

## Article

# Long Eared Owls (*Asio otus* Linnaeus, 1758) as Field-Assistants in an Integrative Taxonomy Survey of a Peculiar *Microtus savii* (Rodentia, Cricetidae) Population

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**Abstract:** Voles from the Genus *Microtus* are rodents with a subterranean lifestyle. Central Italy is populated by two species of the Genus, the North-Western *Microtus savii* and the South-Eastern endemic *Microtus brachycercus*. Although morphometric features able to help distinguish *M. savii* from *M. brachycercus* are lacking, a peculiar morphology of the anterior end of the first lower molar is typical, but not exclusive, of *M. brachycercus*. Since some skulls with a compatible dental morphology were collected from Long-eared owl pellets in Umbria (Central Italy), a genetic assessment was deemed necessary to verify the possible presence of *M. brachycercus* outside of its known distribution range. Hemimandibles were isolated and used for both genetic and morphometric dental analyses. Despite the fact that this could suggest the presence of a *M. brachycercus* population, genetic analyses confirmed the species attribution to the *M. savii* complex, showing peculiar genetic polymorphisms that can either be due to the wide sampling area, represented by the owls' hunting grounds, or to the recent evolutive history of the analysed population. This study, once again, underlines the importance of integrative taxonomy, suggesting the key role of predators as field-assistants to minimize the impact of sampling campaign on natural populations.

**Keywords:** *Microtus*; owl pellets; comparative morphology; morphometric analysis; dental morphology; mtDNA



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## 1. Introduction

As observed in many species from the Genus *Microtus* (Schränk 1798), the systematics of the *Microtus savii* group have long raised controversies to the extent that some authors ascribe it to the Genus *Terricola* [1]. Therefore, even its geographical distribution has been differently reported by different authors throughout the years. In this study, the term *Microtus savii* group is used to regroup three species: *Microtus brachycercus* (Lehmann 1961), *Microtus nebrodensis* (Minà-Palumbo 1868) and *Microtus savii* (de Selys-Longchamps 1838). *M. savii* is distributed throughout continental Italy, whereas *M. nebrodensis* is reported from Sicily. Amori and Castiglia [2] include *M. nebrodensis* in the list of Italian endemic species, considering it a separate species, distributed throughout Sicily, with the exclusion of the Etna's slope [3]. *M. s. brachycercus*, from Calabria (Southern Italy) was at first described as

a new subspecies of *M. savii* and lately considered a valid species based at first on karyological differences and later with an integrative taxonomy approach [4–6]. Several studies based on mtDNA haplotype characterizations showed that the northernmost limit of *M. brachycercus* reaches Central Italy [3,6]. The taxonomical situation was further complicated when the *M. savii* group was found throughout the Iberian Peninsula where Ellerman and Morrison-Scott [7] reported a series of subspecies, the possible presence of the group in Greece and reported *M. s. savii* in continental Italy and *M. s. nebrodensis* in Sicily. Corbet [8] reported *M. savii savii* in continental Italy (except Calabria), *M. s. pyrenaicus* in France and Northern Spain, *M. s. nebrodensis* in Sicily, *M. s. brachycercus* in Calabria and *M. s. felteni* in Macedonia and former Yugoslavia. However, he stated that: “The allocation of all these to *Pitymys savii* [= *M. savii*] is very tentative” and he highlighted the French subspecies, considered as a valid species by Saint Girons [9], and the Balkan subspecies, according to Petrov et al. [10] “may be specifically distinct”. Amori et al. [11] consider *M. savii* and *M. brachycercus* as valid species but do not report the distribution map of the two species because haplotypes of *M. brachycercus* were also found in other Italian regions [3]. On the other hand, accounting for the presence, at least in Calabria, of *M. brachycercus*, and accounting for the significant genetic divergence given by geographical isolation of Sicilian populations, the affinity of the latter with *M. savii* does not seem very plausible. Shortly after, other authors [3,6] also considered *M. savii*, *M. brachycercus* and *M. nebrodensis* as valid species. According to these authors, *M. savii* is distributed in Central (mainly in the Western part) and Northern Italy, *M. brachycercus*, in Central (mainly in the Eastern part) and Southern Italy, and *M. nebrodensis* is a Sicilian endemic species with distributions confirmed by other literature [2,12,13]. According to the above-reported data, specimens collected throughout the Umbria region (Central Italy) may assume particular importance as Central Italy represents an interesting field of research to better delineate the northern limit of *M. brachycercus*. To better understand this phenomenon, large samples from several geographical regions are required but, being both nocturnal and hypogean animals, collecting samples of *Microtus* requires significant and invasive trapping efforts. On the other hand, several nocturnal raptor species rely on the daily consumptions of rodents, voles in particular [14–16]. Collecting owl pellets represents therefore an opportunistic, cost effective and non-invasive way to retrieve rodent samples to better study vole populations also because skulls retrieved from pellets allow for both morphological and genetic analysis without the need to purposely trap and sacrifice wild rodents, allowing researchers to reduce their impacts on wild populations [17,18].

A preliminary analysis (Gaggi and Paci, unpublished material) found some specimens from Umbria (Central Italy) with an open anterior loop of the first lower molar ( $m_1$ ), a feature widely represented but not exclusive to Southern vole populations nowadays ascribed to *M. brachycercus*. The anterior loop of the first lower molar ( $m_1$ ) is generally more open in the *M. savii* southern group ( $28.08 \pm 7.94\%$ ) than in the *M. savii* northern group ( $24.80 \pm 7.38\%$ ) [19]. Therefore, if a sample with a closed anterior loop consists predominantly of *M. savii* and a sample with an open anterior loop consists predominantly of *M. brachycercus*, the palatine length should be greater in a sample with an open anterior loop than in a sample with a closed anterior loop. This scenario is further supported by the findings of Bezerra and colleagues [6] that reported a significant difference in the palatine length between *M. brachycercus* ( $12.06 \pm 0.85$  mm) and *M. savii* ( $11.24 \pm 0.61$  mm). Further odontometric, craniometric and genetic analyses on a designated subsample were considered necessary as voles show great variability in dental morphology, and it is frequent that some individuals share morphometrical traits typical of other species [20–22].

According to these data, Central Italy represents a possible site of sympatry for *M. savii* and *M. brachycercus* and interesting area of research to better delineate the northern limit of *M. brachycercus* and to unravel the taxonomy of these local populations. A joint effort involving morphometrical and genetic analyses carried out on skulls retrieved from owl pellets was deemed necessary to assess the possible presence of a population of *M. brachycercus* out of its known distribution range. In fact, to carry out animal biology studies of elusive

cryptic populations, the first step must be the specific identification of the animals, reliably attributing them to defined OTUs.

## 2. Materials and Methods

Skulls and mandibles morphologically ascribable to the *Microtus savii* group were retrieved from Barn owl (*Tyto alba* Scopoli 1769), Little owl (*Athene noctua* Scopoli 1769), Long-eared owl (*Asio otus* Linnaeus 1758) (Figure 1) and Common kestrel (*Falco tinnunculus* Linnaeus 1758) pellets collected in different locations selected for morphological analysis.



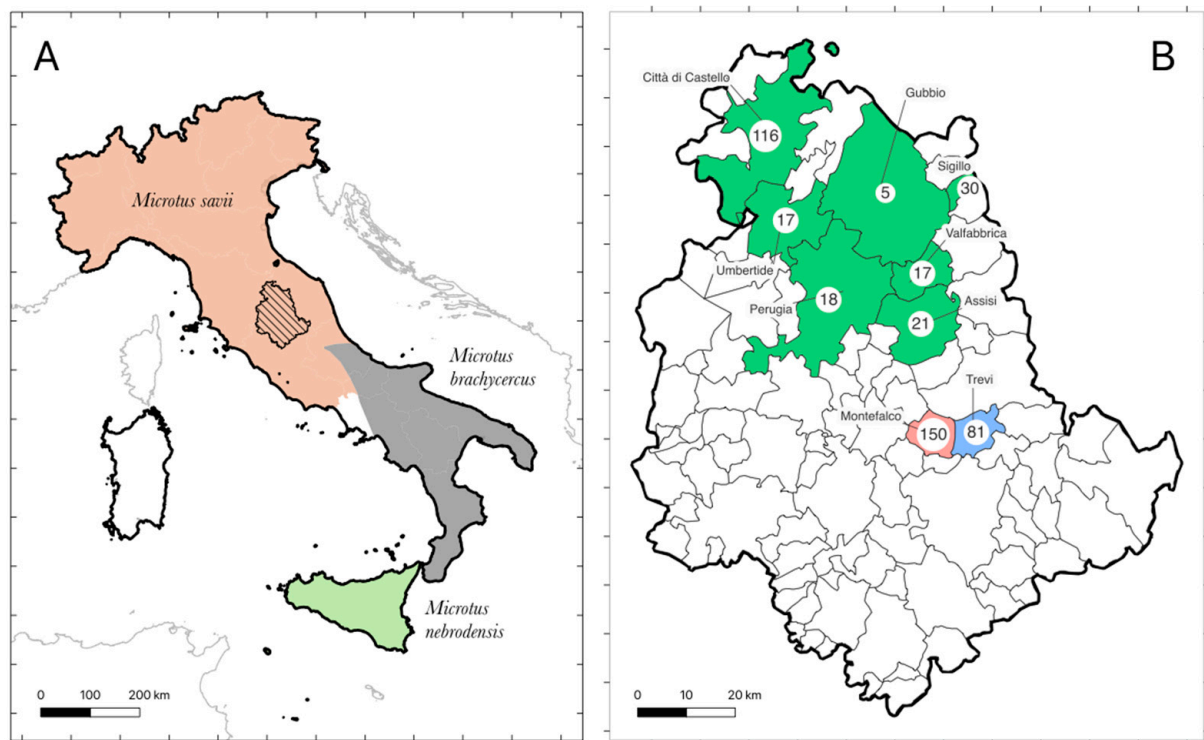
**Figure 1.** (A) Entire Long-eared owl pellet sampled under the Montefalco roost; (B) bones of at least two prey items retrieved from pellets (scale bar is 2 cm).

Bones found in pellets from all the raptor species were used for morphometrical analyses, whereas a subsample of bones found in pellets from *A. otus* was used for genetic analysis of a specific population.

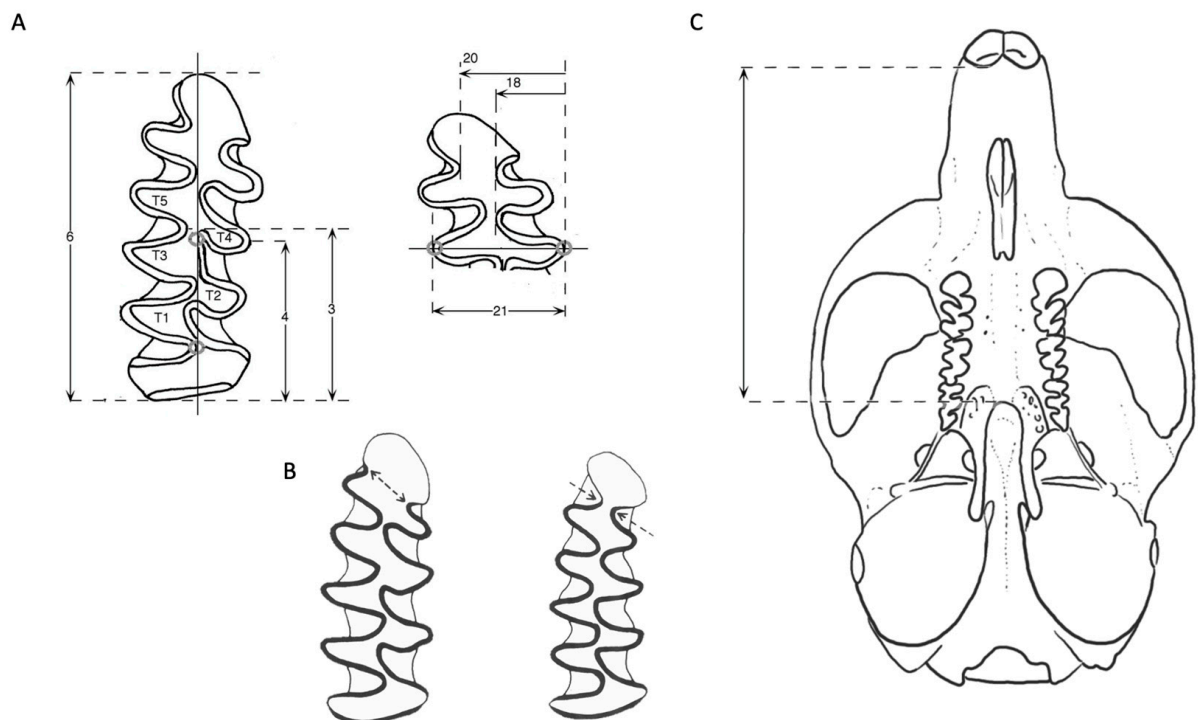
For the odontometric analysis, a total of 455 first lower molars (both right and left) belonging to 3 geographical populations sampled across the Umbria region (Figure 2) were analysed.

Six measurements, v3, v4, v6, v18, v20 and v21 (Figure 3A), were taken on the occlusal surface of m<sub>1</sub> [19,23,24] using a Nikon Measuring Microscope MM-60 (precision 1 µm) [25]. Three indexes were calculated: the development of the anterior part of m<sub>1</sub> [ $AP = (v6 - v3)/v6 * 100$ ], the tilt of the pitomyan rhombus ( $PR = v4 - v3$ ) and the closure of the anterior loop ( $AL = (v20 - v18)/v21 * 100$ ). Four criteria were analysed by means of a PCA analysis combined with a multivariate analysis of variance (MANOVA) and one-way analysis of variance (ANOVA) completed by a Bonferroni's test when necessary (ANOVA  $p < 0.05$ ): total length of m<sub>1</sub> (TL), the development of the anterior part (AP), the tilt of the pitomyan rhombus (PR) and the closure of the anterior loop (AL) (Figure 3B). For the palatilar length analysis (Figure 3C) a subsample of 41 specimens retrieved between 2011 and 2012 under an *A. otus* roost from Montefalco was analysed.

The comparison of the palatilar length between anterior loop groups (open AL vs closed AL) was performed using the Wilcoxon–Mann–Whitney test. In that subsample, 12 specimens presented an m<sub>1</sub> with a closed anterior loop ( $\leq 20\%$ ), 10 specimens presented an m<sub>1</sub> with a moderately closed anterior loop ( $[20, 25] \%$ ), 8 specimens presented an m<sub>1</sub> with a moderately open anterior loop ( $[25, 30] \%$ ) and 11 specimens presented an m<sub>1</sub> with an open anterior loop ( $\geq 30\%$ ). Within the subsample, 33 specimens (Figure 4) were destined for mtDNA analysis in order to see if specimens with open anterior loops of m<sub>1</sub> belonged to *M. brachycercus*.

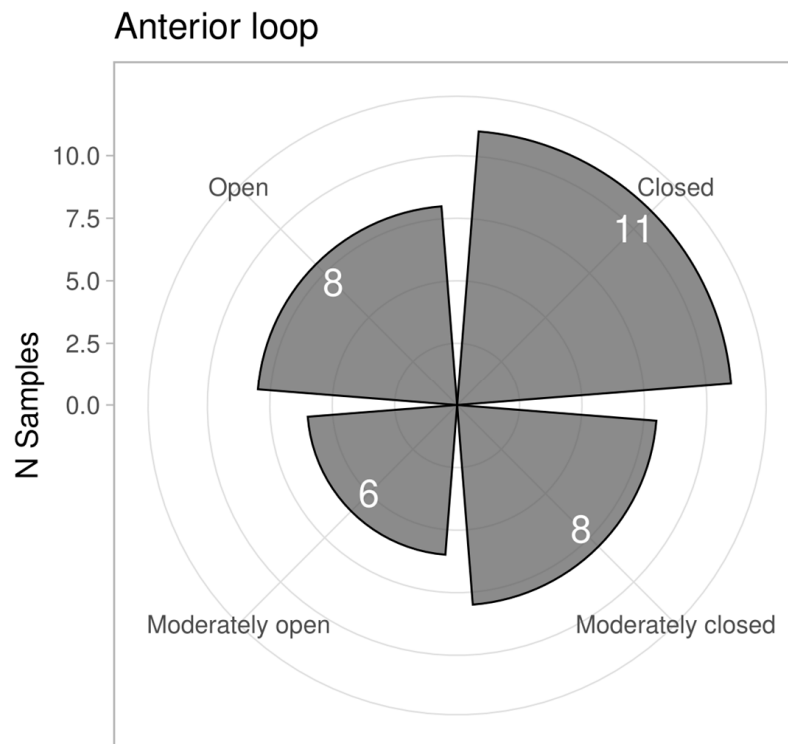


**Figure 2.** (A) Presumptive distribution of *Microtus savii* (orange), *M. brachycercus* (gray) and *M. nebrodensis* (green) (redrawn from [2]). (B) Map of the municipalities of the Umbria region. *Microtus* sp. Geographical populations are shown with different colours: red (Montefalco), light blue (Trevi), and green (North and Central provinces of Perugia).



**Figure 3.** (A) Measurements taken on the occlusal surface of the first lower molar in the studied specimens. (B) First lower molars of *Microtus (Terricola)* with open (left) and closed (right) anterior loops. (C) Measurement of the palatilar length in *Microtus (Terricola)* skull (redrawn from [6]).





**Figure 4.** Samples collected in the Montefalco sampling site and genotyped through mtDNA analysis.

Statistical analyses and graphical elaborations were carried out with the R statistical framework ([cran.r-project.org](https://cran.r-project.org)) (accessed on 14 March 2023) within the RStudio Development Environment ([rstudio.com](https://rstudio.com)) (accessed on 14 March 2023).

To rule out contamination from operator DNA, all laboratory procedures were carried out under a laminar flow hood using disposable nitrile gloves, disposable FFP-2 face masks and proceeding with frequent and thorough cleaning of all possible surfaces with denaturated ethyl alcohol.

To prevent cross-contamination, all samples were handled individually with researchers cleaning the workspace before and after each processed sample. To prevent the genotyping of the same individual, only the left hemimandible from each skull was selected for DNA extraction. Hemimandibles were put in sterile plastic bags and fractured, producing bone fragments to use for downstream application of the protocol. Extraction was preceded by PBS washings of bone fragments to rehydrate the samples and eliminate possible PCR inhibitors and fixatives [26]. Bone fragments were transferred in 1.5 mL sterile tubes, and 250 µL of PBS were added. Each tube was vortex-mixed, and fluid was recovered with sterile pipette tips and discarded. This procedure was repeated until the fluid was clear, at which point the fluid was discarded and then 620 µL of a mix made of 500 µL of Nuclei Lysis Solution (Promega) and 120 µL EDTA, to be stored in the freezer until cloudy, and 20 µL of Proteinase K (Promega) was aliquoted in each sample, which were then vortex-mixed for 25" and incubated at 65 °C for 10'. Samples were incubated at 55 °C for 24 to 72 h with occasional vortex mixing, adding 20 µL of Proteinase K every 12 h until complete lysis of the bone fragments was achieved. The obtained solution was then processed using the Wizard Genomic DNA Purification Kit (Promega) following a modified protocol [27,28]. For the final step, 50 µL of DNA Rehydration Solution were aliquoted in each sample, which were then incubated at 37 °C for 20' to resuspend DNA.

PCR amplification targeting the mitochondrial gene cytochrome *b* (*cytb*), widely used for similar molecular analyses [3,6,29,30], was performed using four sets of primers that enabled the amplification of the whole gene and of three partially overlapping fragments of the gene, the latter were used to amplify samples that failed to

amplify or that presented fade bands upon verification on 2% agarose gel. Primers L14725 (forward) 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' and H15915 (reverse) 5'-AACTGCAGTCATCTCCGGTTTACAAGAC-3' [29] were used to produce a first amplification of the complete gene, to be used as a template for the amplification of the smaller fragments, using the sets of primers: L14841 (forward) 5'-CCATCAAATATTTTCATCATGATGAAA-3' and H15408 (reverse) 5'-TGGAACGGGATTTTGTCTGC-3'; L15162 (forward) 5'-GCTACGTACT-TCCATGAGGACAAATATC-3' and H15549 (reverse) 5'-AAACTGCAGCCCCCTCAGAATG-ATATTTGTCCTCA-3'; L15408 (forward) 5'-GCAGACAAAATCCCGTTCCA-3' and H15915 (reverse) 5'-AACTGCAGTCATCTCCGGTTTACAAGAC-3' [30].

Three different Nested or SemiNested were performed: a SemiNested of the 5' fragment was performed with primers L14725 [29] and H15408M [3]; a central Nested was performed with primers L15162M2 and H15549M [3] and another SemiNested of the 3' fragment was performed with L15408M [30] and H15915 [29] primers. Amplifications were performed using 2 µL of the template DNA, 12.5 µL of 2× PCR Master Mix (Promega), 1 µL for each of 10 µM primers and nuclease-free H<sub>2</sub>O (Promega) to attain a final volume of 25 µL using the following amplification scheme: initial denaturation at 94 °C for 2' followed by 30 cycles with denaturation at 94 °C for 1' with annealing at 50 °C for 1' and elongation at 72 °C for 1'30" and a final extension at 72 °C for 8'. The amplification products were checked using electrophoresis in TBE buffer and 2% agarose gel containing SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, Cambridgeshire, UK) and visualized under UV lamps. Amplified fragments were purified using ExoSap-IT® (USB Corporation, Cleveland, OH, USA), and Sanger sequencing was outsourced for both ends of amplicons to Eurofins Genomics. The entire gene was sequenced from 10 samples using a Forward and Reverse strategy for the three overlapping fragments to ensure coverage of all the gene lengths and comparing them with already published sequences of *M. brachycercus* and *M. savii* (AY513824.1–AY513828.1; EU158776.1–EU158804.1; KT896517.1–KT896526.1) and *Microtus duodecimcostatus* (de Selys-Longchamps 1839) (KT896526.1) as outgroups. For the remaining 22 samples, sequencing procedures were limited using a Forward and Reverse strategy on the second and third fragments to allow for comparison with already deposited samples (KT896517.1–KT896526.1).

Electropherograms were handled and edited using MEGA 11 [31]. The taxonomic identities of the obtained sequences were evaluated using BLAST+ [32]. Phylogenetic relationships were estimated through Maximum Likelihood (ML) and reconstructions; an ML tree was obtained using MEGA 11 [31], supporting nodes by 1000 bootstrap replicates. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes–Cantor model and then selecting the topology with a superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.2461)). The trees were drawn to scale with branch lengths measured in the number of substitutions per site. Phylogenetic analyses were performed on a dataset including the entire set of samples genotyped for the present study (Montefalco specimens) together with the *cytb* sequences of the *Microtus savii* group available in GenBank by February 2023 (Supplementary Table S1).

Sequences that did not show double signals in the electropherogram were tested through the TCS v.1.21 program [33,34], setting network the connection limit at 95% and gaps as “missing”. TCS v.1.21 subdivided the identified haplotypes into haplogroups, describing the relationships between haplotypes/haplogroups found and clarifying the significant number of substitutions connecting haplotypes [35]. The network was visualized and plotted with tcsBU [36]. Statistical selection of best-fit models of nucleotide substitution was performed by means of jModelTest [37,38]. On the basis of these analysis, the Jukes–Cantor model was used to assess the evolutionary history of the *Microtus* specimens; Maximum Likelihood and Neighbour Joining methods were inferred estimating standard error with a bootstrap procedure (500 replicates). For the Maximum Likelihood method,

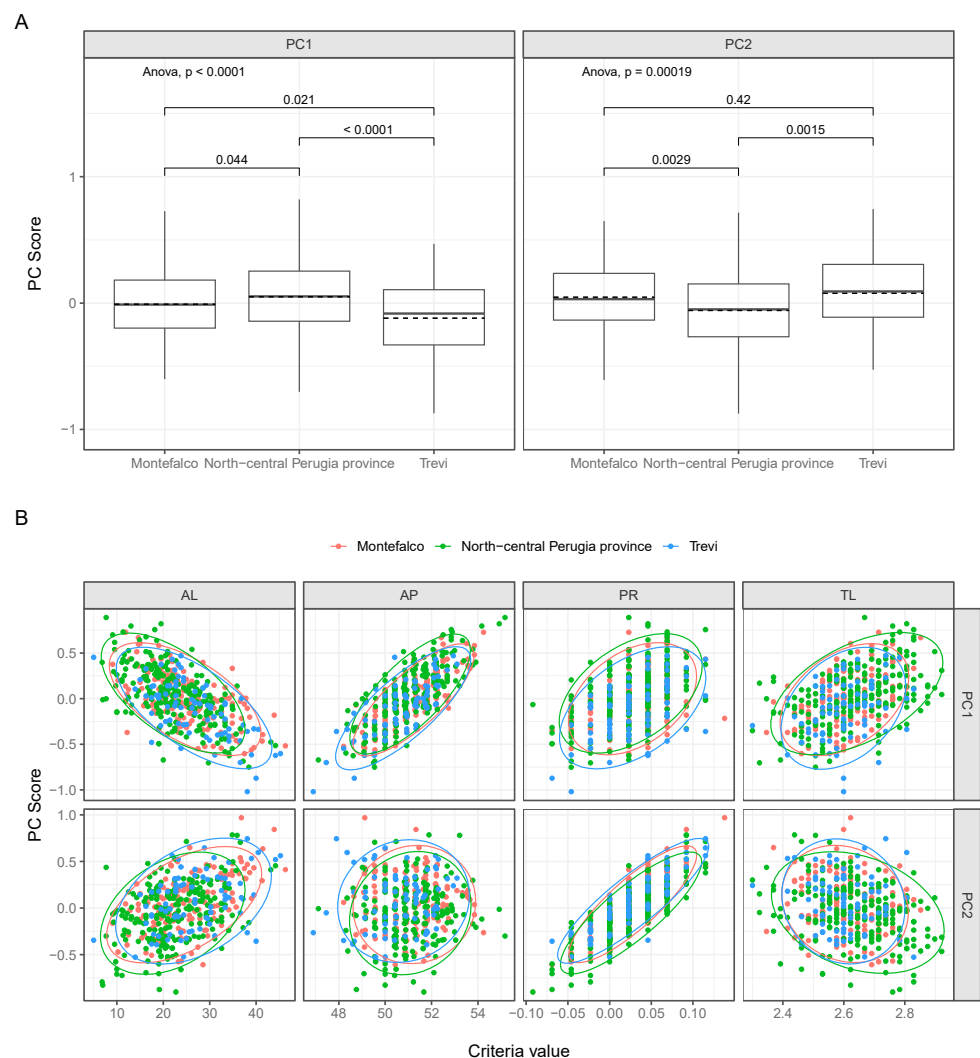
a discrete Gamma distribution was used to model evolutionary rate differences among sites (G categories = 4).

Spatial or demographic expansion was estimated through Tajima's D neutrality test [39], and rate homogeneity for nucleotide substitutions among lineages was tested through MEGA 11.

The risk of analysing nuclear insertions of mtDNAs (NUMTs) instead of mitochondrial original sequences was taken into account and prevented using bones as the DNA source [40,41]. By any chance, prior purification and sequencing, each amplicon was run on 2.5% agarose gel, and the entire sample set clearly showed only a single and discrete band with no evidence of smaller amplicons, excluding the possible presence of NUMTs that are usually shorter than the corresponding mitochondrial gene [42]. Furthermore, NUMTs frequently show several amino acid variations [40] absent in translating the obtained sequences.

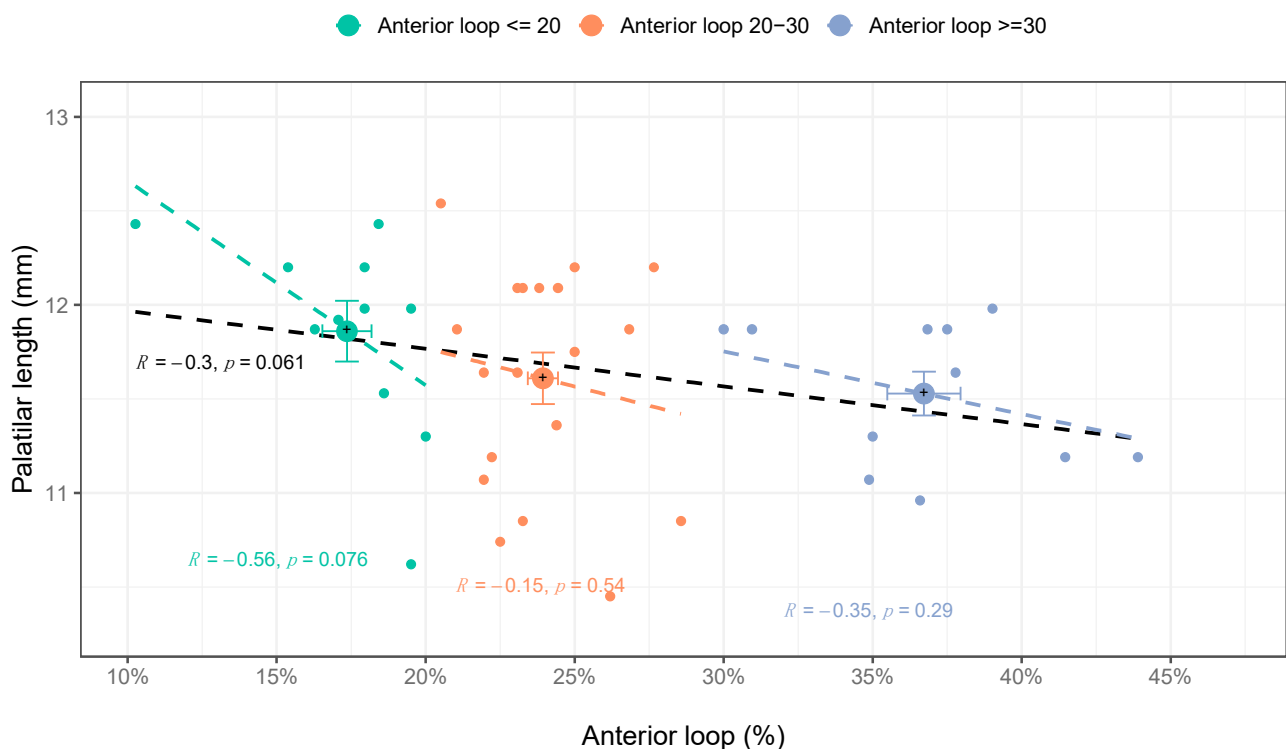
### 3. Results

The results of the odontometrical analysis are summarized in Figure 5.



**Figure 5.** (A) Distribution of the row scores of the first two components of PCA. The  $p$  value of Bonferroni tests reveals whether significant differences are present between the geographical populations. Colours are consistent with those used in Figure 2. (B) Individual PC scores and odontometric variables. The ellipses contain 95% of the data.

The examination of the morphospace obtained through a PCA analysis and testing the differences among the populations with a MANOVA analysis revealed significant differences among the three geographical districts (Pillai's Trace = 0.082;  $p < 0.001$ ). The first two PCs were statistically significant and accounted for 59% of the variance (Figure 5A). The following correlation analyses showed that the first axis (34% of explained variance) had a positive correlation with AP, PR and TL, but a negative correlation with AL ( $p < 0.01$ ). The second axis (25% of explained variance) was positively influenced by AL and PR ( $p < 0.01$ ) (Figure 5B). In the palatilar length analysis, we considered that the Montefalco sample consisted of 11 specimens with anterior loops  $\leq 20\%$ , 11 specimens with anterior loops  $\geq 30\%$  and 19 specimens with anterior loops within the 20–30% interval. The main results are shown in Figure 6.

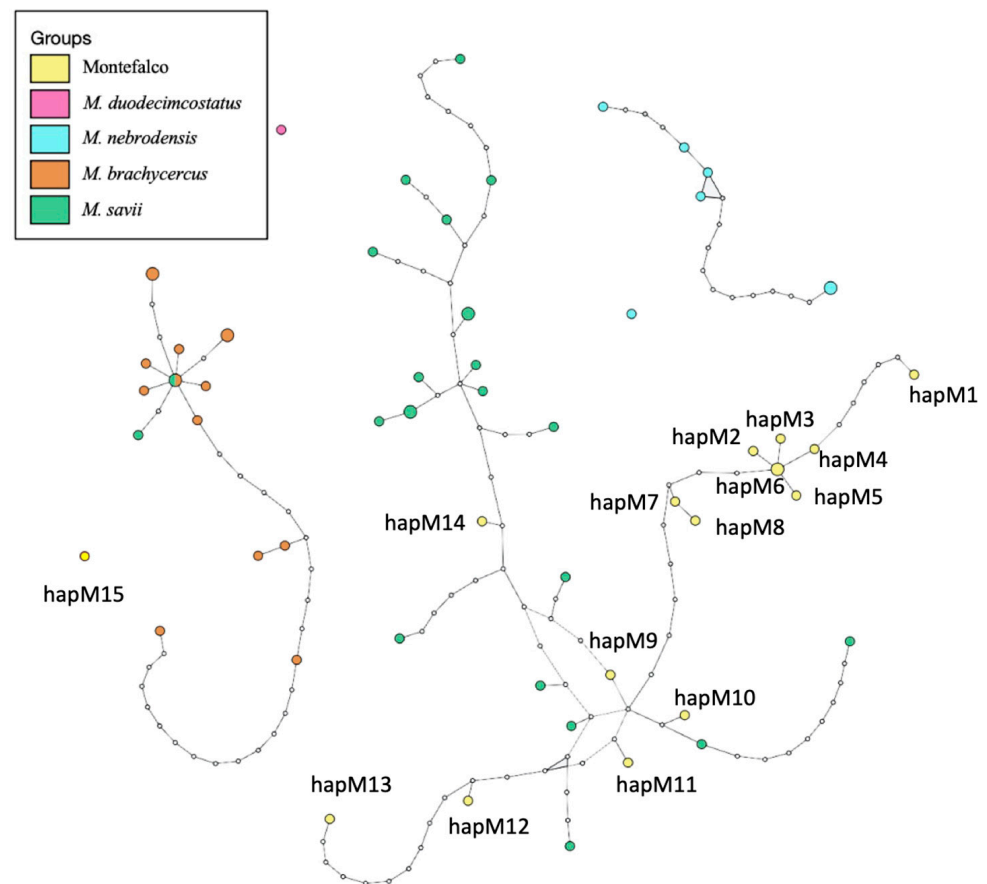


**Figure 6.** Regression and correlation analysis of palatilar lengths (mm) and the anterior loop percentages among the  $m_1$  groups. The largest points (+) depict the centroids of each group with the standard errors for both variables. The black line represents the common regression line while the coloured ones, those of each individual group.

No significant differences in palatilar lengths were found between individuals with more-or-less-opened anterior loops (ANOVA  $F = 1.194$ ;  $p = 0.059$ ) nor were statistically significant correlations between palatilar lengths and anterior loop percentages found ( $R = -0.3$ ,  $p = 0.061$ ).

Regarding the genetic analyses, 32 sequences of the *Microtus cytb* gene of specimens belonging to Montefalco sampling site were obtained. Ten sequences were performed overlapping the three fragments and covered the entire *cytb* (1143 bp). The others were obtained using only fragments 2 and 3 (629 bp in total) and ranged from 588 to 629 bp in length due to a poor electropherogram quality of three samples. All sequences were deposited in GenBank under the Accession Numbers ON815278–ON815292. Every polymorphism was inspected to check that the theoretical protein translation was possible and all alternative codons were *sensu*. A total of 17 haplotypes were found and desegregated, as reported in Figure 7.





**Figure 7.** Haplotype minimum spanning network. Each circle represents a unique haplotype, each *Microtus* species is colour-coded, as reported in the figure key, with Montefalco specimens identified in yellow. The size of the circle is proportional to the number of samples represented by each haplotype.

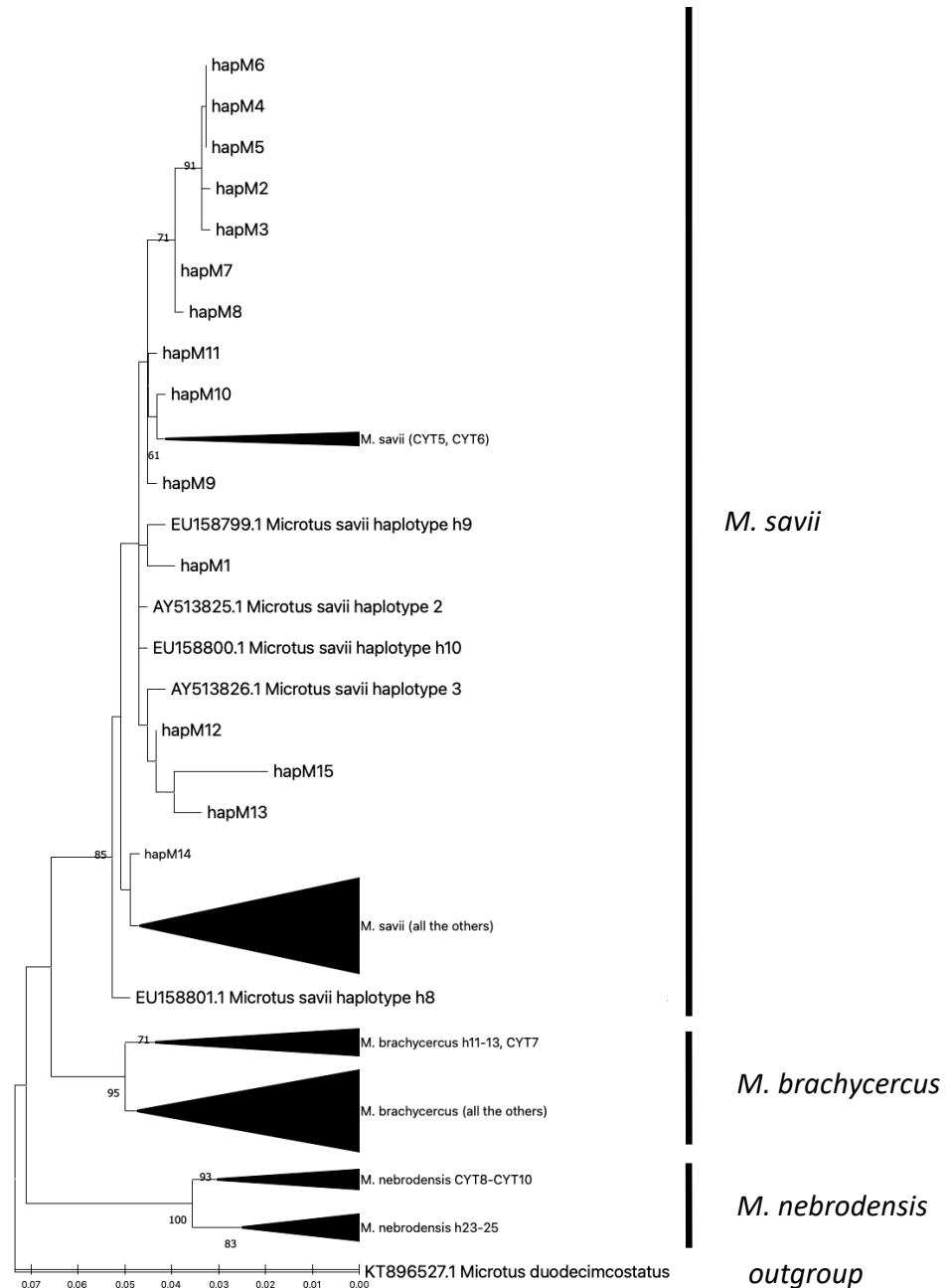
No specimen belonging to the Montefalco sample was attributed to the *M. brachycercus* haplogroup (orange in Figure 7). New haplotypes found in Montefalco described two main ramifications: one including only two haplotypes (hapM12 and hapM13) and the other grouping eight haplotypes (hapM1–hapM8). The other four haplotypes were dispersed across the *M. savii* network (hapM9–hapM13) or represented as an independent haplogroup, as was the case with hapM15.

The high number of haplotypes was caused by an abundance of rare mutations whose presence was noticed in just one or few individuals at a time. This scenario was confirmed by the Tajima Neutrality test. Montefalco's sample pool showed a negative Tajima's D, suggesting an excess of low-frequency polymorphisms, i.e., fewer haplotypes that influenced an observed value lower than the expected one ( $\Theta\text{-Pi} < \Theta\text{-k}$ ). This can be due to an abundance of rare alleles presumably caused by a recent population evolving after expansion.

The evolutionary history was inferred using the Maximum Likelihood (ML) method and Jukes–Cantor model [43] on 60 nucleotide sequences; the tree with the highest log likelihood (−2024.87) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Codon positions included were 1st + 2nd + 3rd + Noncoding.

The ML tree showed three main branches; one clade corresponds to *M. nebrodensis*, the taxon geographically distributed in Sicily. In contrast, the other two branches clustered samples of *M. savii* and *M. brachycercus*. The branch that corresponds to *M. savii* was constituted by samples belonging to Montefalco and were from Northern and Central Italy. No sample belonging to Montefalco emerged in the branch referable to *M. brachycercus*.

Interestingly, using these statistical conditions, there were two *M. savii* haplotypes (hap 4 and hap 5) that fell within the *M. brachycercus* clade. Furthermore, Montefalco samples did not cluster together, but formed at least three main sub-branches (Figure 8).



**Figure 8.** Maximum Likelihood performed using the Jukes–Cantor model. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [44]. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st + 2nd + 3rd + Noncoding for 552 positions in the final dataset.

#### 4. Discussion

As reported in the Materials and Methods section, several samples showed double signals in the electropherogram and therefore were not included in the haplotype counts as described by Dąbrowski and colleagues [45]. This condition is presumably caused by heteroplasmy, i.e., the co-occurrence of different mtDNA haplotypes within the same

individual. This condition can stem from several causes, including somatic or germline mutations or mitochondrial paternal leakage [46], a phenomenon already described within the Genus [45]. In this case, each double signal was visually scanned, and the two bases called by the sequencer were always typical of two of the already known haplotypes. These phenomena may have originated with hybridization, either between different species or subspecies, well documented in the *Microtus* Genus [5,47,48]. In the Montefalco population, both the genetic variability and the frequency of heteroplasmic individuals are very high. The differentiation of some individuals of this population, if compared to other *Microtus* genetic data from Italy, is well-supported by the presence of unique mutations never reported before. Furthermore, the mutations are not attributable to known haplotypes of *M. savii* or *M. brachycercus*, even though these individuals seem phylogenetically related to the *M. savii* complex. Literature analysis revealed a high variability in haplotype numbers present in different sampling pools of the *Microtus* species. Data ranged from several haplotypes, each represented by a few individuals [49,50], to a small number of haplotypes, each represented by many individuals [45,51]. The number of different sequences is higher than those already documented for congeneric species at local geographic scale [45,51] and might be due either to the coexistence of different genetic pools in the same area or to the sampling method. In fact, samples from each site could be gathered all around the owls' hunting grounds that may extend several miles from their roosts [52].

As suggested by some authors [19,53–55], the analysis of small mammal remains recovered from raptor pellets offers many resources for several kind of studies. Our results confirm the presence of *M. savii* in Umbria, as previously reported by Gaggi and Paci [56], whereas no solid data were found to support the presence of *M. brachycercus*, which shows the northernmost distribution near the regional border. Generally, a greater value of the anterior loop breadth on the first lower molar is a feature that is typical of *M. brachycercus* [57]. Therefore, the morphological analyses performed on the Montefalco subsample gave reasons to believe that individuals of *M. brachycercus* were present well above their northernmost limit of distribution. The morphometric findings were not supported by genetic data that did not support the morphological species identification but highlighted the presence of a population of *M. savii*, which presented an array of mutations that distanced the studied samples from already known *M. savii* haplotypes. This peculiar biodiversity might stem from the different partitioning of voles' home ranges that are small when compared to owls' hunting grounds. Yletyinen and Norrdhal [58], using radiotelemetry data, found significant differences in the habitat use of field voles (*Microtus agrestis* Linnaeus 1758) with females being more philopatric than males that had home ranges five times wider. Likewise, *A. otus* home ranges exceed 9.69 Km<sup>2</sup> [52], making the roost a communal collection of remains from voles of different populations or subpopulations, giving a possible explanation for this enhanced variability.

The genotyped samples were collected in 2015. Therefore, a new re-assessment of the genetic asset of contemporaneous living voles is required and will be carried out along with widening the sample area and performing karyotypic analyses to assess the possible presence of hybrids. Galleni et al. [48] have not only documented the possibility of *M. savii* x *M. brachycercus* hybridization, but have also demonstrated that hybrid karyotypes underline a relationship between the severity of meiotic aberrations in the hybrids and the degree of disturbances in spermatogenesis. Even though the karyotype analysis might represent an important upgrade to this research, this work demonstrates, yet again, how useful and user-friendly the analysis of bones retrieved from pellets to collect data on the rodent community can be. Approaching the analysis using conservative samples has also given an interesting insight for future investigations as molecular analyses open new perspectives on the role of museal collections in the study of biodiversity [59]. Museum specimens from which DNA can be extracted represent, in fact, an archive of biodiversity that is an actual window on the genetic assets of both wild and domestic species from the past.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app13084703/s1>, Table S1: GenBank codes and entry short descriptions of the sequences used for phylogenetics analysis.

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## References

1. Abramson, N.I.; Bodrov, S.Y.; Bondareva, O.V.; Genelt-Yanovskiy, E.A.; Petrova, T.V. A mitochondrial genome phylogeny of voles and lemmings (Rodentia: Arvicolinae): Evolutionary and taxonomic implications. *PLoS ONE* **2021**, *16*, e0248198. [CrossRef] [PubMed]
2. Amori, G.; Castiglia, R. Mammal endemism in Italy: A review. *Biogeographia* **2017**, *33*, 19–31. [CrossRef]
3. Castiglia, R.; Annesi, F.; Aloise, G.; Amori, G. Systematics of the *Microtus savii* complex (Rodentia, Cricetidae) via mitochondrial DNA analyses: Paraphyly and pattern of sex chromosome evolution. *Mol. Phylogenet. Evol.* **2008**, *46*, 1157–1164. [CrossRef] [PubMed]
4. Galleni, L.; Stanyon, R.; Tellini, A.; Giordano, G.; Santini, L. Taxonomy of *Microtus savii* (Rodentia, Arvicolidae) in Italy: Cytogenetic and hybridization data. *J. Mammal.* **1994**, *75*, 1040–1044. [CrossRef]
5. Galleni, L.; Stanyon, R.; Contadini, L.; Tellini, A. Biogeographical and karyological data of the *Microtus savii* group (Rodentia, Arvicolidae) in Italy. *Bonn. Zool. Beitr.* **1998**, *47*, 277–282.
6. Bezerra, A.M.R.; Annesi, F.; Aloise, G.; Amori, G.; Giustini, L.; Castiglia, R. Integrative taxonomy of the Italian pine voles, *Microtus savii* group (Cricetidae, Arvicolinae). *Zool. Scr.* **2015**, *45*, 225–236. [CrossRef]
7. Ellerman, R.; Morrison-Scott, T.C.S. *Checklist of Palearctic and Indian mammals 1758–1946*, 2nd ed.; British Museum of Natural History: London, UK, 1966; 810p.
8. Corbet, G.B. *The Mammals of the Palearctic Region: A Taxonomic Review*; British Museum of Natural History Press: London, UK; Cornell University: Ithaca, NY, USA, 1978.
9. Saint Girons, M.C. *Les Mammifères de France et du Benelux (Faune Marine Exceptée)*; Doin: Paris, France, 1973.
10. Petrov, B.M.; Zivkovic, S.; Rimsa, D. Über die Arteigenständigkeit der Kleinwühlmaus, *Pitymys felteni*. *Senckenberg. Biol.* **1976**, *57*, 1–10.
11. Amori, G.; Contoli, L.; Nappi, A. Mammalia II. Erinaceomorpha, Soricomorpha, Lagomorpha, Rodentia. In “Fauna d’Italia” Series; Edizioni Calderini: Milano, Italy, 2008; Volume XLIV.
12. Pardiñas, M.F.J.; Myers, P.; León-Paniagua, L.; Garza, N.O.; Cook, J.A.; Kryštufek, B.; Haslauer, R.; Bradley, R.D.; Shenbrot, G.I.; Patton, J.L. Family Cricetidae (true Hamsters, Voles, Lemmings and New World Rats and Mice). In *Handbook of the Mammals of the World Vol. 7. Rodents II*; Wilson, D.E., Lacher, T.E., Jr., Mittermeier, R.A., Eds.; Lynx Edicions: Barcelona, Spain, 2017; pp. 204–535.
13. Loy, A.; Aloise, G.; Ancillotto, L.; Angelici, F.M.; Bertolino, S.; Capizzi, D.; Castiglia, R.; Colangelo, P.; Contoli, L.; Cozzi, B.; et al. Mammals of Italy: An annotated checklist. *Hystrix* **2019**, *30*, 87–106.
14. Petty, S. Diet of tawny owls (*Strix aluco*) in relation to field vole (*Microtus agrestis*) abundance in a conifer forest in northern England. *J. Zool.* **1999**, *248*, 451–465. [CrossRef]

15. Cecere, F.; Vicini, G. Micromammals in the diet of the Long-eared Owl (*Asio otus*) at the W.W.F.'s Oasi San Giuliano (Matera, South Italy). *Hystrix* **2000**, *11*, 2.
16. Sergio, F.; Marchesi, L.; Pedrini, P. Density, diet and productivity of Long-eared Owls *Asio otus* in the Italian Alps: The importance of *Microtus voles*. *Bird Study* **2008**, *55*, 321–328. [\[CrossRef\]](#)
17. Taberlet, P.; Fumagalli, L. Owl pellets as a source of DNA for genetic studies of small mammals. *Mol. Ecol.* **1996**, *5*, 301–305. [\[CrossRef\]](#)
18. Turpen, E.R.; Vrla, S.C.; Matlack, R.S.; Bradley, R.D. Evidence from Owl Pellets Indicate that Prairie Voles (*Microtus ochrogaster*) are Broadly Distributed in the Texas Panhandle. In *Occasional Papers*; Museum of Texas Tech University: Lubbock, TX, USA, 2022; n. 382.
19. Nappi, A.; Brunet-Lecomte, P.; Montuire, S. The systematics of *Microtus (Terricola) savii* group: An odonthometrical perspective (Mammalia, Rodentia, Cricetidae). *J. Nat. Hist.* **2019**, *53*, 2855–2867. [\[CrossRef\]](#)
20. Angermann, R. Die Zahnvariabilität bei Microtinen im Liechte von Vavilov's "Gesetz der homologen Serien". In *Proceedings of the Symposium Theriologicum II—International Symposium on Species and Zoogeography of European Mammals*, Brno, Czech Republic, 22–26 November 1971; Accademia, Publishing house of the Czechoslovak Academy of Sciences: Praha, Czech Republic, 1974; pp. 61–73.
21. Contoli, L.; Amori, G.; Nazzaro, C. Tooth diversity in *Arvicolidae* (Mammalia, Rodentia): Ecochorological factors and speciation time. *Hystrix* **1993**, *4*, 2.
22. Navarro, N.; Montuire, S.; Laffont, R.; Steimetz, E.; Onofrei, C.; Royer, A. Identifying past remains of morphologically similar vole species using molar shapes. *Quaternary* **2018**, *1*, 20. [\[CrossRef\]](#)
23. Brunet-Lecomte, P. Les Campagnols Souterrains (*Terricola*, *Arvicolidae*, Rodentia) Actuels et Fossiles d'Europe Occidentale. Ph.D. Dissertation, Université de Bourgogne, Dijon, France, 1988.
24. Laplana, C.; Montuire, S.; Brunet-Lecomte, P.; Chaline, J. Révision des *Allophaiomys* (*Arvicolidae*, Rodentia, Mammalia) des Valerots (Côte-d'Or, France). *Geodiversitas* **2000**, *22*, 255–267.
25. Nappi, A.; Brunet-Lecomte, P.; Montuire, S. Intraspecific morphological tooth variability and geographical distribution: Application to the Savi's vole, *Microtus (Terricola) savii* (Rodentia, Arvicolinae). *J. Nat. Hist.* **2006**, *40*, 345–358. [\[CrossRef\]](#)
26. Iudica, C.A.; Whitten, W.M.; Williams, N.H. Small bones from dried mammal museum specimens as a reliable source of DNA. *BioTechniques* **2001**, *30*, 732–736. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Brustenga, L.; Chiesa, S.; Maltese, S.; Consales, G.; Marsili, L.; Lauriano, G.; Scarano, V.; Scarpato, A.; Lucentini, L. A Reliable and Cost-Efficient PCR-RFLP Tool for the Rapid Identification of Cetaceans in the Mediterranean Sea. *Sustainability* **2022**, *14*, 16763. [\[CrossRef\]](#)
28. Fontaneto, D.; Viola, P.; Pizzirani, C.; Chiesa, S.; Rossetti, A.; Amici, A.; Lucentini, L. Mismatches between Morphology and DNA in Italian Partridges May Not Be Explained Only by Recent Artificial Release of Farm-Reared Birds. *Animals* **2022**, *12*, 541. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Irwin, D.M.; Kocher, T.D.; Wilson, A.C. Evolution of the cytochrome b gene of mammals. *J. Mol. Evol.* **1991**, *32*, 128–144. [\[CrossRef\]](#)
30. Jaarola, M.; Searle, J.B. Phylogeography of field voles (*Microtus agrestis*) in Eurasia inferred from mitochondrial DNA sequences. *Mol. Ecol.* **2002**, *11*, 2613–2621. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Tamura, K.; Stecher, G.; Kumar, S. MEGA 11: Molecular Evolutionary Genetics Analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinf.* **2009**, *10*, 421. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Templeton, A.R.; Crandall, K.A.; Sing, C.F. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **1992**, *132*, 619–633. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Clement, M.; Posada, D.; Crandall, K.A. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* **2000**, *9*, 1657–1659. [\[CrossRef\]](#)
35. Chiesa, S.; Lucentini, L.; Chainho, P.; Plazzi, F.; Angélico, M.M.; Ruano, F.; Freitas, R.; Costa, J.L. One in a Million: Genetic Diversity and Conservation of the Reference *Crassostrea angulata* Population in Europe from the Sado Estuary (Portugal). *Life* **2021**, *11*, 1173. [\[CrossRef\]](#)
36. dos Santos, A.M.; Cabezas, M.P.; Tavares, A.I.; Xavier, R.; Branco, M. tcsBU: A tool to extend TCS network layout and visualization. *Bioinformatics* **2016**, *32*, 627–628. [\[CrossRef\]](#)
37. Guindon, S.; Gascuel, O. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Syst. Biol.* **2003**, *52*, 696–704. [\[CrossRef\]](#)
38. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [\[CrossRef\]](#)
39. Tajima, F. Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. *Genetics* **1989**, *123*, 585–595. [\[CrossRef\]](#)
40. Sorenson, M.D.; Quinn, T.W. Numts: A Challenge for Avian Systematics and Population Biology. *Ornithology* **1998**, *115*, 214–221. [\[CrossRef\]](#)
41. Triant, D.A.; DeWoody, J.A. Extensive mitochondrial DNA transfer in a rapidly evolving rodent has been mediated by independent insertion events and by duplications. *Gene* **2007**, *401*, 61–70. [\[CrossRef\]](#) [\[PubMed\]](#)



42. Sun, J.; Feng, Z.; Liu, Y. Nucleotide Substitution Pattern in Mitochondrial Cytochrome b Pseudogenes of Ten Species in Galliformes. In *Information Technology and Agricultural Engineering. Advances in Intelligent and Soft Computing*; Zhu, E., Sambath, S., Eds.; Springer: Berlin/Heidelberg, Germany, 2012; Volume 134, pp. 269–270.
43. Jukes, T.H.; Cantor, C.R. Evolution of protein molecules. In *Mammalian Protein Metabolism*; Munro, H.N., Ed.; Academic Press: New York, NY, USA, 1969; pp. 21–132.
44. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [[CrossRef](#)]
45. Dąbrowski, M.J.; Pomorski, J.J.; Gliwicz, J. Cytochrome b gene (*cytb*) sequence diversity in a *Microtus oeconomus* population from Białowieża Primeval Forest. *Acta Theriol.* **2013**, *58*, 119–126. [[CrossRef](#)]
46. Gandolfi, A.; Crestanello, B.; Fagotti, A.; Simoncelli, F.; Chiesa, S.; Girardi, M.; Giovagnoli, E.; Marangoni, C.; Di Rosa, I.; Lucentini, L. New Evidences of Mitochondrial DNA Heteroplasmy by Putative Paternal Leakage between the Rock Partridge (*Alectoris graeca*) and the Chukar Partridge (*Alectoris chukar*). *PLoS ONE* **2017**, *12*, e0170507. [[CrossRef](#)] [[PubMed](#)]
47. Kovalskaya, Y.M.; Savinetskaya, L.E.; Aksenova, T.G. Experimental hybridization of voles of the genus *Microtus* s. l. *M. socialis* with species of the group *arvalis* (Mammalia, Rodentia). *Biol. Bull. Russ. Acad. Sci.* **2014**, *41*, 559–563. [[CrossRef](#)]
48. Galleni, L.; Tellini, A.; Cicalò, A.; Fantini, C.; Fabiani, O. Histological examination of the male gonad of hybrid specimens: *Microtus savii* x *Microtus brachycercus* (Rodentia-Arvicolinae). *Bonn. Zool. Beitr.* **1998**, *48*, 1–8.
49. Tougaard, C.; Renvoisé, E.; Petitjean, A.; Quéré, J.P. New insight into the colonization processes of common voles: Inferences from molecular and fossil evidence. *PLoS ONE* **2008**, *3*, e3532. [[CrossRef](#)] [[PubMed](#)]
50. Stojak, J.; McDevitt, A.D.; Herman, J.S.; Kryštufek, B.; Uhlíková, J.; Purger, J.J.; Lavrenchenko, L.A.; Searle, J.B.; Wójcik, J.M. Between the Balkans and the Baltic: Phylogeography of a Common Vole Mitochondrial DNA Lineage Limited to Central Europe. *PLoS ONE* **2016**, *11*, e0168621. [[CrossRef](#)]
51. Francl, K.E.; Glenn, T.C.; Castleberry, S.B.; Ford, W.M. Genetic relationships of meadow vole (*Microtus pennsylvanicus*) populations in central Appalachian wetlands. *Can. J. Zool.* **2008**, *86*, 344–355. [[CrossRef](#)]
52. Emin, D.; Toxopeus, A.G.; Groen, I.T.A.; Kontogeorgos, I.; Georgopoulou, E.; Xirouchakis, S. Home Range and Habitat Selection of Long-Eared Owls (*Asio otus*) in Mediterranean Agricultural Landscapes (Crete, Greece). *Avian. Biol. Res.* **2018**, *11*, 204–218. [[CrossRef](#)]
53. Nappi, A. L'analisi delle borre degli uccelli: Metodiche, applicazioni e informazioni. Un lavoro monografico. *Picus* **2011**, *37*, 106–120.
54. Buś, M.M.; Żmihorski, M.; Romanowski, J.; Balčiauskienė, L.; Cichocki, J.; Balčiauskas, L. High efficiency protocol of DNA extraction from *Micromys minutus* mandibles from owl pellets: A tool for molecular research of cryptic mammal species. *Acta Theriol.* **2013**, *59*, 99–109. [[CrossRef](#)]
55. Gomes-Rocha, R.; Justino, J.; Reis-Leite, Y.L.; Pires-Costa, L. DNA from owl pellet bones uncovers hidden biodiversity. *Syst. Biodiver.* **2015**, *13*, 403–412. [[CrossRef](#)]
56. Gaggi, A.; Paci, A.M. *Atlante degli Erinaceomorfi, dei Soricomorfi e dei piccoli Roditori dell'Umbria*; Regione Umbria: Perugia, Italy, 2014; 211p.
57. Nappi, A.; Brunet-Lecomte, P.; Montuire, S. Dental morphology of *Microtus (Terricola)* voles from Calabria (Southern Italy) and relationships with *M. (T.) savii* (Rodentia, Arvicolidae). *Hystrix* **2005**, *16*, 75–85.
58. Yletyinen, S.; Norrdahl, K. Habitat use of field voles (*Microtus agrestis*) in wide and narrow buffer zones. *Agric. Ecosyst. Environ.* **2008**, *123*, 194–200. [[CrossRef](#)]
59. Barili, A.; Gentili, S.; Lucentini, L. DNA, archivi della biodiversità e musei scientifici: Alcune esperienze presso la Galleria di Storia Naturale dell'Università degli Studi di Perugia. *Museol. Sci. Mem.* **2017**, *17*, 97–100.

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