Evolution of vertebrate endemics in oceanic islands follows a predictable pattern, known as the island rule, according to which gigantism arises in originally small-sized species and dwarfism in large ones. Species of extinct insular giant rodents are known from all over the world. In the Canary Islands, two examples of giant rats, \( \dagger \text{Canariomys bravoi} \) and \( \dagger \text{Canariomys tamarani} \), endemic to Tenerife and Gran Canaria, respectively, disappeared soon after human settlement. The highly derived morphological features of these insular endemic rodents hamper the reconstruction of their evolutionary histories. We have retrieved partial nuclear and mitochondrial data from \( \dagger \text{C. bravoi} \) and used this information to explore its evolutionary affinities. The resulting dated phylogeny confidently places \( \dagger \text{C. bravoi} \) within the African grass rat clade (\( \text{Arvicanthis niloticus} \)). The estimated divergence time, 650,000 years ago (95% highest posterior densities: 373,000 – 944,000), points toward an island colonization during the Günz–Mindel interglacial stage. \( \dagger \text{Canariomys bravoi} \) ancestors would have reached the island via passive rafting and then underwent a yearly increase of mean body mass calculated between 0.0015 g and 0.0023 g; this corresponds to fast evolutionary rates (in darwins (d), ranging from 7.09 d to 2.78 d) that are well above those observed for non-insular mammals.

1. Introduction

The Canary Islands are located northwest off the coast of Africa, with their nearest island (Fuerteventura) being only separated from the continent by about 100 km (figure 1b). Although this volcanic archipelago was never connected to the mainland by any land bridge or island chain, colonization of
terrestrial organisms from the mainland was favoured by dominant oceanic currents. This archipelago offers a unique opportunity to study the colonization and diversification of multiple groups of organisms, such as birds, reptiles or small mammals [1]. Among the latter, there are three known striking examples of gigantism: the lava mouse of Fuerteventura and Lanzarote (†Malpaisomys insularis) and the extinct giant rats of Tenerife and Gran Canaria (†Canariomys bravoi and †Canariomys tamarani, respectively).

The Tenerife giant rat was described by Crusafont-Pairó & Petter [2] after the discovery of numerous specimens in Quaternary sites. Subsequent studies explored its diet, ecology, body mass and extinction causes, and also tentatively assessed its phylogenetic affinities based on dental traits (e.g. [3,4]). †Canariomys bravoi shows a set of traits characteristic of insular rodents, including gigantism, a robust skeleton and high-crowned teeth (figure 1c). It became extinct after the fourth century BCE, likely in relation to the arrival of Canarian indigenous people [4]. †Canariomys tamarani also became extinct soon after the arrival of the first settlers while †M. insularis survived until the beginning of the fourteenth century, when Europeans reached the archipelago [5]. Ancient mitochondrial DNA (mtDNA) from †M. insularis, showed close affinities to the extant genus Mus and pointed to a 6.9 Ma divergence date (genetic data were obtained by means of the traditional polymerase chain reaction method).

Hot and humid thermal conditions hamper the retrieval of ancient genetic data [6]. Without this information, it is difficult to unravel the affinities of highly modified extinct species such as †C. bravoi to their mainland smaller relatives. Here, we managed to retrieve partial nuclear and mtDNA data from two †C. bravoi specimens; we subsequently used this information to provide divergence age estimates and phylogenetic relationships for this lineage and determine the rate of increase in body size of this insular rodent.

2. Material and methods

(a) The samples

We performed DNA extraction from 12 mandibles: two from Barranco Moraditas and 10 more from Cueva del Viento (figure 1b). Specimens used for extractions were deposited in the Vertebrate collection (DZUL) of Departamento de Biología Animal, Edafología y Geología de la Universidad de La Laguna (Tenerife) with the following inventory numbers: CB-1 (DZUL 3200); CB-2 (DZUL 3201); CB-3 (DZUL 3202); CB-4 (DZUL 3203); CB-5 (DZUL 3204); CB-6 (DZUL 3205); CB-7 (DZUL 3206); CB-8 (DZUL 3207); CB-9 (DZUL 3208); CB-10 (DZUL 3209); CB-11 (DZUL 3210) and CB-12 (DZUL 3211).

Cueva del Viento site is a 17 km-long system of volcanic lava tubes formed 0.17–0.13 Ma [7] and situated in the north side of Tenerife at 700 m above sea level. The animals went into the cave through a small pit fall that acted like a trap. Bones were found in connection, showing the absence of transport after deposition. Previous calibrated radiocarbon ages of †C. bravoi samples from this site are between 17 300 and 2150 cal BP [4,8]. The samples from Barranco de las Moraditas were recovered from a small cave infilling in basaltic materials of Quaternary age at the east of Tenerife [7]. The median age reported for another †C. bravoi sample from this site is 2310 cal BP [4].

(b) DNA extraction, mitochondrial DNA capture and library preparation

All DNA extraction and initial library preparation steps were performed in a dedicated clean laboratory, physically isolated from the laboratory used for post-PCR analyses; no previous work on extinct or extant rodents was ever conducted in our laboratory. Strict protocols were followed to minimize the amount of human DNA in the ancient DNA laboratory, including wearing a full-body suit, sleeves, shoe covers, clean shoes, facemask, hair net and double gloving.
First, teeth samples were UV irradiated (245 nm) for 10 min and the outermost surface of the teeth was scraped off with a drill engraving cutter, followed by another UV irradiation in order to exclude the surface DNA contamination. Second, approximately 30 mg of tooth cementum was obtained by drilling at low speed (5000 r.p.m.) with a new engraving cutter.

DNA extraction from teeth powder was performed following the method of Dabney et al. [9]. The teeth powder samples, including an extraction blank, were added to 1 ml of extraction buffer (final concentrations: 0.45 M EDTA, 0.25 mg ml\(^{-1}\) proteinase K, pH 8.0), resuspended by vortexing and incubated at 37°C overnight on rotation. The remaining tooth powder was then pelleted by centrifugation in a bench-top centrifuge at maximum speed (16 100g). The supernatant was added to 10 ml of binding buffer (final concentrations: 5 M guanidine hydrochloride, 40% (vol/vol) isopropanol, 0.05% Tween-20 and 90 mM sodium acetate (pH 5.2)) and purified on a High Pure Extender column (Roche). DNA samples were eluted with 45 µl of EDTA TE buffer (pH 8.0). However, 10 samples failed to yield quantifiable DNA after extraction and only two from Cueva del Viento (CB-4 and CB-10) were further selected for library building.

A total of 35 µl of each DNA extract was used for library preparation in three sequential reactions: end-repair, adapter ligation, and nick fill-in; following the BEST protocol [10]. DNA extract from CB-4 was used for DNA-library preparation prior to Illumina sequencing; the resulting library was amplified by PCR with two uniquely barcoded primers and used for shotgun sequencing. Both libraries were purified with a 1× AMPure clean (Beckman Coulter) and eluted in 25 µl of EDTA TE buffer (pH 8.0). Library size and concentration were determined with the Agilent DNA 7500 Kit on the 2100 BioAnalyzer instrument. The DNA libraries were sequenced using HiSeq400 of Illumina platform from CB-4 and CB-10) were further selected for library building.

We calculated evolutionary rates for the body mass (in grams) increase from \(A.\ niloticus\) to \(C.\ bravoi\). Mean body mass for \(A.\ niloticus\) (sexes combined) is taken from Monadjem et al. [24], while estimated mean body mass for \(C.\ bravoi\) is taken from Moncunill-Solé et al. [25] and is based on multiple regressions considering dental, cranial and postcranial measurements.

We calculated evolutionary rates using the simple classic equation by Haldane [26]:

\[
\frac{\Delta r}{\Delta t} = \ln(x_1) - \ln(x_2)
\]

where, \(r\) is the rate of change (in darwins, d); \(x_1\) and \(x_2\) are the initial and final value of the analysed variable, respectively; and \(\Delta t\) is the amount of time elapsed. The calculations are carried for the minimum and maximum divergence dates between \(A.\ niloticus\) and \(C.\ bravoi\). The age of the oldest \(C.\ bravoi\) fossils (17 300 cal BP [4,8]) is taken as the endpoint of the size increase trend. Calculated evolutionary rates are compared to those derived for other mammals (e.g. [27,28]) as well as to those for \(tM.\ insularis\), which is included because it represents another case of murid gigantism in the same archipelago. Body mass for \(tM.\ insularis\) is estimated from published cranial and postcranial measurements [29], applying described equations [25]. Estimated mean body mass for \(tM.\ insularis\) is compared to
that of its closest relatives, *Mus (Coelomys) pahari* and *Mus (Coelomys) crociduroides* from Southeastern Asia [30] with calculations formulated for minimum and maximum divergence dates between †*M. insularis* and *Mus (Coelomys)* spp. The oldest †*Malpaisomys* fossils have been dated to 32 000 cal BP [30], which is taken as the age for the end of the size increase trend.

### 3. Results and discussion

A total of 1 616 176 nucleotides mapped to *Arvicanthis* nuclear genome, representing only a 0.0006× depth of coverage but proving that genomic retrieval of †*C. bravoi* is a possible—albeit a challenging—task.

**Table 1.** Mapping statistics of †*C. bravoi* mitochondrial and nuclear DNA reads. CB-4 corresponds to shotgun sequencing and CB-10 to mtDNA capture and sequencing. mtDNA reads were mapped following the procedure described in the Methods section; nuclear reads from CB-4 were mapped against the *Arvicanthis niloticus* nuclear genome (NCBI:txid61156).

| specimen (mtDNA) | sequenced read pairs | mapped reads | unique reads | q20 reads | BLAST reads | mapped bases | reference recovered |
|------------------|-----------------------|--------------|--------------|-----------|-------------|--------------|---------------------|
| CB-4             | 119 102 486           | 281          | 104          | 41        | 1826        | 10.78%       |
| CB-10            | 3 746 833             | 10 745       | 55           | 45        | 3387        | 7.21%        |

| specimen (nuclear DNA) | sequenced read pairs | mapped reads | unique reads | q20 reads | unique reads | mapped bases | average coverage |
|------------------------|----------------------|--------------|--------------|-----------|--------------|--------------|-----------------|
| CB-4                   | 119 102 486          | 111 084      | 40 434       | 35 593    | 1 616 176    | 0.0006X      |

**Figure 2.** Molecular phylogenetic tree of the Muridae with the mitochondrial DNA data. Median ages are indicated at the nodes while error bars (grey shading) at nodes correspond to the 95% highest posterior density (HPD) intervals of age estimates. Purple circles at nodes indicate posterior probabilities greater than 95%. NCBI codes for each mitogenome can be found at electronic supplementary material, table S1.
A total of 2627 mapped mtDNA nucleotides were aligned to a large dataset of rodent mitogenomes (table S1) and subsequently used for the phylogenetic analysis (table 1). The resulting dated phylogeny confidently places tC. bravoi within the Arvicanthus genus, in the A. niloticus species complex [30] (figure 2); it is closely related to a specimen from Masai Mara (Kenya) and is more distinctly related to a specimen belonging to the C2–C4 lineage that is distributed across the Sahel (both Arvicanthus specimens correspond to cryptic species in the A. niloticus species complex [31]). This unexpected placement parallels that of M. insularis, which was found to cluster between members of the genus Mus [32], despite its uncommon dental and skeletal traits. +Canariomys and +Malpaisiomys belong to different murine tribes (Murini and Arvicanthini), thus indicating that their origin must be traced to different ancestors and likely different colonization events (the former being much more recent).

The expansion of Arvicanthus species through North Africa was heavily influenced by Pleistocene climatic fluctuations [33,34]. When environmental conditions changed and the Sahara region dried up, different Arvicanthus populations were cornered in areas of grassland and savannah habitats far apart each other. The current patchy distribution of members of the A. niloticus species complex includes the Nile River up to the great African lakes, the whole strip of the Sahel and some isolated populations surviving in Pleistocene refuges such as the Hoggar mountains (southern Algeria) (figure 1b). A previous molecular study indicated that the A. niloticus species complex likely originated in eastern Africa as early as 2 Ma and differentiated in genetically distinct lineages from east to west as a result of Pleistocene climatic cycles [33].

The divergence time between tC. bravoi and its closest Arvicanthus relative is estimated at 650 000 years ago (95% HPD intervals: 373 000–944 000 years ago) (figure 2). This interval basically includes the Günz and Mindel glaciations, as well as the Günz–Mindel interglacial. This interglacial appears as the most probable period for the colonization of Tenerife by +Canariomys ancestors. Interglacial periods altered the monsoon regime and increased rainfall across Africa. Satellite images of the Draa ancient river bed, which altered the monsoon regime and increased rainfall across Tenerife (figure 1c) are assuming that the body size of the garden dormouse Eliomys quercinus [11] and generally well above values inferred for Pleistocene giant murines from several Mediterranean islands (usually 2–3 times heavier than their mainland ancestors [34,35]). It is also far greater than that calculated for +Malpaisiomys, which is almost four times heavier (90 g) than its mainland relatives (24 g) [30]. It is difficult to assess when the process of size increase was achieved because the age of the oldest tC. bravoi fossils is not well constrained and their earliest date for the start of gigantism is necessarily after the splitting from a mainland Arvicanthus lineage. According to our data, the resulting temporal range would suppose a yearly increase of mean body mass of just between 0.0015 g and 0.0023 g (considering maximum and minimum splitting dates, respectively). This corresponds to evolutionary rates (in darwins (d)), ranging from 7.09 d to 2.78 d that are well above those observed for non-insular mammals (usually less than 1 d). By contrast, calculated evolutionary rates for +Malpaisiomys are in the range of those of mainland mammals (0.22–0.16 d). We must remark that our +Canariomys evolutionary rate estimates represents a minimal estimate and assumes a constant rate of change. However, large body size may have not been achieved at a constant rate but soon after colonization and stabilized from then on. This would imply even a faster initial growing-size rate for +Canariomys.

However, only the retrieval of additional +Canariomys nuclear genome-wide data would further clarify its evolutionary history and would also allow the identification of the genomic regions under selection that might be responsible for the conspicuous physical differences observed between this extinct lineage and its living relatives.

Data accessibility. Genetic data generated are deposited in the Dryad database (project https://doi.org/10.5061/dryad.9s4mw6mhn).

Competing interests. The authors declare no conflict of interest.

Funding. C.L.-F. is supported by a PGC2018-095931-B-I00 grant (MICIU/AEI/FEDER, UE) of Spain; T.M.-B. is supported by funding from the European Research Council (ERC) (grant agreement no. 864203), BFU2017-86471-P (MINECO/FEDER, UE), ‘Unidad de Excelencia María de Maeztu’, funded by the AEI (CEX2018-000792-M), Howard Hughes International Early Career and Generalitat de Catalunya (CERCA Programme and 2017 SGR 880); I.C.-V. is supported by grant nos. 1-D1-2020-117289 (GB00 funded by MCIN/AEI/10.13039/501100011033/, the MINECO (RYC-2013-12470) and the Generalitat de Catalunya (CERCA Programme and 2017 SGR 116).

References

1. Juan C, Emerson BC, Oromi P, Hewitt GM. 2000 Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. Trends Ecol. Evol. 5, 104–109. (doi:10.1016/S0169-5347(99)01776-0)

2. Crusafont-Pairo M, Petter F. 1964 Un muriné géant fossile des îles Canaries: Canariomys bravoi gen. nov., sp. nov. (Rongeurs, Muridés). Mammalia 28, 608–611. (doi:10.1515/mamm.1964.28.4.607)

3. Michaux J, Hautier L, Hutterer R, Lebrun R, Guy F, García-Talavera F. 2012 Body shape and life style of the garden dormouse Eliomys quercinus (Rodentia, Muridae). J Zool Syst Evol Res 50, 17–28. (doi:10.1111/jzs.12003)
extinct rodent Canariomys bravoi (Mammalia, Murinae) from Tenerife, Canary Islands (Spain). C. R. Palevol. 11, 485–494. (doi:10.1016/j.crpv.2012.06.004)

4. Rando JC, Alcover JA, Galván B, Navarro JF. 2014 reappraisal of the extinction of Canarius bravoi, the giant rat from Tenerife (Canary Islands). Quat. Sci. Rev. 94, 22–27. (doi:10.1016/j.quascirev.2014.04.013)

5. Rando JC, Alcover JA, Navarro JF, García-Talavera F, Hutterer R, Michaux J. 2008 chronology and causes of the extinction of the lava mouse, Malpaisomys insularis (Rodentia: Muridae) from the Canary Islands. Quat. Res. 70, 141–148. (doi:10.1016/j.yqres.2008.04.012)

6. García-Garcérra M, Gili G, Sanchez-Quido F, Ramírez O, Calafell F, Coví S, Lahuzca-Fox C. 2011 fragmentation of contaminant and endogenous DNA in ancient samples detected by shotgun sequencing; prospects for human palaeogenetics. PLoS ONE 6, e24161. (doi:10.1371/journal.pone.0024161)

7. Ancochea E, Fuster JM, Ibarrola E, Cendrero A, Coello J, et al. 2018934111) Sci. USA Res. 1073/pnas.1314445110) reconstructed from ultrashort DNA fragments. Proc. Natl Acad. Sci. USA 111, 2229–2234. (doi:10.1073/pnas.1318934111)

8. Aghová T, Kimura Y, Bryja J, Dobigny G, Granjon L, Kergoat G. 2018 fossils know it best: using a new set of fossil calibrations to improve the temporal phylogenetic framework of murid rodents (Rodentia: Muridae). Mol. Phylogenetics Evol. 128, 98–111. (doi:10.1016/j.ympev.2018.07.017)

9. Bouckaert R et al. 2019 BEAST 2.5: an advanced software platform for Bayesian evolutionary analysis. PLoS Comput. Biol. 15, e1006650. (doi:10.1371/journal.pcbi.1006650)

10. Carøe C, Gopalakrishnan S, Vinner L, Mak SST, et al. 2010 endogenous ancient DNA from modern day contamination in a Siberian Neandertal. Science 329, 772–780. (doi:10.1038/molbev/MSt010)

11. Katoh K, Standley DM. 2013 Mafft multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 770–780. (doi:10.1093/molbev/msx10)

12. Posada D. 2008 JModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256. (doi:10.1093/molbev/msn083)

13. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. 2006 Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, e88. (doi:10.1371/journal.pbio.0040088)

14. Heled J, Drummond AJ. 2009 Bayesian inference of species trees from multilocus data. Mol. Biol. Evol. 27, 570–580. (doi:10.1093/molbev/msp274)

15. Jacobs LL, Flynn LJ. 2005 of mice again: the Siwalik rodent record, murine distribution, and molecular clocks. In Interpreting the past: essays on human, primate, and mammal evolution in honor of David Pilbeam (eds DE Lieberman, RJ Smith, J Kelley), pp. 373–408. New York: Columbia University Press.

16. Gonaads D. 2001 Rongeurs du Mioéenne supérieur de Ch’torona, Ethiopie: Murininae, Dendromurinae et Conilurinae. J. Zool. Syst. Evol. Res. 39, 241–249. (doi:10.1111/j.1471-7214.2001.tb00004.x)

17. Kitchan ES, Cazalla A, Colombo A, Coelho J, Hamén F, Cantargel JM, Jamond C. 1990 Volcanic evolution of the island of Tenerife (Canary Islands) in the light of new K-Ar data. J. Volcanol. Geotherm. Res. 44, 231–249. (doi:10.1016/0377-0273(90)90019-C)

18. Crowley BE, Yanes Y, Moshes SG, Rando JC. 2019 Revisiting the foraging ecology and extinction history of two endemic vertebrates from Tenerife, Canary Islands. Quaternary 2, 10. (doi:10.3390/qua2010010)

19. Dabney J et al. 2013 Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. Proc. Natl Acad. Sci. USA 110, 15 758–15 765. (doi:10.1073/pnas.1304451110)

20. Carre C, Gopalakrishnan S, Vinner L, Mak SST, Sindling MHS, Samaniego JA, Wales N, Sicelitz-Pontén T, Gilbert MTP. 2018 Single-tube library preparation for degraded DNA. Methods Mol. Biol. Evol. 9, 410–419. (doi:10.1111/j.2041-210X.12871)

21. Kircher M, Sawyer S, Meyer M. 2012 Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res. 40, e3. (doi:10.1093/nar/gkr771)

22. Skoglund P, Northoff BH, Shunkov MV, Demirvani AP, Pääbo S, Krause J, Jakobsson M. 2014 Separating endogenous ancient DNA from modern day contamination in a Siberian Neandertal. Proc. Natl Acad. Sci. USA 111, 2229–2234. (doi:10.1073/pnas.1318934111)

23. Monadjem A, Taylor PJ, Denys C, Cotterell FFP. 2015 Rodents of Sub-Saharan Africa. A biogeographic and taxonomic synthesis. Berlin, Germany: De Gruyter.

24. Moncunill-Solé B, Jordana X, Marin-Moratalla N, Moyá-Sola S, Köhler M. 2014 How large are the extinct giant insular rodents? New body mass estimations from teeth and bones. Integr. Zool. 9, 197–212. (doi:10.1111/1749-4877.12063)

25. Haldane JBS. 1949 Suggestions as to quantitative measurement of rates of evolution. Evolution 3, 51–56. (doi:10.1111/j.1558-5646.1949.tb00004.x)

26. Gingerich PD. 1983 Rates of evolution: effects of time and temporal scaling. Science 222, 159. (doi:10.1126/science.222.4620.159-a)

27. MacFadden BJ. 1986 Fossil horses from ‘Eophippus’ (Hyracotherium) to Equus: scaling, Cope’s Law, and the evolution of body size. Paleobiology 12, 355–369. (doi:10.1006/palo.2000.03109)

28. Hutterer R, López-Martínez N, Michaux J. 1998 A new rodent from quaternary deposits of the Canary Islands and its relationships with Neogene and recent murids of Europe and Africa. Palaeovertebrata 24, 241–262.