

Catch-All No More: Integrative Systematic Revision of the Genus Allolobophora Eisen, 1874 (Crassiclitellata, Lumbricidae) with the Description of Two New Relict Earthworm Genera

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Research Article

Catch-All No More: Integrative Systematic Revision of the Genus Allolobophora Eisen, 1874 (Crassiclitellata, Lumbricidae) with the Description of Two New Relict Earthworm Genera

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The taxonomy of earthworms has been riddled by instability, lack of systematically useful characters, and lax diagnoses of some genera. This has led to the use of some genera such as *Allolobophora* Eisen, 1874 as taxonomic wastebaskets, blurring their evolution and biogeographical history. The implementation of molecular techniques has revolutionized the systematics of the genus; however, some of its species have not been previously included in molecular phylogenetic analyses. Thus, the molecular markers COI, 16S, ND1, 12S, and 28S were sequenced for six endemic species including several taxa of *Allolobophora* and *Aporrectodea* Örely, 1885 (another related catch-all genus). Phylogenetic relationships determined by Bayesian inference and maximum likelihood analyses support the status of two of the six taxa examined (*Allolobophora burgondiae* Bouché, 1972 and *Aporrectodea icterica* Savigny, 1826) as part of *Allolobophora* sensu stricto and a presumed synonymy between *Allolobophora* and *Heraclescolex* Qiu and Bouché, 1978. Branch lengths and average pairwise genetic distances support the transfer of *Allolobophora satchelli* Bouché, 1972 to the genus *Panoniona* Mršić and Šapkarev, 1988 and the emergence of two new genera, *Heraultia* gen. nov. and *Vosgesia* gen. nov., endemic to France, hosting *Allolobophora tiginosa* Bouché, 1972 and *Allolobophora zicsii* Bouché, 1972, respectively. The aforementioned changes of status and the diagnosis for *Heraultia* and *Vosgesia* are presented. These results provided more evolutionarily and biogeographically coherent earthworm groups and highlighted that the Maghreb and the area around the Alps are potential key locations for the diversification of *Allolobophora* and several lineages of Lumbricidae.

1. Introduction

The taxonomy of earthworms has proven to be very unstable over the last two centuries. This is due to a lack of systematically useful morphological characters and the fact that most of them are homoplasious or symplesiomorphic [1]. Within Lumbricidae Rafinesque-Schmaltz 1805, the most speciose family of the Palaearctic, some genera have been inadvertently used as taxonomic wastebaskets due to excessively loose diagnoses. As shown by the molecular phylogenetic analyses of Dominguez et al. [2], such catchall taxa, e.g., *Aporrectodea* Örley, 1885, *Allolobophora* Eisen, 1874, or *Helodrilus* Hoffmeister, 1885, contain independent evolutionary lineages which should be described as different genera.

Allolobophora was established by Eisen in 1874 based on external morphological characters and included seven species, but without selecting a type species. Further species were progressively added to a somewhat refined Allolobophora (as internal characters were added to its diagnosis), but after the revision of Pop [3], it became a catch-all genus [1]. Later works suggested that Allolobophora should be divided into subgenera or even different genera [4-8], but with little consensus.

Only the designation of Allolobophora chlorotica (Savigny, 1826) as its genus type allowed the delimitation of Allolobophora sensu stricto and Allolobophora sensu lato, which includes most of its species [1]. The inclusion of All. chlorotica in the phylogenetic analysis of Domínguez et al. [2] corroborated this view: All. chlorotica and other green pigmented species (Allolobophora dubiosa Örley, 1881, Allolobophora moebii Michaelsen, 1895, and Allolobophora molleri Rosa, 1889) formed a clade separated from the species assigned to the subgenus Allolobophora (Gatesona) Bouché, 1972 and to Allolobophora sensu lato (or Karpatodinariona Mršić and Šapkarev, 1988 and Serbiona Mršić and Šapkarev, 1988). Further molecular phylogenetic works cemented this separation by establishing the genus Gatesona [9] and by amending the genus Cernosvitovia Omodeo, 1956 to include several Balkanic species of Allolobophora sensu lato [10].

Eophila Rosa, 1893 is in strong conflict with *Allolobophora*, as numerous species have been transferred between the two genera in successive taxonomic revisions. It has also been considered synonymous with *Heraclescolex* Qiu and Bouché, 1998, a genus consisting mainly of green pigmented earthworms with little distinction from the other two genera. The inclusion of the type species of the genus *Eophila*, *Eophila tellinii* Rosa, 1888, in molecular phylogenetic analysis by de Sosa et al. [11] has eliminated the confusion: *Eophila* should be restricted to *Eo. tellinii, Eophila gestroi* (Cognetti de Martiis, 1905), and *Eophila crodabepis* [12]. Furthermore, the genotype of *Heraclescolex* (*All. moebii*) was nested with *All. chlorotica*, thus suggesting the synonymy of the latter genera.

Koinodrilus Qiu and Bouché, 1998 was created to accommodate species previously assigned to Allolobophora and/or Aporrectodea. Its type species, Aporrectodea georgii Michaelsen, 1890, and a few other representatives (Aporrectodea jassyensis (Michaelsen, 1891), Aporrectodea limicola (Michaelsen, 1890), Aporrectodea rosea (Savigny, 1826), and Allolobophora oliveirae (Rosa, 1894)) were included in phylogenetic trees by Domínguez et al. [2], but the results did not strongly support this genus. On the contrary, Ap. georgii and Ap. jassyensis formed a clade, but the rest of the species appeared scattered within other well-supported clades or behaved as rogue taxa (with unstable positions).

Despite the significant advances in the taxonomy and systematics of the French Lumbricidae in recent years, the phylogenetic relationships of some rare endemic species remain uncertain. For example, *Allolobophora burgondiae* (Bouché, 1972) and *Allolobophora satchelli* (Bouché, 1972) were originally placed within *Allolobophora* sensu stricto by Bouché [4] and retained within the genus by Qiu and Bouché [8], and their relationships were not been questioned; yet they were never been included in molecular phylogenetic analyses. *Allolobophora tiginosa* (Bouché, 1972) and *Aporrectodea icterica* (Savigny, 1826) were transferred from *Allolobophora* sensu stricto to *Koinodrilus* and *Heraclescolex*, respectively, in Qiu and Bouché [13], while *Allolobophora zicsii* Bouché, 1972 was transferred from *Allolobophora* sensu lato to *Koinodrilus* [14]. Finally, *Apor-* rectodea pseudoantipai (Qiu and Bouché, 1998) was originally placed within Koinodrilus.

This paper places the aforementioned species in a molecular phylogenetic context to (i) place the last few *incerta sedis* French endemic species into their appropriate genera, (ii) interrogate whether *Allolobophora* and *Heraclescolex* are synonymous, and (iii) test the validity of *Koinodrilus*.

2. Materials and Methods

2.1. Samplings and Studied Specimens. This study follows the methodology of Marchán et al. [9, 15], and the method description partially reproduces their wording. 15 specimens of six species of Lumbricidae (genera *Allolobophora* and *Aporrectodea*) were collected during two sampling surveys in southeastern and northeastern France in spring 2021 and autumn 2021, respectively. The list of species and the sampling localities are given in Table 1. Individuals were collected by digging up the soil and sorting by hand, rinsing with water, and fixing in pure ethanol to allow further molecular analyses. Sampling and handling of specimens were done ethically and in accordance with Directive 2010/ 63/EU.

Species classification and morphological diagnoses were made under a binocular stereomicroscope using the set of external and internal morphological characters used by Qiu and Bouché [16] and following the format established by Domínguez et al. [17]. The following main external morphological characters were considered: mean length, mean number of segments, mean weight, pigmentation, type of prostomium, setal arrangement, position of papillae, position of first dorsal pore, nephridial pore arrangement, position and development of male pores, position and development of female pores, position of spermathecal pores, position of clitellum, and position of tubercula pubertatis. The main internal anatomical features were position of oesophageal hearts, position and morphology of calciferous glands, position of crop, position of gizzard, type of typhlosole, shape of nephridial bladders, number and position of seminal vesicles, and number and position of spermathecae.

2.2. DNA Extraction and Sequencing. After morphological identification, total genomic DNA was extracted from ventral integument samples of approximately 5×5 mm by using the SpeedTools Tissue DNA Extraction kit (Biotools). Regions of the nuclear gene 28S rRNA and mitochondrial 16S rRNA, 12S rRNA, NADH dehydrogenase (ND1), and cytochrome oxidase subunit 1 (COI) were amplified by polymerase chain reaction (PCR), using the primers described in Pérez-Losada et al. [18] and Folmer et al. [19]. PCR reactions were performed using a GeneAmp Multicycler Ep gradient (Eppendorf) under the following conditions: an initial denaturation step (5 min at 94°C); 40 cycles (35 for ND1) consisting of denaturation at 95 °C for 30 s, annealing (between 45°C and 55°C) for 45 s, and extension at 72°C for 1 min; and a final extension step (5 min at 72°C). The amplified PCR products were purified using the Multiscreen PCR μ 96 purification kit (Millipore) and sequenced in Macrogen (Spain). DNA sequences

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Species	Ν	Collectors	Sampling location	Date	Coordinates
Allolobophora burgondiae	3	Marchán, Decaëns, Della Vedova	Bouze-lés-Beaune, Côte-d'Or, France	30-Oct-2021	47.062, 4.760
Allolobophora satchelli	3	Marchán, Decaëns, Della Vedova	Saint-Louis, Haut-Rhin, France	04-Nov-2021	47.618, 7.538
Allolobophora tiginosa	1	Hedde, Marsden, Gerard, Beauchesne	Puéchabon, Hérault, France	09-Feb-2021	43.713, 3.605
Aporrectodea icterica	3	Marchán, Decaëns, Della Vedova	Plombiêres-les-Bains, Vosges, France	02-Nov-2021	47.988, 6.512
Allolobophora zicsii	3	Marchán, Decaëns, Della Vedova	Luxeuil-les-Bains, Haute-Saône, France	02-Nov-2021	47.836, 6.359
Aporrectodea pseudoantipai	2	Marchán, Decaëns, Della Vedova	Trépot, Doubs, France	31-Oct-2021	47.170, 6.168

TABLE 1: Sampling information for the target species of this work.

obtained in this study are available in GenBank, under accession numbers OQ224974-OQ224979 for COI, OQ236700-OQ236705 for ND1, OQ225516-OQ225521 for 28S, OQ225522-OQ225527 for 12S, and OQ225532-OQ225537 for 16S; COI sequences, together with metadata, are publicly available in the dataset DS-ALLREV in BOLD (doi:10.5883/DS-ALLREV).

2.3. Phylogenetic Analyses. Sequences were aligned using MAFFT v.7 [20] with default parameters and concatenated in BioEdit [21], obtaining a sequence of 3,260 base pairs for each species. Sequences reported by Dominguez et al. [2], Domínguez et al. [17], Pérez-Losada et al. [18], Pérez-Losada et al. [22], Pérez-Losada et al. [23], Paoletti et al. [12], de Sosa et al. [11], Bozorgi et al. [24], Jiménez Pinadero et al. [25], and Marchán et al. [26] with representatives of most Lumbricidae genera and two members of the closest families (Hormogastridae and Criodrilidae) were downloaded from GenBank and used as a reference dataset. The included species and their source publications are listed in Supplementary Table 1.

The best-fitting evolutionary model for each partition was selected with jModelTest v. 2.1.3 [27] following the Akaike Information Criterion (AIC; [28]) and the Bayesian Information Criterion (BIC; [29]). GTR+I+G was selected as the best evolutionary model for COI, 28S, and ND1, whereas GTR+G and HKY+I+G were chosen for the molecular markers 12S and 16S, respectively. Phylogenetic relationships were inferred by Bayesian inference analysis (BI) using MrBayes v.3.2.6 [30] implemented in CIPRES Science Gateway V. 3.3 [31]. Parameters were set to 50 million generations and sampled every 5,000th generation (10,000 trees). Two independent runs of four chains each were performed, and 20% of the trees were discarded as burn-in. The remaining trees were combined and summarized on a 50% majority-rule consensus tree. Maximum likelihood phylogenetic inference was performed using RAxML-NG [32], starting from 10 random initial trees and 1000 fast bootstrap replicates. Clade support values over 70% and 90% (for bootstrap and posterior probability, respectively) were considered high.

Uncorrected average pairwise distances between the studied species for the molecular markers COI and 16S were calculated in MEGA 11 [33] to support their status as separate species and to investigate genetic distances within and between genera.

3. Results

3.1. Morphological Study. The studied specimens fitted the diagnoses of their respective species [4, 13, 14].

3.2. Molecular Phylogenetic Analysis. Phylogenetic relationships determined by Bayesian inference (Figure 1) and maximum likelihood (Supplementary Figure 1) were congruent with previous phylogenetic analyses and between them, with the main internal branches resolved receiving high statistical support. The results do not support the monophyly of Allolobophora as currently defined, as the studied species appeared scattered throughout different clades. However, All. burgondiae and Ap. icterica were recovered in a clade with All. chlorotica and the species formerly assigned to Heraclescolex (All. moebii-designated as its type-All. molleri, Ap. icterica, and Ap. dubiosa). In contrast, the four remaining species were recovered as more distantly related taxa, not sharing the most recent common ancestor with Allolobophora. Although with low support, All. zicsii was recovered in both phylogenetic analysis as a sister species of Avelona ligra (Bouché, 1969), the only known species of the genus Avelona Qiu and Bouché, 2000; both species formed a clade sister to the strongly supported genus-level clade Gatesona. A similar scenario was found for All. satchelli, who was recovered as a sister taxon to Panoniona leoni Michaelsen, 1891. With respect to Allolobophora tiginosa, this taxon was placed in a large clade comprising Castellodrilus Qiu and Bouché, 1998; Zophoscolex Qiu and Bouché, 1998; Ethnodrilus Bouché, 1972; Prosellodrilus Bouché, 1972; and Cataladrilus Qiu and Bouché, 1998, forming a well-supported clade with the last two mentioned genera. However, the precise phylogenetic relationship between them remained unsolved. Finally, the results for Aporrectodea pseudoantipai show that it belongs in a clade with the representatives of Aporrectodea (except for Aporrectodea rosea, which is considered a rogue taxon), with Aporrectodea handlirschi (Rosa, 1897) as its closest relative. Aporrectodea georgii (type species of Koinodrilus) showed no affinity with the species once assigned to Koinodrilus (zicsii, pseudoantipai, rosea, jassyensis, limicola, and oliveirae). Allolobophora bartolii (Bouché, 1970), although with minor support, does not appear to be related to Allolobophora sensu stricto. Instead, it is more closely related to Eophila Rosa, 1893 and Imetescolex Szederjesi et al., 2022.

The four taxa not placed within the Allolobophora sensu stricto clade showed high distance values for both markers with representatives of that clade (including All. burgondiae and All. icterica), with All. satchelli showing the smallest distances with the species of the genus (Table 2). However, All. satchelli displayed its lowest distance values for both markers with P. leoni (14.71% for COI, the lowest value for this marker, and 6.00% for 16S). For All. zicsii, UAPG distances for the molecular markers COI and 16S were high between the species and every taxon included in the analysis, even with its closest relative Av. ligra (19.17% for COI and 14.02% for 16S). All. tiginosa showed its lowest distance with the type species of the genus Cataladrilus, C. monticola (10.61% for 16S), while Ap. pseudoantipai showed generally lower distances with Ap. handlirschi than with any other species of Allolobophora.

4. Discussion

4.1. Systematic Implications. The inclusion of All. burgondiae and Ap. icterica within a well-supported clade with All. chlorotica, All. molleri, and All. dubiosa and the low UAPG distance values among them suggest that both species should be placed within Allolobophora. The phylogenetic position of Ap. pseudoantipai with the representatives of Aporrectodea,



FIGURE 1: Detail of the clades recovered by Bayesian inference that includes the species of study. The phylogenetic analysis was performed using the concatenated sequence of the five molecular markers (COI, ND1, 16S, 12S, and 28S). Vertical grey lines show differences in topology according to a maximum likelihood analysis. Support values are shown besides corresponding nodes (posterior probability, left; bootstrap, right). The taxa included in this work are shown in bold. *Allolobophora* sensu stricto clade is shaded in green. The tree containing all phylogenetic relationships is shown in Supplementary Figure 2.

all of them forming a well-supported genus-level clade, suggests that it should remain within that genus. The possibility that *Eiseniona* Omodeo, 1956 (with *Eiseniona handlirschi* as type species) is a valid genus and that *Ap. pseudoantipai* should belong to it cannot be discarded without sampling additional representatives.

On the other hand, the nonmonophyly of All. satchelli, All. zicsii, and All. tiginosa with the species of Allolobophora sensu stricto implies these species should be assigned to other genera. For All. satchelli, the Bayesian inference and the UAPG distances support the inclusion of the species within the genus Panoniona Mršić and Šapkarev, 1988, changing its status to Panoniona satchelli. Interestingly, All. bartolii was placed by Qiu and Bouché [8] within Panoniona, which was not supported by molecular phylogenetics results. This highly polymorphic species (probably being a species complex) requires further study before a new genus can be proposed for it. The high distance values between All. zicsii and all the studied species and the long branches that separate this taxon from its closest relative suggest the designation of a new genus for this species. Similarly, All. tiginosa has been shown to be closely related to the genera

Prosellodrilus and *Cataladrilus* but without belonging to the same genus-level clade, thus justifying the creation of its own genus.

The nonmonophyly of Aporrectodea georgii with the other species included in Koinodrilus suggests that it would be a monospecific genus if retained. Also, Aporrectodea georgii does not appear to be related to Aporrectodea sensu stricto, the closest relatives of Aporrectodea trapezoides (Dugès 1828), so it should not be classified within Aporrectodea. Therefore, Koinodrilus would be a valid name, and a new one would not be necessary. Interestingly, Marchán et al. [34] recovered a close phylogenetic relationship between Ap. georgii and All. chlorotica when using the novel phylogenomic technique AHE (Anchored Hybrid Enrichment, [35]): this relationship was recovered with low support in the maximum likelihood analysis based on the traditional Sanger-sequenced markers. As the taxonomic sampling was limited (43 species), an increased number of species could confirm or deny such an evolutionary affinity.

Proposed taxonomic changes Phylum Annelida Lamarck, 1802 Subphylum Clitellata Michaelsen, 1919 Class Oligochaeta Grube, 1850 Superorder Megadrili Benham, 1890 Order Haplotaxida Michaelsen, 1900 Family Lumbricidae Rafinesque-Schmaltz, 1815 Tribe Prosellodrilini Qiu and Bouché, 1998 Genus *Heraultia* Martínez Navarro and Marchán, 2022 Type species: *Heraultia tiginosa stat. nov.* (Bouché, 1972) Material studied: 1 adult specimen; BOLD sample ID: ALL-016; Occitanie, Hérault, Puéchabon; latitude/longitude:

43.713/3.605; 09-Feb-2021; M. Hedde, Marsden, Gerard, Beauchesne leg. Specimen deposited in UCM-LT.

Diagnosis:

Small-sized (50-75 mm and 0.2-0.29 gr) Prosellodrilini with less than 200 segments and absence of pigmentation. Prostomium is epilobous. Setae are closely paired. First dorsal pore in 4/5. Male pores in 1/2 15 with well-developed porophores. Spermathecal pores in 10/11, 11/12, and 12/13. Nephridial pores aligned. Clitellum in 21-36. *Tubercula pubertatis* in 27-29, winglet shaped. Oesophageal hearts in 6-11; Calciferous glands poorly developed in 13 and 14, without diverticula or dilations. Crop in 15-16. Glizzard in 17-18. Typhlosole is bifid. Three pairs of globular spermathecae in 11, 12, and 13, rarely double. Glandular papillae in 10, 11, and 26-32. Two pairs of seminal vesicles in 11 and 12, poorly developed. Nephridial bladders are U-shaped (incurvate and reclinate). Cross section of longitudinal musculature elementary (sensu Bouché, 1972).

Differential diagnosis:

Heraultia shares with *Prosellodrilus* and *Cataladrilus* (its closest relatives) the anterior position of the clitellum and the number and position of seminal vesicles (two pairs in 11 and 12), but it is clearly separated from both by the poorly developed calciferous glands (well-developed and usually with diverticula or dilations in 11 in *Prosellodrilus* and *Cataladrilus*) and by the position of the spermathecae (10/11, 11/12, and 12/13 vs. (12/13), 13/14, and 14/15 in *Prosellodrilus* and 9/10 and 10/11 in *Cataladrilus*). For the diagnoses of the compared genera, see Table 3.

Other species included: none.

Tribe Lumbricini Qiu and Bouché, 1998

Genus Allolobophora Eisen, 1874

Type species: Allolobophora chlorotica (Savigny 1826)

Material studied: (a) *Allolobophora burgondiae*: 3 adult specimens; BOLD sample ID: ALL-002, ALL-003, ALL-004; Bourgogne, Côté d'Or, Bouze-lés-Beaune; latitude/longitude: 47.062/4.760; 30-Oct-2021; R. Della Vedova, T. Decäens, D. Fernández Marchán leg. Specimen deposited in UCM-LT; (b) *Allolobophora icterica*: 3 adult specimens; BOLD sample ID: ALL-010, ALL-011, ALL-012; Grand Est, Vosges, Plombiêres-les-Bains; latitude/longitude: 47.988/ 6.512; 02-Nov-2021; R. Della Vedova, T. Decäens, D. Fernández Marchán leg. Specimen deposited in UCM-LT.

Diagnosis:

Small-to-medium-sized Lumbricini with green pigmentation (sometimes absent). Prostomium is epilobous, no transversal furrows. Setae are closely paired. First dorsal pore in 4/5 (rarely 3/4, 5/6, and 7/8). Male pores in 1/2 15 with well-developed porophores. Presence of spermatophores. Spermathecal pores in variable position and number between 7/8, 8/9, 9/10, and 10/11, simple. Nephridial pores irregularly distributed (*en solfège*). Clitellum moderately posterior to extremely posterior, starting between 28 and 73 and ending between 36 and 78. Oesophageal hearts in 6-11. Calciferous glands in 10-14 with diverticula in 10. Crop in 15-16. Glizzard in 17-18. Typhlosole is usually trifid (sometimes bifid or with 4 lamellae). Variable number of simple spermathecae, usually 3 or 4 pairs. Four pairs of seminal vesicles in 9, 10, 11, and 12. Nephridial bladders are Ushaped (incurvate and reclinate or proclinate). The main morphological characters of the species of *Allolobophora* sensu stricto (see below) can be found in Table 4.

Differential diagnosis:

Even though *Cernosvitovia* as redefined by Popović et al. [10] is a morphologically heterogeneous genus, *Allolobophora* can be distinguished from it by its pigmentation (if present: green vs. brown) and either the position of the male pore (1/2 15 vs. 25-30) or the position of the spermathecae (variable in 7/8, 8/9, 9/10, and 10/11 vs. 9/10, 10/11, 11/12, and (12/13)).

Allolobophora is clearly separated from *Gatesona* by the absence of pigmentary dots in cephalic segments, by its general pigmentation (if present: green vs. brown), by the absence of very conspicuous genital papillae between segment 9 and the end of the clitellum and by the start of the clitellum (28-73 vs. 23-27). They also differ in the position of the spermathecae (variable in 7/8, 8/9, 9/10, and 10/11 vs. 9/10, 10/11, and (11/12)).

Allolobophora differs from *Panoniona* by the more posterior position of the clitellum, frequently green pigmentation vs. absence of pigmentation, repetition of spermathecae (simple vs. double our multiple), number of seminal vesicles (four pairs in 9, 10, 11, and 12 vs. two pairs in 11 and 12), and shape of nephridial bladders (U-shaped vs. J-shaped).

Allolobophora can be separated from *Eophila* by its generally smaller size, its pigmentation (if present: green vs. purplish-brown in bands or brown), and the position of the spermathecae (variable in 7/8, 8/9, 9/10, and 10/11 vs. 9/10 and 10/11). The comparison of the mentioned genera is shown in Table 3.

Species included the following:

- (i) Allolobophora chlorotica (Savigny, 1826)
- (ii) Allolobophora burgondiae Bouché, 1972
- (iii) Allolobophora icterica (Savigny, 1826)
- (iv) Allolobophora molleri Rosa, 1889
- (v) Allolobophora moebii Michaelsen, 1895
- (vi) Allolobophora dubiosa Örley, 1881

Remarks: the species assigned to *Heraclescolex* by Qiu and Bouché [13] form a remarkably homogenous group in terms of morphology and anatomy. Since four species formerly assigned to *Heraclescolex* (including the type species) have been recovered within *Allolobophora* by phylogenetic analyses, it is likely that the rest will also belong to this genus. The taxonomic status of the *Allolobophora molleri*-

TABLE 2: Uncorrected average pairwise genetic (UAPG) distances between the species of study and representatives of the genus Allolobophora, Cataladrilus, Prosellodrilus, Avelona, Panoniona, and Aporrectodea, for 16S (above the diagonal) and COI (below the diagonal). The cell could be shown in green (low genetic distance between the compared taxa), yellow (medium), or red (high).

інэгтівпра .qA	11.46	14.19	14.91	10.65	9.00	9.98	10.83	11.87	11.65	12.65	12.65	10.15	9.18	13.81	
nzonipit.IIA	10.61	15.00	14.91	14.17	13.52	15.17	13.52	15.69	16.17	17.17	15.78	14.67	12.88		16.36
illədətnə .llA	11.52	15.53	12.71	6.00	7.36	10.67	11.69	11.54	11.52	12.35	14.52	11.00		15.04	18.02
Ap. icterica	13.98	15.69	16.22	11.30	7.49	6.15	5.82	12.71	9.30	14.84	14.14		16.36	17.19	20.13
iisəiz IIA	14.43	17.00	16.72	14.81	14.02	14.98	14.45	14.02	15.64	17.82		20.83	19.34	17.85	20.99
inqiinnobuseq .qA	16.13	17.74	17.46	14.00	13.37	15.68	14.70	17.26	14.86		18.02	18.51	18.84	17.69	18.35
snibnogrud .llA	13.98	16.03	15.41	12.81	7.65	10.15	10.17	13.38		19.87	17.55	16.86	17.38	17.88	20.17
Av. ligra	13.33	16.97	14.36	13.55	11.06	12.71	12.23		17.85	19.67	19.17	20.13	18.18	18.02	18.15
insllom .llA	13.83	15.38	15.41	11.81	8.51	7.82		19.37	17.70	18.56	19.73	16.69	18.56	17.89	18.03
nsoidub .llA	13.31	15.36	14.55	11.63	7.67		15.19	17.66	18.68	18.84	19.34	16.01	18.84	18.35	19.64
All. chlorotica	10.17	13.88	11.07	9.33		17.99	19.20	18.48	19.01	20.33	18.02	18.32	17.36	17.85	18.65
P. leoni	12.98	16.36	14.55		20.46	19.80	20.37	18.65	19.67	18.68	19.83	20.63	14.71	18.02	17.99
Pr. biauriculatus	12.02	14.67		18.15	19.47	18.98	18.86	18.65	20.33	17.52	19.17	20.30	18.02	16.36	17.49
susinnsvyq.r ^q	11.94		17.00	19.97	21.45	20.13	20.03	19.64	19.83	19.34	20.33	21.95	19.50	17.52	19.31
Cat. monticola		18.32	16.01	20.63	17.99	19.80	20.87	19.31	19.83	18.68	18.84	19.80	17.69	16.53	17.16
	Cat. monticola	Pr. pyrenaicus	Pr. biauriculatus	P. leoni	All. chlorotica	All. dubiosa	All. molleri	Av. ligra	All. burgondiae	Ap. pseudoantipai	All. zicsii	Ap. icterica	All. satchelli	All. tiginosa	Ap. handlirschi

	Allolobophora Eisen, 1874	<i>Gatesona</i> Qiu and Bouché, 1998	<i>Eophila</i> Rosa, 1893	<i>Cernosvitovia</i> Omodeo, 1856	<i>Panoniona</i> Mršić and Šapkarev, 1988	<i>Vosgesia</i> Martínez Navarro and Marchán, 2022	Avelona Qiu and Bouché, 2000	<i>Heraultia</i> Martínez Navarro and Marchán, 2022	Prosellodrilus Bouché, 1972	<i>Cataladrilus</i> Qiu and Bouché, 1998
Length (mm)	30-150	68-192	100-360	42-250	40-151	50-75	40-80	50-75	35-335	25-220
N. segments	60-210	118-197	139-341	138-304	115-181	125-140	100-125	156-172	130-350	124-384
Pigmentation	Green (sometimes absent)	Pigmentary dots	Purplish- brown in bands or brown	Light grey to brown	Absent	Absent	Brown	Absent	Absent	Absent
Prostomium	Epilobous	Epilobous	Epilobous	Epilobous or prolobous	Epilobous	Epilobous	Epilobous	Epilobous	Epilobous, prolobous or tanilobous	Epilobous
First dorsal pore	(3/4), 4/5, (5/6), (6/7)	4/5-7/8	5/6	9/10, 10/11, 12/13, 13/14	4/5	4/5	8/9	4/5	4/5-6/7	4/5-11/12
Spermathecal pores	Simple in 7/8, 8/9, 9/10, 10/11	Simple in 9/10, 10/11	Paired in 9/10, 10/11	Position and number highly variable, from 9/10 to 19/20	Double or multiple in 9/10, 10/11	Simple or double in 9/10, 10/11	Simple in 9/10, 10/11	Simple in 10/11, 11/12, 12/13	Simple in 12/13, 13/14, and 14/15 or simple, double, or multiple in 14, 15	Simple in 9/10, 10/11
Clitellum	28-78	1/2 22-36	24-44	21-65	23-34	26-34	29-36	21-36	(19) 20-27 (34)	22-46
Tubercula pubertatis	30-78	27-26	29-40	21-62	28-34	27-33	(31) 32-35 (36)	27-29	Variable	28-45
Oesophageal hearts	6-11	5, 6-11, 12	6-11	5, 6-11, 12	6-11	6-11	6-11	6-11	6, 7-11	6, 7-11
Calciferous glands	10-14 with diverticula in 10	10-12 (14) with diverticula in 10	10-12 with diverticula in 10	10-13	10-14 with diverticula in 10	10-14 with diverticula in 10	10-14 with diverticula in 10	13-14	11-15 with diverticula in 11	11-15 with diverticula in 11
Crop	15-16	15-16	15-16	15-16	15-16	15-16	15-16	15-16	15-16	15-16 (17, 18)
Gizzard	17-18	17-18	17-19	17-19, 20	17-18	17-18	17-18	(17) 18	17-18	17 (18, 19)-18 (21)
Typhlosole	Bifid or trifid	Bifid	Trifid	Trifid	Trifid	Bifid	Bifid	Bifid	Bifid or multifid	Simple, bifid, trifid, or multifid
Seminal vesicles	9-12	(9), (10), 11, 12	9-12	9-12 or 11-12	11-12	11-12	11-12	11-12	11-12	11-12
Spermathecae	Simple in 7/8, 8/9, 9/10, 10/11	Simple, globular in 9, 10	Simple in 9/10, 10/11	Position and number highly variable, from 9/10 to 19/20.	Double or multiple in 10, 11	Simple or double in 10, 11	Simple in 10, 11	Globular in 11, 12, 13	Variable in (12/13), 13/14, 14/15	9/10, 10/11

TABLE 3: Comparison of the main morphological characters of the four revised genera and its closest genera.

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	TABLE 4	: List of the species included	l in Allolobophora sensu sti	ricto and its main morpholo	ogical features.	
	All. chlorotica	All. burgondiae	All. icterica	All. molleri	All. moebii	All. dubiosa
Length (mm)	30-80	75-80	20-90	150-200	60-125	126-240
N. segments	60-140	150-170	140-200	145-210	104-122	121-303
Pigmentation	Green (sometimes absent)	Absent/brownish	Absent	Green	Green	Absent
Prostomium	Epilobous	Epilobous	Epilobous	Epilobous	Epilobous	Epilobous
First dorsal pore	4/5 (5/6)	4/5	4/5	4/5 (5/6)	4/5 (5/6)	4/5
Spermathecal pores	8/9, 9/10, 10/11	8/9, 9/10, 10/11	(7/8), 8/9, 9/10, 10/11	7/8, 8/9, 9/10, 10/11	7/8, 8/9, 9/10, 10/11	(7/8), 8/9, 9/10, 10/11
Clitellum	(28) 29-37	(27) 28-38 (39)	(33) 34-43 (44)	(48) 49-59	(52) 53-61 (62)	(36) 38-46 (49)
Tubercula pubertatis	30-36	30-37	(35) 36-42 (43)	50-57	(55) 56-61	(43) 44-47 (48)
Oesophageal hearts	6-11	6-11	6-11	6-11	6-11	6-11
Calciferous glands	10-14 with diverticula in 10	10-14 with diverticula in 10	10-14 with diverticula in 10	10-12 with diverticula in 10	10-12 with diverticula in 10	10-14 with diverticula in 10
Crop	15-16	15-16	15-16	15-16	15-16	15-16
Gizzard	17-18	17-18	17-18	17-18	17-18	17-18
Typhlosole	Bifid	Trifid	Bifid	Trifid	Trifid	Bifid
Nephridial bladders	U-shaped	U-shaped	U-shaped	U-shaped	U-shaped	U-shaped
Seminal vesicles	9, 10, 11, 12	9, 10, 11, 12	9, 10, 11, 12	9, 10, 11, 12	9, 10, 11, 12	9,10, 11, 12
Spermathecae	9, 10, 11	9, 10, 11	9, 10, 11	7/8, 8/9, 9/10, 10/11	7/8, 8/9, 9/10, 10/11	8/9, 9/10, 10/11

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moebii complex and closely related species is very controversial [36–38] and should be addressed by including the different species and subspecies in molecular phylogenetic analyses.

Genus Panoniona Mršić and Šapkarev, 1988

Type species: Allolobophora leoni Michaelsen, 1891

Material studied: *Panoniona satchelli*, 3 adult specimens; BOLD sample ID: ALL-013, ALL-014, ALL-015; Alsace, Haut-Rhin, Saint-Louis; latitude/longitude: 47.618/7.538; 04-Nov-2021; R. Della Vedova, T. Decaëns, D. Fernández Marchán leg. Specimen deposited in UCM-LT.

Diagnosis:

Small-to-medium-sized Lumbricini with absence of pigmentation. Prostomium is epilobous. Setae are closely paired. First dorsal pore in 4/5. Male pores in 1/2 15 with well-developed porophores. Spermathecal pores in 9/10, 10/11, double or multiple. Nephridial pores aligned or irregularly distributed (*en solfège*). Clitellum between 23 and 34. *Tubercula pubertatis* between 28 and 34. Oesophageal hearts in 6-11. Calciferous glands in 10-14 with diverticula in 10. Crop in 15-16. Glizzard in 17-18. Glandular papillae in 11-13, 16, (17), 26-29, and (34). Typhlosole is trifid or with two large and two small lamellae. Spermathecae double or multiple in 10, 11, globular and intracelomic. Two pairs of seminal vesicles in 11 and 12. Nephridial bladders are Jshaped with or without loop.

Differential diagnosis:

Panoniona can be differentiated from *Allolobophora* by the more anterior position of the clitellum and absence of pigmentation vs. frequently green pigmentation, repetition of spermathecae (double our multiple vs simple), number of seminal vesicles (two pairs in 11 and 12 vs. four pairs in 9, 10, 11, and 12), and shape of nephridial bladders (Jshaped vs. U-shaped).

Even though *Cernosvitovia* as redefined by Popovic et al. [10] is a morphologically heterogeneous genus, *Panoniona* can be distinguished from it by the type of spermathecae (double our multiple vs. simple) and either the position of the male pore (1/2 15 vs. 25-30) or the position of the spermathecae (9/10 and 10/11 vs. 9/10, 10/11, 11/12, and (12/13)).

Panoniona is clearly separated from *Gatesona* by the absence of pigmentary dots in cephalic segments, absence of very conspicuous genital papillae between segment 9 and the end of the clitellum, shape of nephridial bladders (J-shaped vs. sigmoid), repetition of spermathecae (double our multiple vs. simple), and number of seminal vesicles (two pairs in 11 and 12 vs. three to four pairs in (9, 10), 11, and 12).

Panoniona differs from *Eophila* by its generally smaller size, its pigmentation (absent vs. purplish-brown in bands or brown), and repetition of spermathecae (double our multiple vs. simple; Table 3).

Species included the following:

(i) Panoniona leoni Michaelsen, 1891

(ii) Panoniona satchelli stat. nov. Bouché, 1972

Genus Vosgesia Martínez Navarro and Marchán, 2022 Type species: Vosgesia zicsii stat. nov. (Bouché, 1972) Material studied: 3 adult specimens; BOLD sample ID: ALL-007, ALL-008; ALL-009; Bourgogne; Haute-Saône, Luxeuil-les-Bains, latitude/longitude: 47.836/6.359; 02-Nov-2021; R. Della Vedova, T. Decäens, D. Fernández Marchán leg. Specimens deposited in UCM-LT.

Diagnosis:

Small-sized (50-75 mm and 0.2-0.29 gr) Lumbricini with less than 150 segments and absence of pigmentation. Prostomium is epilobous. Setae are closely paired. First dorsal pore in 4/5. Male pores in 1/2 15 with well-developed porophores. Spermathecal pores in 9/10, 10/11, simple or double. Nephridial pores aligned. Clitellum in 26-34. *Tubercula pubertatis* in 27-33, with maximum development in 30-33. Oesophageal hearts in 6-11. Calciferous glands in 10-14 with diverticula in 10. Crop in 15-16. Glizzard in 17-18. Glandular papillae in 13, 27, and 30-32. Typhlosole is bifid. Spermathecae simple or double in 10, 11, globular and intracelomic. Two pairs of seminal vesicles in 11 and 12. Nephridial vesicles are J-shaped (incurvate and reclinate).

Differential diagnosis:

Vosgesia shares with *Avelona* (their closest relatives) the position of spermathecae (9/10 and 10/11) and seminal vesicles (11 and 12), typhlosole shape (bifid), and several external character states, but it is clearly separated by the position of the dorsal pore (4/5 vs. 8/9), the position of the clitellum (26-34 vs. 29-36), the nephridial pore disposition (aligned vs. irregular), the type of spermathecae (simple or double vs. simple), and the shape of the nephridial bladders (U-shaped vs. digitoid). The complete diagnoses of both genera are listed in Table 3.

Other species included: none.

4.2. Evolutionary and Biogeographic Implications. Historically, the genus Allolobophora includes a large number of species that are often taxonomic unrelated, resulting in a genus with a wide distribution and high diversity (the second largest one within the Lumbricidae), but with weak evolutionary ties. Unfortunately, this has complicated evolutionary and biogeographic interpretations. The gradual implementation of molecular techniques has revolutionized the systematics of the genus, with the elevation of redefined *Cernosvitovia* [10] and *Gatesona* [9], previously considered subgenera of Allolobophora, to generic status. The present work has continued this trend by supporting the elevation of *Panoniona* to genus status and the assignment of two of the studied species to newly described genera.

The resulting monophyletic clade comprising the former *Allolobophora* sensu stricto (with *All. chlorotica* and *All. burgondiae*) and *Heraclescolex* (comprising *All. moebii* and *All. molleri*, *All. icterica*, and *All. dubiosa*) appears more coherent in its morphological variability and distribution than the loosely defined *Allolobophora* of old. Qiu and Bouché [16] already indicated the strong morphological similarity between *Allolobophora* sensu stricto and *Heraclescolex*; in spite of that, surprisingly, they proposed to place them in different tribes. Domínguez et al. [2] showed for the first time that *All. molleri*, *All. moebii*, and *All. dubiosa* are closely related taxa to *All. chlorotica* by molecular phylogenetic

methods. The presence of All. burgondiae and All. icterica within that clade reinforces the idea that Allolobophora and Heraclescolex are synonyms. An alternative interpretation of the topology of the phylogenetic trees might be that Allolobophora sensu stricto (with All. chlorotica and All. burgon*diae*) and *Heraclescolex* do not constitute the same genus but are very closely related, independent genera. Even though both scenarios are likely, their extremely similar morphology (including the widespread green pigmentation) advises to synonymize them. Their main difference, the orientation of the nephridial bladders, is a rather feeble taxonomic character within a strongly variable structure within genera [9, 24]. Examination of the remaining species of Heraclescolex and their inclusion in the molecular phylogenetic analysis could recover them within Allolobophora, which would further support our hypothesis.

Interestingly, the genotypes of both merged genera (*All. chlorotica* and *All. moebii*) constitute species complexes on opposite poles of the morphological variability spectrum.

Allolobophora chlorotica has been shown to comprise several cryptic lineages [39, 40], some of which are widespread or even cosmopolitan, while a few are endemic to Provence and the Alps [39, 41]. Moreover, several subspecies have been described, but their association with those genetic lineages is still unknown. The integration of both sources of information with the new phylogenetic frame is essential to understand their phylogeography and the geographic origin of the genus.

The molleri-moebii species complex is a particularly controversial group that has been assigned to the genera Allolobophora, Eophila, Aporrectodea, or Heraclescolex. Trigo et al. [37, 38] found specimens with intermediate character states with respect to the position and extension of the clitellum in a continuum between the previously described species, which blurred the boundary between them. Barros [36] concluded that All. molleri and All. moebii constitute a single species named All. molleri. However, these results were not accepted by Qiu and Bouché [8, 13, 14, 16], who described additional subspecies and closely related species (the Maghrebian Heraclescolex kionionus Qiu and Bouché, 1998, Heraclescolex rifanus Qiu and Bouché, 1998, and Heraclescolex postsellis Qiu and Bouché, 1998). Our results appear to support the former view, as All. molleri and All. moebii do not have sufficient genetic divergence to be considered separate species. In order to solve the decade-old riddle of the taxonomy of this species complex, a systematic sampling of different populations of All. molleri, All. moebii, and the other species within this complex (Allolobophora fernandae Graff, 1957, Allolobophora monchicana Trigo, Mascates, Briones and Diaz Cosin, 1990, and Allolobophora opisthosellata Graff, 1961) will be performed in the near future; this will allow integrative species delimitation and will shed light on the evolutionary significance of the extraordinary clitellar variability of this group.

Although several questions remain to be solved, our current knowledge allows us to propose a rough scenario for the evolutionary origin and historical biogeography of *Allolobophora*. All the species assigned to the newly defined *Allolobophora* (plus the currently unstudied *Heraclescolex* species)

are native to the western Iberian Peninsula, France, Switzerland, Italy, and central-eastern Europe. The center of origin could be near the western Alps, as suggested by the presence of "endemic" lineages of All. chlorotica as well as All. burgondiae and All. icterica (Figure 2). The less intuitive distribution corresponds to the All. moebii-molleri complex and *Heraclescolex* species, which show a remarkable disjunction. However, the highest diversity of species and subspecies in southwest of the Iberian Peninsula, Morocco, and Algeria suggests an origin of this species group in the AlKaPeCa (Alboran-Kabylian-Peloritan-Calabrian) terrane, whose fragments migrated to their current positions in the Iberian Peninsula and northern Africa [42]. This terrane showed continuity with alpine Corsica and the developing Alps, allowing the dispersal and diversification of the ancestors of the extant Allolobophora species to their current range. An increased taxonomic and phylogeographic sampling of the areas and species involved would allow to refine and test this hypothesis, offering valuable insights into the link between the paleogeography of the western Mediterranean and its diversity.

The small, isolated genera Heraultia and Vosgesia and the occurrence of Panoniona in northeastern France and Switzerland also provide interesting biogeographic and evolutionary insights (Figure 2). *Heraultia tiginosa* represents a phylogenetic and biogeographic link between the French Zophoscolex and Ethnodrilus and the mostly Pyrenean Prosellodrilus and Cataladrilus. While the latter two are remarkably diverse genera, Heraultia comprises a single species known from a single location: this suggests that Heraultia is a relict genus whose diversity and range might have been reduced by environmental and faunistic changes (as inferred for Galiciandrilus, Compostelandrilus, and Vindoboscolex [15]). Although new species belonging to Heraultia might be discovered in the future extending its distribution area, its presence highlights the high conservation value of Herault (and the area around Montpellier) as a hotspot of Mediterranean earthworm diversity.

Similarly, V. zicsii appears as an isolated distant relative of Av. ligra and Gatesona and the most northeastern representative of their clade (Figure 1). Its presence attests to a wider distribution of such lineage in the past and putatively to a greater diversity that is no longer present in France. Panoniona satchelli is isolated from its eastern relative P. leoni (present in Italy, Slovenia, Croatia, and further east) by the Alps (and about 400 km in a straight line) (Figure 2). In the case of Panoniona, the cause of such disjunction could indeed be the progressive elevation of the mountain chain, with an associated change in climate and vegetation, which could have led to a large unsuitable area between the current species. However, there is another cause that could explain the present range of Vosgesia zicsii and P. leoni. As stated by Bouché [4], endemic species in France appear to be restricted to the south of the Loire River: towards the North, they would have been eliminated by the presence of permafrost during the Last Glacial Maximum (LGM, ca. 33,000-15,000 years ago [43]). Even though most recent models suggest that permafrost would have not been present south of 49° N in France [44], the dominant



FIGURE 2: Sampling localities of the species studied in this work; for all species except *Allolobophora icterica*, this corresponds to their known ranges. The main ranges of the closest relatives according to phylogenetic analyses are shown as coloured shapes.

biome in most of France and the rest of Europe would have been steppe-tundra [45] which is unsuitable for most lumbricid earthworms. The almost complete absence of endemic earthworm species (and low diversity) in Germany, Czech Republic, Poland, Slovakia, and countries further north supports this hypothesis. Nevertheless, Bouché [4] noted the presence of a few strictly endemic species in a small region (Val de Saône-Vosges) within the "glaciated" area. Allolobophora burgondiae, P. satchelli, V. zicsii, and Ap. pseudoantipai (as well as Ap. gogna Bouché, 1972 and Ap. velox (Bouché 1967)) occur there. Thus, the isolation of these species relative to their closest southern or eastern relatives would be explained by the disappearance of intermediate species during the LGM or other unsuitable climatic events. Interestingly, some models show that the aforementioned region roughly corresponds to the presumed distribution of different vegetation types (such as ombrotrophic bogs) during the LGM [46], which supports the role of the Val de Saône-Vosges area as a glacial refuge for endemic Lumbricidae. Further efforts should be devoted to biodiversity assessment and conservation of this unique hotspot, which has been generally neglected in favour of the southern regions.

The biogeographic inferences drawn from *Allolobophora* and the relict species from northeastern France illustrate the relevance of the Alpine region for the evolution of several lineages of Lumbricidae, with the effects of the LGM likely disrupting the remaining evidence. This is probably also true for other genera such as *Aporrectodea* sensu stricto, *Lumbricus* Linnaeus, 1758 or *Octodrilus* Omodeo, 1956. A greater focus on the implementation of phylogeography and molecular phylogenetics to the earthworm faunas of Switzerland, Italy, Austria, Slovenia, Croatia, and Serbia would greatly enhance our understanding of the evolution and historical biogeography of Lumbricidae.

5. Conclusions

Molecular phylogenetic analyses revealed that the representatives of *Allolobophora* sensu stricto and *Heraclescolex* belong to a clearly delimited clade, which suggest their synonymy. On the other hand, other species formerly assigned to *Allolobophora* were recovered within *Panoniona*, or as isolated, genus-level branches. Therefore, the revised definitions for *Allolobophora* and *Panoniona* were provided, and the new genera *Heraultia* and *Vosgesia* were described. Although several species of *Heraclescolex* remain to be included in this molecular phylogenetic framework, it is likely that most of them will be nested within the newly defined *Allolobophora*. If so, the synonymy between both genera would be proven.

Systematic revision of the aforementioned earthworm groups revealed evolutionarily more coherent groups that provide information on their diversification and historical biogeography. The Maghreb and the areas around the Alps were highlighted as relevant targets for expanding our knowledge on this topic, and the importance of the Val de Saône-Vosges (northeastern France) as a glacial refugium with relict earthworm lineages was confirmed.

Data Availability

DNA sequences obtained in this study are available in Gen-Bank with their corresponding accession numbers. COI sequences, together with metadata, are publicly available in BOLD.

Conflicts of Interest

The authors declare that there are no conflicts of interest or personal relationships that could have influenced the work reported in this article.

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Supplementary Materials

Additional information can be found in the online version of the article on the publisher's website. Table S1: list of species included in the phylogenetic analyses with reference to the original publication from which the sequences were taken. Figure S1: phylogenetic tree estimated by maximum likelihood, based on the concatenated sequence of COI, 16S, 28S, 12S, and ND1. Figure S2: complete Bayesian inference of the phylogenetic relationships of the studied taxa and some Lumbricidae representatives. (*Supplementary Materials*)

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