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Genetic Variability and Family Relationships in a Reintroduced Osprey (*Pandion haliaetus*) Population: A Field-Lab Integrated Approach

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Abstract: Reintroductions represent an opportunity to restore local biodiversity and reverse the effect of taxa extinction. However, they need feasibility and monitoring plans before and during their implementation to ensure concrete and lasting results. During the 20th century, the osprey (*Pandion haliaetus*) underwent a severe population decline in many European countries due to direct persecution and coast exploitation. In the 1960s–1970s, it was declared extinct as a breeder in Italy. In 2004, the Maremma Regional Park (Tuscany, central Italy) started a reintroduction project by capturing and releasing, from 2006 to 2010, 33 juvenile Corsican ospreys on the southern coast of Tuscany. The settlement of the first breeding pair in 2011 was the initial sign of the success of the reintroduction project, then further pairs settled from 2011 onward. A total of 81 feather or blood samples were collected for DNA extraction from both translocated (2006–2010) and newborn individuals (2011–2021). Individuals were analyzed at 16 microsatellite loci to verify any changes in genetic variability over time and to set out a protocol for the reconstruction of kinship for conservation and management purposes. We did not observe a reduction in genetic variability between the two sampling periods, although we found a slight sign of the founder effect in the reestablished population. A strong genetic differentiation was observed between this Mediterranean population and an injured osprey from a Northern European population, thus confirming the importance of considering the local genetic pool in any reintroduction project. Monogamous behavior was confirmed by family reconstruction, which allowed the identification of clear kinship relationships. Our findings indirectly inform on the genetic variability of the population during the 16-year period from the start of the project and provide useful insights for its long-term conservation.

Keywords: osprey; reintroduction; central Italy; genetic variability; family relationships

1. Introduction

Extinction is a natural process when viewed on a geological scale, but the increased human-mediated loss of species at presumed rates of extinction exceeding that by more than two orders of magnitude is a threat to biodiversity conservation [1]. At the same time,

the dramatic decline in abundance and diversity and the concurrent increasing homogenization resulting in low levels of genetic differentiation are further influencing the biodiversity crisis at various levels, including population and individual levels within species [2]. When extinction interests only partially the whole distribution range of a species, a population restoration program [3] can help to reconstitute an extinct local population. Several alternative approaches have been used to achieve this goal for the conservation of target species [4]. Restoration techniques include the construction of artificial nests to promote birds' settlement and reproduction, aiming at population increase [5], captive-breeding programs using eggs or specimens from donor populations [6], even possibly assisted by artificial incubators or insemination [7], followed by chick hand-rearing [8], control of predators and pathogens and enhancement of habitats, among others. In the framework of conservation translocation programs (e.g., reintroduction and restocking [9]), it is critical to provide individuals with similar genetic composition and evolutionary histories from non-threatened source populations to be released into the wild [9]. However, it is well known that reintroductions (the release of an organism into an area that was once part of its range, but from which it was extirpated [9,10]), even when correctly planned and carried out, can produce a reduction in genetic variability, due to the founder effect (e.g., [11,12]). By combining this outcome with the reduced number of individuals that usually characterize reintroduction projects, the main threat arising in this context is potentially restarting the vortex of the extinction process [13]. Genetic and demographic founder effects may thus have serious consequences for colonizing populations and their long-term establishment (e.g., [3,14,15]). To limit these risks, it is therefore important that the population resulting from a reintroduction program interconnects with other populations through the exchange of individuals and consequent gene flow, thus fostering genetic diversity and, in turn, the self-sustainability of the population in the long term. In any reintroduction project, standardized monitoring of the pre- and post-release phases is of fundamental importance, as well as that of the reproductive individuals of the new established population [16]. The implementation of extensive ecological and genetic monitoring programs is key to evaluating the effectiveness of the actions undertaken and correcting any errors in plan management and conservation strategies aimed at increasing the success of the project [6,16]. In this perspective, it has been proven that combining different and integrative tools can represent a more powerful approach than using a single technique [17]. While ringing and modern tracking techniques, such as satellite telemetry, represent critical tools for elucidating patterns of animal movements (e.g., [18]), video recordings allow monitoring the trend of a reintroduced population by describing the dynamics and estimating changes in population size (e.g., [19–21]). Moreover, the genetic characterization of individuals (e.g., microsatellite genotyping) can allow researchers to describe the genetic variability and identify any modifications, both increasing or decreasing, as well as record the presence of any genetic flow among populations [22,23]. For example, the trend of several reintroduction projects has been documented by using single nucleotide polymorphisms (SNPs) or microsatellite loci (STRs), allowing the description and the recording of the genetic variability in several taxa (e.g., [22,24,25]). Integrated long-term ecological and genetic monitoring programs thus offer a holistic approach to the management and conservation of target species (e.g., [21]).

Here, we focus on the osprey (*Pandion haliaetus*), an iconic raptor species of high conservation concern and subject of several long-term reintroduction programs across America and Europe [26]. During the 19th and early 20th centuries, the species suffered a severe population decline [26,27], which resulted in the extinction of breeding populations in several countries [28]. In Europe, it is included in Annex I of the European Directive (2009/147/EC) on the conservation of wild birds and it is hence considered a priority species for conservation along its whole distributional range. Direct management actions and both international and national law enforcement allowed a partial recovery of the species across Europe, although numbers remained lower than historical populations (e.g., [26,29]). Some populations, such as those of the Mediterranean basin, where the total population size is estimated at ca. 100 breeding pairs only [23,30], are still considered in danger. In addition,

the osprey populations living in the Palearctic are not genetically homogeneous: past and recent population genetic studies found evident genetic structuration between northern (long-distance migratory) and southern (mostly sedentary or short-distance migratory) populations within the Western Palearctic [21,23,31,32]. Native birds from Corsica, the Balearics, northern Africa, and the Canary Islands are genetically different from ospreys breeding in Central and Northern Europe, and show distinct migratory strategies with respect to both temporal and spatial components of migration. They hence represent a separate management unit, characterized by wider gene exchanges among its populations than with those occurring in the rest of Europe (gene flow < 4%; [23]). These populations thus deserve attentive management and priority of conservation efforts, while maintaining their characteristics and evolutionary potential [32,33]. In the region, several reintroduction projects have been carried out in the last few decades (e.g., [34–36]). In Italy, osprey became extinct as a breeding species during the late 1960s–early 1970s, mainly due to direct persecution [37–40]. In 2004, a reintroduction project was developed in collaboration between the Maremma Regional Park (Italy) and the Natural Regional Park of Corsica (France) to restore a viable nesting osprey population in central Italy that would interconnect with the neighboring Corsican one to ultimately secure its future conservation [41]. From 2006 to 2010, a total of 33 Corsican osprey chicks were captured at their nest and translocated in the Maremma Regional Park, where, after a short period of permanence in hacking pens, were released into the wild [36]. During the project, several artificial nests were built within the park at key different sites in the extensive coastal wetland system of southern Tuscany and on the islands of the Tuscan Archipelago National Park, placed midway between Corsica and coastal Tuscany. After the first settlement of a breeding pair in one of the artificial nests in 2011, other nests were gradually occupied by adult ospreys in the following years (i.e., a total of seven to eight territorial pairs in 2022 [41]). Ecological studies showed that floating individuals tend to occupy vacant nests in the proximity of other breeding pairs, thus showing a "semi-coloniality" habit [29,42]. However, although the species can reach sexual maturity already at the age of 2 years, the recruitment in the breeding population can require more time, sometimes with first successful breeding attempts occurring after 3–5 years (e.g., [43]) and with an estimated mean generation time of 9.6 years [44]. For this reason, the settlement of the first breeding pair in the framework of a reintroduction project is an important milestone that can be interpreted as a chance for the success of the project itself [34]. However, in the early stages of a newly established population, genetic variability may be low, especially when it originates from a limited stock of translocated individuals. Furthermore, the phenomenon could be much more marked in monogamous species, where mates tend to be faithful to the same partner, year after year, further limiting any possible gene exchange.

We genotyped 16 loci in individuals belonging to both the source population of the translocated birds (2006–2010) and those belonging to the newly established population (2011–2021) to: (i) assess the genetic variability and (ii) verify any modification of the genetic composition in the long term compared to the early stages of the reintroduction program to 2021. Moreover, notwithstanding the monogamy of the species, we obtained evidence from video camera recordings of cases of extra-copulation, a behavior that has been already described in the species [43,45], but never recorded in the early stage of a population settlement [43,45]. As we could not assess if this behavior resulted in genetically diverse brood composition, we carried out parentage tests (iii) to infer direct relationships among individuals as well as full relationships among individuals and enable the verification of any occurrence of chicks associated with non-mate individuals.

2. Materials and Methods

2.1. Sample Collection and Study Area

A total of 81 samples (74 feather and 7 blood samples) were collected in the study period. Sampling was carried out taking into account the two phases of the project: in the first phase, called "translocation" (which took place between 2006 and 2010), 33 individuals

of Corsican origin were sampled to obtain genetic information on the founder stock that would contribute to the future breeding population. During this phase, thirteen different nests located along the west coast of Corsica were used to collect chicks for translocation (Table S1). Each year, depending on the active nests available, a total of 6–8 chicks were taken by selecting only one chick per nest and taking care to collect the biggest and oldest one of the clutches (i.e., while the younger chicks were left in the nest, where their chances of survival were increased in the absence of one sibling). Chicks were then transported by helicopters to the hacking pens located in Maremma Regional Park (42°39′55.47″ N, 11°1′33.70″ E), where they were kept for approximately three weeks until release. Two of these 33 individuals (a male ringed I1-E4647 in 2006 and a female ringed S5-E1146 in 2010) successively settled in different years in two different nests located in the study area (see below), leading to a series of breeding events, starting from 2011 and 2014, respectively. In the second phase, called "population after reproduction" (between 2011 and 2021), a total of 46 individuals out of the 56 that had hatched was captured at the nests and sampled to obtain information on the genetic composition of the reestablished new population and monitor any possible immigration of breeding individuals from other populations. Remarkably, this sample includes two individuals hatched in 2011 and 2016, settled and bred in two new different nests in Diaccia Botrona Natural Reserve and WWF Natural Reserve Orti-Bottagone Marsh in 2014 and 2019, respectively. In this second phase, an unringed adult female of unknown origin that settled in the area in 2015 (see [41]; for further details, see also Figure 1 and 2) was also captured, ringed and sampled for subsequent genetic analysis.
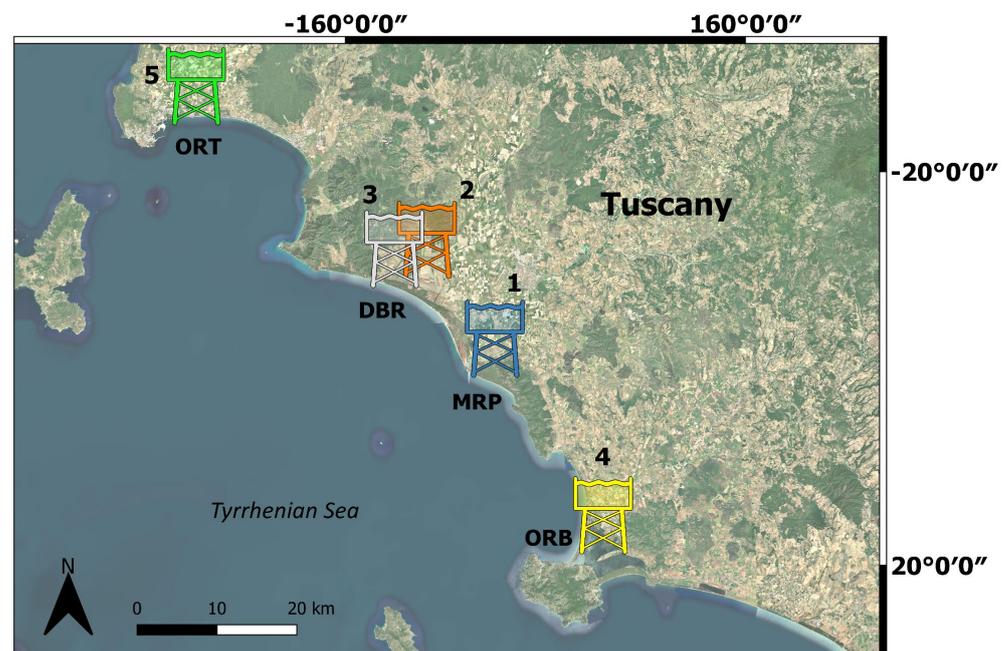
Figure 1. Geographical representation of the study area where osprey samples were collected. The four protected areas within the extensive coastal wetland system in southern Tuscany (central coastal Italy) and hosting the five osprey nest sites were: Maremma Regional Park (MRP−NEST_1, in blue), Diaccia Botrona Natural Reserve (DBR−NEST_2 and NEST_3, in orange and grey, respectively), Orbetello Lagoon (ORB−NEST_4, in yellow) and Orti-Bottagone Marsh (ORT−NEST_5, in green). The numerical values for latitude and longitude in decimal number format are: ° for degrees, ′ for minutes and ″ for seconds.

Furthermore, to test whether in the current Italian population there could be individuals of Central–Northern Europe origin [21,23], we included in the analyses the feather samples of an adult individual who was found injured in March 2019 on a shore of Elba

Island (Tuscany). Following a short period in a recovery center, it was released, after being equipped with a GPS/GSM transmitter (model Duck-4, Ecotone, Gdynia, Poland), which allowed us later to identify both the breeding area (Latvia) and the wintering ground (Nigeria) (unpublished data).

All chicks were handled at an age of 5–8 weeks (38–60 days according to the bird's growth rate), measured, and marked with both a metal and a colored ring. Capture, handling, and tagging procedures were carried out under the supervision of the Italian Institute for Environmental Protection and Research (ISPRA) in accordance with Law 157/1992 [Art.4(1) and Art 7(5)], which regulates research on wild bird species. Field work was also conducted with the permission of management bodies of the nature reserves involved: Maremma Regional Park, Tuscany Region, WWF Natural Reserves. Blood and feather samples for genetic analyses were collected from osprey chicks during ringing activities at nests. About 0.5 mL of blood was taken by venipuncture from the wing of each individual and stored in Eppendorf tubes containing a Longmire solution [46]. Feathers were conserved in 2.0 mL tubes in ethanol 95%. Bird handling (from capture to release) lasted a total of 30–40 min.

The study area was made up of four protected areas within an extensive coastal wetland system in southern Tuscany (central coastal Italy). The nest sites were located in: Maremma Regional Park (MRP−regional park; 1 breeding pair; NEST_1), Diaccia Botrona Natural Reserve (DBR−natural reserve; 2 breeding pairs; NEST_2 and NEST_3); Orbetello Lagoon (ORB−WWF natural reserve; 1 breeding pair; NEST_4), Orti-Bottagone Marsh (ORT−WWF natural reserve; 1 breeding pair; NEST_5) (Figure 1). The mean distance among nests sites is 32.7 km (range: 1.1−73.9 km). In these sites, ospreys reproduce in artificial nests built on three-pole structures equipped with video surveillance systems installed in the framework of the project. Internet protocol (IP) cameras with a 3X optical zoom, 2- and 4-megapixel sensors and full-HD resolution 1920×1080 were used. The camera registry program (CRP) was set on video mode recording nonstop 24 h/day. Cameras recorded in color during daylight hours and switched to black and white (using infrared vision) at night, allowing constant monitoring of the ospreys' activity. The Tyrrhenian Sea, lagoons, rivers, saltwater marshes and channels characterizing the study area provide highly suitable fishing grounds for ospreys [41]. At these sites, population monitoring takes place routinely. Breeding events are kept under daily surveillance through nest video recordings, accompanied by weekly field surveys, allowing the recording of key breeding dates and parameters.

2.2. Criterion of the Marker Choice

We used a combined marker panel from two previous studies [23,47]. From the first one [47] we selected 12 markers (PHA04, PHA11, PHA12, PHA13, PHA14, PHA16, PHA27, PHA28, PHA29, PHA35, PHA36, PHA37) that have been described as successful, reliable, and polymorphic in the three tested populations (England, Scotland and Norway), assuming they will be polymorphic also in the southern Corsican population. Marker size was also used as a further criterion to allow for marker multiplexing. From the second study [23], we tested 17 markers from the first four multiplexes (Balbu11, Balbu15, Balbu18, Balbu40, Balbu12, Balbu25, Balbu30, Balbu35, Balbu37, Balbu14, Balbu21, Balbu23, Balbu28, Balbu29, Balbu31, Balbu10, Balbu17). A total of 11 out of 12 markers from the first study [47] were polymorphic (PHA35 was retained, although it was polymorphic only in the injured individual), while only 5 were retained from the second study [23] because of the lack of genetic variability inside the Corsican Italian population (see the following paragraph).

2.3. DNA Extraction and Amplification

According to the manufacturer's instructions, DNA was isolated from feather or blood samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Hilden, Germany) and amplified at 16 nuclear microsatellite loci, Balbu17, Balbu18, Balbu23, Balbu28, Balbu40 [23], PHA04, PHA11, PHA13, PHA14, PHA16, PHA27, PHA28, PHA29, PHA35, PHA36, PHA37 [47] in an 8 µL final volume reaction. Amplifications were carried out as follows: $1\times$ reaction buffer, 0.02% BSA, 1.5 mM $MgCl_2$, 0.125 mM of dNTPs, Hot Start Taq Polymerase 0.025U (Qiagen, Inc., Hilden,

Germany), 0.125 mM primer (forward and reverse), 1 µL DNA template and nuclease-free water to reach the final volume. Details on multiplex PCR primer sets are reported in Table S2.

According to melting temperature and reference bibliography, molecular sexing DNA was performed by amplifying informative regions on ZZ/ZW chromosomes with the following thermal profile: 94 °C for 15′, followed by 35 cycles at 94 °C for 40″, 55 °C for 40″, ending with a final extension at 72 °C for 10′ [48].

Autosomal and sexual amplicons were separated through capillary electrophoresis in an ABI 3130xl genetic analyzer (Thermo Fisher Scientific, Waltham, MA, USA); alleles were scored in GeneMapper 4.0 using GeneScan 500 ROX size standard (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Dataset Identification

Statistical analyses were conducted on three different data sets, including (1) the whole sample set (WSS, $n = 81$); (2) two populations, the first consisting of individuals released in the study area during the "translocation" phase from 2006 to 2010 (POP_1; $n = 33$) and the second being composed of individuals hatched in the study area during the "population after reproduction" phase from 2011 to 2021, the unringed adult female of unknown origin that settled in the area in 2015, and the two translocated individuals that, once adults, settled in the area and reproduced in 2011 and 2014 (POP_2; $n = 49$); (3) one population, including only the individuals hatched in the study area from 2011 to 2021 (POP_3; $n = 46$).

2.5. Genetic Variability

Genetic variability was computed in WSS ($n = 81$), POP_1 ($n = 33$), and POP_2 ($n = 49$). Allele number (*Na*), effective allele number (*Ne*), number of private alleles (*Pa*), observed and expected heterozygosity (*Ho*, *He*) were assessed using GenAlEx 6.41 [49], while the allelic richness (*Ar*) was computed using Fstat [50] based on a minimum sample size of 32 individuals. Significant differences between translocated and newborn individuals were computed using an ANOVA test in Past [51]. Hardy–Weinberg equilibrium (HWE) was tested in Genepop on the web software [52,53], using the probability test (Dememorization = 1000; Batches = 100; Iterations per batch = 1000). The significance of departure from HWE was checked by applying a Bonferroni correction for multiple comparisons.

We tested the presence of genetic drift due to the founder effect mediated by the reintroduction by using Bottleneck v.1.2.02 [54] in the whole dataset and in the two populations. Both infinite allele (IAM) and two-phase (TPM) mutation models were applied by using 1000 iterations. We did not use the strict stepwise mutation model (SMM) because only a few loci usually follow this model strictly [55]. Significance was computed by one- and two-tailed Wilcoxon tests ($p < 0.05$). TPM was computed firstly by using default parameters (70% SMM and 30% variance for TMP), then by setting SMM at 0.00 and variance at 35%, as suggested in the manual for STRs.

An additional computation between the translocated and re-established populations (POP_1 and POP_2) and the outgroup individual from Northern Europe was carried out to record private alleles distinguishing the Mediterranean and Northern European populations.

2.6. Analysis of the Genetic Structure

F*st* and AMOVA tests were calculated in Genetix v. 4.05 [56] and in GenAlEx, respectively, to record any difference in the genetic composition between the translocated population (POP_1) and the population after the reintroduction (POP_2). A factorial correspondence analysis (FCA) was carried out in Genetix and visualized in an Excel graph to infer any genetic structure in the sampling.

A Bayesian clustering procedure was tested in Structure 2.3.4 [57,58] to identify any substructure or difference in the genetic composition between POP_1 and POP_2. Simulations were run by using a burn-in period of 40,000 replicates, followed by 400,000 Monte Carlo iterations, and assuming that the number of clusters K could range from 1 to 10. All simulations

were independently replicated five times for each K, using the "admixture" and the "correlated" allele frequency models [59]. The optimal number of populations K was set at the value that maximized the increase in the posterior probability of the data LnP(D) according to the formula LnP(D)k–LnP(D)k–1 [60] that was computed and plotted in Structure Harvester [61]. Best cluster bar plots were visualized and printed by using Clumpak (https://clumpak.tau.ac.il, accessed from 15 November 2022 to 28 February 2023 [62]).

Finally, the Northern European osprey sample was included in the FCA to infer any structure between Northern and Southern populations or detect any migrants from other European populations.

2.7. Family Relationship Confirmation

Family reconstructions were conducted to verify the application of the marker panel to record parentage relationships in absence of field information. Individual relationships were tested using Colony 2.0 [63] by applying the non-inbreeding data and monogamy models and by setting the genotyping error rate value at 0.0001. Genotypes of all breeding pairs were not available, so, at first, we looked for the confirmation of the known family relationships between the translocated individuals (POP_1) and the newborn chicks (POP_2). To address all the family relationships, we included in POP_1 also the 3 adults from the POP_2: the two wild-born chicks that reproduced afterward and the unringed female from NEST_3 ($n = 33 + 3$). Then, we conducted a full sibling test only considering the newborn chicks (POP_3; $n = 46$). As two individuals were both chicks and reproductive individuals (Figure 2), we also conducted a new test after having removed their genotypes from the analysis ($n = 44$). The results obtained from this analysis were compared with known data for each nest and used to verify the reliability of the marker panel and also to record any modification in the monogamy behavior, as indicated from video camera surveillance.
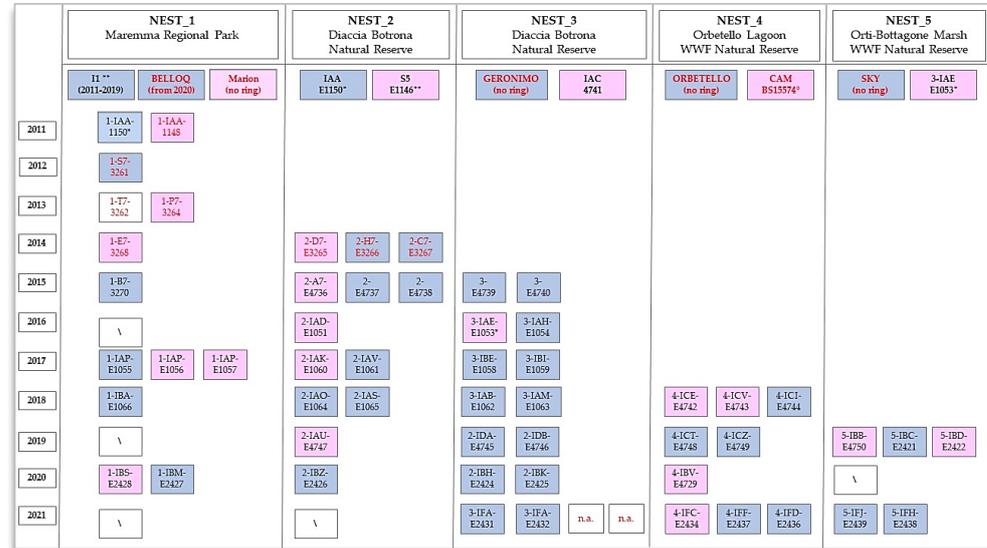
Figure 2. Graphic scheme of nests and reproductive events between 2011 and 2021. BELLOQ is the unringed male that in 2020 replaced I1-E4647 in NEST_1 and the father of solely the two chicks hatched in 2020 in NEST_1 (1-IBM-E2427 and 1-IBS-E2428). Two individuals hatched in 2011 and in 2016 in NEST_1 and NEST_3 (indicated by *), upon reaching sexual maturity, reproduced in the NEST_2 and NEST_5 (see notes below), respectively. In black are the ospreys sampled, while in red are reported other individuals of the population that have not been sampled for this study. Blue and pink squares indicate male and female birds, respectively. ** = individuals translocated during 2006–2010 that became successful breeders; ° = non-translocated Corsican adult female (ringed in Corsica as a *pullus*) that naturally settled in NEST_4 in 2018; n.a. = two individuals hatched in NEST_3 in 2021 but not ringed/sampled. \ = indicates unsuccessful breeding events (e.g., eggs did not hatch or *pullus* died before fledging).

Finally, Bayesian and multivariate analyses were computed on POP_2 to obtain information about the distribution of the genetic components between and within the nests.

3. Results

3.1. Genetic Variability in the Long Term

All 81 individuals were fully genotyped. Biomolecular sexing determined that 12 out of 33 translocated individuals and 33 out of the 46 post-translocation wild-born chicks were males, which is evidently a different sex ratio between the two project sampling phases. A strong departure from Hardy–Weinberg equilibrium was found in WSS and in POP_2, while no disequilibrium was recorded in POP_1. One (Balbu23) and three loci (PHA11, PHA13, PHA36) were in HW disequilibrium in POP1 and in POP_2, respectively, while Balbu23 showed a departure when considering the WSS. As the disequilibrium affected these loci differently in the different sample sets, they were retained in the following analyses.

Allele number was 3.4 ± 0.4 in the WSS, with no significant differences between the two periods (3.4 ± 0.4 in POP_1 and 3.0 ± 0.4 in POP_2); allelic richness was computed on a minimum sample size of 32 individuals and did not differ significantly in the two populations, resulting in 3.4 ± 0.4 and 3.0 ± 0.4. Detailed values on Na and Ar are listed for each locus in Table S3a. Neither observed nor expected heterozygosity was different between the two sample sets, and their values were $Ho = 0.458 \pm 0.068$ in WSS, 0.451 ± 0.066 in POP_1, 0.462 ± 0.072 in POP_2 and $He = 0.445 \pm 0.058$ in WSS, 0.444 ± 0.056 in POP_1, 0.433 ± 0.060 in POP_2. Detailed results are shown in Table 1. Five private alleles with a frequency ranging from 0.15 to 0.31 were found in POP_1 (Table S3b). When comparing the Northern European osprey individual with POP_1 and POP_2, it showed six alleles not recorded in the samples from the other two populations (Table S3c).

Table 1. Variability indices in the two sampling periods (Na = number of alleles; Ar = Allelic richness; Ho = observed heterozygosity, He = expected heterozygosity; $P_{(HWE)}$ = significance of departure from HWE).

Pop	Na	Ar	Ho	He	P (HWE)
POP_1 (n = 33)	3.4 ± 0.4	3.4 ± 0.4	0.451 ± 0.066	0.444 ± 0.056	-
POP_2 (n = 49)	3.0 ± 0.4	3.0 ± 0.4	0.462 ± 0.072	0.433 ± 0.060	$<1.33 \times 10^{-10}$
WSS (n = 81)	3.4 ± 0.4	3.4 ± 0.4	0.458 ± 0.068	0.445 ± 0.058	$<1.61 \times 10^{-7}$

Significant sightings of genetic drift were identified from Bottleneck software in POP_2 and confirmed by both IAM and TMP models (Table S4).

3.2. Structure Analysis

Fst recorded a significant difference between POP_1 and POP_2 (Fst = 0.02102; P = 0.000), although AMOVA computation confirmed that almost the whole recorded variance (98%) was within individuals (Figure S1). By analyzing the FCA plot, the main variance was described by Axis 1 (14.63%), although no clear differentiation was found between POP_1 and POP_2 (Figure S2).

Best clustering was defined at K = 4 by delta K procedures and following the mean likelihood probability plot. Bar plots of clusters from K = 2 to K = 5 in Figure S3 evidence the lack of clear differentiation between POP_1 and POP_2, although this also manifested in the presence of different percentages in some genetic components (e.g., violet, orange, and green components that showed a different distribution in the bar plots of K = 3 and K = 4).

After adding the Northern Europe osprey genotype to the FCA plot (Figure S4), it was separated from the other samples, and the variance explained by Axis 1 (16.48%) was between the Northern individual and the other populations from this study.

3.3. Family Relationship Confirmation

Although we were able to confirm some association between adults and chicks, some attributions failed in the family reconstruction (Table S5). Conversely, the full-sibling analysis, conducted by considering only the relationships between chicks hatched post-translocation, allowed us to correctly assess which individuals were hatched in the same nest, except for individuals hatched in NEST_5, which were divided into two different clusters. However, when removing the individuals hatched in NEST_1 and NEST_3 that subsequently reproduced in NEST_2 and NEST_5, respectively, all the individuals were correctly assigned to single clusters with probability values ranging from 0.96 (NEST_2) to 1.00 (the remnant nests).

Two individuals (1-IBM-E2427 and 1-IBS-E2428) that were hatched in NEST_1 after the male of the breeding pair was replaced by another individual, were assigned to different genetic clusters than their half-siblings from the previous years (see Table 2), thus explaining the presence of six clusters with respect to the five nests.

Table 2. Family group reconstructions using the software colony. Full-sibling relationships were found within and between the nests. The two individuals listed in cluster n. 6 were hatched in NEST_1 in 2020. After that, the male was replaced by another individual in the breeding pair. In this analysis, we removed the two chicks that had hatched in 2011 and 2016 in the framework of the reintroduction project and that successively reproduced in 2014 and 2019 (see the text and Figure 2 for further details).

Family Cluster 1 Prob (inc) 1.00 NEST_1	Family Cluster 2 Prob (inc) 0.96 NEST_2	Family Cluster 3 Prob (inc) 1.00 NEST_3	Family Cluster 4 Prob (inc) 1.00 NEST_4	Family Cluster 5 Prob (inc) 1.00 NEST_5	Family Cluster 6 Prob (inc) 1.00 NEST_1
1-B7-E3270	2-IAD-E1051	3-E4739	4-ICE-E4742	5-IBB-E4750	1-IBS-E2428
1-IAP-E1055	2-E4738	3-E4740	4-ICV-E4743	5-IBC-E2421	1-IBM-E2427
1-IAT-E1056	2-E4737	3-IAH-E1054	4-ICI-E4744	5-IBD-E2422	
1-IAZ-E1057	2-A7-E4736	3-IBE-E1058	4-ICT-E4748	5-IFJ-E2439	
1-IBA-E1066	2-IAK-E1060	3-IBI-E1059	4-ICZ-E4749	5-IFH-E2438	
	2-IAV-E1061	3-IAM-E1063	4-IBV-E2429		
	2-IAO-E1064	3-IAB-E1062	4-IFC-E2434		
	2-IAS-E1065	3-IDA-E4745	4-IFF-E2437		
	2-IAU-E4747	3-IDB-E4746	4-IFD-E2436		
	2-IBZ-E2426	3-IBK-E2425			
		3-IBH-E2424			
		3-IFA-E2431			
		3-IFB-E2432			

The Bayesian analysis identified the best clusters at K = 3 and K = 4. A similar genetic composition was shared between NEST_1–2, and between NEST_3–5 (Figure 3); these similarities are expected because the male IAA-E1150 and the female IAE-E1053 that hatched in the NEST_1 and NEST_3 reproduced with the respective breeding mates in the NEST_2 and NEST_5, respectively.

The factorial analysis of correspondence (FCA) produced similar results to the main proximity in the plot of NEST_1–2 and NEST_3–5 (Figure 4).
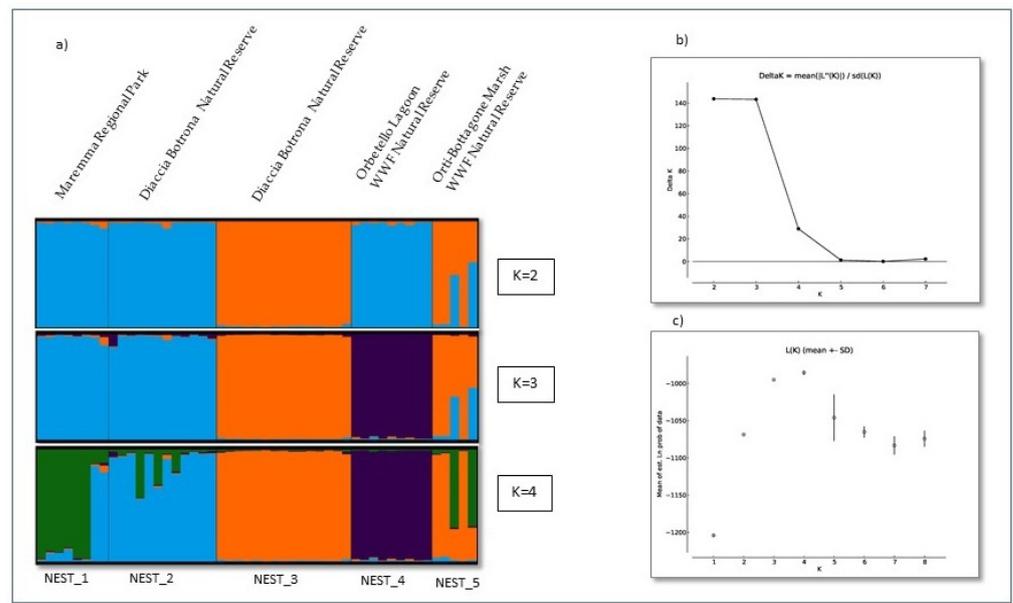
Figure 3. Results from the Bayesian analysis conducted in POP_2. Following the delta K procedure, the best clusters were described at K = 3 and K = 4. Genetic components reflect what is known about the two chicks hatched in NEST_1 after the male replacement, IAA-E1150 that originated in NEST_1 and reproduced in NEST_2, and IAE-E1053 that was hatched in NEST_3 and reproduced in NEST_5. (**a**) Bar plots at K = 3, K = 4 and K = 5; (**b**) delta K and (**c**) mean likelihood curve to identify the best K.
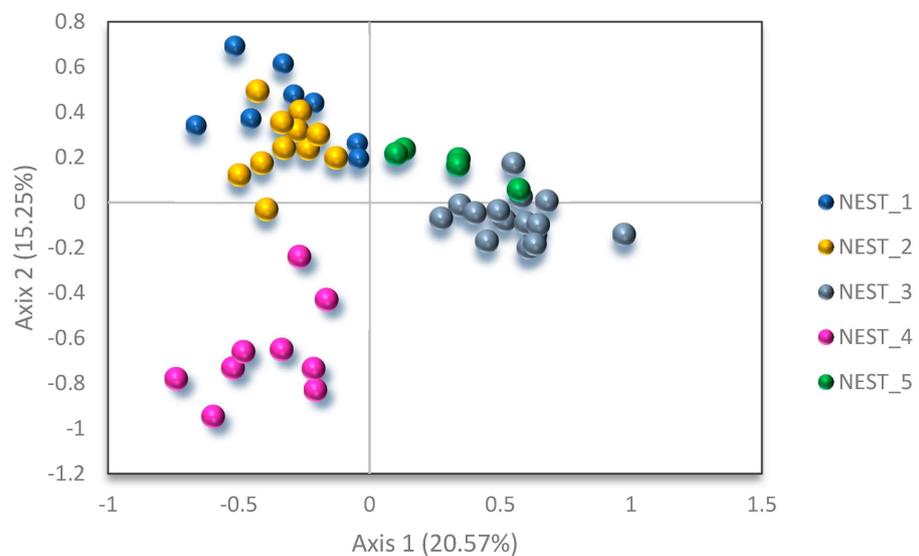
Figure 4. Results from the factorial correspondence analysis conducted in POP_2. In the plot, the proximity of NEST_1 with NEST_2 and of NEST_3 with NEST_5 is evident.

4. Discussion

The multidisciplinary and integrated long-term monitoring approach of our osprey reintroduction project in central Italy proved to be essential to clarify the dynamics of the new population. It provided relevant insights from different disciplines and fields of competence. After recording the behavior of the breeding pairs in the nesting sites with video cameras, accompanied by field survey and satellite tracking, it was possible to obtain information about egg laying and chick fledging, and, remarkably, to achieve relevant information on the genetic pattern and family relationships of the reestablished population in the long term, ultimately obtaining a reliable evaluation on the success of the project.

4.1. Genetic Variability

We analyzed the DNA of 49 individuals from the reestablished population (POP_2) that included 46 individuals hatched from the reproductive pairs, an unringed adult female of unknown origin that settled in the area in 2015 and the two translocated individuals that, once adults, settled in the area and reproduced in 2011 and 2014, respectively. The levels of genetic variability from this sample set (POP_2) did not differ significantly from those recorded in the released populations (POP_1) made up of the 33 translocated young individuals. The values of allele number, allele richness and observed heterozygosity were slightly lower than those retrieved in a recent investigation carried out on Welsh ospreys (probably originating by a wider pool of mixed founding populations of Scottish and Northern European ancestry) by Skujina and colleagues [21] that used a combination of the panel markers from two former studies [23,47], but higher with respect to the values observed in Monti and colleagues [23], in which only a single primer panel was used. Since the comparable combination of the two marker sets used in Skujina et al. [21] and in the present study, the lower diversity levels in Italian populations found in this study can be considered a real biological difference and excludes this being merely a consequence of different marker choice.

A modification of genetic variability has been recorded in several raptor reintroduction projects as a consequence of the reintroduction programs ([64] in *Gyps fulvus*; [65] in *Gypaetus barbatus*; [66] in *Falco naumanni*; [67] in *Falco punctatus*). In 2011, Jamieson [12] conducted a study dealing with the reintroduction of four different monogamous bird species, focusing on the effects produced by a limited number of individuals released into the wild. The main derived issues described in that study regarded inbreeding and loss of genetic variability, which seemed worsened by the presence of a skewed sex ratio of founders (see also [68]). In our study, notwithstanding the deviated sex ratio in the released individual pool, we did not note a further reduction in genetic variability. This mitigated effect might be explained by the recorded natural integration of the reproductive stock with wild individuals coming from surrounding colonies (e.g., a non-translocated Corsican female (CAM-BS15574) who naturally set in the NEST_4, as well as other unringed adults of unknown origin that actively contributed to reproduction in other nests; Figure 2). Reintroduction programs with a high number of released animals seem to be less sensitive to changes in genetic variability ([69] in *Falco femoralis septentrionalis*). However, respecting the genetic origin and evolutionary history of the reestablished population it is also extremely (or even more) important, and the selection of the source populations is crucial to provide individuals for translocation that are genetically as close as possible to the original population. Post-release monitoring of the genetic variability allows to record and foresee any sights of inbreeding and reduced variability, informing on the need to release additional individuals [64].

Although we did not find significant differences in variability indices between the two project phases, we recorded a departure from Hardy–Weinberg equilibrium in POP_2. However, these effects could have been amplified by the absence of all the adults in the genetic analysis. Moreover, if considering the presence of private alleles found in POP_1, it can be assumed that the departure from Hardy–Weinberg equilibrium might be a biased consequence of a misrepresented population and uncomplete allele and genotype frequencies in the HWE computation, other than the consequences determined by the founder effect.

4.2. Genetic Structure

Although a slight sign of the founder effect was recorded in the reestablished population, the AMOVA identified that 98% of the variance was distributed within individuals, 2% among populations and 0% among individuals. However, Bayesian analysis also showed divergent genetic compositions in the two different time samplings. In Figure S3, it is evident from the bar plot that the main components are different before and after the first reproduction event. This result could be explained by the fact that only two of the translo-

cated individuals were able to successfully reproduce in the study area and contribute to the genetic composition. At this point, it is not possible to affirm whether this success is attributed to a random chance or better adaptative genomic variants of these breeding individuals. The FCA plot referring to the POP_2 in the lowest part of the graph (Figure S4) and the color patterns in the Bayesian plot at K = 4 (Figure S3) also suggest a variation in the frequencies of genetic components with respect to POP_1. Again, this agrees with the contribution in the breeding pairs of new individuals different from the released stock, likely originating from the closest surrounding colonies.

Two studies [23,31] identified the presence of genetic differentiation between the Mediterranean and Central European osprey populations. When the osprey from Northern Europe was compared with genotypes from this study, both allele composition and the FCA plot confirmed a strong differentiation with respect to the Mediterranean samples. This aspect underlines the importance of considering the gene pool in any reintroduction project to hinder the release of genetically maladapted individuals in specific habitats and belonging to populations that have gone through different evolutionary histories (e.g., [70,71]). The lack of structure in this study between pre- and post-released populations accounts for the correctness in identifying the source of the population to be released [32]. Following the IUCN guidelines, the Corsican populations belonging to the Mediterranean clusters were used as a source, enhancing the chances of creating a new population adapted to the local climate, thus saving the genomic adaptive variants generated by the different evolutive lineages.

The lack of a genetic structure and gene differentiation accounts also for the presence of gene flow between restocked and neighboring populations, ensuring connectivity between colonies, and hampering inbreeding incidence in the newly reestablished population.

4.3. Family Relationships

Family relationships obtained in Colony matched the observations by video cameras and those recorded through sightings of the ringed birds. Interestingly, however, the low genetic variability did not allow for the assessment of the known direct relationships; the genetic protocol was able to identify the correct full sibling through the years without using preliminary information. This aspect is relevant when considering its application in the monitoring of wild populations or in the choice of source individuals for reintroduction. Genetic analysis and parentage analysis clearly identified the substitution of a male in a breeding pair after the departure of the previous one, showing the power of being also informative in the discovery of any potential entry of new breeders into the population or extra-copulation events.

Osprey is described as a strictly monogamous species; however, cases of extra-pair copulation events have also been recorded (e.g., [44,45]). In our study, video cameras recorded several extra-pair copulation attempts of at least a male and a female, but the genetic analysis excluded the occurrence of a successful extra-copulation event and confirmed the full-sibling relationships. The role of these behaviors, therefore, remains doubtful. A possible explanation could be that attempts of extra-pair mating potentially inform about the good quality of an individual as a partner, but do not necessarily result in a change of the partner, or even a mechanism of sperm competition as a part of sexual selection. A study in Sweden [45] identified a positive correlation between extra-copulation incidence and population density, and this matches with an observation in Minnesota [43], which ascertained 14 events of extra-pair copulation during the behavior recording of marked birds in 306 occupied nests. The presence of different mating behavior observed by video cameras in the absence of successful extra-copulation events in our study suggests continuing observations to verify any further occurrence in the presence of a future demographic increase.

5. Conclusions

This project represents one of the longest monitoring projects dealing with the conservation of the osprey in Europe carried out with an integrated approach.

The first successful breeding event was recorded 5 years after release started, mostly due to the average age at the first known successful nest which, in this species, is ca. 4.39 years for males and 3.64 years for females [43]. These times are in line with those of other projects: in the Twin Cities project (Minneapolis–Saint Paul Minnesota, USA), 143 birds were released between 1984 and 1995 and the first breeding success took place after 4 years [72]; in the Pocono project (north-eastern Pennsylvania, USA), 111 birds were released from 1980 to 1986 and the first breeding success took place after 6 years [73]; at Rutland Water Natural Reserve (Rutland, England;), 64 birds were released from 1996 to 2001 and the first breeding success took place after 5 years [74]. The notable feature of this project is to have taken into strict account the phylogenetic histories of the species, by referring to a source population whose genetic pattern and migratory strategies are most likely better representative of the extinct genetic pool of the osprey populations in central Italy [32].

The video camera monitoring carried out over the long period allowed us to describe breeding and mate behavior in the period starting from osprey releasing into the wild to the first nest settlement until now, in which five nests have been occupied and 56 chick births have been recorded, of whom 46 have been also genotyped.

The monitoring of the genetic variability in the long term has been possible by carrying out an annual sampling of the new chicks and the optimization of a marker panel able to maximize the genetic variability in the two osprey populations. This panel has been demonstrated to be useful also in describing the genetic variability of Northern osprey populations [21,23,47], so it could be considered for future genetic monitoring of other European populations and for evaluating connectivity or differences in the genetic variability.

Mating habits have been demonstrated to be driven by MHC genes [75–77], although some studies were not able to clearly describe this association [78,79]. The analysis of these genes could verify the presence of any association and, eventually, explain the mating behavior in the populations. Moreover, as osprey is described also as a migrant species, the analysis of the gene responsible for migrating behavior over the long distance (gene clock [80,81]) could contribute also to investigating individual migrating habits with respect to the origin population and other Northern populations. The analysis along the period of the project would also permit verification of any modified variability at these genes so as to explain any mutated behavior due to the reintroduction or in response to climate changes.

Integrated information and monitoring approaches can be crucial for the management of remnant populations or for the reconstitution of extinct populations so as to recreate interconnected viable populations, mainly in the Mediterranean basin, where past anthropogenic factors have caused the important demographic decline of the species.

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G.S.; supervision, N.M., F.M. and A.S.; project administration, A.S. and N.M. All authors have read and agreed to the published version of the manuscript.

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