



Research Article

New endemic mammal species for Europe: *Sciurus meridionalis* (Rodentia, Sciuridae)

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Abstract

Combining genetic, morphological and geographical data, we re-evaluate *Sciurus meridionalis*, Lucifero 1907 as a tree squirrel species. The species, previously considered a subspecies of the Eurasian red squirrel, *Sciurus vulgaris*, is endemic to South Italy with a disjunct distribution with respect to *S. vulgaris*. The new species has a typical, monomorphic coat colour characterized by a white ventral fur and a very dark-brown to blackish fur on the back, sides and tail. Specimens of *S. meridionalis* have a larger hind foot length and weigh about 35% more than live-caught *S. vulgaris* from northern Italy. *S. meridionalis* is larger than *S. vulgaris* specimens from three other regions in Italy for mandible length, skull width and skull (condylobasal) length, and principal component scores indicate significant shape differences of specimens from the Calabria population (*S. meridionalis*) compared to all other specimens (*S. vulgaris*). These morphological differences are further supported by genetic evidence at three mitochondrial markers (D-loop, cytochrome b and the DNA barcoding region COI) using the widest molecular dataset ever assembled for *Sciurus vulgaris* and *S. meridionalis*. All the investigated markers revealed exclusive haplotypes for *S. meridionalis* well separated from those of *S. vulgaris* and previously published results based on nuclear markers further support our taxonomic hypothesis. We suggest Calabrian black squirrel as common name for this new taxon.

Introduction

The Quaternary has experienced important climatic changes that deeply modified the distribution range of species within the Palaearctic region (Webb and Bartlein, 1992). In Europe, during the glacial events of the Quaternary, central regions tended to be covered by steppe-tundra, whereas deciduous forests were generally confined to the Mediterranean peninsulas (Zagwijn, 1992). For temperate forest mammal species, such as squirrels, it is predicted that they modified their range according to the shifts of their habitat, so that they would have survived glacial maxima in the Mediterranean peninsulas (Iberian, Italian and Balcanic) and extra-Mediterranean refugia of forests and forest fauna detected based on fossil record and phylogeographic studies (e.g. Sommer and Nadachowski, 2006; Stewart et al., 2010). Mediterranean glacial refugia have frequently been the source of postglacial and interglacial distributions of species in central and northern Europe (Taberlet and Bouvet, 1994; Hewitt, 1999, 2001; Michaux et al., 2003). But rather than contributing to the postglacial colonisation of Europe, Mediterranean populations of widespread small mammals may represent long-term isolates undergoing allopatric speciation. This could explain the high incidence of endemisms of small mammals associated with the Mediterranean peninsulas (Bilton et al., 1998; Randi, 2007; Stewart et al., 2010). Typical examples for Italy are *Talpa romana* (Filippucci et al., 1987) and *Lepus corsicanus* (Italian hare, Pierpaoli et al., 1999). The Italian hare is an endemic species confused with the

widespread *Lepus europaeus* (brown hare) but phylogenetically closer to *Lepus timidus* (mountain hare). *L. corsicanus* is present in central-southern Italy and Sicily and was first described and classified, using a morphological approach, as a true species (De Winton, 1898), later considered a subspecies of *Lepus europaeus* to be finally re-evaluated at the end of 20th century using an integrated approach of morphology and genetics (Palacios, 1996; Pierpaoli et al., 1999; Riga et al., 2001). Moreover, phylogeographic studies mainly based on mtDNA have revealed a complex genetic structure found in several Calabrian mammals, that are very often clearly distinct from the rest of peninsular Italy (for *Neomys* see Castiglia et al., 2007; for *Talpa romana* Canestrelli et al., 2010; for *Myodes* Colangelo et al., 2012; for *Muscardinus* Mouton et al., 2016). This is certainly due to the complex Quaternary paleogeographic history of Calabria, an archipelago for much of its history (Bonfiglio et al., 2002).

The Italian Peninsula is characterised by high species richness, hosting not less than 58000 known animal species (Italy's 5^o National report to the Convention on Biological Diversity, 2009–2013).

Vertebrates includes almost 1300 species, and 672 species have been assessed in the recent "IUCN Red List of Italian Vertebrates" (Rondinini et al., 2013). It has been calculated that about 10% of Italian invertebrates and about 5% of vertebrates are endemic (Rondinini et al., 2013).

Species richness in the Mediterranean peninsulae is also influenced by recent debate on the species concept in taxonomy. Some schools are in favour of less inclusive species concepts (thus raising the status of previously considered subspecies to species level, e.g. Gippoliti

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and Groves, 2012), while others are against what they call the “taxonomic/species inflation” (e.g. Zachos et al., 2013).

The Eurasian red squirrel (*Sciurus vulgaris*) is the only naturally occurring tree squirrel species throughout its wide range, which stretches from the British Isles to Eastern Siberia, China and parts of Japan (Hokkaido island) and from the northern boreal forests to the Mediterranean peninsulas. There is recent (genetic) evidence that at least two, but possibly more, allopatric species were erroneously classified under one species name (Grill et al., 2009; Gomes Rocha et al., 2014).

In Southern-Italy, a distinct subspecies, *S. v. meridionalis* occurs in Calabria (Lurz et al., 2005). The biogeography of this region has been strongly influenced by its isolation from the rest of the Italian peninsula, in particular during the Quaternary. At the beginning of the Middle Pleistocene, the tectonic evolution of southern Calabria was characterised by the uplifting of three (four if also Sila/Catena Costiera, in the northern part of the region, is considered) main blocks around which a marine basin persisted during the Early Pleistocene (Fabbri et al., 1980; Bonfiglio et al., 2002). Various taxa that became isolated in Calabria show distinct genotypes (Castiglia et al., 2007; Canestrelli et al., 2008, 2010, 2012; Colangelo et al., 2012). This may also be the case of the Calabrian squirrel. Specifically, in 2009, Grill and colleagues, using a mitochondrial DNA-based survey concluded that individuals from *S. v. meridionalis* did not contribute to the postglacial recolonization of populations farther north, but became isolated in Calabria and never reconnected to central Italian populations, forming an endemic evolutionary unit (Grill et al., 2009). Because most conservation legislation assigns priority based on some index of species diversity, endemism or on the distribution and/or rarity of species, species delimitation is critical to conservation planning.

Molecular techniques, and more recently the DNA barcoding approach using cytochrome oxidase I, triggered a small revolution inside taxonomy and conservation issues, especially concerning mammals (Galimberti et al., 2012, 2015), including sciurids (Gabrielli et al., 2014; Ermakov et al., 2015; Hope et al., 2016; Mazzamuto et al., 2016). Unfortunately no DNA barcoding data have been produced and published for the *S. vulgaris* complex except for only two sequences stored in GenBank (last accessed on November 1st 2015), with no data at all for the Calabrian population.

The aim of this study is to use for the very first time molecular sequence data mined from Genbank, new DNA barcoding sequence and biometric data to examine whether the squirrel sub-species *Sciurus vulgaris meridionalis* deserves species status. Since lack of accurate information on population trends and threats is a general problem in sciurid conservation (The IUCN Red List of Threatened Species 2014), we also discuss this taxon from the conservation point of view.

Material and methods

Source of specimens and documentation

Lectotypes

Three adult specimens from the type locality, deposited at the “Museo La Specola” Florence, Italy (MZUF). They are not cited in the published catalogue of “types” of the Florence Museum (Vanni et al., 1991). *Lectotype* (Fig. 1) – MZUF 11481 (mounted skin and skull), male adult, collected at Potilia Policastro (now Petilia Policastro), locality Cerigliana (Sila massif), Crotone province, Calabria, Italy (Lat. 39°7'39.97" N; Long. 16°41'26.27" E), 1552 m a.s.l., 24.XII.1895, Antonio Lucifero *legit*. *Description*: Skull: condylobasal length 51.9 mm, zygomatic breadth 34.3 mm, nasal length 17.3 mm, maxillary tooththrow 10.0 mm, diastema 13.3 mm, mandible length 32.5 mm, mandibular tooththrow 10.0 mm, interorbital constriction n.a. (damaged skull). No body measurements are available for this specimen. Specimen in winter coat. Body entirely black (dorsal part of the body, head, neck, tail, feet, pads and claws), with the exception of the ventral region, from inner part of the forelegs to genital area, pure white.

Paralectotypes – MZUF 11743 female adult, MZUF 11480 male adult (mounted skins with skulls inside), collected at Potilia Policastro (now Petilia Policastro), locality Cerigliana (Sila massif), Crotone province, Calabria, Italy (Lat. 39°7'39.97" N; Long. 16°41'26.27" E),



Figure 1 – Lectotype of *Sciurus meridionalis* (MZUF 11481): (A) mounted skin; (B) dorsal, (C) ventral and (D) lateral view of the skull.

1552 m a.s.l., 24.XII.1895, Antonio Lucifero *legit*. No body measurements are available for these two specimens.

Other material

Photograph of living specimen from the type locality (Fig. 2). In addition to the type series, we examined further 13 specimens preserved in the Theriological Collection “G. Aloise” (measurements in Table S2).

Biometrics

For skull measurements we used the data of adult specimens reported in Cavazza (1913) and reanalyzed by Amori et al. (2014). Following Cavazza (1913) specimens were divided into four Operational Taxonomic Units (OTUs): (a) Alps, (b) northern and central Italy including Latium and excluding Abruzzi, (c) southern Italy including Abruzzi and Campania, and (d) Calabria (for localities see Fig. 1 in Amori et al., 2014). As reported by Amori et al. (2014) skull measurements were log transformed prior to analysis and each measurement was compared among OTUs by one-way ANOVA. Next, a cluster analysis was performed to explore dissimilarities among all of Cavazza (1913) specimens in terms of their skull measurements. Dispersion of specimens in a multivariate space was analysed by Amori et al. (2014) with Principal Components Analysis (PCA) using the covariance matrix.

We also analysed some body size data: length of right hind foot and body mass of adult specimens of *S. meridionalis* were compared to those of adults *S. vulgaris*, trapped during long-term studies on demography of populations in the Italian Alps (Wauters et al., 2007). To make our analyses conservative, we used data from the alpine populations with the largest size and mass. We used General Linear Models (GLM), with either foot length or body mass as dependent variable, to test the effects of species (*S. meridionalis* vs. *S. vulgaris*), sex and the species×sex interaction. We also used a GLM with body mass as dependent variable and foot length, species and the species×foot length interaction as explanatory variables in order to test whether an increase of body mass with size differed between the two species.

DNA barcoding characterization and updated revision of available genetic data

Five tissue samples of *Sciurus meridionalis* were collected from Calabrian individuals at Spezzano della Sila, Cosenza province, Sila National Park (Lat. 39°25'79" N; Long. 16°55'84" E). In addition, five samples of *Sciurus vulgaris* were collected in northern Italy and specifically at



Figure 2 – *Sciurus meridionalis*, in summer coat, from Sila massif, Calabria, Italy. Photograph by Antonio Mancuso.

Bormio, Sondrio province, Stelvio National Park (Lat. 46°26'9.27" N, Long. 10°25'32.20" E).

Animals were trapped, manipulated and morphologically identified prior to sequencing by expert field operators. A 4 mm diameter sample of skin was taken from one ear for genetic analysis using a biopsy punch. Samples were vouchered and then stored in 99% ethanol at –20 °C. Voucher codes are listed in supplementary material (Table S1).

DNA extraction, amplification, sequencing and sequences check and correction were conducted as described in Mazzamuto et al. (2016). Barcode COI sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI) (see Table S1 for accession numbers).

To clarify biogeographical isolation phenomena, mtDNA could be very useful (Hung et al., 2016). Grill and co-workers used mtDNA data for the case of the Calabrian *S. meridionalis* (Grill et al., 2009). Consequently, three distinct datasets of mitochondrial DNA sequences were assembled for this study in order to provide an updated overview of mitochondrial nucleotide sequence variation between the Calabrian *S. meridionalis* and other Palearctic *S. vulgaris* populations. Apart from the DNA barcoding dataset based on a region of the mitochondrial COI, we recovered D-loop and cytochrome *b* (abbreviated as *cyt b*) sequences of the *S. vulgaris* complex from GenBank. This choice was motivated because they were historically used for exploring the molecular structure of Eurasian *S. vulgaris* populations and therefore, a huge number of D-loop and *cyt b* nucleotide sequences is available for comparison (see references in Table S1). Moreover, the three markers typically show high mutation rate. This is an essential condition to better identify similarities and differences among different species (COI and *cyt b*) and even populations (D-loop). Molecular datasets

were organized as follows (see also Table S1). DNA barcoding dataset (COI) includes 12 sequences, 10 of which produced in this study with five samples belonging to the Calabrian *S. meridionalis* and the other five belonging to *S. vulgaris*. The remaining two sequences were mined from GenBank and refer to *S. vulgaris* from European localities out of Italy.

D-loop dataset included 456 sequences from GenBank encompassing data from Calabria and other Italian localities as well as other European and Asiatic populations (see Table S1 for further locality details and references).

Cyt b dataset included 50 sequences from GenBank, belonging to several European and Asiatic populations. These sequences also encompass records from Calabria and other Italian localities (see Table S1 for further locality details and references).

Moreover, in order to explore sequence variation among different species of the genus *Sciurus*, nucleotide sequences for *S. niger* and *S. carolinensis* were also retrieved from Genbank and included in the datasets (see Table S1 for further details). Other species of the genus *Sciurus* occur in GenBank, however, the above-mentioned taxa are the only ones for which sequences of the three mitochondrial regions used in this study were available.

Bioinformatics analyses were conducted separately on the three datasets (i.e. COI, D-loop and *cyt b*). Sequences of each marker were aligned using MUSCLE online (<http://www.ebi.ac.uk/Tools/msa/muscle/>; Edgar, 2004) with default options. For all datasets, the number of haplotypes and nucleotide diversity per locality group and per species were computed with DnaSP v. 5.10.1 software (Librado and Rozas, 2009). Average genetic sequence divergences *p*-distance (and relative standard errors, SE) between and within distinct locality groups and *Sciurus* species were also calculated. Values were obtained for the three mitochondrial markers by using MEGA 6 with the same settings described in Galimberti et al. (2012).

Sampling localities (i.e. country of provenance) reported in Table S1 were grouped in different locality groups depending on the analyzed parameter. Specifically, for haplotype variation, we considered the following groups: overall, Italy (including Calabria), Italy (except Calabria), Calabria, Europe, Europe without Calabria and Asia. Concerning the calculation of genetic sequence divergence values, the locality groups considered were: Calabria, Italy (except Calabria), rest of Europe (European localities excluding Italy and Calabria) and Asia.

This strategy was decided taking into account the almost complete lack of phylogeographical structure at mitochondrial markers across Europe and People's Republic of China reported for *S. vulgaris* by Grill et al. (2009) and successive studies (Dozieres et al., 2012; Liu et al., 2014; Lucas et al., 2015).

Results

Family **Sciuridae** Fischer, 1817.

Genus **Sciurus** Linnaeus, 1758.

Sciurus meridionalis, Lucifero 1907.

Source of specimens and documentation

In 1907 Lucifero described a new species of Eurasian squirrel that he named *Sciurus meridionalis*, collected from forests in Calabria, in the southern tip of the Italian peninsula. Miller (1912) overlooked this taxon because he did not examine specimens from Southern Italy, whereas at the same time Cavazza (1913) recognised *meridionalis* as a valid taxon based on a morphometric study of Italian *Sciurus*. Such condition was further supported by Ghigi (1950), who accorded it species status. Ellerman and Morrison-Scott (1951) treated *S. meridionalis* as a synonym of *Sciurus vulgaris italicus* Bonaparte, 1837 but accepted as a valid subspecies *Sciurus vulgaris silanus* Heinrich, 1934 (terra typica: Sila Mountains), that was clearly a junior synonym of *S. meridionalis*. Since then, *S. meridionalis* had for more than half a century been considered as a quite distinct subspecies of the polytypic *Sciurus vulgaris* L., 1758 (Toschi, 1965; Sidorowicz, 1971; Wiltafsky, 1973; Corbet, 1978; Amori et al., 2008). Recently, it was preliminary

considered a distinct species by Gippoliti (2013) following the phylogenetic species concept.

In his paper, Lucifero (1907) stated that his father had sent some specimens of Calabrian squirrels to Prof. Enrico Giglioli at Florence for a study that, however, was never published. Effectively, three specimens are recorded in the General Catalogue of the Museum of Natural History of Florence University (Zoological Section) donated by the Marquis Antonio Lucifero in 1895.: “3 *Sciurus vulgaris* 2M 1F var. nera, Cerigliana (Sila) Potilia Policastro Calabria”). All three specimens are still present in Florence as mounted skins: the lectotype (MZUF 11481, male) and the two paralectotypes (MZUF 11743, female; MZUF 11480, male).

The original description of *Sciurus meridionalis* Lucifero, 1907 is very brief. According to Lucifero, subspecies *meridionalis* is said to differ from *vulgaris* one “nelle dimensioni, nel colore del pelame e nel complesso generale delle sue forme” [in body size, fur colour and general body shape]. Interestingly, Lucifero emphasizes that only black squirrels were known from Calabria “in human memory”.

External parameters

S. meridionalis shows no sexual dimorphism in size or fur colour. There is no marked seasonal variation in fur colour and no other colour than black has been recorded. The winter coat is bright black all over the dorsal part of the body, head and neck. The tail is black, and so are the feet, pads and claws. The abdomen is pure white from above the genital area to the chestnut inner part of the forelegs. Between the white abdomen and black flanks there is often a slate-grey line.

In the summer or transitional pelage there are often dark brown areas on the head, around the eyes and on the back. The underfur is generally dull black or slate-grey, rarely brownish. The hind foot has four tubercles at the base of the toes, against five in the forefoot. There are 8 mammae (pectoral 1–1, abdominal 2–2, inguinal 1–1 = 8), in agreement with Miller (1912).

Hecht (1931), on the evidence of three specimens (one in winter coat and two transitional), reported the almost total absence of ear tufts in *S. v. silanus*, a feature that has been confirmed recently in the iconography of the taxon (Spagnesi and De Marinis, 2002; Amori et al., 2008). From examination of four specimens in winter coat, four in summer coat and four in a transitional phase, we found that in winter there are always ear tufts, which are absent in summer. Obviously, different developmental stages occur in the transitional phase and in some cases the ears showed no tufts except for a few long hairs, the last vestige of the winter coat.

According to the available data, *S. meridionalis* has considerably larger body size than the Italian populations of *S. vulgaris* (Toschi, 1965; Wiltafsky, 1973).

Biometrics

Mean and standard deviation for each skull measurement of the 74 Italian specimens measured by Cavazza (1913) divided by OTU are reported in Tab. 3 in Amori et al. (2014). There were significant differences among OTUs in skull length, skull width, skull height and mandible length (One-way ANOVA all $p < 0.0001$). Calabrian specimens, which can be attributed to *S. meridionalis*, differed significantly from every other group for mandible length (Post-hoc Tukey test, all $p < 0.01$), and for skull width (all $p < 0.001$), while for skull length they differed from Alpine and central Italian specimens (all $p < 0.01$), but not from Campania specimens ($p = 0.088$). For skull height, they differed from Campania ($p = 0.024$) and Alpine specimens ($p = 0.018$), but not from central Italian specimens ($p = 0.43$). Principal component scores indicated that there were significant statistical shape differences among the four OTUs (one-way ANOVA: $F_{3,70} = 30.4$; $p < 0.0001$) with the Calabrian population significantly different from all others (Tukey test, all $p < 0.001$).

Foot length of the entire sample ($n = 100$) varied between 56.0 and 66.2 mm and body mass ($n = 101$) between 278 and 530 g. There was no significant difference between the sexes in foot length and no species by sex interaction (sex effect $F_{1,96} = 1.40$; $p = 0.24$; interaction $F_{1,96} = 2.29$; $p = 0.13$), but *S. meridionalis* had larger hind foot length than *S. vul-*

garis (Tab. 1, species effect $F_{1,96} = 70.0$; $p < 0.0001$). For body mass there was a significant species ($F_{1,97} = 173.4$; $p < 0.0001$) and sex effect ($F_{1,97} = 4.19$; $p = 0.043$), while the interaction was not significant ($F_{1,97} = 1.52$; $p = 0.22$): *S. meridionalis* was on average 35% heavier than *S. vulgaris* and females of both species tended to be slightly heavier than males (Tab. 1). Finally, the relationship between hind foot length and body mass did not differ between species (foot length by species interaction $F_{1,96} = 0.08$; $p = 0.78$); and in both species body mass increased significantly with foot length (estimate \pm SE = 12.0 ± 1.6 ; effect of foot length $F_{1,97} = 56.0$; $p < 0.0001$; species effect $F_{1,97} = 51.0$; $p < 0.0001$).

Multi-marker genetic characterization

Amplification of the COI barcode region with the selected primer pairs was successful and resulting nucleotide sequences were of high quality. Regarding the COI DNA barcoding dataset, no sequence contained insertion/deletions (indels), stop codons or were biased by NUMT interference. The same alignment characteristics occurred for the *cyt b* whereas, as expected, the D-loop sequence alignment showed different sites that were gaps or missing.

D-loop dataset

Concerning the D-loop region (252 bp), among the 456 sequences included in the dataset, 250 haplotypes were found (Tab. S1 and Tab. 2). Ninety-eight sites were variable, 71 parsimony informative and 27 were singleton variable sites. Every sampling locality had at least one exclusive D-loop haplotype (Tab. S1) and, as already reported by Grill et al. (2009) and successive studies, several haplotypes were shared between different countries (see for example haplotype HDL98 that is found in three European and one Asian countries, Tab. S1). Multiple cases of shared haplotypes between Eurasian countries, the reduced nucleotide variability within Europe and Asia (Tab. 2) and the low p -distance values within and between European and Asian locality groups (Tab. S3) confirm the homogeneous genetic structure across the entire *S. vulgaris* range. The only exception to this pattern is represented again by the Italian peninsula and specifically by squirrels inhabiting the region of Calabria. As already showed by Grill et al. (2009), these individuals contribute to the rather high nucleotide diversity value (0.02979 ± 0.00279) of the Italy locality group, especially when compared to the overall value (0.02432 ± 0.00083) and to the one of Italy (except Calabria) group (0.0184 ± 0.00119). Average genetic divergence values were higher among *Sciurus* species (p -distance ranging between 0.206 and 0.267, see Tab. S3), however, the group of Calabria was clearly differentiated from other *S. vulgaris* inhabiting the rest of the Italian peninsula (p -distance: 0.067 ± 0.014), European countries (p -distance: 0.067 ± 0.013) and Asia (p -distance: 0.063 ± 0.013). Analysis of the comprehensive dataset of D-loop sequence data here assembled confirms that the two Calabrian haplotypes found by Grill et al. (2009) are again unique to this region and genetic divergence values further remark the neat differentiation of *S. meridionalis* from all of the other *S. vulgaris* populations (even including different morphological subspecies) sampled by the same authors.

cyt b dataset

For *cyt b* sequence alignment (336 bp), among the 50 sequences included in the dataset, 33 haplotypes were found (Tab. S1 and Tab. 2). A total of 38 sites were variable, of which ten parsimony informative and 28 were singleton sites. All but three sampling locality groups (i.e., Albania, Korea and Portugal) had unique haplotypes and the Calabrian haplotype (HCB29) was again unique to this region and specific of *S. meridionalis*. Overall nucleotide diversity was lower than at D-loop (0.00621 ± 0.00095), however, similarly to D-loop, nucleotide diversity values resulted higher for Italy (0.01247 ± 0.00419) and about two times lower for Italy without Calabria (0.00655 ± 0.00085). Table 2 shows that also in the case of *cyt b*, Calabrian haplotypes have a key role in shaping the genetic structure of Eurasian *S. vulgaris* complex populations. The highest average divergence values were those among the different species of *Sciurus* (*vulgaris*, *niger* and *carolinensis*) and ranged between 0.127 and 0.156 (p -distance values, see Tab. S3). Individuals from Calabria (*S. meridionalis*) showed rather high divergence val-

Table 1 – Mean±SE body size (hind foot length) and body mass of *S. vulgaris* from the Italian Alps and of *S. meridionalis*. Data per sex and for sexes combined (total), sample sizes in parentheses.

Measurement	Species	Males	Females	Total
Hind foot length (mm)	<i>S. vulgaris</i>	(51) 58.4±1.0	(39) 58.3±1.2	(90) 58.4±1.1
	<i>S. meridionalis</i>	(5) 61.5±3.1	(5) 62.7±2.8	(10) 62.1±2.8
Body mass (g)	<i>S. vulgaris</i>	(51) 318.0±21.0	(39) 325.0±23.0	(90) 321.0±22.0
	<i>S. meridionalis</i>	(6) 420.0±54.0	(5) 448.0±54.0	(11) 433.0±53.0

ues with other Italian (p -distance: 0.027 ± 0.008), European (excluding Italy) (p -distance: 0.026 ± 0.007) and Asian (p -distance: 0.025 ± 0.007) populations. Values for p -distance equal or lower than 0.007 were instead observed between all of the other locality groups inhabited by *S. vulgaris*.

COI (DNA barcoding) dataset

For COI, among the 12 sequences analysed (657 bp), ten of which obtained in this study, three haplotypes were found (Tab. S1 and Tab. 2). A total of six sites were variable, of which five were parsimony-informative and one was singleton variable. Only Calabria group contained a unique COI haplotype (HCO2), whereas the other haplotype found in Italy (HCO1) was shared with a Russian individual. The third European haplotype (HCO3) was also unique but no information on its exact provenance is available. Overall and Europe nucleotide diversity was 0.00477 ± 0.00063 , a value that is about five times higher than that of the locality group Europe (without Calabria) (0.00091 ± 0.00027). Such a discrepancy indicates the strong contribution (in terms of molecular variability) provided by *S. meridionalis* to the global variability of the *S. vulgaris* complex, as well as the marked differentiation of Calabrian squirrels from the other Italian and European populations (Tab. 2). Considering average genetic variation (Tab. S3), p -distance values between the Calabria group and Italy (except Calabria) and rest of Europe groups were 0.008 ± 0.003 and 0.009 ± 0.004 respectively. As expected, the lowest genetic divergence value was that between Italy (except Calabria) and rest of Europe (p -distance: 0.001 ± 0.001) whereas the highest values were those between *S. niger*, *S. carolinensis* and the other locality groups (p -distance range: 0.102–0.144).

Geographical distribution

The range of *S. meridionalis* Lucifero, 1907 includes the three main mountain blocks of Calabria: the whole Pollino massif (including Lucanian side) at the border between Calabria and Lucania, the Sila massif and the Aspromonte massif, with three once disjunct populations. Only recently the Pollino and Sila populations have become connected by colonization of the Catena Costiera, which was made possible by replanting of conifers (Rima et al., 2009). The species has not been reported from the Serre Massif (Fig. 3).

Discussion and conclusions

To date, many studies have addressed the genetic structure and the biogeography of *S. vulgaris* populations by using different approaches, ranging from the analysis of mitochondrial to hyper variable nuclear genome regions. After pioneer researches, among which the one con-

ducted by Grill et al. (2009), other authors examined the genetic variability of different European and Asian red squirrel populations (Dozieres et al., 2012; Liu et al., 2014; Lucas et al., 2015; Madsen et al., 2015) and all of these found little or no genetic heterogeneity at both mitochondrial and nuclear DNA marker regions within the entire species distribution range, even when comparing different recognized subspecies (Grill et al., 2009; Madsen et al., 2015). No studies except the one conducted by Grill and co-workers (2009) considered the Calabrian *S. meridionalis* and although these authors provided well-supported genetics evidence for its segregation, they concluded that it undoubtedly forms an endemic evolutionary unit, but did not discuss its taxonomic status.

Most of subspecies determination of *S. vulgaris* has been mainly based on morphological variation, mainly in fur colour polymorphism and skull measurements (Sidorowicz, 1971; Wiltafsky, 1973; Bosch and Lurz, 2012) and less on potential barriers between ranges of different subspecies (Sidorowicz, 1971; Lurz et al., 2005). However, degree of variation in fur colour polymorphism and/or skull measurements within a given subspecies (e.g. *S. v. fuscoater*) can be as great as that between some subspecies (e.g. Wiltafsky, 1973; Wauters et al., 2004). Nevertheless, the large body size and skull of *S. meridionalis*, in particular when compared with specimens from the subspecies present in the rest of Italy, is obvious. In fact, we clearly showed that specimens of *S. meridionalis* were about 6.3% larger and even 35% heavier than those from populations of *S. vulgaris* from northern Italy. Similarly, they were larger and heavier than individuals of *S. vulgaris* studied in Belgium (Wauters and Dhondt, 1989). Moreover, a larger skull size of *S. meridionalis*, in comparison with specimens of *S. vulgaris* only from Italy, was confirmed by re-analyses of skull measurements available in the literature and taken from museum collections (Amori et al., 2014), underlining the important role of museum collections as an often overlooked source of biological data (Andreone et al., 2014; Gippoliti et al., 2014), allowing the comparison of the different red squirrels morphs occurring in the Italian peninsula.

Allocation of divergence patterns into species level versus subspecies ranks is critical for conservation and management (Hoisington-Lopez et al., 2012). This is especially true in the case of indigenous squirrels for which conservation status is strongly jeopardized by the spread of other invasive alien invasive sciurids (Bertolino et al., 2008, 2014; Santicchia et al., 2015; Mazzamuto et al., 2016), or by habitat loss and fragmentation (Celada et al., 1994; Wauters et al., 1994). Because most conservation strategies assigns priority based on species diversity, species delimitation is critical to conservation, and modern taxonomy requires the integration of several source of information to ad-

Table 2 – Haplotype characteristics: number of sequences, number of haplotypes and nucleotide diversity Π values (with standard deviation SD) in the locality groups hosting populations of *Sciurus vulgaris* and *S. meridionalis* at the three mitochondrial markers analyzed in this study.

Locality group	D-loop				Cyt b				COI			
	n. seq	n. hap	Π	SD	n. seq	n. hap	Π	SD	n. seq	n. hap	Π	SD
Overall	456	250	0.02432	0.00083	50	33	0.00621	0.00095	13	3	0.00477	0.00063
Italy including Calabria	82	36	0.02979	0.00279	7	8	0.01247	0.00419	10	2	0.00362	0.0013
Italy except Calabria	71	34	0.0184	0.00119	6	7	0.00655	0.00085	5	1	-	-
Calabria	11	2	0.00507	0.004	1	1	-	-	5	1	-	-
Europe	412	211	0.02381	0.00089	26	24	0.00832	0.0015	13	3	0.00477	0.00063
Europe without Calabria	401	209	0.02178	0.00072	25	23	0.00683	0.00067	8	2	0.00091	0.00027
Asia	44	42	0.02771	0.00159	23	12	0.00383	0.00084	0	-	-	-

dress species delimitation purposes (Dayrat, 2005; Padial et al., 2010; Galimberti et al., 2012).

The present work is the first specifically aiming at clarifying the taxonomic status of the divergent Calabrian black squirrel. The integrative approach employing molecular and morphometric data allowed a more complete view on the identification of the two species. The molecular dataset here analyzed is the widest ever assembled for the study of the *Sciurus vulgaris* complex and, in addition, we produced the first COI DNA barcoding sequences for Italian (and Calabrian) populations. The molecular analysis conducted by pooling all the available data deposited in GenBank and considering the newly produced DNA barcoding data, highlighted two main findings supporting the status of Calabrian *S. meridionalis* as a separate species.

The first result is that *S. meridionalis* DNA sequences showed exclusive haplotypes at the three mitochondrial regions considered in this study that are markedly differentiated from those of other Italian, European and Asian populations of the *S. vulgaris* complex. The second evidence supporting our taxonomic hypothesis is that the comparisons at the three genetic markers among *S. vulgaris* populations and subspecies throughout the entire areal reveals an almost complete lack of phylogeographical structure, as also outlined using different datasets by other researchers (Dozieres et al., 2012; Liu et al., 2014; Lucas et al., 2015; Madsen et al., 2015). Previous published works, such as Grill et al. (2009) demonstrated the uniqueness and differentiation of Calabrian black squirrels from other Italian and Eurasian populations using also nuclear microsatellites markers, thus further supporting our taxonomic view.

DNA barcoding analysis based on sequence differences at the mitochondrial COI region show a neat distinction between *S. v. vulgaris* and *S. v. meridionalis*, although genetic divergence values are lower than those occurring between other mammalian species (Galimberti et al., 2012; Ermakov et al., 2015). However, considering the exclusive haplotypes of Calabrian squirrels, the complex dynamics shaping sciurids mitochondrial variability (Ermakov et al., 2015; Galimberti et al., 2015) and similar cases occurring at COI marker between closely related mammalian species such as the Italian *Eptesicus* (Chiroptera: Vespertilionidae), we can reasonably affirm that also DNA barcoding confirms the distinction of *S. meridionalis* as a separate species. Moreover, in the case of cyt *b*, the genetic divergence values between *S. meridionalis* and the various *S. vulgaris* populations are greater than the thresholds conventionally used to flag the occurrence of different species following the genetic species concept proposed by Bradley and Baker (2001) and Baker and Bradley (2006). Also D-loop genetic divergence values were in line with those found across different sciurids species, as in the case of the genus *Spermophilus* (see for example Gündüz et al., 2007).

The unique and monomorphic coat colour pattern and morphometric variables (larger body size and skull size) support these genetic evidence. We suggest Calabrian black squirrel as a common name for the species *S. meridionalis*.

Biogeography and geographical distribution

The taxon *S. meridionalis* has occasionally been reported from the Apennines north of the Calabria-Pollino massif, for instance in the Abruzzo Lazio and Molise National Parks (e.g. Krapp, 1975). Black squirrels from Mt. Somma (Vesuvio) and the Abruzzo mountains were ascribed by Costa (1839) to *Sciurus alpinus* Cuvier, 1821, at the time considered the black squirrel of the Pyrenees. However, Central Apennine squirrels are clearly distinct, genetically and by their smaller size, from the true “*meridionalis*” (Cavazza, 1913; Grill et al., 2009; Amori et al., 2014). Moreover, *S. vulgaris fuscoater* from the Alps can also have dark brown to black dorsal fur (e.g. Wauters et al., 2004). Finally, in contrast with Calabria, other colour morphs are also known from the Central Apennines (Cavazza, 1913).

In conclusion, until recently the northern edge of the distribution range of *S. meridionalis* was the northern slope of the Pollino massif (Lucanian side) with no apparent geographical or ecological barriers towards the Lucanian Apennines. In fact observations from the past

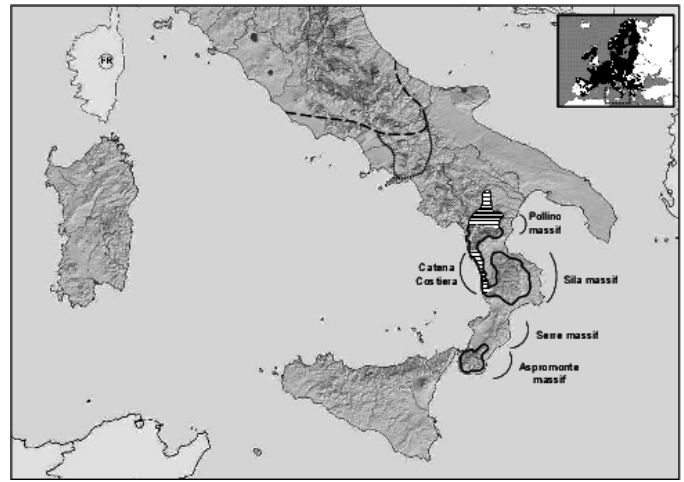


Figure 3 – Geographical distribution of *S. meridionalis* in continuous line with latest area of colonization (hatched area). Current known southern distribution limit of *S. vulgaris* (dashed line); historical presence of *S. vulgaris* (from Cavazza, 1913) (dotted line). Inset: European distribution of *S. vulgaris*.

decade suggest that *S. meridionalis* is colonising new northward areas of the Lucanian Apennines. Recent data indicate a range expansion toward the Southern and Central part of Lucania (region Basilicata) and suggest that these populations are geographically connected with those from the Western and Northern side of the Pollino massif (Bartolomei, Sgrosso and Aloise, unpublished data) (Fig. 3). It must be underlined that in this area, as well as in region of Puglia, *S. vulgaris* is absent and, as far as we know, there is a gap of more than 100 km between the most southern edge of *S. vulgaris* range in Italy (marginally present in northern parts of regions Campania and Basilicata, based on records from past 30 years) and the most northern records of *S. meridionalis*. The data available suggest that the range of *S. meridionalis* has an almost complete overlap with that of the endemic but little-known *Dryomys nitedula aspromontis* Lehman, 1964 (Kryštufek, 1999; Aloise pers. obs.).

Conservation

There are no data on the population status but, despite the colonization of some new areas, we can assume that population size is stable. Studies using indirect methods (mainly drey (nest) counts) to reveal presence/absence or estimate relative densities showed marked effects of landscape structure and forest habitat composition on densities and occurrence (Cagnin et al., 2000; Rima et al., 2009). Squirrels were more likely to occur in mature forests with conifers and drey density increased with tree species diversity (Rima et al., 2009). The area of occurrence of *S. meridionalis* is approximately 7000 km² which would suggest an IUCN classification as Vulnerable. However, no other sub-criteria are available to confirm the VU status. Pending further more detailed information on population trends and range of densities of this species we believe the most appropriate category is NT. However, it must be stressed that the introduction, and subsequent spread, of *Callosciurus finlaysonii* in areas close to the distribution of *S. meridionalis* (Aloise and Bertolino, 2005) may become a significant threat for this endemic species. Thus, *S. meridionalis* is yet another example of the several endemic mammal species, or unique genetic lineages, that have been identified in the last two decades using phylogeographic studies (e.g. Pierpaoli et al., 1999; Mucedda et al., 2002; Randi, 2007; Galimberti et al., 2012; Castiglia et al., 2016; Gippoliti, 2016; Mouton et al., 2016). These papers represent a valuable reappraisal of the Italian mammal fauna which should be considered in European and national conservation policy. ☞

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Supplemental information

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

Table S1 Molecular datasets of the *Sciurus* species analyzed in this study.

Table S2 Measurements of *S. meridionalis*.

Table S3 Intra- and inter-group genetic divergence values (*p*-distance and standard error) at the three mitochondrial markers based on the *p*-distance calculation.