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A Noninvasive Genetic Insight into the Spatial and Social Organization of an Endangered Population of the Eurasian Otter (*Lutra lutra*, Mustelidae, Carnivora)

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Abstract: The Eurasian otter is endangered in Italy, only surviving in southern river basins. The spatial and social structure of a population living at the border of the current range was explored through a noninvasive genetic study along 174 km of the Sangro river. Sampling was conducted in 2011 and 2012, collecting spraints and anal jellies at 62 marking sites. Samples were successfully genotyped at 13 nuclear microsatellites and the ZFX/ZFY locus for molecular sexing, resulting in 14 distinct genotypes (4 females, 2 possible females, 8 males), from 35 marking sites. Mean captures/recaptures rate was 3.8 captures/individual, with males being recaptured more frequently than females. Spatial overlap among individuals was analyzed through a linear regression model fitted against sibship categories and sex pairing. Nine out of the fourteen genotyped individuals belonged to three full-sib clusters, while five individuals had no full-sibs in the population. Full-sibs overlapped more than half-sibs, while male–male pairs showed significantly higher spatial overlap than both male–female and female–female pairs. Estimated mean density was 0.152 otters/km and 2.4 individuals/10 × 10 km grid cell. Accordingly, the 3440 grid cells of otter occurrence in Italy could likely host about 8000 otters, suggesting the current population has become larger than the minimum viable population size.

Keywords: population size estimate; *Lutra lutra*; scent marking; sibship analysis

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1. Introduction

Successful conservation and management of threatened species require accurate information on population abundance, structure, and genetic variability. Nevertheless, such data are often difficult to obtain for wild populations, especially for elusive and rare species. During the last century alone, Eurasian otter (*Lutra lutra*) populations have declined throughout Europe and have experienced a hard habitat reduction and fragmentation all over their distribution range [1–3]. In Italy, the Eurasian otter population survived only in the southern part of the peninsula [4–7]. Following strict protection and the ban of polychlorinated biphenyl (PCB) compounds in the EU since 1987, the species is now recovering in most European countries [2,3]. Since 2000, the small Italian population started to slowly recover and repopulate areas in south-central rivers of the peninsula that had been part of their historic distribution range [6–8]. Otter individuals also reappeared in 2011 in the north-eastern Alps following range expansion into Austria and Slovenia [9–12]. However, the Italian core population is geographically and genetically isolated from other European populations [13], and the species is still one of the most threatened mammals in Italy, classified as Endangered in the national red list [6,11,14]. Diet composition, habitat preference, distribution pattern, and genetic variation of the Italian otters have been explored

by various authors [4–13,15–26]. However, in spite of its isolation and genetic uniqueness, the spatial organization and structure of the Italian population are still largely unknown, the only available information being from one male and one female otter radiotracked in southern Italy [27].

Traditional techniques used to gather information on population structure, density, and social and spatial behavior of wild mammals involve their capture and handling to fit tracking devices [28,29]. As for otters, these invasive techniques are particularly risky and controversial, since they employ the use of invasive capture devices and surgery to implant intra-peritoneal transmitters [29]. Technical advances in molecular ecology offer an alternative way to monitor these rare and elusive species in a noninvasive way. Specifically, the analysis of variable microsatellite loci allows obtaining a genetic fingerprint that can be used to estimate population abundance, genetic structure, and degree of relatedness in many species, including otters [4,30–37], as well as to gather information on territorial and social behavior [38–49]. Most noninvasive genetic sampling on otters is based on DNA extraction from spraints (i.e., feces) and anal jelly produced by their anal glands and used for scent marking [49–51]. Although this technique has been applied to gather information on the population density and genetic structure of otters in various European countries [4,34–45], its use in exploring spatial and social organization has yet to be examined except by a few studies [38–40,42,43,45]. Specifically, noninvasively collected sample material has been used to explore the marking behavior of male and female otters in Germany [49] and individual distribution and dispersal in Spain, Germany, Portugal, France, and the Netherlands [38,39,43–45].

Information on spatial and social behavior is especially valuable for rare and elusive species, such as the Italian population of the Eurasian otter, as they help in devising accurate conservation strategies that might fail if based on data derived from different geographic and environmental contexts [52]. This study was aimed at gathering first-hand insight into the population density and spatial dynamics of an otter metapopulation living at the northernmost limit of its range in southern Italy. Specifically, the sampling design was constructed to answer the following questions: (1) Is the otter density at the range boundary similar to the density found in the range core in southern Italy [4]? (2) Is the home-range extent similar to that observed in other Mediterranean areas [27,53,54]? (3) Is there any spatial overlap among individuals, and between or within sexes, that could account for a social organization other than the solitary and polygynous mating system known for this species?

2. Materials and Methods

2.1. Study Area

The study was conducted in southern Italy along 174 km of the Sangro river and its main tributaries (Figure 1). The river represents the northernmost limit of the otter range in Italy (not considering the recent return in the Alps). The river basin was recolonized by the Eurasian otter in 2007 after its extinction during the 1980s [11,55], and it is included among the priority areas of the otter national action plan [6]. The main course of the river flows to the Adriatic Sea from 1441 m a.s.l. for about 122 km. The whole basin covers an area of 1545 km^2.

2.2. Sample Collection and Storage

Field sampling was conducted from April to September 2011 and from June to September 2012. Spraints and anal jelly samples were regularly collected at 62 known marking sites previously identified during a four-month pilot study [51] (Figure 1). Geographic coordinates were recorded at each sampling site to implement spatial analyses. Although high temperatures could represent a critical issue for genotyping success [51,56], samples were also collected during summer to reach marking sites that were inaccessible in other seasons (Figure 1). To guarantee the collection of fresh samples (within 24 h from deposition), marking sites were checked daily after clearing older spraints [51]. At sites

that were less often marked by otters, we also collected samples of medium freshness and unknown deposition time. Spraints and anal jelly were stored in 96% ethanol in 1.5 mL tubes preserved at −20 °C until DNA extraction.

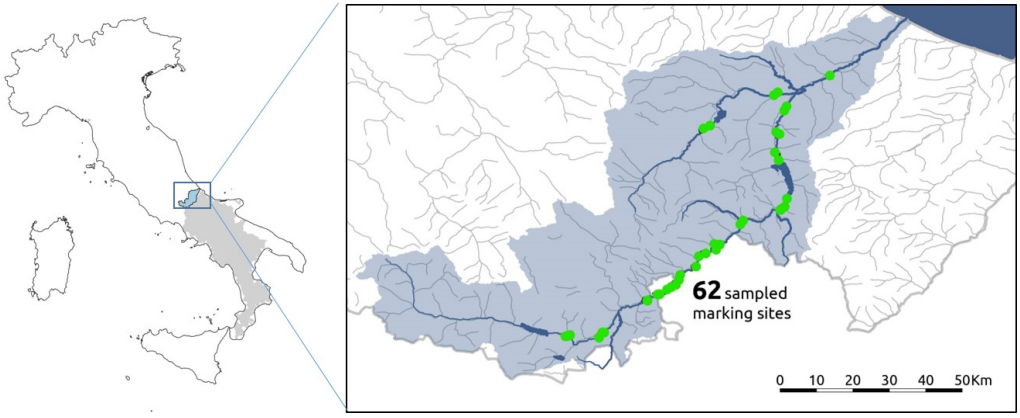

Figure 1. Location of the study area within the otter range in Italy (grey) and distribution of the 62 sampling sites (green circles) surveyed along 174 km of the river Sangro in 2011 and 2012.

2.3. DNA Extraction and Analyses

DNA was extracted from otter spraints and anal jelly samples using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer protocol and then amplified according to [51]. Genotypes were initially obtained by GeneMapper v. 4.0 (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). Matches were checked using GIMLET software [57], where genotypes matched at all but one or two alleles. We reviewed electropherograms and, when required, performed an additional four PCR repeats for uncertain loci. To carry out individual identification, DNA samples were analyzed through a panel of 13 nuclear microsatellite loci—Lut453, Lut604, Lut701, Lut832, Lut833, Lut902 [58] and OT04, OT05, OT07, OT14, OT17, OT19, OT22 [51,59]. Genotyped samples were also analyzed at ZFX/ZFY locus for molecular sexing [60]. In all analyses, contamination risks were minimized using a laboratory dedicated to the pre-polymerase chain reaction (PCR) handling of noninvasively collected samples [61]. Working with noninvasive samples, we decided to perform a multiple tube approach with a minimum number of four replicates per sample in order to assess the rate of allelic dropout (ADO) and false alleles (FA) [61]. Using RELIOTYPE [62], we determined the reliability value for each sample and checked if further replicates were needed.

We used GENALEX v. 6.1 [63] to estimate the allele frequencies by locus and population, mean number of alleles per locus (Na), observed (Ho) and expected unbiased (UHe) heterozygosity, and the related chi-square test (χ^2) for deviations from Hardy–Weinberg equilibrium (computation was adjusted using the Bonferroni correction) [64]. False allele (FA) and allelic dropout (ADO) rates were estimated using GIMLET software v.1.3.3. Observed (HO) and unbiased expected (HE) [65] heterozygosity values were computed for combinations of all loci; Hardy–Weinberg and linkage disequilibrium were then tested in ARLEQUIN 3.5.1.2 [66] through the Markov chain exact test with a chain length of 100,000 and 3000 dememorization steps, AMOVA, and F-statistics (testing the null hypothesis of no differentiation by permuting genotypes between populations with 10,000 replicates at $p < 0.001$). Population size estimate was based on genotypes identified during 2011 to allow capture–mark–recapture estimates for a closed population [67]. We grouped identical multilocus genotypes to produce individual capture–recapture histories over the sampling season. Population size was estimated using the CAPWIRE R package [68], a platform specifically designed for DNA-based capture–recapture data and well-performing with small populations ($n < 100$). Within CAPWIRE, population size and confidence intervals were computed using the TIRM model for heterogeneous capturability rates. To infer

the degree of relatedness among genotyped individuals, we performed the sibship analysis [69,70] on the whole sample of genotyped individuals in the two years, using the software COLONY (Ver. 2.0.5.8) [71].

For individuals that were only detected once, we considered a minimum range extent of 7 km (centered on the detection site), corresponding to 97% of probability of otter presence from a marking site [34]. This was also applied to individuals captured twice within 1 km. Marking sites of those individuals that were recaptured at least twice at a distance >1 km were interpolated following the main river course, between neighboring capture/recapture sites. Spatial overlap among individual pairs was analyzed through linear regressions where the length of spatial overlap was used as the response variable, while sibship category (i.e., full-sibs, half-sibs, unrelated) and sex pairing (i.e., male–male, male–female, female–female) were included in turn as the explanatory variable. Regression goodness-of-fit was assessed by calculating the coefficient of determination R^2.

3. Results

A total of 191 fresh spraints (67 in 2011 and 44 in 2012) were collected at 62 marking sites along 174 km of the Sangro river. A total of 74 spraints from 35 sampling sites (covering 64 km) were successfully genotyped with a mean genotyping success of 31.5% (Figure 2). The success rate was higher for pure anal jelly (79.3%), followed by mixed spraint/jelly (28.0%) and spraints (27.0%). The mean frequencies of ADO and FA among samples were 0.144 and 0.337, respectively (for details on allele frequencies and microsatellite loci performances, see [51]). All loci but Lut453 were polymorphic (range 2–4 alleles). After the Bonferroni correction, all loci except OT17 were at Hardy–Weinberg equilibrium, and only 9 out of 78 comparisons among loci indicated a linkage disequilibrium (see [51] for details).

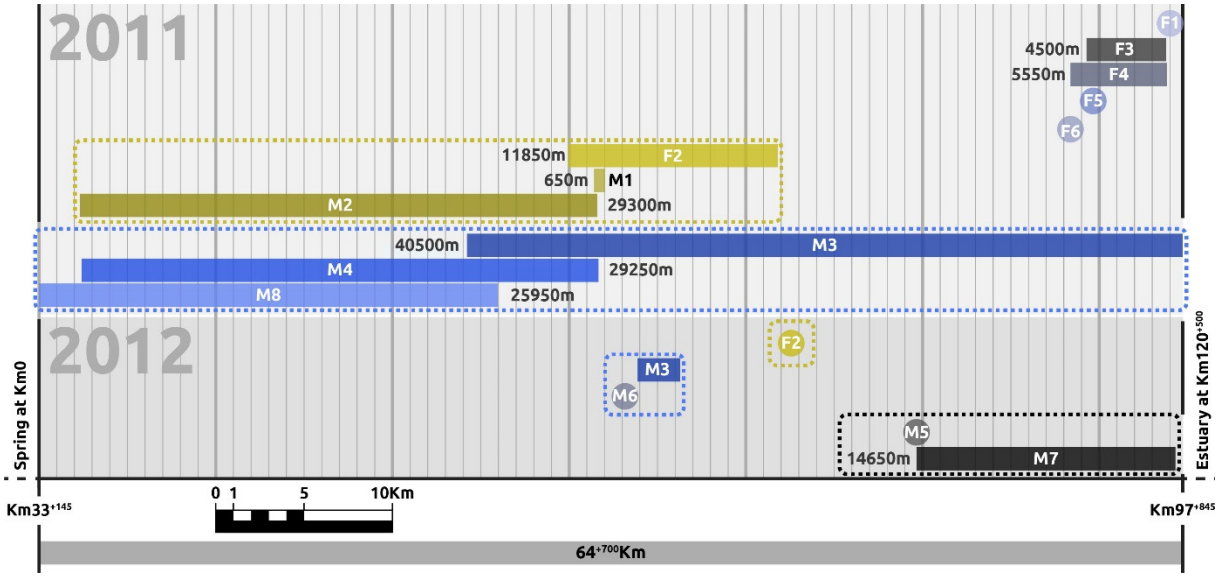

Figure 2. Pattern of spatial overlap among individuals genotyped in 2011 and 2012. Dotted lines indicate family groups (see text).

Overall, 14 individuals were identified from the whole sample. Among these, we identified 8 males and 4 females. For two specimens, PCR results were inconclusive. Based on the prevalence of males in most of the results (8 out of 12), the two unidentified individuals were more likely to be females (F5 and F6). For two otters sharing the same genotype (M2 and F2), we completed four extra PCR replicates and analyzed four extra loci (Lut715, Lut733, Lut782, Lut818). Since the latter were monomorphic, the distinction between the two individuals was inferred by combining molecular sexing, spatial location, and dates of sample collection. The probability of identity critical value was 0.001 (microsatellite loci) for unrelated individuals (PID) and 0.003 (13 loci) for related individuals (PIDsibs).

Individual capture/recaptures rates varied from 1 (n = 2) to 14 (n = 1; Table 1), with a mean of 3.8 captures per individual. Males were recaptured slightly more often than females (mean males = 5.4, mean females = 3.4) but the difference was not significant (t = 1.010, p-value = 0.356). Distances between recaptures are reported in Table 1, whereas potential full-sib families are listed in Table 2. For individuals captured once or recaptured at distances less than 1 km, we added 7 km of river stretch, considering that within this length there is an 89.67% probability of the individual's occurrence [34].

Table 1. Details on captures/recaptures (*N*) of otters sampled in the study area during the sampling season 2011. For individuals recaptured at distances less than 1 km we added 7 km of river stretch where the probability of occurrence of the same individual is about 90% [34].

Code	First Capture	Last Recapture	*N.* Captures/Recaptures	River Length Covered (M)
M1	3 June 2011	5 August 2011	2	650 (7650 corrected)
M2	3 June 2011	16 September 2011	7	29,300
M3	5 April 2011	23 September 2011	14	40,500
M4	15 September 2011	23 September 2011	2	29,250
M8	18 September 2011	21 September 2011	2	25,950
F1	2 May 2011	6 May 2011	2	25 (7025 corrected)
F2	3 June 2011	21 September 2011	7	11,850
F3	28 April 2011	15 July 2011	4	4500
F4	28 April 2011	21 August 2011	2	5550
F5 likely female	22 July 2011	-	1	7000 (corrected)
F6 likely female	24 September 2011	-	1	7000 (corrected)

Table 2. Each row represents a potential full-sib family. The inclusive probability, Prob (Inc.), is the probability that all individuals listed on the family row are full-sibs (i.e., share both parents), while the exclusive probability, Prob (Exc.), is the probability that all individuals of the full-sib family and no other individuals are full-sibs with this family.

FullSibIndex	Prob (Inc.)	Prob (Exc.)	Member 1	Member 2	Member 3	Member 4
2	0.9846	0.983	M1	F2	M2	
4	0.9986	0.9986	M3	M4	M6	M8
5	0.995	0.995	M5	M7		

3.1. Spatial Organisation and Kinship

The longest distances covered by a single individual were 40.5 km for a male (M3; Table 1) that was recaptured 14 times and 11.85 km for a female recaptured 7 times (F2; Table 1). The range of M3 was not the result of a temporal shift, as the same otter was recaptured in 2012 within the same range detected in 2011 (Figure 3). Recaptures of M3 were therefore consistent with the activity of an adult resident male. The longest distance covered during a single night was 14.65 km, recorded for male M7 in August 2012 (Figure 2). For individuals with a longer capture history, we could infer not only a broad temporal overlap with other otters but, in some cases, we also recorded the presence of more individuals during the same night at the same sampling site or a few meters apart (Figure 2, Table S1). This is the case of M1–F2 (full siblings, 3 May 2011 and 5 August 2011), F2–M3 (unrelated, 3 June 2011 and 2 August 2011), M1–M3 (unrelated, 5 August 2011), F3–F4 (unrelated, 28 April 2011), and M3–M4 (full siblings, 23 September 11).

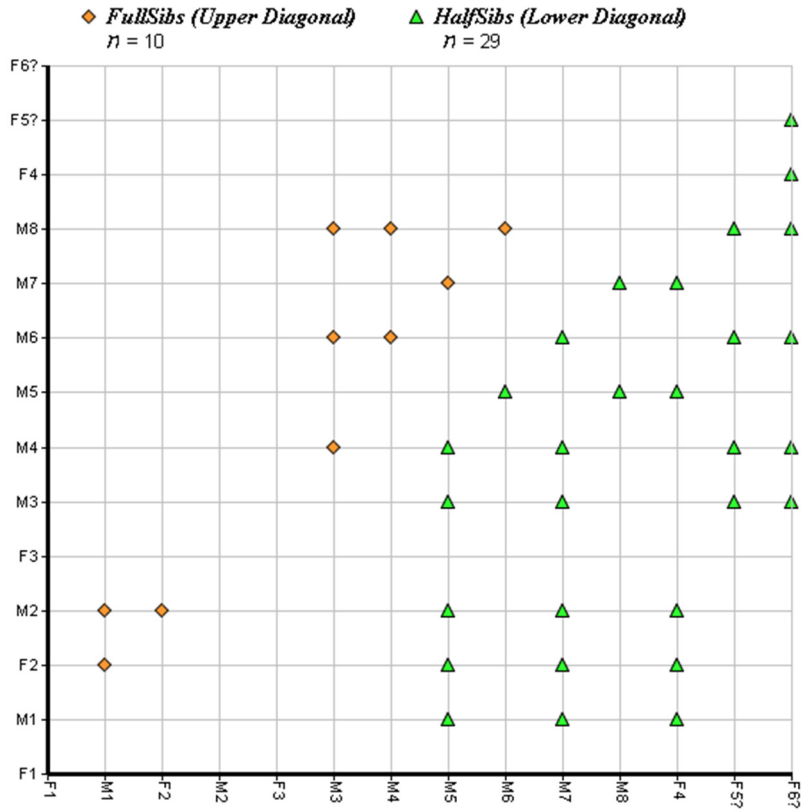

Figure 3. Graphic representation of relationships obtained from COLONY: full-sibs are shown above the diagonal (orange diamonds), half-sibs below the diagonal (green triangles). As an example, M1, M2, and F2 are full-sibs, whereas they are half-sib with M5, M7, and F4.

As expected by a polygynous species, several overlaps were observed between male and female ranges (M3–all females, M1–F2, M2–F2, M4–F2) (Figure 2). However, our results also detected both related and unrelated male–male (M1–M2–M3–M4, M8–M2–M4) and female–female (F3–F4–F5–F6) overlaps (Figure 2).

For three individuals (F2, M2, M3), we obtained comparable capture histories distributed over several months, allowing us to infer their activity ranges. F2 continuously marked at least 11.85 km, while M2 and M3 marked at least 29.3 and 40.5 km, respectively. Linear regression fitted between spatial overlap and sibship category indicated full-sibs overlapped significantly more than half-sibs (coefficient = 6262, $p = 0.034$), while this difference is only close to significance considering unrelated individuals (coefficient = 4833, $p = 0.058$). In addition, there is no significant difference in spatial overlap between half-sibs and unrelated individuals (coefficient = −1430, $p = 0.743$). As for sex pairing, male–male pair exhibited a significantly higher overlap than both male–female (coefficient = 8845, $p < 0.0001$) and female–female (coefficient = 8612, $p < 0.0001$) pairs, while the latter two did not show any significant difference in their spatial overlap (coefficient = −233, $p = 0.987$). In particular, the variation in spatial overlap resulted more explained by sex pairing, where the R^2 is equal to 0.34, than by sibship category ($R^2 = 0.08$).

To investigate possible mechanisms underlying spatial interactions within and between sexes, all genotypes were tested for sibship using COLONY [70] to identify potential full-sibs families. COLONY takes into account allele frequencies and error rates, using a group approach (i.e., the simultaneous analysis and comparison of the entire sample) to infer full-sibs families. For sibship analysis, we considered the whole sample set. No evidence of inbreeding was found, and 9 out of the 14 genotyped individuals belonged to three full-sib clusters, while five female individuals (F1, F3, F4, F5, and F6) had no full-sibs in the population (Figure 3).

Full-sibs individuals also showed to be half-sibs to other individuals presented in Figure 3. An important aspect to underline is that parent–offspring and full-sibs dyads share the same relatedness coefficient, measuring the dyad overall identity by descendant (IBD). The inability to directly observe otters and to obtain age estimates from the scat samples prevent the ability to infer age-related relationships among the individuals.

3.2. Population Size, Density, and Structure

To estimate population density, we considered 64.75 km of river stretches where we obtained positive genotyping results and added a 7 km buffer at both ends, corresponding to 97% probability of otter's presence from a sampling site [56], obtaining a total length of 78.75 km. Population size analyses provided an estimate of 12 otters (CI95% = 11–14), corresponding to a mean otter density of 0.152 otters/km of watercourse (CI95% 0.139–0.177). Considering that the hydrographic river network of the area covers five 10 × 10 km grid cells, these values correspond to a density of 2.4 individuals/cell.

4. Discussion

During the two sampling years, we successfully genotyped 14 individual otters along 64 km of the river Sangro, corresponding to 0.152 individuals/km. This value falls within the estimates provided for the European range, spanning from 0.012 otters/km in England to 1.14 otters/km in Germany [49,72–75]. Specifically, the values from our study were close to those reported by [4] in southern Italy (0.18–0.20 otters/km) and by [38] in Germany (0.21 individuals/km), but lower than those reported by [50] along pond shores in Germany (0.34 to 0.48) or by [76] for the Bohemian forest (0.032 individuals/km). Furthermore, the density of 2.4 individuals/10 × 10 km grid cell can be used for population size and trend estimation according to the European regulation 92/43/EC (Habitat Directive), since reporting cycles are based on number and distribution of 10 × 10 km grid cells occupied by the species. Accordingly, the 3440 grid cells of otter occurrence in Italy reported in the last reporting cycle (i.e., 2013–2018) likely host around 8250 otters, which suggests that the current Italian population is now larger than the minimum viable population size [77].

Additionally, the high number of recaptures and the degree of relatedness of the recaptured individuals provided new relevant information on the density, structure, and social organization of the Eurasian otter metapopulation living in the Sangro river basin. As expected for a polygynous species, we observed ranges of related individuals to partly or totally overlap, and ranges of unrelated males to overlap with those of several females. These results are also congruent with previous studies that reported solitary male otters having extremely large ranges overlapping with several females, while related females share group territories with individual exclusive core areas [49,50,54,72]. However, we also observed large spatial overlaps of both unrelated males and females, and a higher influence of sex-pairing on explaining spatial overlap compared to sibship. This phenomenon was especially marked in males, where male–male overlaps were significantly higher compared to both male–female and female–female ones. These high overlaps among unrelated males have never been reported in previous studies, suggesting an even more flexible social system than previously expected for a solitary and territorial polygynous species.

In fact, we recorded the presence of more individuals during the same night at the same sampling site, or a few meters apart. This was not only the case of both full sibling and unrelated male–female dyads that could indicate the temporary bond of a mother and son (full sibling) or reproductive pair (unrelated male–female), but also the case of unrelated male–male and female–female pairs. We are aware that spatial overlap does not necessarily equal true dyad encounters and that in populations where dispersal is limited, wandering males continue to overlap each other [78]. As interactions form the basis for relationships between individuals and are the foundation for analyzing social structure [77–81], this evidence needs further confirmation, likely by other approaches such as camera trapping or radiotracking [82]. We are also aware that, unless coupled with hormone detection [38,39], noninvasive genetic analyses are unable to provide information about age and reproductive

status of individuals. Thus, we cannot exclude that these overlapping individuals were either juveniles or subadults detected during one-way dispersal from their natal area.

Moreover, related females were only detected downstream, where fish abundance is likely higher, although the lack of females upstream could be due to a failure in genotyping spraints in this portion of the study area. As a matter of fact, although in a completely different scenario (Shetlands) [83], male otters were observed to mark/spraint more frequently than females, especially during summer. If seasonality was a cause, a male bias in the sampling could have been caused by the low female marking/sprainting rates. In addition, higher marking frequencies might be related to the higher dispersal movements of males compared to females [43,54]. Another explanation could be inferred from the classical mustelid sociobiology which predicts resource-based female home-ranges [84]. The absence of females in the upper watercourse of the Sangro river could be related to a shortage of food resources in this portion of the study area. Although the lower course of the Sangro river is known to host higher fish diversity compared to upstream (see for example [85]), this latter hypothesis deserves further investigation to assess any differential feeding resource abundance along the Sangro river basin.

Finally, for one female and two male Eurasian otters in the study, we obtained comparable capture histories distributed over several months, which allowed us to infer their range use and size. Specifically, one female was detected leaving spraints along at least 11.85 km of river stretches, whereas two males scent-marked along 29.3 and 40.5 km of river stretches, respectively. These values are coherent with home-range estimates recorded in previous studies, i.e., 12–30 km for female otters and 21–67 km for male otters [27,86]. Moreover, larger distances covered by the males are in accordance with the general pattern observed in polygynous carnivores [87–89]. However, these values were larger than those reported for the seasonal home-range extent of the only two individual otters tracked in Italy, which were 20 and 30 km for the female and the male, respectively [27]. In accordance with the resource dispersion hypothesis [83], these larger distances might be either related to the lower availability of feeding resources or to lower densities that characterize the periphery of the range [25].

Since extensive variations in social organization, habits, and spatial distribution have been recorded for several mustelids among different study areas, seasons, and years [27,53,87,90–93], we are aware that further studies are still needed from different parts of the range to ultimately describe the behavioral ecology of this small and isolated population of Eurasian otters. In addition, the extension of the same approach to the neighboring rivers will likely contribute to evaluating gene flow and degree of isolation among river basins [26,27], as well as the dispersal patterns at the boundary of the Italian range, which were out of the scope of this work. Nevertheless, our combined approach gives the first relevant contribution to the comprehension of range dynamics and social structure of the isolated and endangered population of the Eurasian otter occurring in Italy. We also highlight the noninvasiveness and success of the method that could be suitable for other taxa that are also lacking such data.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14041943/s1, Table S1: Details on dyads that were detected at the same site at least once (F = full-sibs, H = half-sibs, U = unrelated; 1 = male–female couple, 2 = male–male couple, 3 = female–female couple). Full-sibs are defined as progenies having the same male and female parents, while half-sibs are progenies having only one parent in common.

Author Contributions: Conceptualization, A.L. and L.L.; methodology, A.L., L.L. and C.M.; software, L.L., C.M. and M.D.F.; validation, L.L., C.M. and A.L.; formal analysis, L.L.; investigation, L.L.; resources, A.L.; data curation, L.L.; writing—original draft preparation, L.L. and A.L.; writing—review and editing, H.K.; visualization, L.L.; supervision, A.L.; project administration, H.K.; funding acquisition, A.L. and L.L. All authors have read and agreed to the published version of the manuscript.

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