



## Research Article

## Revising museum collections help to fill knowledge gaps in the Italian mammal fauna: the case of *Sorex araneus* and *Sorex antinorii* from South Tyrol

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### Abstract

In Italy, after the elevation to species rank of the Valais shrew *Sorex antinorii*, all collection specimens from the museums previously attributed to the Eurasian shrew *Sorex araneus* were assigned to the former species. But no official verification of this “automatic” species attribution has ever been attempted. In our work we did the revision of 14 specimens of the *Sorex araneus* group from the Autonomous Province of Bolzano-South Tyrol in the north-east of Italy, by using molecular markers. The study was based on cytochrome b sequences, that allowed to assign unambiguously the analyzed specimens to one of the *Sorex* species. Among these South Tyrolean *Sorex* records we unexpectedly found only one specimen of *S. antinorii*, whereas the other 13 specimens could all be assigned to *S. araneus*. In this way, we were able to prove the occurrence of both *S. antinorii* and *S. araneus* for South Tyrol. Our work furthermore shows that *S. araneus* in Italy was never “gone” and it has to be added to the list of Italian mammals again. These findings thus stress the importance of a revision of museum collections by using modern technologies, to estimate the correct level of diversity of the small mammal fauna at regional and national level and, as in the case of *S. antinorii*, to better define its actual northern distribution limit in the Alps.

## Introduction

The genus *Sorex* (Linnaeus) includes numerous species, whose status has not yet been sufficiently clarified (Spitzenberger, 1990). Therefore, information on the number of species in the literature varies considerably, ranging from 64 species reported in the first mammalian checklist of the world (Honacki et al., 1982) to 70 in Wilson and Reeder (1993) and 77 in Wilson and Reeder (2005), to the current 86 (Wilson and Mittermeier, 2018).

The Eurasian shrew *Sorex araneus* is widespread throughout the northern Palearctic range and shows numerous karyotypic races, all parapatric (Searle and Wojcik, 1998). In the past, it was also given as present on the Italian territory (Corbet and Hill, 1991; Toschi, 1965). In the course of time, many of its chromosomal races raised to species level and were included in the so called *Sorex araneus* group, a monophyletic group of morphologically similar species, but with marked chromosomal differences (Searle and Wojcik, 1998; Zima et al., 1996). *Sorex coronatus* (Meylan and Hausser, 1978) and *Sorex antinorii* (the “Valais race”), for instance, have been shown to be clearly different from all other chromosome races of *S. araneus* not only karyologically, but also morphologically, biochemically and genetically (Brünner et al., 2002). Specimens of *S. araneus* s.l. from all over Italy, analysed by Brünner et al. (2002), showed clear evidence of *S. antinorii*, but not of *S. araneus sensu strictu*. As a consequence, all specimens previously attributed to *S. araneus* in Italy were classified as *S. antinorii* and *S. araneus s. str.* was not recognized anymore for the Italian peninsula (Loy et al., 2019; Hutterer and Kryštufek, 2016; Amori et al., 2008). However, a complete review of Italian *Sorex* sp. material has never been officially attempted.

Amori et al. (2008) reported five *Sorex*-species for Italy: *Sorex alpinus*, *S. antinorii*, *S. arunchi*, *S. minutus* and *S. samniticus*. Among these species, two can be assigned to the *S. araneus* group: the sub-endemic *S. antinorii* and the endemic *Sorex samniticus*. The latter is considered a phylogenetic basal species of the *S. araneus* group, characterized by not-rearranged fully acrocentric chromosomes (Mackiewicz et al., 2017). In the latest checklist of Italian mammals (Loy et al., 2019), only four *Sorex*-species are recognized, as *Sorex arunchi*, described by Lapini and Testone (1998), was retracted after molecular genetic studies by Yannic et al. (2012) had shown its conspecificity with *S. antinorii*.

Although *S. araneus s. str.* is not listed in the current checklist of Italian mammals (Loy et al., 2019), there is some evidence of its presence, at least in some areas in the Northeast. In fact, preliminary analyses of the mandibles of *Sorex* sp. from the Italian Alps (Friuli-Venezia Giulia and Veneto regions), suggest the occurrence of populations with a closer morphology to *S. araneus* than to *S. antinorii* (Lapini and Casol, 2017; Dorigo et al., 2016). Such observations make revision of collection material coupled with analysis of genetic data mandatory to support the actual occurrence of *S. araneus s. str.* in Italy (Loy et al., 2019).

Bertolino et al. (2015) already emphasized the importance of genetic studies to better understand the taxonomy and genetic diversity of Italian small mammals. Italy is an endemism-rich area for small mammals but for different reasons species richness and distribution, as well as possible contact zones between different species, are far from being fully described (Amori and Castiglia, 2018).

In some Italian museums, especially in the North, such as the Museum of Nature South Tyrol, there are still numerous specimens labeled as *S. araneus*. As a matter of fact, small mammal diversity in South Tyrol, a region in the far north of northeastern Italy, is largely neglected in scientific literature, although its peculiar geographical posi-

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**Figure 1** – Map showing the geographical origin of the 14 analyzed *Sorex* spp. specimens from South Tyrol (circle = *S. araneus*, triangle = *S. antinorii*). In the box a part of the Alpine distribution range of *S. araneus* is reported (in grey), according to the last IUCN assessment by Hutterer and Kryštufek (2016). The shaded area shows the study area in north-eastern Italy, at the border of the currently known distribution range of *S. araneus*.

tion makes this region a putative contact zone between Italian endemic species and genetic lineages and their European relatives.

In this study, we employed molecular analyses to verify which of the species from the *Sorex araneus* group occur in the Autonomous Province of Bolzano - South Tyrol. Extracting DNA from preserved museum specimens, we compared South Tyrolean material with sequences available in GenBank of individuals of *S. araneus* and *S. antinorii*, collected in different sites of their current known European distribution range. In contrast to the hardly reliable discrimination of these species on the basis of preserved specimens' morphology (skulls and bellows), molecular markers offer the opportunity to re-evaluate the species attribution, to define the distribution boundaries of *S. antinorii* and eventually confirm or reject the occurrence of *S. araneus* in north-eastern Italy.

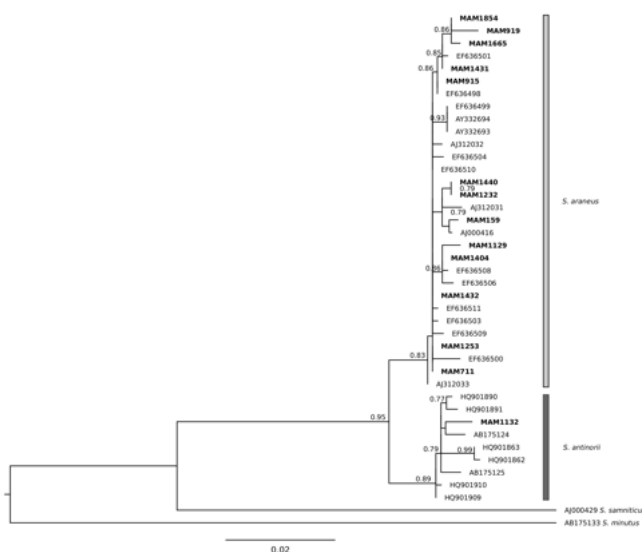
## Materials and methods

We examined 14 specimens of *Sorex* sp. preserved in alcohol at the Museum of Nature South Tyrol (Bolzano), collected in different localities of the province (Fig. 1). Total genomic DNA was extracted by standard salting-out procedure (Aljanabi and Martinez, 1997) and a

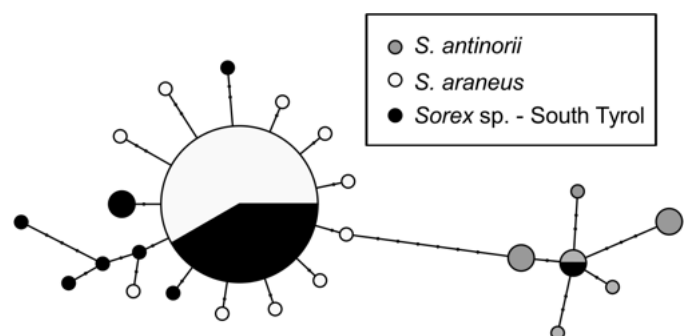
fragment of the mitochondrial cytochrome b gene was amplified for all the *Sorex* spp. specimens. Cytochrome b sequences were obtained using the primers L14734 (Ohdachi et al., 2001) and H15906 (Lebedev et al., 2007). PCR reactions were carried out in 25  $\mu$ l reaction volume including 200 ng of each primer, 2.5  $\mu$ l of 10 $\times$  buffer, 2.5  $\mu$ l MgCl<sub>2</sub> 50 mM, 0.2 mM dNTP, 2 U Taq polymerase (BioLine), and 50–500 ng of template DNA. Double stranded PCR products were purified using Sure Clean (BioLine) and prepared for automated sequencing using the same primers as used for the amplification. The products of amplification were sent for sequencing to an external service (Microsynth). Raw sequences were quality checked and trimmed using FinchTV v1.5 (Geospiza Inc.) and successively deposited on GenBank (Accession numbers from MW389518 to MW389531).

The obtained sequences (637 bp) of *Sorex* spp. from South Tyrol were aligned and compared respect to available sequences of both *S. antinorii* and *S. araneus* archived in GenBank. The alignment was performed by using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm (Edgar, 2004), implemented in the software Seaview version 5.0.4 (Gouy et al., 2010). In order to properly assign the individuals to one of the two species of *Sorex* spp. putatively present in the area, our 14 sequences were aligned with a small set of cytochrome b from individuals from Switzerland and France (accession numbers in Fig. 2), i.e. the closest localities to South Tyrol available in GenBank. The aligned sequences were used to build a maximum likelihood phylogenetic tree using the software PhyML 3.0 (Guindon et al., 2010) and a Bayesian tree with MrBayes 3.2.4 (Ronquist and Huelsenbeck, 2003). Both the maximum likelihood (ML) and bayesian analyses (BA) were performed under the assumption of a HKY model with gamma rates. This model was chosen among 56 different evolutionary models using modelgenerator v 0.85 (Keane et al., 2006). The best fitting model was chosen by comparing the likelihood by Bayesian Information Criteria (BIC). The robustness of the ML tree was assessed using the Approximate Likelihood-Ratio (aLRT; Anisimova and Gascuel, 2006) implemented in PhyML. Bayesian inference was performed using two independent searches of one million of generations and sampling every 100 steps. After a burn-in of the first 25% genealogies, a 50%-majority rule consensus tree was built. Finally, the same dataset (n=39 after removing the two outgroup species) was used to obtain a parsimony network using the R package Pegas (Paradis, 2010).

Successively a larger dataset (n=277) gathered from GenBank was used to compare mtDNA diversity indexes for *S. araneus* in South Tyrol respect to other countries for which cytochrome b sequences were available. Genetic diversity indexes, i.e. the number of haplotype and haplotypic and nucleotide diversity, were estimated using the R package Pegas (Paradis, 2010).



**Figure 2** – Maximum likelihood phylogenetic tree of *Sorex* species based on Tamura-Nei model with gamma distributed rates. Numbers at nodes are aLRT support. Museum IDs of specimens from South Tyrol are bolded, whereas for individuals gathered from GenBank the accession number is reported.



**Figure 3** – Statistical parsimony network of *Sorex* sp. haplotypes for South Tyrol, Italy, Switzerland and France. Two main haplogroups were found, corresponding to the two species, *S. araneus* and *S. antinorii*. Only one of South Tyrolean haplotypes fall within the *S. antinorii* haplogroup.

**Table 1** – Collection localities of the 14 examined specimens of *Sorex* spp., the name of the municipalities is given in German and Italian. All specimens are deposited in the Museum of Nature South Tyrol (Bolzano).

Museum ID	Species	Collection year	Locality (municipality)	Altitude	Longitude	Latitude
MAM 159	<i>S. araneus</i>	1996	Eyrs (Laas/Lasa)	875	10.65490	46.62430
MAM 711	<i>S. araneus</i>	2007	Meransen (Mühlbach/Rio di Pusteria)	1510	11.65540	46.81870
MAM 915	<i>S. araneus</i>	2013	Laugenalm (Unsere liebe Frau im Walde-St. Felix/Senale San Felice)	1780	11.09350	46.50700
MAM 919	<i>S. araneus</i>	2012	Weißenstein (Deutschnofen/Nova Ponente)	1500	11.41300	46.38800
MAM 1129	<i>S. araneus</i>	2014	Obere Gostalm (Moos in Passeier/Moso in Passiria)	1990	11.15870	46.88410
MAM 1232	<i>S. araneus</i>	2014	Traiersee (Sand in Taufers/Campo Tures)	2009	11.91410	46.92260
MAM 1253	<i>S. araneus</i>	2002	Monte Morel (Altrei/Anterivo)	1265	11.37854	46.28566
MAM 1404	<i>S. araneus</i>	2015	Hinterstein (Brenner/Brennero)	1456	11.32210	46.96800
MAM 1431	<i>S. araneus</i>	2015	Zanser Alm (Villnöss/Funes)	1950	11.77790	46.63600
MAM 1432	<i>S. araneus</i>	2015	Karthaus (Schnals/Senales)	1280	10.91110	46.70530
MAM 1440	<i>S. araneus</i>	2016	Streitmoos (Karneid/Cornedo all’Isarco)	1275	11.44330	46.46780
MAM 1665	<i>S. araneus</i>	2017	Simelemüller (Mölten/Meltina)	1136	11.24340	46.59900
MAM 1854	<i>S. araneus</i>	2019	Gurndinalm (Aldein/Aldino)	2034	11.43569	46.35019
MAM 1132	<i>S. antinorii</i>	2014	Tramin (Tramin a.d. Weinstraße/Termenon s.s.d.Vino)	870	11.22950	46.36050

## Results

After the alignment the cytochrome b sequences (n=41) includes 520 complete sites (no gaps, no ambiguities), 91 variable sites (17.5%) and 34 informative sites (6.5%). Both ML and BA phylogenetic analyses give a clear evidence of the presence of both *S. araneus* and *S. antinorii* in South Tyrol (Fig. 2 and Fig. S1 in supplementary information). The two species are monophyletic and 13 specimens (nine haplotypes) can be attributed to *S. araneus* on the basis of their phylogenetic relationships (Fig. 2). Conversely, only one specimen can be assigned to *S. antinorii* (Fig. 2). The two species show an average uncorrected genetic distance equal to 0.015. According to the SP network the 10 South Tyrolean haplotypes are clustered in the two species that are separated by six mutational steps (Fig. 3). Genetic diversity indexes in Tab. 2 suggest that the observed haplotypic diversity in South Tyrol is comparable to that in other countries within the distribution area of *S. araneus*. On the other hand, nucleotide diversity is much lower than the one observed in other countries.

## Discussion

In Italy, after the elevation to species rank of the Valais shrew *S. antinorii*, all specimens previously attributed to *S. araneus* were assigned to *S. antinorii*. However, some preliminary studies conducted by Lapini et al. (2001), Lapini and Cassol (2017) and Dorigo et al. (2016) on northern Italian *Sorex* specimens suggest that *S. araneus* was probably always present, at least in north-east Italy. Our data support this hypothesis and show how important a genetic revision of the *S. araneus* group in Italy would be: in our study on South Tyrolean *Sorex* spp. we unexpectedly found only one specimen assignable to *S. antinorii*, whereas the other specimens all belong to *S. araneus*. The *Sorex antinorii* specimen comes from one of the southernmost and lowest sample sites, located in the Lower Adige Valley. The broad valley, open to the south, is possibly a corridor of this sub-endemic Italian species to the north. The species was also found in the southern Swiss and French

Alps (Müller, 2018; Brünner et al., 2002), proving that the Alps are not an impassable barrier for *S. antinorii*. Further studies should clarify the actual northern distribution limit in South Tyrol and in the Alps in general. In addition, questions need to be answered as to whether interspecific competition with other *Sorex* species plays a role in limiting the dispersal of *S. antinorii* to the north, whether it is still spreading northwards or whether climate change might favour this southern species in its expansion.


Regarding *S. araneus*, the 13 identified individuals do not form a monophyletic clade, despite they were collected from relatively close localities compared to the overall range of the species. The lack of a South Tyrolean monophyletic clade would suggest that the presence of this species in the southern Alps is not very recent. However, the *S. araneus* population from the small area of South Tyrol shows a remarkable level of haplotypic diversity, comparable to that estimated for whole countries, but it is coupled with a lower level of nucleotide diversity. This could reflect a recent expansion following a bottleneck (i.e., during the last glaciation) or a relatively recent colonization event. However, a sampling bias cannot be excluded at this stage of the research and further analyses are required to assess the historical demography of this Italian population.

Another important and intriguing issue that needs to be carefully considered is the potential hybridization between *S. antinorii* and *S. araneus*. The existence of hybrids between the two species is evident from the literature (Yannic et al., 2008; Basset et al., 2006; Balloux et al., 2000; Brünner et al., 2002). Generally, the identified hybridization zones were very narrow (a few kilometers) and the number of admixed individuals was very low, suggesting a strong reproductive isolation between *S. araneus* and *S. antinorii*, which proves the good species status of the latter. These findings further imply that the mitochondrial DNA attributable to *S. araneus* in South Tyrolean specimens is unlikely to be due to large introgression within *S. antinorii*. In any case, this aspect deserves further investigations by using other markers, such as microsatellite and SNPs, in order to exclude potential hybridization.

In summary, the following conclusions can be drawn from our data. First of all, with the finding of *S. antinorii* a new species may be added to the small mammal species list of South Tyrol. Furthermore, basing on morphological, allozymic (Lapini et al., 2001) and molecular data (our study) we should assume a definite occurrence of *S. araneus* in Italy. On the other hand, this makes it necessary to update the Italian mammal fauna checklist recently published by Loy et al. (2019) re-including *S. araneus*. According to current knowledge, the presence of *S. araneus* in most parts of South Tyrol can be confirmed on the basis of molecular data, and it is very likely that the species also occurs elsewhere in the Italian Alpine territory. Our findings also underline the importance of revising the existing collection material from South Tyrol and the entire Alpine arc in order to properly assess the diversity level of the small mammal fauna in this region.

**Table 2** – Number of *Sorex araneus* individuals (n), number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) and their variances.

	n	H	Hd	$\pi$
Italy	13	9	0.872±0.0079	0.004282e-03±7.98e-06
Switzerland	9	6	0.834±0.0149	2.520479e-03±3.81e-06
France	8	6	0.893±0.0104	3.308129e-03±5.94e-06
Sweden	7	5	0.857±0.0161	3.240616e-03±6.00e-06
Finland	13	5	0.628±0.0205	1.405652e-03±1.55e-06
Hungary	17	10	0.919±0.0016	6.810853e-03±1.65e-05
Poland	31	8	0.454±0.0124	1.089497e-03±1.01e-06
Russia	138	56	0.862±0.0007	4.821935e-03±8.33e-06
UK	54	26	0.816±0.0029	2.605023e-03±3.27e-06

The importance of using museum collections to better describe past and current mammal biodiversity is recognized in the literature (Gippoliti et al., 2014), but in practice the reality is often different. Indeed, museum collections are rarely reanalyzed with modern technologies (i.e. molecular markers), due to lack of funding or technical difficulties. However, analysis of museum collections can improve our knowledge of mammal diversity and the combination of genetic and morphometric approaches also provides opportunities to fill knowledge gaps. 

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## Supplemental information

Additional Supplemental Information may be found in the online version of this article:

**Figure S1** Bayesian consensus tree.