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## **First asserted record of the house mouse in Morocco: application of a multidisciplinary approach to the site of Rirha (5th − 1st c. BC)**

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#### **Abstract**

The biogeographic and demographic history of the house mouse, *Mus musculus domesticus*, has been closely linked to human history since pre-Neolithic times – this species first appeared in the Near East and subsequently dispersed westward with humans into regions with local *Mus species*. However, if this issue is now well known for the eastern Mediterranean, data are still lacking for the western Mediterranean, and especially for north-western Africa. In the present study, we combine morphological, geometric morphometric and genetic analyses to *Mus* remains from the Moroccan site of Rirha, dating to the Mauretanian period (5th to 1st century BC). Thanks to this multidisciplinary approach, we were able to confirm the first asserted record of the house mouse in a well-established archaeological context in the Maghreb. The morphometric and genetic results are largely congruent, with a single discrepancy observed for one specimen, which may be linked to possible hybridization between *M. m. domesticus* and *M. spretus* species. Further analyses are required on material from other North African sites dating to earlier chrono-cultural periods to better document the oldest appearance and the dispersal of the house mouse in the south-western Mediterranean through time.

**Keywords :** *Mus musculus domesticus,* Molar morphology, Geometric morphometrics, Palaeogenetics, Morocco, Mauretanian period.

#### **INTRODUCTION**

The biogeographic and demographic history of certain animal species can be closely linked to human history. This is the case, for example, of anthropophilic and commensal species, which have been able to adapt to new ecological niches offered by humans, and then expand geographically and demographically thanks to humans (e.g., Dobson 1998; Grayson 2001; Hulme-Beaman et al. 2016).

Probably one of the best-known examples among vertebrates is the house mouse (*Mus musculus domesticus*), which is thought to have begun developing commensal behaviour around 15,000 years ago in the Levant, shortly before the development of agriculture in the region (Weissbrod et al. 2017). The emergence of commensal behaviour was probably linked to the sedentarization of human populations and the development of villages, bringing several advantages for these small animals, such as access to waste and food stocks and some protection from predators and weather (Weissbrod et al. 2017). *Mus musculus domesticus* would then have dispersed from the Levant in the eastern Mediterranean region, spreading to Cyprus and Anatolia between 11,000 and 9,000 cal BP and towards Transcaucasia between 5,000 and 4,000 cal BP (Cucchi et al. 2005, 2020). For the western Mediterranean region, both the fossil record and genetic data of extant populations indicate that it expanded after 3,000 years ago ( $\approx$  1000 BC), favoured by the intensification of trade and navigation along the Mediterranean by Phoenicians, as well as human population expansion (Bonhomme et al. 2011; Cucchi et al. 2005, 2020; Jones et al. 2013; Domínguez García et al. 2019). However, recent studies suggest that the house mouse could have arrived even earlier (4,400–4,100 cal BP) to northern Iberia, following an alternative route through inland Europe (Álvarez-Vena et al. 2023). The dispersal to north-western Africa could then have occurred either by sea from Iberia, or through parallel dispersal events on both sides of the Mediterranean. While these dispersal processes are already well known for the eastern Mediterranean (Cucchi et al. 2020), this is not the case for the western Mediterranean, in particular for the southern shore (North Africa), for which palaeontological data are scarce.

In north-western Africa, another mouse species occurs today: the Algerian mouse or short-tailed mouse, *Mus spretus*. The biogeographic history of this species is also related to the dispersion of human groups. Originating from north-western Africa, and known in this region since the Middle Pleistocene (Stoetzel 2013), *Mus spretus* would have colonized south-western Europe during the Middle Holocene, probably translocated by maritime transport through the Strait of Gibraltar (e.g., Domínguez García et al. 2019; Lalis et al. 2019). In some areas, prior to the arrival of the house mouse, some wild species, such as the Algerian mouse (*Mus spretus*) or wood mice (*Apodemus* spp.), are thought to have developed anthropophilic behaviours before being supplanted by the much more competitive house mouse (Cucchi et al. 2005; Weissbrod et al. 2017).

Concerning the palaeontological record of north-western Africa, studies published prior to the 2000s have mistakenly attributed the remains of the Algerian mouse (*Mus spretus*) to *Mus musculus*, contributing to the absence of ascertained identifications of the house mouse from securely dated archaeological sites in this region (Stoetzel 2013; Stoetzel et al. 2013). Moreover, the morphological proximity of the two species can sometimes complicate identifications, when based on conventional morphology alone, meaning further analyses are required, notably geometric morphometric methods (Cucchi et al. 2006, 2011; Michaux et al. 2007; Valenzuela-Lamas et al. 2011; Stoetzel et al. 2013; Moclán et al. 2023). In addition to the morphometric methods, palaeogenetic analyses have become a powerful tool for archaeological and palaeontological research in the last decades (e.g., Utge et al. 2020), particularly for small mammal records, and numerous studies have provided valuable insights concerning their taxonomy and phylogeography (e.g., Baca et al. 2019, 2020; Cucchi et al. 2020; García-Rodríguez et al. 2021; Alfaro-Ibañez et al. 2023).

A recent paper focusing on excavated layers dating to the 1st century BC from the site of Rirha in northern Morocco mentions the presence of both *M. spretus* and *M. m. domesticus* on the basis of conventional morphological characters (Oueslati et al. 2020). This would represent the first asserted record of the house mouse in a well-established archaeological context in the Maghreb, except that the identifications remain uncertain because of the method used.

The present study aims to validate the identification of *Mus* spp. remains from Rirha by applying a multidisciplinary approach using geometric morphometric (GMM) analyses of the first lower molar (m1) outline shape combined with Machine Learning (ML) models (Moclán et al. 2023). In addition, palaeogenetic analyses were performed to verify the morphometric-based taxonomic identification of the material, with the final objective being to better document the dispersal of the house mouse in south-western Mediterranean and its co-occurrence with the Algerian mouse.

#### **MATERIALS AND METHODS**

#### **The site of Rirha**

The site of Rirha (34°18'37"N 5°55'52"W) lies on an artificial mound, created by successive layers of human occupations through the centuries, in a meander of the wadi Beht in the Gharb plain (Fig. 1). Ongoing excavations have been undertaken every year since 2005 (Callegarin et al. 2016). The earliest Mauretanian levels have been dated by associated ceramics to the 5th century BC, and the occupation continued without interruption until the 1st century BC. Radiocarbon dating has provided ages of between  $\sim$  410 and  $\sim$  110 cal BC (Table 1). Excavation has revealed that the structures are directly overlaid by Roman walls.

The Mauretanian-era levels consist of a group of buildings constructed exclusively from unfired brick corresponding to a residential block. This sector has benefited from detailed excavation, systematic sampling for palaeoenvironmental studies and soil analysis through micro-morphology. Currently, levels older than the 2nd century BC are yet to be excavated; therefore, we can expect new data to appear from an older time span during successive campaigns.

#### **Study material**

The studied material comes from the Mauretanian Tell at Rirha. Small murid assemblages were sampled from well-stratified strata and the sediments were sieved using 0.5 mm mesh. The material was often associated with living floors or building and construction levels with characteristic mud brick architecture containing well-dated ceramics.

Although all the cranio-dental elements were counted, for this study we only focused on the molars – in archaeological and palaeontological contexts whole skulls and mandibles are rare or absent and cannot be used for reliable identification to species level. The first attempt at species attribution was made on the basis of molar morphological characters according to Darviche and Orsini (1982) and Darviche et al. (2006). The main characteristics to distinguish *M. spretus* and *M. m. domesticus* can be summarized as follows: the major axis of the t1 of the M1 is rather oblique in *M. m. domesticus*, while it is much less oblique and backwardly oriented in *M. spretus*; the anterior part of the m1 is trilobate in *M. m. domesticus* (tE reduced compared with tF) and tetralobate in *M. spretus* (tE well individualized); the presence of a cingulate margin with an accessory tubercule (c1) on the labial side of the m1 is frequent in *M. spretus* and rare in *M. m. domesticus*; on the m2, a cingulum (vestigial tE) is sometimes observed on the antero-labial base of the crown in *M. m. domesticus*. This preliminary identification work was published in Oueslati et al. (2020) and is here compared to our geometric morphometric and genetic data.

A modern reference sample was built, including 375 first lower molars (m1) of *M. m. domesticus*  and *M. spretus* (Table 2) – m1 was chosen as it has been proved to be the most useful tooth for identifying mice taxa of the genus *Mus* (Cucchi et al. 2006; Darviche et al. 2006). We photographed specimens of different populations from south-western Europe (France, Iberian Spain, Canary Islands, Mallorca and Switzerland) and North Africa (Algeria, Morocco and Tunisia), stored in the collections of various institutions: Estación Biológica de Doñana (EBD - Sevilla, Spain), Institut des Sciences de l'Evolution de Montpellier (ISEM – Montpellier, France), Museo Nacional de Ciencias Naturales (MNCN – Madrid, Spain) and the Muséum national d'Histoire naturelle (MNHN – Paris, France), as well as those captured during field campaigns in Morocco by the French ANR project "Modern Human installation in Morocco, influence on the small terrestrial vertebrate biodiversity and evolution" (MOHMIE), stored at the MNHN. Some of these specimens are genotyped (ISEM and MOHMIE), thus providing precise taxonomic identification. Classification of the reference material was carefully reviewed following the cranial and external criteria commonly used to discriminate between both species (Britton et al. 1976; Darviche et al. 2006; Darviche and Orsini 1982; Gerasimov et al. 1990). See supplementary material (Table S1) for detailed catalogue information for each specimen.

The archaeological material studied in this work consisted of 38 first lower molars (m1) from the site of Rirha (Table 2). Only complete and unaltered molars were selected. Broken teeth or those showing advanced tooth wear were excluded from the GMM analysis. Nine m1s were selected for palaeogenetic analyses.

#### **Geometric morphometrics**

To obtain reliable taxonomic identifications of the archaeological material assigned to *Mus* spp. from the site of Rirha, Geometric Morphometric (GMM) analysis was performed. To minimize wearrelated variation, GMM techniques were applied to 2D images of the occlusal view to obtain the outline of each m1 included in this study (Renaud et al. 1996). To ensure consistency in the shape of outlines and the positioning of the landmark and semi-landmarks, particular attention was given to maintaining the occlusal surface of the tooth in a horizontal orientation during the photographic process (Fox et al. 2020). Following the procedure described by Cucchi et al. (2013, 2020) and Moclán et al. (2023), we digitized one landmark located at the furthermost point in the anterior axe and 63 sliding semi-landmarks equally spaced along the crown's external outline of the molar using tpsUtil v.1.76 (Rohlf 2018) and tpsDig v.2.32 (Rohlf 2010). Generalized Procrustes Analysis (GPA) was then carried out using the minimum bending-energy criterion for sliding semi-landmarks (Green 1996; Bookstein 1997) using tpsRelw v. 1.70 (Rohlf 2019). To explore the relationship between shape and taxonomy, Principal Component Analysis (PCA) was conducted on the Procrustes coordinates of each specimen, resulting in the PC scores being employed as molar shape variables in the subsequent analyses.

As previously shown (Courtenay et al. 2019; Bellin et al. 2021), ML algorithms are a powerful system of data classification when combined with GMM techniques, particularly in *Mus* classification tasks (Moclán et al. 2023). Here, we combined GMM data with ML techniques following the proposal of Moclán et al. (2023) to classify the archaeological material from Rirha. A complete description of the methods is available in the original paper of Moclán et al. (2023). The supplementary code was developed in R (R Core Team 2022) with 'caret' (Kuhn et al. 2020) and 'caretEnsemble' (Deane-Mayer and Knowles 2019; Deane-Mayer 2019) libraries.

A series of 11 algorithms were first trained with 82 PC scores obtained from the PCA – the PC scores were selected based on the number that did not exhibit collinearity. The algorithms used were Neural Networks (NNET), Linear Support Vector Machines (SVMl), Radial Support Vector Machines (SVMr), *k*nearest neighbour (*k*NN), Logistic Regression (LG), Decision Trees using the 5.0 algorithm (DTC5.0), Random Forest (RF), Gradient Boosting (GB), Naïve Bayes (NB), Linear Discriminant Analysis (LDA) and Partial Least Squares (PLS). Ensemble learning (EL) techniques were then employed to train new metalearners from the 'caret' models, as these techniques usually produce a better performance than "simple" base learners (Opitz and Maclin 1999; Dietterich 2000; Rokach 2010; Sagi and Rokach 2018). Thus, we trained a Generalized Linear Model (Ensemble: GLM) and three stacked models: a Neural Network, a Random Forest and a Gradient Boosting algorithm.

The training process was developed using a typical sequence – dividing the sample into training (70%) and testing (30%) subsamples and using cross-validation as the control method (base learners = 10-folds, 10 repeats; meta-learners = 11-folds, 10 repeats). Accuracy and kappa values were considered in combination with sensitivity, specificity and balanced accuracy to evaluate the algorithm performance. Note that as we have improved the modern sample of *Mus* in comparison with Moclán et al. (2023), we have also retrained the models using the 'tunelengh = 20' function of 'caret'.

The final classification of *Mus* remains from Rirha was achieved using a combination of the results provided by the ensemble/stacked models following the criteria of Moclán et al. (2023). As a further precaution, we classified the difference between those samples with a posterior probability value higher than 0.9 (Cucchi et al. 2013).

#### **Palaeogenetic analyses**

The methodology used for the taxonomic identification of archaeological remains was based on the use of real-time Polymerase Chain reactions (PCRs) and TaqMan MG probes (Kutyavin et al. 2000), which allowed an amplification of the specific and diagnostic genomic regions for the determination or comparison between the study species (Elalouf 2022; Utge et al. 2020). Compared to conventional PCRs based on the use of primers targeting the amplified DNA region, the TaqMan method also includes a probe that hybridizes to the recopied DNA fragment. The DNA polymerase, which copies the DNA from the primer, has a 5'–3' exonuclease activity that cleaves the probe. Once this cleavage has taken place, the suppressor in the probe is far enough away from the fluorophore for the latter to emit a signal when agitated. The advantage of this method is the increased specificity compared with a simple PCR. While oligonucleotides allow the amplification of fragments other than the one required, these undesirable fragments will not hybridize the probe and therefore no spurious signal will be produced. In principle, this approach should detect only the desired fragment, thus avoiding the need for subsequent manipulations (gel purification, cloning, sequencing).

Primers and probes for the TaqMan MG assays (Table 3) were designed with the help of Geneious Primes 2021 software. In total, 95 reference sequences of the cytochrome B (cytB) for the genus *Mus*, within different haplotypes of *M. spretus* and *M. m. domesticus*, were aligned and used as a reference to obtain the TaqMan assays (Supplement material, Table S2).

Validation of the assays was conducted by qPCR with the DNA extracted from the tissue samples, preserved in alcohol, of contemporaneous specimens from the MNHN collection. The tissue sample of *M. m. domesticus* (ID: SPOT1308) came from a specimen collected in Marcq-en- Bareuil (France), and the tissue sample of *M. spretus* (ID: 21,001) came from a specimen collected in Montbazin (France). The DNA of both samples were extracted using the NucleoSpin Tissue kit (Macherey Nagel), following the steps suggested by the manufacturer and with a final elution of 100 μl. The concentrations of DNA obtained were measured with a Nanodrop 2000 (ThermoScientific) (Table 4).

The specificity and efficiency of the probe were then measured. The specificity of the probes was tested in a real-time PCR using the DNA of both samples with the two TaqMan assays. The efficiency was tested in a real-time PCR using, for each TaqMan probe, a DNA dilution series of 1:10 covering five dilution points for each sample. The real-time PCR was carried out in a 20-μl reaction volume containing 10 μl of 2X TaqMan fast advanced master mix (Thermo Fisher Scientific), 900 nM of forward and reverse primers, 250 nM of TaqMan probe, and water (PCR blank), with the DNA diluted extracts. Amplification was performed in a BioRad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratoires) in 50 °C, 2 min; 95 °C, 20 s; then 40 PCR cycles (95 °C, 3 s; 60 °C, 30 s). Data were analysed using CFX Maestro Software (Bio-Rad Laboratories) set with default parameters to determine the cycle threshold (*C*T), i.e., the number of PCR cycles required for the fluorescent signal to exceed the background level. Only samples with a CT value below or equal to 37 cycles were considered positive for DNA content.

Once the assays were verified, DNA extraction and the pre-PCR steps using the nine archaeological rodent remains were performed in a laboratory dedicated to ancient DNA studies – the Plateau de Paléogénomique et de Génétique Moléculaire at the Musée de l'Homme (P2GM platform). Ancient DNA (aDNA) samples were extracted from rodent hemi-mandibles. The total quantity of material per specimen ranged from 10 to 40 mg. Lysis was carried out by digesting the extracted complete teeth (without prior grinding), placing them in 1 ml of DNA extraction buffer (0.25 M EDTA, 10 mM Tris-EDTA (pH 8), 0.2% N-lauryl-sarcosyl, 200 μg/ml proteinase K) and then incubating for 16 h at 42 °C under constant agitation. The tubes were then centrifuged (5,000 g; 5 min) in an Eppendorf microcentrifuge, and the supernatant was transferred into an Amicon ultra- 0.5 ml 30 kDa centrifugal filter unit (Millipore; Burlington, MA, USA). The filter unit was centrifuged (14,000 g; 7 min), rinsed 4 times by adding 500 μl of ultrapure DNase/ RNase-free distilled water (Thermo Fisher Scientific) and centrifuged again (14,000 g; 7 min). The DNA extract was recovered as a  $\sim$  50-µl sample volume by centrifugation (1,000 g; 1 min) of the filter unit in the reverse position. The extract was further purified using a Qiagen minelute PCR purification kit (Qiagen; Venlo, Netherlands), according to the manufacturer's instructions, and eluted in 60  $\mu$ l of 10 mM Tris, pH 8. Quantification of the extracted DNA was performed using a Qubit HS kit (Invitrogen) (Table 5).

Real-time PCR was carried out in a 20-μl reaction volume, as before, using the TaqMan fast advanced master mix, which contains dUTP (instead of dTTP) and uracil- N-glycosylase to prevent carryover contamination from one experiment to another. For each sample, 3 to 4 serial dilutions corresponding to between 2 and 0.03 μl of the DNA extract were analyzed for each TaqMan assay. Only samples for which a CT value below or equal to 37 cycles were considered positive and the taxonomic identification was confirmed.

#### **RESULTS**

#### **Geometric morphometrics and machine learning**

The PCA applied to the shape data of modern samples and the archaeological material from Rirha shows that the first two principal components (PC1-PC2) captured 59.1% of the shape variation, providing a separation between both species with some overlapping (Fig. 2). The major shape difference in this plot is the relative development of t1 in the anterior part of the tooth. *M*. *m. domesticus* specimens are mainly located at the right part of the plot, showing positive values in PC1, while *M. spretus* clusters at the left part of the *x*-axis with mainly negative values. The archaeological specimens from Rirha show a non-clear distribution overlapping with both modern subsamples.

The results obtained from the ML algorithm training (Table 6) show high-classification ratios (Accuracy > 0.9; kappa = > 0.8) for the modern testing subsample. The highest accuracy value is shown by LDA (0.973), while the lowest is presented by SVMl (0.90) and DTC5.0, GB and NB (0.91). In general, the values of sensitivity (0.852–0.963) and specificity (0.912–1) are also very high. The final hyperparameter configuration of each algorithm is shown in Table 7.

When the correlation among algorithmic classification was tested (Table 8), the results showed that a general correlation between classifications is a rarity. Those correlations identified by the test were found between SVMl and LG (*r* = 0.77), SVMl and LDA (*r* = 0.58), LDA and LG (*r* = 0.61) and RF and GB (*r* = 0.78). These results allow us to consider ensemble and stacking techniques as extremely useful for classifying the archaeological samples.

The training process of the meta-learners provided extremely similar results to those delivered by the base learners. The lowest accuracy value is shown in the stacked RF (accuracy =  $0.946$ ; kappa = 0.892), while the highest value was provided by the ensembled GLM (accuracy = 0.964; kappa = 0.928). All the results provided perfect specificity values, high sensitivity values (0.88–0.926) and balanced accuracy values (0.944–0.963). In this sense, the highest accuracy value provided by a base learner had to be discarded (LDA accuracy = 0.973) due to the possible presence of overfitting in that specific model; therefore, the ensemble/stacked models were used to classify the archaeological samples.

The taxonomic assignment of the m1s from Rirha provided similar results among the different ensemble/stacked models (Table 9). The ensembled GLM indicated the presence of 16 *M. spretus*  specimens against 22 *M. m. domesticus* ones, out of the 38 analysed specimens. On the other hand, the stacked models highlighted the presence of 17 *M. spretus* and 21 *M. m. domesticus*. In all models, the vast majority of molars analysed were classified to one or another species with posterior probabilities (p) above the 0.9 threshold (GLM = 97.37%; NNET and GB =  $100\%$ ; RF = 92.11%). Even those specimens classified with *p* < 0.9 by some models were assigned by at least two to the same species with *p* > 0.9. Discrepancies between models were found in only one specimen (RHA06 US109 2), which was classified as *M*. *m*. *domesticus* by the ensembled GLM (*p* = 1), whereas stacking models assigned it to *M*. *spretus* (*p* = 0.79–0.99); it should therefore be considered as indeterminate.

To summarize the results of the ML classification, a total of 21 specimens were reliably assigned to *M. m. domesticus*, and 16 were classified as *M. spretus*, while just one specimen should be considered indeterminate. Considering the laterality of the analysed specimens, these belong to a minimum number of individuals (MNI) of 12 *M*. *m*. *domesticus* and 9 *M*. *spretus*.

#### **Palaeogenetic analyses**

DNA obtained from each of the nine archaeological samples was used in a real-time PCR where taxonomic identification was performed using the two proposed TaqMan assays. The results obtained from the qPCR (Table 10) show a strong correlation between GMM + ML and genetic determination. Four specimens had the same taxonomic assignment, while one specimen had a conflicting assignment. Three samples were not taxonomically determined using the proposed assays, as there was no qPCR amplification of the chosen citB gene region, possibly due to poor DNA preservation.

#### **DISCUSSION AND CONCLUSION**

This study is the first attempt to approach combining GMM + ML and palaeogenetics for the taxonomic identification of the genus *Mus* in the western Mediterranean region, following the model developed by Cucchi et al. (2020) for the Near East. It has provided a new case supporting the usefulness of this procedure in studying fossil small mammals.

The presence of both species, *M. spretus* and *M. m. domesticus*, at Rirha is clearly confirmed by GMM combined with ML, as well as palaeogenetics. GMM + ML analyses provided high-accuracy values for discrimination between both species (0.946–0.955, Table 6). These results are slightly lower than those achieved by Moclán et al. (2023) who reported a perfect classification of the testing/modern samples with a stacked NNET. However, this decrease in accuracy seems to be caused by the increase in the number of modern mice m1s employed in this study compared to the previous one. An effort has been made to add 72 new *Mus* spp. remains, including new populations such as *Mus spretus* from Algeria and Tunisia, and *Mus m*. *domesticus* from Switzerland. At the same time, the sample size of populations with low specimen numbers (*M*. *spretus* and *M*. *m*. *domesticus* from France) was increased. This approach, therefore, provided a greater morphometric variability, allowing us to improve the validity/applicability of the models. Nonetheless, the high accuracy values obtained are similar or even greater than those obtained by previous studies applying elliptic Fourier analysis to the outline shape and cross-validated linear discriminant analysis for classification (Michaux et al. 2007; Valenzuela-Lamas et al. 2011; Stoetzel et al. 2013), employing lower sample sizes for the modern referential dataset in some cases (Valenzuela-Lamas et al. 2011; Stoetzel et al. 2013).

Palaeogenetic analysis provided a taxonomic identification for one specimen that was excluded from the GMM + ML analysis (RHA05-US0029-5, Table 10), since the m1 was partially broken and displayed signs of digestion. However, another excluded specimen (RHA06- US104-3), which also showed signs of light digestion, was not identified by palaeogenetics. The remaining specimens that did not provide positive results by aDNA analysis (RHA06-US104-2, RHA06-US109-1) were classified as *Mus m*. *domesticus* by GMM + ML according to their m1 shape, as those molars were complete and displayed a good preservation status.

Overall, the two approaches concur, evidencing the high reliability of these methods for taxonomic identification. Among the analysed teeth (some examples are shown in Fig. 3), only two are problematic: RHA06 US109-2 (Fig. 3e), which was classified as indeterminate by GMM + ML and for which no palaeogenetic analysis was performed; and RHA06 US1106-1 (Fig. 3f), which showed discrepancies between the GMM + ML and palaeogenetics assignments (Table 10). This indicates that the classification achieved by GMM + ML is not perfect. Despite the high accuracy of ensemble/stacking models employed, low misclassification percentages (3.6–5.4%) were also obtained due to the elevated intraspecific variability of the two species (Darviche and Orsini 1982; Cucchi et al. 2002). If we analyse the morphology of this m1 (RHA06 US1106-1) in a traditional way, it displays a

moderately reduced t1 and a cingular margin showing an incipient c1 (Fig. 3f), which is rare in *M*. *m*. *domesticus* populations (Darviche et al. 2006). However, the modern reference database includes similar morphotypes, meaning that traditional morphological criteria must be used with caution. In addition, it is striking that all models provided a posterior probability of 1 for assignment as *M*. *spretus*  for RHA06 US1106-1 (Table 9). Another methodological issue that can explain this discrepancy is related to the incorrect positioning of the tooth when taking the picture. Although special care was taken in this regard, since changes in tooth orientation may lead to shape alterations (Fox et al. 2020), this tooth seems to have partially shifted to its anterior region. However, although we considered that this small shift in tooth orientation had not caused any significant changes in the shape of the tooth outline, this possibility cannot be ruled out. In addition, the possible hybridization between *M. spretus*  and *M. domesticus* (Orth et al. 2002; Banker et al. 2022), a phenomenon with poorly known effects on the phenotype (notably the dental morphology), may also explain the discrepancy between morphometric and genetic results in this specimen. This possibility could represent a new research direction. In conclusion, this single case of discrepancy in the classification observed between GMM + ML and palaeogenetics represents a challenging result, reinforcing the complexity in discriminating between sibling species characterized by overlapping morphometric traits. These results also highlight the importance of some methodological considerations such as the low misclassification rate or accurate tooth orientation, as well as the potential influence of the hybridization process between both species.

The site of Rirha indicates the existence of a strongly anthropized context, since it comprises a succession of consecutive settlements from the Mauretanian period to Roman times, and two medieval phases (Oueslati et al. 2020). The house mouse would have already benefited from the presence of a commensal niche at Rirha since 390–200 cal BC, providing a food supply and protection against climatic variations and predation. This is also consistent with the presence at the site of other commensal rodents such as *Rattus rattus* and the domestic exploitation of chickens since the first half of the 1st century BC (Oueslati et al. 2020). In addition, the accumulation of mouse remains and other small vertebrates including *Lemniscomys barbarus*, shrews and reptiles is linked to predation, probably by domestic cats (Oueslati et al. 2020). These wild taxa are represented in very low abundance in the assemblage, which also shows a lower diversity compared to the modern natural community of Morocco (Mediani et al. 2015; Aulagnier 2017). Following a comparison of both classification approaches, our sample shows a higher abundance of the house mouse (MNI = 13; %MNI = 61.9%) compared to the Algerian mouse (MNI = 8; %MNI = 39.1%). This abundance pattern can be attributed to their distinct ecological behaviours: *M. m. domesticus* is a typically commensal taxon, inhabiting areas near or within human settlements; conversely, *M. spretus*, while still benefitting from anthropogenic environments, tends to avoid direct proximity to human settlements (Denys et al. 2017). This ecological distinction appears to result in a gradual displacement of the Algerian mouse from the human-made habitats in the Rirha site and its immediate surroundings.

The multidisciplinary approach adopted by this study allowed us to confirm the first asserted record of the house mouse in a well-established archaeological context in the Maghreb. However, European genetic and archaeological data assume that the arrival of *M. m. domesticus* in the western Mediterranean should be dated to at least the 1st millennium BC (Bonhomme et al. 2011; Cucchi et al. 2005, 2020), much earlier than the Rirha occupations (1st − 4th century BC). We must therefore continue our investigations by applying a similar multidisciplinary approach to the older levels of Rirha, as well as other sites dated to earlier chrono-cultural periods (from the Phoenician up to the Neolithic), with well-established archaeological, stratigraphic and chronological contexts, to verify this hypothesis in north-western Africa.

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**Author contributions** A.C.D.G., J.U. and E.S. designed, conceptualized the research, and wrote the original draft. A.C.D.G., J.U., C.L. and A.M. performed the analyses. T.O., L.C., C.A.D.C. and E.R. carefully proofread the article and helped to write certain sections. T.O. conducted the zooarchaeological identification of micromammals and provided the photos for the GMM analysis. M.K.A., L.C., C.A.D.C. and E.R. are the directors of the excavation team at the Rirha site. E.S. supervised and coordinated the project. All authors approved the final version of the manuscript and agree to be responsible for its content.

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**Data availability** All data supporting our results are given in the manuscript and in the supplementary material.

## **Declarations**

**Competing interests** The authors declare no competing interests.

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## **FIGURES**



**Figure 1.** Location of the Rirha site in northern Morocco (top left) and plan of the remains with location of the main Mauretanian quarter 'Ensemble 5' (© Rirha Project).



**Figure 2**. Biplot of the first two Principal Components of the modern samples and the specimens from Rirha.

### **Principal Component Analysis**



**Figure 3.** Selected *Mus* spp. m1 from Rirha in occlusal view. *Mus musculus domesticus*: a) RHA07- US5143-16, b) RHA07 US5143-6, f) RHA06-US1106-1; *Mus spretus*: c) RHA06-US5044-3, d) RHA06 US5044-1; indeterminate (GMM+ML, palaeogenetic analysis not performed): e) RHA06 US109-2; coincident classification between GMM+ML and palaeogenetic: a) and c); palaeogenetic analyses not performed: b) and d); discrepancy between GMM+ML and palaeogenetic identifications: f). Scale bar  $= 1$  mm.

### **TABLES**



**Table 1.** Radiocarbon ages obtained for Rirha (Poznań Radiocarbon Laboratory). Here, intervals of calendar age are provided, where the true ages of the samples encompass the probability of ca. 68% and ca. 95%. Calibration was made using the Intcal 20 curve of atmospheric data (Reimer et al. 2020) and OxCal v4.4 (Bronk Ramsey 2009).



**Table 2.** Modern reference and archaeological samples used in this study, according to species, populations (location), collections and number of specimens studied (*n*).



**Table 3.** TaqMan assays designed for the taxonomic identification of *Mus spretus* and *Mus musculus domesticus* archaeological remains.



**Table 4.** MNHN collection tissue samples used for DNA extraction.



**Table 5.** DNA extraction from archaeological remains. *M. m. d.* = *Mus musculus domesticus, M.s.* = *Mus spretus*.



**Table 6.** Results provided by ML and EL algorithms with the modern samples of *M. m. domesticus* and *M. spretus*.

<b>NNET</b>	
Size	11
Decay	0.00464159
<b>SVMI</b>	
C	$\overline{1}$
<b>SVMr</b>	
Sigma	0.00677087
C	32768
kNN	
k	17
RF	
mtry	14
<b>GB</b>	
n.trees	700
interaction.depth	2
shrinkage	0.1
n.minobsinnode	10
<b>NB</b>	
fl	0
usekernel	False
adjust	$\mathbf{1}$
<b>PLS</b>	
ncomp	4
<b>Stacking: NNET</b>	
Size	11
Decay	0.03162278
<b>Stacking: RF</b>	
mtry	4
<b>Stacking: GB</b>	
n.trees	350
interaction.depth	16
shrinkage	0.1
n.minobsinnode	10

**Table 7.** Hyperparameter configuration of the best tunned ML algorithms.



**Table 8.** Results obtained after calculating the pairwise correlation of the different algorithms applied to the modern sample. \* = presence of correlation between algorithms.





**Table 9.** Classification results of the different *Mus* individuals from Rirha using each of the ensemble/stacking models. *M. s.* = *Mus spretus*; *M. m. d.* = *Mus musculus domesticus.* Specimens showing discrepancies in their taxonomic attribution are highlighted in grey.



**Table 10.** The qPCR results for the nine archaeological remains using the TaqMan assays and comparison with GMM+ML classification results. *M. s.* = *Mus spretus*; *M. m. d.* = *Mus musculus domesticus.*, - = indeterminate, \* = morphotype according to traditional morphology (Darviche & Orsini, 1982; Darviche et al. 2006) of specimen excluded from GMM+ML analyses. The specimen showing a discrepancy in its taxonomic attribution is highlighted in grey.

## **SUPPLEMENTARY MATERIAL**

**Table S1. Specimen ID number and data** of modern *Mus musculus domesticus* and *Mus spretus* material used in this study – EBD: Estación Biológica de Doñana, Sevilla; ISEM: Institut des Sciences de l'Evolution de Montpellier; MNCN: Museo Nacional de Ciencias Naturales, Madrid; MOHMIE: project MOdern Human installation in Morocco; MNHN: Museum National d'Histoire Naturelle.



















**Table S2.** Ninety-five cytB reference sequences of *Mus* genus used to design the TaqMan probes of *Mus musculus domesticus* and *Mus spretus*.





