

Taxonomic status of southern Iberian *Neomys* populations with evolutionary and conservation implications

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Running title: Taxonomy of southern Iberian *Neomys*

Abstract

The genus *Neomys* contains four species, three of which are present on the Iberian Peninsula. Recent phylogeographic studies have thoroughly assessed the evolutionary history of this genus in this region. However, perhaps due to its rarity, the isolated and endangered populations of southern Iberia have never been included in these studies. Thus, the current taxonomic status of these apparently isolated populations of *Neomys anomalus* is currently based on morphological evidence. In order to test whether southern Iberian *Neomys* represent a recently diverged relict population from recent expansions of previously described phylogroups, or a highly divergent lineage due to isolation in an ancient southern refugia, we genetically analyze four museum individuals of *Neomys anomalus* from this southern population in Andalucia, Spain. Our results based on cytochrome *b* sequences from two individuals suggest that the southern Iberian *Neomys* population represents a relatively recently diverged lineage with a unique haplotype. This is in contrast to other phylogeographic studies on Iberian small mammals. These findings might suggest potential recent extinctions throughout the species' southern range and/or the need for additional sampling in this region in order to properly assess the distribution and conservation status of this Iberian endemic species.

Keywords

Baetic System; Sierra Nevada; phylogeography; systematics; water shrew; Soricidae

The water shrew genus *Neomys* contains four species distributed throughout the Palearctic: *N. fodiens*, which extends from northern Spain to eastern Russia and China; *N. teres*, which is restricted to the Caucasus and adjacent areas in Turkey and Iran; *N. milleri*, which has a patchy distribution over the western and continental Palearctic, and *N. anomalus* which is endemic to the Iberian Peninsula (Burguin et al. 2018, Igea et al. 2015). The Iberian Peninsula hosts the highest diversity of this genus, with *N. fodiens* distributed throughout the north, *N. milleri* restricted to the northeast, and *N. anomalus* present in three disjunct populations: (1) the northwest and central system main distribution core, (2) the southwestern Sierra de Aracena (Huelva), and (3) eastern Baetic range, from Sierra de Camarolos (Málaga) to the southeast to Sierra de Cazorla (Jaén) to the northwest (**Figure 1**; Burguin et al. 2018, Querejeta and Castresana 2018, Igea et al. 2015, Haro et al. 2004). While *N. anomalus* is considered fairly common in its main north-central distribution core, it is very rare in southern Iberia and cataloged as endangered (Haro et al. 2004, Franco and Rodriguez 2001). Such rarity is not surprising given that it is the only Soricine reaching this latitude in Iberia and that species distribution modelling suggest a low probability for these southern patches at the present time (Querejeta and Castresana 2018, Haro et al. 2004). This rarity may explain why none of the comprehensive phylogenetic/phylogeographic studies carried out on Iberian *Neomys* have sampled southern populations (**Figure 1**; Balmori-de la puente et al. 2019, Querejeta and Castresana 2018, Igea et al. 2015). Thus, the current taxonomic status of these apparently isolated populations is currently based solely on morphological evidence. Shrews are characterised by high levels of cryptic diversity and extreme morphological conservatism (Burguin et al. 2018). Despite general morphological conservatism, some studies in *Neomys* and *Crocidura* shrews have suggested adaptive ecophenotypic variation in morphological traits (Hinckley et al. 2021, Balmori-de la puente et al. 2019). For these reasons, taxonomic assessments are much more robust when they combine phenotypic and genetic evidence. Although it is likely that SE Iberian *Neomys* populations are conspecific with central *Neomys anomalus* populations, alternative scenarios such as a relict population of *Neomys milleri* or a distinct Baetic endemic species cannot be discarded. The strong structure detected in northern populations of *N. anomalus* suggests that this lineage evolved independently in the Iberian Peninsula and is not a recent European colonizer, potentially enabling the Baetic lineage to diverge in allopatry for a long time period (Querejeta and Castresana 2018, Igea et al. 2015). Due to Late Quaternary climate stability in the Iberian

Peninsula, it is considered a cradle for genetic diversity and endemisms and a source for recolonization of Europe after glacial periods (Abellán and Svenning 2014, Gomez and Lundt 2007). Patterns of endemism and cross-taxa concordant phylogeographic patterns support a refugia-within-refugia scenario in which the Iberian refugium hosted several refugial areas throughout the Ice Ages (Abellán and Svenning 2014, Gomez and Lundt 2007). In order to test the alternative hypotheses that (1) southern Iberian *Neomys* represent a recently diverged relict population from recent expansions of the phylogroups detected in Querejeta and Castresana (2018), or (2) a highly divergent lineage due to isolation in an ancient southern refugia, we genetically analyze four museum individuals of *Neomys anomalus* from this southern population in Andalucía, Spain.

Skin and/or inner skull tissue crusts were sampled from four specimens hosted in Estación Biológica de Doñana-CSIC scientific collection (EBD3530, EBD3541, EBD3519 and EBD3508). These specimens were collected in Carataunas, Sierra Nevada range, Granada (**Figure 1**; SE population) in 21-09-1981. External measurements are as follows: Weight 10.8-11.8 g, total length 139-142 mm, tail length 60 mm and hindfoot length 16-17 mm. The only specimen from Huelva (**Figure 1**; SW population) was on loan and thus unavailable to this study. DNA was extracted in an isolated, dedicated low quality DNA lab with a UV for pre-PCR steps and negative controls were included in DNA extraction and PCR batches following the standards and protocols of Hinckley et al. (2021). We developed the following primers to amplify short but variable areas of two loci (116 and 207 bp of cytochrome *b* (Cyt *b*) and 216 bp of nuclear intron *PRPF31*) from highly-fragmented historic DNA specimens skins/inner skull tissue crusts in single reactions with the same PCR conditions:

Cytb1.Neo.F (5'-CAGTTATAGCYACTGCCTTTATAGG-3'),

Cytb1.Neo.R (5'-GTGAGRGTGCTTTGTCTACGG-3'),

Cytb2.Neo.F (5'-CTACCATTCATYATYGCAGCC-3'),

Cytb2.Neo.R (5'-TGAGGGGGTGTGTTKAGTGG-3'),

PRPF31.Neo.F (5'-TTTCACAGCACACAGAGATTGC-3'),

PRPF31.Neo.R (5'-TCCCTAAATGAGTCAGCTCCG-3').

PCR conditions were the same for all primer sets: initial denaturation at 95°C for 5 min, followed by 9 cycles of 45 sec at 94°C, 60 sec starting at 62 °C with a touchdown of -1°C per

cycle, 45 sec at 74 °C, and then 27 cycles of 45 sec at 94°C, 60 sec at 53 °C, 45 sec at 74 °C and a final extension at 72 °C for 5 min. Four replicate PCRs were run per sample in order to identify apparent mutations caused by damage or degradation of the DNA. All primers used in this study were designed with a tail to add Illumina barcodes and sequencing primers in a second round PCR following Forcina et al. (2021). DNA amplification failed in two of the specimens which showed a highly fragmented DNA (less than 100 bp; EBD3519 and EBD3508) and worked in the other two specimens (EBD3530 and EBD3541). Dual indexed amplicon libraries were sequenced on a Illumina MiSeq machine at Johns Hopkins University (Baltimore, Maryland, USA). Specimens EBD3530 and EBD3541 yielded identical sequences at both loci. The assembled two non-overlapping *Cyt b* fragments were 323 bp, which is shorter than the fragment analyzed in modern DNA studies but still phylogenetically informative. Unfortunately, *PRPF31* was not informative, since not even *N. anomalus* and *N. milleri* were reciprocally monophyletic, so it was not included in downstream analyses but was deposited in GenBank. Data from another 268 samples from all four *Neomys* species were downloaded from GenBank for comparison. Adaptor removal, quality trimming, read mapping, consensus calling and genotyping was performed as in Hinckley et al. (2020). Trimmed reads were mapped to the closest homologous sequences found in Genbank (DQ991052, *Cyt b*; LK936894, *PRPF31*; coverage depth: 545-1884 reads). We constructed haplotype networks with HaplowebMaker (Spöri and Flot 2020). The median joining network algorithm was selected, with columns with missing data masked. We computed uncorrected pairwise genetic distances on the *Cyt b* gene following Hinckley et al. (2020). The sequences generated in this study are available in GenBank: MW862007-MW862010.

The *Cyt b* haplotype network clearly highlights that southern Iberian *Neomys* are nested within the diversity of *Neomys anomalus* (**Figure 2**). *Cyt b* uncorrected genetic distances among southern and northern Iberian *Neomys* were less than 2.5% and in the range of intraspecific sequence divergence of *N. anomalus* and other species of this genus (**Figure 3**).

These data suggest that the southern Iberian *Neomys* population may represent an isolated (due to its unique haplotype) but relatively recently diverged lineage. This scenario is consistent with a previous study which showed a higher mitochondrial genetic diversity and higher habitat

suitability during the Last Interglacial in the northern part of the main core distribution of *N. anomalus* than the south (Querejeta and Castresana 2018). This result is, however, in contrast with other phylogeographic studies on small mammals such as European rabbit, Cabrera vole, or Iberian mole (Nicolas et al. 2017, Barbosa et al. 2017, Ferrand and Branco 2007), the high levels of plant endemism (Gómez-Campo et al. 1984), and the description of multiple endemic vertebrates in the last decades (Ceriaco and Bauer 2018, Doadrio and Carmona 2006, Doadrio et al. 2002, Sánchez-Herráiz et al. 2000, Arntzen and García-París 1995, Mateo and Castroviejo 1990) which have supported southern Iberia as an important glacial refugia. Analyses based on multiple nuclear markers will be required to rule out any possibility that this remarkably shallow divergence between southern and central Iberian *N. anomalus* is the result of other processes, such as mitochondrial introgression.

Lack of multiple, independently inherited informative markers hinders the interpretation of potential colonization routes by the ancestors of SE Iberian populations. However, the Cyt *b* haplotype network shows that southern Iberian populations are clearly nested within the variation of lineage A (Querejeta and Castresana 2018), more specifically two mutational steps from a haplotype shared by A1 and A2b specimens (**Figure 2**; Querejeta and Castresana 2018). This might suggest a colonization route between the western Central System (Serra da Estrela and Sierra de Gredos) and southeast Iberia, but further geographic and gene sampling will be required to address this. Our results on the evolutionary origin of this population have several conservation implications. Firstly, the shallow divergence of southern Iberian populations supports potential recent local extinctions throughout southern-central Spain or undetected populations in this region. The water shrew *Neomys anomalus* is highly elusive, so the latter hypothesis seems plausible. The WC-SE colonization route scenario could suggest the presence of this species in different central and southern ranges connecting the two southern distribution patches and these with the Sierra de Gredos, such as Montes de Toledo, Sierra Morena, Sierra Bermeja, and Sierra de las Nieves. A recent unpublished record in Arroyo de La Fuensanta, Sierra de las Nieves (12/03/2016), confirmed by J. Molino and J.A. Cortés (R. Haro personal communication; **Figure 1**) is in line with this hypothesis. A combination of lack of sampling and local extinctions is also possible. A targeted live-trapping survey in 2017 in Turvillas river, Canillas de Albaida, Málaga one of the few known southern populations, did not catch this

species, nor detect any indirect signs (faeces) perhaps suggesting a local extinction (authors unpublished data). Finally, the confirmed conspecificity of southern populations with northern ones could potentially allow reintroducing individuals from northern sources if the former become extinct in the future. Our findings suggest the need for additional sampling in southern Portugal, Andalucía, Extremadura and Castilla La Mancha regions in order to properly assess the distribution and conservation status of this endemic Iberian species.

Authors contribution

AH initiated project, performed genetic analyses, and wrote first draft of manuscript which was edited by JAL, who also supervised and funded the project.

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Fig. 1. Map highlighting the distribution range of *Neomys anomalus* and the populations sampled in previous studies (Querejeta and Castresana 2018, Igea et al. 2015) and this research. EBD stands for Estación Biológica de Doñana. Personal communication records come from personal observations made by J. Molino, J.A. Cortés and R. Haro.

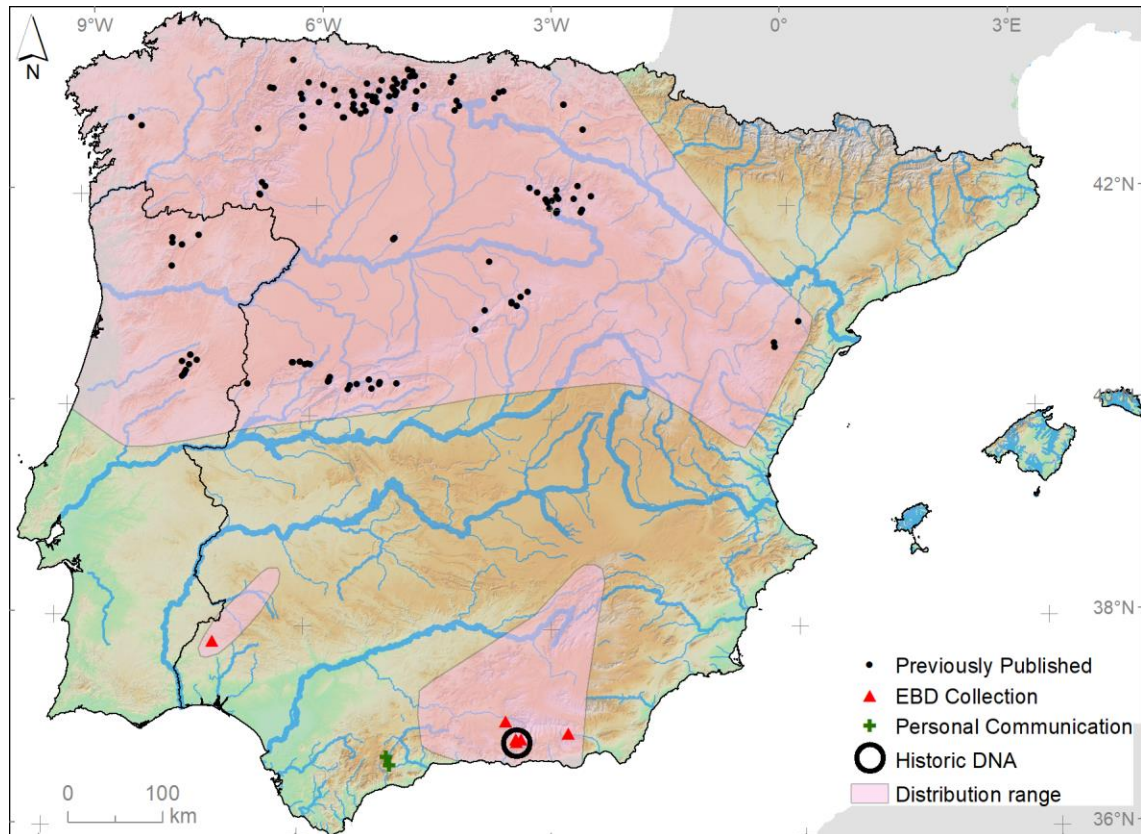


Fig. 2. Cytochrome *b* haplotype median joining network of *Neomys*. Branch lengths are proportional to the number of mutations and the number of mutations has been specified as hatch marks. Arrows point southern *Neomys* populations haplotypes. The two major *N. anomalous* lineages shown in Querejeta and Castresana (2018) and the sublineage of the closest haplotype to southern *Neomys* have been specified for the sake of comparison.

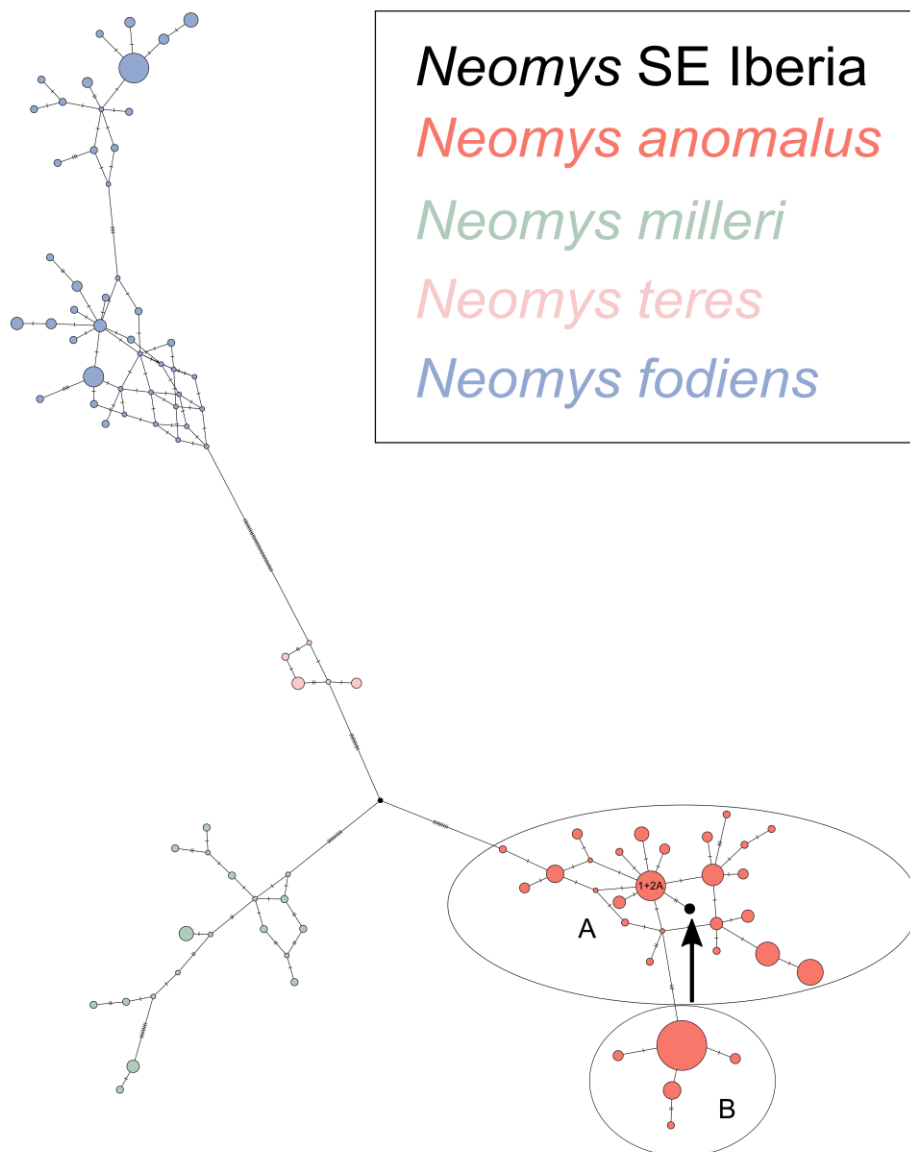


Fig. 3. Cytochrome *b* pairwise uncorrected genetic distances among species and populations of *Neomys*.

