



Biodiversity of the tundra vole, *Alexandromys oeconomus*: ancient mitogenomes and the identification of a novel European haplogroup

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LETHAIA



The palaeontological record for the tundra vole *Alexandromys oeconomus* indicates a reduction in geographical range and population size since the Last Pleistocene. Up to four different mitochondrial haplogroups are currently identified in modern and recent historical specimens: Central European (CE), Northern European (NE), Central Asian (CA) and Beringian (BE). Here, we generated ancient mitochondrial genomes from five Late Pleistocene palaeontological samples (mandibles) from El Mirón Cave (Cantabria, Spain), one of the last Iberian strongholds of *A. oeconomus*, to investigate the relationship to present-day populations. The analysis of the mitochondrial genome data obtained from the five palaeontological samples of *A. oeconomus* allowed the identification of a fifth novel haplogroup, currently extinct, that was present at least during the Late Pleistocene-Holocene of the Iberian Peninsula. A tentative time-calibrated phylogenetic analysis suggests that the novel haplogroup could have separated from the CE-NE haplogroups around 0.050–0.1790 Ma, probably before the separation of the CE-NE haplogroups (0.017–0.136 Ma). □ Ancient DNA, mitochondrial genome, population dynamics, Iberian Peninsula, haplogroups, biodiversity

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Being important Cenozoic biostratigraphical and palaeoclimatic markers, voles and lemmings are well studied in the palaeontological record. However, phylogenetic relationships and systematics of Arvicolinae (Rodentia, Mammalia) are complex in understanding how the speciation process took place. This is, in part, due to the rapid radiation rate of these mammals during the Quaternary (Chaline *et al.* 1999), with a total of three radiation events identified in the last 7.67 (7.07–8.53) Ma BP (calculated by means of RNA-seq by Abramson *et al.* 2025). In addition, the classification of the vole species in the fossil record is based almost exclusively on the morphology of the first lower molars or m1 (Chaline 1972; van der Meulen 1973; Fejfar 2011). Therefore, relationships within genera and species included in this subfamily have been under constant debate. One of these cases is observed with *Microtus sensu lato* (*s.l.*). Most of the Arvicolinae species corresponding to the third

radiation of this subfamily have been identified as belonging to the genus *Microtus* (Jaarola *et al.* 2004). Many of them have been separated into different genera as new methodologies provided a new taxonomic framework (e.g. Abramson *et al.* 2021). This is the case for some species, such as the snow vole (*Chionomys nivalis*) (Chaline & Graft 1988) and most recently the tundra vole (*Alexandromys oeconomus*) (Abramson & Lissovsky 2012).

The genus *Alexandromys* is included within the Asian lineages of voles, which diverged within the Arvicolini tribe during the late Pliocene-early Pleistocene transition (Abramson *et al.* 2021). This Arvicolinae genus presents great morphological variability in the first lower molar (m1), the most abundant and easily identifiable anatomical element, and therefore most used in the identification of fossils and subfossil remains of the subfamily. For this reason, most previous systematic studies on the genus

Alexandromys have focused on the morphological differences of the *A. oeconomus* m1 from the other species of the genus (Lissovsky et al. 2017). *A. oeconomus* and *A. limnophilus* present a more primitive morphology of the m1, with a four-triangle structure, with six closed dentine fields on the occlusal surface of the m1. The remaining *Alexandromys* species have a five-triangle structure, with seven closed dentin fields, and only occasionally display the ‘*oeconomus*’ morphotype (Gromov & Polyakov, 1992; Bannikova et al. 2010; Lissovsky et al. 2017) (Fig. 1).

As in the case of many small mammalian species, *A. oeconomus* was initially classified in a completely different genus, *Mus*, by Pallas (1776). However, Galewski et al. (2006) classified the species as *Microtus oeconomus* based on genetic evidence. Abramson & Lissovsky (2012) attributed this species to the genus *Alexandromys*, being the most accepted classification by the scientific community today (e.g. Abramson et al. 2021; Withnell & Scarpetta 2024). Based on mitochondrial cytochrome B (*CYTB*) sequences, Bannikova et al. (2010) included *A. oeconomus* within the subgenus *Pallasiinus*. These authors separated *Alexandromys sensu stricto* from the *Pallasiinus* subgenus, indicating a split between *Pallasiinus* and *Alexandromys* around 1.2 Ma, that

encompasses the species *A. oeconomus*, *A. montebelli* and *A. kikuchii*.

Identifying the first appearance of *A. oeconomus* is complicated because of the morphological similarities between this species and its putative ancestors, *Microtus protoeconomus*, and the *M. oeconomus-ratticepoides* group. Maul & Markova (2007) separated *M. protoeconomus* and *A. oeconomus* species on the basis of the a/L index [a = length of the anterior part of the molar, L = total length of the molar, according to van der Meulen 1973)], considering *A. oeconomus* those individuals with values $a/L \geq 50$.

The first records of *A. oeconomus* in Central Europe were observed in early middle Pleistocene levels, on the palaeontological sites of the Stránská skála in Brno, Czech Republic (~600,000 years or ~MIS 17–16) (Kučera et al. 2009; Bartík et al. 2019; Szymanek & Julien 2018). The palaeontological record of Gran Dolina (Atapuerca, Burgos, Spain) includes a species with a similar m1 morphology, *Microtus oeconomus-ratticepoides*, an important biostratigraphical marker in the Atapuerca complex defining the Faunal Unit 5b (= *M. ratticepoides* Biozone in the early middle Pleistocene layers of Gran Dolina site, Cuenca-Bescós et al. 2016). For some authors, the first appearance of the true *A. oeconomus* in Central Europe correspond with the Saalian Glaciations

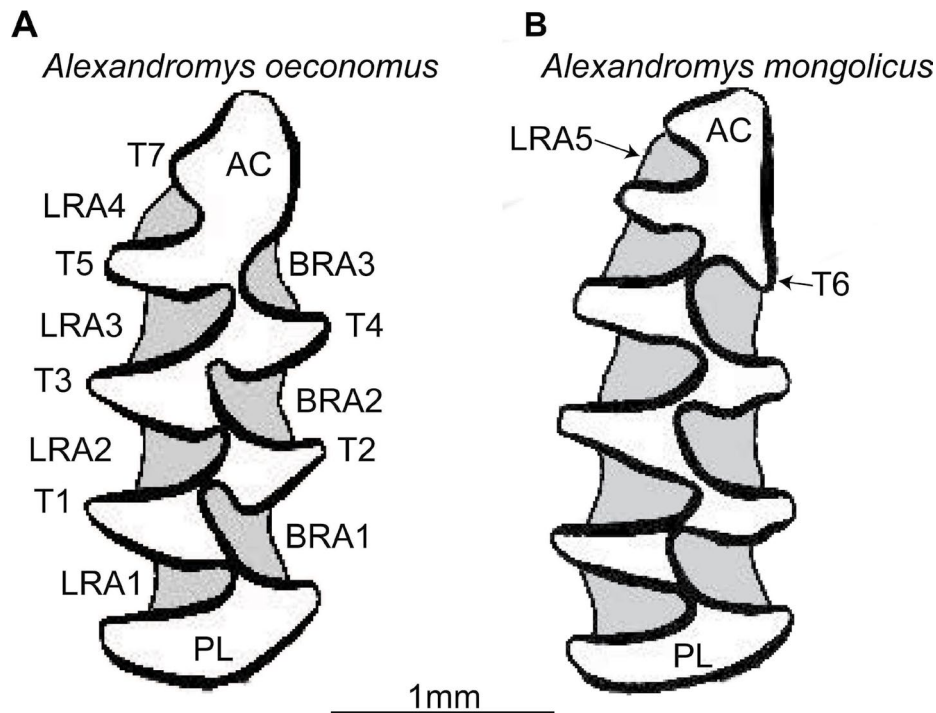


Fig. 1. Images of the morphology of the occlusal surface of the species *Alexandromys oeconomus* (A) and *Alexandromys mongolicus* (B). Triangles: T; Buccal Re-entrant Angles: BRA; Lingual Re-entrant Angles: LRA; Posterior Lobule: PL, Anterior Cusp: AC. Scale 1 mm. Modified from Kryštufek & Shenbrot (2022).

(MIS 8 – MIS 6) in Solymár (Hungary), and in eastern Europe to the Cromerian (MIS 20 to MIS 17) (Maul & Markova 2007; Krokmal *et al.* 2023).

The species *A. oeconomus* is present today in northern regions of North and Central Europe, North America, and Asia. This is the only *Alexandromys* species with a widespread distribution (i.e. observed in Western Europe, east to the Alaska Peninsula and adjacent regions of North America, Lissovsky *et al.* 2017). It is assumed that the colonization of the American continent by *Alexandromys* was via the Bering Land Bridge around 55,000 years ago (Rausch 1963; MacPherson 1965; Zakrzewski 1985). During the Pleistocene, the distribution of *A. oeconomus* reached the south of Europe, being present in various palaeontological sites of the Iberian Peninsula (e.g. Cuenca-Bescós *et al.* 2009; Bañuls-Cardona *et al.* 2014; Laplana *et al.* 2016; Cuenca-Bescós & Morcillo-Amo 2022; Alfaro-Ibáñez *et al.* 2023). At present, *A. oeconomus* is globally classified as a ‘least concern’ species, however, many of its isolated haplogroups are classified as ‘critically endangered’ or ‘vulnerable’ (Linzey *et al.* 2016). Even so, in some countries, as in the case of Germany, *A. oeconomus* is considered an endangered species (Meinig *et al.* 2020). Brunhoff *et al.* (2003) identified a total of four different haplogroups of *A. oeconomus* using complete sequences of the mitochondrial gene *CYTB*: Central European (CE) (central Sweden, southern Norway, the Netherlands, Poland, Hungary, Slovakia and Lithuania); Northern European (NE) (northern Fennoscandia – Sweden, Norway and Finland, Russia west of the Ural Mountains and Belarus), Central Asian (CA) (Russia between the Ural Mountains and the 151° E longitude); and Beringian (BE) (easternmost Russia, northwest Canada and Alaska).

In this study, we generated the first Late Pleistocene mitochondrial genomes for *A. oeconomus* from El Mirón Cave (Cantabria, Spain). With this data, we investigated the phylogenetic relationships of our ancient *A. oeconomus* to the present-day populations, to determine if these ancient Iberian mitogenomes form a novel haplogroup.

Material and methods

Study site and species

Five left mandibles samples with laboratory numbers 132, 281, 282, 284 and 286 identified as *A. oeconomus* (Table 1) from the palaeo-archaeological site of El Mirón Cave (Cantabria, Spain) (Fig. 2 B-P) were used for the palaeogenomic analyses. All samples were

broken in the posterior part of the mandible, but sample 132 had this part better preserved. All the samples presented the first (m1) and second (m2) lower molars, except in the case of sample 281 which only preserved the m1. Samples 132, 284 and 286 also preserved the incisor (Fig. 2).

The selected individuals were classified according to m1 morphology, where *Alexandromys oeconomus* displays a total of four closed triangles (T), with five well developed dentine fields, with a non-existent or poorly developed T6 and their corresponding buccal re-entrant angles (BRA5) (Chaline 1972; Markova 2013) (Fig. 1). This characteristic differentiated this species from other Arvicolinae observed in El Mirón Cave (Cuenca-Bescós *et al.* 2008). Furthermore, the *A. oeconomus* individuals from El Mirón Cave display the morphotype ‘oeconomus’, the most common morphotype observed in the extant and in the Late Pleistocene individuals of this species (Lemanik *et al.* 2022), in which T5 converges with T7 and T5 is communicated with the anterior cusp (AC) (Fig. 1).

The generally good preservation of the fossil remains from this site already allowed to obtain ancient DNA (aDNA) data from other Arvicolinae species (e.g. Baca, *et al.* 2022, 2023; Alfaro-Ibáñez *et al.* 2023, 2024). Each mandible of *A. oeconomus* used here was excavated from a different stratigraphical level of El Mirón Cave, ranging in age from 46,890–33,160 to 17,810–13,930 cal BP (Table 1) (Hopkins *et al.* 2021). However, *A. oeconomus* has been recorded in the whole stratigraphical sequence from El Mirón Cave, being present until level 3 (4250–3720 cal BP) (Hopkins *et al.* 2021; Alfaro-Ibáñez, *et al.* 2023). This allowed us to analyse the morphology and genetic evolution of the specimens of *A. oeconomus* of El Mirón Cave before, during and after the LGM.

DNA extraction, library construction and sequencing

All the laboratory tasks were performed in the dedicated laboratory of Palaeogenomics of the University Institute for Research in Environmental Sciences of Aragon (IUCA) of the University of Zaragoza (Spain).

All mandibles were UV-irradiated for 15 minutes on each side, and the surface of the mandibles was wiped with bleach 0.5%, and then subsequently submerged in water for 10 minutes and in 80% ethanol for 10 min. After air drying, each mandible was UV-irradiated again for 15 minutes on each side and placed in 2 ml screw-cap tubes, being finally mechanically pulverized using sterile tweezers inside the screw-cap tubes. All the mandibles were fully used in this step. A total amount of 115, 23, 42, 77 and 101 mg,

Table 1. Age information of the analysed mandibles of *Alexandromys oeconomus*. MIS: Marine Isotopic Stages.

Lab ID	Stratigraphic Level	Age (cal BP) (Hopkins <i>et al.</i> 2021)	MIS
281	12	17810–13930	2
282	122	22270–21660	2
286	126	23580–22510	2
132	128	39010–24800	3
284	129	46890–33160	3

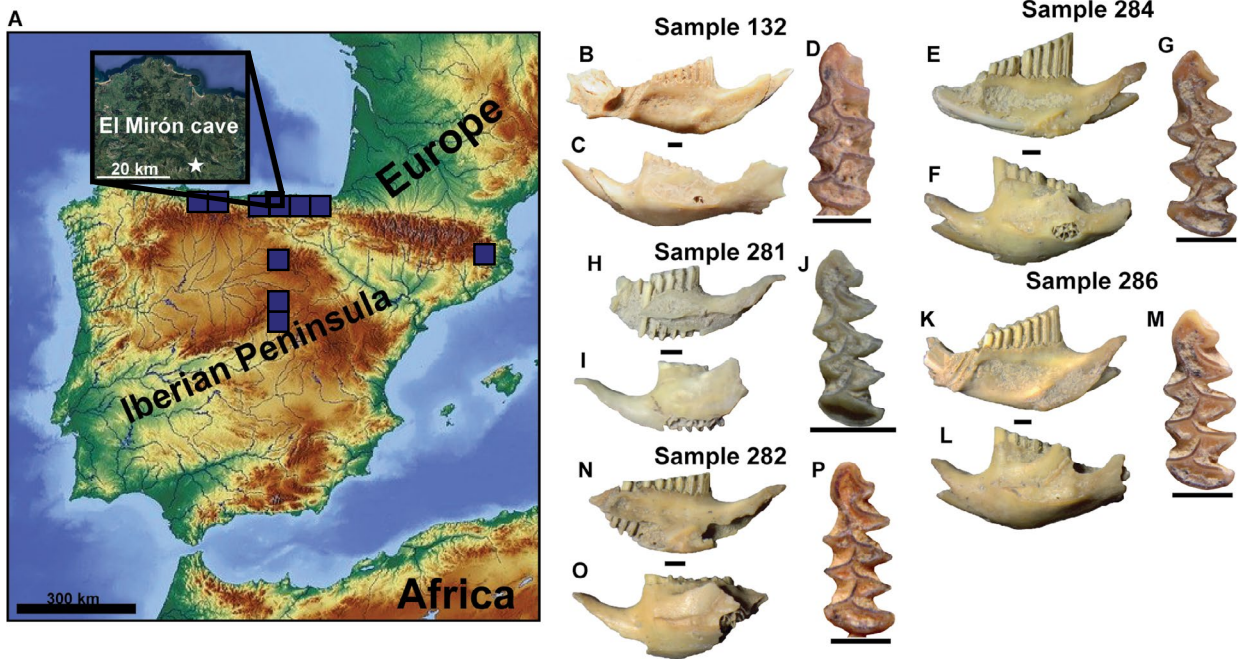


Fig. 2. A, geographical location of the palaeontological site of El Mirón Cave in the Iberian Peninsula (Spain), and the distribution of *A. oeconomus* in the Iberian Peninsula during the MIS 2 according to Sevilla *et al.* (2011). B–P, fossil samples used in this work depicting both sides of the mandible and m1 of each sample. Sample 132: mandible in lingual view (B) and buccal view (C); occlusal surface of the m1 (D). Sample 284: mandible in lingual view (E) and buccal view (F); occlusal surface of the m1 (G). Sample 281: mandible in lingual view (H) and buccal view (I); occlusal surface of the m1 (J). Sample 286: mandible in lingual view (K) and buccal view (L); occlusal surface of the m1 (M). Sample 282: mandible in lingual view (N) and buccal view (O); occlusal surface of the m1 (P). Scale 1 mm.

for the complete mandibles 132, 281, 282, 284 and 286 were used for DNA extraction, respectively.

DNA from the five mandibles was extracted (extracts A) following the silica suspended protocol of Brotherton *et al.* (2013) with the binding buffer modification of Bover *et al.* (2019) based on Dabney *et al.* (2013) protocol. After a predigestion step using 1 ml EDTA on a rotary wheel for one hour at room temperature, the bone powder was digested overnight using ~1 mL of a digestion buffer (900 μ L EDTA 0.5M pH8.0 and 20 μ L of 20 mg/mL Proteinase K) on a rotary wheel at 55°C. The DNA binding step was performed in 13 mL of a modified binding buffer (13.6 mL LPB buffer [Qiagen], 420 μ L Sodium Acetate 3M and 7 μ L Tween-20) and 100 μ L of suspended silica particles. The DNA was finally eluted in 100 μ L of TLE buffer after three

washes (1 \times 900 μ L of remaining binding buffer and 2 \times 900 μ L 80% ethanol). Negative controls were extracted along the samples (Extraction Blank Controls, EBC).

Double-stranded libraries were constructed following Meyer & Kircher (2010), with modifications of Llamas *et al.* (2016) and using a partial uracil-DNA glycosylase treatment (Rohland *et al.* 2015), except for sample 132, which was not treated/repaired. The purified and quantified libraries were sequenced in an Illumina HiSeqX platform (Paired End, 2 \times 150 bp).

Mitochondrial genome assembly and phylogenetic analyses

Mitochondrial genome assembly.— Sequencing read quality was initially analysed using fastQC v0.11.2

(<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Sabre v.1.0 (<http://github.com/najoshi/sabre>) was used to demultiplex the reads from each sample by filtering both 7-bp P5 and P7 internal barcodes, allowing only one mismatch. AdapterRemoval v.2.3.0 (Schubert *et al.* 2016) was used to trim the adapter sequences using the following parameters: 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 discarded.

The collapsed reads for each of the five samples were iteratively mapped to the mitochondrial genome of *Alexandromys oeconomus* (GenBank Accession Number ON165345) using BWA v.0.7.17 backtrack algorithm (Li & Durbin 2009) and the parameters widely accepted for ancient data mapping (i.e. $aln -n 0.01, -o 2, -l 1024$). Reads with a minimum mapping quality of 25 ($-q 25$) were removed using SAMtools v.1.11 (Li *et al.* 2009) and duplicate reads were filtered using FilterUniqueSAMCons.py (Kircher, 2012).

After each round of mapping, an intermediate 75% majority consensus sequence was generated for each sample using Geneious Prime v.2022.0.2 (Biomatters, <http://www.geneious.com>, Kearsse *et al.* 2012), calling the reference nucleotides for sites with read-depth $< 3\times$. This new consensus was then used as the new reference and the process was iterated until no additional reads were mapped (see Table 2). A final 75% majority consensus sequence for the iterative mapping of each sample was then generated in Geneious, calling nucleotides only at sites with read-depth $\geq 3\times$ for each sample, and calling Ns otherwise. Number of iterations and unique reads mapped are listed in Table 2. Ancient DNA misincorporation and fragmentation patterns were assessed using mapDamage v.2.1.1 (Jónsson *et al.* 2013).

Phylogenetic analyses.— The mitochondrial genome sequences of *A. oeconomus* from El Mirón Cave were annotated using the mitochondrial genome of an extant individual of *A. oeconomus* as a template and adjusting changes in Protein Coding Genes (PCGs) using Geneious.

The five partial mitochondrial genome sequences of *A. oeconomus* obtained here were aligned with 39 arvicoline mitochondrial genomes from 26 species available at GenBank (see Supplementary Table 1). All available *Alexandromys* mitochondrial sequences (19 sequences from 6 species) were selected as well as one individual per species from genera *Iberomys* ($n = 1$), *Microtus* ($n = 8$),

Mynomes ($n = 8$) and *Terricola* ($n = 3$) [in this work we followed the genera and subfamily nomenclature proposed by Abramson *et al.* (2021)], and the Cricetinae *Cricetulus kamensis* and *C. longicaudatus* were used as outgroups. The 46 mitochondrial genomes were aligned using MUSCLE implemented in Geneious, and the alignment was partitioned in Protein Coding Genes (PCGs), *tRNAs*, *12S_rRNA* and *16S_rRNA*, excluding the Control Region (CR) for further analyses. PCGs were divided by codon positions using DAMBE v.7.0.5 (Xia, 2017) and ambiguously aligned regions of *tRNAs* and *rRNAs* were removed using default parameters in Gblocks v.0.91.1 (Castresana 2000) available at Phylogeny.fr (Dereeper *et al.* 2008), retaining 895 out of 967 (92%) positions for *12S_rRNA*, 1439 out of 1599 (89%) for *16S_rRNA* and 1436 out of 1528 (93%) positions for *tRNAs*. The final length of the alignment was 15,089 bp. Partitioning scheme and nucleotide substitution models were inferred using the implemented ModelFinder (Kalyaanamoorthy *et al.* 2017) in IQTREE2 v.2.2.0 (Minh *et al.* 2020) (see Supplementary Table 2). A partitioned Maximum Likelihood analysis was performed using IQTREE2, with node support values estimated by performing 1000 ultrafast bootstrap replicates.

Two additional unpartitioned datasets were constructed using the Cytochrome B (*CYTB*) gene sequences. First, to further evaluate the taxonomic identity of the ancient samples using as many *Alexandromys* species as possible, all ($n = 808$) *CYTB* sequences from *Alexandromys* species available in GenBank as in July 2024 (see Supplementary Table 3) were aligned to the five newly generated sequences of ancient *A. oeconomus* and four outgroups (*Clethrionomys glareolus*, *Microtus agrestis*, *M. arvalis* and *M. schelkovnikovi*), with a total length of the alignment of 1140 bp. Second, a dataset just including the *CYTB* sequences for 478 *A. oeconomus* individuals (473 modern + 5 ancient, see Supplementary Table 3) was also analysed to identify the possible haplogroup of the ancient individuals among the *A. oeconomus* haplogroup classification by Brunhoff *et al.* (2003). The phylogenetic trees for both datasets were inferred using IQTREE2 and node support estimated by using 1000 ultrafast bootstrap replicates. The phylogenetic tree obtained using the dataset of the *A. oeconomus* *CYTB* was used to generate a haplogroup network using Fitchi v1.1.4 (Matschiner, 2016).

This second dataset using the *A. oeconomus* *CYTB* sequences was used to perform a tentative calibrated analysis under relaxed lognormal molecular clock

Table 2. Statistics of the AdapterRemoval adapter trimming (raw versus collapsed reads) and BWA iterative mapping of each El Mirón Cave individual to the reference *Alexandromys oeconomicus* ON165345. Numbers in red indicate last round of mapping (i.e., no more reads were mapped)

	132A	281A	282A	284A	286A
Total raw reads	54118538	20507933	18271650	18433785	21235679
Total collapsed reads	42665560	11069968	14386417	13031639	14090964
% collapsed	78.8	54.0	78.7	70.7	66.4
ITERATIVE MAPPING STATS					
Unique reads mapped on each iteration					
Iteration	132A	281A	282A	284A	286A
1	6282	1298	1551	915	2726
2	7326	1393	1687	960	2962
3	7376	1398	1708	967	2971
4	7385	1400	1717	968	2974
5	7385	1400	1718	969	2974
6			1718	969	
Ref-Seq coverage (%)	99.8	98.5	99.8	94.7	99.9
Coverage depth (X)	25.3	5.9	7.9	3.3	10.9
Std. Dev. coverage (X)	10.1	2.7	3.0	1.9	4.0
Mean read length (bp)	55.9	66.4	71.1	54.2	55.1
5' Damage (C→T, %)	37.7	6.7	8.4	9.3	12.9

using BEAST v.2.7.7 (Bouckaert *et al.* 2014) and using a substitution/site/year of $3.2E-7$ estimated for the *CYTB* of Arvicolinae species following Herman & Searle (2011) and Martinková *et al.* (2013).. The correct model test was inferred using the bModel test package implemented in BEAST and using Coalescent Constant Population tree prior. Tip dates were calibrated using the chronology ranges of the stratigraphical layer for each of the samples (Table 1) and using a uniform distribution. We ran two analysis for 10^9 generations sampling every 10^4 generations, discarding the first 10% of samples as burn-in. Convergence was assessed using Tracer v.1.7.2. Individual run outputs were combined using LogCombiner v.2.7.7 and a final maximum clade credibility tree was generated using TreeAnnotator v.2.7.7 implemented in BEAST.

Results

Alexandromys oeconomicus mitochondrial genome assembly

We generated five mitochondrial sequences of Late Pleistocene *A. oeconomicus*. The final mitochondrial sequences displayed a total of 108 unknown (N)

positions (0.7% of the estimated length of the mitochondrial genome) for extract 132A, 1687 (10.3%) for extract 281A, 386 (2.4%) for extract 282A, 5761 (35.3%) for extract 284A and 199 (1.2%) for extract 286A (Supplementary Table 4). The average coverage depth of the obtained mitochondrial genomes ranged between 25.3X (sample 132A) and 3.3X (sample 284A) (Table 2), whereas the mean read length (bp) of the mapped reads ranged between 71.1 bp (282A) and 54.2 bp (284A) (Table 2).

The ancient DNA misincorporation and fragmentation patterns (Supplementary Figure 1) displayed the expected damage pattern observed in fossil samples for sample 132 (unrepaired library), and in partial uracil-DNA glycosylase treated libraries for the rest of the samples (Rohland *et al.* 2015).

Phylogenetic analyses

In the mitochondrial genome phylogenetic tree, the new five *A. oeconomicus* sequences form a fully supported clade (Maximum Likelihood Bootstrap, MLB = 100) with the three mitochondrial sequences of *A. oeconomicus* available in GenBank (Fig. 3A), confirming the attribution of the El Mirón Cave samples to *A. oeconomicus*. The sister clade (MLB = 100) of *A. oeconomicus* is the fully supported clade formed

by *A. limnophilus*, *A. maximowiczii* and *A. fortis* as already suggested in phylogenies using mitochondrial genomes and large nuclear genes datasets (e.g. Wang *et al.* 2022).

In the case of the phylogeny using *Alexandromys* *CYTB* sequences (Fig. 3B) *A. oeconomus* forms a supported clade with *A. kikuchii* (MLB = 96) with *A. montebelli* as sister clade (MLB = 95), whose position in the mitochondrial genome tree is not fully solved. The topology of this tree already shows the four *A. oeconomus* haplogroups identified by Brunhoff *et al.* (2003). Although it is beyond the scope of this paper, the results obtained using the *CYTB* sequences highlight the complex taxonomy of some of the *Alexandromys* species such as *A. maximowiczii* and *A. mongolicus* not being monophyletic taxa. This observation might be due to the misclassification of some individuals or could be related to recent taxonomic reviews. This is the case of the differentiation of *A. mongolicus* already

observed by Bannikova *et al.* (2019), that separated this species into two different lineages, *A. mongolicus* and *A. alpinus*. On another issue, up to three sequences of individuals in the GenBank database are placed in different species in our analysis. The individual AM910620, initially classified as *Alexandromys limnophilus* is in fact an *A. oeconomus*, as well as other two individuals classified as *A. mongolicus* (MF099566 and MF099567).

The *CYTB* tree also shows that our *A. oeconomus* sequences constituted a fully supported (MLB = 100) different haplogroup within the species (Fig. 3B), with a closer relationship (MLB = 100) with the European haplogroups (Northern and Central as sister taxa to each other with MLB = 97). This fully supported relationship of the - now three - European haplogroups is also confirmed by the specific *CYTB* phylogeny of *A. oeconomus* (Fig. 4 and Supplementary Figure 2), but with low support in the case of the calibrated tree

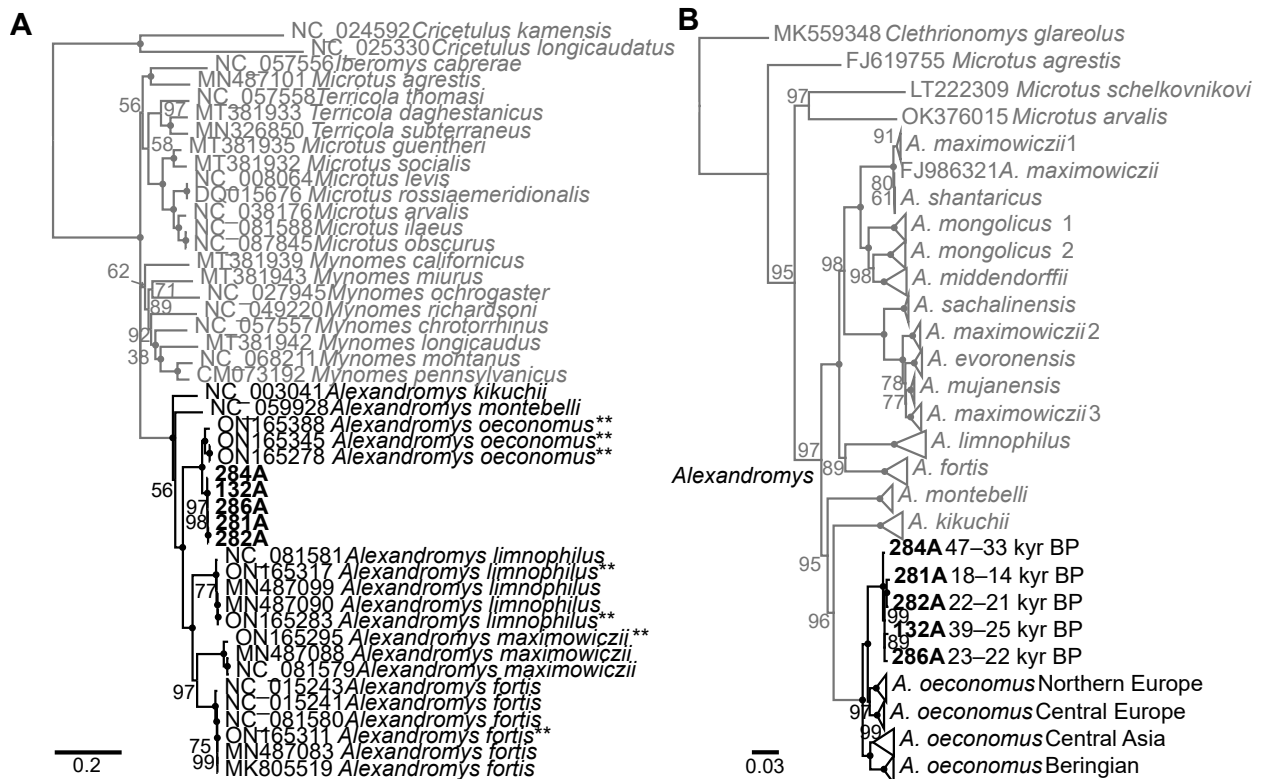


Fig. 3. Maximum Likelihood (ML) phylogenetic trees using IQTREE2. A, the mitochondrial genome sequence of 26 species of Arvicolinae, and the five partial mitochondrial genome sequences obtained from the fossil samples of El Mirón Cave (length of the alignment 15,089 bp). B, the available *CYTB* sequences of *Alexandromys* species available in GenBank and including the *CYTB* sequences for the *A. oeconomus* from El Mirón Cave (length of the alignment 1140 bp), in which monophyletic nodes have been collapsed by species, except for modern *A. oeconomus*, which has been collapsed by population. The new ancient *A. oeconomus* sequences are indicated in both trees with laboratory numbers 132, 281, 282, 282, 284 and 286. Numbers in nodes indicate Maximum Likelihood Bootstrap (MLB) values, whereas circles indicate MLB = 100. GenBank accession numbers are listed in Table S1 for the mitochondrial genome dataset and in Table S4 for the *CYTB* dataset. Partitioning scheme for the mitochondrial genome phylogenetic tree can be observed in Table S2. ** = sequences labelled as 'Unverified' in GenBank.

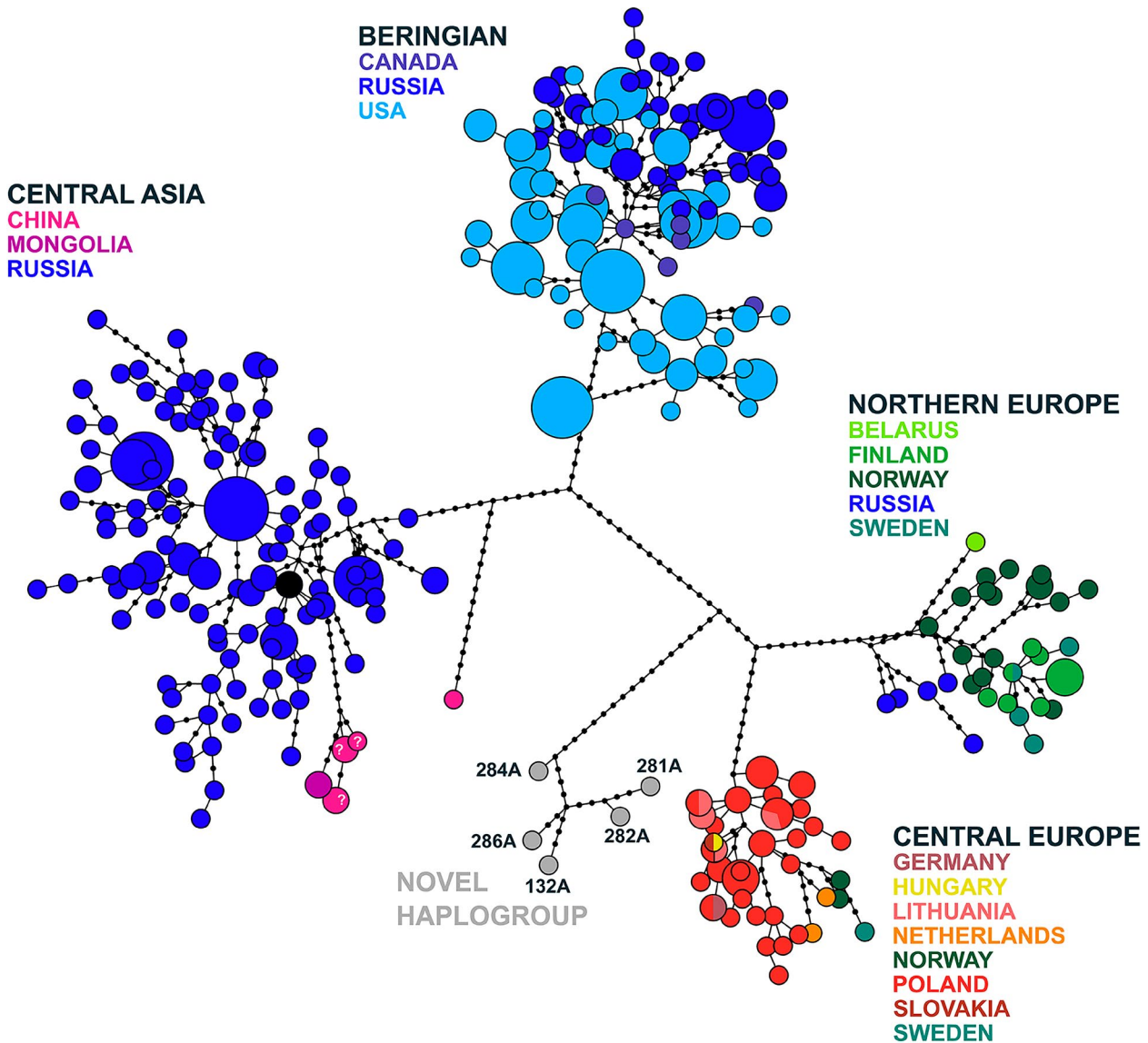


Fig. 4. Haplogroup network using the *CYTB* sequences of available *Alexandromys oeconomicus* sequences available in GenBank (see Supplementary Table 3, *A. oeconomicus* individuals) and the *CYTB* sequences of the ancient individuals from El Mirón Cave (indicated in grey circles and laboratory numbers 132, 281, 282, 284 and 286). ? = uncertain origin of individuals. Haplogroups of modern individuals follow Brunhoff *et al.* (2003).

[Posterior Probability (PP) = 0.71], and the support value for the Central and Northern European sister relationships is lower (PP = 0.82; MLB = 90).

We are aware that using short sequences (such as *CYTB*) to reconstruct calibrated phylogenies infers very wide ranges of Highest Posterior Density (HPD) values. In addition, the support (PP) of the nodes indicating the relationship between European haplogroups is < 0.95. For these reasons drawing conclusions from this data must be taken with caution. Tentatively, the chronology for the node separating the newly identified extinct haplogroup from

the extant European haplogroups can be inferred in around 111,300 years (95% HPD ~ 0.050–0.190 Ma) (Fig. 5), which corresponds to a time frame that encompasses from MIS 7 (Middle-Upper Pleistocene) to MIS 3 (Late Pleistocene). In the case of the split CE-NE, our data situates this split later, at 0.017–0.136 Ma, and after the differentiation of our samples from the CE-NE haplogroups. Within the extinct Iberian haplogroup the oldest chronological sample (i.e. 284, 46,890–33,160 cal BP, Table 1) is placed basal to the other more modern palaeontological samples in the phylogeny.

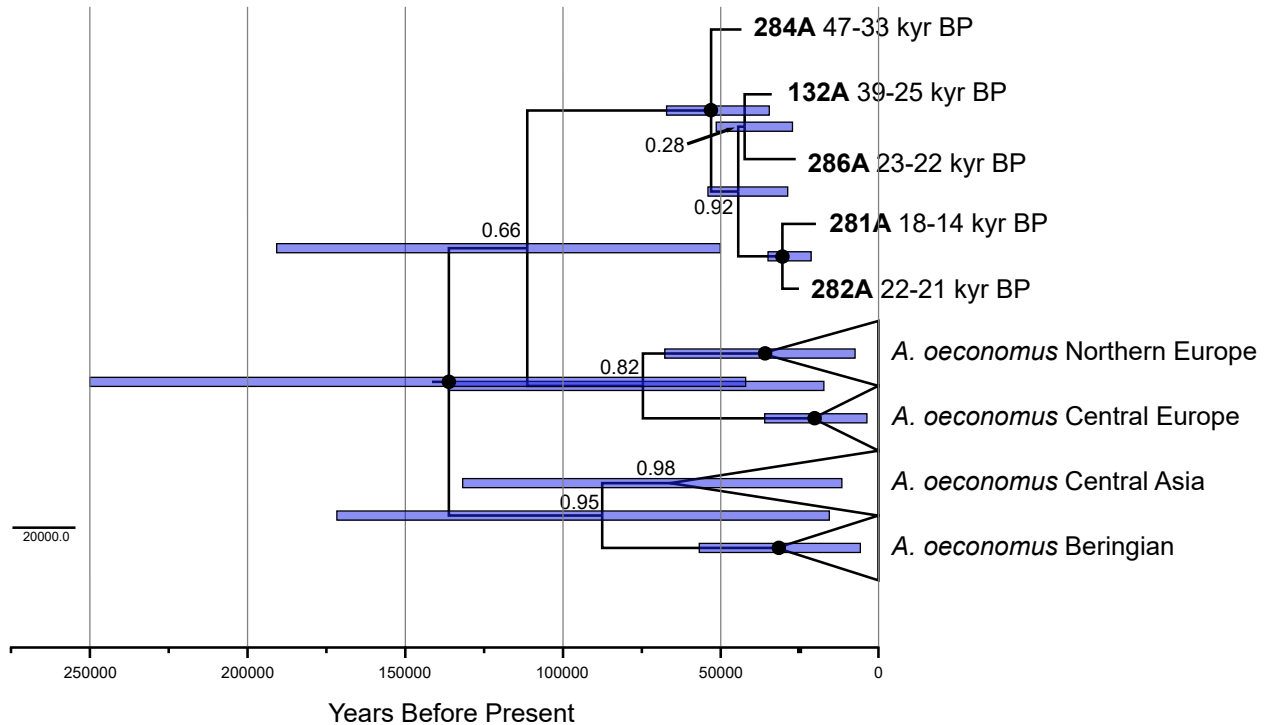


Fig. 5. Calibrated Bayesian tree using all the *Alexandromys oeconomus* CYTB sequences (length of the alignment 1140 bp) available at GenBank, and the five CYTB sequences obtained from the fossil samples from El Mirón Cave. Number in nodes indicate the Posterior Probability (PP), and circles indicate total support of the node (PP = 1).

Discussion

Phylogeographical distribution of Alexandromys oeconomus

In our analysis we differentiate the four extant haplogroups of *A. oeconomus* described by Brunhoff *et al.* (2003), together with the new extinct haplogroup. As mentioned above, these authors identified two split moments for the haplogroups during the last two to five glaciations. The first one, corresponding to the NE and CA diverged around 0.29–0.49 Ma (95% CI = 0.19–0.66 Ma). The second one corresponds to CE and NE groups (0.20–0.33 Ma (95% CI = 0.13–0.46 Ma)) and the CA and BE groups [0.21–0.35 Ma (95% CI = 0.13–0.48 Ma)] divergence times. However, fossil record and mtDNA data shown that an earlier colonization of the Beringian region could have taken place during the Illinoian glacial period, 200–130 Ka (Jopling *et al.* 1981; Zakrzewski, 1985; Brunhoff *et al.* 2003). This chronological framework is included within the 95% HPD of the divergence time obtained in our calibrated phylogenetic tree, except in the case of the CE-NE split, where we obtain a younger divergence age (Fig. 5). These extant populations of *A. oeconomus* could have their origin in diverse refugia areas during the Last Glacial

Maximum (LGM). The Southern Mediterranean and Caucasus served as an LGM refugia for many species (e.g. Taberlet *et al.* 1998; Hewitt 1999, 2000) but not *A. oeconomus*. Based on modern, historical and fossil data, the CE group may have their refugia area near the Balkans and Carpathians (Brunhoff *et al.* 2003), meanwhile the refugia area of the NE groups may have been near the Black Sea or in the south Russian Plain (Brunhoff *et al.* 2003). The CA groups may have their refugia around the NW Altai, in the Chita region or closer to Vladivostok (Brunhoff *et al.* 2003). Finally, the BE refugia area may have been in Beringia (Hewitt, 2011). *A. oeconomus* populations further reached classical southern refugia, as the case of the Cantabrian region in Spain (e.g. Cuenca-Bescós *et al.* 2008, Alfaro-Ibáñez *et al.* 2023). However, this population is currently extinct and therefore their relationship with the extant populations remains uncertain. The final extinction of *A. oeconomus* in the Iberian Peninsula is commonly correlated with the increase of temperatures during the postLGM period, and their presence in this area is usually restricted to cooler phases and regions with high latitude and altitude (Sesé, 1994). This is in concordance with the current distribution habitat of this species, in areas with colder temperatures and usually found in areas with constant vegetation near the

edges of lakes, or even in tundra or taiga forests (IUCN Red List, 2025)

The group identified in El Mirón Cave belongs to one of the last populations of *A. oeconomus* observed in the Iberian Peninsula (Cuenca-Bescós *et al.* 2008). This study demonstrates that this population constitutes a novel Southern European haplogroup during the Late Pleistocene, highlighting the loss of biodiversity probably associated with the Pleistocene-Holocene boundary (Fig. 4). This novel haplogroup has been detected through MIS 2, but the presence of *A. oeconomus* in the Iberian Peninsula is observed since the interval MIS 7- MIS 6, in the level K of the Lezetxiki II Cave (TT-OSL dated 215.7 ± 15.1 ka) (García-Ibaibarriaga *et al.* 2018).

During the Pleistocene, including the postLGM, the distribution of *A. oeconomus* in Europe was far more extensive, expanding south and west in Europe during the LGM reaching the centre part of the Iberian Peninsula (Sevilla *et al.* 2009). However, the presence of *A. oeconomus* in Central Iberia has been observed since the MIS 4, as recorded in the Buena Pinta Cave (Madrid, Spain) (Laplana *et al.* 2016). During the postLGM the Tundra vole expanded further northward, as the ice melting took place. This southern expansion during the glacial periods would constitute sources for the subsequent northward migration during the postLGM, as the case of the western Sweden population (Brunhoff *et al.* 2003). However, the novel extinct population recorded in Northern Iberia apparently did not present this northern expansion during the postLGM. There are records of *A. oeconomus* in Italy and the southern part of France during the LGM, disappearing at the onset of the Holocene in France (Royer *et al.* 2016) and earlier in Italy during the Epigravettian (Bartolomei *et al.* 1988; Berto *et al.* 2018). However, this relationship between the French and Italian populations and the one observed in Northern Spain cannot be currently confirmed, as additional genomic data from other populations (from inside and outside Iberia) is not currently available.

The recolonization from refugial areas of novel haplogroups, that can lead to the current extant, or to extinct, populations was a fact already observed in different Arvicolinae rodents. Baca *et al.* (2020, 2022), observed that the *Microtus arvalis* extant populations had their origin in different refugial areas during the LGM as well as during previous glacial periods. These authors also observed the presence of different extinct haplogroups of *M. arvalis* in various areas of their current distribution, that were substituted by the current populations after the LGM. In this case, the novel haplogroup of *A. oeconomus* was not substituted by the NE or CE populations in the Iberian

Peninsula, probably due to the climatic characteristics present today in southern Europe, which are not the ideal for this species (Linzey *et al.* 2016). Our data suggest that novel haplogroup of *A. oeconomus* disappeared after the LGM, probably due to the warmer temperatures, as was the case of other cold adapted species such as the true lemmings (*Lemmus*), collared lemmings (*Dicrostonyx*) and *Stenocranius gregalis* in southern Europe (Lagerholm *et al.* 2014; Royer *et al.* 2016; Berto *et al.* 2022; Baca *et al.* 2023). Cold adapted species populations present today in Europe seem to have their refugial areas in northern Europe which allowed their expansion to areas with more favourable climatic conditions, with the corresponding extinction of the middle or more southern European populations, as the case of the *A. oeconomus* novel haplogroup, (e.g. Lagerholm *et al.* 2014; Lord *et al.* 2022). Contrary to that observation, other species more adapted to warmer temperatures experienced an expansion of their distribution after the LGM and other glacial periods (e.g. Baca *et al.* 2025).

Relationships between the extinct and extant haplogroups of A. oeconomus

The extinct population of *A. oeconomus* of El Mirón Cave has a closer relationship with the CE-NE haplogroups clade, but it can be clearly differentiated from genetic perspectives. The morphology of the molars of the fossil *Alexandromys* population of El Mirón Cave share the predominant morphology of the extant *A. oeconomus*, presenting the morphotype 'oeconomus' (Lemanik *et al.* 2022), which is also in concordance with the age of the palaeontological site (Late Pleistocene-Holocene) (Hopkins *et al.* 2021). This 'oeconomus' morphology is characteristic for presenting five closed dentine fields, with T5 and the anterior cusp well communicated, and an absent or a not well-developed T6. Only one m1 with the morphology 'nivalis', differentiated from the 'oeconomus' morphotype by the anterior cusp isolated from T5 (Lemanik *et al.* 2022), has been identified in El Mirón Cave. This 'nivalis' morphotype is also found in extant CE populations of *A. oeconomus*, being a rare morphology and less abundant than the 'oeconomus' one (Lemanik *et al.* 2022). Therefore, the morphotypes observed in El Mirón Cave are the same that we can observe in the extant European populations.

The divergence time obtained of the extinct population of El Mirón Cave from the NE and CE populations (0.050–0.190 Ma) would place the separation of this extinct group not much later of the split CE-NE groups [0.20–0.33 Ma (95% HPD = 0.13–0.46 Ma)] by Brunhoff *et al.* (2003), or around the same time or

a bit earlier (0.017–0.136 Ma) according to our data (Fig. 5). This relationship of our sample with the CE-NE clade can also be observed in the obtained Network (Fig. 4). The presence of *A. oeconomus* in Northern Iberia since 215.7 ± 15.1 ka (García-Ibaibarriaga *et al.* 2018) is closer to the estimated separation date of the CE and NE populations. It is possible that during the cold period at the end of MIS 7 - start of MIS 6, the *A. oeconomus* population that would have given rise to the haplogroup observed in El Mirón Cave and to the CE-NE haplogroups, expanded until reaching the northern part of the Iberian Peninsula. This population would correspond with the first representatives of *A. oeconomus* observed in Central Europe during the MIS 8 - MIS 6 according to Maul & Markova (2007). Later on, according to our results, during the MIS 6 - MIS 3 (0.050–0.190 Ma), a split event took place, differentiating the novel haplogroup identified in El Mirón Cave, from the NE-CE haplogroups.

Conclusions

The extinct populations of *A. oeconomus* of the Iberian Peninsula have a closer relationship with the European populations observed today, both by the morphological m1 characteristics and by genetic data. This novel haplogroup identified from the fossil record, represents a now-extinct southern European haplogroup. However, their relationship with the extinct populations of other south European countries it is still uncertain, as in the case of the Italian and French fossil populations. This new haplogroup could have separated from the CE-NE haplogroups around 0.050–0.190 Ma, probably before the separation of the CE-NE haplogroups (0.017–0.136 Ma). This fact indicates that in the past the diversity of this species was higher than observed in the present, and that a decrease in the biodiversity of this species has taken place in the recent past.

Acknowledgements. - Thanks are due to B. Bauluz (Universidad de Zaragoza, Spain, Departamento de Ciencias de la Tierra and Research Institute of Environmental Sciences, Spain) for their support of the Paleogenomics Laboratory. We acknowledge the Laboratory of Sequencing and Functional Genomics and the Laboratory of Proteomics of Servicios Científico-Técnicos of CIBA (IACS-Universidad de Zaragoza, Spain), and the directors of excavation (L.G. Straus, University of New Mexico, USA) and M. González Morales (Universidad de Cantabria, Spain) and the excavation team of the Universidad de Cantabria that carry out the excavations of El Mirón Cave. The research reported in this paper is included in the project PGC2018-093925-B-C33 of the Spanish Ministerio de Ciencia, Innovación y Universidades - Generación de Conocimiento and 'Aragosaurus: recursos geológicos y paleoambientes, REF: E18_23R' of the Gobierno de Aragón. MPAl is supported by a grant from the Ministerio de Universidades of Spain (FPU20/02031). The authors declare no competing interests.

Data Accessibility Statement. - Obtained mitochondrial genome sequences for *Alexandromys oeconomus* are available at GenBank under Accession Numbers PX230039 (132A), PX230040 (281A), PX230041 (282A), PX230042 (284A), PX230043 (285A). Alignment datasets used in each dataset for phylogenetic analysis are indicated in the Supplementary material. Reference material of *A. oeconomus* from El Mirón Cave can be found at the "Museo de Ciencias Naturales de la Universidad de Zaragoza", under the references numbers MPZ 2025/277 and MPZ 2025/278.

Supplementary information

Supplementary Table 1. GenBank accession numbers of the Arvicolinae and outgroups mitochondrial genomes used in the phylogenetic analyses (Fig. 3A).

Supplementary Table 2. Best partitioning scheme and substitution models for the Maximum Likelihood (ML) analysis (Fig. 3A) using mitochondrial genomes inferred by ModelFinder.

Supplementary Table 3. GenBank accession numbers of the *Alexandromys* and outgroups *CYTB* sequences used in the phylogenetic analyses (Figs 3B, 5 and S2) and haplogroup network (Fig. 4) analyses using this gene.

Supplementary Table 4. Details of the mitochondrial genomes for *Alexandromys oeconomus* obtained in this study, including length, number of degenerate nucleotides, GC content and percentage of the genome available.

Supplementary Figure 1. MapDamage report of the BWA iterative mapping for each of the samples analysed. Substitution panels can be observed on the top part, whereas misincorporations patterns (middle) display the expected accumulation of 5' C-to-T (red) and 3' G-to-A (blue) for unrepaired (sample 132) and partial-UDG treated (samples 281, 282, 284 and 286) libraries. On the bottom part, fragment size distribution of the mapped unique reads.

Supplementary Figure 2. Maximum Likelihood (ML) tree of *CYTB A. oeconomus* sequences used to infer the haplogroup network (Fig. 5). Numbers in nodes indicate Maximum Likelihood Bootstrap (MLB) values.

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