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APPLICATION ESSAYS

Faecal DNA-based genetic survey of a relict Eurasian otter (*Lutra lutra*) population (Sila Massif, S Italy)

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Abstract

Faecal DNA-based genetic analysis is a suitable tool for assessing both population size and genetic diversity of threatened and elusive species. We applied microsatellite analysis and mtDNA sequencing for investigating the southernmost Italian (Sila Massif, Calabria Region) population of the Eurasian otter (*Lutra lutra*). This relict population, filed as extinct in the mid-1980s, is currently expanding but still quite isolated. On the two main rivers hosting otters permanently since 2014, we collected 47 spraints, out of which 24 (51.1%) were successfully genotyped (on average 2.0 alleles per *locus*). Thirteen individuals were identified: seven females and three males (sex identification success of 76.9%). Population size was assessed as 16 individuals (13–22), corresponding to a density of 0.15 (0.13–0.21) ind/km. Successfully amplified mtDNA samples (N = 16) confirmed the occurrence of a haplotype—H10—which had been previously reported only for Southern Italy, bringing new evidence of the unicity of the Italian otter population. Although density values complied with those reported for the core area of otter Italian range, the small size and genetic isolation of this population require special attention. To assist the ongoing re-colonisation of the Sila Massif, habitat management should aim to enhance fish availability and connectivity with the core area.

Keywords Population size · Density · Microsatellites · Heterozygosity · Haplotype

Introduction

Ongoing Eurasian otter (*Lutra lutra*) natural expansion, as well as reintroduction programs carried out across Europe (Wayre 1985; Fernández-Morán et al. 2002; Koelewijn et al. 2010; Balestrieri et al. 2021), require a detailed evaluation of the genetic structure of the different populations to enhance genetic variability while preserving "unique" haplotypes (Weeks et al. 2016).

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Sub-fossil records and genetic analyses agree in supporting that otter colonization of central and northern Europe occurred in the Holocene (5500−3000 years BC), from otters spreading from a single or few refugial areas (Ferrando et al. 2004; Sommer and Nadachowski 2006). By analysing the mtDNA control region of otter samples collected throughout its wide distribution range, Mucci et al. (2010) suggested a single glacial refugium, possibly coinciding with the Italian peninsula. They recorded a widespread haplotype (H3), occurring in almost all sampled populations across Europe, from the Iberian Peninsula to Scandinavia and Russia, while the other 19 identified haplotypes were restricted to relatively small geographical areas, differing from H3 by only 1–2 mutations over more than 1500 nucleotides (Mucci et al. 2010). Consistently with previous studies, these unusually low levels of mtDNA variability matched with moderate microsatellite allelic diversity and heterozygosity, as assessed by the analysis of 11 autosomal loci (Mucci et al. 2010).

Otters from southern Italy (N = 3) showed a single haplotype (H10) and the lowest average number of alleles per

locus (A_e =1.7) and heterozygosity ($H_e = H_o = 0.37$), suggesting that they may represent an Evolutionarily Significant Unit (ESU). Anyway, these findings call for more detailed analyses using a larger sample size (Mucci et al. 2010).

In the second half of the twentieth century, the Eurasian otter suffered a dramatic decline throughout the Italian peninsula. The national-scale survey carried out in 1984–1985 (Cassola 1986) pointed out the upcoming extinction of the species throughout the northern and central sectors, while a small residual population, probably consisting of less than 250 specimens, was recorded to occur in the South (Campania and Basilicata regions; Balestrieri et al. 2016). Starting from the 1990s, much evidence of the recovery of this otter nucleus has been recorded, progressively leading to a continuous extent of occurrence from southern Abruzzo to Calabria regions (Giovacchini et al. 2018; Loy and Duplaix 2020).

Recently, otters have been newly recorded also on the Sila Massif (Calabria Region; Gariano and Balestrieri 2018), where the species had been claimed as extinct in the 1980s (Arcà 1986). Currently, this area represents the southernmost tip of the Italian otter range and, although the species has been occasionally recorded on the River Crati, which may connect the core area of otter range to the Sila Massif (see Loy et al. 2015), this "Lazarus population" is likely isolated and did not originate from a recent recolonisation event (Gariano and Balestrieri 2018). Consistently, in the core area of the Italian otter range, populations from different river catchments are genetically differentiated, suggesting that geographical barriers can hinder otter dispersal even between neighbouring watersheds (Buglione et al. 2020).

Between 2014 and 2017, the yearly monitoring of the Massif allowed to record the stable occurrence of the otter on a 29 km long stretch of the River Savuto and a 15 km long stretch of the River Neto-Lese (Gariano and Balestrieri 2018). Since 2017 otters also occur on a 13 km long stretch of the River Amato, while in spring 2020 otter presence was confirmed for the mid-course (18.8 km) of the River Tacina (authors' unpublished data; Fig. 1).

The taxonomy status and genetic variability of populations, as well as size, are pivotal for predicting their viability and expansion potential and develop effective

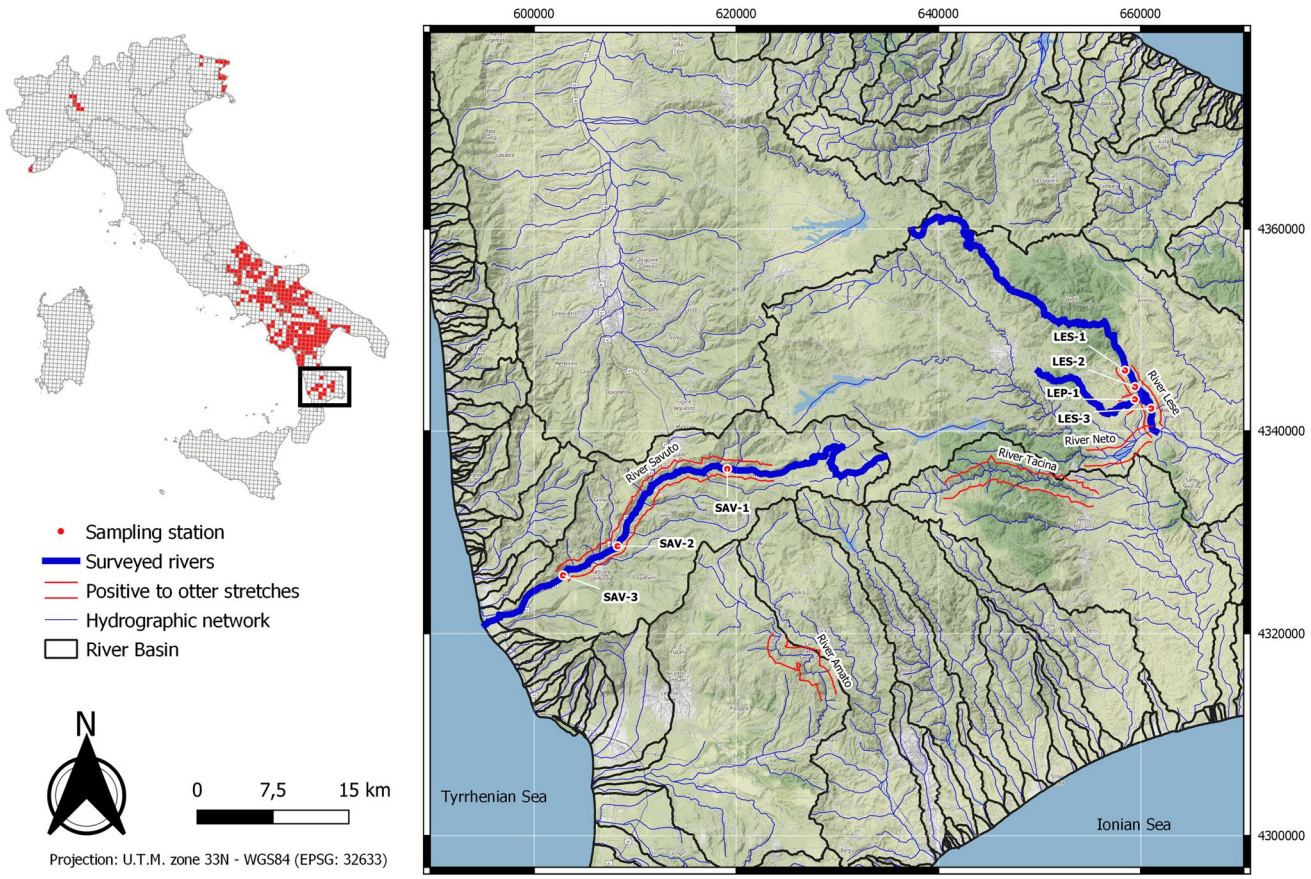

Fig. 1 Location of the river stretches (sampling stations) surveyed for fresh otter spraints on the Sila Massif (rivers Savuto, Lese and Lepre) in December 2017 and July 2018. The map also shows the river stretches where otter occurrence has been regularly recorded since 2014. The national-scale distribution of the otter is reported according to a 10 × 10 km UTM reference grid

conservation-aimed management strategies. To assess the abundance and genetic variability of the otter population of the Sila Massif, we applied faecal DNA-based genetic analysis. Although genotyping success is usually low and error rates high (Lampa et al. 2013), this non-invasive method has been largely applied to monitor such elusive species as the Eurasian otter (e.g.: Prigioni et al. 2006a; Koelewijn et al. 2010; Vergara et al. 2014). Fresh-looking otter spraints were collected on the two main positive-to-otter rivers and analysed using both autosomal microsatellite loci and mtDNA control region.

The specific aims of this study were: (i) to assess otter minimum population size and density, (ii) estimate the genetic variability of the Sila Massif population with respect to other Italian populations and (iii) verify the unicity of the mtDNA haplotype of Italian otter populations (Mucci et al. 2010).

Study area

The hydrographic network of the south-facing slopes of the Sila Massif (3300 km^2, Calabria region, S Italy) includes eight main river catchments: Savuto (60 km) and Amato (56 km), flowing into the Tyrrhenian Sea, Corace (48 km), Alli (46 km), Simeri (42 km), Crocchio (38 km) and Tacina (65 km), flowing into the Squillace Gulf (Ionian Sea), and Neto (92 km), with River Lese (43 km) as its main tributary.

Altitude ranges from the sea level to 1928 m a.s.l. (Mount Botte Donato). Climate is Mediterranean—cool and rainy in winter while hot and dry in summer. About 76% of the Massif area is covered by forests, mainly consisting of Calabrian pine *Pinus nigra laricio* (28%), beech *Fagus sylvatica* (17%) and mixed forests of these two species (26%); minor forests include chestnut *Castanea sativa* and fir *Abies alba* stands, while 24% of the area is covered by small villages and crop fields (Nicolaci et al. 2014).

Materials and methods

Sampling

In mid-December 2017 and mid-July 2018, the rivers Savuto and Lese were surveyed for fresh-looking otter spraints. Sampling stations consisted of three river stretches for each watercourse (mean length ± SD = 978 ± 35 m, min–max: 940–1010 m; Fig. 1), chosen based on accessibility and the results of previous surveys (Gariano and Balestrieri 2018). A further sampling station (1.5 km) was surveyed on the River Lepre (15 km), starting from its confluence in the River Lese (Fig. 1).

Riversides were surveyed in dry periods and mainly in the morning, to prevent DNA degradation during the warmer hours of the day. Spraint freshness was assessed visually, based on colour and moistness. Fresh looking samples were immediately preserved in 95% ethanol and stored at − 20 °C until analysis.

Based on marking intensity, we expected to find about twelve individuals on the sampled river stretches (Gariano and Balestrieri 2018).

Genetic analysis

DNA was extracted using the QIAamp® DNA Stool Mini Kit, after evaporating ethanol traces under vacuum. As spraints can be confused only with mink scats (Dettori et al. 2021), and neither European mink (*Mustela lutreola*) nor American mink (*Neovison vison*) occur in the study area, we did not deem necessary to confirm species identification by mtDNA analysis.

Genotyping was performed using 11 polymorphic autosomal microsatellite *loci* (Dallas and Piertney 1998; Dallas et al. 1999; Huang et al. 2005; Kalz et al. 2006; Hájková et al. 2006; Bonesi et al. 2013; Lerone et al. 2014; Vergara et al. 2014; Table 1). Two multiplex PCRs were conducted, splitting microsatellites based on fragment size and labelling by fluorescent dyes, and using the QIAGEN Multiplex PCR Kit protocol (15 min at 95 °C; 35 cycles of three steps: 30 s at 94 °C, 90 s at 57–63 °C, and 60 s at 72 °C; 30 min at 62 °C; the final volume was reduced to 25 ul).

To lower the probability of retaining false homozygotes or false allele errors, a multitube-approach of 4 independent replicates was used (Taberlet et al. 1996). To construct consensus genotypes heterozygotes were accepted only when the two alleles were recorded in ≥ 2 replicates, while a single allele had to be recorded in ≥ 3 replicates to confirm homozygosity (e.g., Frantz et al. 2003; Brzeski et al. 2013).

Amplified fragments were analysed by Macrogen (Seoul, South Korea) and electropherograms were inspected using GeneMarker V.2.2.0 (SoftGenetics, State College, PA, USA). Variation in genotyping success between the two sampling periods was tested by the chi-squared (χ^2) test.

Sex identification was accomplished by typing a fragment of the Zink-finger protein genes ZFX/ZFY following the protocol used by Mucci and Randi (2007). A multi-tube procedure (four replicates) was used, accepting sex identification only when at least two successful amplifications were consistent.

A 578 bp long sequence spanning from the last part of the cytochrome b to the first region of the mitochondrial DNA control region (positions 15167–15745 of the GenBank available for Europe; accession No. LC094961) was amplified using the primers LlucybL996 and H16498 (2 min at 94 °C; 40 cycles of three steps: 30 s at 94 °C, 30 s at 55 °C,

Table 1 Allele number (N_A) and size and observed heterozygosis per *locus* (H_O)

River Savuto				River Lese/Lepre		
Locus	N_A	Size (bp)	H_O	N_A	Size (bp)	H_O
OT-14	5	117–121–125–129–132	1	2	117, 125	0.23
OT-04	1	206		1	205	
Lut 453	2	127–135	0.57	1	127	
OT-17	3	151–155–159	0.71	2	155–159	1
Lut 833	2	151-159	0.71	2	151–159	0.75
Lut 701	3	206–210–214	0.28	2	211–215	0.85
Lut 818	2	174–178	0.67	3	172–174–178	0.2
OT-19	3	211–218–226	1	3	222–226–230	0.27
Lut 435	1	123		1	123	
Lut 715	2	203–219	0.67	1	203	
Lut 902	1	149		1	149	
Mean	2.3		0.51	1.7		0.30

30 s at 72 °C; 10 min at 72 °C) (Mucci et al. 2010; Shields and Kocher 1991). Sanger sequencing was carried out using the forward primer LlucybL996. Amplicons were separated on an ABI 3130XL automated sequencer and visualized and corrected using Seqscape v. 5.0. Results were compared in Bioedit with all the sequences from GenBank aligning with the amplified fragment (Accession numbers: EF672696; FJ236015; LC049377; LC049953; LC049954; LC049955; LC050126; LC049961; MN122838; MW027025; MW027026; MW027027; MW027028; MW573979; NC01358) (Hall 1999). A maximum likelihood phylogenetic tree was drawn by MEGA 11 using the Tamura-Nei model and 1000 bootstrap (Tamura et al. 2021). The initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with the highest log likelihood value. A sample collected in 1998 in the WWF Oasis of Persano (S Italy), and previously associated with the H10 haplotype by Mucci et al. (2010), was also included in this study. The phylogenetic tree was rooted using *Lutrogale perspicillata* as outgroup (Accession number: NC_035811).

Density estimation

Population size was assessed by Capwire estimators, which have been developed expressly for faecal DNA-based sampling and provide reliable estimates for small populations (Miller et al. 2005).

Data were fitted to two different models to obtain the maximum likelihood estimate (MLE) of each population size. Under the Equal Capture Model (ECM) all individuals were assumed to have an equal probability of being sampled, while under the Two-Innate Rates Model (TIRM) the population was assumed to contain a mixture of easy-to-capture and difficult-to-capture individuals. The fit of the two models was compared using a Likelihood Ratio Test (LRT); the p-value was calculated by using a parametric bootstrap approach to estimate the distribution of the LRT for data simulated under the less-parameterized ECM (Pennell et al. 2013). Confidence intervals (CI) for population size were estimated using a parametric bootstrap approach (Miller et al. 2005). Density was calculated using all consensus genotypes and the total length of surveyed rivers (118 km).

Results

Overall, 47 "fresh" samples were collected, of which 24 (51.1%) were successfully genotyped, 10 on the River Savuto and 14 on the River Lese/Lepre; the mean number of amplified *loci* was 8.8 (80.5%) and 10.0 (90.9%), respectively, while six was the minimum number of amplified *loci* needed for identifying individuals indisputably. Genotyping success did not differ between sampling periods ($\chi^2 = 0.2$, 1 df, $P = 0.66$). A total of 31 different alleles was found, with a mean of 2.8 alleles and a maximum of 5 alleles per *locus*; genetic diversity was higher on the River Savuto (25 alleles, of which 12 private) than on the River Lese/Lepre (19 alleles, of which six private). Respectively, three to five *loci* were monomorphic (Table 1). Observed heterozygosity ($Ho \pm SD$) was 0.37 ± 0.13 (River Savuto: 0.51 ± 0.09; River Lese/Lepre: 0.3 ± 0.09). The average number of heterozygous *loci* was 3.5 (respectively: 4.1, min–max = 2–6 and 2.8, min–max = 1–4).

Overall, 13 individuals were identified, seven on the River Savuto and six on the River Lese/Lepre (Table 2). Sex identification was accomplished for 10 individuals (76.9%), four

females and two males on the latter river and three females and one male on the River Savuto (Table 2; Fig. 2). Male/female ratio was 0.44.

The average number (± SD) of detections (re-samplings) per individual was 1.8 ± 1.1 (min–max: 1–5), with five individuals detected two times and only one individual captured three or five times. The maximum number of otters recorded in the same station/period was three on both rivers (Fig. 2).

Under the best supported model, the ECM, population size was assessed as 16 (CI 13–22) individuals (vs. 21, 13–32 using the TIRM model), corresponding to a density of 0.14 (0.11–0.19) ind/km.

A 341 bp long mtDNA region was obtained for 16 out of the 24 samples that were successfully genotyped. All individuals shared the haplotype "H10" previously identified in southern Italy (Mucci et al. 2010). This haplotype (Fig. 3) differs from all the other GenBank sequences for two mutations in the tRNA-Pro region, at 15,390 (a transition) and 15,392 (a deletion) position (see Accession Number = MN122838 for nucleotide identification). Two additional sequences, consisting of 225 and 169 pb respectively, could be associated to the same haplotype.

Discussion

In the last two decades faecal DNA-based genetic analysis have become increasingly popular as a non-invasive tool to assess Eurasian otter distribution and density; notwithstanding, knowledge about the factors affecting sample quality and thence genotyping success is still insufficient (Weinberger and Winkler 2020). Although both sampling time and period (early in the morning, winter surveys should yield the best samples) are considered key aspects, in our Mediterranean study area no seasonal variation in success rate was recorded. Overall, genotyping success was relatively high compared to previous studies (e.g., 14%, Lanszki et al. 2008; 21%, Ferrando et al. 2008; 41%, Prigioni et al. 2006a; 43%, Vergara et al. 2014; 73% Janssens et al. 2008), balancing sample size, which was rather low (N = 47 vs. 156, 124, 185, 127 and 117,

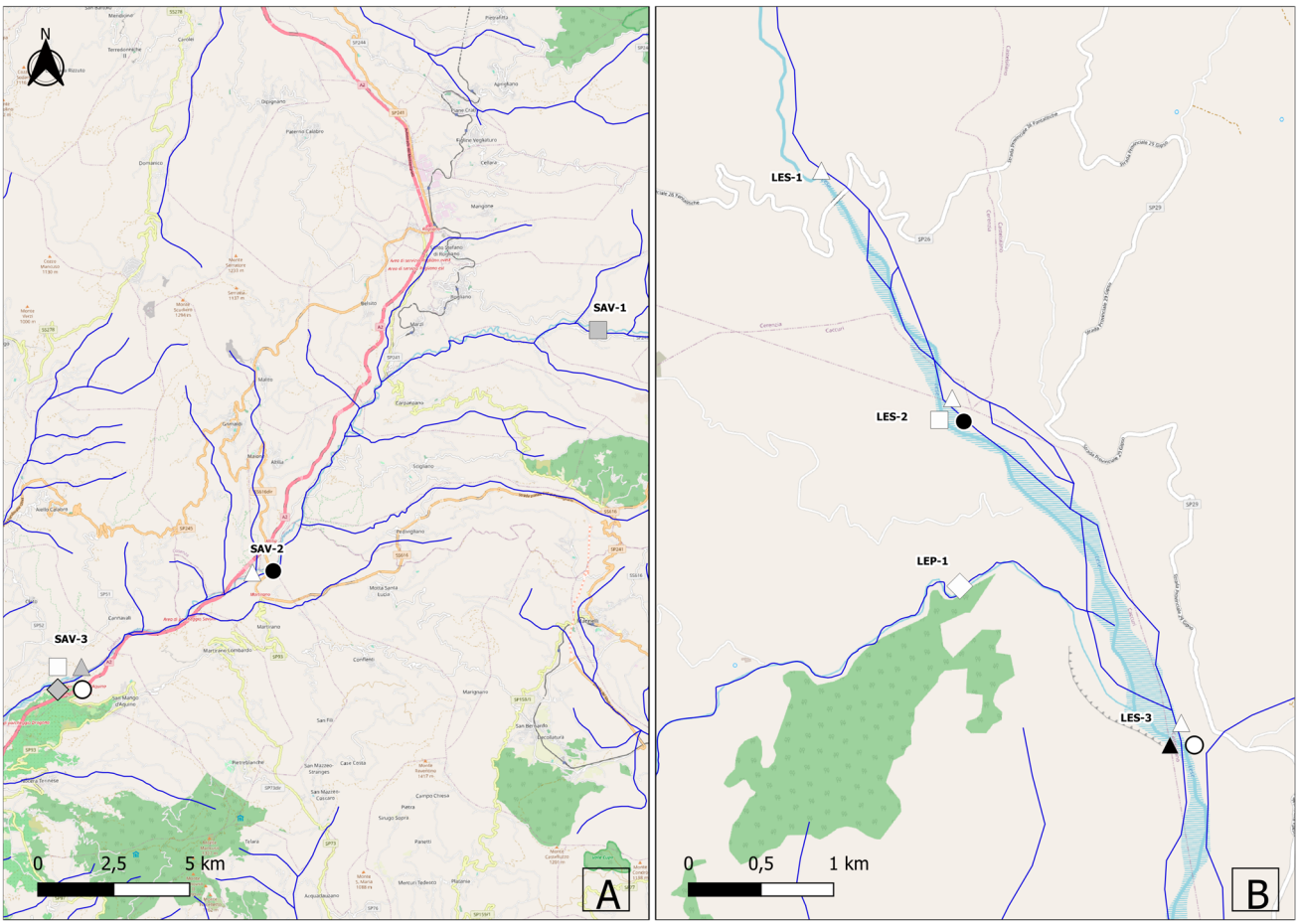

Fig. 2 Distribution and sex (males: black; females: white; undetermined: gray) of genotyped otters on the rivers Savuto (**A**—7 individuals: 1 male, 3 females, 2 undetermined) and Lese-Lepre (**B**—6 individuals: 2 males and 4 females, of which one (Open triangle)—recorded in all the sampling stations on the R. Lese)

Table 2 Sex (F: female; M; male; NA: not amplified), number of re-samplings (N_R), number of amplified *loci* (N_L), amplification success (% Amp), number (N_H) and percentage ($\%_H$) of heterozygous *loci* and number of polymorphic *loci* (N_P) for the 13 otters genotyped on the rivers Savuto and Lese/Lepre

Individual	Sex	Station	N_R	N_L	% Amp	N_H	$\%_H$	N_P
Otter 1	NA	Savuto1	2	6	54.5	3	50.0	5
Otter 2	F	Savuto 2	2	6	54.5	2	33.3	5
Otter 3	M	Savuto 2	1	9	81.8	3	33.3	7
Otter 4	NA	Savuto 3	2	11	100	6	54.5	8
Otter 5	F	Savuto 3	1	9	81.8	4	44.4	6
Otter 6	NA	Savuto 3	1	11	100	6	54.5	8
Otter 7	F	Savuto 3	1	10	90.9	5	50.0	7
Mean			*1.4*	*8.8*	*80.5*	*4.1*	*45.7*	*6.6*
Otter 1	F	Lese 1, 2, 3	5	10	100	2	18.2	5
Otter 2	M	Lese 3	1	7	63.6	1	14.3	4
Otter 3	F	Lese 3	2	11	100	4	36.4	6
Otter 4	M	Lese 2	1	10	90.9	3	30.0	5
Otter 5	F	Lese 2	3	10	90.9	3	30.0	5
Otter 6	F	Lepre	2	11	100	4	36.4	6
Mean			*2.3*	*10*	*90.9*	*2.8*	*27.6*	*5.2*

respectively), being inevitably affected by otter abundance (Balestrieri et al. 2021). However, following Arandjelovic and Vigilant (2018), the number of spraints analysed was about three times the number of individuals expected to occur in the sampled river stretches. The number of amplified loci needed to identify individuals was consistent with previous studies (Bonesi et al. 2013; Vergara et al. 2014).

Although figures cannot be directly compared, as four of our microsatellites differed from those used by Mucci et al. (2010), both the heterozygosity and polymorphism recorded in the otter population of the Sila Massif agreed with available data for the Italian population (average number of alleles per locus = 2.0 and 2.6, respectively; $H_o = 0.37$ for both). The lower heterozygosity and allele richness found on the River Lese/Lepre may suggest that its catchment was colonised by otters dispersing from the River Savuto, where otter occurrence went probably undetected during the census carried out in the 1980s (Gariano and Balestrieri 2018).

As for previous studies (e.g., 5 mos., Prigioni et al. 2006a; 7 mos., Vergara et al. 2014), sampling period was rather long. While the effect of births can be discarded, as in southern Italy they mostly occur in spring-summer (Prigioni 1997) and young start marking when they are 4–5 months old (Polotti et al. 1995), the influence of mortality cannot be ruled out.

Although estimations must be considered indicative of otter actual density, the values recorded are close to those reported for the core area of the Italian otter range (0.18–0.20 ind/km; Prigioni et al. 2006a), suggesting that population size may be next to carrying capacity; dispersal from these two catchments may support the ongoing colonisation of neighbouring watersheds.

Considering that otters have also been recorded on the upstream half of the River Neto and on the rivers Tacina and Amato (total length: 170 km), where mean marking intensity is about one-third of that recorded on the River Savuto, following Prigioni et al. (2006b) we may expect the occurrence of further 7–12 individuals, for a total population size of 20–34 otters.

The success of the sex identification was rather high and similar to previous studies (79%, Mucci and Randi 2007; 85%, Vergara et al. 2014). Despite non-invasive surveys have been claimed to overestimate the number of male individuals because of the higher marking activity of otter males respect to females (Ferrando et al. 2008; Koelewijn et al. 2010; Bonesi et al. 2013), male/female ratio was consistent with that recorded in northern Spain (0.55, Vergara et al. 2014), and agreed with the basic spacing pattern of otters (intrasexual territoriality), with each male home range including one or more female ranges and little overlap between neighbouring ranges of same-sex individuals (Powell 1979).

The maximum likelihood tree of the haplotypes sharing the informative region confirmed the unicity of the Italian haplotype, the occurrence of which had been previously inferred by the examination of a small sample of individuals (Mucci et al. 2010). The unicity of the Italian otter population will need to be carefully considered whenever planning translocations to enhance its recolonisation of the whole Peninsula, as the admixture of historically isolated populations may increase genetic diversity but also lead to outbreeding depression (Frankham et al. 2017). Guidelines for translocations ask for the genetic assessment of both donor and recipient populations to prevent introgression between subspecies (see Balestrieri et al. 2021 about the contribution of the Asiatic *L. lutra barang* to the genetic pool of otters

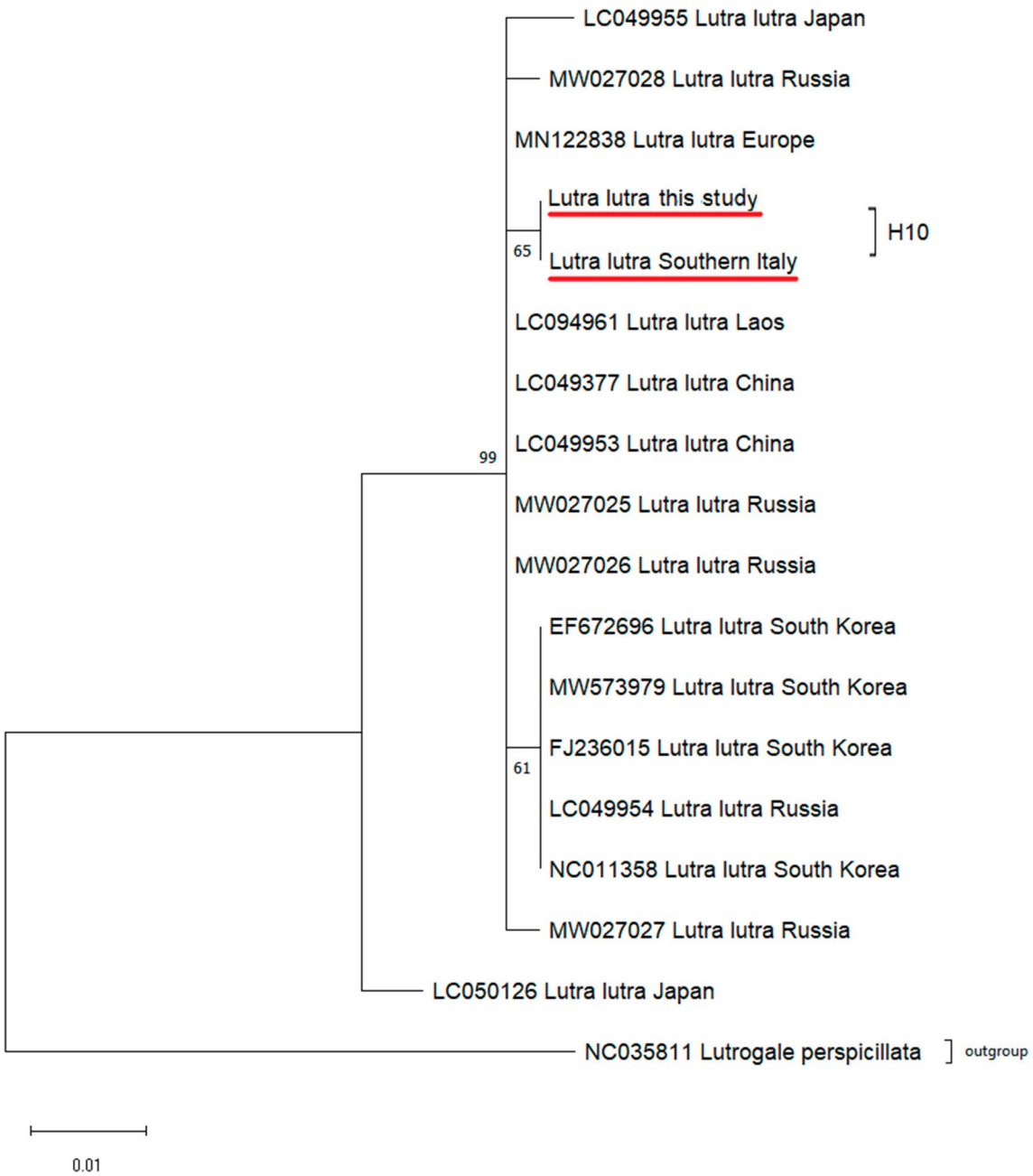

Fig. 3 Relationship between the Italian otter haplotype H10 (this study and Southern Italy, both marked in red) and the GenBank sequences, as inferred by the Maximum Likelihood method and Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

reintroduced in northern Italy). While this study confirmed the spread of H10 in southern Italy, further studies are needed to highlight, through the analysis of museum specimens, the genetic structure of the extinct sub-population of the River Po catchment, as the recent expansion of the Austrian and French populations demonstrates the existence of corridors crossing the Alps (Giovacchini et al. 2021).

Despite low sample size, our pilot study allowed to gain useful information on the current abundance of a relatively isolated, long neglected population of the Eurasian otter. Both density and distribution data (Gariano and Balestrieri 2018) suggest that the trend of this southernmost otter population may comply with the general positive conservation status of the Eurasian otter in the core area of its Italian range. However, population size is still small and the quality of freshwater habitats of the Sila Massif needs to be enhanced by specific management actions in order to enhance otter expansion and stable occupancy of some of the

river catchments currently hosting otters (Rivers Amato and Tacina). Particularly, food availability should be increased by the effective management of fish resources (Smiroldo et al. 2019). Finally, genetic isolation asks for the improvement of habitat quality on the rivers Crati and Mucone, which may connect the Sila population to the otter's core area (Pollino Massif).

Author contributions All authors contributed to the study conception, material preparation, data collection and analysis. The first draft of the manuscript was written by AB and PT. All authors commented on previous versions of the manuscript and approved the final manuscript. All data analysed during this study are included in this published article.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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