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The identity of an allochthonous Pyrenean population of *Speleomantes* cave salamanders

Enrico Lunghi[1,2,3,4], Raoul Manenti[5,6] & Roberta Cimmaruta[7]

[1] Key Laboratory of the Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

[2] Museo di Storia Naturale dell'Università degli Studi di Firenze, "La Specola", Firenze, Italy

[3] Natural Oasis, Prato, Italy

[4] Unione Speleologica Calenzano, Calenzano, Firenze, Italy

[5] Department of Environmental Science and Policy, Università degli Studi di Milano, via Celoria, 26, 20133 Milano, Italy

[6] Laboratorio di Biologia Sotterranea "Enrico Pezzoli", Parco Regionale del Monte Barro, 23851 Galbiate, Italy

[7] Dipartimento di Scienze Ecologiche e Biologiche, Università della Tuscia, Largo dell'Università snc, 01100 Viterbo, Italy

Corresponding author: Raoul Manenti, e-mail: raoulmanenti@gmail.com

Salamanders of the genus *Speleomantes* are the only members of the family Plethodontidae occurring in Europe: seven species (*S. ambrosii*, *S. italicus*, *S. flavus*, *S. supramontis*, *S. imperialis*, *S. genei*, *S. sarrabusensis*) are endemic to Italy, while the distribution of *S. strinatii* also extends to France (Lanza et al. 2006). A few records of *Speleomantes* exist from beyond their natural range. Individuals of *S. ambrosii* and *S. italicus* have been introduced to a cave in southern Tuscany (Italy) for an experiment of artificial syntopy (Forti et al. 2005), which has resulted in the formation of new hybrids (Cimmaruta et al. 2013). In other cases, individuals of *S. ambrosii* have been released in the eastern part of the range of *S. strinatii* (Liguria, Italy), while individuals of this latter species have been moved to the Italian Karst (northeastern Italy) (Lanza et al. 2006). Outside Italy, allochthonous populations of *Speleomantes* are known from Germany and France. In the first case, a population of about twenty *S. italicus* has been known from a quarry in a forested area in central Germany since 2013 (Ginal et al. 2021, Schulz et al. 2021). One of the alien French populations was discovered in central France in 2015, when about thirty individuals of *S. strinatii* were observed inside a natural cave (Lucente et al. 2016). The second French allochthonous population was introduced to a mine in the Pyrenees in 1970 (Lunghi et al. 2018) in the shape of individuals from a rearing program conducted at the Subterranean Laboratory of Moulis (Ariège, France) (Durand 1967a, b). The old registers of this experimental program report that the *Speleomantes* reared in Moulis had been collected from

about fifteen localities ranging from the Maritime Alps to the Tuscan Apennines, an area that embraces the distribution of all the three mainland *Speleomantes* species (Guillaume & Durand 2003, Lunghi et al. 2018).

Considering the interfertility that characterizes the mainland *Speleomantes* spp. (Cimmaruta et al. 2013, Ficetola et al. 2019), it was recently hypothesized that the introduced population of the Pyrenees may consist of a new generation of hybrids (Lunghi et al. 2018). This hypothesis was also supported by the peculiar dorsal colour patterns observed in some individuals, which seemed a mix of those characterizing the three mainland species (Lunghi et al. 2020b). The scant existing information on the origin of the Pyrenean allochthonous population is mostly anecdotal and refers to *S. strinatii* (Raffaëlli 2007); however, no studies have been performed yet to shed light on the identity of this introduced population. In this study we assess the genetic structure of the Pyrenean *Speleomantes* population and compare it with the available information in order to unravel the identity of its components and verify whether or not these cave salamanders represent hybrids.

In August 2018, we surveyed the allochthonous *Speleomantes* population living in a mine situated within the Parc Naturel Régional des Pyrénées Ariégeoises, in France (Lunghi et al. 2018). As in previous papers, no further information on the exact location of this site is provided to ensure the protection of this population (Lunghi et al. 2019). We collected tissue samples from 31 specimens of

Speleomantes captured inside the mine. Using a scalpel, we removed about 10 mm of the tail tip, placed it in a 2-ml plastic tube filled with absolute ETOH right after the cut. Before each cut, the scalpel was held over a flame until it became red hot; this ensured thorough sterilization and an easy cut. Total DNA was isolated from the tissue sample according to a slightly modified CTAB protocol (Doyle & Doyle 1987). Two mitochondrial regions of the genes cytochrome-b (cytb) and NADH dehydrogenase subunit 2 (ND2) were amplified using the primers and PCR conditions as reported in Cimmaruta et al. (2015). PCR products were submitted to Macrogen Inc. (www.macrogen.com) for purification and sequencing. Electropherograms were visually checked and the obtained sequences were deposited in GenBank (accession number MZ666133). A 524-bp fragment of the nuclear gene sodium–calcium exchanger (NCX1) was also sequenced using the primers NCX1_F (5'-GACTGTCTCCAACTTGACCTTGAT-3') and NCX1_R (5'-CTTTGAGGATTCTGGCCATGT-3'), as originally reported in Roelants et al. (2007). PCR re-

actions were performed according to Rovito (2010), in a total volume of 25 μl, containing 1 unit of Taq DNApolymerase (Promega), 1 unit of reaction buffer 10X, 2.5 μl of MgCl2, 0.2 μl of dNTPs, 0.2 of each primer, and 2 μl of DNA template. Thermal cycling conditions were the following: initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 s, 1-minute annealing at 51°C, 1-minute elongation at 72°C, and final elongation for 10 minutes at 72°C. The obtained sequences were deposited in GenBank (accession numbers MZ666135–MZ666137). The analyzed NCX1 fragment was used to confirm the mitochondrial taxonomic assignment of the samples since it showed two diagnostic SNPs (positions 3 and 192) between the three mainland species of *Speleomantes*: TC *S. strinatii*, TT *S. ambrosii*, CT *S. italicus*. To assign the obtained sequences to a species of *Speleomantes*, concatenated mitochondrial haplotypes were compared to those of the three mainland species: *S. strinatii*, recovered from Cimmaruta et al. (2015); *S. italicus* and *S. ambrosii ambrosii*, recovered from Lucente et al. (2016), with *S. imperialis* and *S. supra-*
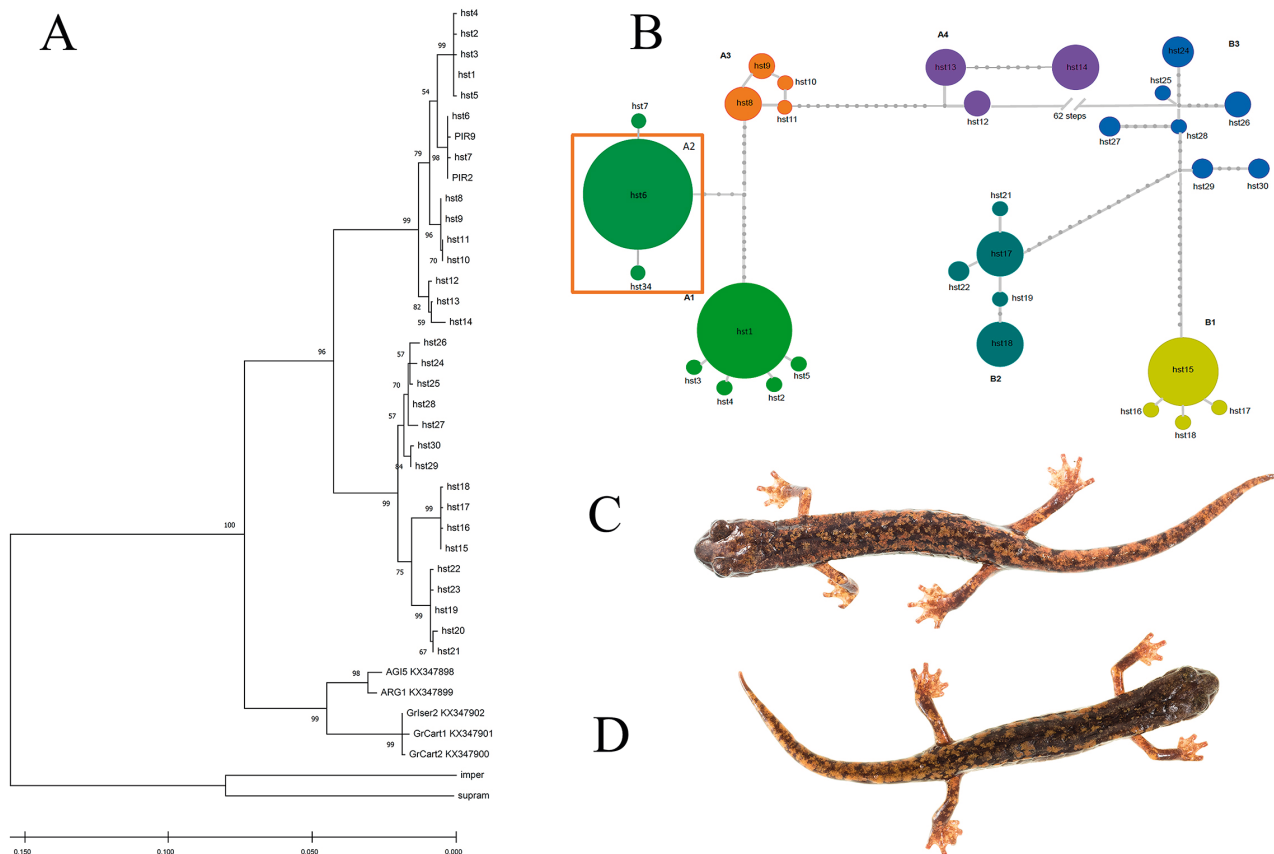

Figure 1. (A) Phylogenetic relationships between *S. italicus, S. ambrosii* and *S. strinatii* from concatenated sequences of cyt*b* and ND2. Neighbor Joining tree; bootstrap values above 85% are shown on each node. The Sardinian species *S. supramontis* and *S. imperialis* serve as outgroups. (B) Median-joining network including 194 sequences of *S. strinatii* from Cimmaruta et al. (2015) and the 31 sequences from the Pyrenees analyzed in this study (squared). Haplogroups A1–4 and B1–3 are named and coloured as in Cimmaruta et al. (2015). (C) An individual from the introduced Pyrenean population (Lunghi et al. 2020b). (D) An individual from the identified source population located in the southern part of Genoa Province (Lunghi et al. 2021); the dorsal pattern is similar to that from the Pyrenean population (Lunghi et al. 2020b).

montis for further comparison. After finding the best substitution model (TN93+G), these sequences were used to build a Neighbor Joining (NJ) tree based on Tamura & Nei (1993) distances with set gene partitions and one thousand bootstrap replicates. All analyses were run in the software MEGA X (Kumar et al. 2018). A median-joining network was built on the mt concatenated alignment, consisting of the whole *S. strinatii* mitochondrial dataset from Cimmaruta et al. (2015) and the concatenated sequences from the Pyrenees, using Network v. 10.0 (http://www.fluxus-engineering.com) under the Greedy FHP criterion (Bandelt et al. 1999, Foulds et al. 1979).

The fragments obtained from the 31 individuals from the Pyrenees were 638 bp in length for cytb and 675 bp for ND2, providing a final concatenated alignment of 1313 bp. A single haplotype was found for the cytb gene; at ND2, two haplotypes were recovered that differed in a single mutation, with the same haplotype found in all the sampled individuals except one. Both NJ and ML analyses carried out on concatenated sequences provided the same topology, with the Pyrenean haplotypes nesting within *S. strinatii* (Fig. 1A), and clustering with the samples from the environs of Genoa (southeastern Liguria). The same result was obtained in the outcome of the median-joining network (Fig. 1B), where the haplotypes from the entire species range of *S. strinatii* were included to represent all the subclades found in this species. One of the two haplotypes from the Pyrenees was the same as the most frequently recovered one from the caves in the area of Rapallo, Bargagli and Isoverde (haplotype hst6), while the second haplotype (hst34) was a single mutational step away. All of the 31 Pyrenean individuals showed the same bases (TC) of the other *S. strinatii* samples at the two NCX1 SNP positions.

The genetic data obtained clearly identify all individuals examined from this Pyrenean locality as *S. strinatii*. The mitochondrial haplotypes are identical to the most common one found in southerneast Liguria (haplotype hst6; Cimmaruta et al. 2015). The source area of the introduced Pyrenean population is therefore most likely located in the southern parts of Genoa Province, where the same haplotype is largely dominant and even exclusive in the local gene pool in the cave salamanders of the caves of Rapallo, Bargagli and Isoverde. The only differentiated haplotype (hst34) diverges from hst6 by a single mutation at the ND2 gene. The analysis of the nuclear NCX1 gene fragment also confirms that the sampled individuals belong to *S. strinatii*, without any sign of admixture.

The different haplotypes characterizing the two French allochthonous populations so far known (that showed up in this study and the one described in Lucente et al. 2016) highlight two independent introduction events, with founders coming from different clades of *S. strinatii*, whose gene pool is subdivided into two main groups that inhabit the western and eastern parts of its range, respectively (Cimmaruta et al. 2015). The individuals sampled in the Pyrenees and analysed for this study have haplotypes belonging to populations of the eastern clade, while those from Poitou-Charente province had haplotypes nested within the western clade of *S. strinatii* (Lucente et al. 2016). Among *S. strinatii* from the source populations (Rapallo, Bargagli and Isoverde), patterns that diverge from the "classic" yellowish coloration of this species are sometimes found (Lanza et al. 1995, Lunghi et al. 2021) in specimens that look like the more "reddish" ones observed in the Pyrenean population (Lunghi et al. 2020a) (Figs 1C–D). However, such reddish coloration seems to be more frequent and have greater intensity in the allochthonous Pyrenean population, potentially suggesting the occurrence of stochastic events that have boosted the spread of a particular phenotype. On the other hand, additional influences (e.g., environmental conditions or diet) cannot be excluded; indeed, *S. strinatii* has a relatively wide distribution range across different environments (Lanza et al. 2006), which is a condition that likely promotes phenotypic plasticity (Auld et al. 2010, Lunghi & Zhao 2020).

The data presented here negate the hypothesis of a hybrid Pyrenean *Speleomantes* population at the investigated site. Actually, none of the known hybrid populations has *S. strinatii* as a parental species (Cimmaruta et al. 2013, Ficetola et al. 2019). Furthermore, a study analysing an area of parapatric distribution between *S. strinatii* and *S. ambrosii*, as well as the results of an experiment of artificial syntopy in a cave in the same area, suggested *S. strinatii* as the more successful competitor and able to relegate *S. ambrosii* to the less suitable environmental sectors of the cave (Cimmaruta et al. 1999). Therefore, even if individuals of all three mainland *Speleomantes* species were originally released in the Pyrenees, *S. strinatii* would have outcompeted *S. ambrosii* and *S. italicus* in terms of adaptability, competition, or even by its higher tolerance of suboptimal microclimatic conditions (Ficetola et al. 2018) and would therefore have persisted as the "last survivor" of the originally introduced multispecies assemblage.

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