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First genetic survey on the Montagu's Harrier *Circus pygargus* breeding population in Italy

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ABSTRACT

Investigating genetic variability and differentiation in target populations of conservation concern is pivotal, as poor genetic variability may further threaten their survival and persistence over time. Here we present the first genetic data for the Italian breeding population of the Montagu's Harrier *Circus pygargus*, a declining migratory raptor species typical of steppe and agricultural landscapes, ranked as vulnerable at the national level. We analyzed four mitochondrial genes, the cytochrome oxidase gene (*COXI*), control region (*D-loop*), 16S RNA subunit gene (*16S*), and NADH dehydrogenase 2 gene (*ND2*), in six individuals from central Italy to estimate their phylogenetic relationships with European populations and assess local genetic variation. We recorded low genetic nucleotide diversity ($\pi = 0.0009$), but relatively high haplotype diversity ($h = 0.8$) compared to Central European populations and a close phylogenetic relationship of Italian Montagu's Harriers with both western and eastern European populations. This study can serve as a baseline to study genetic variability and conservation of this vulnerable raptor species in Italy.

Keywords: Accipitridae, breeding population, *Circus pygargus*, migration, mitochondrial DNA.

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INTRODUCTION

Assessing genetic variability in target populations of conservation concern is important to detect limited genetic variation (e.g. Frankham et al. 2002). Low genetic variability can further threaten the survival and persistence of populations already affected by anthropogenic threats or in decline (e.g. Lande 1998), making their conservation even more challenging. Conservation efforts should therefore focus on reducing both direct and indirect human threats while promoting the maintenance of genetic variability and structure among populations. Raptors represent one of the most threatened groups of birds (Buechley et al. 2019), with 52% of global species in decline (McClure et al. 2018). Among them, migratory populations are especially vulnerable (Bildstein 2006; De Pascalis et al. 2020), mainly because of the high mortality they often experience during hazardous long-distance migratory journeys (Strandberg et al. 2009; Klaassen et al. 2014).

The Montagu's Harrier (*Circus pygargus*) is a long-distance migratory raptor species typical of steppe habitats and open agricultural landscapes, which is declining across its range because of habitat loss and fragmentation (Corbacho et al. 1997; Butet & Leroux 2001) and other human-related threats (De Pascalis et al. 2020). It breeds throughout most of the Western Palearctic and part of the Maghreb in North Africa (Garrido et al. 2021) and extends as far East as Central Asia (BirdLife International 2024). In the Western Palearctic, the largest concentration of Montagu's Harriers occurs in Spain and France (e.g. Soutullo et al. 2006), accounting for approximately two-thirds of the entire European population (excluding Russia: Simmons 2000). Within this region, 45–90% of breeding pairs utilize cultivated crops as their nesting grounds. Being a ground-breeding species, it is highly susceptible to impacts arising from harvesting activities and land-use changes (Simmon 2000; Limiñana et al. 2006; Gameiro et al. 2024), particularly in Europe where nearly 40% of the land area is occupied by farmlands (Rigal et al. 2023).

In Italy, the Montagu's Harrier has a fragmented distribution across several central-northern regions (Brichetti & Fracasso 2018, Ravasini 2022; Fig. 1). Breeding nuclei are present in Emilia Romagna, Tuscany, northern Latium and the hilly areas of western Marche (Morelli et al. 2012, Ravasini 2022). Other breeding pairs have also been reported in the Po plain and North-Eastern areas, whereas it is more localized and/or with discontinuous breeding in Piedmont, Sardinia and Apulia (Ravasini 2022). At the country level, the breeding population is estimated at 260 to 380 breeding pairs (Ravasini 2022) and is ranked as vulnerable (VU), due to the reduced number of mature individuals and the presence of threats, such as those linked to cereal and fodder threshing operations, destroying nests (IUCN RedList - Rondinini et al. 2022).

Previous satellite telemetry studies have identified three main migratory routes at the Afro-Palearctic scale, with Italy crossed by the central route (Trierweiler et al. 2014, Schlaich & Klaassen 2021). Rutkowski et al. (2014) provided a preliminary analysis of genetic variability for Spanish and Polish Montagu's Harrier populations using molecular markers and found a low but significant level of genetic differentiation between these two populations. Successively, Rutkowski et al. (2015) analyzed part of the mitochondrial control region in 158 individuals from Central Spain, Germany, the Czech Republic and Poland and observed high genetic diversity in the European breeding population of Montagu's Harrier. This finding aligns with the species' extensive distribution range, low level of philopatry and high dispersal capabilities (Limiñana et al. 2012; but see Janowski et al. 2018). Garcia et al. (2011) recorded the highest genetic variation in Southern Europe, i.e., where migration routes of individuals from different northern European countries concentrate. No genetic information is available yet from Montagu's Harrier populations breeding in Italy.

As low levels of genetic variability within a population may threaten its survival and viability over time, understanding the level of genetic variation represents an important useful basis for planning effective conservation measures (e.g. Monti et al. 2018). This study aimed to provide the first baseline genetic data on the Italian Montagu's Harrier breeding population. We analyzed fragments of four mitochondrial genes: the cytochrome oxidase gene (*COXI*), control region (*D-loop*), 16S RNA subunit gene (*16S*), and NADH dehydrogenase 2 gene (*ND2*), in six samples from the breeding population of Central Italy (Tuscany), an area located in the middle of the central migratory route at the Afro-Palearctic scale (Schlaich and Klaassen 2021). We also investigated the phylogenetic relationship of Italian Montagu's Harriers with other European populations and assessed the genetic variation at the local level as a starting point for further sampling campaigns.

MATERIALS AND METHODS

Study area and population monitoring

In Tuscany, the breeding population of Montagu's Harrier is estimated at 50-100 pairs (thus 19.2-26.3% of the entire Italian population), with strongholds mainly located in the Pisan mountains in the north and the hill areas of the province of Grosseto in the south (Tellini Florenzano et al. 1997; Ravasini 2022). Our study was carried out in the latter area (Fig. 1a).

Starting from sites of known historical presence (e.g. Tellini Florenzano et al. 1997), suitable areas for the species were checked from 0 to ca. 1500 m above sea level, from mid-April to mid-late September, between 2020-2023. Further information on the presence of the species was obtained through citizen science records (e.g. www.inaturalist.org; Accessed on 08 March 2020). When the presence of a harrier was recorded, we checked on the area through repeated daily stakeouts (by binoculars

Figure 1. Montagu's Harrier breeding range in Italy and study area in Tuscany. a) The species' breeding range (light yellow) (BirdLife International 2022; IUCN 2023); the shaded grey area shows Tuscany (regional administrative boundaries), while the red triangle is for the study area location within the region. b) Unatched eggs from the same nest found after nest abandonment in 2022, in the province of Grosseto (Tuscany). c) Flight interaction between a breeding male and a juvenile in the proximity of a nesting site in Grosseto's District. All pictures by Marco Brandi ©.

©Leica - Geovid R 10×42) with a minimum hourly interval of four hours, to confirm the utilization of the area by the harriers over time. Checks were repeated twice a week for at least two months before excluding an area. Following Berger-Geiger et al. (2019), nests were located by observing the females landing at the nest site after a food pass. When nesting was ascertained (e.g. Fig. 1b), daily surveys were organized and repeated at least once a week from vantage points at a distance of > 500 m from nest sites, throughout the season (Fig. 1c). The census of the areas and the search for nest sites involved volunteer ornithologists that, overall, monitored ca. 2000 km^2 between 2020 and 2023. Eleven breeding pairs/nest sites were found (mean nest sites per year: 6.5 ± 3.6 s.d.) during the study, but only a few of them at the end of the breeding season contained samples suitable for molecular analyses (see below).

Sample collection and molecular analyses

To limit disturbance to breeding individuals, samples were collected at nest sites only at the end of the breeding season, as accessing active nests to collect a high number of samples would have been challenging and would have required specific authorizations. Biological monitoring samples, collected through non-destructive methods, were utilized to assess mitochondrial lineages and investigate levels of genetic variation at the local level. Three unhatched eggs were found and collected from the same nest that was abandoned in July 2022. Only one of these eggs (randomly chosen) was used for subsequent analyses. After collection, eggs were kept at -20 °C until lab analysis. Five shed feathers from different nests were found and stored in 96% ethanol in 15 ml tubes at room temperature until analyses. In addition, a road-killed individual was found in the surroundings of a territory in 2023 (Tab. 1), and feathers were kept and stored in 96% ethanol for being analyzed.

For the amplification of four genes, the total genome DNA was extracted from six individuals/samples of the nine collected: one unhatched egg, four feathers and one road-killed individual. These samples come from six different nests from four different localities/sites within the study area. DNA was extracted using the Qiagen Qiamp Tissue and Blood kit (©Qiagen, Inc, Tokyo, Japan). For feathers, we extracted DNA from blood clots as remnants of the axial artery (when available; Proctor & Lynch 1993, Horvàth et al. 2005) or from calamus tips. As for the unhatched egg, the DNA of the adult breeding harrier was extracted by scraping the outside of the egg's shell with a razor blade. After DNA extraction, the 5'end of the mitochondrial DNA control region (hereafter, *D-loop*), cytochrome oxidase I (*COXI*), 16S RNA subunit (*16S*), and NADH dehydrogenase 2 (*ND2*) genes were amplified using primers already used for Montagu's Harriers and other birds (Hebert et al. 2004; Spicer and Dunipace 2004; García et al. 2011): CIRCRF (5'-CACTAACCGGAGCCCTAGAA-3') and CIRCRR (5'-CATATGTGAGGGCCACCTGT-3') for D-loop, BirdF1 (5'- TTCTCCAACCACAAAGACATTGGCAC-3') and BirdR1 (5'-

Table 1. Samples of *Circus pygargus* used for this study and collected in the Grosseto District (Tuscany). Acc N: Genbank Accession Number

Collection year	Location	Acc N (COXI)	Acc N (ND2)	Acc N (16S)	Acc N (D-loop)
2021	Site1 – [feather]	OR523521	PQ238359	PQ238358	PQ238361
2021	Site1 – [feather]	OR523526	PQ238359	PQ238358	PQ238360
2021	Site2 – [feather]	OR523520	PQ238359	PQ238358	PQ238360
2022	Site2– [feather]	OR523724	PQ238359	PQ238358	PQ238360
2022	Site3 – [egg]	OR523594	PQ238359	PQ238358	PQ238360
2023	Site4 – [road- killed]	OR523522	PQ238359	PQ238358	PQ238360

ACGTGGAGATAATTCCAAATCCTGG-3′) for COXI, 16SAR (5′- CGCCTGTTTATCAAAAACAT-3′) and 16SBr (5′-CCGGTTTGAACTCAGATCACGT-3′) for 16S, H6313 (5′-ACTCTTRTTTAAGGCTTTGAAGGC-3′) and L5758 (5′-GGCTGAATRGGMCTNAAYCARAC-3′) for ND2. PCR reactions were carried out with an Applied Biosystems 2700 thermal cycler in 25 µl mix, including 100 ng of each DNA sample, buffer 10X, 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, and one unit of Taq polymerase (©Life Technologies, Waltham, Massachusetts, USA). PCR conditions included initial denaturing at 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 sec, annealing at 50 °C (*COXI*), 51 °C (*16S* and *ND2*) and 53 °C (*D-loop*) for 30 sec, extending at 72 °C for 1 min, and a final extension to 72 °C for 10 min. PCR products were run by electrophoresis on 1.5% agarose gel, containing 0.5 mg/ml of SYBR gel staining. PCR amplifications were then purified (ExoSAP-IT PCR clean-up Kit, ©Applied Biosystems, Foster City, California, USA) and sequenced through the Sanger method at the BMR Genomics (https://www.bmr-genomics.it/, Padua, Italy). Electropherograms were displayed on the Chromas 1.45 software (http://www.technelysium.com/au). Sequences were manually corrected and analyzed with the Mega XI software (Tamura et al. 2021).

The dataset analyzed for Montagu's Harrier is shown in Tab. S1. (Supplementary Electronic Material). Nucleotide diversity, haplotype diversity, and number of polymorphic and parsimony-informative sites were computed through DNAsp vers. 5 (Librado & Rozas 2009). Nucleotide pairwise genetic divergences were calculated for *COXI* data using K2P distances and including populations of *C. pygargus* from other European regions (Tab. 2). Median-joining networks were built up for *C. pygargus* Italian haplotypes using all genes (*16S+D-loop+ND2+COXI*) and for the entire dataset including *C. pygargus COXI* Italian sequences and all available on GenBank, through the TCS software (Clement et al. 2000) based on Maximum Parsimony and graphically visualized by TCBuntu (dos Santos et al. 2016). JModelTEST 304 (Posada & Crandall 1998) was used to test the most accurate model of substitution using the Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC), corrected for the heterogeneity between sites (gamma) (Schwarz et al. 1978). Sequences obtained in this study were aligned with previously published Montagu's Harrier genetic sequences available in GenBank (http://www.ncbi.nlm.nih.gov). Since Genbank sequences of *Circus* spp. comprising *COXI+16S+Dloop+ND2* are very few (four mitochondrial whole genomes, which did not include *C. pygargus*), a separate dataset involving all *Circus* sequences available in Genbank was created. A phylogenetic reconstruction Bayesian (hereafter, BI) analysis was performed separately using only the *COXI* and the *D-loop*, given that they were the most represented genes on GenBank for *C. pygargus*. The BI analysis was performed with MrBayes v.3.12 (Ronquist & Huelsenbeck 2003), using the best model selected for the two genes separated. We used the Markov chain Monte Carlo (MCMC) theory approach in BI, running 10,000,000 generations, drawing one sample every 1000, running four chains per analysis, and removing the top 25% of the data with the other parameters as the default. The results were checked using Tracer 1.7.2 (Rambaut et al. 2018). We obtained effective sample size (ESS) values > 250 for both genes. We inferred the phylogenetic relationships of the haplotypes related to this study with sequences of all the other *Circus* species obtained from Genbank. Related accession numbers are reported in the phylogenetic trees. Other *C. pygargus*, Western Marsh-Harrier *C. aeruginosus*, Hen Harrier *C. cyaneus*, Long-winged Harrier *C. buffoni*, Swamp Harrier *C. approximans*, Eyles's Harrier *C. teauteensis* (extinct) and Pallid Harrier *C. macrourus*, were included. The Eurasian Sparrowhawk *Accipiter nisus* was used as an outgroup (Tab. 1).

RESULTS

The *D-loop*, *16S*, *ND2* and *COXI* gene portions (2089 bps in total, five polymorphic sites, five parsimony-informative sites, four for *COXI* and 1 for *D-loop*) for the six samples showed four haplotypes (Tab. 1; Fig. 2). The nucleotide diversity (π) for the whole 2089 bp fragment was 0.0009 and the haplotype diversity (*h*) was 0.800. The *ND2* and *16S* genes were monomorphic in our population (Accession numbers: PQ238358- PQ238359). Calculating the genetic diversity for the two other genes, *COXI* and *D-loop*, for Italian Montagu's Harriers was *h* = 0.948 and π = 0.002 for fragment *COXI* while it was *h* = 0.4 and π = 0.0009 for *D-loop*. Considering all available haplotypes, the nucleotide diversity of *C. pygargus* in Europe (Italy excluded) was similar to Italy for *COXI* (*h* = 0.985, π = 0.002) and quite higher for *D-loop* (h = 1, π = 0.004). The average number of nucleotide differences among haplotypes was low (k = 1.47, Tab. 1). The K2P distances were low for all pairwise comparisons, also between different described *Circus* species (see between *C. aeruginosus* and *approximans*); the distances among different Italian localities/sites were 0.2-0.3%. Genealogical relationships shown in the net-

Table 2. Mean K2P pairwise genetic among Montagu's harrier populations and *C. approximans* and *aeroginosus* for an interspecific comparison. Rate variation among sites was modelled with a gamma distribution (shape parameter = 1). There was a total of 626 positions in the final dataset

Circus	***aeruginosus***	***approximans***	**pygSWEurope**	**pygNEEurope**
approximans	0.9%			
Pygargus SW Europe	6%	6%		
Pygargus NE Europe	6%	6.6%	0.5%	
Pygargus Italy	6%	6.5%	0.5%	0.5%

works confirmed the close relations between the different localities/sites of South Tuscany, except for the second haplotype found in Site 1 (red circle on the right) that was more separated from the other Italian samples (Fig. 2a). In the *COXI* network including other European sampling localities, the Italian haplotypes are in close relationships with the other SW European samples sharing the same haplotypes with these populations (Fig. 2b).

Haplotypes showed the same relationships in the phylogenetic reconstructions. Montagu's Harriers from Central Italy were strongly phylogenetically related to other populations from Southern Europe. All *C. pygargus* sequences from Europe

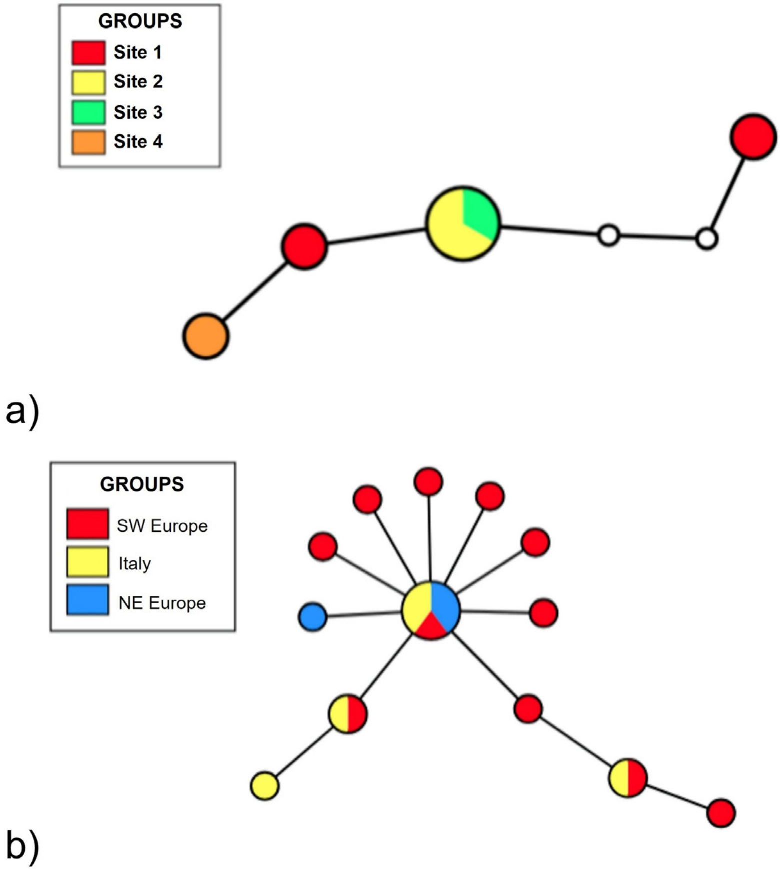

Figure 2. TCS Parsimony Network showing the relationship among a) all mitochondrial (16S+*D-loop*+*ND2*+*COXI*) haplotypes of *C. pygargus* and b) *COXI* data including other available sequences of *C. pygargus* named as in Garcia et al. (2011). Circles represent different haplotypes. Circle size is proportional to the number of samples for each haplotype. Median vectors, which represent extant either unsampled or extinct ancestral sequences, are indicated by white circles.

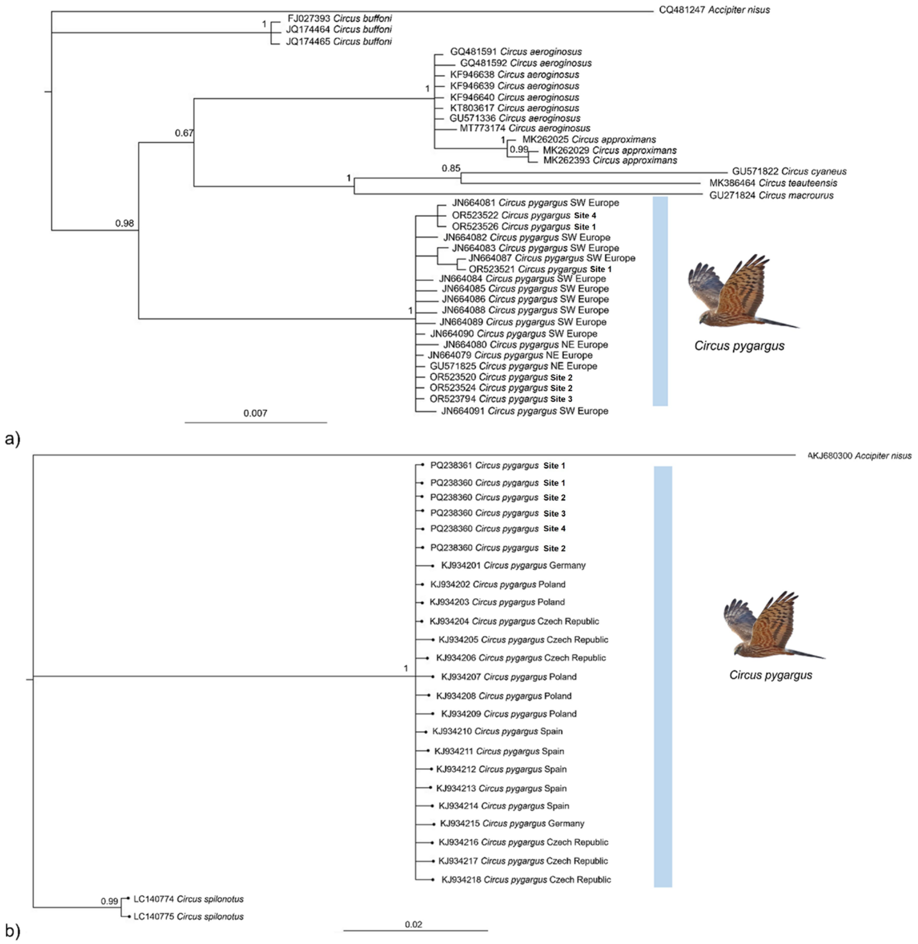

Figure 3. Phylogenetic relationships within the *Circus* species, estimated by a) *COXI* (633 bp) and b) *D-loop* (526 bp) sequences. Posterior probabilities (BI MCMC=10,000,000) are indicated at each node. The blue band indicates the clade *Circus pygargus*. Sequences from this study are red framed. Image of the Montagu's Harrier is by Wikimedia Commons: CC-BY-SA-3.0.

formed a unique highly supported monophyletic clade (Fig. 3).

DISCUSSION

The nucleotide diversity found in this study was lower than that usually reported for both threatened and non-threatened birds (0.001-0.006; e.g., Bartolomé et al. 2011, Arbabi et al. 2014, Petit-Marty et al. 2021), including *C. pygargus* (García et al. 2011; Rutkowski et al. 2015), for all genes. The only exception was for the *COXI* marker which showed similar values throughout all of Europe. However, pair haplotypes differed by a single substitution, on average, as found for other Montagu's Harrier populations (Rutkowski et al. 2015). This indicates that there is little variation in the population at the nucleotide sequence level among the sampled individuals. This could be in line with the species' vulnerable status nationwide and the multiple human-related threats currently affecting its populations. On the other hand, however, despite the low nucleotide diversity, haplotype diversity was relatively high in our sample, indicating some variability among them at the population level, similar to values of other European populations (e.g. García et al. 2011; Ruktowski et al. 2015). Despite the small sample size (six) preventing any strong conclusion, these preliminary data suggest that Central Italy is an admixture of different haplotypes of Montagu's Harrier, which may contribute to the genetic variability recorded, comparable with Spanish and French populations. In this sense, our preliminary results may indicate that the Montagu's Harrier population of Central Italy could be an important genetic resource for the species. Different not-mutually exclusive hypotheses may explain such variability: in the Holocene, Southern Europe (including Italy) was mostly characterized by open areas, while most of the rest of Europe was covered with forests (Mitchell et al. 2005). This may have secured remnant populations of *C. pygargus* in these southern regions, contributing to the preservation of their genetic pool (e.g. García et al. 2011). At the same time, the species is known to have a high natal dispersal capacity (for both sexes) potentially favouring high genetic mixing between populations (Limiñana et al. 2012). For example, in a review of five tagging programs in different sites in continental Spain, the percentage of philopatric individuals was lower than 5% (Limiñana et al. 2012). Furthermore, the Italian breeding population is in the middle of one of the main migratory routes at the Afro-Palearctic scale, which is mostly used by central-northern and central-eastern Montagu's Harriers (Schlaich & Klaassen 2021), during both autumn and spring migration (García & Arroyo 1998; Schlaich et al. 2017). This may promote encounters between individuals from even distant populations. All these points probably contribute to explaining the co-presence of genetically different individuals of both Western and Eastern origin.

In contrast, the low nucleotide diversity found would indicate genetically limited variability at the sequence level, thus potentially worrying from a conservation point of view for this population. In the future, polymorphic nuclear markers, possibly on a larger sample size, should be considered together with current results, to deepen knowledge of the current genetic structure of this and other Italian breeding nuclei.

The genetic variability observed in this study suggests a potential for local breeding population increase if good management strategies could be applied. As Tuscany hosts relatively high concentrations of confirmed breeding occurrences compared to other parts of the country (Ravasini 2022), this population may be highly important for the conservation of this threatened species not only for its size but also from a genetic point of view.

This study provides a preliminary baseline for addressing the challenges related to genetic variability and conservation of the Montagu's Harrier. The Italian Red List classified *C. pygargus* as "Vulnerable" both in 2013 and 2022, suggesting that its conservation status has not improved in the last ten years (Rondinini et al. 2022). The continuing population decline, the decrease in natural habitats and the mechanization of agriculture call for alternative agricultural practices and urgent increased conservation efforts for securing the long-term preservation of this declining ground-nesting species.

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Conflicts Of Interest

The authors have no competing interests to declare that are relevant to the content of this article.

Author Contributions

Conceptualization, E.G. and M.Br.; methodology, E.M., A.V. and M.Ba.; software, M.Ba.; validation, E.G. and M.Ba.; formal analysis, M.Ba.; investigation, E.G. and M.Br.; resources, M.Ba.; data curation, E.M.; writing-original draft preparation, E.M.; writing-review and editing, E.M, E.G. and M.Ba; visualization, M.Ba.; supervision, M.Ba.; project administration, E.G.; All authors have read and agreed to the published version of the manuscript.

Ethics and Animal Welfare

Authors certify that no living animal was used for this research.

Data Availability Statement

All sequences used in this work have been uploaded to GenBank (accession numbers are reported in Tab. 1 and Tab. S1) (Supplementary Electronic Material).

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