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Genomic Insights Into the Origin, Decline and Recovery of the Once Critically Endangered Iberian Lynx

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ABSTRACT

The Iberian lynx was at the brink of extinction by the year 2000 but has since then, and thanks to intensive conservation measures, gone through a remarkable recovery, providing a much-welcomed and encouraging conservation success story. Genetic issues have probably contributed to the decline in the past, and the genetic management of inbreeding and genetic diversity is likely contributing to its recent recovery. The species was an early adopter of genetic and genomic approaches, and the combination of an extreme decline, an intensive monitoring and management programme and extensive genomic resources and data makes the Iberian lynx an excellent model for conservation genomics. Here, we review how genetic and genomic data have contributed to the knowledge of the species evolutionary and demographic history, the evaluation of the genetic status of the species through time, including historical and ancient data, and how this information has prompted and guided conservation actions. In the process, genomics provided valuable insights into the dynamics of functional variation in bottlenecked populations and the consequences of intraspecific and interspecific admixtures. In more applied terms, the species is subjected to an ambitious genetic monitoring and management programme, covering captive, remnant and reintroduced populations, which has succeeded in improving the genetic status of the species and thereby contributed to its recovery. Current genomic work aims at expanding these contributions with novel genomic resources and data while capitalising on extensive demographic and genealogical data provided by the ongoing non-invasive genetic monitoring programme.

1 | Introduction

The Iberian lynx, *Lynx pardinus* (Temminck, 1827), was considered the most endangered felid and was classified as 'Critically Endangered' in the IUCN red list by the end of the 20th century, becoming an enormous challenge for species conservation in Europe (Cat Specialist Group 2002; Nowell et al. 1996). Habitat loss, direct persecution and prey decline have been identified as the main factors driving the species to the brink of extinction.

The implementation of ambitious in situ and ex situ conservation measures since the early 2000s avoided an imminent extinction and promoted a spectacular recovery. The latest census (2023) reports over 2,000 individuals in the wild distributed among remnant and reintroduced populations in Spain and Portugal, with six populations hosting more than 100 individuals (https://www.miteco.gob.es/content/dam/miteco/es/biodiversidad/temas/inventarios-nacionales/lince/censolinceiberico2023.pdf) (Figure 1). The current demographic status and trend prompted its reclassification as 'Vulnerable' in the latest Red List assessment (Rodríguez 2023) and its inclusion in the first Green List assessment as Largely Depleted (Salcedo et al. 2023). The species that was once a symbol of biodiversity loss has now become an example of successful species recovery.

Such conservation success has been possible thanks to an impressive effort co-funded by the European Commission LIFE programmes and regional and national administrations in Spain and Portugal. The intensive management of the species spanning the past two decades has integrated in situ measures, addressing the main threats identified at the time (e.g., habitat loss, prey unavailability and non-natural mortality), and ex situ measures, including a successful conservation breeding programme, which in recent years has supported the reintroduction of the Iberian lynx in selected areas. As a result, the total number of individuals in the wild has experienced a 20-fold increase during the last 22 years, from less than 100 in 2002 to more than 2,000 in 2023. At the same time, the associated intensive monitoring and management efforts provided ample access to samples and auxiliary information on life history traits and fitness and created the opportunity for incorporating genetic principles and data into species management.

Genetic studies starting in the early 21st century have progressively expanded the suite of samples—from modern to historic to ancient—and genetic data—from microsatellite markers to SNPs and whole-genome sequences (WGS). The Iberian lynx was one of the first endangered species to have a draft reference genome and genome-wide variation data.

The combination of a well-documented decline that reached a near-extinction scenario, an intensive monitoring and management programme,and extensive genomic resources and data make the Iberian lynx an excellent model for conservation genomics. Here, we review how genomics contributed to the knowledge of the evolutionary and demographic history of the Iberian lynx, allowed the evaluation of the impact of bottlenecks

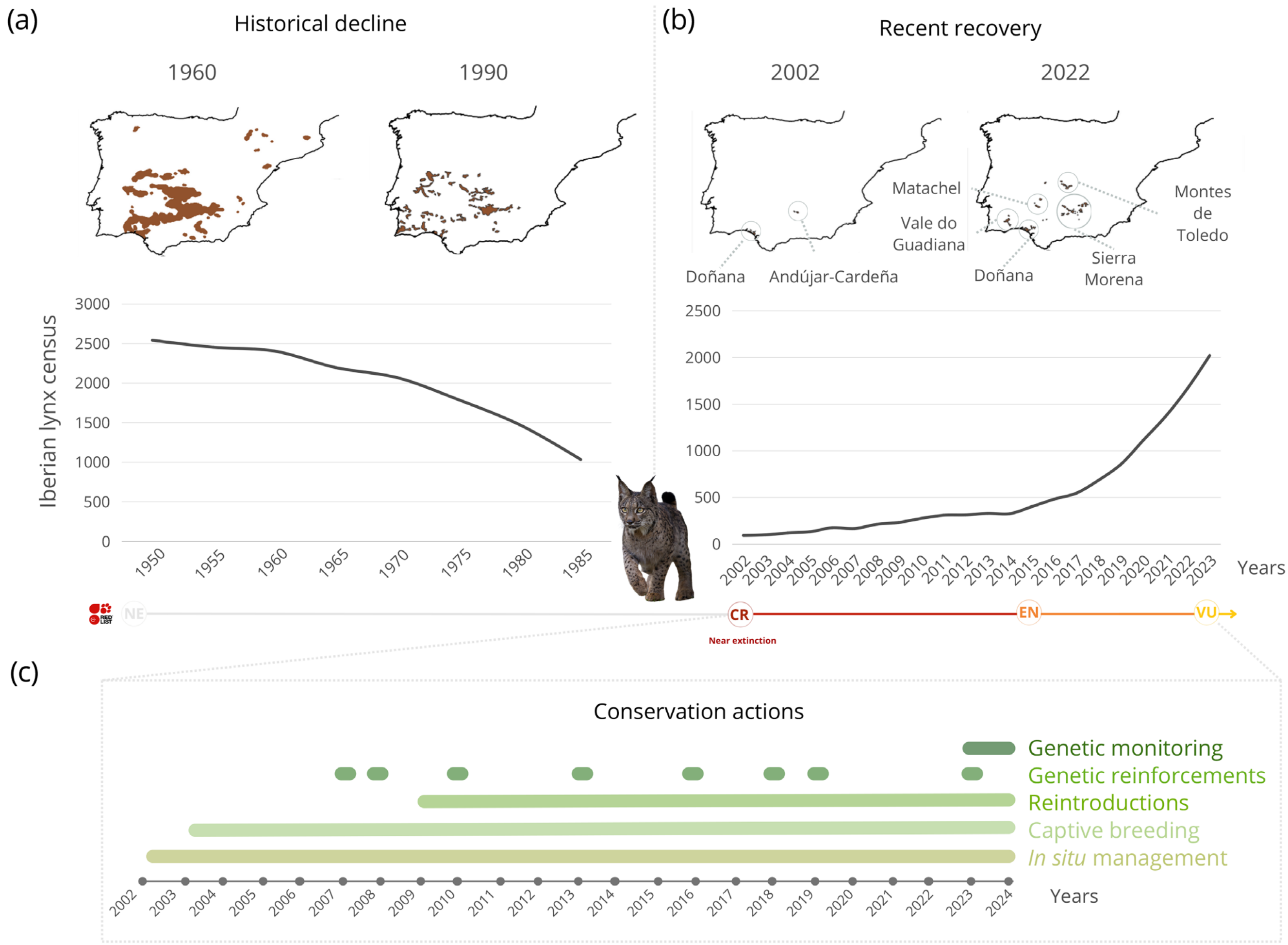

FIGURE 1 | History of the decline (a) and recovery (b) of the Iberian lynx, along with a timeline of main conservation actions (c). (a) and (b) panels show the maps of the distribution range above, and a graph of population sizes through time below, along a timeline of IUCN red list classifications. Decline data refer mostly to the Spanish part of the distribution, obviating the range in Portugal, whereas the recovery panel includes the whole species distribution.

on genomic variation, and is contributing to the ongoing species management and monitoring. We also discuss current genomic research and the prospects of additional impacts of genomics in the conservation of the Iberian lynx.

2 | Evolutionary History

The Iberian lynx is one of the four species comprising the *Lynx* genus, together with the bobcat, *L. rufus*, the Canada lynx, *L. canadensis*, and the Eurasian lynx, *L. lynx*. The history of the genus and of the Iberian lynx in particular is far from being clear, but genomic data have provided valuable insights into this issue. Paleontological studies suggest *L. issiodorensis* (Croizet and Jobert, 1828), as the ancestor of all remnant *Lynx* lineages (Werdelin 1981). The origin of the species has been placed by different authors in Africa, Europe or North America. In any case, the species was present in Europe in the late Pliocene and throughout the Pleistocene, and extended from the Iberian Peninsula to Eastern Europe and Asia. *L. rufus* might have evolved from *L. issidorensis* in North America, either natively or following its early colonisation from Asia through the Bering Strait. In Asia, *L. issidorensis* gave rise to the Eurasian lynx, a species that expanded during the Eemian interglacial into Europe and to North America, where it evolved into *L. canadensis* (Werdelin 1981). In the Iberian Peninsula, *L. issidorensis* gave rise to the Iberian lynx, with earliest fossil records of a smaller body and teeth size resembling *L. pardinus* dated around 2 million years before present (Cuccu et al. 2023) and a later earliest specimen unequivocally ascribed to *L. pardinus* dated around 1.6 million years ago (Boscaini et al. 2016, 2015). Lynx remains in southwestern Europe during the Middle to Late Pleistocene, with sizes intermediate between extant Iberian and Eurasian lynxes, have been classified either as a distinct lineage of lynx, the cave lynx, *Lynx spelea* Boule, 1919, *L. pardinus* (e.g., Boscaini et al. 2016; Mecozzi et al. 2021), or one of its subspecies (Werdelin 1981).

Although phylogenetic and phylogenomic analyses throw some light on the history of the genus, they fail to provide a definitive picture because two competing topologies are recurrently recovered. The first of these topologies (T1) places *L. pardinus* as sister species to a clade that comprise both the Canada lynx, *L. canadensis*, and the Eurasian lynx, *L. lynx*, while the second (T2) is characterised by a sister relationship between *L. pardinus* and *L. lynx*, with *L. canadensis* diverging earlier from the common ancestor of the two (Figure 2). Older analyses of fossil morphology recovered the T1 topology, inferring *L. pardinus* to have diverged from the European population of *L. issodorensis*, well before the divergence of *L. lynx* and *L. canadensis* from the Asian population of *L. issodorensis* (Werdelin 1981). Later studies based on molecular data, including both mitochondrial and nuclear DNA sequences, recovered the T2 topology, dating the divergence of *L. pardinus* from *L. lynx* at around 1.2 Mya (Johnson et al. 2006).

More recently, an extensive phylogenomic analysis of the Felidae family found T2 topology to be the more frequently retrieved, but the T1 topology to be prevalent in low recombination regions of the X chromosome (Li et al. 2019). This led the authors to conclude that T1 is the true species topology, while the more common T2 reconstructions in the rest of the genome are the result of extensive post-divergence gene flow between *L. pardinus* and *L. lynx* (Li et al. 2019) (Figure 2). This finding is consistent with the overwhelming support for a model of speciation between the two species, including a period of gene flow following divergence and extending until very recent times (CoalHMM, Abascal et al. 2016), and with evidence from ancient Iberian lynx genomes indicating introgression from Eurasian lynx occurring during the last 2,000–4,000 years (Lucena-Perez et al. 2024). A recurrent admixture with Eurasian lynx during most of the Iberian lynx history seems thus to be an important factor shaping its current genomic variation. Extensive and frequent admixture is indeed a realistic scenario, as the distributional ranges of the two species recurrently overlapped in southern France and Italy during the Late Pleistocene (Sommer and Benecke 2006) and within the Iberian Peninsula until as recently as 200 years ago (Lucena-Perez et al. 2022; Rodríguez-Varela et al. 2016). Future studies using phylogenomic data, and demographic modelling of introgression might help to shed light on the complex relationship between these two lynx species and the demographic and evolutionary consequences of admixture.

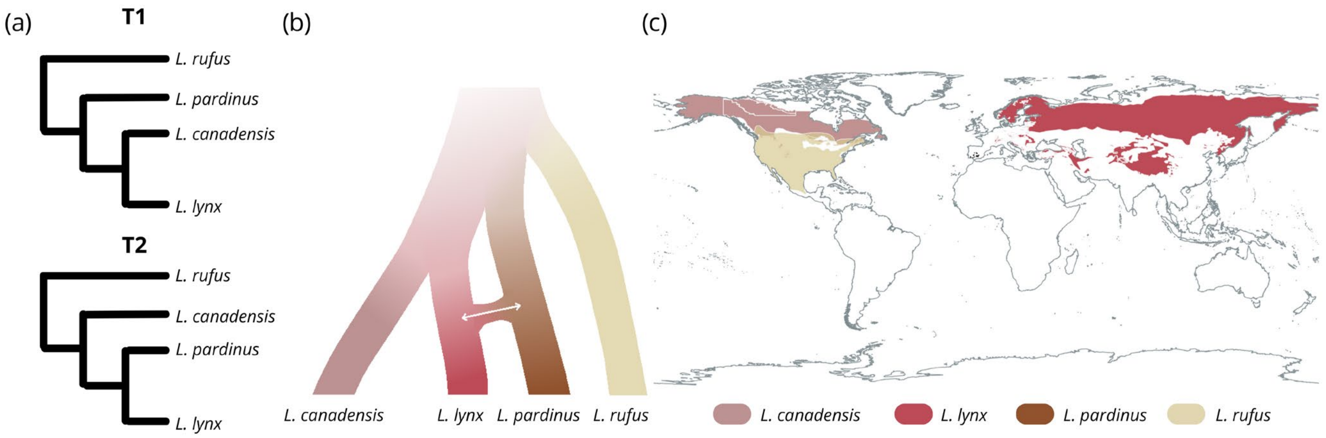

FIGURE 2 | History of the *Lynx* genus. (a) Two alternative and similarly frequent gene topologies are recovered from phylogenomic analyses, yielding conflicting sequences of divergence between *L. canadensis*, *L. lynx* and *L. pardinus*. (b) The two topologies might be reconciled by a species history involving an old divergence of *L. pardinus* followed by extensive admixture and introgression with *L. lynx*. (c) Contemporary ranges of the four lynx species (The IUCN Red List of Threatened Species. Version 2024-2).

Genomic analyses have also provided insights into the adaptive evolution of the *Lynx* genus by finding a significant expansion of genes related to sensory perception of smell and signatures of positive selection in genes involved in hearing and vision, consistent with the exceptionally acute vision and hearing attributed to lynxes (Abascal et al. 2016). Studies on adaptive divergences among lynx species are, however, lacking.

3 | Demographic History

Paleontological data suggest that the Iberian lynx was continuously present and quite common in Mediterranean climate areas of the Iberian Peninsula during the Middle and Late Pleistocene and extended to Southern France and northwestern Italy and even southeastern Italy through the late Pleistocene and early Holocene (Boscaini et al. 2016; Mecozzi et al. 2021; Rodríguez-Varela et al. 2015). Reconstructions of Iberian lynx ranges based on citations in historical written sources since the 16th to the early 20th century depict an already fragmented distribution, mostly but not exclusively restricted to the southwestern quadrant of the Iberian Peninsula (Clavero and Delibes 2013; Jiménez et al. 2018), a distribution similar to that reconstructed for the 1960s (Rodríguez and Delibes 1992). The Iberian lynx range was further contracted by 45%–81% with respect to the 1960s range by 1980, with an estimated census of 1,100 individuals (Rodríguez and Delibes 1992, 2002). The subsequent intensification of the decline and the extirpation of most subpopulations left only two isolated populations in the Doñana area and in Andújar–Cardeña by 2002, with around 100 individuals in total (Guzmán et al. 2004; Rodríguez and Delibes 2003) (Figure 1).

Consistent with historical reconstructions, microsatellite, mitochondrial and whole-genome SNP data converge to suggest a scenario of effective population sizes of only a few thousand individuals during the last 20,000 years, followed by a drastic decline to a few hundred around 300–400 years ago, predating the more recent drastic decline during the second half of the 20th century (Abascal et al. 2016; Casas-Marce et al. 2013, 2017) (Figure 3). The reconstruction of recent demography with GONE (Santiago et al. 2020) using more extensive genome-wide population data also illustrates the continuous decline of the two remnant populations during the last 100 generations (ca. 500 years), while showing the different trajectories of the two: the Doñana population being consistently smaller with around 50 individuals during the last 150 years, and Andújar–Cardeña hosting over 700 individuals until the start of its decline ca. 200 years ago, which intensified around 90 years ago (Figure 3). Overall, genomic data suggest that the combination of small long-term effective sizes and serial historical bottlenecks is the cause of the extremely low levels of genomic diversity observed today.

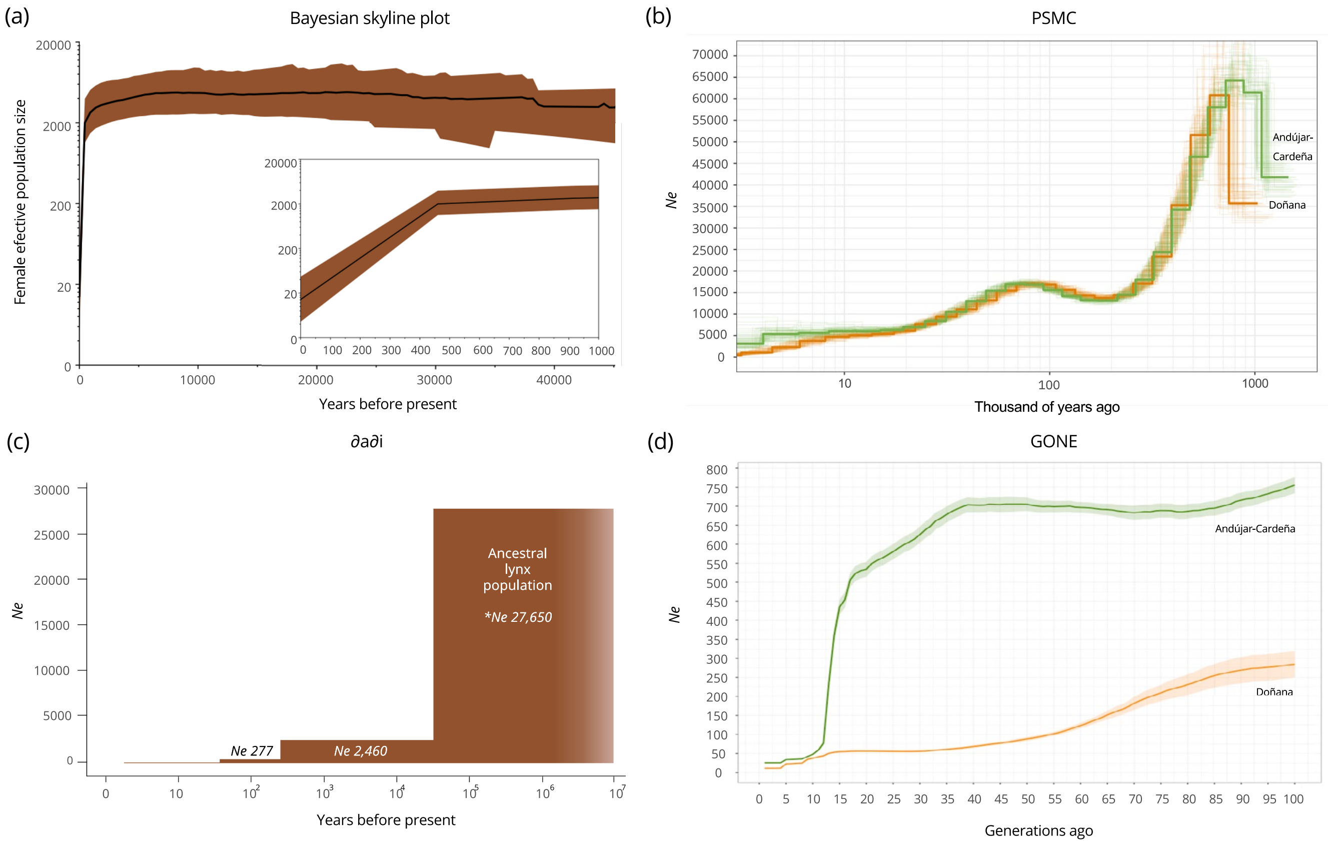

FIGURE 3 | Reconstructions of demographic history of Iberian lynx obtained by different inference methods and datasets. (a) Bayesian skyline plot using whole mitogenomes from modern, historical and ancient samples. The inset is a zoom into the last 1,000 years. (b) PSMC, using data from one individual from each of the two remnant populations, sequenced at around 50X and aligned to the *L. pardinus* reference genome (mLynPar1.2). (c) ∂a∂i, based on the allele frequency spectrum obtained from 1 million SNPs in a sample of seven individuals from remnant Andújar–Cardeña. (d) GONE, using whole-genome resequencing data to ~20× depth of 23 and 29 lynxes with pure Doñana and Andújar–Cardeña ancestry, respectively. Mean values (thick lines) and 95% confidence intervals (shaded regions) of 50 independent replicates are represented.

4 | Genetic Patterns Across Space and Time

4.1 | Near-Extinction Scenario by 2000

Early genetic analysis using microsatellite markers already indicated that by 2002 the remnant Iberian lynx populations displayed extensive signatures of genetic erosion, including levels of genetic diversity lower than those of other critically endangered species, and high levels of inbreeding, especially in the smallest and longest isolated population of Doñana (Casas-Marce et al. 2013; Johnson et al. 2004) (Figure 4). These analyses also confirmed that the two populations were genetically differentiated ($F_{ST} = 0.42$) and harboured a high proportion of private alleles, particularly in Andújar–Cardeña, the largest remnant population (Casas-Marce et al. 2013) (Figure 4).

More recent analysis of whole-genome variation corroborated the critical genetic status of the Iberian lynx by 2002. The average genome-wide heterozygosity rate in the species is among the lowest genome-wide and species-wide diversity reported (Abascal et al. 2016; Lucena-Perez et al. 2021). Again, the genome-wide diversity of the Doñana population stands out as extremely low, with around half of the diversity of Andújar–Cardeña ($\pi_{And} = 2.5 \times 10^{-4}$; π_{Don}: $\pi = 1.3 \times 10^{-4}$; Lucena-Perez et al. 2021) (Figure 4), a pattern also confirmed by other genomic elements such as polymorphic transposable element insertions and copy-number variants (Abascal et al. 2016). Whole-genome analyses also revealed an abundance of long runs of homozygosity (ROH) in individual Iberian lynx genomes. ROH larger than 1 Mb were more abundant in Doñana than in Andújar–Cardeña, indicating a more recent inbreeding in concordance with its known demographic history ($F_{ROH\text{-}Don} = 0.32$; $F_{ROH\text{-}And} = 0.16$) (Abascal et al. 2016). Despite the extremely low overall genomic diversity, some functional diversity was observed in certain gene families like the olfactory receptors and the MHC (Abascal et al. 2016; Marmesat et al. 2017), more likely as the consequence of functionally divergent alleles being retained across paralogous loci, rather than through high allelic diversity within loci (Marmesat et al. 2017).

4.2 | Historical and Ancient Genetic Patterns

While the analyses of modern samples indicated alarming levels of genetic erosion, the question remained whether this was a long-term pattern or the direct consequence of the most recent decline. This question was addressed through the analysis of 245 samples from the 20th and 19th centuries, and 10 ancient samples dated 2.5–4.3 ka ago using microsatellites and whole mitogenome sequences (Casas-Marce et al. 2017). This study indicated that the Iberian lynx populations transitioned from a near panmictic population in the ancient past, through a genetically structured population in the more recent past (global $F_{ST} = 0.27$), to the two genetically eroded and highly differentiated remnant populations by 2002 ($F_{ST} = 0.42$) (Figure 4). The historical populations of Montes de Toledo (MT) and Eastern Sierra Morena (eSM), which remained large and connected until a steep decline resulted in the sudden extinction of the former and the contraction of the latter to remnant Andújar–Cardeña, had a considerably higher diversity than the two remnant populations by 2002 ($H_{E\_MT} = 0.58$ and $H_{E\_eSM} = 0.61$ versus $H_{E\text{-}Don02} = 0.32$ and $H_{E\text{-}And02} = 0.46$) (Casas-Marce et al. 2017). Other historical subpopulations showed varying levels of genetic diversity and differentiation in relation to their size and time in isolation. Several populations, including Doñana and Andújar–Cardeña, showed a progressive reduction in individual heterozygosity and an

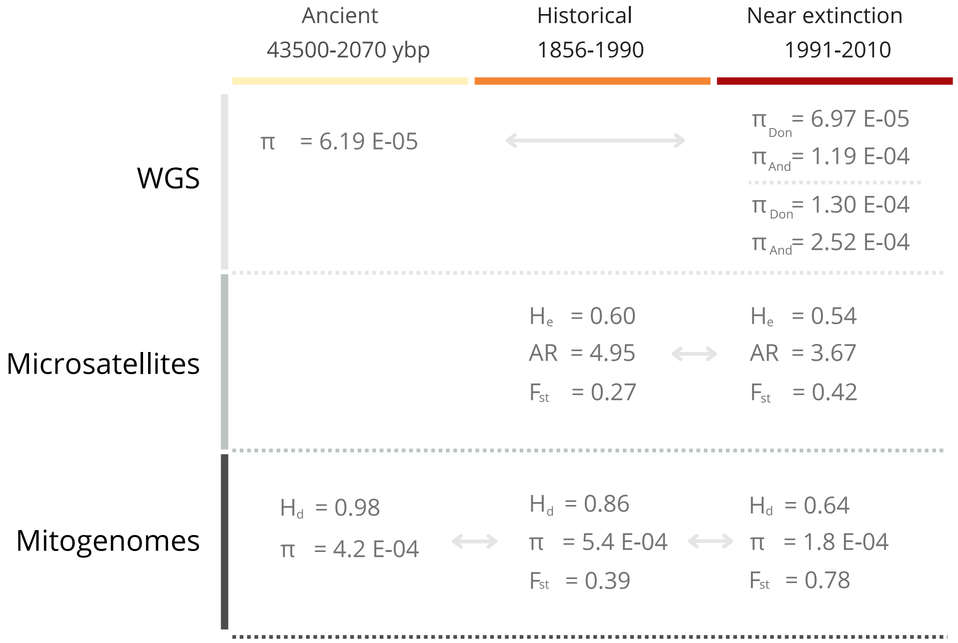

FIGURE 4 | Genetic population parameters estimated for different periods and using different genetic datasets. Two estimates of WGS are provided for the near-extinction period: the first one corresponds to a sample of three individuals sequenced at ≈3× depth to allow the direct comparison with the estimate of the ancient sample, whereas the second provides the best estimate available, using more samples ($N_{Don} = 12$; $N_{And} = 19$) and higher depth of coverage (mean 5.9×). Arrows connect estimates that are directly comparable across periods.

increase in relatedness through time, providing a direct illustration of the effects of genetic drift. This retrospective genetic study also provided a direct estimation of the proportion of genetic diversity lost during the recent species decline. By 2002, the proportion of the historical diversity (H_E) maintained was 75% in Doñana and 89% in Andújar–Cardeña (Casas-Marce et al. 2017). Importantly, this study dated the isolation of the two remnant populations around 200 years ago, indicating that the risks of outbreeding depression were low and validating their admixture and conjunct management in captivity and in the wild (Casas-Marce et al. 2017).

Mitogenome sequences also revealed a sharp decline in genetic diversity from historical to modern times, as reflected in both haplotype diversity ($H_{d\_historical} = 0.86$; $H_{d\_modern} = 0.64$) and nucleotide diversity ($\pi_{\_historical} = 5.4\ 10^{-04}$, $\pi_{\_modern} = 1.8\ 10^{-04}$). However, ancient mitogenomic diversity was not significantly higher than historical ($H_{d\_ancient} = 0.98$; $\pi_{\_ancient} = 4.2\ 10^{-04}$) (Casas-Marce et al. 2017) (Figure 4). Ancient and historical mitogenomic diversity in Iberian lynx, although higher than modern, were still among the lowest ever reported for any mammal. The sharing of mitogenome haplotypes across large distances also suggested that genetic differentiation was low in ancient times, probably related to a more contiguous distribution and fewer barriers to dispersal.

Subsequent work allowed the obtention of whole-genome data from three ancient Iberian lynx samples ranging in age from 2.0 to 4.3 ka ago (Lucena-Perez et al. 2024). These ancient nuclear data corroborated a shallower structure among the ancient Iberian lynx, based on both principal component (PCA) and individual-based clustering analyses, which also showed a closer relationship of the ancient Iberian lynx samples to modern Andújar–Cardeña than to modern Doñana (Lucena-Perez et al. 2024). However, contrary to the expected pattern of decreasing diversity over time, whole-genome ancient genetic diversity was lower than that of contemporary lynxes, as reflected both in observed individual heterozygosity and in population diversity (Lucena-Perez et al. 2024). Such differences in population genomic diversity could not be attributed to differences in coverage depth or ancient DNA damage, and were found to be consistent across different genomic regions, such as coding sequences (CDS) or untranslated regions (UTRs), suggesting that the difference in diversity is not related to the relaxation of purifying selection in bottlenecked lynx populations (Lucena-Perez et al. 2024). Interestingly, this study also reported a higher level of introgression from Eurasian lynx in modern than in ancient Iberian lynx individuals, and in the more recent ancient samples (2.0 and 2.5 ka, ca. 2% higher introgression) than in the oldest ancient sample (4.2 ka, 1.2%), indicating a quite continuous introgression process in the last 4,000 years. The admixture between the two palearctic lynx species seemed also asymmetrical, as alleles introgressed from Eurasian lynx into Iberian lynx outnumbered the opposite pattern (Lucena-Perez et al. 2024).

5 | Fitness Effects of Genomic Erosion

The patterns of deleterious variation in the Iberian lynx were first examined as part of a broader analysis comparing patterns of genetic diversity across the genome in smaller (more bottlenecked, e.g., Doñana) vs. larger (less or non-bottlenecked, e.g., Andújar) populations that also included Eurasian lynx populations (Lucena-Perez et al. 2021). This study found that the relative reduction in genetic diversity in the bottlenecked populations with respect to their closely related non-bottlenecked population was smaller in selectively constrained features such as CDS than in putatively neutral genomic features (e.g.: intergenic regions, introns). In the extreme case of ultra-conserved non-coding elements (UCNE), Watterson's θ_W was actually higher in Doñana than in Andújar–Cardeña, which points to a relative excess of mildly deleterious variants in evolutionary conserved genomic regions in Doñana as the consequence of the efficiency of natural selection being reduced by the overwhelming effect of genetic drift (i.e., relaxation of purifying selection). In the smaller populations of the Eurasian lynx, where bottlenecks have been somewhat milder, the authors found excess diversity mostly in genes relatively tolerant to the accumulation of functional mutations (Petrovski et al. 2013), suggesting reasonable levels of purifying selection, whereas in the Iberian lynx comparison, which involves a more intense bottleneck, mutation accumulation extended to genes more intolerant to mutations (Lucena-Perez et al. 2021).

Another separate study formally assessed the genomic patterns of deleterious burden in the two palearctic lynx species by comparing their derived count, that is, the number of putatively deleterious (as inferred using bioinformatics tools) derived alleles per individual (Kleinman-Ruiz et al. 2022), a proper genomic proxy of genetic load (Bertorelle et al. 2022). These authors found significantly lower derived counts in the Iberian than in the Eurasian lynx in mutation categories predicted to have a high impact on fitness but not in putatively neutral ones. Such relative depletion was particularly notable for loss-of-function mutations and the more deleterious subset of missense variants (Kleinman-Ruiz et al. 2022). This pattern is best explained by purging, i.e., the elimination of highly deleterious recessive alleles facilitated by increased exposure as homozygotes in inbred or small populations (Dussex et al. 2023; Hedrick and Garcia-Dorado 2016). It also implies a historical alleviation of inbreeding depression in the Iberian lynx that may have somewhat facilitated its persistence to the present day in spite of its extensive genetic erosion. The study did not yield noticeable differences in the count of derived deleterious alleles between larger or smaller populations of the same species, likely as a consequence of the time at small sizes being too short for extensive purging. Notwithstanding, smaller populations, like the Doñana population of Iberian lynx, showed higher homozygosity and increased fixation of deleterious alleles, predicted to result in lower fitness (Kleinman-Ruiz et al. 2022).

In summary, genomic research indicates that the smaller historical population size of Iberian lynx relative to Eurasian lynx has enabled the purging of part of its genetic load, which might have contributed to its current resilience. Notwithstanding, the dramatic population declines that the species has experienced in the very recent past have also led to drift-driven accumulation and expression of deleterious variants, likely resulting in reduced fitness. The study by Kleinman-Ruiz et al. (2022) also provides a database of several thousand potentially deleterious mutations, which may include those responsible for negative fitness consequences of inbreeding (i.e., inbreeding depression) and the deleterious traits affecting Iberian lynx populations.

Several lines of evidence for reduced fitness in remnant Iberian lynx populations with likely genetic bases have accumulated over the years. A recent decrease in litter size and an increase in disease-associated mortality in Doñana, concomitant with reductions of genetic diversity, suggested that the Doñana population had already entered extinction-vortex dynamics by 2008 (Palomares et al. 2012). This disease-associated mortality was mostly due to the high virulence of a feline leukaemia virus (FeLV) outbreak that killed six males, including all five males in the most important reproductive nuclei (López et al. 2009; Meli et al. 2010). Subsequent research showed that the viral strain was not particularly virulent in domestic cats, suggesting that Doñana lynxes were highly susceptible, possibly due to low immunological competence (Geret et al. 2011). The high incidences of glomerulonephritis and lymphoid depletion in Iberian lynx, otherwise rare diseases in other felids, were also suggested to be associated with genetic erosion and inbreeding (Jiménez et al. 2008; Peña et al. 2006). Several other deleterious traits have been observed segregating in the captive population, including cryptorchidism and idiopathic juvenile epilepsy (Martínez et al. 2013).

Providing a more direct evidence of inbreeding depression, individual microsatellite heterozygosity was shown to correlate negatively with semen quality in both Iberian lynx remnant populations (Ruiz-López et al. 2012), and inbreeding is negatively correlated with early survival in the captive population (Cuenca-Cambronero et al. 2019). Still, the more robust evidence for fitness reductions in remnant populations is coming from the observation of increased reproductive success of individuals of admixed ancestry both in captivity (Cuenca-Cambronero et al. 2019) and in Doñana following translocations for genetic reinforcement (López et al. 2015).

6 | Genetic Management and Monitoring

Evidence of extreme levels of genetic erosion and signs of reduced fitness raised concerns about the viability of the species due to inbreeding depression and reduced adaptive potential. Measures to reduce inbreeding and to restore genetic diversity were progressively implemented in conservation actions, including captive breeding, translocations and reintroductions (Figure 5) (Godoy et al. 2009, 2024).

6.1 | Captive Breeding

The Iberian lynx ex situ conservation breeding produced its first litter in 2005 and has since quickly expanded to include four exclusive breeding centres hosting close to 100 reproductive individuals and producing 30–40 cubs per year since 2012 (Serra et al. 2024). The programme has implemented a marker-assisted genetic management to achieve its stated goal of capturing and maintaining the maximum genetic diversity possible and producing fit individuals for reintroduction (Godoy et al. 2024).

A first objective was to assure a good representation of the remnant genetic diversity through an adequate number and distribution of founders coming from the two remnant populations. Using the estimates of microsatellite genetic diversity within and

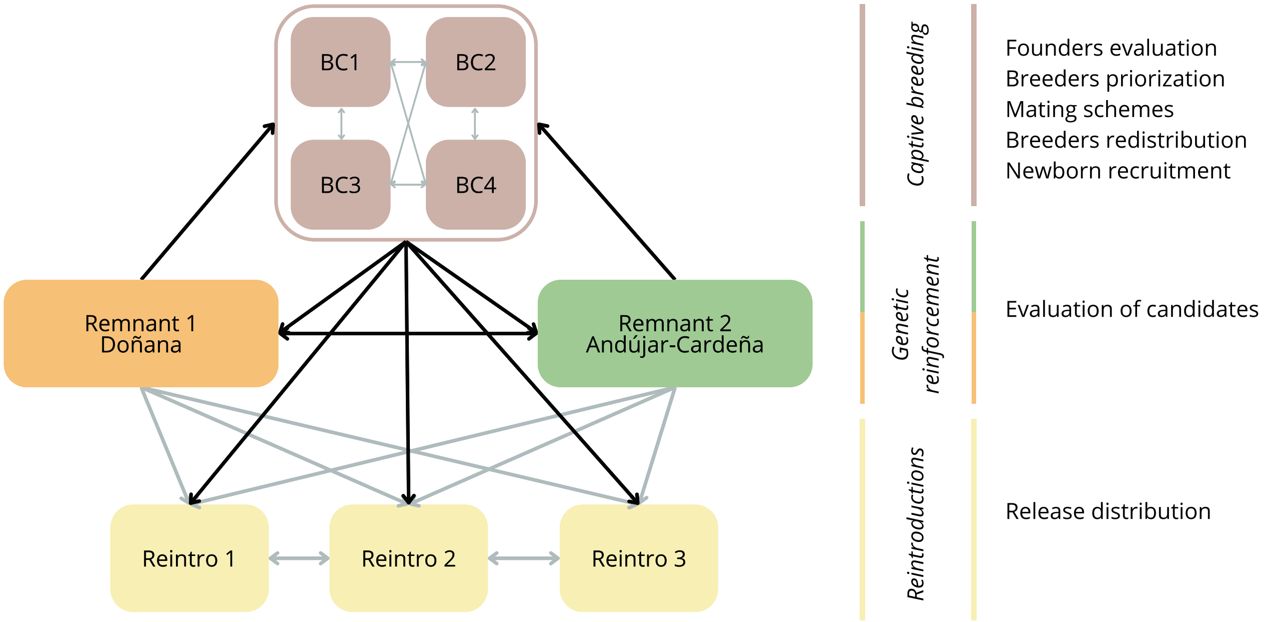

FIGURE 5 | Iberian lynx conservation actions and their associated genetic management tasks. The diagram depicts the three types of populations (captive, remnant and reintroduced) and the potential (grey arrows) or actual (black arrows) intentional movements of individuals between them. There are four main breeding centres (BC), that are, however, co-ordinately managed by a single captive breeding programme, two remnant populations (Doñana and Andújar–Cardeña) and three main reintroduction areas (Montes de Toledo, Matachel and Vale do Guadiana). Other reintroductions performed in areas close and well connected to Andújar–Cardeña are considered part of this larger population.

between populations, and following the methodology of Toro and Caballero (2005), the highest levels of genetic diversity (measured as expected heterozygosity, *He*) that could be captured in the core population would imply taking 36% of the animals from Doñana and 64% from Andújar–Cardeña, which would result in an expected maximum $H_E = 0.54$. This optimal proportion and maximum H_E were progressively approached by guiding the incorporation of new founders and through subsequent genetic management based on the minimum-kinship strategy (Kleinman-Ruiz et al. 2019). For this purpose, a kinship matrix between the founders is estimated from marker genotypes using the WEDS estimator, recommended for structured populations (Oliehoek et al. 2006), and estimates are then made congruent (coancestries within the usual range of 0–1) by adjusting to a virtual genealogy of three generations above founders (Fernández and Toro 2006). Each time a new founder is incorporated into the programme, the kinship matrix is updated using the same methodology. Starting in 2022, the 36 microsatellite marker panel has been replaced by a panel of 283 SNPs, also used for the monitoring of reintroduced populations (Kleinman-Ruiz et al. 2017), but the procedure for the construction of the kinship matrix between founders has remained the same. The kinship of the animals born in captivity is calculated from the fully known pedigree, but taking into account the marker-based empirical kinship matrix among founders, as implemented in the software PMx (Lacy et al. 2012). This kinship matrix is the primary data used in the application of a minimum-kinship strategy to management decisions (Ballou and Lacy 1995), which routinely include founder evaluation, breeders prioritisation, design of mating schemes, breeders redistribution among centres, and selection of captive-born kittens for recruitment (Figure 5).

Thanks to the genetic management implemented, the ex situ programme has been able to maintain reasonably high levels of heterozygosity and low levels of inbreeding. Expected heterozygosity has progressively increased, soon surpassing that present in either of the two remnant populations and approaching the maximum possible value. Regarding inbreeding, after an initial increase to 0.2, due to the recruitment of wild-born animals, management kept levels around 0.1 with almost no increase over generations, an indicator that may be more relevant than the actual absolute value (Kleinman-Ruiz et al. 2019).

Genetic management has also addressed the occurrence of deleterious traits with a likely genetic basis, including cryptorchidism and juvenile idiopathic epilepsy. The latter has only been observed in the captive population, where it reached a prevalence of 7.44% in the period 2005–2012 (20 affected individuals out of 121 cubs) (Minguez et al. 2021). A threshold model estimated high levels of heritability, and a segregation analysis supported a major gene with recessive inheritance as the cause of the juvenile epilepsy in the Iberian lynx (Jesús Fernández, unpublished). This is further confirmed by the fact that current management, consisting of avoiding reproduction of affected animals and crosses between potential carriers, assuming a monogenic recessive inheritance, has effectively prevented new cases since its implementation in 2017 (Minguez et al. 2021). Notwithstanding, a more precise determination of the genetic basis of juvenile epilepsy and other deleterious traits could help to implement a more effective management of these diseases, which might eradicate them without provoking a large loss of genetic diversity (see below).

6.2 | Genetic Reinforcement of Doñana Population

The accumulating evidence of extreme levels of genetic erosion and signs of reduced reproduction and survival in the Doñana population prompted the translocation of individuals from Andújar–Cardeña in an attempt to restore genetic diversity and, ideally, fitness and population growth. This was done, rather opportunistically, in 2007, following the FeLV outbreak that left several females with no local males for the reproductive season. A single male born in the Andújar population, named Baya, was released into the male-empty area in January 2008 (Ruiz et al. 2009). Baya successfully reproduced with three females in the following breeding season, and some of its descendants have been highly successful. At least seven more wild-born and four captive-born Iberian lynx have been released in the area until 2023, although reproduction has been confirmed for only three of them (Lopez et al. 2024; Simón et al. 2012).

Genetic and pedigree data confirmed a significant increase in average genetic diversity and a reduction in average inbreeding following translocations, mostly driven by the high heterozygosity and low inbreeding of F1 individuals and subsequent backcrosses to pure Doñana individuals (Mora 2023). Baya also introduced MHC Class I and Class II haplotypes that were absent in the Doñana population, potentially improving immune response (Marmesat, unpublished). However, the mating between close relatives sharing Baya as their common ancestor is resulting in high inbreeding in F2 and backcrossed individuals (Mora 2023), as observed in other small genetically eroded populations following limited natural or assisted migration (Hasselgren 2022; Hedrick et al. 2019; Lotsander et al. 2021). Concomitantly, the population size of Doñana has progressively increased from around 50 during most of the last decades to over 100 in the 2023 census (https://www.miteco.gob.es/content/dam/miteco/es/biodiversidad/temas/inventarios-nacionales/lince/censolinceiberico2023.pdf). Given that substantial conservation efforts during most of the recent history of the population did not result in significant population growth, post-translocation growth indicates a successful genetic rescue of the Doñana Iberian lynx population. An early analysis revealing a significantly higher reproductive success of F1 individuals provided support for this hypothesis (López et al. 2015).

6.3 | Reintroductions

The Iberian lynx reintroductions started with the release of the first individuals in 2009, initially in localities close to the remnant Andújar–Cardeña population (Gualdamellato and Guarrizas, Jaen province, Andalusia, Spain), later extending to more distant areas in other regions (Matachel, Extremadura, Spain; Montes de Toledo, Castilla-La Mancha, Spain; Campo de Montiel, Castilla-La Mancha, Spain; Vale do Guadiana, Portugal) and to three other Spanish localities since 2020 (Sierra Arana, Andalusia; Lorca, Murcia; Campos de Hellín, Castilla-La Mancha). Up to 441 individuals have been released, with a great majority being captive-born lynxes (402) and the rest being animals translocated from the remnant Andújar-Cardeña population (Lopez et al. 2024). Iberian lynx were released at a rate of 30–40 individuals per year (8–10 individuals per year per locality), allowing for the genetic selection of released individuals based on minimal kinship to animals currently living at the receiving population, according to annually updated census and genealogy data.

With the goal of complementing traditional monitoring practices based on radiotracking and camera trapping, and of generating the precise individual-based information required for the genetic management of ongoing reintroductions, an ambitious non-invasive genetic monitoring programme is being implemented as part of the *Lynxconnect* LIFE project (Figure 6). Early work with microsatellite markers established most of the technical procedures and validated the feasibility of the approach, which was, however, never implemented in a systematic and extensive way (Palomares et al. 2011). The current non-invasive genetic monitoring is based on a set of 343 highly informative autosomal SNP markers ($\mathrm{MAF} > 0.4$), which surpass the power to identify individuals, assign parentage and estimate relatedness of the previously used microsatellite panel (Kleinman-Ruiz et al. 2017). These SNP can be seen as an important direct contribution of early genomic information to conservation practice, as finding such highly variable markers in such a genetically eroded species would have been impossible without extensive genome-wide variation data. Faeces are being sampled in known territories across the current Iberian lynx range, with a particular focus on genetically managed populations (reintroduced and reinforced). Faeces surface is rubbed with a cotton swab that is then placed in barcoded tubes containing lysis buffer (Ramon-Laca et al. 2015), and the sample code and associated metadata, including geographical coordinates, is recorded using a custom CyberTracker sequence (https://cybertracker.org/). Once in the lab, DNA is extracted using a silica-based method in a 96-well plate format, and the DNA is first genotyped for a panel of 96 SNPs, including two species-specific sex markers (SRY and ZF), in a microfluidic platform (von Thaden et al. 2017, 2020). The power of this panel is very high for discriminating between individuals (probability of identity $<10^{-39}$ and $<10^{-20}$ for unrelated individuals and sibs, respectively) and high for parentage analyses (combined non-exclusion probability $<10^{-5}$, $<10^{-8}$ and $<10^{-13}$, for first parent, second parent and trios, respectively), based on the allelic frequencies of the source captive population. Genotypes are then assigned to individuals through comparisons among them and with reference genotypes. Samples with unique genotypes are further genotyped for two additional 96 SNP panels, and the 3-panel genotypes are used for genealogical reconstructions (Huisman 2017), kinship estimation and population genetic analyses (Figure 6). The plan is also to use the non-invasive samples assigned to individuals for the estimation of abundance and population density through spatial capture–recapture (SCR) (Jiménez et al. 2021) or close-kin mark–recapture (CKMR) methods (Bravington et al. 2016; Lloyd-Jones et al. 2023), as a potentially more viable and cost-effective approach to be used as a complement or as an alternative to current census estimations based almost exclusively on camera trapping (Garrote et al. 2014).

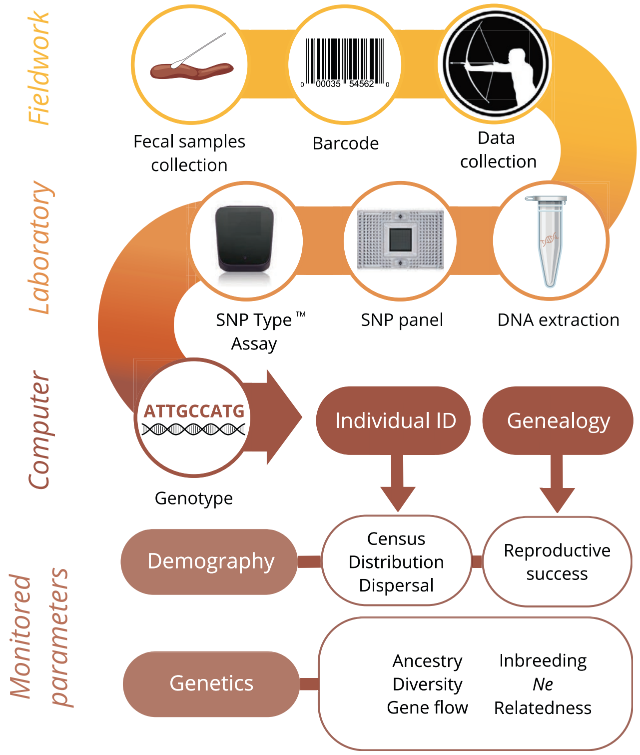

FIGURE 6 | Non-invasive genetic monitoring of Iberian lynx populations. The diagram shows the workflow as well as the individual- and population-level demographic and genetic parameters estimated from the individual genotypes and the inferred genealogy.

As of September 2024, around 5,000 DNA extracts from ca. 4,600 faecal samples have been processed, of which around 3,900 (78%) were molecularly confirmed to be Iberian lynx scats (Palomares et al. 2002). Of these, around 2,500 extracts (64%), which successfully amplified a nuclear microsatellite marker, were targeted for SNP genotyping. Around 2,200 reasonably complete genotypes (>85% loci typed) were obtained, translating into an overall genotyping success rate of ca. 58% of Iberian lynx faecal extracts. Almost 200 of the observed unique genotypes matched individuals previously included in our reference genotype database containing >1,700 genotyped individuals. The remaining represent novel genotypes, with most of them corresponding to animals born in situ at reintroduction areas. In the most intensively sampled populations in Castilla-La Mancha (Montes de Toledo and Campos de Montiel) around 60% of genotypes were genetically assigned to both parents and 80% to at least one parent.

The genetic management of reintroductions has consisted of the selection of animals to be released at each site. Once the number and sex of Iberian lynxes to be released in each area are agreed upon among the involved administrations, the optimal distribution of animals among release sites is decided based on genetic criteria. When the animals are released to an empty area, the aim is to maximise the starting diversity by sending animals minimally related to one another (i.e., distribute the cubs in groups of minimum kinship). In subsequent releases, the objective is to select animals that are minimally related to those already living in the release area, as informed by field (radio-tracking and camera traps) or non-invasive genetic monitoring. Such intensive individual-based genetic management will become unfeasible as populations grow and expand, so the plan is that once the releases stop, the genetic monitoring and management will shift from an individual- to a population-based approach, in which the need for further reinforcements or assisted gene flow is periodically evaluated based on population genetic parameters (diversity, inbreeding and differentiation).

Preliminary data coming from the non-invasive genetic monitoring suggest that the main reintroduction sites at Matachel, Montes de Toledo and Vale do Guadiana have low differentiation ($F_{ST} \approx 0.01$) and diversity levels similar to the captive source population, suggesting that genetic management has been effective in buffering founder effects. The remnant population of Andújar–Cardeña is now the central core of a well-connected population incorporating the reintroduction sites of Guadalmellato, Guarrizas and Campos de Montiel, similar to the Eastern Sierra Morena population described in the 1980s (Lopez et al. 2024; Rodríguez and Delibes 1992). The improvement of demographic and genetic connectivity between these main geographically separated nuclei is a major focus of ongoing conservation measures (Lopez et al. 2024). While proper estimates are still lacking, the observation of a few effective spontaneous dispersals between nuclei provides encouraging evidence that reasonable levels of connectivity are possible (Lopes-Fernandes et al. 2024).

7 | Genetic Viability and Favourable Reference Population

The long-term viability of the Iberian lynx will still depend on achieving effective population sizes that assure sufficient adaptive potential. A recent study evaluated through simulations the metapopulation effective size of current and potential future metapopulation scenarios differing in the number of subpopulations, census sizes and gene flow matrices (Pacin et al. 2024). The study concluded that achieving an $Ne \geq 500$ in a 100-year time frame would require at least three populations with effective sizes above 150, plus 10 subpopulations with Ne over 40, and migration rates close to 0.1 between neighbouring subpopulations, implying a global population of 2,165 reproductive individuals (ca. 1,100 breeding females) (Pacin et al. 2024). This represents about three times the 2023 population size, so although there is still a long way to go to achieve long-term genetic viability, the goal does not seem out of reach if we consider that, so far, conservation actions managed to multiply numbers by 20 in the last 22 years.

8 | Prospects for Functional Conservation Genomics

Genomic analyses so far have allowed a description of patterns of genetic variation, including functional variation, across the Iberian lynx genome, and provided a deeper understanding of the evolutionary processes shaping them, as well as a precise diagnostic of the genetic status of the species. At the same time, there is compelling evidence suggesting that genetic factors have affected fitness and potentially hampered conservation efforts in the past, and that the subsequent management through the admixture of the two populations has restored fitness and contributed to the spectacular species recovery. Current genomic research aims to move a significant step towards the identification of the genomic regions, genes and variants affecting fitness most. This will require a high-quality and fully annotated reference genome and extensive individual-based whole-genome data with associated fitness or phenotype data.

Whereas most previous work on Iberian lynx genomics was based on an assembly of moderate quality (Abascal et al. 2016), a second version of the Iberian lynx reference genome (mLynPar1.2) has been recently generated using a combination of long Oxford Nanopore Technologies (ONT) reads, 10X linked-reads and Hi-C reads (Omni-C). The novel assembly is 2.42Gb long and has 99.87% of the total base pairs in chromosomal pseudomolecules . This new chromosome-level sequence has been thoroughly curated, is highly collinear to other Felidae genomes, shows excellent gene completeness (96.1% complete, 0.6% duplicated, 1.1% fragmented and 2.8% missing; out of a total of 9,226 Mammalian BUSCOs), consensus quality value (QV = 46.8), kmer completeness (97.52%) and low false duplicate rates (0.30%) (F. Cruz, personal communication). Overall, this new reference genome meets by far the minimum assembly quality standards required by the Eukaryotic Biogenome Project (EBP) (https://www.earthbiogenome.org/report-on-assembly-standards) and provides an excellent resource for ongoing and future genomic studies.

We have also generated a high-density genome-wide phased genotype dataset from the high-coverage whole-genome resequencing of 50 individuals with significant genetic relevance to current populations, including Iberian lynx from Andújar–Cardeña and Doñana (both before and after the translocations), many of which are founders of the captive population, translocated individuals, or captive-born individuals released into the wild in reintroduction programmes (L. Mayor, unpublished). This will be used as a reference dataset for the genotype imputation of hundreds of additional samples sequenced at a lower coverage (~1–2×) (Lou et al. 2021; Watowich et al. 2023). This approach is being used for trait mapping in farmed (Wang et al. 2022), domestic (Wragg et al. 2024), and increasingly in wild species (Puckett et al. 2023; Wu et al. 2024). The kinship structure and high linkage disequilibrium within the Iberian lynx are expected to improve the phasing and imputation accuracy. Additionally, the availability of extensive genealogical data of captive and wild populations generated by the ongoing monitoring programme will provide the means to validate the quality of the imputation.

Phased whole-genome data will grant increased power for conservation genomics, including highly accurate individual inbreeding estimates based on runs of homozygosity (ROHs), which are expected to be more correlated with fitness than marker-based estimates (Kardos et al. 2015; Leitwein et al. 2020). Most importantly, ROH distribution along the genome holds the potential for identifying particular genomic regions contributing to fitness reductions through inbreeding depression (Duntsch et al. 2023; Hill et al. 2022; Kardos et al. 2016; Stoffel et al. 2021). For example, ROH deserts (regions in the genome that never appear in ROHs) are suggested to be enriched for loci of critical function (Hedrick and Garcia-Dorado 2016; Hedrick et al. 2016). Several studies have successfully applied this approach to identify potential haplotypes harbouring prenatal lethal recessive loci (Hoff et al. 2017; Todd et al. 2020; Wu et al. 2020), so it could also shed light on the underlying factors contributing to early and perinatal mortality in Iberian lynx. ROHs and haplotypes can also be employed to pinpoint loci associated with recessive deleterious traits via homozygosity and linkage mapping (Hildebrandt et al. 2009; Murgiano et al. 2019).

One noteworthy part of the ongoing project focuses on identifying structural variants (SVs) from long read sequencing and evaluating their role in traits and diseases related to inbreeding depression (Chakraborty et al. 2019). Structural variants are expected to have a greater impact on gene expression (Chiang et al. 2017) and to be more deleterious than non-synonymous SNPs (Ho et al. 2020). Structural variants segregating in the Iberian lynx will initially be identified in 10 individual genomes HiFi sequenced on the PacBio Revio system to produce highly accurate long read data. The individuals sequenced are representative individuals from both the Doñana and Andújar–Cardeña populations. In subsequent steps, we also aim at constructing a pangenome based on the same individuals and use it to reliably identify and genotype SVs in a larger dataset of short-read sequenced individuals (Gong et al. 2023). Documenting the role of SVs involved in inbreeding depression remains a promising field of study within conservation genomics in general and in the Iberian lynx in particular.

We are currently working on applying these approaches to elucidate the genetic architecture of juvenile idiopathic epilepsy, which previous analyses identified as a highly heritable monogenic disease. The whole-genome sequencing of affected individuals alongside their first-degree relatives will allow us to combine family-based approaches with population-level genome-wide association analyses (as in Dechow et al. 2022; Murgiano et al. 2020) to try to identify variants that are either causally or tightly linked to the disease. This would allow the identification of the true carriers of the deleterious allele(s), which will improve the efficiency of current genetic management while minimising the impact on overall genetic diversity (Minguez et al. 2021).

We also aim at investigating the genomic bases of genetic rescue by addressing the relative contribution of ancestry and heterozygosity to increased fitness. Extensive whole-genome data will be used to assess the effects of genetic ancestry on individual fitness. Furthermore, we will try to identify local ancestry throughout the genome and detect ongoing selection as departures from patterns expected under neutrality, through the use of Bayesian genomic cline models (Gompert and Buerkle 2011) and pedigree-based analyses (Chen et al. 2019). A particular focus will be on assessing the occurrence of ongoing balancing selection, including associative overdominance, i.e., the potential positive effects due to the masking of possible deleterious alleles in low recombining regions (Leitwein et al. 2021).

9 | Conclusions and Take-Home Messages

Conservation genomics of the Iberian lynx has led to a deeper understanding of the species history, and a thorough characterisation of the extent and fitness effects of genetic erosion, while notably contributing to its conservation through effective genetic monitoring tools and a sound genetic management programme. The Iberian lynx success story might serve as a model to illustrate, and hopefully encourage, the application of genomic tools and approaches to species conservation and restoration. In particular, the collection of genomic studies of the Iberian lynx highlights the following ideas regarding conservation genomics, which were crucial for this species and may therefore be of relevance for other endangered species:

- Genomic data provide critical information about the species evolutionary history, including divergence dates and admixture rates, which, in the case of the Iberian lynx, support its status as a distinct, diverged species that has extensively hybridised with the Eurasian lynx, a relevant factor to take into account in the discussion of the effects of hybridisation and its consideration as a potential management tool.
- Long-term and short-term demographic reconstructions based on genomic data are key for establishing baselines in conservation and have helped in identifying the causes of the observed low levels of genomic diversity in the Iberian lynx.
- Historical and ancient genomic data allow the reconstruction of the recent genetic history and provide a direct estimation of realised genetic erosion. This information was important for the establishment of genetic baselines and to validate the mixing of remnant populations of the Iberian lynx.
- The analyses of genetic diversity patterns across genomic features subjected to different selective constraints and the assessment of genetic load allow a first evaluation of the fitness consequences of genetic erosion and the relative impacts of the relaxation of purifying selection and purging. These also provided a catalogue of genetic variants which are candidates for causing inbreeding depression and genetic disorders.
- Genome-wide data allow the selection of highly informative neutral genetic markers which, in combination with cost-effective genotyping technologies, provide efficient and minimally invasive tools for species demographic and genetic monitoring and management.
- Marker-assisted genetic management of highly eroded populations can have a large positive impact on fitness and bolster demographic recovery by reducing inbreeding depression and increasing adaptive potential.
- The combination of high-density individual-based genomic and fitness data, only likely to occur in intensively monitored and managed species, allows the exploration of the genomic basis of genetic disorders, inbreeding depression and genetic rescue, providing a significant step forward in conservation genomics.

The conservation of Iberian lynx managed to partially bridge the widely acknowledged gap between genetic research and application, and may thus provide an encouraging example for other species (Klütsch and Laikre 2021; Shafer et al. 2015; Taylor et al. 2017). At the same time, the increasing availability of genomic and fitness data, which remain rare in most other endangered species, creates a good opportunity to advance the roadmap of conservation genomics, e.g., by addressing the genetic basis of inbreeding depression and genetic diseases, and incorporating this information into genetic management (Schmidt et al. 2024; Theissinger et al. 2023). As long as the current in situ and ex situ conservation efforts are maintained, genomics might continue to help the Iberian lynx reach the final goal of full demographic and genetic viability.

Author Contributions

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Disclosure

Benefit-Sharing Statement: Benefits from this research accrue from providing scientific information relevant to conservation, as well as by sharing our data and results on public databases.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

This article reviews previously published results, and the corresponding data were made available as described in the original articles.

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