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Toward Scientific Clarity in the Evolutionary Puzzle of *Austropotamobius* Crayfish

Lucian Pârvulescu[1,2] | David Livadariu[2,3] | Alexandru E. Mizeranschi[2,3,4] | Marinela A. Chețan[5] | Andreea M. Lamoly[2] | Mihaela C. Ion[6] | Ana-Maria Neacă[7] | Márta S. Mészáros[8] | Ana Molnar[2] | Ecaterina Mocioacă[3] | Alina Satmari[3] | Lucian Fusu[9] | Cristian Panaiotu[10,11] | Miguel Clavero[12] | Dan V. Palcu Rolier[13,14]

[1]Department of Biology, Faculty of Chemistry, Biology, Geography, West University of Timisoara, Timisoara, Romania | [2]Crayfish Research Centre, Institute for Advanced Environmental Research, West University of Timisoara, Timisoara, Romania | [3]Department of Informatics, Faculty of Mathematics and Computer Science, West University of Timisoara, Timisoara, Romania | [4]Research and Development Station for Bovine – Arad, Arad, Romania | [5]Department of Geography, Faculty of Chemistry, Biology, Geography, West University of Timisoara, Timisoara, Romania | [6]Institute of Biology Bucharest, Romanian Academy, Bucharest, Romania | [7]Department of Taxonomy and Ecology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania | [8]Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania | [9]Research Group in Invertebrate Diversity and Phylogenetics, Faculty of Biology, Alexandru Ioan Cuza University, Iasi, Romania | [10]Paleomagnetic Laboratory, Faculty of Physics, University of Bucharest, Măgurele, Romania | [11]Geonomy, Romanian Academy, Bucharest, Romania | [12]Estación Biológica de Doñana–CSIC, Sevilla, Spain | [13]Fort Hoofddijk Paleomagnetic Lab, Utrecht University, Utrecht, the Netherlands | [14]National Institute of Marine Geology and Geo-Ecology (GeoEcoMar), Bucharest, Romania

Correspondence: Lucian Pârvulescu (lucian.parvulescu@e-uvt.ro)

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ABSTRACT

Aim: Illuminating the evolutionary history of *Austropotamobius* crayfish by uncovering patterns of biodiversity and biogeographical processes, thereby providing a foundation for accurate taxonomy and targeted conservation strategies.

Location: Europe.

Time Period: Miocene to present.

Major Taxa Studied: The crayfish genus *Austropotamobius*.

Methods: We synthesised existing knowledge and conducted additional analyses to resolve uncertainties in lineage divergence and distribution. Genetic variation across geographical regions was assessed using mitochondrial COI haplotypes, paired with accurate geolocation data from literature. Divergence times were estimated using a genus-specific molecular clock calibrated to the tectonic-driven separation of *Austropotamobius bihariensis* from its Dinaric relatives, based on 936 base pairs of concatenated COI and 16S mitochondrial sequences. To reconstruct historical connectivity and gene flow direction among regional populations, we applied coalescent-based models comparing alternative scenarios.

Results: Our analysis suggests the common ancestor of *Austropotamobius* diverged ~30 Ma, coinciding with the temporary fragmentation of Balkanatolia from Western Europe, forming two major evolutionary lineages. In the west, between the Alps and Pyrenees, *A. pallipes* showed low genetic diversity and broad distribution. From this lineage, *A. fulcisianus* emerged around

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14 Ma during an Alpine embayment, colonising the forming Italian Peninsula and later expanding into the southern Dinarides during the Messinian Salinity Crisis. Human-mediated translocations further extended its range into Western and Northern Europe. The second major lineage, *A. torrentium*, spans Central and Eastern Europe and includes eight ancient, monophyletic and endemic lineages concentrated in the north-central Dinarides (NCD), except for the most basal branch, *A. bihariensis*, located in the Apuseni Mountains. Two younger lineages expanded across the Balkans and Central Europe, likely beginning around 6.8 Ma with the formation of the Palaeo-Danube system.

Main Conclusions: This study refines the evolutionary trajectory of *Austropotamobius*, highlights taxonomic clarification and underscores the need to reassess several lineages to support genuine, evolutionarily informed conservation strategies.

1 | Introduction

Understanding the evolutionary processes that shape biodiversity is fundamental to comprehending the complexities of nature. Intricate processes, driven by both natural events and human influences, play a critical role in determining the distribution, adaptation and diversification of species across the globe (Campani et al. 2012; Spicer 2017). By combining insights from spatial distribution patterns and genetic analyses, researchers can reconstruct the historical dynamics that have led to present-day biodiversity patterns (Slatkin 1987; Naidoo et al. 2008; Vilaça et al. 2014; Zhang et al. 2024; Ferrer Obiol et al. 2025; Baselga et al. 2025). However, disentangling evolutionary histories is challenging due to the inherent complexity of these processes and the limitations in the available data (Clark et al. 2008). Such updates are crucial not only for scientific knowledge but also for applications in various fields, including conservation and biodiversity management (Stern 2000; Catley 2006; Telford et al. 2016; Ács et al. 2025).

Freshwater crayfish (Crustacea: Decapoda: Astacidea) provide a valuable model for studying evolutionary processes due to their ecological diversity and widespread distribution across various continents (Toon et al. 2010; Bláha et al. 2020; Audo et al. 2023). Most native crayfish species are concentrated in Australasia and Eastern North America, but they are also found in South America, Madagascar, Japan, Eastern Asia and Europe (Ion, Bloomer, et al. 2024). The European indigenous crayfish fauna include three genera—*Astacus* Fabricius, 1775, *Pontastacus* Bott, 1950, and *Austropotamobius* Skorikov, 1907—which have been traditionally considered to include a low number of species (Crandall and De Grave 2017). This apparent low species richness may be an underestimation stemming from the conservative approach traditionally applied to crayfish taxonomy in the region, where divergent lineages are often treated as subspecies or local forms rather than distinct species (Allison et al. 2024). A great uncertainty surrounds the entire genus *Austropotamobius*, composed of two species complexes (*pallipes* and *torrentium*), as there are different schools of thought regarding both the past and recent phylogeographic history, as well as inconsistencies in taxonomy. One direction, initiated by Trontelj et al. (2005), places the common ancestor of the genus within the 15–7 Ma timeframe (Klobučar et al. 2013; Jelić et al. 2016). Meanwhile, approaches based on tectonic or fossil calibrations suggest that the evolutionary events leading to the separation of the two main clades occurred much earlier, around 37 Ma (Bracken-Grissom et al. 2014; Pârvulescu et al. 2019; Stanković et al. 2024). The ongoing debate over *Austropotamobius* taxonomy highlights that, despite molecular and morphological evidence (Maguire et al. 2017; Lovrenčić et al. 2020b), the scientific community remains conservative in accepting the elevation of certain populations to species status (Ghia et al. 2006; Lovrenčić et al. 2020a), even hesitating to fully accept the already described species (Crandall and De Grave 2017). However, a common thread among all these hypotheses is the geographical localisation of the evolutionary centre in the Dinarides—a mountain range in Southeast Europe stretching from Slovenia through Croatia, Bosnia and Herzegovina, Montenegro and into Albania—although intense debates persist concerning the origin of certain remote populations in Europe (Grandjean et al. 2002; Clavero 2022; Clavero and Sempere Marín 2025). Phylogenetic studies suggest that the genus *Austropotamobius* encompasses a complex evolutionary history with deeply rooted lineages (Grandjean et al. 2002; Jelić et al. 2016; Pârvulescu et al. 2019; Stanković et al. 2024). Although the *pallipes* group has been well studied, there is still inconsistency in the use of valid names. It consists of two recognised species: *A. pallipes* (Lereboullet, 1858) and *A. fulcisianus* (Ninni, 1886). The recent description of *Austropotamobius bihariensis* Pârvulescu (2019) as an endemic species found in the Apuseni Mountains of Romania (Pârvulescu 2019), previously included within *Austropotamobius torrentium* (von Paula Schrank 1803), marks a change in the perception of diversity within the *torrentium* group, suggesting the need for a comprehensive review of the entire group (Lovrenčić et al. 2020a; Bláha et al. 2023; Zorić et al. 2025). Moreover, this discovery is particularly important because it provides a robust geological divergence point, which can be used for contextualising evolutionary timelines (Pârvulescu et al. 2019; Stanković et al. 2024). These insights challenge the established theories and underscore the importance of re-evaluating evolutionary timelines and palaeogeographical influences, especially those linked to the complex tectonic and geographical transformations of Southern Europe.

The region known as Balkanatolia, which experienced significant tectonic shifts and fragmentation from the Eocene through the Miocene—including the uplift of the Alpine and Carpathian mountain ranges and the formation of the Paratethys Sea—has been crucial in shaping the biodiversity and endemism observed today (Mertz et al. 2007; Ogg et al. 2016; Licht et al. 2022). Despite significant advancements in understanding *Austropotamobius* crayfish, we still lack an integrated phylogeographic approach encompassing all its lineages. This study aims to address key uncertainties in the evolution of the genus, revisiting pivotal aspects of the group's evolutionary history, such as timeframe calibration, ancestral species split locations and timings, lineage diversification, and past and present geographical dispersal dynamics. We examine the interplay between cosmopolitan and endemic distributions, and propose species diversity refinements to clarify taxonomy.

2 | Methods

To address key evolutionary questions concerning the *Austropotamobius* crayfish group, we implemented an integrative phylogeographic framework that maximises the spatial representativeness of available molecular data. Our approach combines targeted sequence acquisition, geospatial and temporal analysis grounded in geological calibrations, and is complemented by haplotype diversity profiling and model-based inference of colonisation pathways.

2.1 | Molecular Data Collection and Spatial Representativeness

We used the mitochondrial cytochrome c oxidase subunit I (mtCOI) marker exclusively for the spatial visualisation of haplotype distributions, given its broad availability and standardised use across crayfish taxa. While relying on a single locus limits its phylogenetic resolution, previous studies have shown that mtCOI haplotypes reflect major geographical and lineage patterns consistent with multi-locus data in crayfish (Maguire et al. 2014; Pârvulescu et al. 2019). For all other analyses, we used concatenated datasets (see below) to enhance phylogenetic and temporal resolution. Given current gaps in genomic-scale data harmonisation, this marker-specific approach remains effective for broad-scale phylogeographic synthesis (Kress et al. 2015; Theissinger et al. 2023).

Using the *read.GenBank* function from the R package ape v.5.7–1 (Paradis and Schliep 2019), we retrieved sequences from NCBI based on accession codes identified through literature screening. Sequences based on the primer pair LCO-1490/HCO-2198 (Folmer et al. 1994) were found to cover as much as possible *Austropotamobius* crayfish species in records associated with accurate geolocation and were therefore used in this study. Final alignments retained 522 base pairs per sequence. The final dataset comprised 160 sequences for *A. pallipes*, 584 for *A. fulcisianus*, 760 for *A. torrentium* and 35 for *A. bihariensis* (see Table S1 for details). Geographical locations were derived either directly from reported coordinates or inferred based on local toponyms and proximity to described habitats. To assess spatial representativeness, we overlaid occurrence data onto a 100×100 km grid from the European Environment Agency (EEA) and compared genetic coverage against distribution maps from the World of Crayfish platform (https://world.crayfish.ro/). Our dataset covered 81.2% of the known range for *A. bihariensis*, 49.4% for *A. torrentium*, 18.3% for *A. pallipes* and 72.4% for *A. fulcisianus*.

2.2 | Haplotype Delimitation and Spatial Interpolation

To explore haplotype diversity, we aligned sequences using ClustalW v.1.30.1 (Thompson et al. 1994) via the *msa* function (Bodenhofer et al. 2015), followed by trimming for length consistency. The haplotype function from pegas v.1.3 3 (Paradis and Barrett 2010) was used to group sequences into haplogroups, labelled based on accepted (and proposed, see Discussions) taxonomy. This approach yielded 4 haplogroups (AUP—*A. pallipes*, AFM—*A. fulcisianus meridionalis*, AFF—*A. f. fulcisianus* and AFO—*A. f. orientalis*) for the *pallipes* group (Fratini et al. 2004; Jelić et al. 2016); 9 haplogroups (Lovrenčić et al. 2020a; Zorić et al. 2025) for *A. torrentium* (ZV—Zeleni Vir, GK—Gorski Kotar, VOJ—Vojskova; KOR—Kordun, LD—Lika and Dalmatia; ŽPB—Žumberak, Plitvice and Bjelolasica; BAN—Banovina; SB—southern Balkans; and CSE—Central and Southeastern Europe); and one for *A. bihariensis* (AUB, a.k.a., APU lineage). The resulting dataset comprised 160 sequences for *A. pallipes*, 584 for *A. fulcisianus*, 760 for *A. torrentium* and 53 for *A. bihariensis* (see Table S1 for details).

Spatial patterns were assessed using Kernel Density Estimation in ArcGIS Pro 3.0.2. Search radii were defined using the spatial form of Silverman's Rule of Thumb (Silverman 1986), which provides a data-driven estimate of the optimal bandwidth based on the spatial distribution of haplotypes. This resulted in buffers of 108 km for *A. torrentium* + *A. bihariensis* and 146 km for *A. pallipes* + *A. fulcisianus*. Resulting haplotype diversity surfaces were visualised using Ordinary Kriging, with final maps refined in Inkscape 1.3. To further assess spatial structure, we calculated the average nearest neighbour (ANN) index, which compares observed versus simulated nearest-neighbour distances. We standardised analysis areas to 122,000 km^2 for *A. torrentium* and 1,100,000 km^2 for *A. pallipes* groups to ensure comparability. ANN ratios <0.25 were interpreted as highly localised, values between 0.25 and 0.5 as clustered, 0.5 to 1.0 as regionally distributed and values >1.0 as dispersed. Spatial analysis could not be performed for the ZV and VOJ haplogroups of *A. torrentium*, as all available sequences originated from a single locality. A separate analysis was carried out on the ranges of species from which populations known or strongly suspected to result from human introductions were removed, in the case of *A. pallipes* for the United Kingdom, Ireland and Corsica, and for *A. fulcisianus* populations in Spain and France.

To infer plausible early colonisation pathways within intraspecific evolutionary lineages, we applied model selection as implemented in MIGRATE-N v.5.0.4 (Beerli and Felsenstein 2001), a coalescent-based framework suitable for testing alternative population connectivity scenarios. To enhance molecular resolution, we further retrieved available mitochondrial 16S rRNA sequences, mostly obtained with the primer pair 16Sar/16Sbr (Simon et al. 1994), corresponding to the same specimens and sampling sites as the selected mtCOI haplotypes used in spatial pattern analyses, final alignments consisting of 938 base pairs (522 mtCOI and 416 16S) per sequence. For the *A. torrentium* group, sequences were grouped into five clusters based on haplogroup distributions: Cluster 1 the sequences of *A. bihariensis*, Cluster 2 endemic populations from the north-central Dinarides (NCD) containing the haplogroups of ZV, GK, VOJ, KOR, LD, ŽPB, Cluster 3 haplogroup BAN, Cluster 4 haplogroup SB and Cluster 5 haplogroup CSE. The *A. pallipes* group was divided into seven clusters: Cluster 1 grouping sequences of *A. pallipes* populations, Cluster 2 AFMi Italian populations, Cluster 3 AFMd populations from south Dinarides, Cluster 4 AFCi Italian populations, Cluster 5 AFCd populations from south Dinarides, Cluster 6 AFFi Italian populations, Cluster 7 AFFIs Iberian Peninsula populations, respectively. For the populations with uncertain origin out of their native range (i.e., Martínez-Ríos et al. 2023), the sequences were discarded. Multiple colonisation models were tested per group (see Figure 1), each representing different plausible spatial dispersal routes. Simulations were run using a two-locus configuration, treating the mitochondrial COI

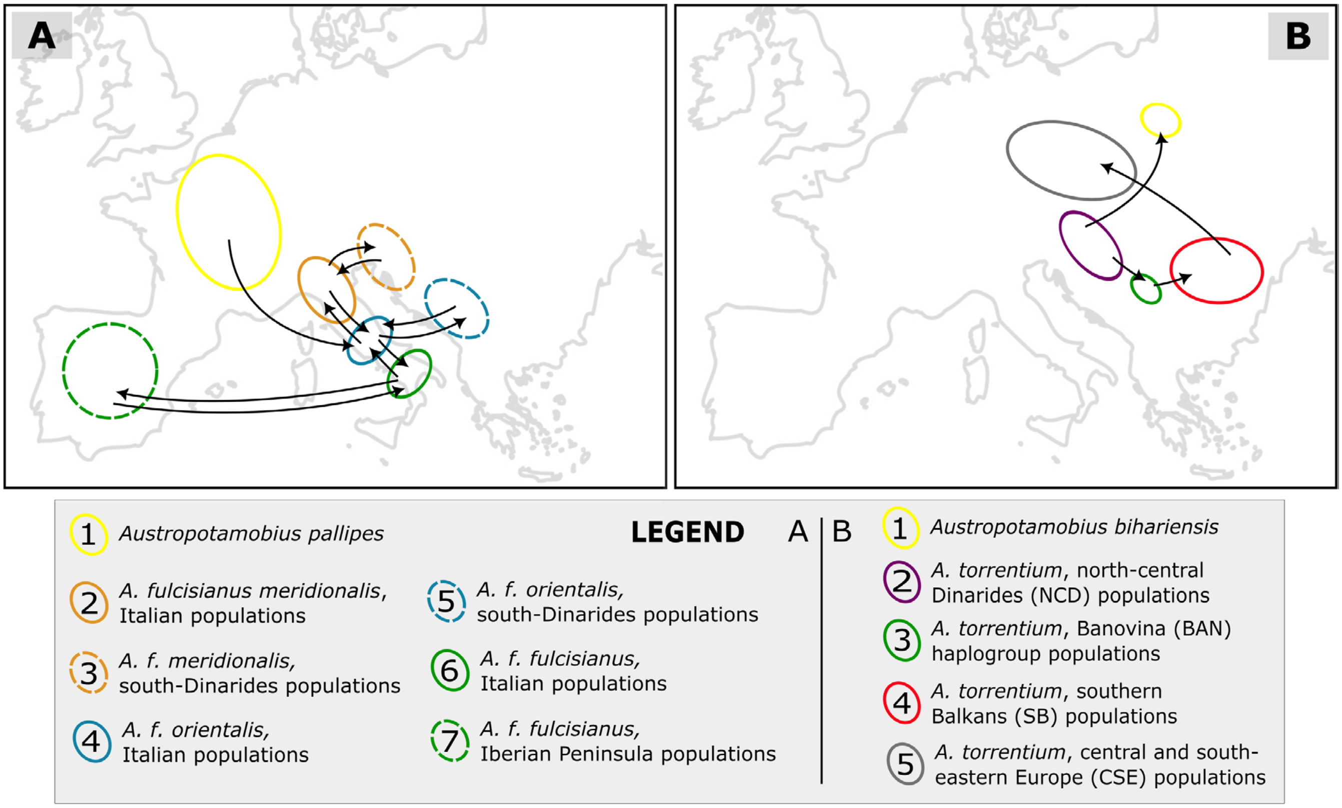

FIGURE 1 | Best-supported colonisation hypothesis model selected by MIGRATE-N for *Austropotamobius* crayfish populations across their geographical regions. (A) *A. pallipes* and *A. fulcisianus* populations. (B) *A. bihariensis* and *A. torrentium* populations, based on concatenated mtCOI and 16S sequences (938 bp). Arrows represent the inferred direction of gene flow. The full set of model comparisons, with Bezier-approximated Thermodynamic Integration (BTI) values, is available in Figure S3A,B. This schematic does not reflect actual lineage distributions; see Figures 4–6 for detailed maps.

and 16S markers as separate but jointly inherited loci within the same mitochondrial genome. Each locus was specified separately in the input file. A single chain per locus was applied with 10,000 steps (1000 burn-in), and parameter estimation was based on 1,000,000 samples. Four heated chains were used to improve sampling across the parameter space. Models were ranked based on Bayes factors estimated via Bezier-approximated Thermodynamic integration—BTI (Beerli and Palczewski 2010; Palczewski and Beerli 2014). Final ranking and diagnostics were conducted using the *mtraceR* package (Pacioni et al. 2015).

2.3 | Evolutionary Calibration and Phylogenetic Analysis

To contextualise the evolutionary history of *Austropotamobius* lineages, we reviewed the palaeogeographical evolution of Southern Europe, with emphasis on the Alpine–Dinaride and Balkan regions, as well as the Rhône and Danube basins. Our synthesis focused on major tectonic shifts and global sea-level changes over the last 45 million years (van Hinsbergen et al. 2020; Westerhold et al. 2020), which shaped the region's freshwater connectivity. During the early Miocene, marine incursions such as the Karpatian Sea established episodic connections between Central Europe and the Eastern Paratethys (Palcu et al. 2019; Harzhauser et al. 2023). A key event for our study is the Mid-Langhian marine transgression (~15 Ma), which inundated the Pannonian Basin and isolated the Apuseni Island (Figure S1), disrupting freshwater corridors across the Pannon–Dinaride area (Kováč et al. 2007; Coric et al. 2009; Sant et al. 2019a, 2019b, 2020; Mandic et al. 2019). This event served as a calibration point for our molecular clock, corresponding to the divergence of *A. bihariensis* from related *A. torrentium* clades in the NCD, as previously proposed (Pârvulescu et al. 2019) and independently corroborated (Stanković et al. 2024).

To reconstruct phylogenetic relationships, we used concatenated alignments consisting of 938 base pairs (522 mtCOI +416 16S) per sequence, with 10 similarly aligned sequences from *Astacus astacus* as an outgroup. Next, we tested substitution models using the *modelTest* function from the R package *phangorn* v.2.12.1 (Schliep et al. 2017), selecting the HKY + G(4) + I model. An ultrametric tree was constructed using the R function *pml_bb* (NNI rearrangement, $k=0$) and bootstrap support estimated from 100 replicates. Assuming comparable evolutionary rates among European crayfish clades (Bláha et al. 2020), we explored the strict clock model (assuming a constant evolutionary rate across groups of related branches), the discrete model (assigning specific rates to designated parts of the tree), the correlated model (assuming evolutionary rates are correlated among neighbouring clades) and the relaxed model (allowing evolutionary rates to vary freely). Among these, the strict clock model consistently provided the largest penalised log-likelihood (*ploglik*) values, indicating the best convergence for our input data. This was corroborated by previous studies, even under scenarios with heterogeneous substitution rates (Paradis 2013). Time calibration was performed with the *chronos* function (R package *ape* v.5.8–1) (Paradis and Schliep 2019), setting minimum and maximum divergence ages at 14 and 16 Ma, respectively, for the most recent common ancestor (MRCA) between

A. bihariensis and north-Dinaric *A. torrentium* clades (divergence centred on Mid-Langhian marine transgression, see above). Model optimisation used standard convergence criteria (e.g., dual.iter.max = 50, iter.max = 1e5, eval.max = 1e5, epsilon = 1e-4 and tol = 1e-4). Confidence intervals (CIs) were estimated via the R function *chronosCI* from the package *chronos* v.0.1–1, based on the default value of 100 bootstrap iterations (B = 100). A consensus tree was derived by assigning node ages to the midpoint of their respective 95% CIs and graphically rendered using *tidytree* v.0.4.2 (Curry and Zhang 2022) and *ggtree* v.3.6.0 (Yu 2020) in R v.3.2.1 (R Core Team 2024). To corroborate species-level clusters, we applied two single-locus delimitation methods: Automatic Barcode Gap Discovery—ABGD (Puillandre et al. 2012) and Bayesian Poisson Tree Processes—bPTP (Zhang et al. 2013).

2.4 | Methodological Considerations

This study relied on mitochondrial markers due to the scarcity and fragmented nature of available nuclear data for this genus. In several cases, sequence truncation or omission was necessary to achieve consistent alignments, which limited direct comparability with certain previously published haplotypes. Although a genome-wide approach would offer higher resolution, its implementation remains logistically challenging, primarily due to limited transnational access to specimens for sample collection. Another limitation stems from uneven sampling effort across the distribution range, with field campaigns being more intensive in some regions while others remain less represented. To minimise oversampling effects, our analyses considered haplotype density rather than the number of individuals per haplotype or per site, but further sampling is clearly needed in underexplored areas.

3 | Results

3.1 | Revisiting the Phylogeny of *Austropotamobius* Main Lineages

The genetic alignments analysed in this study encompass the entire *Austropotamobius* genus. The retained sequences and haplotypes identified for each mtCOI haplogroup, along with their respective geographical distributions, are summarised in Table 1 for *A. pallipes* and *A. fulcisianus*, and Table 2 for *A. bihariensis* and *A. torrentium*. We successfully increased phylogenetic resolution by concatenating the corresponding 16S fragment to the majority (83.2%) of the mtCOI haplotypes, resulting in final sequence lengths of 938 base pairs.

The time-calibrated ultrametric phylogenetic tree of the entire *Austropotamobius* genus (Figure 2) indicates that the divergence between the two main lineages, *A. torrentium* and *A. pallipes*, is estimated to have occurred between 22.0 and 39.1 Ma (95% CIs). In comparison, the CIs for all other nodes were narrower, mostly ranging between 1 and 5 Ma, with strong statistical support across the tree (Table S1).

There is significant genetic diversity within the *torrentium* group, with up to nine monophyletic lineages corresponding to distinct Molecular Operational Taxonomic Units (MOTUs). These may warrant formal taxonomic recognition in the future. Among them, *A. bihariensis* is the only lineage formally described as a species to date and represents the oldest clade in the group. In contrast, the most recent MOTUs (SB and CSE) are not strongly supported as distinct species, but rather as potential subspecies (Figure 2). Within the *pallipes* group, the two described species—*A. pallipes* and *A. fulcisianus*—correspond to distinct MOTUs (Figure 2), with the latter further subdivided into several genetically differentiated clades traditionally recognised in earlier work (i.e., Fratini et al. 2004), which are discussed in more detail below.

3.2 | Spatial Patterns of *Austropotamobius* Main Lineages

The highest haplotype diversity within *Austropotamobius* is found in the Dinarides region (Figure 3). Other areas with notable haplotype diversity include the Italian Peninsula south of the Alps, the Balkans and the southern Pannonian Basin.

With respect to the spatial distribution of *A. pallipes* and *A. fulcisianus* (Table 1), our results indicate that all *A. fulcisianus* haplogroups (AFF in the native range, AFM and AFO) exhibit clustered distribution patterns. However, when considering introduced populations, the distribution of the AFF shifted to a

TABLE 1 | Basic and the average nearest neighbour (ANN) statistics for the analysed haplogroups of *A. pallipes* and *A. fulcisianus*.

	AFM	AFF	AFF–n	AFO	AUP	AUP–n
Sequences	206	345	66	33	160	144
Haplotypes	47	18	9	11	8	6
Area of occurrence (km^2)	550779.5	1146755.8	33663.1	61816.5	581363.6	287341.1
Observed mean distance	20683.6	29296.4	13012.4	11658.1	70042.1	65818.5
Expected mean distance	33907.5	26877.43	61963.8	64767.2	66706.8	95389.1
Nearest neighbour ratio	0.61	1.09	**0.21**	**0.18**	1.05	0.69
z score	−9.88	−6.44	−7.75	−7.74	−3.04	−5.02
p value	<0.005	<0.005	<0.005	<0.005	<0.05	<0.005

Note: Bold values show significant clustering.
Abbreviations: AFF, *A. f. fulcisianus*; AFM, *A. f. meridionalis*; AFO, *A. f. orientalis*; AUP, *A. pallipes*; n, native area only.

TABLE 2 | Basic and the Average Nearest Neighbour (ANN) statistics for the analysed haplogroups of *A. torrentium* and *A. bihariensis*. *A. torrentium*.

	AUB	ZV	GK	KOR	VOJ	LD	ŽPB	BAN	SB	CSE
Sequences	35	8	56	4	2	26	45	43	123	453
Haplotypes	7	5	13	3	1	9	13	13	37	94
Area of occurrence (km^2)	2450.7	NA	883.3	67.5	NA	3681.1	24376.6	4201.2	84220.3	604893.9
Observed mean distance	16822.9	NA	4201.6	12278.7	NA	19640.5	6876.3	12873.3	32068.3	20960.7
Expected mean distance	70095.4	NA	43852.9	94451.5	NA	37057.5	38201.7	55970.9	32060.1	15412.3
Nearest neighbour ratio	**0.25**	NA	**0.10**	**0.13**	NA	0.53	**0.18**	**0.23**	0.97	1.36
z score	−3.46	NA	−6.24	−2.87	NA	−3.40	−6.47	−4.43	−0.25	7.03
p value	< 0.005	NA	< 0.005	< 0.005	NA	< 0.005	< 0.005	< 0.005	0.80	< 0.005

Note: The ZV (Zeleni Vir) and VOJ (Vojskova) haplogroups were not rated due to the lack of spatiality. Bold values show significant clustering.
Abbreviations: BAN, Banovina; CSE, Central and Southeastern Europe; GK, Gorski Kotar; KOR, Kordun, LD, Lika and Dalmatia; SB, southern Balkans; ŽPB, Žumberak, Plitvice and Bjelolasica.

dispersed pattern. In contrast, *A. pallipes* consistently exhibited a dispersed distribution, regardless of whether introduced (nonnative) locations were included in the analysis. Interestingly, while widely distributed *A. torrentium* haplogroups maintain relatively high haplotype diversity, *A. pallipes* exhibits notably low genetic diversity.

The *A. torrentium* haplogroup with the most dispersed spatial distribution was CSE, with an observed average distance of 20.9 km and a ratio of 1.36, accompanied by a very low p value and high z score (Table 2). In contrast, most *A. torrentium* haplogroups (ZV, BAN, VOJ, GK, KOR, LD and ŽPB) and *A. bihariensis* populations exhibited clustered distributions (Figure S2B), with ratios ranging from 0.10 to 0.25 and less than 0.5% probability of occurring by random chance (Table 2). The SB haplogroup showed a tendency toward a clustered distribution; however, based on the p value and z score, the spatial pattern of its populations appeared to be rather random.

The model ranking provided a comparative assessment of colonisation scenarios, helping identify the most plausible evolutionary trajectories within *Austropotamobius*. For *A. pallipes* (Figure S3A), the most supported scenario was model P07 (BTI = −2875.02, probability = 0.99). The model also suggests bidirectional historical gene flow between *A. fulcisianus*, AFM and AFO connecting populations in the Italian Peninsula and the southern Dinarides, as well as between AFF populations from Italy and the Iberian Peninsula. These patterns reflect episodes of reciprocal genetic exchange, indicating recent connectivity within these regional clusters. For *A. torrentium* (Figure S3B), the most strongly supported scenario was model T11 (BTI = −6167.00, probability = 1). This model suggests that the ancestral population originated north of the Dinarides (NCD). From this group of endemic lineages, *A. bihariensis* became isolated, while BAN persisted along the eastern Dinarides, acting as a transitional zone toward Balkan colonisation by the widespread SB, from which the cosmopolitan CSE later emerged.

4 | Discussion

The evolutionary history of the *Austropotamobius* genus has been shaped by a complex interplay of mountain uplift, tectonic isolation, karstic habitat fragmentation, glacial bottlenecks and human-mediated translocations. This multifaceted background has given rise to contrasting biogeographical patterns, ranging from narrowly endemic to widely distributed lineages. To understand these patterns, our narrative focuses on lineages most likely to retain signatures of long-term isolation. Among these, we specifically sought events that could be reliably anchored in geological time. While isolation within karst systems may have persisted over extended timescales, the absence of datable stratigraphic layers and fossil evidence limits the precision of temporal inference. In contrast, divergences associated with marine transgressions offer a more robust framework, such events imposing immediate barriers to gene flow, and their timing can be independently constrained (van Hinsbergen et al. 2020; Westerhold et al. 2020).

4.1 | Toward a Coherent Taxonomy and Conservation Strategy for *Austropotamobius*

The consistent and correct use of species and subspecies names within the genus *Austropotamobius* is essential for effective conservation planning. Although taxonomy is relatively well established for the *A. pallipes* and *A. fulcisianus* (Kawai et al. 2023), the scientific literature still reflects a heterogeneous use of names. A notable example is the repeated misapplication of the invalid species name *A. italicus*. Our analysis confirms the species status of *A. pallipes* and *A. fulcisianus*, the latter comprising three well-supported subspecies. Of these, only two, *A. f. orientalis* (Karaman, 1929) and *A. f. fulcisianus* (Ninni, 1886), are currently recognised (Crandall and De Grave 2017), despite continued inconsistencies in nomenclatural usage. The appropriate name for the lineage previously referred to as '*carsicus*' is *A. f. orientalis*, while *A. f. fulcisianus* includes the synonymous forms '*berndhauseri*', '*italicus*' and '*lusitanicus*', with '*carinthiacus*'

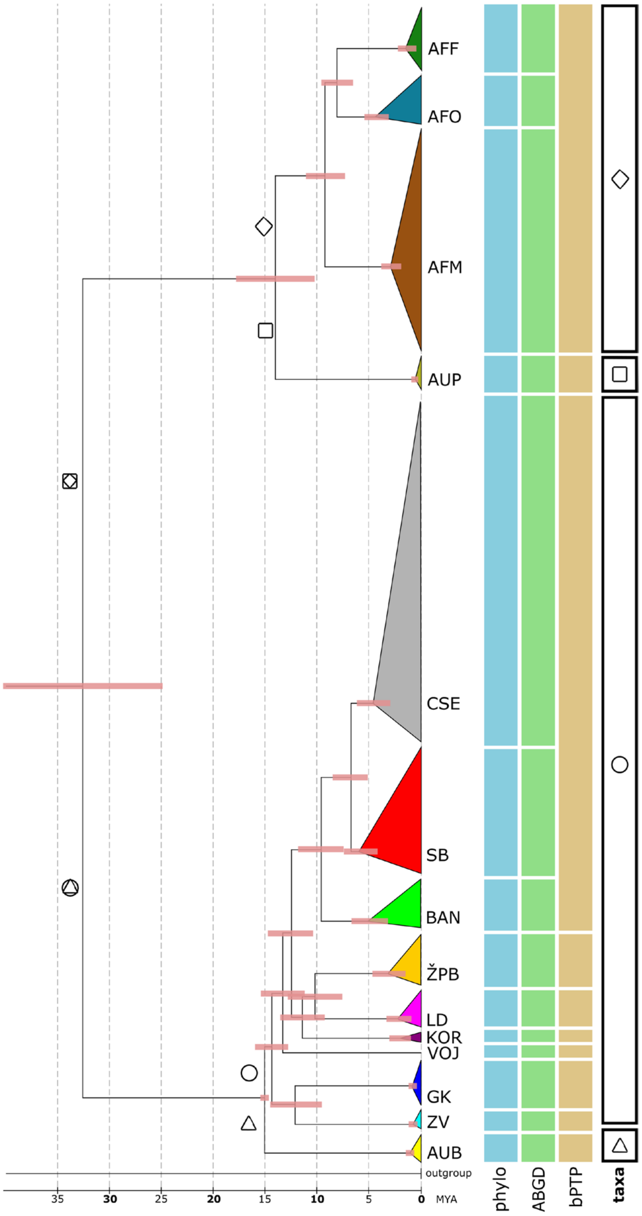

FIGURE 2 | Maximum likelihood ultrametric molecular phylogenetic tree based on concatenated *Austropotamobius* mtCO1 and 16S sequences (938 base pairs). The inferred ages of nodes corresponding to the most recent common ancestor (MRCA) of various clades, along with their bootstrap values and confidence intervals (CIs), can be found in Table S1. Vertical bars correspond to molecular operational taxonomic units (MOTUs) according to two different species delimitation methods, next to the phylogroups. The coding legends used for described taxa are as follows: Triangle for AUB—*A. bihariensis*; circle for *A. torrentium* lineages ZV (Zeleni Vir), GK (Gorski Kotar), VOJ (Vojskova), KOR (Kordun), LD (Lika and Dalmatia), ŽPB (Žumberak, Plitvice and Bjelolasica), BAN (Banovina), SB (southern Balkans) and CSE (Central and Southeastern Europe); square for AUP—*A. pallipes*; rhomb for AUF—*A. fulcisianus* lineages AFM (*A. f. meridionalis*), AFO (*A. f. orientalis*) and AFF (*A. f. fulcisianus*). Collage was created in Inkscape 1.3.2 (https://inkscape.org).

also clustering clearly within this clade. The third lineage, *A. f. meridionalis*, although not yet formally recognised (i.e., Crandall and De Grave 2017, WoRMS—https://www.marinespecies.org/, DecaNet—https://www.decanet.info/), is strongly supported by both our findings and previous studies. Initially identified by Santucci et al. (1997), this clade should be retained under the name proposed by Fratini et al. (2004).

Our models suggest that *A. f. meridionalis* represents the ancestral lineage from which this species diversified. Bidirectional gene flow was observed among Iberian, Southern Dinaric and Italian subpopulations of *A. fulcisianus* subspecies, likely reflecting relatively recent speciation or contact events. This pattern reflects historical biogeographical processes: during the Messinian Salinity Crisis, populations of *A. f. orientalis* and *A. f. meridionalis* colonised the Adriatic slopes of the Dinarides, where they became geographically isolated. In these karstic regions, habitat fragmentation restricted gene flow and promoted local diversification, contrasting populations outside the Dinarides (Figure 4). The absence of glacial impact in the Dinarides further contributed to the preservation of high genetic diversity in these isolated populations.

Among *Austropotamobius* species, *A. torrentium* stands out for its high genetic diversity and well-resolved clade structure, suggesting the existence of multiple yet undescribed taxa (Maguire et al. 2017; Lovrenčić et al. 2020b). Our results provide strong genetic support for clear separations between species and subspecies. In particular, the NCD endemic lineages likely represent distinct cryptic species deserving formal taxonomic recognition. Among the remaining lineages, the SB and CSE haplogroups stand out as strong candidates for recognition at the subspecies level, the CSE lineage, which includes the type locality of *A. torrentium* (von Paula Schrank 1803) and overlaps geographically with the known distribution of *A. torrentium danubicus* Starobogatov (1996), shows no evidence of genetic or geographical separation; therefore, the name *A. torrentium torrentium* (von Paula Schrank 1803) should be retained based on nomenclatural priority, with *danubicus* treated as a junior synonym. The SB lineage, given its clear genetic distinctiveness and geographical coherence, may warrant recognition as *A. torrentium macedonicus* (Karaman 1929). Although their descriptions were not originally based on molecular data, they align well with the current haplogroup distributions, as the geographical references in the original descriptions correspond to the present-day ranges (Karaman 1929; Starobogatov 1996). Notably, our analyses revealed no evidence of bidirectional gene flow between the SB and CSE lineages, further supporting their taxonomic validity.

From a conservation perspective, our findings suggest that several lineages may warrant reassessment of their conservation status, especially given their high likelihood of being described as distinct taxa in the near future. *A. bihariensis* has already undergone formal evaluation, being classified as 'Endangered' in Romania's Academy Red Book (Pârvulescu 2021) and, following IUCN criteria (Ion, Ács, et al. 2024), listed 'Endangered' on the global IUCN Red List of Threatened Species (Pârvulescu et al. 2025a). This listing has been formally acknowledged in Romanian legislation through its inclusion in Annexes 3 and 5A of Government Emergency Ordinance No. 7/2025 (https://legislatie.just.ro/Public/DetaliiDocument/295259). We encourage similar steps for other candidate taxa, including their formal description and subsequent conservation status evaluation. Notably, the taxonomic revision of *A. bihariensis* also

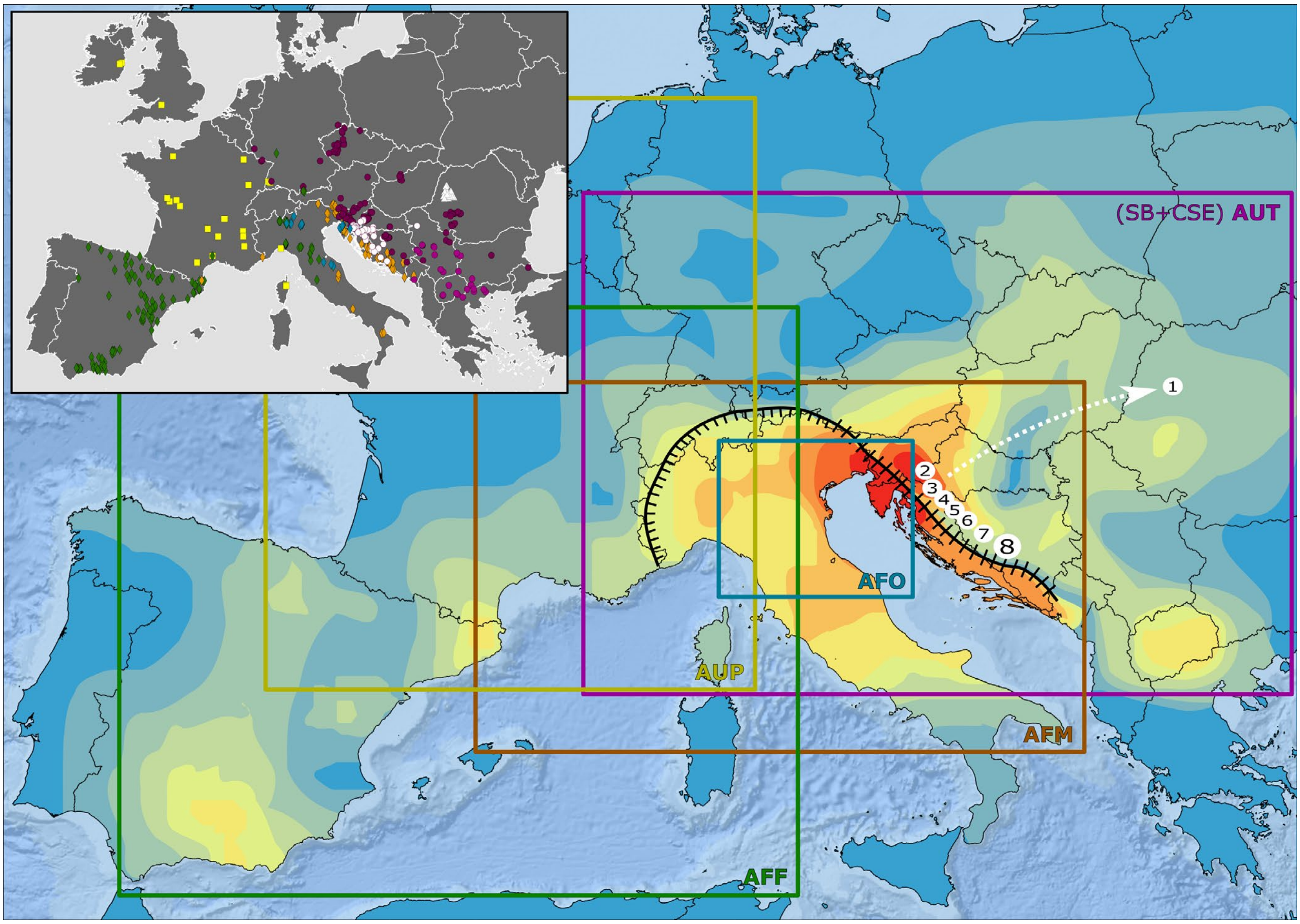

FIGURE 3 | The multiframe heatmap (average nearest neighbour [ANN] method) of Europe based on cosmopolitan lineage distribution. Each window contains a reference to the respective mtCOI haplogroup, which is further detailed in Figures 4–6. Endemic lineages are numbered and emphasised with the annotation of their further dynamics toward the current situation and their encoding legends: 1—*A. bihariensis* (AUB) white triangle, *A. torrentium* lineages 2–ZV (Zeleni Vir), 3–GK (Gorski Kotar), 4–VOJ (Vojskova), 5–KOR (Kordun), 6–LD (Lika and Dalmatia), 7–ŽPB (Žumberak, Plitvice and Bjelolasica), 8–BAN (Banovina) white circles, SB (southern Balkans) and CSE (Central and Southeastern Europe) marked pink circles; for the *A. pallipes* (AUP) yellow squares mark, *A. fulcisianus* lineages, we used orange squares for AFM—*A. f. meridionalis*, blue squares for AFO–*A. f. orientalis* and green squares for AFF–*A. f. fulcisianus*, respectively. The inset map in the top corner shows the geographical origin of individuals according to their mtCOI haplogroup. Collage was created in Inkscape 1.3.2 (https://inkscape.org).

triggered a conservation IUCN status reassessment of *A. torrentium*, which has now been provisionally reclassified as 'Least Concern' (Pârvulescu et al. 2025b).

In contrast, well-established taxa such as *A. fulcisianus* remain absent from conservation legislation, highlighting persistent gaps between scientific taxonomy and legal protection frameworks. Bridging this disconnect will require coordinated efforts to update taxonomic classifications and alignment with current conservation policies. Furthermore, given that some *Austropotamobius* populations have been translocated beyond their native ranges, we recommend that their potential ecological and genetic value be carefully considered. In the context of widespread population declines, such non-native populations may serve as valuable reservoirs of genetic diversity, supporting future conservation and restoration programmes (Oficialdegui et al. 2024). Ensuring that conservation legislation adapts to these realities will be critical for the long-term preservation of *Austropotamobius* diversity.

4.2 | The Palaeogeographical Cradle of *Austropotamobius* Evolution

The palaeogeographical evolution begins during the Eocene (56–34 Ma), with the emergence of Balkanatolia—a composite landmass consisting of Gondwanan and Laurasian terranes—formed by northward subduction of the African Plate under Eurasia (Licht et al. 2022; Palcu and Krijgsman 2023). Separated by warm shallow seas, Balkanatolia acted as an isolated biogeographical island, fostering unique floral and faunal assemblages, including early freshwater taxa (Licht et al. 2022; Montheil et al. 2025). This isolation persisted until the Eocene–Oligocene transition (~33 Ma), when a global sea-level drop and continued tectonic convergence led to the docking of Balkanatolia with Europe and Asia. However, these connections were transient and unstable due to continued fragmentation, resulting in numerous tectonic basins and the disruption of former dispersal corridors (Palcu and Krijgsman 2021).

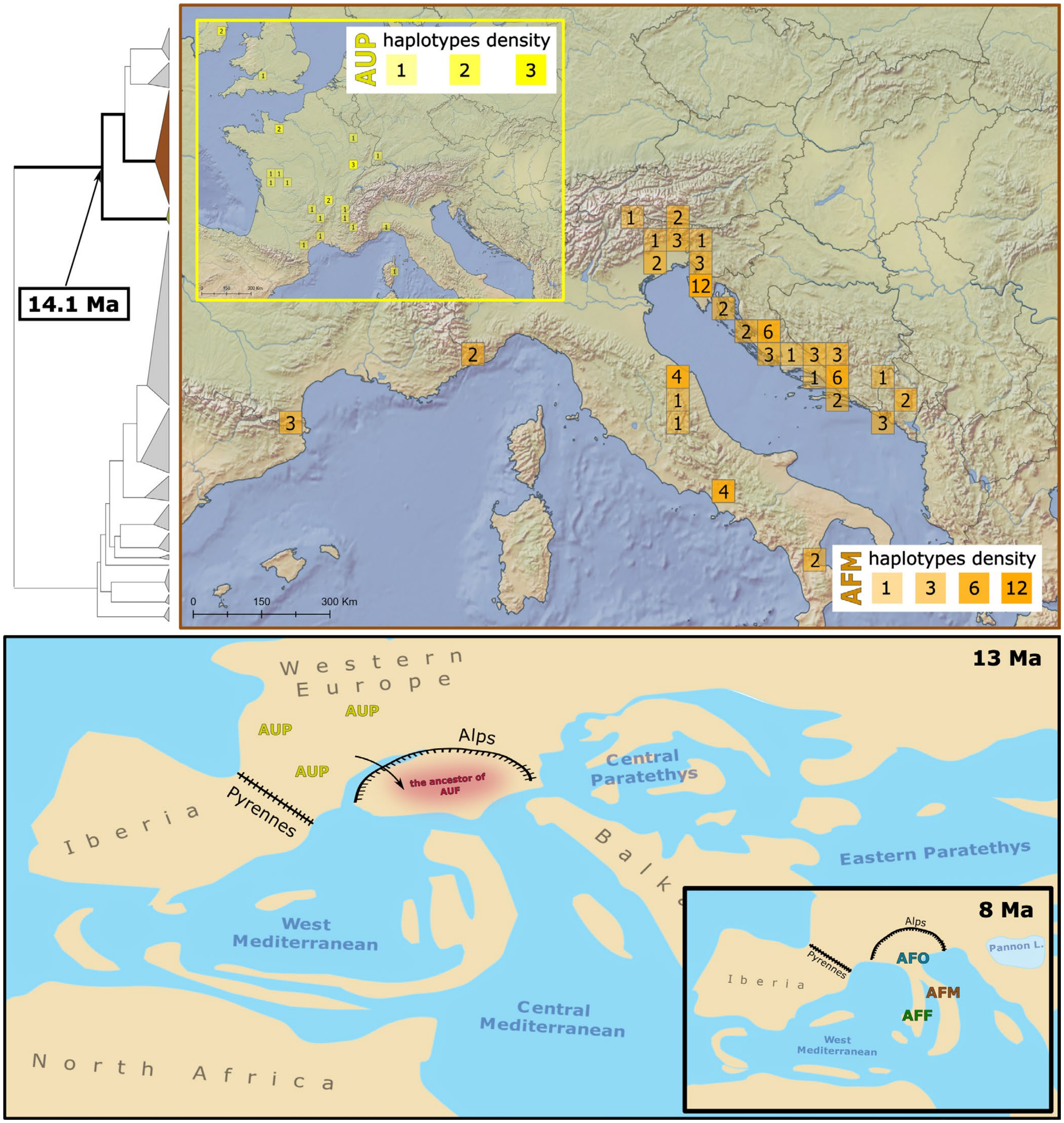

FIGURE 4 | Spatial dynamics and mtCOI haplotype (522 base pairs) distributions within the *A. pallipes* (AUP) and *A. fulcisianus* (AUF) haplogroups. The upper panel displays the current density of haplotypes across these areas, highlighting the concentration of haplotypes in present-day geography. The two lower maps display the palaeogeographical context of their divergence timing, as represented in the phylogenetic tree (see Figure 2 for details): A large map illustrates a reconstructed map of 13 Ma, the opening of an embayment west of the Alps facilitating the early crayfish colonisation South of the Alps, resulting in the *A. fulcisianus* ancestor; a small map displays a reconstructed map of 8 Ma, revealing the forming Italian Peninsula facilitating the spread and diversification of main lineages. This colonisation hypothesis model received the best statistical support (Figure 1A). Palaeogeographical maps were compiled from sources (Palcu and Krijgsman 2021; Kalifi et al. 2023) and collated using Inkscape 1.3.2 (https://inkscape.org).

In the western sector, the retreat of marine environments north and west of the Alps led to the formation of landbridges via the North Alpine Foreland Basin (NAFB), linking the Alpine region with the Massif Central and Aquitaine (Mennecart and Beatty 2015). Faunal turnover events, such as the Microbunodon Event (~24–25 Ma), mark this phase of continental connection (Mertz et al. 2007), but marine incursions during the early Miocene once again inundated these corridors. Only during the Middle Miocene (~16–15 Ma) did continental sedimentation reestablish stable terrestrial habitats in this area (Kalifi et al. 2023).

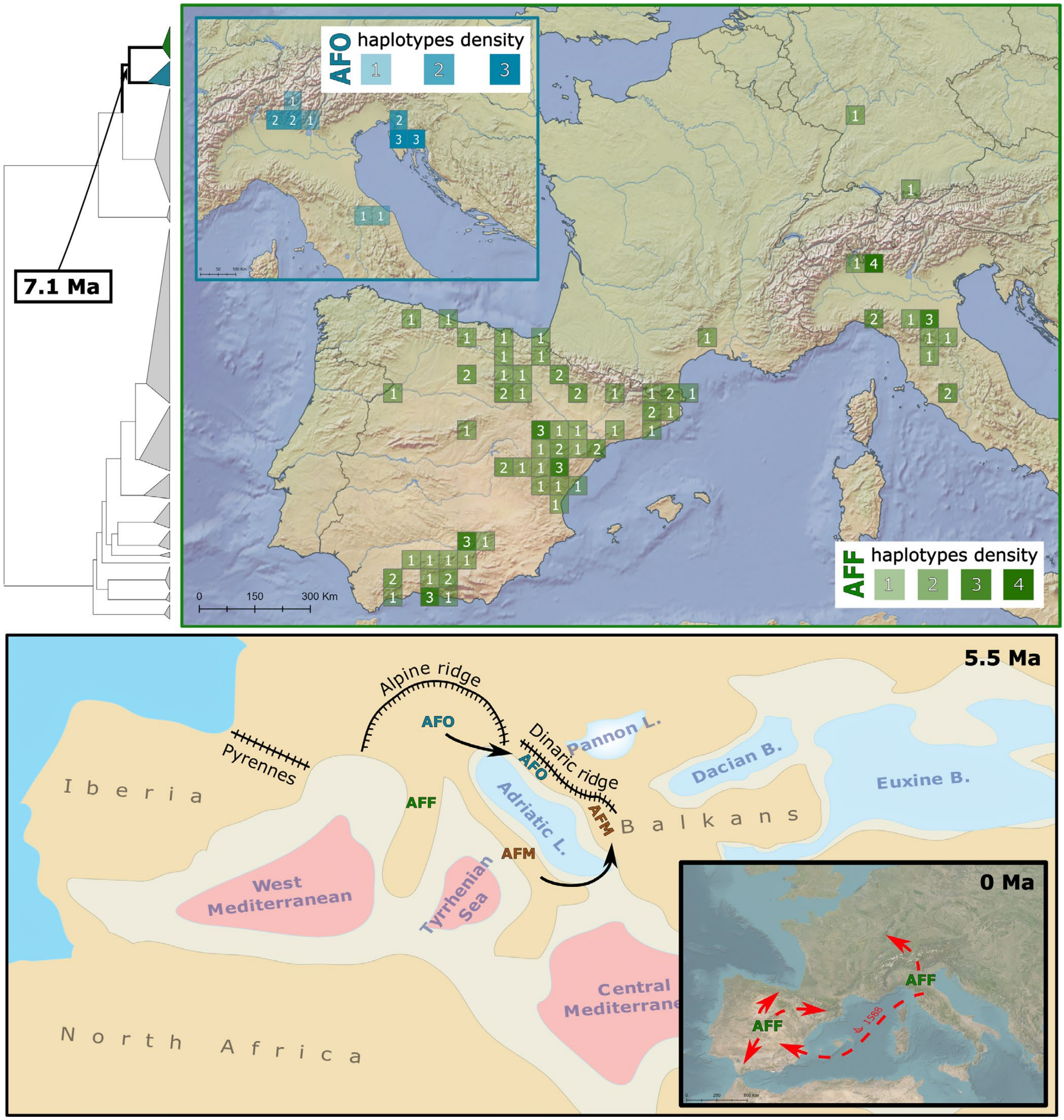

FIGURE 5 | Spatial dynamics and mtCOI haplotype (522 base pairs) distributions within the *A. fulcisianus fulcisianus* (AFF) and *A. fulcisianus orientalis* (AFO) haplogroups. The upper panel displays the current density of haplotypes across these areas, highlighting the concentration of haplotypes in present-day geography. The two lower maps display the dynamics of the AFO, AFF and AFM lineages in the reconstructed palaeogeographical context of their molecular diversification, as represented in the phylogenetic tree (see Figure 2 for details): A large map illustrates a reconstructed map of 5.5 Ma revealing the peak of the Messinian Salinity Crisis, in which AFO colonised through freshwater Adriatic Lake from the Apennine region to Dalmatia before Zanclean Flooding; a small map indicates the 1588 shipment of AFF from Livorno to Alicante, which was subsequently transported to royal gardens around Madrid, arguably originating all Iberian AFF populations through multiple secondary introductions. Other AFF introduction events north of the Alps are also indicated. This colonisation hypothesis model received the best statistical support (Figure 1A). The palaeogeographical map was compiled from sources (Krijgsman et al. 2024) and collated using Inkscape 1.3.2 (https://inkscape.org).

Further east, the gradual closure of the Tethys Ocean and the formation of the Paratethys Sea (~33 Ma) restructured marine basins across Southeastern Europe (Schulz et al. 2005; Palcu et al. 2023). The Paratethys underwent multiple disconnections and reconnections with the global ocean: first with the North Atlantic (~25 Ma), then exclusively with the Mediterranean (~23 Ma), often through ephemeral seaways in the Balkanatolian region (Popov et al. 2019). By the Middle Miocene, intense plate

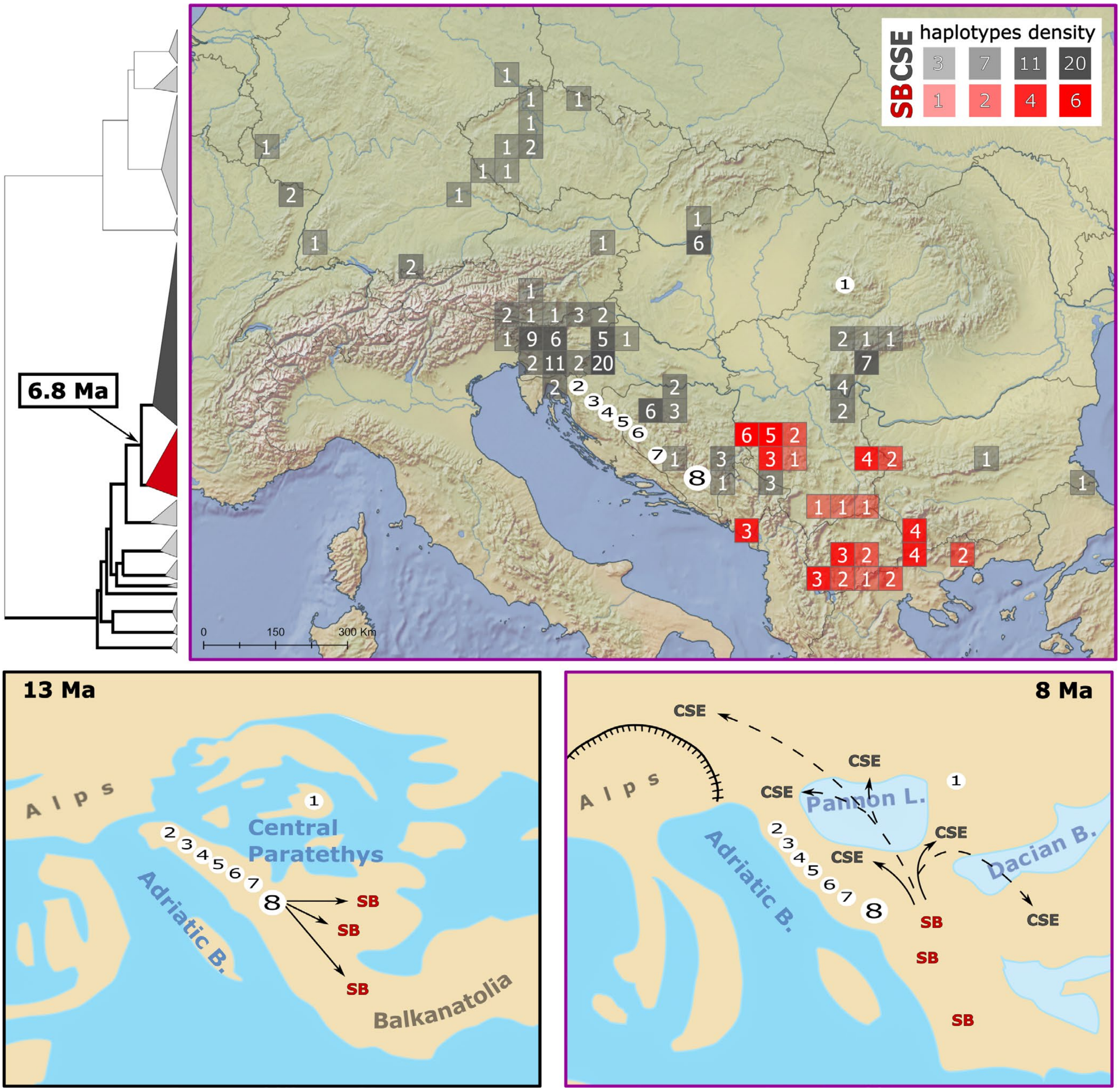

FIGURE 6 | Spatial dynamics and mtCOI haplotype (522 base pairs) distributions of *A. torrentium* within the southern Balkans (SB) and Central and Southeastern Europe (CSE) haplogroups. The upper panel displays the current density of haplotypes across these areas, highlighting the concentration of haplotypes in present-day geography. The two lower panels illustrate the historical dynamics of the SB and CSE lineages in a reconstructed palaeogeographical context, aligned with their molecular divergence timing as represented in the phylogenetic tree (see Figure 2 for details). The left map illustrates the expansion of the SB lineage from the Dinaric Lake systems, which provided conditions favourable for the evolution of endemic lineages (denoted as 2–8), into the Balkans. The right map focuses on the Pannon region, highlighting its transition to the Palaeo Danube freshwater system, which enabled the spread of the most recent and widespread CSE crayfish lineage. This colonisation hypothesis model received the best statistical support (Figure 1B). Haplogroups encoding legend: 1–*A. bihariensis* (AUB), 2–ZV (Zeleni Vir), 3–GK (Gorski Kotar), 4–VOJ (Vojskova), 5–KOR (Kordun), 6–LD (Lika and Dalmatia), 7–ŽPB (Žumberak, Plitvice and Bjelolasica) and 8–BAN (Banovina). Palaeogeographical maps were compiled from sources (Popov 2004, Sant et al. 2017) and collated using Inkscape 1.3.2 (https://inkscape.org).

collisions uplifted the Carpathians and Alps (Schmid et al. 2019), while the Pannonian and Transylvanian basins subsided. This geodynamic setting led to a major marine incursion—the Mid-Langhian transgression (~15 Ma)—that flooded the region and established the Central Paratethys Sea (Kováč et al. 2007; Sant et al. 2019b; a; Mandic et al. 2019). This transgression caused the long-term isolation of emergent highlands such as the Apuseni Mountains, which became a large island surrounded by the brackish waters of the Central Paratethys (Figure 4B), while parts of the Carpathian arc formed an archipelago. The Karpatian Sea, situated across present-day Syria, Transdanubia and the Vienna Basin, was intermittently connected to the Mediterranean via a seaway through western Slovenia and extended eastward toward Transylvania and the Eastern Paratethys (Palcu et al. 2019; Harzhauser et al. 2023). This prolonged marine phase, lasting over five million years, likely severed freshwater connections between the Apuseni and Pannon-Dinaride regions, enabling the allopatric divergence of the *A. bihariensis* clade. The palaeogeographical configuration during this period provides a plausible barrier to gene flow and a robust calibration point for our molecular phylogeny (Pârvulescu et al. 2019; Stanković et al. 2024).

In the late Miocene and Pliocene, ongoing uplift and erosion reshaped the Carpato-Alpine region, carving modern valleys and lake basins and establishing contemporary river networks, including the Danube. The river began discharging into the Black Sea around 4.36 Ma (de Leeuw et al. 2018), marking the final stages of landscape stabilisation.

4.3 | Tracing *Austropotamobius* Evolution Through Palaeogeography

The evolutionary history of *Austropotamobius* crayfish is intrinsically linked to the tectonic and geological evolution of Southern Europe. During the Oligocene-Miocene, the region corresponding to the present-day Dinarides was a northwest-to-southeast-oriented landmass, bordered by salted waters from the Paratethys to the north and from the Mediterranean to the south. This orientation played a crucial role in shaping the direction of river systems, influencing drainage patterns and, consequently, the dispersal and isolation of *Austropotamobius* lineages over time (Audo et al. 2023; Gašparič et al. 2023), with its first split between the groups of proto-*pallipes* and proto-*torrentium* lineages likely occurring at approximately 32–31 Ma (see Figures 2 and 4). This period coincided with significant geological changes during the Eocene to Oligocene extinction event, which was characterised by large-scale turnover of aquatic species (Costa et al. 2011; Ozsvárt et al. 2016).

The proto-*pallipes* lineage eventually diverged into two branches: *A. pallipes*, which became geographically restricted between the Alps and the Pyrenees, and *A. fulcisianus*, which followed a more complex evolutionary trajectory (Figures 2, 5 and 6). Geological changes, particularly the formation of a temporary embayment west of the Alps and a reorganisation of drainage systems, enabled dispersal southward across the Alpine arc (Figure 4). At that time, the Italian Peninsula was still in formation, offering newly available freshwater habitats suitable for colonisation and in situ diversification. This process led to the emergence of the three subspecies of *A. fulcisianus*, with *A. f. meridionalis* diverging first, followed by *A. f. orientalis* and *A. f. fulcisianus*.

A relevant aspect that stands out in this study is the major contrast between some *Austropotamobius* lineages clustered within a small geographical area (i.e., endemic) and the widely dispersed patterns (i.e., cosmopolitan) of others. We assume that this divergence reflects long-term evolutionary processes that have significantly influenced the genetic trajectories of these populations, highlighting the dynamic interplay between geological and ecological factors. Mountains have long played a crucial role in speciation, acting as both barriers and facilitators of diversification (Perrigo et al. 2020), while habitat characteristics influence crayfish diversity through a combination of ecological constraints and opportunities (Barnett and Garrick 2024; Graham and Richardson 2024). In particular, karst relief, with its unique fragmentation across water networks, has driven vicariant diversification (Klobučar et al. 2013; Previšić et al. 2014; Gao et al. 2015). The Dinarides harbours a substantial genetic diversity of *Austropotamobius*, with northern slopes featuring a diverse set of six ancient lineages within the *A. torrentium* complex (most of them arguably pending description as valid species) and southern slopes more recently occupied by two *A. fulcisianus* haplogroups (already described as subspecies). Glaciations had minimal impact on the Dinarides, preserving high genetic diversity in karstic environments.

At approximately 32 Ma, Southern Europe had a palaeogeographical configuration dominated by connected landmasses. The Paratethys Ocean has become almost entirely disconnected from the global ocean and separated from the Mediterranean by a large landmass that amalgamated Iran, Balkanatolia and Western Europe. At approximately 31 Ma, the Southern Europe landmass split into Western Europe and the Alps in the west and Balkanatolia in the east. This event may have acted as a barrier (see Figure S1) separating the ancestors of *A. pallipes* and *A. torrentium*. The directions of the river valleys of Balkanatolia reflect the morphology of the Dinaridic thrust and fold belt, with northeast–southwest-dipping slopes and perpendicular tributary depressions, along thrust faults often associated with small Oligocene–Miocene palaeolake systems, especially in the western part of the Dinarides (de Leeuw et al. 2012). These short valleys flowed directly into the Paratethys or the Mediterranean Sea, suggesting that crayfish individuals cannot exchange genes between patchy subpopulations. In this setting, the early ancestors of *A. torrentium* Dinarides lineages evolved isolated for a considerable period between ~31 and ~6 Ma. Prolonged insularity often generates endemism (Costedoat and Gilles 2009; Neubauer et al. 2015); hence, these events decisively contributed to the current endemic status of these populations.

Within the same timeframe, some *A. torrentium* lineages followed a non-endemic pattern. Certain populations at the eastern margin of the Dinarides gradually expanded into the non-karstic watercourses of the Balkans (Figure 6). Unlike the fragmented karstic habitats of the Dinarides, the Balkans featured larger freshwater basins, such as the Serbian Lake System (Neubauer et al. 2020), enabling broad colonisation and facilitating the development of a cosmopolitan distribution pattern over time. These populations ultimately became part of the SB lineage of *A. torrentium*. However, the most significant expansion occurred only after the regression of the brackish waters of the Pannonian basin, coinciding with the formation of the Danube (Palcu et al. 2021). From the Balkans area, facilitated by the formation of hydrographic connections (presently corresponding to the Morava River), a substantial expansion occurred toward the colonisation of the Danube basin, which fits today's most widespread (CSE) haplogroup. The predominant upstream colonisation of the CSE lineage along the Danube is in line with the behaviour of the species (Kadlecová et al. 2012; Pârvulescu et al. 2016; Daněk et al. 2018). Palaeontological evidence indicates that the Pannon Lake fauna was confined to the

Pannonian basin until the late Miocene, approximately 6 Ma, when the first indications of aquatic faunal dispersal across the Carpathians, from the Pannon basin to the Dacian basin, were identified in the geologic record (Grothe et al. 2016).

4.4 | Tracing Human-Mediated Translocations of *Austropotamobius*

Following the natural evolutionary patterns shaped by palaeogeographical events, human activities have more recently introduced additional complexity to the distribution of *Austropotamobius* across Europe (Souty-Grosset et al. 1997; Largiadèr et al. 2000; Grandjean et al. 2000, 2001; Cataudella et al. 2010; Matallanas et al. 2011; Amouret et al. 2015; Chucholl et al. 2015; Petrusek et al. 2017). In regions such as the British Isles and Iberian Peninsula, we found no evidence from palaeogeographical or phylogenetic reconstructions to support the historical presence of any living *Austropotamobius* species. Introductions of *A. pallipes* and *A. fulcisianus* from continental Europe occurred during medieval and Renaissance times (Largiadèr et al. 2000; Machino et al. 2004; Clavero and Sempere Marín 2025). For instance, *A. pallipes* was likely introduced to Ireland in the medieval period (Gouin et al. 2003), while *A. fulcisianus* populations in Iberia trace their origins to Tuscany (Clavero et al. 2016), introduced under the reign of Spanish King Philip II in the 16th century, who had tried to obtain crayfish for the pools of the royal gardens for at least 25 years (Clavero 2022). Subsequent centuries saw these species' expansion through multiple secondary introductions as they became a popular food item (Clavero and Villero 2014). It has been suggested that the presence of crayfish fossils in Spain (Vía Boada 1971; Garassino 1997) could challenge the non-native status of *Austropotamobius* in the Iberian Peninsula. However, these fossils can not be linked to present-day Iberian crayfish, because they are around 130 My old, and thus predate the geological events leading to European crayfish diversification, discussed here, and even the divergence of Northern Hemisphere crayfish families (i.e., Cambaroididae, Cambaridae and Astacidae) (Wolfe et al. 2019). Recent reviews have cautioned that mitochondrial haplotype diversity on its own may not provide reliable evidence for nativeness, highlighting the risk of overinterpreting such data in the case of *Austropotamobius* in Spain (Clavero et al. 2025). Spanish and Italian crayfish were introduced to France in the 19th century to alleviate population declines (Trontelj et al. 2005), a historical event that would explain the presence of *A. fulcisianus* haplotypes in southern France. The presence of *Austropotamobius* on the Thyrrenian Islands was also due to introductions and reflects political control over the different islands since *A. pallipes* (from France) is present in Corsica, while *A. f. meridionalis* is present in Sardinia, as well as in some neighbouring areas in southern France and northeastern Iberia (Amouret et al. 2015; Jelić et al. 2016). This raises the possibility that even *A. pallipes* may have originally had a more restricted range, with its current widespread distribution and low diversity largely shaped by human-mediated translocations. However, this remains speculative and requires further exploration. Investigations incorporating historical records and genomic data would be crucial to determine the extent and underlying mechanisms of this distributional shift. In most cases, these introductions have led to biogeographical patterns that are challenging to interpret today (Trontelj et al. 2005; Bernini et al. 2016), complicating conservation efforts and necessitating a thorough reassessment of crayfish species' status in these regions.

4.5 | Clarifying Key Hypotheses in *Austropotamobius* Evolution

Karstic landscapes, such as those of the Dinarides, are well known for promoting high biodiversity by fragmenting freshwater habitats and limiting gene flow (Klobučar et al. 2013; Previšić et al. 2014). This natural isolation often generates endemic lineages even across small spatial scales (Hughes et al. 2011; Monegato et al. 2015; Žebre and Stepišnik 2016). In *Austropotamobius*, karstic conditions have fostered substantial local genetic divergence. Particularly, *A. fulcisianus* populations in the southern Dinarides (Figures 4 and 5) likely result from colonisation events during the Messinian Salinity Crisis (~5.5 Ma), when temporary freshwater corridors enabled dispersal from the Italian Peninsula (Palcu et al. 2021), as well as during similar low sea level events that occurred more recently (Storms et al. 2008). Following colonisation, karst fragmentation and the absence of major glaciations allowed these populations to diversify in situ without external genetic input. In contrast, populations outside karstic regions exhibit broader and less structured distributions, reflecting different ecological and geological trajectories. Thus, the high diversity observed in the Dinarides should be interpreted as a consequence of habitat-driven microevolution, not as evidence that the region served as an ancestral radiation centre for the genus. However, gaps in sampling coverage remain an important limitation. Further genetic surveys in regions with limited sampling could reveal additional diversity and refine the interpretation of phylogeographic patterns.

A similar need for clarification arises regarding *A. bihariensis* (also referred as the APU lineage). Some previously proposed hypotheses suggesting random colonisation across the Danube basin followed by glacial contraction (Lovrenčić et al. 2020a) fail to fully explain key biogeographical patterns. If random colonisation had occurred after the Danube's formation, expansion across multiple endemic lineages from the NCD would be expected, yet no such pattern is observed; instead, strong endemism persists. Furthermore, if *A. bihariensis* had sought glacial refuge in the Apuseni karst, similar recolonisation patterns from other karstic refugia, such as the Carpathians or Dinarides, would be expected, which is not the case. Importantly, microsatellite and genomic data (Pârvulescu et al. 2020; Bonassin et al. 2024) reveal no evidence of immigrant genetic influence (sensu Ingvarsson 2001) within *A. bihariensis* populations. The dominance of a single haplotype, accompanied by six conserved local variants (Ion, Ács, et al. 2024), further supports a history of long-term isolation under glacial pressure, contrasting with the greater haplotypic diversity observed in NCD lineages (Table 2).

5 | Conclusion

This study refines our understanding of the evolutionary history and taxonomic structure of the crayfish genus *Austropotamobius*, offering a comprehensive perspective that strengthens both scientific interpretation and conservation efforts. The diversification of the group has been shaped by major palaeogeographical events—such as mountain uplift, karstic habitat fragmentation and marine transgressions—which established long-term barriers to gene flow and fostered the formation of distinct evolutionary lineages. These natural processes have been further complicated by historical and recent human-mediated translocations, partially obscuring original biogeographical signals.

Our findings underscore the urgent need for a consistent, evidence-based taxonomic framework to ensure that all recognised and candidate lineages are adequately represented in biodiversity assessments and legal protection instruments. Establishing a unified, internationally aligned nomenclature is critical for achieving taxonomic stability and scientific clarity. Equally important is the formal evaluation and description of candidate MOTUs, whose recognition is vital for fully capturing the evolutionary and ecological diversity of the genus and for integrating overlooked lineages into effective conservation strategies.

Author Contributions

Conceptualization: L.P. Data curation: L.P., D.L., A.M.L., A.-M.N., M.S.M., A.M., E.M., A.S., M.C.I., L.F. Formal analysis: D.L., A.E.M., M.A.C., A.M.L. Funding acquisition: L.P. Investigation: D.V.P.R., A.E.M., M.C.I., D.L. Methodology: A.E.M., M.A.C., D.L. Project administration: L.P. Supervision: L.P., M.C., C.P. Visualisation: D.V.P.R., A.E.M., D.L., M.A.C., M.C.I. Writing – original draft: L.P. Writing – review and editing: L.P., M.C., D.V.P.R.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

Supporting datasets (mtCOI and 16S sequences ID, location and references) used in this study are available via Elsevier's Mendeley repository platform at DOI: 10.17632/5w6g2vpzgf.3. The distribution data can be visualised via the World of Crayfish platform (https://world.crayfish.ro/) based on accession codes for each species: *A. pallipes* WA0423-0581; *A. fulcisianus* lineage-AFM WA0183-0388, lineage-AFF WA0001-0182 and WK9553-9391, lineage-AFO WA0389-0421; *A. bihariensis* WA1207-1227; *A. torrentium* lineage-ZV WA1530-1537, lineage-ŽPB WA1485-1529, lineage-LD WA1134-1149, lineage-KOR WA1130-1133, lineage-VOJ WK9579-9580, lineage-GK WA1074-1129, lineage-BAN WA0582-0623 and WK9621-9622, lineage-SB WA1425-1484, lineage-CSE WA0627-1026 and WK9556-9578.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Palaeogeographical evolution of Central Europe, emphasising landmass connectivity and isolation. (A) Large-scale and detailed reconstructed maps of the Pannonian Basin at 16 Ma, illustrating the Karpatian Sea and associated basins and highlighting the limited connectivity between Balkanatolia and mainland Europe. This map indicates the potential area of occurrence for the common ancestor of *A. bihariensis* (AUB) and *A. torrentium* (AUT). (B) Large-scale and detailed reconstructed maps of the Central Paratethys at 15 Ma, focusing on the transgressive event driven by tectonic extension and subsidence, which led to the isolation of the large Apuseni Islands. This isolation was a critical event for calibrating the molecular clock, as it triggered the divergence of the *A. bihariensis* lineage (marked as 1) from the ancestor of the *A. torrentium* lineage (marked as 2–8). (C) Reconstructed map of Western Europe and the Balkanatolia landmasses connected into a single Southern European landmass at 32 Ma, providing a general overview of the occurrence of the *Austropotamobius* genus ancestor. The subsequent fragmentation of this landmass at 31 Ma (D) led to the initial split into two main clades across West Alpine Europe and Balkanatolia. Palaeogeographical maps were compiled from sources (Sant et al. 2017) and collated using Inkscape 1.3.2 (https://inkscape.org). **Figure S2:** Average nearest neighbour (ANN) ratio for the analysed haplogroups of the *Austropotamobius* crayfish species. (A) *A. pallipes* and *A. fulcisianus* haplogroups. Encoding legend: AUP—*A. pallipes*, AFM—*A. f. meridionalis*, AFO—*A. f. orientalis*, AFF—*A. f. fulcisianus*, n—native area only. (B) *A. bihariensis* and *A. torrentium* haplogroups. Encoding legends: GK (Gorski Kotar), KOR (Kordun), LD (Lika and Dalmatia), ŽPB (Žumberak, Plitvice and Bjelolasica), BAN (Banovina), SB (southern Balkans) and CSE (Central and Southeastern Europe). The ZV (Zeleni Vir) and VOJ (Vojskova) haplogroups were not rated due to the lack of spatiality. **Figure S3:** Colonisation hypothesis model selection using MIGRATE-N for *Austropotamobius* crayfish species populations based on their geographical regions. (A) *A. pallipes* and *A. fulcisianus* populations. (B) *A. bihariensis* and *A. torrentium* populations, based on concatenated mtCOI and 16S sequences (938 base pairs). Arrows indicate the direction of gene flow, and Bezier-approximated Thermodynamic integration (BTI) values indicate the model fit. **Table S1:** The ultrametric phylogenetic tree of *Austropotamobius* crayfish species. **Data S1:** jbi70059-sup-0005-DataS1.xlsx.