



Short Note

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

Arlo HINCKLEY*, Jennifer A. LEONARD

Estación Biológica de Doñana – CSIC, Spain

Keywords:

Baetic System
Sierra Nevada
phylogeography
systematics
water shrew
Soricidae

Article history:

Received: 01 June 2021

Accepted: 27 September 2021

Acknowledgements

We thank J. Molino, J.A. Cortés and R. Haro, Junta de Andalucía, for permission to mention their personal observations and C. Urdiales and the Scientific Collections at the Estación Biológica de Doñana for access to the specimens. Logistical support was provided by the Laboratorio de Ecología Molecular (EBD-LEM) and the infrastructures offered by Doñana's Singular Scientific-Technical Infrastructure (ICTS-EBD). AH was supported by a Spanish Ministry of Economy contract CGL2014-58793-P. The Spanish Ministry of Science and Innovation grant CGL2017-86068-P to JAL supported this study.

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 Andalucía, 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.

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 (Burgin 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 (Fig. 1; Burgin 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 Rodríguez, 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 (Fig. 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 (Burgin 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., 2022; Balmori-de la Puente et

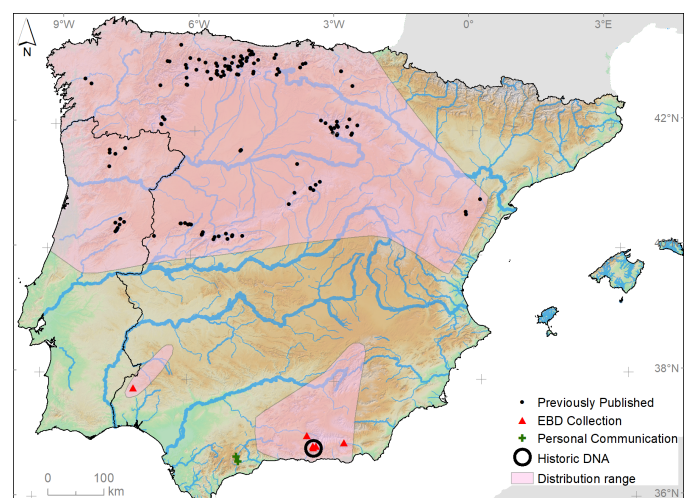


Figure 1 – Map highlighting the distribution range of *Neomys anomalus* and the populations sampled in previous studies (Querejeta and Castresana, 2018; Igea et al., 2015) and this research. EBD stands for Estación Biológica de Doñana. Personal communication records come from personal observations made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

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

*Corresponding author

Email address: arlohinckley90@gmail.com (Arlo HINCKLEY)

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; Gómez and Lunt, 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; Gómez and Lunt, 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 (Fig. 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 (Fig. 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. (2022). 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'–GTGAGRGTTGCTTTGTCTACGG–3'),
- Cytb2.Neo.F (5'–CTACCATTTCATYATYGCAGCC–3'),
- Cytb2.Neo.R (5'–TGAGGGGGTGTGTTKAGTGG–3'),
- PRPF31.Neo.F (5'–TTTCACAGCACAGAGATTGC–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 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

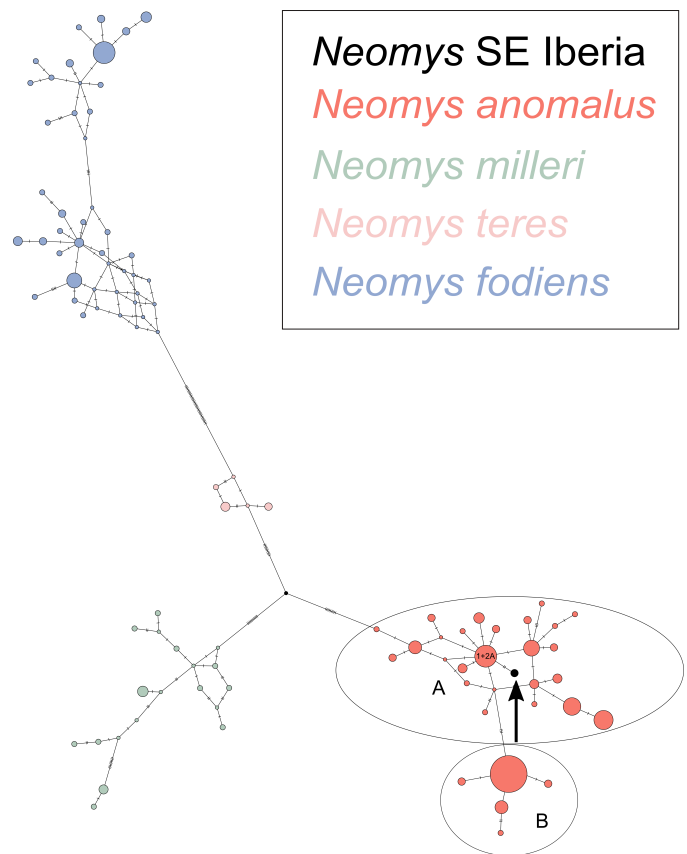


Figure 2 – Cytochrome b haplotype median joining network of *Neomys*. Branch lengths are proportional to the number of mutations and the number of mutations has been specified as hatch marks. Arrows point southern *Neomys* populations haplotypes. The two major *N. anomalus* lineages shown in Querejeta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

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* (Fig. 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 (Fig. 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 (Ceríaco 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 inher-

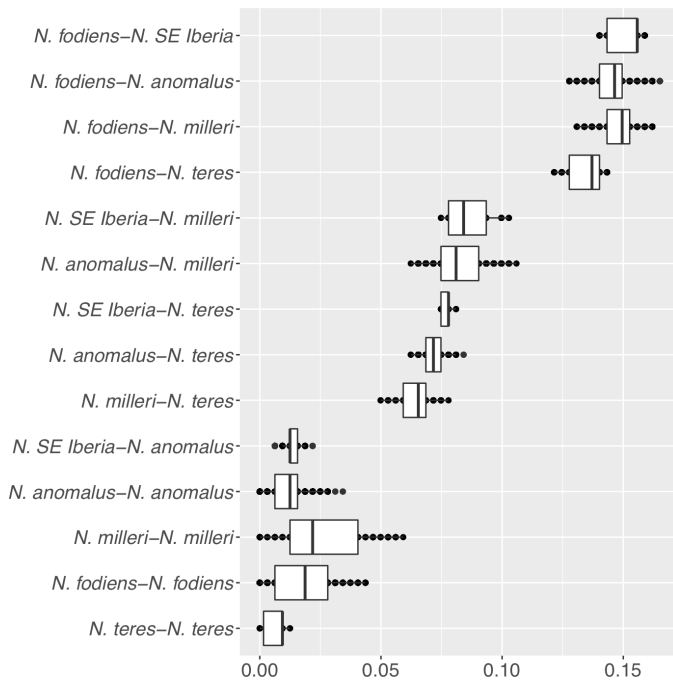


Figure 3 – Cytochrome b pairwise uncorrected genetic distances among species and populations of *Neomys*.

ited 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 (Fig. 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 pers. comm.; Fig. 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. ☞

References

- Abellán P., Svenning J.C., 2014. Refugia within refugia — patterns in endemism and genetic divergence are linked to Late Quaternary climate stability in the Iberian Peninsula. *Biol. J. Linn. Soc. Lond.* 113(1): 13–28. doi:10.1111/bij.12309
- Arntzen J.W., García-París M., 1995. Morphological and allozyme studies of midwife toads (genus *Alytes*), including the description of two new taxa from Spain. *Contrib. Zool.* 65(1): 5–34.
- Balmori-de la Puente A., Norez C., Román J., Fernández-González A., Aymerich P., Gosálbez J., Escoda L., Castresana J., 2019. Size increase without genetic divergence in the Eurasian water shrew *Neomys fodiens*. *Sci. Rep.* 9(1): 1–1. doi:10.1038/s41598-019-53891-y
- Barbosa S., Paupério J., Herman J.S., Ferreira C.M., Pita R., Vale-Gonçalves H.M., Cabral J.A., Garrido-García J.A., Soriguer R.C., Beja P., Mira A., 2017. Endemic species may have complex histories: Within-refugium phylogeography of an endangered Iberian vole. *Mol. Ecol.* 26(3): 951–967. doi:10.1111/mec.13994
- Burgin C.J., He K., Haslauer R., Sheftel B.I., Jenkins P.D., Ruedi M., Hintsche S., Motokawa M., Hinckley A., Hutterer R., 2018. Family Soricidae (Shrews). In: Wilson D.E., Mittermeier R.A. (Eds.) *Handbook of the Mammals of the World Vol. 8. Insectivores, Sloths and Colugos*. Lynx edicions, Barcelona. 332–551.
- Cerfaco L.M., Bauer A.M., 2018. An integrative approach to the nomenclature and taxonomic status of the genus *Blanus* Wagler, 1830 (Squamata: Blaniidae) from the Iberian Peninsula. *J. Nat. Hist.* 52(13–16): 849–880. doi:10.1080/00222933.2017.1422283
- Doadrio I., Carmona J.A., Fernández-Delgado C., 2002. Morphometric study of the Iberian *Aphanius* (Actinopterygii, Cyprinodontiformes), with description of a new species. *Folia Zool. Brno* 51(1): 67–80.
- Doadrio I., Carmona J.A., 2006. Phylogenetic overview of the genus *Squalius* (Actinopterygii, Cyprinidae) in the Iberian Peninsula, with description of two new species. *Cybius* 30(3): 199–214.
- Ferrand N., Branco M., 2007. The evolutionary history of the European rabbit (*Oryctolagus cuniculus*): major patterns of population differentiation and geographic expansion inferred from protein polymorphism. In: *Phylogeography of Southern European Refugia*. Springer, Dordrecht. 207–235.
- Forcina, G., Camacho-Sánchez M., Tuh F.Y.Y., Moreno S., Leonard J.A., 2021. Markers for genetic change. *Heliyon* 7: e05583. doi:10.1016/j.heliyon.2020.e05583
- Franco A., Rodríguez M., 2001. Libro rojo de los vertebrados amenazados de Andalucía. Junta de Andalucía, Sevilla. [in Spanish]
- Gómez A., Lunt D.H., 2007. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S., Ferrand N. (Eds.) *Phylogeography of southern European refugia*. Springer, Dordrecht. 155–188.
- Gómez-Campo C., Bermúdez-de-Castro L., Cagiga M.J., Sánchez-Yéllamo M.D., 1984. Endemism in the Iberian peninsula and Balearic Islands. *Webbia* 38(1): 709–714. doi:10.1080/00837792.1984.10670341
- Hinckley A., Hawkins M.T., Achmadi A.S., Maldonado J.E., Leonard J.A., 2020. Ancient divergence driven by geographic isolation and ecological adaptation in forest dependent Sundaland tree squirrels. *Front. Ecol. Evol.* 8: 208. doi:10.3389/fevo.2020.00208
- Hinckley A., Camacho-Sánchez M., Ruedi M., Hawkins M.T.R., Mullon M., Tuh F., Leonard J.A., 2022. Evolutionary history of Sundaland shrews (Eulipotyphla: Soricidae: *Crocridura*) with a focus on Borneo. *Zool. J. Linn. Soc.* 194(2): 478–501. doi:10.1093/zoolinnean/zlab045
- Igea J., Aymerich P., Bannikova A.A., Gosálbez J., Castresana J., 2015. Multilocus species trees and species delimitation in a temporal context: application to the water shrews of the genus *Neomys*. *BMC Evol. Biol.* 15(1): 1–16. doi:10.1186/s12862-015-0485-z
- Mateo J.A., Castroviejo J., 1990. Variation morphologique et revision taxonomique de l'espèce *Lacerta lepida* Daudin, 1802 (Sauria, Lacertidae). *Zoosystema* 12(3–4): 691–706. [in French]
- Nicolas V., Martínez-Vargas J., Hugot J.P., 2017. Molecular data and ecological niche modelling reveal the evolutionary history of the common and Iberian moles (Talpidae) in Europe. *Zool. Scr.* 46(1): 12–26. doi:10.1111/zsc.12189
- Querejeta M., Castresana J., 2018. Evolutionary history of the endemic water shrew *Neomys anomalus*: Recurrent phylogeographic patterns in semi-aquatic mammals of the Iberian Peninsula. *Ecol. Evol.* 8(20): 10138–10146. doi:10.1002/ece3.4487
- Haro R., Ramírez J., Palomo L.J., 2004. Nuevos datos sobre la distribución del musgano de Cabrera en la provincia de Málaga. *Galemys* 16(1): 69–70. [in Spanish]
- Sánchez-Herráiz M.J., Barbadillo L.J., Machordom A., Sanchiz B., 2000. A new species of pelodytid frog from the Iberian Peninsula. *Herpetologica* 1: 105–118.
- Spöri Y., Flot J.F., 2020. HaplowebMaker and CoMa: two web tools to delimit species using haplowebs and conspecificity matrices. *Methods Ecol. Evol.* 11(11): 1434–1438. doi:10.1111/2041-210X.13454

Associate Editor: M. Scandura