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## Original Article

# Revisiting the taxonomy and biogeography of the largest sigmodontine rodent (Rodentia, Cricetidae) through ancient DNA

Pere Bover[1,2,*], Jorge Brito[3], Francisco X. Castellanos[3,4], Erika Cuéllar Soto[5], Rainer Hutterer[6], María Pilar Alfaro-Ibáñez[7], María Esther Martín González[8], Mauro N. Tammone[9], Ulyses F. J. Pardiñas[3,10,*]

[1]ARAID Foundation, Zaragoza, 50018, Spain
[2]Instituto Universitario de Investigación en Ciencias Ambientales (IUCA) – Grupo Aragosaurus, Universidad de Zaragoza, Zaragoza, 50009, Spain
[3]Instituto Nacional de Biodiversidad (INABIO), Quito, 170506, Ecuador
[4]Department of Biological Sciences, Texas Tech University, Lubbock, TX, 79409, United States
[5]Department of Biology, College of Science, Sultan Qaboos University, Muscat, 123, Oman
[6]LIB Museum Alexander Koenig (ZFMK), Bonn, D-53113, Germany
[7]Aragosaurus-IUCA, Departamento Ciencias de la Tierra, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, 50009, Spain
[8]Museo de Ciencias Naturales, Santa Cruz de Tenerife, Tenerife, 38003, Spain
[9]Instituto de Investigaciones en Biodiversidad y Medioambiente (CONICET-UNComahue), Bariloche, Río Negro, 8400, Argentina
[10]Grupo de Estudio de Mamíferos Australes, Instituto de Diversidad y Evolución Austral (IDEAus-CONICET), Puerto Madryn, Chubut, 9120, Argentina

*Corresponding authors. Instituto Universitario de Investigación en Ciencias Ambientales (IUCA) – Grupo Aragosaurus, Universidad de Zaragoza, C/Pedro Cerbuna, 12, 50009 Zaragoza, Spain; Instituto de Diversidad y Evolución Austral (IDEAus-CONICET), Puerto Madryn, Chubut, 9120, Argentina. E-mail: pbover@unizar.es; ulyses@cenpat-conicet.gob.ar

## ABSTRACT

*Megaoryzomys curioi*, an extinct rodent endemic to the Galápagos Islands (Ecuador), is the largest known member of the diverse Sigmodontinae, a primarily Neotropical subfamily within the Cricetidae (Rodentia, Mammalia). Its tribal affiliation has long been debated, with past classifications assigning it to Oryzomyini, Thomasomyini, or even proposing its placement in a distinct tribe. In this study, we analyse mitochondrial sequences extracted from a topotypical specimen of *M. curioi* (Santa Cruz Island), within a densely sampled cricetid phylogenetic framework. Our results support its inclusion within Oryzomyini. *Megaoryzomys* is recovered as part of a clade that also includes the genera *Aegialomys* and *Nesoryzomys*, encompassing both Galapagoan and mainland representatives. These preliminary findings would support a scenario of two independent colonization events by oryzomyines: one ancient (~4.3 Mya) and another more recent (~0.3 Mya), each followed by insular diversification, to account for the known diversity of Galapagoan rodents (three genera and 11 species, including fossils and living forms). Moreover, the early origin and prolonged persistence of *Megaoryzomys* challenge the notion of the Galápagos as short-lived biogeographic units due to tectonic instability.

**Keywords:** *Aegialomys*; Ecuador; Galápagos; island colonization; *Megaoryzomys*; *Nesoryzomys*; Oryzomyini

## INTRODUCTION

Why islands exert such a powerful influence on human imagination is a complex question. Perhaps it is because they represent a world beyond rules (or a world governed by the rules of a lucky Robinson Crusoe), or they evoke the possibility of mythical beings—from Polyphemus to King Kong. Islands have long been viewed as dreamscapes of escape, as imagined by Homer, Verne, Eco, Hemingway, Saramago, and many others. Biologists, too, are captivated by islands, and the Galápagos—without question—hold a special place in evolutionary thought, with countless contributions exploring nearly every aspect of their unique geobiotic fabric (e.g. Heller 1904, Thornton 1971, Bailey

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1976, Bowman *et al.* 1983, Berry 1984, Christie *et al.* 1992, Pritchard 1996, Grove and Lavenberg 1997, Grant 1999, McMullen 1999, Grehan 2001, Burns *et al.* 2002, Caccone *et al.* 2002, Beheregaray *et al.* 2003, Peck 2006, Harpp *et al.* 2014, Arteaga *et al.* 2019, Poulakakis *et al.* 2020).

Despite this attention, the taxonomy of native Galapagoan rodents has received comparatively little scientific focus (e.g. Orr 1938, Hutterer and Hirsch 1980, Patton and Hafner 1983, Prado and Percequillo 2018, Castañeda-Rico *et al.* 2019, Dowler and Revelez 2021). One genus remains largely overlooked: the cricetid *Megaoryzomys* Lenglet & Coppois, 1979, whose only described species is *Megaoryzomys curioi* (Niethammer, 1964). Initially considered part of the Caribbean *Megalomys* Trouessart, 1881, *Megaoryzomys* is known from two Galápagos islands: *M. curioi* from Santa Cruz and *Megaoryzomys* new species from Isabela (Niethammer 1964, Lenglet and Coppois 1979, Steadman and Ray 1982). It displays several remarkable traits that underscore its distinctiveness. *Megaoryzomys* may represent the largest known member of the Sigmodontinae. While precise mass estimates are lacking, it likely reached approximately 1 kg (Pardiñas *et al.* 2017). Assessments of its postcranial skeleton suggest a unique ecology, possibly adapted to living in lava tubes—a typical Galapagoan geological feature (Hutterer and Oromí 1993). Echoing patterns seen in other large, island-dwelling sigmodontines—such as the striking Caribbean radiation of cricetids (see Brace *et al.* 2015 for a synthesis)—*Megaoryzomys* appears to have gone extinct in very recent times (Steadman *et al.* 1991).

Ronez *et al.* (2021a) revisited Steadman and Ray's (1982) assignment of *Megaoryzomys* to the Thomasomyini and referred this genus to the Oryzomyini. As these authors noted, placing *Megaoryzomys* within Thomasomyini implies not only greater taxonomic complexity but also a greater biogeographical complexity. In fact, it would require at least three independent colonization events of the Galápagos Islands by these rodents, given that all other native cricetids belong to the Oryzomyini (e.g. Dowler 2015, Percequillo 2015). These include the endemic genus *Nesoryzomys* Heller, 1904 and a species of *Aegialomys* Weksler, Percequillo & Voss, 2006, a genus otherwise widespread on the South American mainland (e.g. Dowler 2015, Prado and Percequillo 2018).

The aim of this study is to present the first ancient mitochondrial genome data for *Megaoryzomys*, enabling a phylogenetic analysis to resolve its tribal affiliation and to preliminarily explore the broader biogeographical implications of these findings.

## MATERIAL AND METHODS

### Specimens analysed

A nearly complete skull [including both first upper molars (M1) and right second upper molar (M2), and occipital and basicranial regions and tympanic bullae are missing] identified as *M. curioi* served as the source for DNA extraction (Fig. 1; skull C in Hutterer and Oromí 1993). The specimen is housed in the Colección de Vertebrados Fósiles at the Museo de Ciencias Naturales de Tenerife (Tenerife, Spain) under catalogue number TFMCVF-603. It was collected by Pedro Oromí and colleagues in January 1991 from the surface of the volcanic cave known as Cueva del Cascajo on Santa Cruz Island, Galápagos, Ecuador (see Hutterer and Oromí 1993). According to the refinement proposed by these authors regarding the type locality of *M. curioi*, this specimen qualifies as a topotype. Its geological age remains undetermined. To avoid potential confusion, the Spanish names of the islands comprising the Galápagos archipelago are used throughout this article. Lévêque (1963) provided correspondence on island English and Spanish toponyms.

**Figure 1.** *Megaoryzomys curioi* (Rodentia, Cricetidae): nearly complete skull (specimen TFMCVF-603, Colección de Vertebrados Fósiles, Museo de Ciencias Naturales de Tenerife, Tenerife, Spain) collected on Santa Cruz Island (Galápagos, Ecuador), which served as the source of the ancient DNA analysed in this study. Shown from top to bottom: dorsal, ventral, and lateral views. Scale = 2 cm.

### Molecular analysis

### *Ancient DNA*

All laboratory tasks (sample preparation, DNA extraction, and library construction) were carried out at the ancient DNA dedicated Paleogenomics Laboratory of the University Institute for Research in Environmental Sciences of Aragón (IUCA, Universidad de Zaragoza, Spain). For DNA extraction (laboratory number ZARADNA 336), the right incisor of TFMCVF-603 (Fig. 1) was manually extracted and its root portion cut; its surface was lightly wiped with 0.5% bleach and subsequently washed twice in 2 ml of ultra-pure water and 2 ml of 80% ethanol for 10 minutes for each wash. After air drying, the incisor fragment was UV-radiated on each side for 15 minutes, and finally mechanically

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pulverized in a 2 ml screw-cap tube using sterile tweezers. A final amount of 148 mg of tooth powder was used for DNA extraction.

Tooth DNA was extracted following a silica suspension method (Brotherton *et al.* 2013) using a modified PB buffer (Qiagen) (Bover *et al.* 2019) derived from Dabney *et al.* (2013). Tooth powder underwent predigestion with 1 ml of 0.5 M EDTA (pH 8.0) for 1 hour at room temperature under constant rotation, followed by digestion/decalcification with ~1 ml of buffer (900 µl 0.5 M EDTA pH 8.0 + 20 µl Proteinase K, 20 mg/ml) for 20–24 hours at 55°C. The binding step was performed using 13 ml of modified PB buffer (13.6 ml PB buffer, 420 µl 3 M sodium acetate, 7 µl Tween-20) and 100 µl silica suspension, followed by three washes (the first using 1 ml of binding buffer, and subsequent washes using 80% ethanol), and elution with 100 µl TLE buffer.

Double-stranded genomic libraries were built using the protocol of Meyer and Kircher (2010) with modifications (Llamas *et al.* 2016; i.e. adding internal 7-bp barcodes on each side of the molecules), and using a partial uracil-DNA glycosylase repair treatment (Rohland *et al.* 2015). After amplification (using Platinum Taq HiFi DNA polymerase, Invitrogen), purification, and quantification, the library was sequenced using an Illumina HiSeqX platform (Paired-End, 2 × 150 bp).

Quality control of the 81 743 457 raw sequencing reads obtained was performed with fastQC v.0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Adapter sequences were trimmed using AdapterRemoval v.2.3.0 (Schubert *et al.* 2016) and parameters widely used in ancient DNA data filtering [i.e. 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]. About 91% (74 351 567) of the raw reads were collapsed in a single read and used for subsequent mapping to a reference.

Since *Megaoryzomys* is an oryzomyine (Ronez *et al.* 2021a), the collapsed reads from sample 336 (TFMCVF-603) were iteratively mapped following a Multireference Iterative Mapping Approach (MIMA, e.g. Torres-Roig *et al.* 2021, Alfaro-Ibáñez *et al.* 2024) (see schematic explanation in Supporting Information, Fig. S1). In a first step, the collapsed reads were mapped to the available mitochondrial genomes of three species of this tribe: *Holochilus sciureus* Wagner, 1842 (NC_061914), *Melanomys caliginosus* (Tomes, 1860) (MH939287), and *Oligoryzomys stramineus* Bonvicino & Weksler, 1998 (NC_039723). The mapping to each reference was performed using Burrows–Wheeler Aligner (BWA) v.0.7.17 backtrack algorithm (Li and Durbin 2009) applying the stringent parameters widely accepted to map ancient data (i.e. aln -n 0.01, -o 2, -l 1024) and removing reads with minimum mapping quality (-q 25) using SAMtools v.1.11 (Li *et al.* 2009). Duplicate reads were filtered using FilterUniqueSAMCons.py (Kircher 2012). The alignment of unique mapped reads and each reference was visualized using Geneious Prime v.2022.0.2 (Biomatters, http://www.geneious.com; Kearse *et al.* 2012). The observed pattern of reads mapped to each reference (i.e. reads mostly mapping to conserved regions of the mitochondrial genomes such as ribosomal RNA genes) suggested that the *Megaoryzomys* mitochondrial genome is divergent from the Oryzomyini genomes used as references. Thus, we also iteratively mapped the collapsed reads to each reference but using the more relaxed parameters suggested by Westbury and Lorenzen (2022) to map sequencing data to phylogenetically divergent mitochondrial genomes (i.e. modifying BWA parameter -n to 0.001, and removing reads with minimum mapping quality -q 20). After each round of the iterative mapping using the two different sets of parameters [i.e. stringent (STR) and relaxed (REL)] to each of the three references, we generated intermediate 75% majority consensus sequences using Geneious Prime, retaining the reference nucleotides for sites with a read-depth < 3×. These new consensus sequences were then used as new references for a new round of mapping. This process was iterated until no more reads were mapped. Three final 75% majority consensus sequences (one for each mapping reference) were generated for the iterative mapping using the two different strategies (STR and REL) using Geneious, calling nucleotides only at sites with a read-depth ≥ 3×. All the mapping stats (number of iterations and unique reads mapped) are listed in Supporting Information, Table S1. In the second step of the process, the three consensus reference mitochondrial genomes obtained from each mapping strategy (identical for regions where they overlapped) were aligned using the MUSCLE algorithm v.3.8.425 implemented in Geneious, merging them in a new intermediate consensus by retaining nucleotides called from our sequence data, including unknown positions (Ns) not covered by any of the consensus sequences. In the third step, we aligned these new intermediate consensus sequences (REL and STR) to *M. caliginosus*, as the mapping to this species furnished the best results (with 1362 unique reads mapped vs. 938 mapped to *H. sciureus* and 695 to *O. stramineus* using stringent parameters, and with 1833 unique reads mapped vs. 1588 mapped to *H. sciureus* and 1411 to *O. stramineus* using relaxed parameters). The unknown positions in the intermediate consensus sequences were substituted by the corresponding nucleotides of *M. caliginosus*, and thus obtaining a complete sequence for each mapping strategy to be used as references in a new round of mapping (fourth step) using each set of parameters for a second iterative mapping as above. A final consensus sequence for each mapping strategy was finally generated in Geneious using a 75% majority consensus, retaining the reference nucleotide in positions with coverage depth ≥ 3×, and obtaining ~73% of the *Megaoryzomys* mitochondrial genome using the stringent parameters and around ~89% using the relaxed parameters (Supporting Information, Table S1). Ancient DNA misincorporation and fragmentation patterns were assessed using mapDamage v.2.1.1 (Jónsson *et al.* 2013).

## Phylogenetic analysis

### *Mitochondrial genome*

Currently, only 10 Sigmodontinae mitochondrial genomes from eight species are available in genetic databases. We generated two different datasets aligning one (when two or more available) genome per species (Supporting Information, Table S2) to each of the *Megaoryzomys* mitochondrial genomes (STR and REL mapping strategies) using MUSCLE implemented in Geneious and setting the *Cricetus cricetus* (Linnaeus, 1758) (Rodentia, Cricetidae) mitochondrial genome (accession number NC_037888) as the outgroup. After removing the Control Region, both datasets were partitioned by Protein Coding Gene (PCGs) codon positions, ribosomal RNA genes (12S and 16S), and tRNA genes.

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Codon positions were separated using DAMBE v.7.0.5 (Xia 2017), and ambiguous positions in the alignments of 12S, 16S, and tRNAs were removed using the default parameters in Gblocks v.0.91.1 (Castresana 2000, Talavera and Castresana 2007) available at https://ngphylogeny.fr (Lemoine *et al.* 2019). The Gblocks analysis kept 862 out of 990 nucleotides (87%) for the 12S alignment, 1341 out of 1640 nucleotides (81%) for the 16S alignment, and 1379 out of 1548 nucleotides (89%) for the tRNA alignment. The final dataset comprised 10 individuals and an alignment length of 14 901 bp. A Maximum Likelihood (ML) phylogenetic tree was inferred using IQTREE2 v.2.2.0 (Minh *et al.* 2020), and substitution models inferred by ModelFinder (Kalyaanamoorthy *et al.* 2017) implemented in IQTREE2 (see Supporting Information, Table S3 for partitions and substitution models inferred) and with node support values estimated by performing 1000 ultrafast bootstraps (Hoang *et al.* 2018). Additionally, a Bayesian Inference (BI) phylogenetic analysis was performed in the same two datasets and partitions, inferring substitution models using ModelFinder in IQTREE2 (Supporting Information, Table S3) in MrBayes v.3.2.7a (Ronquist *et al.* 2012) using four independent runs of four Markov chains each, for a total of 10 million generations, sampling every 104 generations and discarding 10% of the trees as burn-in. Convergence of parameter estimates was assessed using Tracer v.1.7.2 (Rambaut *et al.* 2018).

### *Cytochrome B*

A second dataset using the Cytochrome B (*Cytb*) gene sequences longer than 700 bp of representative Sigmodontinae taxa, especially within Oryzomyini, was built to incorporate as many species as possible, using a single sequence per species. Up to 143 complete and partial *Cytb* sequences from GenBank were aligned using the MUSCLE algorithm in Geneious to the *Megaoryzomys* sequence, generating two different datasets using this gene from REL and STR mitochondrial genome sequences, respectively, and setting three Tylomyinae cricetids as the outgroups (Supporting Information, Table S4). The final alignment, partitioned by codon positions using DAMBE, comprised 143 individuals and a length of 1140 bp.

Phylogenetic trees for *Cytb* were constructed under two optimality criteria: Bayesian inference (BI) in MrBayes v.3.2.7a (Ronquist *et al.* 2012) and Maximum Likelihood (ML) in IQTREE2 as for the mitochondrial genome dataset. In both cases, substitution models (Supporting Information, Table S3) were estimated by ModelFinder in IQTREE2 (as above). ML analysis was performed in IQTREE2 estimating node support by 1000 ultrafast bootstraps. BI analysis was run for 20 million generations with sampling every $2 \times 10^4$ generations using four independent runs of four Markov chains each. We considered well-supported clades those with BI posterior probability (PP) $\geq 0.95$ and ML bootstrap value (MLB) $\geq 95\%$.

We tentatively calibrated the Bayesian information *Cytb* tree using the *M. curioi* REL sequence by estimating ancestral node ages with the distance-based least square fast dating method (LSD2, To *et al.* 2016) implemented in IQ-TREE 2 v.2.0.6. As time calibration points, we used fossil (Ronez *et al.* 2021b, Salazar-Bravo *et al.* 2023), mean (Salazar-Bravo *et al.* 2023), and adjusted or median dates (Kumar *et al.* 2022) as second order point calibrations (Supporting Information, Table S5). Additionally, confidence intervals for the time estimates were modelled utilizing the default 0.2 standard deviation of a lognormal relaxed clock and resampling branch lengths 5000 times. To ensure consistency, we used the same partitioning scheme and substitution models for each partition during phylogenetic inference.

### *Combined analysis*

We also ran a combined analysis of molecular (*Cytb*) and morphological data in MrBayes, by adding a morphological matrix as a fourth partition. In this case, we only used the *Megaoryzomys* REL sequence due to the consistent results of the phylogenetic analyses using STR and REL sequences (see Results section) confirming the validity of the REL sequence. *Megaoryzomys curioi* was incorporated into the morphological matrix developed by Weksler *et al.* (2025), accessed via MorphoBank v.3.0 (http://www.morphobank.org). To perform the scoring, we examined not only the incomplete skull here analysed for DNA (TFMCVF-603) and the existing descriptions of the species (Lenglet and Coppois 1979, Steadman and Ray 1982, Hutterer and Oromí 1993), but also a comprehensive collection of digital photographs and anatomical notes generated during a previous project (Ronez *et al.* 2021a). This collection includes dozens of images representing multiple specimens—spanning various ontogenetic stages—held at the Smithsonian National Museum of Natural History (Washington, D.C., USA). A particularly important fossil for this assessment was an almost complete cranium with an associated mandible, housed in the collection of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK; Bonn, Germany) and originally described and illustrated by Hutterer and Oromí (1993: 'Cráneo B'). This specimen (ZFMK 2016_0981) was thoroughly examined using a digital model (see Supporting Information, Fig. S2) generated via micro-CT scanning (Bruker Skyscan 1173) at 70 kV, yielding a resolution of 29 μm. Raw data were reconstructed using NRecon (v.1.7.1.6, Bruker microCT), and 3D models were rendered with CTvox (v.3.0.0 r1114, Bruker microCT; https://www.bruker.com). Following the character definitions and illustrations provided by Weksler (2006), with modifications and updates from Weksler *et al.* (2025), we were able to score 62 of the 103 phenotypic characters for *M. curioi* (60 craniodental and two skeletal) (Supporting Information, Table S6). As expected, characters relating to external morphology (characters 1–18), soft anatomy (89–103), and most of the postcranial skeleton were not scored, given that the available material consists exclusively of fossilized cranial and dental remains. Character 48 (mastoid ossification) could not be scored due to preservation limitations, and character 58 (molars—general pattern) was scored as 'molars planar and hypsodont', although this does not fully capture the condition observed in *Megaoryzomys*, which has planate molar crowns but is not strictly hypsodont. One character—26 (orbital region: surface relief of dorsal and lateral orbital facies of frontal)—was deemed not applicable.

In addition, in this case, and for comparison reasons, we selected the exact same species used by Weksler *et al.* (2025) (Supporting Information, Table S7). The morphological matrix analysed by these authors comprised 103 characters for 63 species to which we added the molecular and morphological information

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for *M. curioi*. The dataset was also partitioned in codon positions of *Cytb* as above, whereas the morphological partition was analysed using the Mkv model (Lewis 2001) with the correction for ascertainment bias (coding = informative) and equal rates across sites. Chains for this combined analysis were run for 20 million generations sampling every $2 \times 10^4$ generations in MrBayes and remaining analyses were performed in the same manner to that of the molecular data alone. A Maximum Likelihood analysis of this same dataset was performed using RAxML v.8.2.11 (Stamatakis 2014) inferring node support by 1000 bootstrap replicates. In this case, as we did not use ultrafast bootstraps, we considered well-supported clades those with a ML bootstrap value (MLB) ≥ 75%.

## RESULTS

### Mitochondrial genome assembly

The final *M. curioi* mitochondrial sequences generated here covered ~ 73% of the reference sequence at mean coverage 4.6× and displayed 6055 unknown positions (37.1% of the estimated length of the mitochondrial genome) using stringent parameters, and ~89% of the reference at mean coverage 5.9× displaying 3180 unknown positions (19.5%) using relaxed parameters (Supporting Information, Table S1). Once aligned, both *M. curioi* consensus sequences displayed identical nucleotides for those regions obtained using the two different mapping strategies. Sequence fragments larger than 50 bp in the relaxed-generated consensus (REL) not present in the stringent-generated consensus (STR) were BLASTn analysed (Altschul *et al.* 1990). The first BLASTn hit of the 20 fragments (ranging from 60–409 bp) were assigned to cricetid (15), murid (3), and heteromyid (1) rodent species (Supporting Information, Table S8) except one 120 bp fragment of the *Cox1* gene assigned to *Sorex* Linnaeus, 1758 (but identity < 90%). In addition, no stop codon or other frame reading codon was observed in mitochondrial PCGs. These results suggest that the BWA mapping strategy using relaxed parameters did not introduce cross-species contamination in the consensus sequence generation as previously stated by Alfaro-Ibáñez *et al.* (2024). The ancient DNA misincorporation and fragmentation patterns (Supporting Information, Fig. S3) for both final sequences displayed the damage pattern usually observed in partial uracil-DNA glycosylase-treated libraries (Rohland *et al.* 2015).

The partial mitochondrial genome for *M. curioi* obtained using the relaxed mapping parameters (REL) (see Supporting Information, Table S9 for mitochondrial genome details) is available in GenBank under accession number PV739406. The sequence obtained using stringent parameters (STR) is available in the Supporting Information, Appendix S1, in fasta format.

### Phylogenetic analyses

The phylogenetic relationships between some Sigmodontinae tribes and species were unsolved and somewhat highly dependent on the morphological and/or molecular dataset used. Solving these relationships was beyond the purpose of this paper as we were mostly using previously published and analysed sequences. For this reason, we focussed on the phylogenetic relationships of *M. curioi* based on the new genetic data obtained here.

The ML and BI trees based on complete mitochondrial genomes strongly supported the placement of *Megaoryzomys* within the tribe Oryzomyini, with *Melanomys caliginosus* recovered as its sister taxon [Maximum Likelihood Bootstrap (MLB) = 100; Posterior Probability (PP) = 1]. Together with *Holochilus sciureus*, these taxa formed a well-supported clade within Oryzomyini (MLB = 100, PP = 1; Fig. 2).

Phylogenetic analyses of *Cytb* sequences—based on both REL (1021 bp) and STR (929 bp) sequences—using BI and ML inferences yielded generally concordant tree topologies (Fig. 3; Supporting Information, Figs. S4 and S5). *Megaoryzomys* was recovered within Oryzomyini, with both sequences forming a well-supported clade (MLB > 98; PP = 1) that also included *Aegialomys* and *Nesoryzomys*, hereafter referred to as the 'Galapagoan–mainland clade' and estimated to have emerged around 4.28 Mya [95% Confidence Interval (CI) = 3.63–4.96 Mya] (Fig. 3). Additionally, this Galapagoan–mainland clade was resolved as sister (but with low support) to a clade comprising the oryzomyine genera *Melanomys*, *Sigmodontomys* Allen, 1897, and

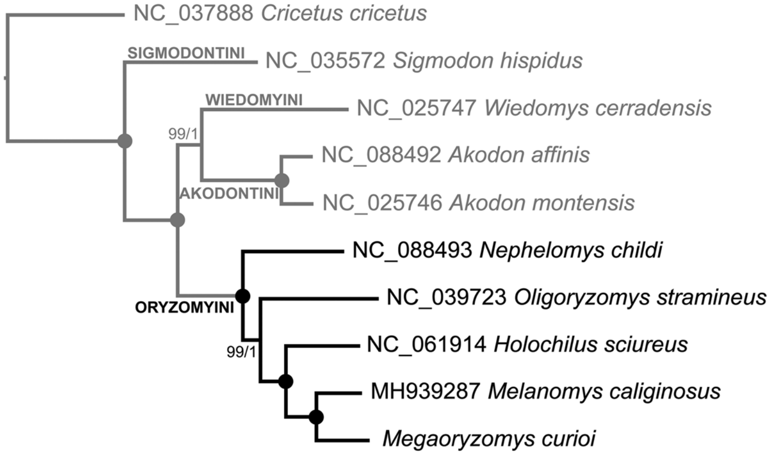

**Figure 2.** Combined Maximum Likelihood and Bayesian Inference tree using Sigmodontinae mitochondrial genomes (excluding the Control Region) available in GenBank (see Supporting Information, Table S2), the newly generated partial mitochondrial genomes for *Megaoryzomys curioi* (representing both STR and REL consensus sequences), and *Cricetus cricetus* as an outgroup. Numbers in nodes indicate Maximum Likelihood Bootstrap values (MLB)/Bayesian Inference Posterior Probability (PP). Circles in nodes indicate MLB = 100 and PP = 1.

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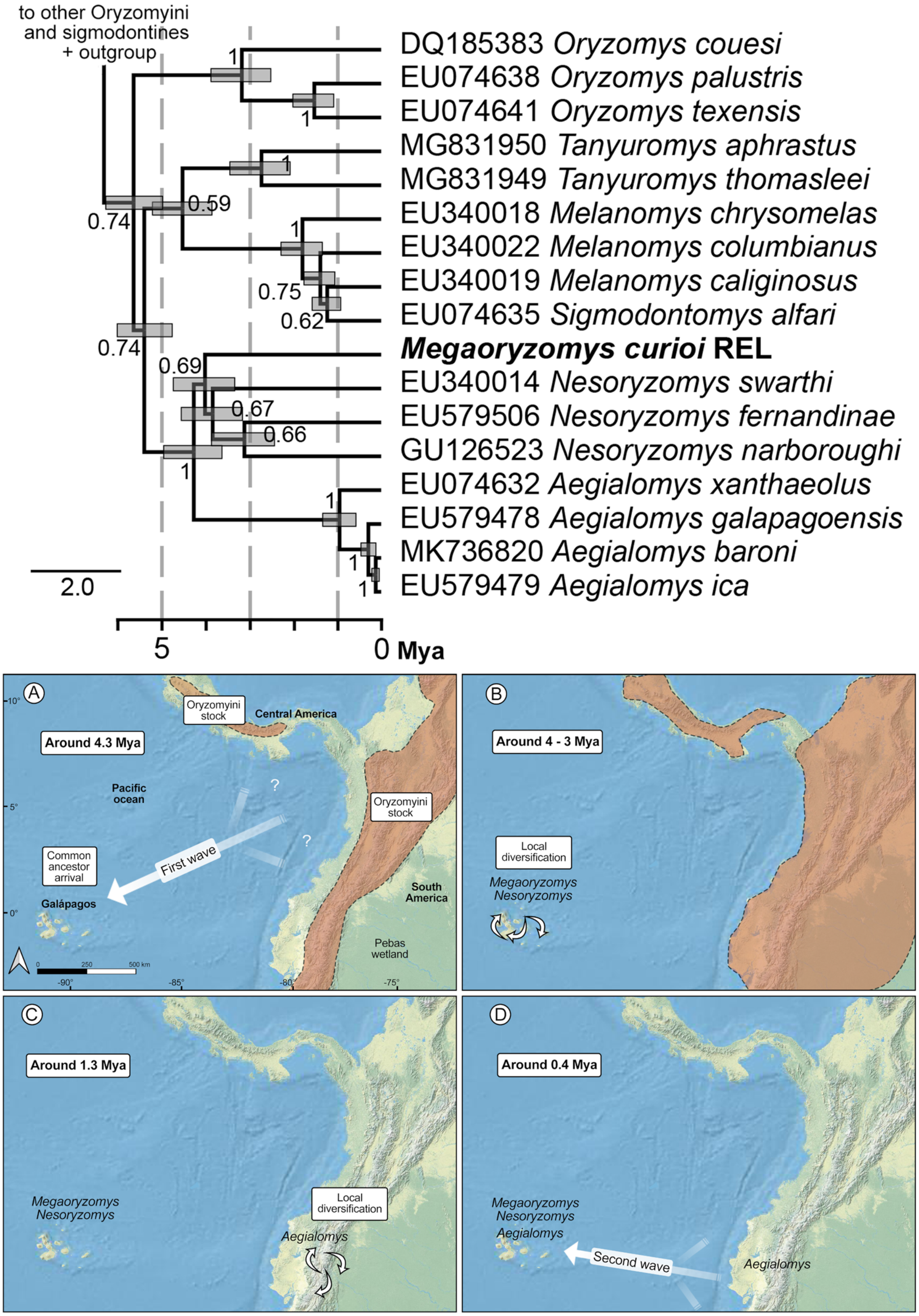

**Figure 3.** Top panel shows a Calibrated Bayesian Inference phylogeny based on *Cytb* sequences, highlighting *Megaoryzomys curioi* (REL consensus sequence) and its closest oryzomyine relatives. Bottom panels depict a schematic representation of the main hypothesized biogeographic events involved in the colonization and radiation of Galápagos rodents: (A) an ancient wave of oryzomyines invading the Galápagos; (B) local diversification of the first arrivals; (C) diversification of *Aegialomys* in the Andean ranges; (D) a second wave of oryzomyines from the mainland, including *Aegialomys*, reaching the Galápagos. Panels (A) and (B) also include silhouettes indicating the approximate configuration of emerged mainland areas during those periods. Base map image from Google Earth.

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*Tanyuromys* Pine, Timm & Weksler, 2012, which was estimated to have split 5.4 Mya (95% CI = 4.77–6.02 Mya) (Fig. 3; Supporting Information, Fig. S6). The *Aegialomys* species shared a common ancestor around 0.96 Mya (95% CI = 0.58–1.34) and *Nesoryzomys* species at 3.85 Mya (95% CI = 3.17–4.56).

The phylogenetic relationships among the three genera comprising the 'Galapagoan-mainland clade' were unresolved, with low support across alternative topologies, except in the ML tree using the *Megaoryzomys* REL sequence, which showed high support for the clade *Megaoryzomys-Nesoryzomys* (ML = 96) (Supporting Information, Fig. S4). Although the clade was supported in this specific analysis, its lack of support in the other three analyses warrants caution in interpreting the results. For this reason, the obtained split between *Megaoryzomys-Nesoryzomys* at 4.02 Mya (95% CI = 3.34–4.74) should be cautiously considered.

Our combined morphology-DNA analysis (Supporting Information, Fig. S6) also fully supported the clade *Aegialomys-Nesoryzomys-Megaoryzomys* in the BI inference analysis (PP = 1) in contrast with the low support (MLB = 66) of this node in the ML analysis.

## DISCUSSION

### Taxonomy of *Megaoryzomys*

The molecular-based clade that includes the three extinct and extant rodent genera found in the Galápagos (i.e. *Aegialomys*, *Megaoryzomys*, and *Nesoryzomys*) is both exciting and expected. Over the past two decades, phylogenetic studies have consistently grouped *Aegialomys* and *Nesoryzomys* within the same clade (e.g. Weksler 2006, Ventura *et al.* 2013), or even placed them as sister taxa (e.g. Hanson 2008, Steppan and Schenk 2017, Brito *et al.* 2020, Percequillo *et al.* 2021, Weksler *et al.* 2025). Many of these studies emphasize the influence of geography in shaping the phylogenetic relationships of sigmodontine rodents, suggesting that geographic proximity plays a significant role in intra-tribal phylogenetic structure (e.g. Parada *et al.* 2013, 2015, Schenk and Steppan 2018, Ronez *et al.* 2023). In this context, the *Cytb* data supporting the inclusion of *Megaoryzomys* in the previously identified Galápagos-mainland clade is a significant result. This finding is particularly important as it further supports a hypothesis previously based on morphological evidence (e.g. Niethammer 1964, Lenglet and Coppois 1979, Ronez *et al.* 2021a).

The tribal affiliation of fossil sigmodontines remains an ongoing debate, and *Megaoryzomys* is no exception. The introduction of molecular data reshaped our understanding of some tribal grouping within the subfamily (e.g. Dowler 2015, Percequillo 2015, Pardiñas *et al.* 2017) and molecular phylogenetics have given us the opportunity to distinguish character convergence from phylogenetic propinquity, placing fossil forms that lack molecular evidence in a precarious position. Many fossil genera, primarily described from Argentina, have been assigned to tribes based on craniodental features—particularly molar morphology (e.g. Reig 1980, Steppan 1996). However, due to the high degree of convergence in molar traits, these tribal assignments are often debated. Classic examples are the Pliocene genera *Olympicomys* Steppan & Pardiñas, 1998, *Panchomys* Pardiñas, 1997, and *Tafimys* Ortiz, Pardinas & Steppan, 2000, which were originally classified within the Phyllotini (e.g. Steppan and Pardiñas 1998, Ortiz *et al.* 2000). More recent studies suggest that these genera represent a past diversity of the current, monotypic Reithrodontini (e.g. Barbière *et al.* 2016, 2022, Barbière 2019).

When *Megaoryzomys* was first described, it was placed within the tribe Oryzomyini, not only because it was considered part of the historically extinct oryzomyine genus *Megalomys*, but also due to shared morphological traits (Niethammer 1964). Even after being recognized as a distinct genus, *Megaoryzomys* remained in Oryzomyini, a tribal affiliation explicitly discussed by Lenglet and Coppois (1979). However, the monograph by Steadman and Ray (1982) proposed an alternative allocation, placing *Megaoryzomys* within the tribe Thomasomyini. This suggestion was based on certain craniodental traits as well as biogeographical considerations, including the proximity of Thomasomyini species on the mainland (Patton *et al.* 2015).

In the following decades, two additional tribal affiliations were proposed for this intriguing Galápagos rodent. One suggestion was that *Megaoryzomys* might represent a distinct tribe that evolved in isolation on the islands (Hutterer and Oromí 1993). Another possibility was its classification as Sigmodontinae *incertae sedis*, following Steadman and Ray's (1982) findings, with Musser and Carleton (2005) noting that 'while not an oryzomyine per se, the relationships and tribal affiliation of *Megaoryzomys* deserve reconsideration within a broader sampling of New World cricetids and from a cladistic perspective'.

The ongoing debate regarding the tribal affiliation of *Megaoryzomys* was revisited by Ronez *et al.* (2021a), who reassessed its craniodental morphology. Their analysis highlighted several key differences: *M. curioi* lacks or has a poorly expressed suspensory process of the squamosal bone, a feature well developed in Thomasomyini (Pacheco 2003). Additionally, *M. curioi* does not exhibit the dorsal aperture condition of the ectotympanic bone found in Thomasomyini, and its mandibular morphology contrasts with the typical structures seen in that tribe (Pacheco 2003). Furthermore, the procingulum of the first lower molar in *M. curioi* lacks the characteristic pattern of two conulids typical of Thomasomyini (Barbière *et al.* 2019). Taken together, these craniodental traits strongly support the classification of *M. curioi* within Oryzomyini (Ronez *et al.* 2021a).

The analysis of mitochondrial DNA from a topotypical specimen of *M. curioi* presented in this study provides further support for its placement within Oryzomyini and significantly contributes to the scarce database of ancient DNA of sigmodontine rodents. However, unilocus phylogenetic approaches, especially in highly speciose groups of muroid rodents, are not free from producing spurious results. For example, when analysed solely on the *Cytb* gene, the oryzomyine *Holochilus* Brandt, 1835 appears paraphyletic despite its conservative morphology, whereas a nuclear gene is required to recover its monophyly (D'Elía *et al.* 2015). The Brazilian cricetid genus *Calassomys* Pardiñas, Lessa, Teta, Salazar-Bravo & Câmara, 2014 presents an even more critical case. Analyses based solely on mitochondrial markers place it within the Wiedomyini, while its current classification within the Phyllotini is supported by a combination of both mitochondrial and nuclear gene data (Pardiñas *et al.* 2014). These examples highlight the need for comprehensive studies that incorporate nuclear and

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mitochondrial markers with morphological and biogeographical analyses to solidify the taxonomic position of *Megaoryzomys*. However, based on the available evidence, combined with previously discussed morphological data, the hypothesis of *Megaoryzomys* as a member of the tribe Oryzomyini remains a defensible one.

This is not the first instance in sigmodontines where ancient DNA has contributed to systematics. A prime example of this approach comes from studies of the Caribbean oryzomyines, where ancient DNA from fossil and historical specimens has been used to construct a robust phylogeny for the genera *Antillomys* Brace, Turvey, Weksler, Hoogland & Barnes, 2023, *Megalomys*, and *Pennatomys* Turvey, Weksler, Morris & Nokkert, 2010 (Brace *et al.* 2015).

## Rethinking cricetid biogeography in the Galápagos

The Galápagos cricetid radiation represents a complex mosaic shaped by lineages of diverse origins. Among the three genera and 11 species recorded to date, the broader pattern reveals a mixture of island-specific distributions, extinct taxa—including both Holocene fossils and historically extirpated species—and extant forms (Fig. 4).

The largest island, Isabela, currently hosts no living oryzomyines but preserves three fossil species from two genera, all known exclusively from palaeontological deposits (Table 1; Fig. 4). Santa Cruz, the second-largest island, also supports three species representing two genera. However, neither *Nesoryzomys darwini* Osgood, 1929 nor *Nesoryzomys indefessus* (Thomas, 1989) have been seen or caught on the island in the past century, and thus they are presumed to be recently extirpated (e.g. Patton and Hafner 1983, Dowler 2015). At the opposite end of the diversity spectrum, Floreana has yielded no oryzomyine fossils or extant records, despite palaeontological excavations that have recovered murid remains from its stratigraphy (Steadman 1982, 1986). In contrast, the smaller islands of Rábida and Baltra each hold a single record—one fossil and one historically extirpated species, respectively. This idiosyncratic distribution of fossil and extant rodents across the archipelago is noteworthy. Interestingly, there appears to be a plausible correlation between the total richness of oryzomyine taxa and island size (surface area in km$^2$; Table 1). However, the palaeontological record remains sparse and is limited to Holocene deposits (e.g. Steadman *et al.* 1991), constraining its utility for reconstructing the deeper evolutionary history of the group.

Recent comprehensive analyses of the Galápagos terrestrial fauna have re-evaluated the cricetid evidence, mostly building upon the first study applying molecular analyses to the Galápagos rodent fauna, allozymes in this case, by Patton and Hafner (1983). A macroregional approach by Heads and Grehan (2021: fig. 12) did not mention *Megaoryzomys* but suggested biogeographic parallels between Galápagos rodents and terrestrial snakes. Ali and Fritz (2021) conducted a more detailed examination of rodent colonization, tracing potential mainland origins and timing. They concluded that 'seven clades [including two lava lizards, two recent geckos, and the three rodents *Aegialomys*, *Megaoryzomys*, *Nesoryzomys*] … could have arrived on an existing island, as the oldest, San Cristóbal, formed *c.* 4 Mya' (Ali and Fritz 2021: 279). Notably, while they cited Castañeda-Rico *et al.* (2019) as supporting three rodent colonization events, that study proposed two colonization events involving the extant taxa (*Aegialomys* and *Nesoryzomys*). In a more recent and refined analysis, Ali and Fritz (2024) reaffirmed the hypothesis of a colonization event at least 4 Mya,

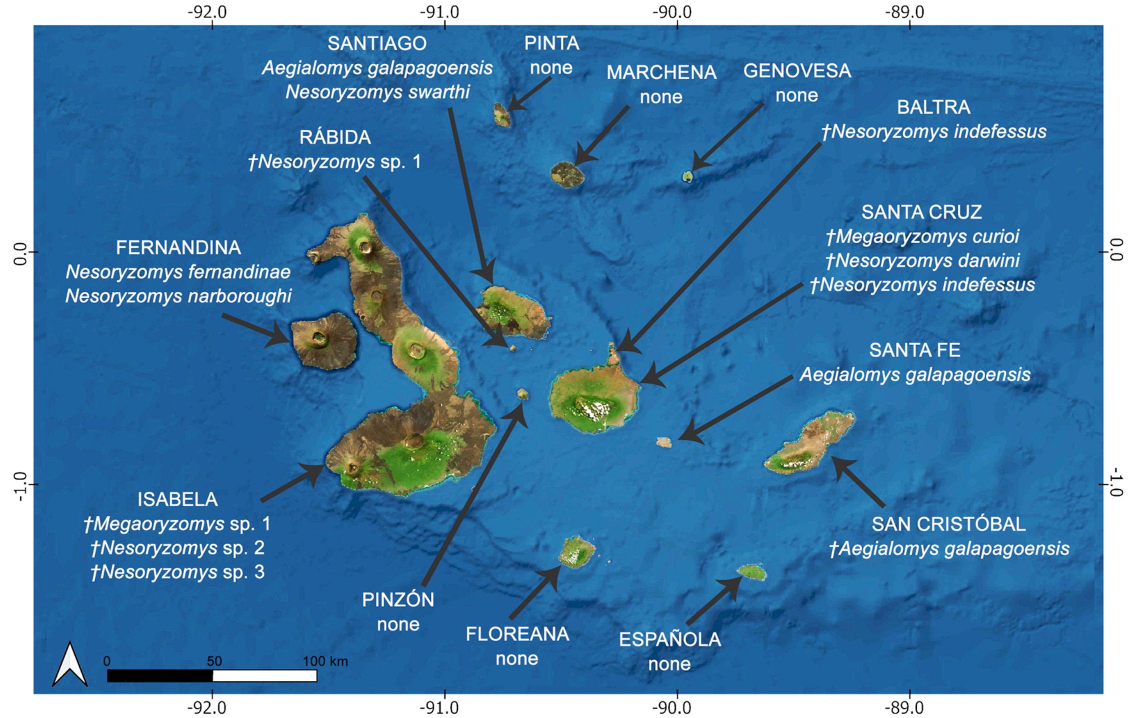

**Figure 4.** The Galápagos archipelago (main islands), showing the total known cricetid assemblages, compiled from several sources. The symbol † denotes extinct (derived from fossil records or historical extirpations). Base map image: satellite view from Google Earth.

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**Table 1.** Basic data for each Galápagos island (arranged in order of increasing surface area) and its oryzomyine assemblage, compiled from multiple sources. 'Fossil' refers to taxa known exclusively from Holocene deposits; 'Extinct' refers to those presumably extirpated since 1835 AD; 'Sites' indicates the approximate number of palaeontological deposits excavated on each island

| Island | Genus | | | | Species | | | | Surface (km$^2$) | Age (Mya) | Sites |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fossil | Extinct | Living | Total | Fossil | Extinct | Living | Total | | | |
| Rábida | 1 | - | - | 1 | 1 | - | - | 1 | 4.95 | ? | 1 |
| Genovesa | - | - | - | 0 | - | - | - | 0 | 14 | 0.3 | 0 |
| Pinzón | - | - | - | 0 | - | - | - | 0 | 18 | 1.7 | 0 |
| Baltra | - | 1 | - | 1 | - | 1 | - | 1 | 21 | 2.3 | 0 |
| Santa Fe | - | - | 1 | 1 | - | - | 1 | 1 | 24 | 2.9 | 0 |
| Pinta | - | - | - | 0 | - | - | - | 0 | 60 | 0.7 | 0 |
| Española | - | - | - | 0 | - | - | - | 0 | 60 | 3.5 | 0 |
| Marchena | - | - | - | 0 | - | - | - | 0 | 130 | 0.6 | 0 |
| Floreana | - | - | - | 0 | - | - | - | 0 | 173 | 2.3 | 3 |
| San Cristóbal | 1 | 1 | - | 2 | 1 | 1 | - | 2 | 558 | 4 | 1 |
| Santiago | - | - | 2 | 2 | - | - | 2 | 2 | 585 | 1.4 | 0 |
| Fernandina | - | - | 1 | 1 | - | - | 2 | 2 | 642 | 0.1 | 0 |
| Santa Cruz | 2 | 1 | - | 3 | 3 | 2 | - | 5 | 986 | 2.3 | 1 |
| Isabela | 2 | - | - | 2 | 3 | - | - | 3 | 4640 | 0.8 | 3 |

involving several lineages of terrestrial vertebrates, including eight reptile and three rodent groups.

Across these studies—including more taxon-focused analyses (e.g. Castañeda-Rico *et al.* 2019)—there is broad agreement on several key assumptions regarding the Galápagos cricetid radiation: (i) the source of colonizing rodents was the mainland, specifically Central and/or South America; (ii) regardless of whether there were one, two, or three colonization events, there is evidence of an initial ancient wave and a much more recent second wave; (iii) various geological processes—such as plate tectonics, island emergence and subsidence, and sea-level fluctuations during glacial cycles (e.g. Geist *et al.* 2014)—facilitated both over-water dispersal and within-archipelago speciation.

From a historical biogeographic perspective, the findings of the present study would support the conclusions of Ronez *et al.* (2021a). Establishing *Megaoryzomys* as an oryzomyine obviates the need to invoke a separate colonization event by a second sigmodontine lineage (e.g. thomasomyines). In this light, Oryzomyini appears to be the only branch of this diverse subfamily capable of successful long-distance overwater dispersal, as demonstrated by its presence on both continental and oceanic islands (e.g. the Lesser Antilles, Fernando de Noronha; e.g. Carleton and Olson 1999, Turvey *et al.* 2010, Ronez *et al.* 2021a).

While limited to a single molecular marker, the present phylogenetic reconstruction could help clarifying aspects of Galápagos cricetid evolution. A single ancient colonization event, pre-dating 4 Mya, would sufficiently explain the two major island rodent lineages: *Megaoryzomys* and *Nesoryzomys*. Their closest mainland relatives (e.g. *Melanomys*, *Tanyuromys*) and their current distributions (e.g. Pine *et al.* 2012, Patton *et al.* 2015, Timm *et al.* 2018), in conjunction with the complex palaeogeography of the region *c.* 4 Mya (see Ali and Fritz 2024 and the references cited therein), leave the exact mainland source ambiguous. Possible routes include dispersal from Central America via the Cocos Ridge or from South America via the Carnegie Ridge (Ali and Fritz 2024: fig. 2). Speciation within *Nesoryzomys*—and possibly *Megaoryzomys*—likely began in the Late Pliocene (Piacenzian), potentially linked to the formation of the present-day major islands, rather than more recent Pleistocene sea-level changes.

A more recent colonization event introduced a third genus, *Aegialomys*, to the archipelago. The presence of *Aegialomys galapagoensis* (Waterhouse, 1839) on the easternmost islands (e.g. San Cristóbal and Santa Fe) appears well supported and suggests that the lineage did not spread to the western islands. The supposed record of this species from Santiago should be disregarded (see discussion in Prado and Percequillo 2018). Moreover, in light of the strong island-specific differentiation observed in *Nesoryzomys* (see Dowler 2015 for a synthesis), the taxonomic status of *Oryzomys bauri* Allen, 1892—originally described from Santa Fe Island and currently considered a junior synonym of *A. galapagoensis* (Prado and Percequillo 2018, 2019)—warrants re-evaluation. Phylogenetic reconstructions (this paper), along with craniodental and chromosomal data (see Prado and Percequillo 2018), would support an origin of the *A. galapagoensis* ancestor from Ecuadorian-Peruvian *Aegialomys* populations.

The early arrival and persistence of the common ancestor to *Megaoryzomys* and *Nesoryzomys* challenges assumptions that island vertebrate faunas are ephemeral due to tectonic instability. Despite rapid island emergence and disappearance during the Plio-Pleistocene (see Geist *et al.* 2014, Ali and Fritz 2024), both endemic genera managed to survive and diversify, suggesting that the Galápagos have functioned as a biological 'museum'—a role mirrored by other island systems worldwide (e.g. Cronk 1997, Rozzi *et al.* 2023, Viñola López *et al.* 2025).

## CONCLUSION

*Megaoryzomys* remains an enigmatic representative of the Galápagos rodent fauna. It achieved remarkable gigantism in about 4 Mya, yet the evolutionary processes, selective pressures, and ecological triggers underlying this transformation remain poorly understood. All known remains of *Megaoryzomys*—from both

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Santa Cruz and the less frequently examined material from Isabela—have been recovered from ossuary assemblages within lava tunnels. While these accumulations were originally attributed to owl predation (Niethammer 1964), this hypothesis appears questionable given the rodent's large body size and the absence of extinct large raptors in the Galápagos (e.g. Steadman 1986). It is more plausible that these lava tubes reflect natural habitats and death sites, supporting the hypothesis of a cave-adapted lifestyle initially proposed by Hutterer and Oromí 1993).

This ecological interpretation invites a broader evolutionary scenario: a probable initial colonization of the archipelago by the ancestor of the Galápagos cricetid lineage around 4 Mya, possibly arriving on a now-submerged or extant island. From this lineage, a descendant adapted to subterranean life in basaltic lava tubes may have emerged as a strategy to cope with the harsh surface conditions. In parallel, a shift towards herbivory may have facilitated the development of larger body size. Stable isotope analyses of dental enamel, similar to those performed on *Antillomys* (Goedert *et al.* 2020), could illuminate the trophic ecology of *Megaoryzomys* and clarify the ecological drivers of its morphological trajectory.

One promising area for future research is the cranial and mandibular morphology of *Megaoryzomys*, especially in relation to its evolution towards increased masticatory efficiency. Shortening of the palate and broadening of the zygomatic plate are two observable trends in an evolution toward an increased bite force at the anterior region of the skull (Ronez *et al.* 2021a). Biomechanical modelling and muscle reconstructions are needed. A valuable comparative case is *Canariomys* Crusafont-Pairó & Petter, 1964, the giant murid from Tenerife and Gran Canaria, which independently evolved significant morphological disparity over a short time span (~0.65 Mya) (Moncunill-Solé *et al.* 2014, Renom *et al.* 2021).

Another area in need of attention is the alpha taxonomy of *Megaoryzomys*. Steadman and Ray (1982) and Steadman *et al.* (1991) proposed the existence of a second species based on specimens from Isabela, yet this taxon remains undescribed decades later. Similarly, three additional undescribed species of *Nesoryzomys* have also gone unstudied. This hidden diversity obscures our understanding of the Galápagos cricetid radiation. Given that at least four Holocene extinctions are documented across two genera within a total pool of just three cricetid genera and 11 known species (including two extinct historical taxa), the need for taxonomic revision is urgent (MacPhee and Flemming 1999).

In a broader context, the Galápagos oryzomyine radiation parallels the cricetid colonization of the Antilles. Although notable differences exist—such as the strictly oceanic nature of the Galápagos vs. the semi-continental character of the Antilles, or the two-step colonization model for the Galápagos compared to at least three steps in the Caribbean—the comparative study of these insular radiations holds significant potential. Caribbean oryzomyines have received considerable attention over the past two decades, revealing high levels of taxonomic and ecological diversity (e.g. Turvey *et al.* 2010, 2012, Mistretta *et al.* 2021). Moreover, their study has provided valuable insights into Holocene extinction patterns across island systems (Turvey 2009, Turvey and Fritz 2011, Cooke *et al.* 2017).

In contrast, Galápagos oryzomyines remain in need of systematic revision. Addressing this knowledge gap requires a collaborative, interdisciplinary effort involving neontologists, palaeontologists, archaeologists, ecologists, and geneticists. Only through such synergy can we fully unravel the evolutionary history and ecological significance of this remarkable insular lineage. Clarifying the evolutionary trajectory of *Megaoryzomys* may ultimately shed light on broader patterns of insular adaptation, extinction dynamics, and mammalian evolution in oceanic archipelagos.

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## SUPPLEMENTARY DATA

Supplementary data is available at *Zoological Journal of the Linnean Society* online.

## CONFLICT OF INTEREST

None declared.

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## DATA AVAILABILITY

Sequences used in this study are all available via GenBank. Voucher materials are all available in the public collections mentioned in the main text.

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