size (Table 3, Figure 4). Male-biased SBSD (SDI<0 in Figure 4) predominated in open habitats whereas female-biased SBSD (i.e. SDI >0) predominated in interstitial habitats. However, habitat size accounted for a smaller proportion of variance in SBSD (Cox-Snell $R^2 = 0.069$) than habitat specialization ($R^2 = 0.110$). We found no interactions between the effect of habitat size and habitat specialization on SBSD.

4 | DISCUSSION

4.1 | The World Asellidae Database (WAD)

Collaborative databasing has become essential to biodiversity sciences because the amount of data and biological material needed to address broad-in-scope questions exceeds the production capabilities of even the most performing laboratories (Nelson et al., 2011; Hobern et al., 2012; Fišer, 2019b). The structure of the database used in the present study and its web application GOTIT have been conceived to provide scientists with an efficient tool to jointly produce multiple-species ecological and molecular resources to study life evolution in groundwater. The tool has been used with success since 2017 to amass worldwide data at an unprecedented rate for the Asellidae. WAD provides to date one of the most important resource of species

occurrence, DNA sequences and biological material for testing eco-evolutionary hypotheses pertaining to groundwater colonization using comparative phylogenetic methods and evolutionary model fitting (Stern et al., 2017; Lefébure et al., 2017; Saclier et al., 2018; Langille et al., 2022).

Beyond Asellidae, the tool offers several desirable features when collaboratively producing species occurrence and sequencing data (Malard et al., 2020). First, the database structure portrays a standard workflow - from field sampling to DNA sequencing - that is common to many laboratories. Second, a user-friendly web application allows implementing that laboratory workflow on a day-by-day basis while simultaneously feeding a centralized database. Third, the database guarantees scientific repeatability by offering a full traceability of field and laboratory protocols and biological vouchers. Fourth, intellectual property rights and citation issues are resolved in a way to encourage information sharing before publishing. Sequence metadata are available to all as DNA sequence production flows, hence well before publicly releasing the latter. Sharing metadata before publishing data is key to minimize duplication of work among producers, thereby promoting sustainable data production. Fifth, the database structure and its web application are free and open-source, so that the developer community can modify the source code to address new user requirements. Four updates of the tool have been released since its publication in 2019, with the last update containing a user-friendly query builder for non-SQL experts to extract large data sets (https://github.com/gotit-dev/gotit).

Current development efforts are following two main directions. The first direction is widening the database structure for housing biological species trait data, including but not limited to morphological traits, which are measured on referenced specimens (see for example Lefèbure

et al. (2017) and Saclier et al. (2018) for data on genome size and rate of molecular evolution, respectively). The second direction consists in providing user-friendly tools to promote expertize sharing among users. One example expert tool could guide sequence producers in selecting the most appropriate primers for sequencing any given species from the hundred primers available in the database (see SI Tables 2 and 3).

4.2 | Species delimitation and dated phylogeny

The presence of highly genetically divergent units (i.e. MOTUs) – often referred to as cryptic species - within morphospecies is a common phenomenon across most animal taxa (Bickford et al., 2007; Pfenninger and Schwenk, 2007), and asellids do not escape the rule (Eme et al., 2013; Morvan et al., 2013). Hence, species molecular delimitation methods based on the COI gene typically provide more species hypotheses – in the present study 1.6 to 1.9 times as many – than morphological delimitation. Although molecular methods typically split asellid morphospecies into several MOTUs, they very rarely reshuffle MOTUs among morphospecies. Using different elementary species units in biodiversity research can provide novel insights into the mechanisms underlying biodiversity patterns (Fišer et al., 2018). In their analysis of the range size pattern of groundwater Asellidae and Niphargidae (Amphipoda) in Europe, Eme and coauthors (2018) showed that using MOTUs instead of morphospecies reinforced the Rapoport effect of increasing range size at higher latitudes and increased the proportion of variance in range size explained by historical climates. In WAD, we are continuously updating the geographic coverages of COI sequences and MOTUs within morphospecies (see SI Figure 3), thereby accumulating data for rigorously testing the hypothesis that groundwater species have a reduced range size compared to their surface counterparts. Despite being a long-standing hypothesis (Malard et al., 2023), the crayfish study by Stern and coauthors (2017) remains the

only phylogenetically controlled test to date, even though the authors used morphospecies rather than MOTU-level data. WAD also provides one of the most comprehensive reference barcode libraries of groundwater taxa for accurately assigning to existing known species the COI sequences that arise from a growing number of DNA-based biodiversity studies (Zagmajster et al., 2022). Such a WAD reference barcode library offers great opportunities to combine environmental DNA sampling, metabarcoding, DNA taxonomy and traditional taxonomy to speed up the acquisition of species occurrence data in difficult-to-access groundwater habitats (Fontaneto, et al., 2015; Saccò et al., 2022; Verdier et al., 2022).

The World Asellidae phylogeny provides one of the most comprehensive phylogenetic frameworks available to date for undertaking comparative studies on evolution to groundwater life (but see also Stern et al., 2017). Here, we highlight key improvements to the phylogeny since a previous version published by Morvan and coauthors (2013). First, the present version of the phylogeny contains 2.5 and 2.4 times more MOTUs and morphological species of asellids respectively, than its previous version. Its geographical coverage is also considerably wider, as it includes not only European species but also many North American and eastern Mediterranean species. Yet, the phylogeny is far from being complete since it presently contains 60 % of described species of asellids, the most species-deficient group being the *Asellus* pattern in Asia with only 13 % of described species included in the phylogeny. Second, we improved dating of divergence times in the phylogeny by adding 14 paleobiogeographic calibration points to the three points originally used by Morvan and coauthors (2013). This addition resulted in overall younger divergence times. Thus, in the present phylogeny, the early diversification of the four *Proasellus* clades is dated to the Paleogene and not to the Upper Jurassic, as estimated by Morvan and coauthors (2013). However, paleobiogeographic calibration points are still

582 relatively unevenly distributed across the phylogeny, with only a single point for the North
583 American, albeit species-rich, clade. Adding new calibration points to this clade would require
584 sampling US regions where species-rich clades might have diversified "on place" following
585 emergence of lands from the sea (e.g., eastern Texas, Florida and Chesapeake Bay). Third, still
586 in comparison with Morvan and coauthors (2013)'s phylogeny, we more than doubled the
587 number of replicates of groundwater evolution by identifying 34 independent pairs of surface
588 and groundwater asellid species, among which 21 within the genus *Proasellus*. Further
589 sampling will likely provide additional species pairs within the *Asellus* pattern and North
590 American asellids, thereby providing a more even distribution of groundwater transitions
591 among three of the four major groups of asellids. Obtaining many replicate species pairs is
592 crucial to robust testing of common principles of groundwater evolution while accounting for
593 the effects of local contingencies. Up to now, comparative studies have relied on few replicates
594 of evolution to groundwater life - i.e., on 3 to 13 independent species pairs - for assessing
595 changes in the evolution of genome size and rate of molecular evolution in asellids (Lefébure
596 et al., 2017; Saclier et al., 2018), vision genes in beetles, crayfishes, and fishes (Stern and
597 Crandall, 2018; Policarpo et al., 2021; Langille et al., 2022), and gene repertoires in beetles
598 (Balart-García et al., 2023).
599
600 Another desirable attribute of a biological study system for understanding trait evolution in
601 groundwater is to have species that have colonized groundwater for different lengths of time.
602 Time is undoubtedly an important factor controlling the evolution of traits, at least those that
603 evolve under relaxed selection, such as the regression of eyes in subterranean animals (Wilkens
604 and Strecker, 2017; Policarpo et al., 2021; Langille et al., 2022). Among the asellids,
605 depigmented and reduced-eye subterranean populations of the surface species *Asellus aquaticus*

colonized groundwater less than one hundred thousand years ago (Protas and Jeffery, 2012; Protas el al., 2023), whereas some eyeless and depigmented species of *Proasellus* have resided in groundwater for over 10 million years (Lefébure et al., 2017). However, in a phylogeny, it is usually unclear at which point along a terminal branch leading to a groundwater species colonization of groundwater exactly occurred. Specifically, groundwater colonization may be much more recent than the speciation event leading to a pair of surface and groundwater species if now-extinct surface species have persisted long after that speciation event. In asellids, a promising approach is to use the pseudogenization of genes coding for opsin light-sensitive proteins to estimate the groundwater colonization time, assuming that loss-of-function mutations accumulate early in the process of groundwater colonization. In a study by Lefébure and coauthors (2017), colonization time was measured for 19 asellid species as a function of the speciation time and an estimate of the pseudogenization of the opsin genes on branches leading to subterranean species. Increasingly sequencing the opsin genes across asellid species (see Table 2) paves the way for accounting for the effect of colonization time on the evolution of phenotype in comparative studies. Of note, however, the pseudogenization approach to dating groundwater colonization times reaches its limits when the gene fails to be amplified, presumably due to a too long period of time a species spent underground (Lefébure et al., 2017; Langille et al., 2022).

4.3 | Comparative phylogenetic analyses of body size and sexual body size dimorphism

Our phylogenetic comparative study of BS and SBSD between surface- and groundwater species illustrates the usefulness of WAD for documenting evolutionary changes during transition to novel habitats. We found that BS in asellids was constrained by the size of habitat in both sexes. This corroborates Pipan and Culver (2017)'s hypothesis that BS within clades

631 containing subterranean species is in part controlled by habitat volume because pore size
632 between rocks can set an upper limit to maximum BS.
633
634 We provide the first, phylogenetically-controlled evidence that the difference in BS between
635 surface- and groundwater species is sex-dependent. Body size of males was significantly larger
636 in surface- than in groundwater species. We propose that competition for synchronously
637 receptive females selects for large males in surface species, while competition for rare, highly
638 asynchronous, receptive females favors small males in groundwater species (Andersson, 1994;
639 Blanckenhorn, 2000; Kelly et al., 2008; Balázs et al., 2021). In precopulatory mate guarding
640 crustaceans, among which many surface asellid species are known (Jormalainen, 2007), large
641 males have a mating advantage because they can more easily displace small guarding males
642 from their guarded females (Ridley and Thompson, 1979). In groundwater asellids, males no
643 longer guard females prior to copulation (Henry, 1976) and selection probably favors small
644 males that are more agile and can attain receptive females more rapidly. In addition, small males
645 can use energy that they do not invest in growth for searching for mates.
646
647 In contrast to male BS, we found that female BS did not differ between surface water and
648 groundwater asellids. Whatever the habitat, strong fecundity selection probably favors large
649 female size because brood size increases with increasing BS (Ridley and Thompson, 1979;
650 Pincheira-Donoso and Hunt, 2017). However, groundwater females take longer to grow than
651 surface water females (Henry, 1976). Life history studies of asellids also showed that
652 groundwater species were long-lived (>> 2 yr) and iteroparous, whereas surface water species
653 had short lifespan (ca. 1 year) and were semelparous (Steel, 1961; Henry, 1976).
654

We found that habitat specialization significantly influenced SBSD. A predominant pattern of male-biased SBSD occurred in surface species, whereas groundwater species were in average monomorphic in BS but exhibited much larger variation in SBSD (Figure 4). We provide two non-mutually exclusive explanations for difference in SBSD between habitats, in addition to selective factors influencing male and female BS discussed above. First, the degree of SBSD decreases in groundwater species because females mate with multiple males and produce multiple clutches of offspring during their life. Both aspects diminish the sex difference in the opportunity for selection and hence the potential for SBSD (Shuster and Wade, 2003; Shuster et al., 2013). Second, in the absence of precopulatory mate guarding, groundwater males may still prefer larger females that produce more eggs, but they no longer have to be bigger than females to carry them prior to copulation (Adams et al., 1985).

A recent morphological study by Balázs and coauthors (2021) investigated sexual dimorphism in 17 morphological traits, including body size, using nine surface and six cave groundwater populations of *Asellus aquaticus* showing various degrees of reduction of eyes and body pigments (see also Biró et al., 2022). The authors showed that several morphological traits were significantly less male-biased in cave than in surface populations (for example the shape of pereopods I). However, contrary to the present study, they found no significant reduction in male-biased dimorphism in body size upon cave groundwater colonization. A potential explanation is that the intraspecific comparative study by Balázs and coauthors (2021) may have been unable to detect a reduction in male-biased SBSD in cave populations of *A. aquaticus* due to insufficient time for BS to evolve. Of note, males were reported to be smaller than females in several depigmented and eyeless subterranean *Asellus* species including *A. amamiensis*, *A. hyugaensis*, *A. primoryensis* and *A. tamaensis* (Matsumoto, 1960, 1961 1963;

679 Henry and Magniez, 1993). A potentially important proportion of the variance in SBSD
680 exhibited by groundwater species might be due to differences in groundwater colonization time
681 among species. If so, using colonization time as a predictor instead of a qualitative present-day
682 biological status (i.e., eyeless and depigmented vs occulated and pigmented) would contribute
683 to a better understanding of trait changes associated with groundwater transitions. This may
684 become possible in a near future as sequences of genes accumulating loss-of-function mutations
685 during colonization (e.g. opsin gene, see Lefébure et al. 2017) become available for a large
686 number of species.
687
688 Dimorphism also significantly depended on habitat size. Groundwater species exhibiting male-
689 biased dimorphism occurred in habitats of larger size than groundwater species exhibiting
690 female-biased dimorphism or monomorphism. A potential explanation is that the mating
691 selective pressure for more agile and hence smaller males is less in cave habitats than in
692 interstitial habitats. Another non-mutually exclusive hypothesis is that even with equivalent
693 mating selective pressures for BS in both habitats, only the smallest specimens of a surface
694 population can colonize interstitial habitats. Hence, even those populations that have recently
695 colonized interstitial habitats would exhibit a weak sexual dimorphism in body size. Yet,
696 populations that have recently colonized cave habitats would exhibit male-biased dimorphism
697 until sexual selection has had time to act.
698
699 Beyond BS, WAD provides many of the necessary resources for testing predictions on how
700 phenotypic traits linked to mating success, fecundity, and survival evolve in response to
701 changes in selective pressures and trade-offs during groundwater colonization. We provide
702 below three example predictions. First, if searching for rather than fighting for mates is key to

703 determining mating success of male groundwater species, then, selection is likely to target
704 sensory organs that improve the ability of males to find females. More specifically, the hundreds
705 of specimens referenced in WAD can be used to test whether males of groundwater species
706 have longer antennae, relative to BS, than surface males and groundwater females, because long
707 antennae are advantageous for detecting receptive females (Bertin and Cézilly, 2003; Balázs et
708 al., 2021). Second, life history theory predicts that relative to their BS, groundwater, iteroparous
709 species should produce fewer but larger eggs per reproductive event than surface, semelparous
710 species (Fišer, 2019a; Venarsky et al., 2023). WAD keeps full record of the number of
711 ovigerous females contained in hundreds of specimen lots for testing this hypothesis. Third,
712 WAD resources can be used to test for the occurrence of a trade-off between transient fecundity
713 (i.e. the number of offspring produced per brood per single reproductive event) and adult
714 survival in long-lived, iteroparous groundwater species. Fecundity selection favors increase in
715 BS, whereas selection for survival may favor narrow and elongated body shapes that allow
716 individuals to withdraw into tiny hiding places to escape predators (Miller, 1933; Fišer et al.,
717 2013; Fišer Ž. et al., 2019). A trade-off may arise because an elongated brood pouch prevents
718 good ventilation of eggs beyond a certain BS. If such a trade-off exists, we predict variation in
719 BS to be more evolutionarily constrained in groundwater females than in surface females. This
720 prediction can be tested by comparing best-fit evolution models of BS and shape between
721 habitats and sexes.
722
723 **5 | CONCLUSION**
724 The asellids fulfill many of the desirable attributes of a model animal system for studying
725 evolution during colonization of a new environment, in particular here groundwater. Recently,
726 Protas and coauthors (2023) synthesized the ecological and molecular resources available for

studying microevolutionary dynamics of groundwater colonization from multiple cave and surface populations of *Asellus aquaticus*. Here, we make available to the scientific community a comprehensive set of taxonomic, distributional and molecular resources and biological material that have been acquired for studying macroevolutionary dynamics of groundwater colonization from multiple-species data. Looking at trait variation among multiple independent colonization events across a wide range of times since colonization can provide better understanding into the temporal dynamics of phenotypic evolution.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

All authors revised the manuscript and approved the publication. Manuscript conception, writing, editing, illustrations: N.S., F.M., C.J.D, D.E. Database structure conception: leaders: F.M., P.G.; contributors: L.K.D., T.L., C.J.D., C.M., C.C., D.E. Writing of code for the database and web application: P.G., L.D. Database management: F.M., L.K.D. Molecular data acquisition and management: L.K.D., C.J.D., T.L., C.F. Phylogenetic and comparative analyses: N.S., C.J.D., F.M. Acquisition of body size data: C.I., F.M., N.S. The authors hereinafter largely contributed to sampling and identification of biological material for the following groups and/or regions. North American asellids / North America: J.J.L.; multiple sites in the European Union: N.S., F.M., C.J.D., C.F., D.E., T.L.; coxalis group (*Proasellus*): F.S.; The Balkan Peninsula: B.S., S.G., T.D., M.Z., M.G.; Germany / Luxemburg: D.W.; Portugal: A.S.P.S.R.; Russia: D.P.; Crete: K.P.; United Kingdom: L.R.F.D.K.; Belgium: G.M.; Nouvelle Aquitaine (France): F.L.; Iran: M.J.M.H.; Spain: B.G.D.B., A.I.C.; Cameroon / Stenasellidae: R.P.T.K; Algeria: A.T., N.B.; Italy: D.M.P.G.; Romania: O.T.M.

DATA AVAILABILITY AND BENEFIT SHARING

The sequence data generated as part of this study have been deposited in NCBI. Sequence metadata, sequence alignments, the World Asellidae phylogeny, and data used in comparative

774 analyses are included as Supporting Information at the publisher's website or archived in
775 Zenodo. Access to metadata and data stored in WAD is at https://gotit.univ-lyon1.fr upon
776 request from the corresponding author. Temporary logins in the read mode for the reviewers
777 are as follows: User name: REVIEWER; Password: MOLECOLRES123456. The web
778 application GOTIT and structure of the World Asellidae Database are distributed with full
779 documentation at https://github.com/gotit-dev/gotit under the terms of GNU General Public
780 License. A demo version of GOTIT application is available at https://gotit.cnrs.fr.
781 The work presented herein is from a collaborative group of researchers who is committed to
782 international scientific partnerships, as well as institutional capacity building. Scientific
783 collaborators are included as co-authors and the results of research are shared with the sample
784 provider communities and the broader scientific community via the World Asellidae Database.
785

786 **ORCID**

787 *Nathanaelle Saclier* https://orcid.org/0000-0003-1522-9644
788 *Lara Konecny-Dupré* https://orcid.org/0009-0004-4604-687X
789 Philippe Grison
790 *Louis Duchemin* https://orcid.org/0000-0002-7984-9554
791 *David Eme* https://orcid.org/0000-0001-8790-0412
792 Chloé Martin
793 *Cécile Callou* https://orcid.org/0000-0002-8540-8114
794 *Tristan Lefébure* https://orcid.org/0000-0003-3923-8166
795 *Clémentine François* https://orcid.org/0000-0001-7781-8781
796 Colin Issartel
797 Julian J. Lewis

Fabio Stoch https://orcid.org/0000-0003-4535-3769

Boris Sket https://orcid.org/0000-0002-7398-5483

Sanja Gottstein https://orcid.org/0000-0002-1424-2911

Teo Delić https://orcid.org/0000-0003-4378-5269

Maja Zagmajster https://orcid.org/0000-0003-1323-9937

Michal Grabowski https://orcid.org/0000-0002-4551-3454

Dieter Weber https://orcid.org/0000-0001-7813-842X

Ana Sofia P.S. Reboleira https://orcid.org/0000-0002-4756-7034

Dmitry Palatov https://orcid.org/0000-0002-8826-9316

Kaloust Paragamian https://orcid.org/0000-0001-7372-733X

Lee R.F.D. Knight

Georges Michel

Francois Lefebvre

Mohammad-Javad Malek Hosseini https://orcid.org/0000-0001-7411-2150

Ana I. Camacho https://orcid.org/0000-0003-0596-7678

Gartzia De Bikuña Begoña https://orcid.org/0000-0001-6089-9458

Amina Taleb https://orcid.org/0000-0003-4045-590X

Nouria Belaidi https://orcid.org/0009-0004-2780-8031

Raoul P. Tuekam Kayo https://orcid.org/0000-0002-2578-4898

Diana Maria Paola Galassi https://orcid.org/0000-0002-6448-2710

Oana Teodora Moldovan https://orcid.org/0000-0002-1262-0675

Christophe J. Douady https://orcid.org/0000-0002-4503-8040

Florian Malard https://orcid.org/0000-0001-8037-4464

SUPPORTING INFORMATION

The following additional supporting information may be found in the online version of the article at the publisher's website or at https://doi.org/10.5281/zenodo.6474972 (i.e. SI Tables 4 to 8, plus the World Asellidae phylogeny).

SI Table 1. Metadata for the 2093 COI sequences used in the study.
SI Table 2. List of primers.
SI Table 3. Selection of most often used primers.
SI Table 4. Alignment of the 2093 COI sequences used for the delimitation of MOTUs.
SI Table 5. Alignment of the 424 COI sequences used for the four-gene dated phylogeny.
SI Table 6. Alignment of the 424 16S sequences used for the four-gene dated phylogeny.
SI Table 7. Alignment of the 424 FASTKD4 sequences used for the four-gene dated phylogeny.
SI Table 8. Alignment of the 424 28S sequences used for the four-gene dated phylogeny.
SI Table 9: Metadata for the DNA sequences used for the 4-gene dated phylogeny.
SI Table 10. Paleobiogeographic events used to constrain species divergence times.
SI Table 11. Data on body size, sexual body size dimorphism, habitat specialization and habitat size used in comparative analyses.
SI Table 12. Metadata for the DNA sequences deposited in NCBI as part of this study.
SI Table 13. Results of likelihood ratio tests for testing the effects of habitat specialization and habitat size on body size and sexual body size dimorphism.
SI Figure 1. Simplified logical model of the World Asellidae Database.
SI Figure 2. Full logical model of the World Asellidae Database.
SI Figure 3. Mapping of occurrence data, specimen lots, COI sequences and MOTUs within morphospecies with GOTIT.
SI Figure 4. Timetree of Asellidae (Isopoda, Pancrustacea).
SI Figure 5. Lineage through time plots of Aselloidea.
SI Figure 6. Phylogeny and comparative data set for the analysis of body size and sexual body size dimorphism.

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TABLES AND FIGURES (WITH CAPTIONS)

Table 1: Summary content of the World Asellidae Database (WAD). All items, except references, are for Asellidae (Isopoda, Pancrustacea). Data extraction on 10 March 2023.

Items	Number
Species Occurrence	
Species and subspecies	448
Record	9438
Country	55
Collection material [1]	
Specimen lot	1943
Specimen microscopic slide	1584
Specimen used for DNA extraction	4901
DNA extract	4362
DNA sequencing metadata	
Primer [2]	138
PCR	22743
Chromatogram	12052
Sequence [3]	
16S	3562
COI	2866
FASTKD4	922
28S	1202
Opsin	362
Literature reference	641

[1] All specimens and DNA extracts are stored at -20°C
[2] See SI Tables 2 and 3
[3] Validated sequences. Numbers differ from the number of sequences used in this study because the database is regularly updated with new data.

Table 2: Numbers of surface water (Surf.) and groundwater (Grou.) described species contained in the World Asellidae database (WAD) and numbers of morphospecies and MOTUs included in the Asellidae timetree. Numbers in bold are totals.

Morphospecies groups /genera	WAD		Asellidae timetree				
	Described species		Morphospecies [5]		MOTUs		Species pairs [6]
	Surf.	Grou.	Surf.	Grou.	Surf.	Grou.	
1 - Asellus pattern	**31**	**30**	**7**	**1**	**8**	**1**	**1**
Asellus	28	10	5	1	6	**1**	1
Calasellus		2					NA
Columbasellus		1					NA
Limnoasellus [1]	1		1		1		0
Mesoasellus	1		1		1		0
Nipponasellus		5					NA
Phreatoasellus	1	9					NA
Sibirasellus		2					NA
Uenasellus		1					NA
2 - North American asellids [2]	**68**	**84**	**51**	**24**	**60**	**48**	**10**
Baicalasellus	4		2		2		1
Caecidotea	9	39		9		28	1
Conasellus	21	24	13	9	17	10	6
Gallasellus		1		1		5	0
Lirceolus		6					NA
Lirceus	34	4	36	2	41	2	2
Pseudobaicalasellus		10		3		3	0
3 - *Bragasellus* & *Synasellus*	**3**	**53**	**2**	**20**	**2**	**32**	**1**
Bragasellus	3	18	2	8	2	19	1
Synasellus		35		12		13	0
4 - *Proasellus* [3]	**61**	**113**	**54**	**108**	**51**	**181**	**21**
Others [4]		**5**		**1**		**1**	**1**
Bowmanasellus		1					NA
Oregonasellus		1					NA
Salmasellus		2		1		1	1
Stygasellus		1					NA
Asellidae	**163**	**285**	**114**	**154**	**121**	**263**	**30**

[1] *Nomen nudum* in Hidding et al. (2003)
[2] Genera according to recent revision by Lewis et al. (2023)
[3] Including *Chthonasellus bodoni* Argano & Messana, 1991
[4] Genera that cannot be assigned to any of the four species groups.
[5] Numbers include undescribed morphospecies
[6] Number of independent species pairs containing a surface water and a groundwater asellid species.

1208 Table 3: Results of phylogenetic generalized least-squares regression models for testing the
1209 effects of habitat size and specialization (i.e. surface vs groundwater habitats) on body size of
1210 females and males and sexual dimorphism index. Significant P values are in bold.

1211

Dependent variable	Explanatory variable	Parameter estimate	Standard error	*t*	P
Male body size	Intercept (groundwater)	1.871	0.401	4.662	
	Habitat size	0.186	0.044	4.242	**<0.001**
	Habitat specialization	0.166	0.080	2.084	**0.0387**
	Habitat Size × habitat specialization	-0.039	0.091	-0.427	0.670
Female body size	Intercept (groundwater)	1.768	0.354	4.994	
	Habitat size	0.129	0.039	3.350	**0.001**
	Habitat specialization	-0.008	0.070	-0.111	0.912
	Habitat Size × habitat specialization	-0.008	0.080	-0.098	0.922
Sexual dimorphism index	Intercept (groundwater)	-0.134	0.289	-0.472	
	Habitat size	-0.072	0.032	-2.268	**0.025**
	Habitat specialization	-0.203	0.057	-3.534	**0.001**
	Habitat Size × habitat specialization	0.044	0.065	0.676	0.500

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Figure 1: Distribution of four major species groups of Asellidae (Isopoda, Pancrustacea). Dots are species occurrence data contained in the World Asellidae Database (black dots: surface water species; white dots: groundwater species).

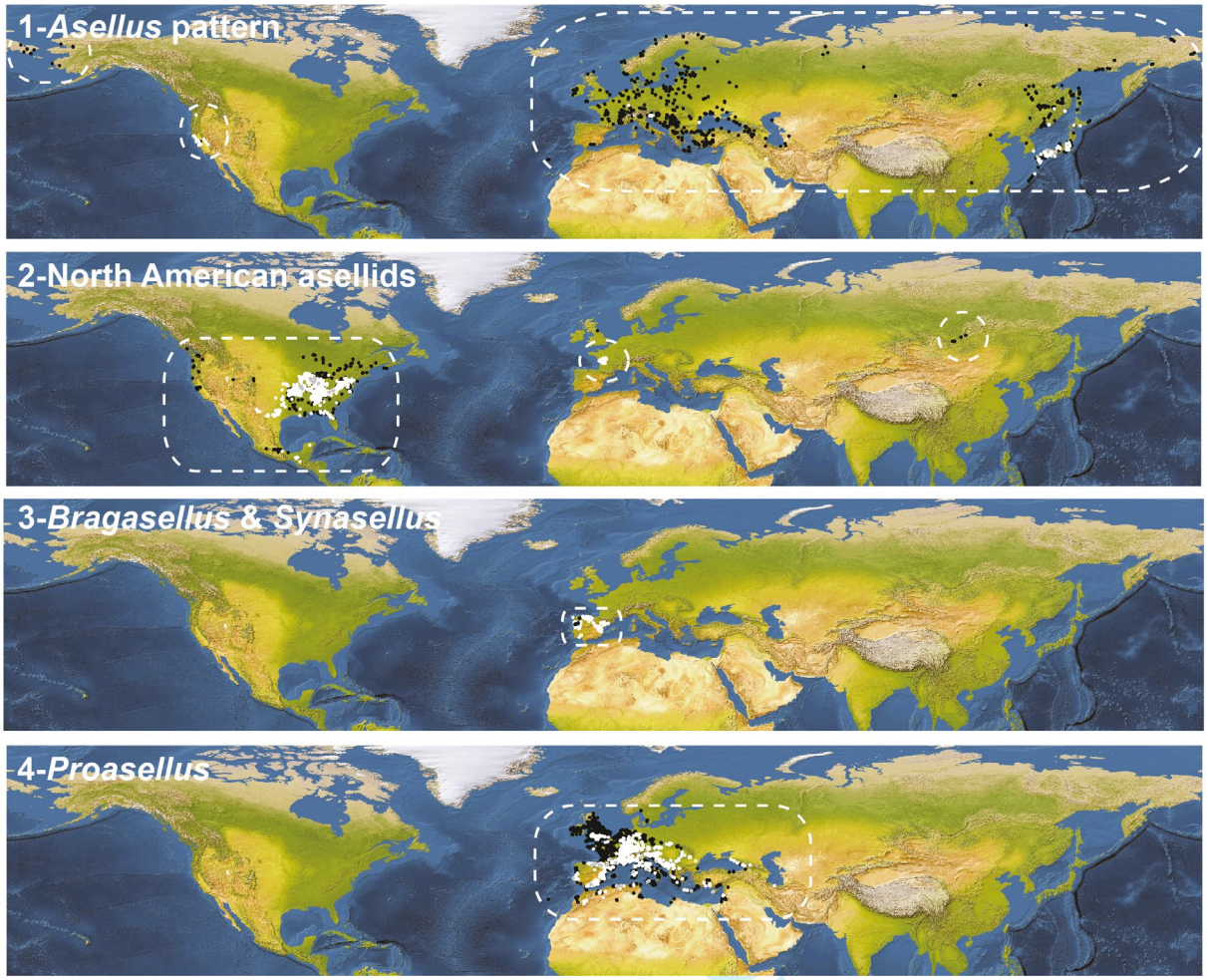

Figure 2: Pairwise taxonomic comparisons between the three different sets of aselloid species hypotheses delimited using morphology (Morph.), a COI divergence threshold (TH), and the Poisson tree processes model (PTP).

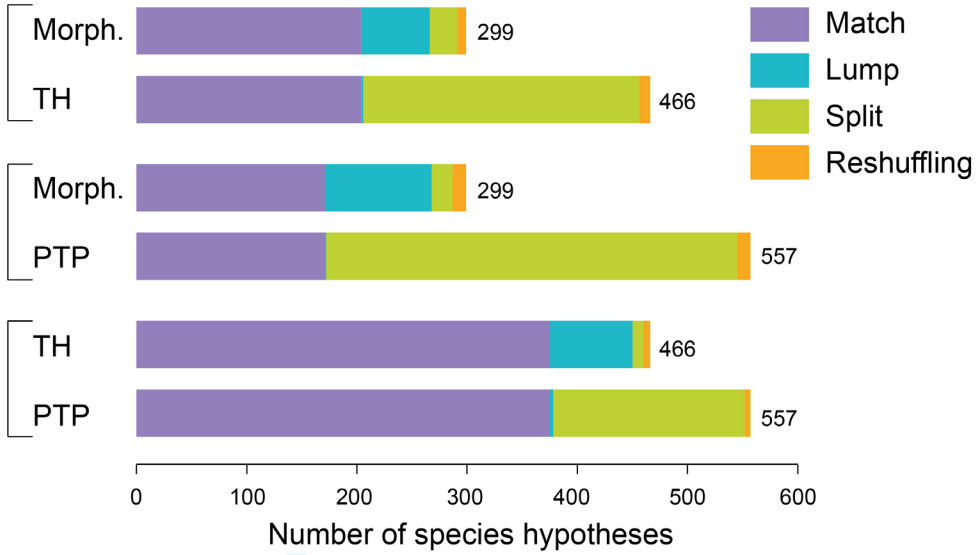

Figure 3: Timetree of Asellidae (Isopoda, Pancrustacea). The tree is rooted using Stenasellidae as outgroup. Terminal nodes are molecular operational taxonomic units (MOTUs) as delimited with the fixed COI threshold method (TH) implemented by Lefébure and coauthors (2006). White terminal nodes are eyeless and depigmented MOTUs; black terminal nodes are occulated and/or pigmented MOTUs. Color rings show time. Red and gray dots show paleobiogeographic calibration points and node supports with posterior probabilities > 0.9, respectively. Black and white squares on the outer ring show independent pairs of surface (black) and groundwater (white) asellid species (see definition of species pairs in materials and methods). Legends show genera and main species groups within the Asellidae family and *Proasellus* genus. Groups are as follows: for Asellidae: 1 – *Asellus* pattern, 2 – North American asellids, 3 – *Bragasellus* + *Synasellus*, 4 – *Proasellus*; for *Proasellus*: slavus – ibero-aquitanian – anophtalmus-coxalis – alpine.

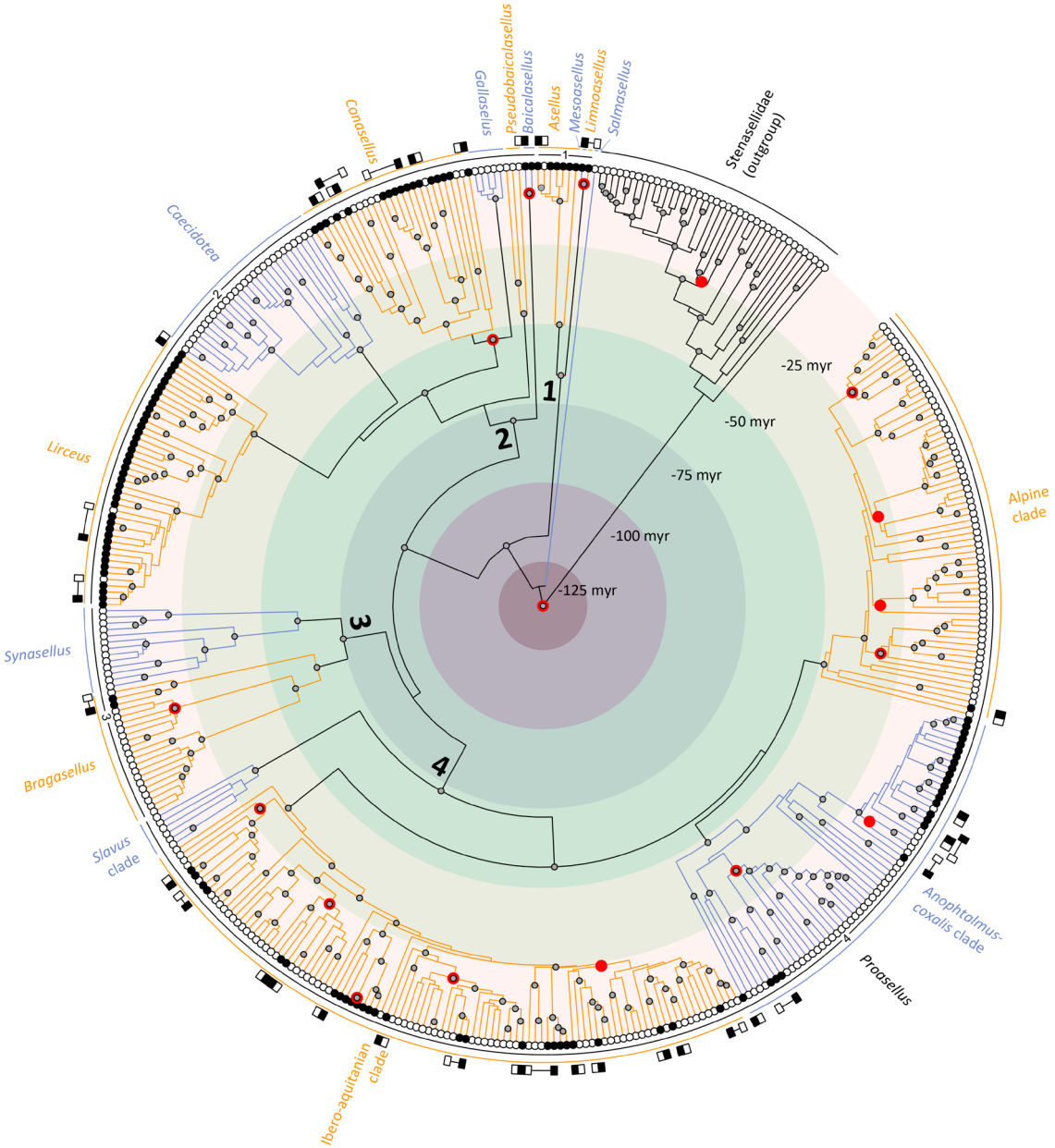

Figure 4: A-C: Relationships between body size (males and females) and habitat size and between sexual dimorphism index (SDI) and habitat size. Data for habitat size correspond to the coordinates of species along the first axis of the fuzzy correspondence analysis performed on the "habitat trait categories per species" matrix (See SI Table 11). SDI is negative when males are larger than females and positive when females are larger than males. The red lines represent the phylogenetic generalized least square regressions. All regressions are statistically significant. D-F: Violin plots showing the difference in body size (males and females) and sexual dimorphism index (SDI) between surface water- and groundwater-habitat specialist species. The white dot, thick black bar, and thin black line show the median value, interquartile range, and 95% of all data, respectively. Significant *P* values are in bold.