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Insights on the evolution of the tribe Pliomyini (Arvicolinae, Rodentia): Ancient DNA from the extinct *Pliomys lenki*

María Pilar Alfaro-Ibáñez, Jaime Lira-Garrido, Gloria Cuenca-Bescós, Joan Pons, and Pere Bover

ABSTRACT

The partially unresolved phylogeny of the Arvicolinae subfamily has been a recurrent topic of discussion in scientific papers. Among the species belonging to this subfamily, *Dinaromys bogdanovi* is one of the species with an unclear phylogenetic position. Paleontologists have traditionally compared and related its molars to the extinct *Pliomys*, whose geologically youngest fossil representative, *Pliomys lenki*, persisted until the Late Pleistocene. Although both genera have been morphologically related, there has always been discussion about when these genera should have separated. In this paper, we use ancient DNA data from *Pliomys lenki* (=*Pliomys coronensis*) to demonstrate the phylogenetic relationship between *Dinaromys* and *Pliomys*, forming the tribe Pliomyini. Additionally, we propose an evolutionary model of the Pliomyini tribe, placing the separation *Dinaromys/Pliomys* around 3.8 Ma (2.6–4.9 Ma).

María Pilar Alfaro-Ibáñez. Aragosaurus-IUCA, Departamento Ciencias de la Tierra, Facultad de Ciencias, Universidad de Zaragoza, C/ Pedro Cerbuna, 12, 50009 Zaragoza, Spain. (Corresponding author.) alfaromp@unizar.es
Jaime Lira-Garrido. Centre d'Anthropobiologie et de Génomique de Toulouse (CAGT), CNRS UMR 5288, Université Paul Sabatier, Toulouse 31000, France and Departamento de Medicina Animal (Área de Medicina y Cirugía Animal), Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain. jaime.e.lira@gmail.com
Gloria Cuenca-Bescós. Aragosaurus-IUCA, Departamento Ciencias de la Tierra, Facultad de Ciencias, Universidad de Zaragoza, C/ Pedro Cerbuna, 12, 50009 Zaragoza, Spain. cuencag@unizar.es
Joan Pons. Institut Mediterrani d'Estudis Avançats, IMEDEA (UIB-CSIC), C/ Miquel Marquès 21, 07190 Esporles, Illes Balears, Spain. jpons@imedea.uib-csic.es
Pere Bover. ARAID Foundation, Avenida de Ranillas, 1-D, planta 2a, Oficina B, 50018 Zaragoza, Spain and Instituto Universitario de Investigación en Ciencias Ambientales (IUCA) – Grupo Aragosaurus, Universidad de Zaragoza, C/ Pedro Cerbuna, 12, 50009 Zaragoza, Spain. (Corresponding author.) pbover@unizar.es

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INTRODUCTION

Arvicolinae is a subfamily of Cricetidae rodents comprising up to 28 extant genera and 151 extant species, with the total number of genera and species continuously increasing through paleontological studies, application of new methods in study of recent and fossil species, and even taxonomic re-analyses (e.g., identification of cryptic species, etc.). The Arvicolinae include the voles sensu lato, such as water vole, snow vole, field vole, etc, as well as the lemmings.

The presence/absence and stratigraphical distribution of Arvicolinae species in paleontological/archaeological deposits have been widely used to analyze paleobiogeographical and paleoenvironmental inferences, and biostratigraphy and paleontological correlation in Quaternary deposits (e.g., Chaline et al., 1999; Fejfar et al., 2011; Cuenca-Bescós et al., 2016; Alfaro et al., 2023). Some examples of the use of this subfamily of rodents can be found in correlation with paleontological localities containing human fossil remains, such as Atapuerca, Orce, and Aroeira, among others, in the Iberian Peninsula, Petralona in Greece, Aragó in France, etc. (e.g., Agustí et al., 2010; Cuenca-Bescós et al., 2015; Lebreton et al., 2016; López-García et al., 2018; Piskoulis et al., 2023). The presence or absence of some Arvicolinae species is also an indicator of environmental characteristics, as is the case of species related to humid environments, such as the *Iberomys* lineage (Cuenca-Bescós et al., 2014), or water bodies, as in the case of the *Arvicola* lineage (e.g., Desclaux et al., 2000; Maul et al., 2000; Cuenca-Bescós et al., 2010a; Mahmoudi et al., 2019). Another biostratigraphic marker is *Pliomys*, which is absent in areas with well-developed forests (Tesakov, 2005). Some species of Arvicolinae are exceptional as fossil guides, as in the case of the *Mimomys* lineage, that marks the biostratigraphic limit of the Early to the Middle Pleistocene (Koenigswald and Kolfschoten, 1996; Maul et al., 2007).

The geologically oldest fossil representatives of Arvicolinae (Cricetidae, Rodentia, Mammalia) are observed in Europe and Asia, and they are most ascribed to *Promimomys* [Miocene-Pliocene boundary, ca. 5.3 million of years (Ma) ago] (Hordijk and de Bruijn, 2009; Martin, 2010). According to Chaline et al. (1999) *Promimomys* gave rise to *Mimomys*, the ancestor of many of the extant vole species, and the ancestor of the Arvicolines present on the North America continent as well. However, some authors considered the microtoid cricetid *Pannonicola* as the first representative of Arvicolinae (Middle Turolian, ca. 7.3 Ma ago) (Kretzoi, 1965; Zazhigin, 1982), that according to Fejfar et al. (2011) would give rise to the Ondatrini and Dycrostonychini species. The arvicolines are characterized by the presence of a higher crown and the reduction and disappearance of roots in a great number of species, developing in this hypsodont or hypselodont molars (or mesodont in the case of early arvicolines as *Promimomys*) that display alternating triangles on the occlusal view.

Within Arvicolinae, *Pliomys* is an extinct genus generally included in the tribe Pliomyini, in which is also included the only living species of this tribe *Dinaromys bogdanovi*, the Balkan vole (Kretzoi, 1969; Kryštufek and Bužan, 2008), a species currently observed in Balkan regions of countries such as Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, and Serbia (Kryštufek, 2018). This *Pliomys-Dinaromys* relationship is based in the description of *Dinaromys bogdanovi* as “a cementum-bearing type of *Pliomys”* due to the characters shared by both species regarding the molar morphology (Chaline et al., 1999). The derived character that makes Pliomyini molars unique is the combination of having roots and a high number of triangles, formed by the morphology of the enamel in salients and re-entrants’ angles on the occlusal view, in the lower first molar (m1), a tooth widely used in Arvicolinae taxonomy (Bartolomei et al., 1975; Terzea, 1983). However, some studies abnegate the relationship of these two genera (Robovský et al., 2008). Pliomyini species also display a third upper molar (M3) with narrow incoming angles in the anterior lobe and with a ‘pliomian islet’ or *‘Pliomys* structure’ in the anterior lobe (Chaline et al., 1999; Yamikova et al., 2022) (Figure 1).

Pliomys includes several extinct species, and for this reason, the origin and evolution knowledge on the *Pliomys* species is based exclusively on the fossil record. There are more than 200 stratigraphic levels in European Quaternary containing *Pliomys* (e.g., Bartolomei et al., 1975; Carls and

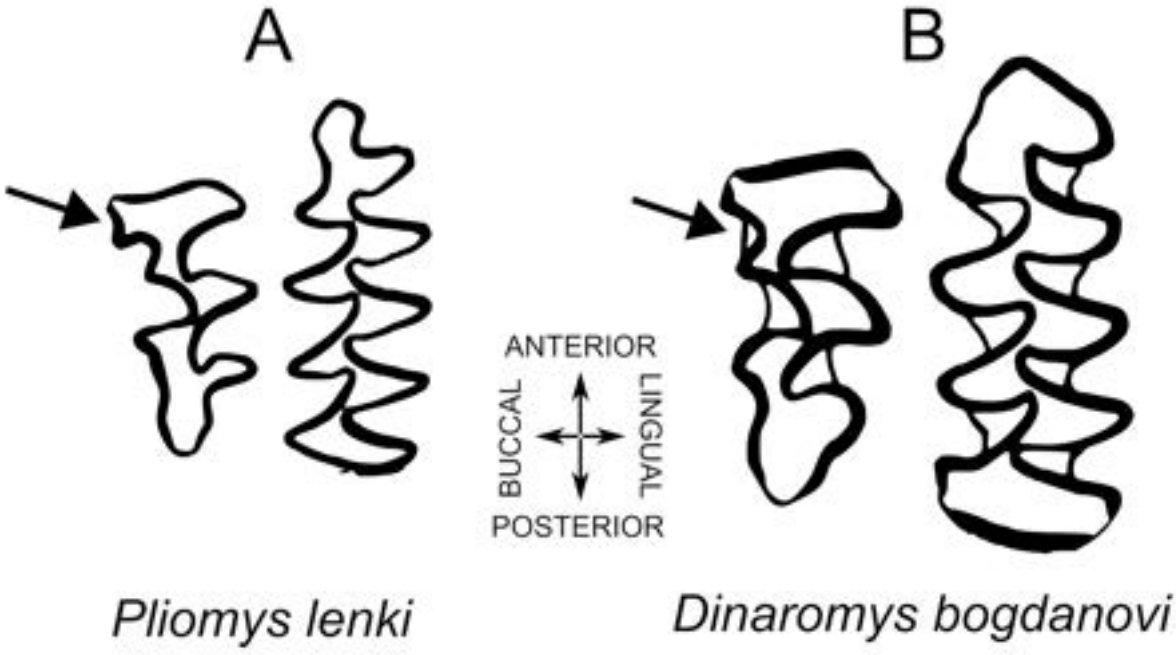

FIGURE 1. Image depicting the first lower (m1, on the right) and third upper (M3, on the left) molars of *Pliomys lenki* (**A**) and *Dinaromys bogdanovi* (**B**). The *Pliomys*, derived, structure is indicated by an arrow in each M3. Modified from Chaline et al. (1999).

Rabeder, 1988; Dema and Rekovets, 2004; Cuenca-Bescós et al., 2008, 2010b). Whereas several species of the genus are known for the European Quaternary fossil record (e.g., Jeannet, 1974; Chaline et al., 1999; Tesakov, 2005), three species have been found in the Iberian Peninsula (western Europe) in the nearly 100 Early Pleistocene to the Late Pleistocene stratigraphic levels containing *Pliomys: Pliomys simplicior, P. episcopalis,* and *P. lenki (=Pliomys coronensis)*. *Pliomys simplicior* was firstly recorded from the earlier Early Pleistocene Razvode site in Croatia (Jánossy, 1986), but the arrival at the Iberian Peninsula has been accurately dated in the Early Pleistocene levels of the Sima del Elefante (lower levels TELRU, TE7 to TE14, in Atapuerca, Spain, Cuenca-Bescós et al., 2016). Other Iberian Early Pleistocene sites with *Pliomys* are Bagur 2, Gran Dolina TD4 to TD6, Casablanca 3 and Chaparral (López et al., 1976; Agustí and Galobart, 1986; López-García et al., 2012; Cuenca-Bescós et al., 2016). *Pliomys episcopalis* was defined in the type site, Somlyó-berg (Kordos and Pazonyi, 2012). This is the type species of the genus *Pliomys,* both described by Mehely in 1914. The last species, *P. lenki*, became extinct at the end of the Quaternary (Marquet, 2001; Cuenca-Bescós et al., 2010b). The well-recorded extinction process of *Pliomys* started in eastern Europe, consequently disappearing westwards with the Iberian Peninsula as final refuge (Cuenca-Bescós et al., 2010b). In general, this genus has been considered of great relevance for the Quaternary biostratigraphy of the Iberian Peninsula since they are abundant in the deposits, and their morphology and size make them easy to classify at the genus level (Cuenca-Bescós and Morcillo-Amo, 2022).

The taxonomy of Arvicolinae is particularly complicated, especially in terms of generic attribution and identification of subgenera as genera or even in the phylogenetic relationships within species, genera, and tribes (e.g., Chaline, 1975; Chaline et al., 1999; Fejfar et al., 2011; Kryštufek and Shenbrot, 2022). Even recent studies using considerably large genomic datasets do not agree in the phylogenetic relationships for some arvicoline taxa (Bužan et al., 2008; Robovský et al., 2008; Abramson et al., 2021; Withnell and Scarpetta, 2024, among others). With the development of ancient DNA methods and high through-output sequencing platforms it is currently possible to obtain a vast amount of molecular information for even small species that can contribute to understand phylogenetic and phylogeographic aspects of zoological taxa such as some Arvicoline (e.g., Baca et al., 2022, 2023).

In this paper we obtained for the first time a partial mitochondrial genome of a Late Pleistocene *Pliomys lenki* individual from the El Mirón Cave deposit (Spain). These sequences allowed us to infer the phylogenetic relationships of the extinct species within the Arvicolinae.

MATERIAL AND METHODS

Study Site and Species

Two right mandibles identified as *Pliomys lenki* from the paleo-archaeological site of El Mirón Cave (Cantabria, Spain) (Figure 2A) were used for the paleogenomic analysis. The identification of the mandibles as belonging to *P. lenki* was based on diagnostic morphological characteristics of the molars such as larger size than those from the other vole species recorded in the Late Pleistocene of the Iberian Peninsula, presence of roots, absence of cement in the re-entrant angles, and thicker continuous enamel. The generally good preservation of the fossil remains from this site has already allowed us to obtain ancient DNA from other Arvicolinae remains (e.g., Baca et al., 2022, 2023; Alfaro-Ibáñez et al., 2023). The first mandible (laboratory number 218) displayed the complete lower molar teeth-row (molars 1 to 3, m1-m3) and one incisor, and the proximal part of the mandible was broken (Figure 2B-D). The second mandible (laboratory number 219) displayed m1, m2, and the incisor, the condylar and coronoid processes were complete but lacked most of the bottom part of the mandible, being the incisor almost fully exposed (Figure 2E-G). Mandible 218 were excavated from level 130 (Spit 27, Square w10, Subsquare B) and mandible 219 from level 129 (Spit 22, Square x10, Subsquare B), with a chronology for these levels of 50,900–39,280 and 46,890–33,160 years cal BP, respectively (Hopkins et al., 2021).

DNA Extraction, Library Construction and Sequencing

All laboratory tasks using the *Pliomys lenki* samples were performed in the dedicated paleogenomics laboratory of the University Institute for Research in Environmental Sciences of Aragon (IUCA) of the University of Zaragoza (Spain). Mandibles 218 and 219 were UV radiated for 15 minutes on each side. Surface of the mandibles was slightly wiped with bleach 1%, and then submerged in water for 10 minutes and subsequently in 80% ethanol for 10 min. After air drying each mandible, they were placed in 2 ml screw-cap tubes. Bone powder was mechanically obtained using sterile tweezers inside the tubes. A total amount of 113 and 116 mg, respectively, was used for the DNA extraction.

DNA from both mandibles were extracted following the silica suspended protocol of Brotherton et al. (2013) and using a modified binding buffer (Bover et al., 2019) based on Dabney et al. (2013) protocol. Shortly, an initial predigestion step using 1 ml EDTA on a rotary wheel for 1 hour at room temperature was followed by an overnight digestion/decalcification using ~1 mL of a digestion buffer (900 µL EDTA 0.5M ph8.0 and 20 µL of 20 mg/mL Proteinase K) on a rotary wheel at 55ºC. DNA extraction was performed using 13 mL of a modified binding buffer [13.6 mL PB buffer (Qiagen), 420 µL Sodium Acetate 3M and 7 µL Tween-20] and 100 µL of suspended silica particles. After three washes (first using 900 µL of remaining binding buffer and two subsequent ones using 900 µL 80% ethanol), DNA was eluted in 100 µL of TLE buffer.

Double-stranded libraries based in protocol by Meyer and Kircher (2010) with modifications described in Llamas et al. (2016) (i.e., ligation of short 7-bp P5 and P7 adapters) and using a partial uracil-DNA glycosylase treatment (Rohland et al., 2015) of sequencing libraries were constructed. Purified and quantified libraries were finally sequenced in an Illumina HiSeqX platform (2 x 150 bp).

Mitochondrial Genome Assembly and Phylogenetic Analyses

Mitochondrial genome assembly. The quality of raw sequencing reads was analysed using fastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). We demultiplexed sequences from both samples by filtering both P5 and P7 barcodes (allowing one mismatch) using Sabre v.1.0 (http://github.com/najoshi/sabre) and adapter sequences were trimmed with AdapterRemoval v.2.3.0 (Schubert et al., 2016) using parameter values as follows: mismatch rate 0.1, minimum Phred quality 4, quality base 33, trim ambiguous bases (N), and trim bases with qualities equal to or less than the given minimum quality, and collapsing (merging) paired reads overlapping by at least 11 bp. Reads shorter than 30 bp were excluded from further analysis. At the end of the process up to 10,196,653 collapsed reads (~70%) and 19,505,962 (~81%) were retained for samples 218 and 219, respectively, for downstream analysis.

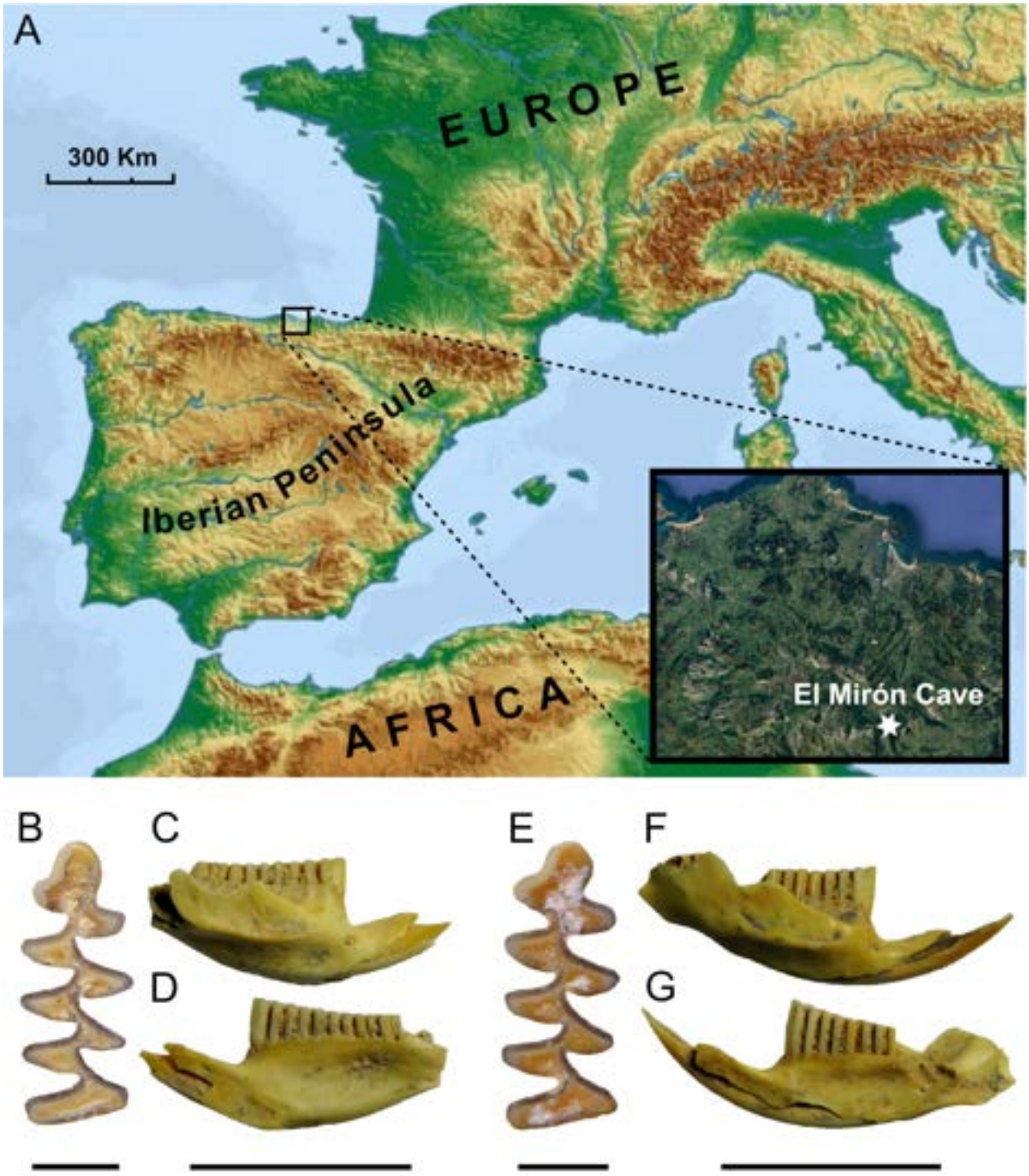

FIGURE 2. A, Geographic location of the Cueva del Mirón paleontological site on the Iberian Peninsula (Spain). **B-G**, *Pliomys lenki* fossil jaws and teeth used in this study. Sample ZARADNA 218: First lower molar (m1) in occlusal view (B); and mandible in buccal (C) and lingual (D) views. Sample ZARADNA 219: m1, occlusal view (E); and mandible in buccal (F) and lingual (G) views. Top and right parts of the teeth are the anterior and buccal margins, respectively. Scale bars in mandibles (C, D, F and G) equal 10 mm. Scale bars in teeth (B and E) equal 1 mm.

We performed a preliminary mapping of collapsed reads for both samples to evaluate the quality of the samples using BWA v.0.7.17 backtrack algorithm (Li and Durbin, 2009) and parameters widely accepted for ancient data mapping (i.e., aln -n 0.01, -o 2, -l 1024), and using the mitochondrial genome of *D. bogdanovi* (GenBank Accession Number MT588182) as reference. Duplicate reads were filtered using FilterUniqueSAMCons.py (Kircher, 2012). The higher number of reads mapped to the reference for sample 219A than the ones for sample 218A (542 versus 55 reads) suggested the selection for this first sample for further analyses.

We constructed two draft mitochondrial genomes of *Pliomys* sample 219A following a "Multi-reference Iterative Mapping Approach" (MIMA, Torres-Roig et al., 2021) using BWA. In both cases, collapsed reads were mapped to three different arvicoline mitochondrial genomes as references: *Dinaromys bogdanovi* (GenBank Accession Number MT588182), *Eolagurus luteus* (GenBank Accession Number MT492448), and *Microtus thomasi* (GenBank Accession Number NC_057558). We used the more stringent BWA parameters for ancient data mapping (i.e., -n 0.01, -o 2) and removing reads with minimum mapping quality (-q 25) using SAMtools v.1.11 (Li et al., 2009) to construct the first draft, but more relaxed parameters (i.e., -n 0.001 and -q 20) suggested by Westbury and Lorenzen (2022) for high phylogenetic divergence mitochondrial genomes as *Pliomys* and the species used as references. *D. bogdanovi* was used as reference due to the morphological similarities that present with *P. lenki*, and specifically for being the only current representative of the Pliomiyni tribe. *Eolagurus luteus* was selected as a reference for being a genus close to *Dinaromys* according to DNA studies, whereas *Microtus thomasi* is a species more phylogenetically distant and morphologically different from *Dinaromys* (Abramson et al., 2021). Duplicate reads were removed as explained above (FilterUniqueSAMCons.py). Using the two different sets of parameters, we performed iterative rounds of mapping. At the end of each round of mapping for each reference, an intermediate 75% majority consensus sequences were generated using Geneious Prime v.2022.0.2 (Biomatters, http://www.geneious.com Kearse et al., 2012), retaining the reference nucleotides for sites with read-depth < 3X. The new consensus was then used as the new reference, and the process was iterated until no more reads were mapped. Final 75% majority consensus sequences for the iterative mapping against each reference were then generated in Geneious, calling nucleotides only at sites with read-depth ≥ 3X. Number of iterations and unique reads mapped are listed in Appendix 1, but a higher number of mapped reads were obtained when using *D. bogdanovi* as reference (i.e., 1,173 unique reads using stringent mapping parameters, covering 58% of the reference at a mean depth of 4.5X and 1,898 unique reads using relaxed parameters, covering 84% of the reference at a mean depth of 7.3X). The consensus sequences generated from this first iterative mapping were identical for regions where they overlapped. As better results were obtained using *D. bogdanovi*, we aligned the three consensus sequences with the mitogenome of this species using the MUSCLE algorithm implemented in Geneious, generating a new merged reference by retaining nucleotides called from our sequence data and filling any gaps with the corresponding nucleotides from *D. bogdanovi*. This new merged reference was then used for another round of iterative mapping (as described above, using both sets of mapping parameters). Using stringent parameters up to six rounds of mapping were needed to map 1,678 unique reads to the reference covering 74% of it at a mean depth of 6.4X, whereas using relaxed parameters 2,175 unique reads mapped covering 93% of the reference at a mean depth of 8.5X). A final consensus for each mapping strategy was generated in Geneious using a 75% majority consensus, retaining the reference nucleotide in positions with coverage depth ≥ 3X, and obtaining ~68% of the mitochondrial genome using the stringent mapping strategy and around ~89% using the relaxed mapping strategy. Ancient DNA misincorporation and fragmentation patterns were assessed using mapDamage v.2.1.1 (Jónsson et al., 2013).

Phylogenetic analyses. The two partial mitochondrial genome sequences of the fossil of *Pliomys* from El Mirón Cave (stringent-STR and relaxed-REL) were annotated using the mitochondrial genome of *Dinaromys bogdanovi* as base and adjusting changes in Protein Coding Genes (PCGs) using Geneious. Each *Pliomys* reference was aligned to 112 arvicoline mitochondrial genomes from 66 species available at GenBank (see Appendix 2). For those species with multiple mitochondrial genomes, we randomly selected five of them after removing incomplete mitogenomes or, in case of just partial genomes available, discarding those with more unknown (N) nucleotides. *Akodon montensis*, *Cricetulus griseus*, and *C. lon-*

gicaudatus were used as outgroups. The 116 mitochondrial genomes were realigned using MUSCLE implemented in Geneious, and after removing the Control Region (CR) we divided the alignment in PCGs, *tRNAs*, *12S_rRNA*, and *16S_rRNA* and were realigned again using MUSCLE. We further divided PCGs by codon positions using DAMBE v.7.0.5 (Xia, 2017), and ambiguously aligned regions of *tRNAs* and *rRNAs* were removed using default parameters using Gblocks v.091b (Castresana, 2000) in Phylogeny.fr (Dereeper et al., 2008), retaining 852 out of 986 (86%) positions for *12S_rRNA*, 1301 out of 1654 (78%) for *16S_rRNA* and 1401 out of 1642 (85%) positions for *tRNAs*. The final length of the alignment was 14,885 bp. Partitioning scheme and nucleotide substitution models were inferred using the implemented ModelFinder (Kalyaanamoorthy et al., 2017) in IQTREE2 (Minh et al., 2020) for RAxML, MrBayes and Beast2 (see Appendix 3). A partitioned Maximum Likelihood analysis was performed using RAxML v.8.2.11 (Stamatakis, 2014), with node support values estimated by performing 100 bootstrap replicates. We also performed a partitioned MrBayes v.3.2.3 analysis (Ronquist et al., 2012), comprising four separate runs of four Markov chains each using default priors. Each chain ran for 25 million generations sampling trees and parameter values every 10^4 generations. Topological convergence was assessed using the average standard deviation of clade (split) frequencies (< 0.02), while convergence in individual parameter values was assessed through broadly overlapping distributions and effective sample sizes > 200 in Tracer v.1.7 (Rambaut et al., 2018). All sampled trees were summarized as a majority-rule consensus tree after discarding the first 10% of trees as burn-in.

A partitioned analysis under relaxed molecular clock was performed using BEAST v1.10.4 (Drummond et al., 2012) and one individual per species (see Appendix 2). We initially followed all calibrations and priors used by Abramson et al. (2021; see table S3 in that paper), except the Arvicolinae calibration (Offset= 5.3 Ma, 95% HPD = 5.4–10.3 Ma), as *Promimomys* is more generally considered the first Arvicolinae in the fossil record at 5.3 Ma (Fejfar et al., 2011). However, all except Dicrostonychii calibrations showed convergence of the posterior values. We decided to exclude the Dicrostonychii constraint in further analyses since conversion of posterior values requires multiple analyses from different prior distributions. Four analyses were run for 10^7 generations sampling every 10^4 generations, discarding the first 10% of samples as burn-in. Convergence was assessed through combined analysis of four independent runs whose parameters showed effective sample sizes > 200 as calculated using Tracer v.1.7. Individual run outputs were combined using LogCombiner v.1.8.4 and a final maximum clade credibility tree was generated using TreeAnnotator v.1.8.4 implemented in BEAST.

Path Sampling (PS)/Stepping Stone sampling (SS) approaches were used to estimate marginal likelihood (MLE) values so they can be directly compared despite the number of parameters of the models, as these methods increase model selection accuracy (Baele et al., 2012). We analyzed the performance of Yule and Birth-Death process competing models using for both scenarios default parameters (Baele et al., 2012). We analyzed 100 path steps with 10^6 length chains and log likelihood every 1000 per path step, with a Beta (α, 1.0) distribution power posterior. Setting α=0.3 has been considered an optimal value for estimations (Xie et al., 2011). Model fit to the data was evaluated calculating Bayes Factor (BF) as a ratio of both marginal likelihoods (Baele et al., 2012).

Some fossil species more commonly identified as being of the genus *Dinaromys*, were ascribed to *Pliomys* by Chaline et al. (1999), as is the case of *Dinaromys dalmatinus* and *Dinaromys pasai* (Kowalski, 2001). To evaluate the position of our *Pliomys lenki* sequences in comparison with the genetic variability of *Dinaromys bogdanovi*, we aligned the available mitochondrial cytochrome b *(CYTB)* data for *D. bogdanovi*, *CYTB* sequences for three *Ellobius* species (sister taxa of *D. bogdanovi* according to Abramson et al., 2021) and the two *CYTB* sequences of *P. lenki* (both obtained using stringent and relaxed mapping parameters) using MUSCLE. The alignment was trimmed to the length of the sequence available for most of the *Dinaromys* sequences (51 out of 55). The final 555-bp *CYTB* alignment comprised a total of 61 individuals (55 *Dinaromys*, one *Ellobius fuscocapillus,* one *E. lutescens*, one *E. talpinus*, the two *P. lenki* generated here, and *Rattus rattus* as outgroup, see Appendix 4). In the case of *P. lenki*, the number of nucleotides unknown for this 555-bp fragment of each *CYTB* gene sequence was seven for the one generated using relaxed parameters, and 78 for the sequence obtained using stringent parameters. A Maximum Likelihood analysis was performed on this dataset using RAxML with node support values estimated by performing 100 bootstrap replicates.

It is beyond the scope of this study to deeper discuss the phylogenetic relationships within Arvicolinae as well as the genus/subgenus identification for some of the species. However, for clarity, we followed the genera and subfamily nomenclature proposed by Abramson et al. (2021), except in the case of *Agricola agrestis*, where we follow the most widely used classification of *Microtus agrestis*.

RESULTS

Pliomys Mitochondrial Genome Assembly

The final *Pliomys* mitochondrial sequences displayed 5,248 unknown positions (32.4% of the estimated length of the mitochondrial genome) using stringent parameters, and 1,768 (11.1%) using relaxed parameters. The alignment of both *Pliomys* consensus sequences showed that they were identical for those mitogenome regions obtained using the two different mapping strategies. Each sequence fragment larger than 50 bp in the relaxed-generated consensus (REL) not present in the stringent-generated consensus (STR) was BLASTn (Altschul et al., 1990) analysed. The first five BLASTn hits of the 16 fragments (ranging from 61-453 bp) were assigned to arvicoline/rodent species (see Appendix 5) indicating that the BWA using relaxed parameters apparently did not introduce random bacterial or other organism sequences in the consensus sequence generation. In both cases, the ancient DNA misincorporation and fragmentation patterns (Appendix 6) display the expected damage pattern observed in partial uracil-DNA glycosylase treated libraries (Rohland et al., 2015).

The new *Pliomys* mitochondrial genome sequence (using relaxed BWA mapping parameters, REL) is available in GenBank under accession number PP873348. The mitochondrial genome sequence using stringent BWA mapping parameters (STR) is provided in Appendix 7. Both partial genomes display the characteristic structure of vertebrate mitochondrial genomes containing genes for 13 proteins (Protein Coding Genes, PCGs), 22 transfer RNAs *(tRNA),* two ribosomal *rRNAs*, and the two non-coding regions Control Region *(CR)* and L-strand origin of replication (see Appendix 8 for full detail).

Phylogenetic Analyses

The topology of the phylogenetic trees obtained from the ML and BI analyses (Figure 3, Appendix 9, 10) widely recapitulates previously published results based on complete or near complete mitochondrial genomes (e.g., Folkertsma et al., 2018; Lamelas et al., 2020; Abramson et al., 2021). All our phylogenetic analyses unequivocally [Maximum Likelihood Bootstrap (MLB)=100, Posterior Probability (PP)=1] placed *Pliomys lenki* as sister taxa of *Dinaromys bogdanovi*, although the relationships of this clade with a clade comprising *Ellobius* (and *Hyperacrius*, depending on analyses,) is not fully supported (MLB < 75, PP < 0.95). The variable position of *Hyperacrius* in our phylogenetic trees agrees with that observed using both mitochondrial, nuclear, and combined data (Abramson et al., 2021; Withnell and Scarpetta, 2024).

As obtained using a larger mitochondrial genes dataset, the phylogenetic analysis of the mitochondrial *CYTB* fragment of *Pliomys* and the available data for *Dinaromys* clearly places (Appendix 11) the fossil taxon as sister taxa of *D. bogdanovi* (MLB = 99), but clearly out of its genetic diversity (MLB = 97). The obtained supported distribution of *Dinaromys* lineages (Northwestern, Southwestern, and Central) according to Kryštufek et al. (2007) and the wide geographic sampling of *Dinaromys* individuals that these authors performed for their study unequivocally indicates that *Pliomys* cannot be considered as a representative of the current *Dinaromys bogdanovi* species.

Bayes Factor (BF) values in both Stepping Stone (SS) and Path Sampling (PS) marginal likelihood estimates (MLE) showed strong and very strong support [according to Kass and Raftery (1995) strength support values, 6 < BF < 10 and BF > 10] for Yule speciation model process, respectively (see Appendix 12). Our time-calibrated BI phylogenetic analysis (Figure 3) is also in agreement with node splits observed in calibrated analyses from Abramson et al. (2021) and Withnell and Scarpetta (2024) (Appendix 13). Our results in general are particularly similar to the node ages obtained by Abramson et al. (2021) using seven calibration ages (i.e., slightly more recent than the other analyses) and to the obtained by Withnell and Scarpetta (2024) for the corresponding nodes. The divergence time between *Dinaromys* and *Pliomys*, using both STR and REL *Pliomys* sequences, is inferred in around 3.8 Ma (95% HPD = 2.6–4.9 Ma approx.), roughly during the Pliocene.

Furthermore, in our analyses we observe that the Pliomyini tribe has a closer relationship with Arvicolini, Ellobiusini, and Lagurini tribes, as already observed by Abramson et al. (2021) and Withnell and Scarpetta (2024). However, these lat-

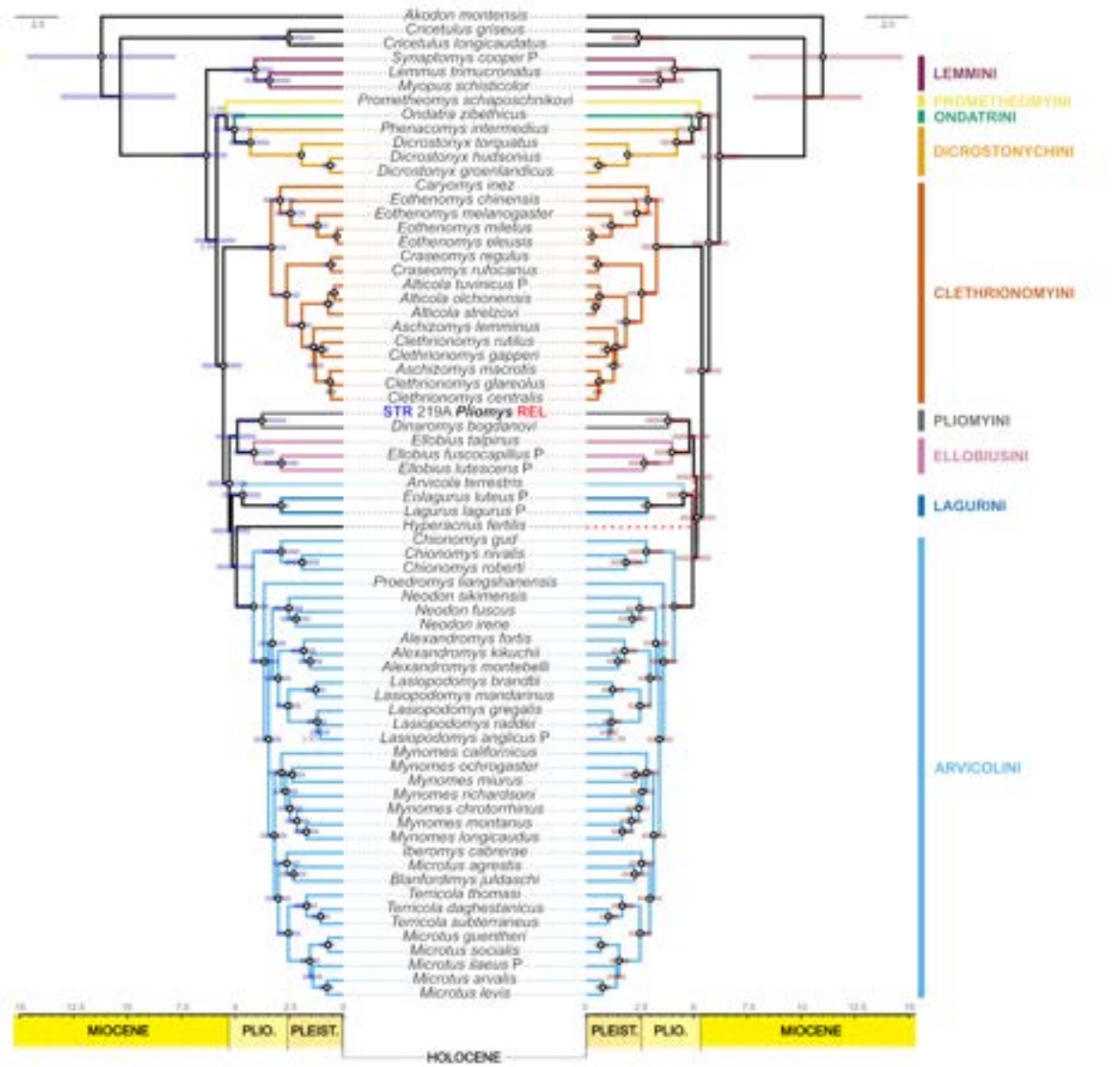

FIGURE 3. Calibrated Bayesian trees using Arvicolinae mitochondrial genomes (excluding Control Region) and both *Pliomys lenki* consensus generated with regular ancient DNA (stringent) mapping parameters (STR sequence, left) and with relaxed parameters (REL sequence, right). Numbers in nodes indicate the Posterior Probability (PP), circles indicating PP=1. Just PP values > 0.95 are displayed. Blue (left) and red (right) bars reflect the 95% highest posterior densities (HPDs) for node age estimates. Dotted red lines in the right topology indicates the different position of *Hyperacrius fertilis* (as sister clade of Pliomyini-Ellobiusini, PP=0.54) in comparison with the topology of the tree on the left side of the figure (as sister clade of Arvicolini, PP=0.53). GenBank accession numbers are listed in Appendix 2 and partitioning scheme in Appendix 3.

ter authors also observed a sister relationship between Pliomyini and Clethriomyini using only discrete mitochondrial data.

DISCUSSION

As mentioned, *Dinaromys* and *Pliomys* share various molar characteristics, which allows clustering them within the Pliomyini tribe of Cricetidae rodents. In general, m1 displays an increased number of triangles (T) on the occlusal surface, T5 and T6 that converge to a greater or lesser degree with the anterior complex that lacks an islet or fold. The third upper molar (M3) displays the so-called '*Pliomys* structure', consisting of an enamel island

in the anterior lobe, lacking an island in the posterior complex (Figure 1). This last structure is a synapomorphy that differentiates the Pliomyini from the other Arvicolinae genera (Kordos and Pazonyi, 2012), with also *Dinaromys bogdanovi* displaying the *Pliomys* structure in M3 and a slightly larger m1 than *Pliomys*. Due to these similarities, both taxa were merged in one single genus, *Dolomys*, considering *Dinaromys* and *Pliomys* as subgenera of it (Hinton, 1926), a view that was later rejected leaving *Dinaromys* and *Pliomys* as valid genera (Kretzoi, 1955; Bartolomei et al., 1975). Although *Dinaromys* was described as "a cementum-bearing type of *Pliomys*" by Chaline et al. (1999), and there are still discussions in the literature about the attribution of some species in *Dinaromys* or *Pliomys* (e.g., Kowalsky, 2001), our results unequivocally demonstrate that *Pliomys* and *Dinaromys*, although closely related by both morphological (Kretzoi, 1969; Chaline et al., 1999) and molecular characters (Figure 3, Appendix 9, 10), should be considered different genera (Appendix 11).

Previous studies included *Dinaromys* in the tribe Clethrionomyini (e.g., Mckenna and Bell, 1997), Ondatrini (Kretzoi, 1955; Corbet, 1978) or Prometheomyini (Pavlinov, 2003). On the other hand, *Pliomys* has been considered as the ancestral clade of Clethrionomyini (e.g., Gromov and Polyakov, 1992; Martin, 2015). Our phylogenetic analyses clearly places both *Dinaromys* and *Pliomys* within the Pliomyini tribe [as observed for *D. bogdanovi* by Abramson et al. (2021) and Withnell and Scarpetta (2024)] as already suggested from a morphological point of view as Clethriomyini is dissimilar in its molar morphology [smaller size, continuous enamel wall, and presence of abundant cement in the reentrant angles (Fejfar et al., 2011)].

Pliomys was initially defined by Lajos Méhely at the beginning of the twentieth century in Hungary, at the Somlyó-hegy (Somlyó-berg) site. The materials had been found by the geologist Theodor Kormos who had initially classified them as *Clethrionomys*, another arvicoline with tooth-roots, but distinguished from it by being smaller and the abundant cement in the enamel folds. These differences encouraged Méhely to describe a new genus (Méhely, 1914). The first appearance datum (FAD) of the genus *Pliomys* is assigned to the species *P. simplicior* in the earlier Early Pleistocene. Nevertheless, some authors considered that the first representatives of *Pliomys* appeared during the Late Pliocene (e.g., Tesakov, 2005; Skandalos et al., 2023). However, these species are more commonly assigned to the genus *Propliomys* (Yamikova et al., 2022), previously described as a subgenus of *Dolomys*, and there is no clear correlation between these two lineages despite displaying morphological similarities. Following Fejfar et al. (2011), the genus *Pannonicola* could have given rise to *Dolomys* and *Propliomys* during the end of the Mammal Neogene zone (MN)14 [ca. 4.2 Ma], both considered by some authors as Ondatrini (Fejfar et al., 2011). On the other hand, Yamikova et al. (2022) refer to the *Propliomys* as the first pliomyine rodent, thus considering it as a Pliomyini, and suggest a species with a promimomyoid morphology as the possible ancestor of the *Propliomys* lineage [*Mimomys/Promimomys moldavicus* following Tesakov (2005)]. These authors also suggest that *Propliomys hungaricus* (MN 15) could potentially be an ancestor of the extant genus *Dinaromys*. Withnell and Scarpetta (2024) proposed that the Pliomyini tribe lineage diverged around 4.2 Ma (95% HPD = 3.15–5.17 Ma), a chronology that widely agrees with the divergence time of *Dinaromys-Pliomys* obtained in the study presented here (3.8 Ma, 95% HPD = 2.6–4.9 Ma approx.). Despite the low number of Pliomyini species with available molecular data, this age would place the rise of this tribe during the Pliocene, probably deriving from *Propliomys* taking in consideration all the morphological similarities between *Propliomys* and *Dinaromys*/*Pliomys* that had been discussed in the scientific literature (Figure 4). In this way, our study is not in disagreement with the most recent works about the possible evolution of the tribe Pliomyini and could be another important piece of information about how the Arvicolinae evolution takes place.

CONCLUSIONS

In the current study, we obtained for the first time genetic information for the fossil species *Pliomys lenki,* and specifically, from a right mandible obtained at the El Mirón Cave (Spain). The specimen that furnished an almost complete mitochondrial genome was excavated from a stratigraphic level with an estimated age of 46,890–33,160 years BP. This partial mitochondrial genome allowed us to confirm the close relationship of *Pliomys* with the current Balkan snow vole (*Dinaromys bogdanovi*) as previously suggested by paleontologists from a morphological perspective based on shared dental characteristics. In addition, we confirmed that *Pliomys* and *Dinaromys* should be considered as different valid genera, which diverged about 2.6–4.9 Ma., and both included in the Arvicolinae tribe Pliomyini. In addition, we show that the

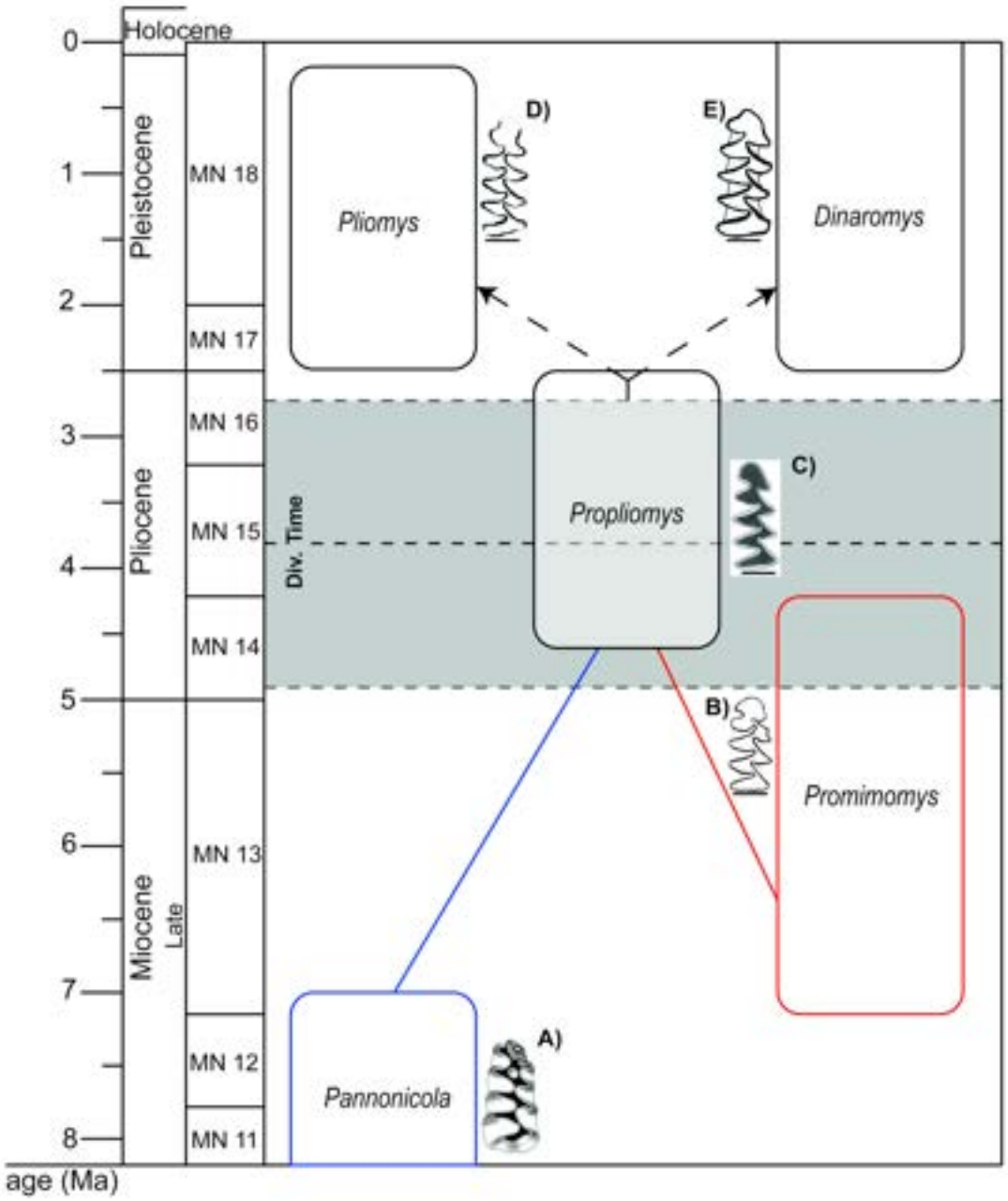

FIGURE 4. Suggested evolution of the Pliomyini tribe, following the works of Fejfar et al. (2011) (blue) and Yamikova et al. (2022) (red). The divergence time between *Dinaromys* and *Pliomys* is located at 3.8 Ma (95% HPD = 2.6–4.9 Ma approx.) during the Pliocene. *Pannonicola brevidens* (A) modified from Fejfar et al. (2011), *Promimomys moldavicus* (B) modified from Rădulescu and Samson (1996), *Propliomys hungaricus* (C) modified from Storch and Fejfar (1990), *Pliomys lenki* (D) modified from Cuenca-Bescós et al. (2010b) and *Dinaromys bogdanovi* (E) modified from Bona et al. (2006).

complex and somewhat controversial taxonomy of Arvicolinae could benefit from the genetic analysis of extinct taxa as well as ancient samples from extant species, even when small and >30 kyr old samples are considered.

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APPENDICES

APPENDIX 1.

Number of mapped reads and comparative statistics of the BWA multireference iterative mapping using stringent (STR) and relaxed (REL) parameters. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 2.

GenBank accession numbers of the Arvicolinae (and outgroups) complete and partial mitochondrial genomes used in the phylogenetic analyses (Figure 3, Appendix 9, 10). Numbers in italics denote partial genomes, whereas numbers in red indicate sequences used in the BEAST analysis (just one sequence per species has been used). (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 3.

Best partition schemes and substitution models for RAxML, MrBayes and BEAST analyses inferred using ModelFinder for both datasets [i.e., using consensus *Pliomys lenki* sequences generated using stringent (STR) and relaxed (REL) BWA mapping parameters]. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 4.

GenBank accession numbers of *Dinaromys bogdanovi* (and outgroups) *CYTB* sequences used to analyze the possible placement of *Pliomys lenki* within the diversity of *D. bogdanovi* (see Appendix 11). (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 5.

First five hits of the BLASTn analyses of the contig fragments >50 bp recovered in the *Pliomys* sequence generated using BWA relaxed parameters (REL) that were not recovered using the stringent parameters (STR). Position of the fragments within the mitogenome, length of the sequences, sequences, and hits statistics are indicated. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 6.

MapDamage report of the BWA iterative mapping using the usual DNA stringent (STR) and relaxed (REL) mapping parameters to generate the consensus sequences for *Pliomys lenki* sample ZARADNA 219. The top four panels show the frequency of purines and pyrimidines just before and after the reads, respectively. The middle two panels show accumulation of 5' C-to-T (red) and 3' G-to-A (blue) misincorporations. Bottom panel displays the read size distribution of mapped reads. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 7.

Partial mitochondrial genome sequence (in fasta format) of *Pliomys lenki* ZARADNA 279 obtained using stringent BWA mapping parameters (STR sequence). (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 8.

Genome characteristics, missing data and PCGs stop codons of the two mitochondrial genomes generated for *Pliomys lenki* using stringent (STR) and relaxed (REL) parameters in the BWA iterative mapping. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 9.

Maximum Likelihood (ML) trees of Arvicolinae mitochondrial genomes (excluding Control Region) and using the *Pliomys lenki* consensus sequences generated with regular ancient DNA mapping stringent parameters (STR sequence, left) and with relaxed parameters (REL sequence, right). Numbers in nodes indicate Maximum Likelihood Bootstrap (MLB) values. Circles indicate MLB=100. GenBank accession numbers are listed in Appendix 2 and partitioning scheme in Appendix 3. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 10.

Bayesian trees of Arvicolinae mitochondrial genomes (excluding Control Region) and using the *Pliomys lenki* consensus sequences generated with regular ancient DNA mapping stringent parameters (STR sequence, left) and with relaxed parameters (REL sequence, right). Numbers in nodes indicate Posterior Probability (PP) values. Circles in nodes indicate PP=1. GenBank accession numbers are listed in Appendix 2 and partitioning scheme in Appendix 3. (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 11.

Maximum Likelihood (ML) trees of a dataset comprising a *CYTB* fragment (555 bp) for *Dinaromys bogdanovi* individuals and the two *Pliomys lenki* sequences generated using stringent (STR) and relaxed (REL) BWA mapping parameters to evaluate the possible inclusion of *Pliomys* in the *D. bogdanovi* variability. Numbers in nodes indicate Maximum Likelihood Bootstrap (MLB) values. Just MLB values in selected nodes are displayed. GenBank accession numbers are listed in Appendix 4. *D. bogdanovi* haplogroups follow Kryštufek et al. (2007). (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 12.

Comparison of Bayesian Factor (BF) values using estimated marginal likelihood (MLE) values and Path Sampling (PS)/Stepping Stone sampling (SS) approaches for the calibrated dataset. Yule and Birth-Death process competing models were analyzed, being the Yule process strongly supported (following value ranges of Kass and Raftery, 1995). (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)

APPENDIX 13.

Comparison of node age estimates and their 95% highest posterior densities (HPDs) between the different estimates obtained by Abramson et al. (2021), Whithnell and Scarpetta (2024) and in this paper (for both *Pliomys* sequences generated). (All appendices are available for download at https://palaeo-electronica.org/content/2024/5329-pliomys-lenki-ancient-dna)