Genetic evaluation of the Iberian lynx *ex situ* conservation programme

Daniel Kleinman-Ruiz[1], Laura Soriano[1], Mireia Casas-Marce[1], Charles Szychta[1], Iñigo Sánchez[2], Jesús Fernández[3] & José A. Godoy[1]

1 Departamento de Ecología Integrativa, Estación Biológica de Doñana (EBD-CSIC), Calle Americo Vespucio 26, 41092 Sevilla, Spain
2 Zoobotánico Jerez, Calle Madreselva s/n, 11408 Jerez de la Frontera, Spain
3 Departamento de Mejora Genética Animal, INIA, Carretera de la Coruña Km. 7, 28040 Madrid, Spain

Correspondence: José A. Godoy, Estación Biológica de Doñana (EBD-CSIC), Calle Americo Vespucio 26, 41092 Sevilla, Spain. Fax: 34-954621125. E-mail: godoy@ebd.csic.es

Running title (< 50 characters): Genetic evaluation of *ex situ* Iberian lynx

Word count: 6 286 (abstract included; references excluded)

Abstract

Ex situ programmes have become critical for improving the conservation of many threatened species, as they establish backup populations and provide individuals for reintroduction and reinforcement of wild populations. The Iberian lynx was considered the most threatened felid species in the world in the wake of a dramatic decline during the second half of the 20th century that reduced its numbers to around only 100 individuals. An *ex situ* conservation programme was established in 2003 with individuals from the two well-differentiated, remnant populations, with great success from a demographic point of view. Here we evaluate the genetic status of the Iberian lynx captive population based on molecular data from 36 microsatellites, including patterns of relatedness and representativeness of the two remnant genetic backgrounds among founders, the evolution of diversity and inbreeding over the years, and genetic differentiation among breeding facilities. In general terms, the *ex situ* population harbours most of the genetic variability found in the two wild populations and has been able to maintain reasonably low levels of inbreeding and high diversity, thus validating the applied management measures, and potentially representing a model for other species in need of conservation.

Keywords

Conservation, Iberian lynx, *ex situ* programme, captive population, molecular kinship

Introduction

Human activity is driving species to extinction at exceptionally high rates (Pimm *et al.*, 2014). Ever-growing threats to biodiversity, such as overexploitation, habitat destruction, and pervasiveness of invasive species (Wilson, 1992), pose a challenging ordeal for species persistence (Butchart *et al.*, 2010). At the same time, it is becoming evident that conservation actions can have a positive impact (Hoffmann *et al.*, 2010). *In situ* conservation is the main focus of biological conservation based on the multiple benefits derived from the integrated protection of habitats and ecosystems. However, in the current scenario of rapid climate change and increased habitat destruction, reliance solely on *in situ* approaches may not ensure the long-term –or, under critical scenarios, even the short-term– persistence of many species. In contrast, the potential of *ex situ* conservation, once relegated to educational, research and fund-raising roles (Pritchard *et al.*, 2012), to contribute more directly to biological conservation is rapidly increasing, thanks to improved global coordination, infrastructures, technical knowledge and practices (Pritchard *et al.*, 2012; Redford *et al.*, 2012).

Captive populations act as an insurance reserve in case disaster strikes wild populations. Individuals in captive populations are protected from threats such as predation and poaching, they are subject to continuous surveillance, and they can receive timely medical assistance. As collateral benefits, *ex situ* programmes provide valuable insights into species' biology by supporting and enhancing research on behaviour, reproduction, and

50 physiology, among others (McGowan *et al.*, 2017). Indeed, *ex situ* conservation
51 has played a major role in the improved conservation of many vertebrate
52 species, including the California condor (*Gymnogyps californianus*), Arabian
53 oryx (*Oryx leucoryx*), whooping crane (*Grus americana*), and blackfooted ferret
54 (*Mustela nigripes*) (Hoffmann *et al.*, 2010). Still, *ex situ* conservation should be
55 regarded as an additional means of preserving species, so whenever possible
56 it should be the source for individual reintroductions into the wild and be part
57 of an integrative approach to species-wide management in conjunction with *in*
58 *situ* actions (Russello and Amato, 2007; Pritchard *et al.*, 2012; Redford *et al.*,
59 2012; McGowan *et al.*, 2017).
60 More importantly, captive populations can be used to boost genetic
61 diversity and minimize inbreeding through managed and monitored
62 admixture of differentiated remnant populations when available. This is
63 particularly relevant since species under extinction risk are often genetically
64 eroded (i.e. show loss of genetic diversity and accumulation of inbreeding), as
65 the consequence of species decline and fragmentation. However, captive
66 populations –which are generally founded by a small number of individuals,
67 are usually small themselves, and have an environment different from wild–
68 carry their own additional genetic risks, namely: i) loss of genetic diversity, ii)
69 accumulation of inbreeding, iii) incidence of genetic diseases and other
70 deleterious traits, and iv) adaptation to captivity (Frankham, 2008; Frankham
71 *et al.*, 2010).
72 Genetic risks in captivity, though, can be somewhat ameliorated
73 through adequate genetic management. Towards this goal, the commonly

recommended and optimal strategy (as proven both theoretically and by simulations) is based on the minimisation of average kinship (Ballou and Lacy, 1995; Meuwissen, 1997; Fernández and Toro, 1999; Caballero and Toro, 2000; Fernández *et al.*, 2004). The implementation of the minimum kinship strategy has traditionally been based on studbook records and pedigree analyses (Ballou *et al.*, 2010). However, molecular data can assist and improve genetic management by assessing the representativeness of founders, detecting studbook errors and, most importantly, unravelling the typically unknown relatedness among founders. By doing so, they allow for higher accuracy in routine tasks such as identifying optimal breeding pairs, exchanging individuals between facilities, or selecting suitable candidates for reintroduction (Frankham *et al.*, 2010).

Of the four extant species in the lynx genus, the Iberian lynx (*Lynx pardinus*) has the smallest distribution area and population size. During the second half of the 20th century the species underwent a drastic decline that saw the majority of its populations become extinct in the span of five decades (Rodríguez and Delibes, 1992, 2002; Calzada *et al.*, 2007). By 2002, less than 100 individuals remained, distributed among two isolated populations in Andalusia (southern Spain): a peripheral population in the protected area of Doñana (DON), and a central one in Sierra de Andújar in the Sierra Morena range (AND). Separated by ca. 240 km, the two populations have been effectively isolated for almost two centuries (ca. 40 generations), until the start of translocations in 2007 (Guzmán *et al.*, 2004; Casas-Marce *et al.*, 2017).

98 This scenario led to the recognition of the Iberian lynx as the most endangered
99 felid in the world (Nowell and Jackson, 1996) and to its classification as
100 "critically endangered" in the 2002 and 2008 IUCN red lists (Rodríguez and
101 Calzada, 2015).
102 Genetic erosion in the Iberian lynx has been well documented. Studies
103 based on mitochondrial, microsatellite and genome-wide data have revealed a
104 high inbreeding rate, high differentiation between the two populations, and
105 extremely low whole-genome level diversity ($\pi = 0.026\%$), which is among
106 the lowest ever reported – in the same ballpark as that of the cheetah
107 (*Acinonyx jubatus*) and notably lower than that of other felids and humans
108 (Johnson *et al.*, 2004; Palomares *et al.*, 2012; Casas-Marce *et al.*, 2013;
109 Dobrynin *et al.*, 2015; Abascal *et al.*, 2016). Moreover, several observations
110 indicative of inbreeding depression have been reported, including an increase
111 in the proportion of abnormal sperm with individual inbreeding (Ruiz-López
112 *et al.*, 2012), a decrease in survival and litter size in DON (Palomares *et al.*,
113 2012), a trend that may have reversed following translocations (Simón *et al.*,
114 2012), and a high incidence of membranous glomerulonephritis and lymphoid
115 depletion (Peña *et al.*, 2006; Jiménez *et al.*, 2008). Besides, a number of
116 deleterious traits believed to have a genetic basis are currently segregating at
117 moderate frequencies in the captive population (Martínez *et al.*, 2013).
118 Such a negative outlook encouraged the implementation of active
119 conservation measures with the main funding coming from four successive
120 European LIFE projects in the period 1994-2012 (Simón *et al.*, 2012). *In situ*
121 actions have included the improvement of natural habitats, an increase of prey

abundance, the reduction of non-natural mortality, the reconnection of the two remnant populations through translocations, and awareness-raising measures, to name a few (Simón *et al.*, 2012). In parallel, an *ex situ* breeding programme was established in 2003 with the specific goals of: i) maintaining a genetically and demographically managed captive population that encompasses and safeguards the extant genetic diversity present in the two wild populations; and ii) providing individuals for reintroduction programmes aiming to recover the species' historical distribution in Spain and Portugal (Vargas *et al.*, 2008; Vargas, Breitenmoser, *et al.*, 2009; Vargas, Rivas, *et al.*, 2009).

The initial Iberian lynx captive population was founded with animals from the two genetically differentiated populations. Genotypes for 36 microsatellite markers (Godoy *et al.*, 2009; Casas-Marce *et al.*, 2013) have been routinely used to estimate the unknown relatedness among founders, which then informs the pedigree-based kinship minimisation management strategy. On a yearly basis, a list with the optimal contributions and mating pairs is developed for each centre, becoming the blueprint on which crosses are attempted. Every few years, translocations of animals between facilities take place to avoid reduced diversity within centres and differentiation between them. Finally, since 2010, when carrying capacity was reached, most captive-born animals have been released at reintroduction sites. The selection and distribution of animals among reintroduction sites have also been informed by minimum average kinships.

Global conservation efforts have helped to recover the number of free-living lynx to over the 500-individual milestone (589 in the latest census from 2017; http://www.iberlince.eu/images/docs/3_InformesLIFE/Informe_Censo_2017.pdf), which has resulted in the down-listing of the species from "Critically Endangered" to "Endangered" in the 2015 International Union for Conservation of Nature (IUCN) Red List (Rodríguez and Calzada, 2015).

The aim of this study is to evaluate the genetic status of the captive Iberian lynx population based on pedigree and genotype data for 36 microsatellites. We specifically aim to answer the following questions: i) how well is the genetic diversity of wild populations represented by founders, and ii) how have diversity and genetic structure statistics changed over the years, as measured by marker- and pedigree-based analyses. In short, we evaluate how successful the Iberian lynx *ex situ* programme has been from a genetic point of view.

Material & Methods

Populations

Here we studied the captive population (CAP) of Iberian lynx from its conception up to 2015. For most analyses, captive individuals were categorised into two groups: wild-born founders (from here on CAP-WB)

belonging to either the DON (CAP-WB-DON) or the AND (CAP-WB-AND) populations, and captive-born (CAP-CB).

The Iberian lynx *ex situ* programme was founded in 2003 with seven CAP-WB individuals that had been captured starting in 2001, and up to 50 have since been recruited (Supplementary Figure S1a). Currently, the programme comprises four main breeding facilities: i) El Acebuche, located within the boundaries of Doñana National Park (Andalusia, Spain), with 18 enclosures; ii) Centro Nacional de Reprodução de Lince Ibérico, in Silves (Algarve, Portugal), with 16 enclosures; iii) Granadilla, located north of Cáceres (Extremadura, Spain), also with 16 enclosures; and iv) La Olivilla, in Santa Elena (Andalusia, Spain), with 23 enclosures. Five additional enclosures in the Zoobotánico Jerez facility, in Jerez de la Frontera (Andalusia, Spain), complete the breeding network (https://www.lynxexsitu.es/programa-en.php?sec=centro).

For the yearly analyses, two different sets of criteria were adopted to define the yearly datasets based on a version of the studbook dated the 21st of April, 2017 (Supplementary Table S1). Yearly cohorts include CAP-CB individuals born in the year (typically between March and May) that were still alive on the 1st of November (i.e. only each year's surviving newborns were included; Supplementary Figure S1b). Yearly censuses include all living captive individuals on the 1st of November of each year (Supplementary Figure S1c).

Additionally, free-living wild-born individuals (FRL-WB) coming from the remnant populations of AND (FRL-WB-AND) and DON (FRL-WB-DON)

were used for comparison with CAP-WB individuals. A map showing the location of both remnant populations as well as the breeding facilities is provided in Supplementary Figure S2.

Samples & DNA extraction

A total of 239 captive individuals, sampled during routine check-ups or necropsies between 2003 and 2015, were genotyped for this study, including an exhaustive sampling of wild-born individuals (49 out of 50 CAP-WB individuals; the unsampled one died before contributing any descendants). Thirty-six of these individuals were of pure AND genetic background, nine of pure DON genetic background, and the remaining four, which were born in the DON population following translocations of AND animals, were first backcrosses to DON individuals (descendants of DON x F_1 matings). With regard to the 190 CAP-CB individuals, the sampling was comprehensive until 2011, with the exception of some rare cases of abortions and a few other individuals that died before breeding. From 2012 to 2015, the genotyped samples covered between 63% and 94% of each year's cohort.

Whole blood from living animals was mixed with four volumes of lysis buffer (0.1 M Tris–HCl, pH 8.0; 0.1 M Na-EDTA; 0.01 M NaCl, 0.5% SDS) immediately after collection and stored at 4 °C. Tissue samples (mainly muscle) from dead animals were frozen and kept at -80 °C. DNA was extracted from all samples using standard phenol–chloroform methods (Sambrook and Russell, 2006).

As for the remnant populations, genotypes previously reported in Casas-Marce *et al.* (2013) from a total of 50 FRL-WB-DON and 54 FRL-WB-AND individuals were used to gauge representativeness of CAP-WB individuals.

Microsatellite genotyping

Samples were genotyped for 36 heterospecific microsatellite loci as previously reported (Casas-Marce *et al.*, 2013). Fluorescently labelled products were amplified in separate PCRs containing 1x Bioline PCR buffer, 2 mM MgCl2, 0.25 mM dNTPs, 0.01 mg/mL BSA, 0.4 μM of each primer, 0.4U of Bioline Taq polymerase and 50 ng of template in a total volume of 14 μL and were run for 2' at 92 ºC, followed by 39 cycles of 30'' at 92 ºC, 30'' at 55 ºC and 30'' at 72 ºC, and a final extension of 5' at 72 ºC. Products were analysed in an ABI 3130xl Genetic Analyzer (Applied Biosystems), and the scoring of alleles was carried out using GENEMAPPER, version 3.7 software (Applied Biosystems).

A previous study of wild populations did not yield evidence of null alleles, high error rates, or deviations from Hardy-Weinberg equilibrium for both populations for any of the markers (Casas-Marcé et al., 2013). Here we used the software CERVUS 3.0.7 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007) and the completely known *ex situ* pedigree to tally Mendelian inconsistencies across all 190 CAP sire-dam-offspring triads as a proxy for error rate. All genotypes obtained in this study are reported in Supplementary Table S2.

239 Molecular data analyses

240 *Genetic diversity and differentiation.* With the aim of comparing wild
241 and captive genetic variation, genetic diversity statistics were estimated in R
242 (The R Core Team, 2015) using the PopGenReport package (Adamack and
243 Gruber, 2014), including observed (H_O) and expected heterozygosity (H_E),
244 mean number of alleles per locus (NA), and allelic richness (AR; the average
245 number of alleles per locus rarefied to the smallest number of alleles seen in a
246 sample across all combinations of population and locus). These statistics were
247 obtained for the CAP population, CAP-WB individuals, subsets that included
248 only pure captive individuals born in Doñana (CAP-WB-DON) or Andújar
249 (CAP-WB-AND), and the aforementioned FRL-WB-DON and FRL-WB-AND
250 populations. F_{IS} (population inbreeding coefficient) values were estimated
251 with FSTAT v2.9.3 (Goudet, 1995, 2002). Four admixed (backcross DON) CAP-
252 WB-DON individuals were excluded from most analyses pertaining to
253 founders. Significance of differences in average H_O, H_E, N and AR between CAP-
254 WB and FRL-WB individuals were tested for both DON and AND using a two-
255 tailed paired Wilcoxon signed rank test. To illustrate the distribution of alleles
256 between these two groups, Venn diagrams were drawn using the R package
257 VennDiagram (Chen and Boutros, 2011) for all observed alleles and for a
258 reduced dataset in which all alleles with a single occurrence were pruned out.

259 We assessed differentiation between the CAP-WB sets and their
260 respective source FRL-WB population for each locus and across loci by
261 estimating F_{ST} (Weir and Cockerham, 1984), as implemented in FSTAT, and
262 Jost's D (Jost, 2008) as given by PopGenReport. Additionally, we obtained F_{ST}

263 values and 99% confidence intervals among the four main breeding centres
264 (A, S, G & O) at the end of our sampling period (year 2015). We considered
265 significant those F_{ST} values that did not include zero in their 99% confidence
266 interval.

267 We also estimated the variation over time in H_O and H_E, per locus and
268 across loci, for CAP-CB individuals (cohorts approach), or for the whole CAP
269 population as well as the CAP-WB and CAP-CB subsets of individuals (census
270 approach) using the advanced frequency-based analysis module in GENALEX
271 6.5 (Peakall and Smouse, 2012). To assess the evolution of inbreeding we
272 estimated F_{IS} for CAP-CB individuals (cohorts and census approaches) and the
273 whole CAP population (census approach).

274 *Relatedness and relationships*. A two-step approach was used for the
275 estimation of relatedness (R) between CAP-WB individuals from molecular
276 marker genotypes. First, we used the *weighted equal drift similarity* (WEDS)
277 estimator proposed by Oliehoek *et al.* (2006) and implemented in the
278 software REA v0.2, with the option of β2 correction. The WEDS estimator
279 adjusts observed molecular similarities so that the increase in co-ancestry
280 since the base population is equal at all loci. The β2 correction regresses
281 relatedness estimates to their mean using β2 (the regression coefficient of
282 pedigree relatedness on estimated relatedness) empirically predicted from
283 the amount of molecular information. The corrected WEDS estimator was
284 shown to conserve a higher proportion of diversity than other estimators
285 under an optimal contribution scheme, particularly so in structured
286 populations (Oliehoek *et al.,* 2006). However, kinships estimated with this

method do not range from zero to one as expected for a true kinship. Thus, we also used the methodology implemented in the software MOLCOANC v3.0 (Fernández and Toro, 2006) to obtain a congruent kinship matrix. This software creates a virtual genealogy for the founders in such a way that the correlation between the genealogical kinship calculated from that virtual pedigree has the highest correlation with a provided matrix (the one from REA v0.2 in our case). The methodology is able to account for any *a priori* known relationship (e.g. a couple of individuals sharing a parent) and to detect Mendelian incompatibilities in the proposed families. The number of generations above the founders was set to three, which provided a correlation between matrices of around 98%. Any time a new founder is added to the *ex situ* population the kinship matrix is re-evaluated in order to accommodate the relationships of the new incorporation.

Additionally, with the aim of assessing the occurrence of close relatives among founders from the same population, we used the software ML-RELATE (Kalinowski *et al.*, 2006) to calculate maximum likelihood estimates of relatedness and relationship, to compare between alternative relationship categories (PO: parent-offspring; FS: full-siblings; HS: half-siblings; UR: unrelated) and to identify a "confidence set" of categories for each pair of individuals. On the basis of the latter, we conservatively considered as related only those pairs for which the 95% confidence set did not include the UR category, as unrelated those with only UR included in the set, and as ambiguous those with any combination of unrelated and related categories. For this purpose, we analysed only intra-population pairs using the

311 empirical allele frequencies of each population, so that these estimates reflect
312 only the most recent genealogy.
313
314 Pedigree analyses
315 Pedigree analyses for a version of the studbook dated the 21st of April,
316 2017 (Supplementary Table S1) were carried out with the software
317 Population Management x (PMx; Lacy *et al.*, 2012). We estimated values for
318 gene diversity (GD; measured as the reduction in H_E with respect to the
319 founding population) and mean inbreeding (F) over time taking into account
320 or not the kinships among founders (empirical kinships ON vs. empirical
321 kinships OFF, respectively), which were estimated from molecular marker
322 data as detailed in the previous section.
323
324 Optimal and realised ancestry proportions among founders and theoretical
325 maximum H_E
326 We estimated the optimal proportion of founders (i.e. the contribution
327 of each genetic stock to the pool that would maximise its genetic diversity)
328 following the equation on page 1,369 of Toro & Caballero (2005), and on the
329 basis of the genetic diversity within, and Nei's minimum distance (Nei, 1973)
330 between, the two remnant populations empirically estimated from
331 microsatellite genotypes. We often refer to the H_E of this ideal mix as the
332 theoretical maximum H_E, and use it as a reference point to gauge the actual
333 estimated H_E.

For comparison purposes, we also inferred from the census the actual proportion of CAP-WB individuals from each pool each year, as well as the proportion of contributing founders (defined as those CAP-WB individuals that had offspring before the end of 2017). For all practical purposes, CAP-WB individuals coming from a first backcross of an F_1 individual with an individual of DON ancestry contributed to calculations as 0.75 DON and 0.25 AND founders.

Results

Genotyping

All samples were genotyped at a minimum of 25 (out of the 36 total) loci, with 96% of them genotyped at 30 or more. Thus, no sample was discarded because of a high proportion of missing data. The proportion of genotypes with Mendelian inconsistencies across all CAP sire-dam-offspring trios in the dataset was 1.35%, indicating a low overall error rate.

Comparing wild and captive genetic variation

Genetic diversity and differentiation. Average number of alleles observed per locus for CAP (3.81; range: 2-11) was similar to previously reported values for wild populations (3.75; Casas-Marce *et al.*, 2013), although the average H_O was higher (0.509; range: 0.103-0.792) than in any of the remnant populations (0.313 and 0.467 in FRL-WB-DON and FRL-WB-AND,

358 respectively), as expected for an admixed population (Table 1). As previously
359 reported, the genetic diversity of CAP-WB-AND was higher than that of CAP-
360 WB-DON (Casas-Marce *et al.*, 2013). We found no significant differences in any
361 of the diversity statistics in comparisons between CAP-WB-AND and FRL-WB-
362 AND populations (Wilcoxon test: $P > 0.05$), but NA and AR were significantly
363 lower in CAP-WB-DON than in FRL-WB-DON ($P < 0.012$ and $P < 1.226 * 10^{-06}$,
364 respectively).
365 The sets of alleles in each pool of CAP-WB individuals and in their
366 respective FRL-WB population were largely overlapping, more so for AND
367 (where only two and three alleles were private to the FRL-WB and CAP-WB
368 populations, respectively) than for DON (where 12 alleles sampled in FRL-WB
369 were not represented in CAP-WB, and one was exclusive to the latter).
370 Differences between FRL-WB and CAP-WB sets were noticeably reduced when
371 alleles with only one occurrence (singletons, most likely arising from
372 genotyping errors or mutations) were ignored: only one and three alleles
373 were private to FRL-WB-AND and FRL-WB-DON populations, respectively,
374 and none was private to the CAP-WB population (Fig. 1).
375 Finally, genetic differentiation was extremely low between CAP-WB-
376 DON and FRL-WB-DON populations (F_{ST} = -0.001; 99% CI [-0.022, 0.029]), and
377 even lower for AND (F_{ST} = -0.009; 99% CI [-0.011, -0.006]). These slightly
378 negative values suggest that variance is higher within than between groups.
379 In summary, lack of structure between founders and their source
380 population indicate that both genetic stocks are reasonably well represented

in captivity, whereas diversity statistics suggest a slightly incomplete representation of wild allelic variation in founders from DON.

Optimal and realised ancestry proportion among founders and theoretical maximum H_E. According to theoretical predictions, and given the unbiased expected homozygosity of each genetic stock (DON: 0.703; AND: 0.541) and observed Nei's minimum distance between the two (0.3), a proportion of 0.36 founders from DON and 0.64 from AND (~1:2 DON:AND) would yield the theoretical maximum H_E of 0.54 (Supplementary Figure S3). The actual proportion of founders from each remnant population has changed over the years and is approaching –but has not yet reached– this optimum, showing a relative deficit of CAP-WB-DON individuals for most of the study period (Supplementary Figure S4). This may have contributed to a realized H_E a little below the theoretical maximum at the end of the study period (see below).

Molecular estimation of relatedness among CAP-WB individuals. Relatedness values among CAP-WB individuals are reported in Supplementary Table S3. Average R was notable (R = 0.098 after excluding self-relatedness). The actual distribution exhibited a marked bimodality in which the mode closer to zero amassed relatedness between DON and AND individuals and the higher mode encompassed relatedness within each genetic pool (Fig. 2). The subsequent shoulder and a long tail suggest the existence of certain pairs of

moderately related founders within both stocks, but mainly within the DON subset.

To characterise relationships between founders better, we obtained maximum likelihood inferences of relatedness among pairs within each genetic CAP-WB stock using the observed allelic frequencies of each population (Supplementary Tables S4 and S5). We obtained moderate levels of ambiguous kinship; in other words, many individuals could not be assigned unambiguously as unrelated or related, especially in the DON stock, where diversity was lower and relationships are known to be more complex (Lucena-Perez *et al.*, 2018). Nevertheless, both stocks were confirmed to include a number of related pairs (resolved with a confidence of 95%), including some instances of FS and PO relationships (Supplementary Figure S5).

Evolution of diversity over the years

Molecular statistics. Average H_O of the CAP-CB cohorts fluctuated over the years, mostly driven by the degree of admixture of the two differentiated genetic stocks of DON and AND (Fig. 3a). H_O peaked in years when most of the offspring were F_1, decreased later when successive generations of admixture (backcrosses) became more frequent, and then stabilized in more recent years around the theoretical maximum value for H_E. H_O consistently stayed above H_E, resulting in negative F_{IS} throughout the entire history of the captive population, an expected outcome of the inbreeding avoidance strategy

implemented by the genetic management programme, which has favoured the mating of least related couples (Fig. 3a and 3b).

The whole population (census approach) followed a pattern similar to that of cohorts: while H_O exceeded H_E in CAP-CB individuals, reaching higher values in years with a higher proportion of F_1 individuals, CAP-WB individuals exhibited the opposite pattern, i.e. H_E –which seemed to flatten above 0.5 following the stabilisation of allelic frequencies– was higher than average H_O (Fig. 4a and 4b). Since the CAP-WB group resulted from the pooling of two differentiated populations, such a Wahlund effect (Wahlund, 1928) was expected. Consistent with this, global F_{IS} for the whole CAP population was positive in the early phase of the *ex situ* programme (when CAP-WB was the major, or even only, component), and subsequently steadily decreased as the proportion of admixed individuals continued to increase, finally reaching a negative value in the last year of our study (Fig. 4c).

Regarding the structure between facilities in 2015, F_{ST} values were low between all pairs of breeding facilities, although still significant for the Acebuche-Granadilla, Granadilla-Olivilla, and Acebuche-Olivilla pairs. The highest value (0.018; 99% CI [0.009, 0.028]) was found between Acebuche and Granadilla, while the lowest (-0.005; 99% CI [-0.012, 0.001]) was observed for the Silves and Olivilla comparison (Supplementary Figure S6).

Pedigree statistics. GD increased swiftly in the first few years of the breeding programme as average kinship diminished with new recruitments, but stabilised from 2007 onwards at a value higher than 0.97. When

accounting for the empirical relatedness between founders, however, GD tends towards a value around 0.87 (Fig. 5a).

Under the assumption of zero relatedness between founders, the breeding programme has been able to keep inbreeding at bay ($F < 10^{-4}$ up to 2015). When considering molecular kinships, however, the picture is starkly different (Fig. 5b). In earlier years values up to nearly 0.2 were reached, likely reflecting recruitment of wild-born individuals with varying degrees of inbreeding. In later years, inbreeding has fluctuated around 0.1.

Discussion

Since its inception in 2003, the Iberian lynx *ex situ* programme has become a major pillar on which conservation efforts to save the species from extinction rest. Although its course of action has been clearly validated in demographic terms, evaluating its success from a genetic point of view is not as straightforward. A thorough study was necessary to either back the adopted set of measures and decisions or shed light on potential concerns or amendable issues. In this study, we discovered that the species' remnant genetic diversity is fairly well represented in the CAP population, more so for the AND genetic background than for the DON one. Considering that the heterozygosity of the CAP population has been approaching the theoretical maximum level, and its mean inbreeding has been constantly shrinking, the programme's genetic management has been satisfactory. Moreover,

differentiation between breeding facilities is also negligible. In short, the programme has thus far been able to achieve its goals from a genetic perspective.

1. To mix or not to mix

A first important decision to be made when genetically differentiated populations persist is whether to mix them and manage them as a single unit or to keep them separated in captivity. In those cases where remnant populations have different local adaptations or a long history of isolation, mixing stocks could potentially lead to a reduction in the biological fitness of the hybrid offspring (outbreeding depression) due to extrinsic or intrinsic factors (Templeton, 1986; Lynch, 1991; The SSC Re-introduction Specialist Group, 1995). In contrast, when differentiation is due to stochastic processes, and/or when the risk of inbreeding in the source populations far outweighs the risk of outbreeding, mixing affords the potential advantages of maximising genetic diversity and boosting fitness of hybrid individuals (Chesser, 1983; Lacy, 1987). For the Iberian lynx *ex situ* programme, it was decided that the two remnant populations of DON and AND should be mixed in order to restore the highest possible amount of historical diversity and to limit inbreeding depression. This decision, originally informed by accumulated signs of inbreeding depression (Peña *et al.*, 2006; Jiménez *et al.*, 2008; Palomares *et al.*, 2012; Ruiz-López *et al.*, 2012) and historical range reconstructions showing likely connections in the recent past, has recently been further validated by an extensive analysis of the historical variation in the species, which showed a

499 progressive loss of genetic diversity within populations and the accumulation
500 of genetic differentiation among them, resulting from the contraction and
501 fragmentation that took place in the latter half of the 20th century (Casas-
502 Marce *et al.*, 2017). Even though the estimated date of divergence of the two
503 remnant populations (ca. 200 ybp) was earlier than previously thought based
504 on range reconstructions (around 50 ybp), the absence of major
505 environmental differences and the evidence of gene flow in the recent past do
506 conclusively support the management of the two remnant populations as a
507 single unit in captivity as well as in the wild (Frankham *et al.*, 2011). These
508 points also validate the genetic reinforcement of DON through the
509 translocation of animals from AND which was initiated in 2007 (Simón *et al.*,
510 2012).

511 The fitness consequences of admixture in the Iberian lynx have not
512 been formally analysed yet, but as often observed (e.g., Frankham, 2016), the
513 fitness of outbred individuals coming from admixture of the two inbred lines
514 appears to be higher, and at the very least it is not lower than that of their
515 pure, inbred counterparts, both in captivity and in the wild (Simón *et al.*,
516 2012).

517

518 2. Representativeness.

519 One of the main goals of *ex situ* conservation is to safeguard genetic
520 diversity of threatened species or populations, and so capturing as much of
521 that variation as possible should be the first objective. Both genetic stocks of
522 DON and AND seem to have been represented well in captivity, as revealed by

523 our analyses on private alleles and particularly by the extremely low F_{ST}
524 values between each founder subset and their source population. As expected,
525 our results suggest that of the two CAP-WB stocks, the smaller CAP-WB-DON
526 (N=9 pure and 4 backcross individuals) provides a slightly worse
527 representation of its source than its counterpart from AND (N=36), as
528 indicated by its higher proportion of unsampled alleles (Figure 1) and
529 significant differences in NA and AR (Table 1). Thus, recruiting pure DON
530 individuals should be a priority in the near future. On the other hand, once a
531 source population is well represented genetically, as is now the case for SMO,
532 randomly selected new founders will likely make a relatively small
533 contribution to the global genetic diversity and may rank low in breeding
534 priority. Given this situation, together with current space limitations, carefully
535 selecting potential founders based on their average kinship to the current
536 captive population becomes a priority.
537 A related but different matter concerns the proportions in which the
538 two genetic stocks should be mixed in captivity, considering the difference in
539 their genetic diversity and the level of genetic differentiation observed
540 between them. Our estimated optimal contribution of about two AND lynx for
541 every DON individual (0.64 vs. 0.36) has not quite yet been realised, as the
542 AND stock has been overrepresented since 2003 to the detriment of the DON
543 one. Empirical proportions did approach the optimal ones in the last few years
544 of the study (0.71 vs. 0.29 in 2015, when they were the closest), and do so
545 even more at present (data not shown), so it is expected that the H_E of the
546 captive population will continue to close in on the theoretical maximum in the

547 near future. For the time being, this observation underlines the importance of
548 prioritising DON founders versus AND ones in the short term.
549
550 3. Effectiveness of genetic management
551 Management in *ex situ* conservation programmes typically invokes
552 pedigree-based kinships assuming unrelated founders. However, such an
553 assumption dismisses the possible genetic structure between, and kinship
554 structure within, the source populations (e.g. Astle and Balding, 2009), which
555 are common situations when dealing with recently declined and fragmented
556 populations, such as those of the Iberian lynx. Molecular markers, on the other
557 hand, can provide insights into these processes, and marker-based relatedness
558 estimators have been developed specifically for structured populations
559 (Oliehoek *et al.*, 2006). Microsatellite genotypes have been previously used in
560 breeding programmes to augment pedigree-based management (Jones *et al.*,
561 2002; Gonçalves Da Silva *et al.*, 2010; Henkel *et al.*, 2012), while more
562 recently, SNP-derived kinships have informed the genetic management of the
563 whooping crane (*Grus americana*; Boardman *et al.*, 2017) and Tasmanian devil
564 (*Sarcophilius harissii*; Hogg *et al.*, 2017). Unfortunately, these are among the
565 few existing examples of direct usage of molecular kinships within breeding
566 programmes (Norman *et al.*, 2019).
567 Currently, the Iberian lynx captive population is genetically managed
568 using a mixture of both pedigree- and molecular-based approaches, where
569 kinships among all founders are estimated based on genotypes for the 36
570 microsatellite set, and pedigree-based kinships are calculated for the

571 remainder of the population taking the estimated founder kinships into
572 consideration. Here we found moderate levels of relatedness among founders,
573 evidence of the recent structure within both wild populations, and particularly
574 in DON. It is therefore likely that a hypothetical alternative management
575 assuming unrelated founders would have yielded a suboptimal outcome.
576 Results from PMx show that ignoring relationships between founders can lead
577 to a gross overestimation of GD and underestimation of mean inbreeding in
578 the population. To be more specific, under the assumption of zero relatedness
579 among founders, no inbreeding accumulated during the period of our study,
580 but when the empirical matrix is accounted for, the average population
581 inbreeding fluctuated around a non-negligible value of 0.1 (Figure 5). It must
582 be noted that ignoring relatedness among founders will generally lead to the
583 underestimation of inbreeding for any given genealogy, but more importantly,
584 to suboptimal mating schemes (i.e. a different genealogy) that would result in
585 an otherwise avoidable accumulation of inbreeding. Overall, our findings
586 argue against the founder assumption whenever a certain level of inbreeding
587 and/or relatedness among founders cannot be discarded, as typically occurs
588 in admixed captive populations.
589 In such instances, a natural consequence of applying a minimum
590 kinship based strategy is that genetic structure between the two (or more)
591 source populations weighs heavily in the design of the matings, so
592 interpopulation crosses that generate hybrids are often favoured.
593 Genetic management in breeding programmes should be planned in
594 advance. Particularly, the consideration of kinship among founders is most

595 necessary at an early stage, as allelic frequencies in a population managed by
596 minimum mean kinship equalise after four to five generations, so that
597 information on founder relationships may not have any significant effect on
598 genetic trends at these later stages (Schäfer and Reiners, 2017).
599 Molecular kinship holds the convenience of reflecting realised instead
600 of average expected kinship; however, it has been argued to be preferable to
601 genealogical information only when estimated from high-density molecular
602 data (Gómez-Romano *et al.*, 2013; Kardos *et al.*, 2015). Indeed, estimates of
603 relatedness and other genetic parameters based on a limited number of
604 hypervariable markers such as microsatellites have been argued to have high
605 sampling variances and can be inaccurate (Blouin *et al.*, 1996; Csilléry *et al.*,
606 2006), particularly so in genetically eroded populations (Tokarska *et al.*,
607 2009). However, our set of 36 microsatellites, which is larger than the average
608 size used in wildlife studies (Witzenberger and Hochkirch, 2011), has a low
609 overall genotype error rate as judged by the small proportion of Mendelian
610 errors in the pedigree, and holds moderate power to classify individuals in
611 kinship categories, properly discriminating full-siblings from half-siblings in
612 75% of the cases and from unrelated individuals in over 99% of them
613 (Kleinman-Ruiz *et al.*, 2017). Furthermore, we only used molecular
614 information to assess the relatedness among founders, for which no genealogy
615 is available, but relied on the fully known genealogy otherwise.
616 The Iberian lynx *ex situ* programme is arranged in breeding facilities as
617 a security measure in case of catastrophes. Since matings are performed
618 within centres, genetic management also pursues the minimisation of genetic

619 structure among the facilities to restrict the rise of inbreeding. The nearly
620 negligible but still significant levels of structure that we observe here support
621 the maintenance of translocations of individuals at current or even slightly
622 higher levels.
623
624 4. Needs, challenges and recommendations
625 Deleterious traits with a likely genetic basis are a pervasive challenge
626 for *ex situ* conservation programmes (Laikre, 1999 and the references
627 therein), and the Iberian lynx' is no exception. Idiopathic epilepsy occurring at
628 moderate frequency in captivity causes a number of cubs at the age of around
629 two months to go through episodes of seizures and eventually die unless
630 treated (Martínez *et al.*, 2013). Its segregation pattern suggests a simple
631 genetic basis with recessive inheritance, but without further knowledge,
632 management of this trait has consisted of excluding affected individuals from
633 the pool of breeders, and avoiding crosses between any two identified
634 carriers. Not only can these restrictions interfere with minimum kinship
635 breeding recommendations, they also imply a general lessening of the
636 breeding programme's performance as unbreedable individuals add up and
637 occupy more enclosures. While relocating this surplus to external parties
638 (such as zoos) can alleviate the saturation, it does not tackle the root cause of
639 the problem. Further research is encouraged to narrow down the cause of this
640 and other genetic disorders, such as cryptorchidism, and to identify carriers
641 molecularly with the objective of incorporating selection measures against the

642 responsible alleles, as is being done to manage chondrodystrophy in the
643 California condor (Ryder *et al.*, 2016).
644 Beyond its role as an insurance population, the Iberian lynx *ex situ*
645 population has also provided the majority of individuals for reintroduction.
646 Between 2010 and 2017, a total of six populations were founded across the
647 southern half of the Iberian Peninsula by sub-adults released from captivity on
648 a yearly basis. Under these circumstances, it is necessary to strike a balance
649 between the number (and composition) of released and kept individuals, in
650 order not to distort the age pyramid nor the allelic frequencies of the
651 insurance population. Moreover, since all reintroduced populations are meant
652 to be representative of the captive stock and, eventually, act as a single
653 metapopulation, the distribution of individuals among release sites should
654 ideally be based on up-to-date allelic frequencies and/or genealogical data at
655 each site. This scenario requires an intensive and integrative individual-based
656 genetic monitoring that records survival, reproduction and dispersal in all
657 reintroduced populations. To fulfil this requirement, current practices based
658 on camera-trapping and radio and GPS telemetry should be complemented
659 with non-invasive genetic monitoring, which would allow for the assessment
660 of progress towards the final objectives of the interactive *in situ*/*ex situ*
661 strategy (Attard *et al.*, 2016).
662 Following the publication of the Iberian lynx genome (Abascal *et al.*,
663 2016), small panels of optimal, genome-wide SNPs were selected based on the
664 allelic frequencies of the captive population and compared to the set of 36
665 microsatellites upon which genetic management of the species has relied so

666 far (Kleinman-Ruiz *et al.*, 2017). According to this study, these SNP panels
667 provide more power than the current 36-microsatellite panel to carry out
668 routine conservation tasks such as the identification of individuals, parentage
669 assignment, or relatedness estimation, and they are also best suited for
670 application to non-invasive samples. Hence, they are positioned as the most
671 convenient and effective tool on which to base the genetic monitoring and
672 integrative management of all Iberian lynx populations in the future.
673 It is important to keep in mind *ex situ* conservation strategies involve
674 huge resource investments (Conway, 1986), which many authors argue
675 should rather be diverted into *in situ* measures (Caughley, 1994; Snyder *et al.*,
676 1996). Moreover, *ex situ* actions will not be applicable to most threatened
677 species (Rahbek, 1993; Balmford *et al.*, 1995; Snyder *et al.*, 1996). And yet the
678 IUCN's Conservation Breeding Specialist Group have recommended captive
679 breeding for one third of vertebrate taxa at risk of extinction (Seal *et al.*,
680 1994). The opportunity, feasibility, and priority of an *ex situ* conservation
681 programme should be considered carefully case by case, and when
682 implemented, it must be scientifically managed and integrated into a
683 multifaceted species conservation plan (Russello and Amato, 2007; Bowkett,
684 2009).
685 The Iberian lynx *ex situ* breeding programme has been a key piece in
686 the multifaceted conservation of the Iberian lynx. After 12 years, it has
687 accomplished the two main goals that were set at the start. Firstly, it has
688 succeeded in establishing a demographically and genetically healthy *ex situ*
689 population that may act as a safeguard for the species in case of extinction in

690 the wild. Secondly, after a few years of internal growth, the population has
691 been serving as the almost exclusive source of individuals for reintroduction
692 in the wild. Besides, the programme has assembled a set of experts in many
693 different fields as well as fostered and coordinated research on issues of high
694 relevance for the conservation of the species, while becoming a major driver
695 of awareness and dissemination. Here we show how the Iberian lynx *ex situ*
696 programme has been quite effective in representing, boosting and maintaining
697 the low genetic diversity that survived the latest severe bottleneck of the
698 species while minimising the accumulation of inbreeding, which should
699 ultimately result in increased adaptive potential and average fitness. In this
700 regard, the Iberian lynx *ex situ* programme could serve as an example of
701 marker-assisted pedigree-based management for other species at similar
702 conservation stages.
703
704
705 Acknowledgments:
706 Iberian lynx samples were contributed by the Iberian lynx *Ex Situ* Breeding
707 Programme, the Centro de Análisis y Diagnóstico de la Fauna Silvestre and the
708 LIFE projects LIFE06NAT/E/000209 and LIFE10NAT/ES/570, with
709 authorization of the Consejería de Medio Ambiente de la Junta de Andalucía.
710 Logistical support was provided by the Laboratorio de Ecología Molecular,
711 Estación Biológica de Doñana, CSIC (LEM-EBD). The study was funded by the
712 contract between the CSIC and the Consejería de Medio Ambiente de la Junta
713 de Andalucía "Caracterización genética de individuos y poblaciones andaluzas

714 de lince ibérico" and by the Spanish Dirección General de Investigación
715 Científica y Técnica through projects CGL2006–10853/BOS, CGL2010-
716 21540/BOS and CGL2013-47755-P. DKR was supported by a PhD contract
717 from the Programa Internacional de Becas "La Caixa-Severo Ochoa". MCM was
718 supported by a PhD JAE grant from the CSIC (Spanish National Research
719 Council). CS was supported by a Ciência Sem Fronteiras grant from the CNPq
720 (Brazilian National Research Council). Special thanks to David Aragonés from
721 Laboratorio de Sistemas de Información Geográfica y Teledetección, Estación
722 Biológica de Doñana, CSIC (LAST-EBD) for his help with the map, and to P.
723 James Macaluso Jr. for his assistance in the English editing of the manuscript.
724 We appreciate useful comments and suggestions from Maria Lucena-Perez,
725 Elena Marmesat, Ana Píriz, and three anonymous reviewers.

726

727 Conflict of Interest

728 The authors declare no conflict of interest.

729

730 Data archiving

731 The datasets analysed in this study are available in our institutional repository
732 DIGITAL.CSIC (http://digital.csic.es).

733

734 References

735

736 Abascal F, Corvelo A, Cruz F, Villanueva-Cañas JL, Vlasova A, Marcet-Houben
737 M, *et al.* (2016). Extreme genomic erosion after recurrent demographic
738 bottlenecks in the highly endangered Iberian lynx. *Genome Biol* **17**: 251.
739 Adamack AT, Gruber B (2014). PopGenReport: Simplifying basic population

genetic analyses in R. *Methods Ecol Evol* **5**: 384–7.
Astle W, Balding DJ (2009). Population structure and cryptic relatedness in genetic association studies. *Stat Sci* **24**: 451–71.
Attard CRM, Möller LM, Sasaki M, Hammer MP, Bice CM, Brauer CJ, *et al.* (2016). A novel holistic framework for genetic-based captive-breeding and reintroduction programs. *Conserv Biol* **30**: 1060–9.
Ballou JD, Lacy RC (1995). Identifying genetically important individuals for management of genetic diversity in pedigreed populations. In: Ballou JD, Gilpin M, Foose TJ (eds) *Population management for survival and recovery*, Columbia University Press: New York, pp 76–111.
Ballou JD, Lees C, Faust LJ, Long S, Lynch C, Lackey LB, *et al.* (2010). Demographic and genetic management of captive populations. In: Kleiman DG, Thompson K, Kirk-Baer C (eds) *Wild mammals in captivity, principles and techniques*, Univ. of Chicago Press: London & Chicago, pp 219–52.
Balmford A, Leader-Williams N, Green MJB (1995). Parks or arks. *Biodivers Conserv* **4**: 595–607.
Blouin MS, Parsons M, Lacaille V, Lotz S (1996). Use of microsatellite loci to classify individuals by relatedness. *Mol Ecol* **5**: 393–401.
Boardman K, Mace M, Peregoy S, Ivy JA (2017). Population analysis & breeding transfer plan, whooping crane (Grus americana). AZA Species Survival Plan.
Bowkett AE (2009). Recent captive-breeding proposals and the return of the ark concept to global species conservation. *Conserv Biol* **23**: 773–6.
Butchart SHM, Walpole M, Collen B, van Strien A, Scharlemann JPW, Almond REA, *et al.* (2010). Global biodiversity: Indicators of recent declines. *Science* **328**: 1164–8.
Caballero A, Toro MA (2000). Interrelations between effective population size and other pedigree tools for the management of conserved populations. *Genet Res* **75**: 331–43.
Calzada J, Guzmán JN, Rodríguez A (2007). Lynx pardinus (Temminck, 1827). Ficha libro rojo. In: Palomo L, Gisbert J, Blanco J (eds) *Atlas y libro rojo de los mamíferos terrestres de España*, Dirección General para la Biodiversidad-SECEM-SECEMU: Madrid, pp 345–7.
Casas-Marce M, Marmesat E, Soriano L, Martínez-Cruz B, Lucena-Perez M, Nocete F, *et al.* (2017). Spatiotemporal dynamics of genetic variation in the Iberian lynx along its path to extinction reconstructed with ancient DNA. *Mol Biol Evol* **34**: 2893–907.
Casas-Marce M, Soriano L, López-Bao J V, Godoy JA (2013). Genetics at the verge of extinction: insights from the Iberian lynx. *Mol Ecol* **22**: 5503–15.
Caughley G (1994). Directions in conservation biology. *J Anim Ecol* **63**: 215–44.
Chen H, Boutros PC (2011). VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* **12**: 12–35.
Chesser RK (1983). Isolation by distance: relationship to management of genetic resources. In: Schonewald-Cox C, Chambers S, MacBryde B,

787 Thomas W (eds) *Genetics and conservation: a reference for managing wild*
788 *animal and plant populations*, Benjamin Cummings Publishing Co.:
789 London & Amsterdam, pp 66–77.
790 Conway WG (1986). The practical difficulties and financial implications of
791 endangered species breeding programmes. *Int Zoo Yearb* **24**: 210–9.
792 Csilléry K, Johnson T, Beraldi D, Clutton-Brock T, Coltman D, Hansson B, *et al.*
793 (2006). Performance of marker-based relatedness estimators in natural
794 populations of outbred vertebrates. *Genetics* **173**: 2091–101.
795 Dobrynin P, Liu S, Tamazian G, Xiong Z, Yurchenko AA, Krasheninnikova K, *et*
796 *al.* (2015). Genomic legacy of the African cheetah, Acinonyx jubatus.
797 *Genome Biol* **16**: 277.
798 Fernández BJ, Toro MA (1999). The use of mathematical programming to
799 control inbreeding in selection schemes. *J Anim Breed Genet* **116**: 447–
800 66.
801 Fernández J, Toro MA (2006). A new method to estimate relatedness from
802 molecular markers. *Mol Ecol* **15**: 1657–67.
803 Fernández J, Toro MA, Caballero A (2004). Managing individuals'
804 contributions to maximize the allelic diversity maintained in small,
805 conserved populations. *Conserv Biol* **18**: 1358–67.
806 Frankham R (2008). Genetic adaptation to captivity in species conservation
807 programs. *Mol Ecol* **17**: 325–33.
808 Frankham R (2016). Genetic rescue benefits persist to at least the F3
809 generation, based on a meta-analysis. *Biol Conserv* **195**: 33–6.
810 Frankham R, Ballou JD, Briscoe DA (2010). *Introduction to conservation*
811 *genetics*, 2nd edn. Cambridge University Press: Cambridge.
812 Frankham R, Ballou JD, Eldridge MDB, Lacy RC, Ralls K, Dudash MR, *et al.*
813 (2011). Predicting the probability of outbreeding depression. *Conserv*
814 *Biol* **25**: 465–75.
815 Godoy JA, Casas-Marcé M, Fernández J (2009). Genetic issues in the
816 implementation of the Iberian lynx ex situ conservation programme. In:
817 Vargas A, Breitenmoser C, Breitenmoser U (eds) *Iberian lynx ex situ*
818 *conservation: an interdisciplinary approach*, Fundación Biodiversidad in
819 collaboration with: International Union for Conservation of Nature,
820 Species Survival Commission (IUCN /SSC) Cat Specialist Group: Madrid,
821 pp 86–99.
822 Gómez-Romano F, Villanueva B, de Cara MA, Fernández J (2013). Maintaining
823 genetic diversity using molecular coancestry: the effect of marker density
824 and effective population size. *Genet Sel Evol* **45**: 38.
825 Gonçalves Da Silva A, Lalonde DR, Quse V, Shoemaker A, Russello MA (2010).
826 Genetic approaches refine ex situ lowland tapir (Tapirus terrestris)
827 conservation. *J Hered* **101**: 581–90.
828 Goudet J (1995). FSTAT (version 1.2): a computer program to calculate F-
829 statistics. *J Hered* **86**: 485–6.
830 Goudet J (2002). FSTAT, a program to estimate and test gene diversities and
831 fixation indices (version 2.9.3).
832 *https://www2.unil.ch/popgen/softwares/fstat.htm*.
833 Guzmán JN, García FJ, Garrote G, Pérez de Ayala R, Iglesias C (2004). *El lince*

834 *ibérico (Lynx pardinus) en España y Portugal. Censo diagnóstico de sus*
835 *poblaciones.* Dirección General para la Biodiversidad: Madrid.
836 Henkel JR, Jones KL, Hereford SG, Savoie ML, Leibo SP, Howard JJ (2012).
837 Integrating microsatellite and pedigree analyses to facilitate the captive
838 management of the endangered Mississippi sandhill crane (Grus
839 canadensis pulla). *Zoo Biol* **31**: 322–35.
840 Hoffmann M, Hilton-Taylor C, Angulo A, Böhm M, Brooks TM, Butchart SHM, *et*
841 *al.* (2010). The impact of conservation on the status of the world's
842 vertebrates. *Science* **330**: 1503–9.
843 Hogg CJ, Grueber CE, Pemberton D, Fox S, Lee A V, Ivy JA, *et al.* (2017). "Devil
844 Tools & Tech": a synergy of conservation research and management
845 practice. *Conserv Lett* **10**: 133–8.
846 Jiménez MA, Sánchez B, Alenza MDP, García P, López J V, Rodríguez A, *et al.*
847 (2008). Membranous glomerulonephritis in the Iberian lynx (Lynx
848 pardinus). *Vet Immunol Immunopathol* **121**: 34–43.
849 Johnson WE, Godoy JA, Palomares F, Delibes M, Fernandes M, Revilla E, *et al.*
850 (2004). Phylogenetic and phylogeographic analysis of Iberian lynx
851 populations. *J Hered* **95**: 19–28.
852 Jones KL, Glenn TC, Lacy RC, Pierce JR, Unruh N, Mirande CM, *et al.* (2002).
853 Refining the whooping crane studbook by incorporating microsatellite
854 DNA and leg-banding analyses. *Conserv Biol* **16**: 789–99.
855 Jost L (2008). GST and its relatives do not measure differentiation. *Mol Ecol*
856 **17**: 4015–26.
857 Kalinowski ST, Taper ML, Marshall TC (2007). Revising how the computer
858 program CERVUS accommodates genotyping error increases success in
859 paternity assignment. *Mol Ecol* **16**: 1099–106.
860 Kalinowski ST, Wagner AP, Taper ML (2006). ML-RELATE: a computer
861 program for maximum likelihood estimation of relatedness and
862 relationship. *Mol Ecol Notes* **6**: 576–9.
863 Kardos M, Luikart G, Allendorf FW (2015). Measuring individual inbreeding in
864 the age of genomics: marker-based measures are better than pedigrees.
865 *Heredity (Edinb)* **115**: 63–72.
866 Kleinman-Ruiz D, Martínez-Cruz B, Soriano L, Lucena-Perez M, Cruz F,
867 Villanueva B, *et al.* (2017). Novel efficient genome-wide SNP panels for
868 the conservation of the highly endangered Iberian lynx. *BMC Genomics*
869 **18**: 556.
870 Lacy RC (1987). Loss of genetic diversity from managed populations:
871 interacting effects of drift, mutation, immigration, selection, and
872 population subdivision. *Conserv Biol* **1**: 143–58.
873 Lacy RC, Ballou JD, Pollak JP (2012). PMx: software package for demographic
874 and genetic analysis and management of pedigreed populations. *Methods*
875 *Ecol Evol* **3**: 433–7.
876 Laikre L (1999). Hereditary defects and conservation genetic management of
877 captive populations. *Zoo Biol* **18**: 81–99.
878 Lucena-Perez M, Soriano L, López-Bao JV, Marmesat E, Fernández L,
879 Palomares F, *et al.* (2018). Reproductive biology and genealogy in the
880 endangered Iberian lynx: implications for conservation. *Mamm Biol* **89**:

881 7–13.
882 Lynch M (1991). The genetic interpretation of inbreeding depression and
883 outbreeding depression. *Evolution* **45**: 622–9.
884 Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998). Statistical confidence
885 for likelihood-based paternity inference in natural populations. *Mol Ecol*
886 **7**: 639–55.
887 Martínez F, Manteca X, Pastor J (2013). Retrospective study of morbidity and
888 mortality of captive Iberian lynx (Lynx pardinus) in the ex situ
889 conservation programme (2004–june 2010). *J Zoo Wildl Med* **44**: 845–52.
890 McGowan PJK, Traylor-Holzer K, Leus K (2017). IUCN guidelines for
891 determining when and how ex situ management should be used in
892 species conservation. *Conserv Lett* **10**: 361–6.
893 Meuwissen TH (1997). Maximizing the response of selection with a
894 predefined rate of inbreeding. *J Anim Sci* **75**: 934–40.
895 Nei M (1973). Analysis of gene diversity in subdivided populations. *Proc Natl*
896 *Acad Sci* **70**: 3321–3.
897 Norman AJ, Putnam AS, Ivy JA (2019). Use of molecular data in zoo and
898 aquarium collection management: benefits, challenges, and best
899 practices. *Zoo Biol* **38**: 106–18.
900 Nowell K, Jackson P (1996). *Wild cats: status survey and conservation action*
901 *plan.* IUCN/SSC Cat Specialist Group. IUCN: Gland.
902 Oliehoek PA, Windig JJ, Van Arendonk JAM, Bijma P (2006). Estimating
903 relatedness between individuals in general populations with a focus on
904 their use in conservation programs. *Genetics* **173**: 483–96.
905 Palomares F, Godoy JA, López-Bao JV, Rodríguez A, Roques S, Casas-Marce M,
906 *et al.* (2012). Possible extinction vortex for a population of Iberian lynx
907 on the verge of extirpation. *Conserv Biol* **26**: 689–97.
908 Peakall R, Smouse P (2012). GenAlEx 6.5: Genetic analysis in Excel. Population
909 genetic software for teaching and research – an update. *Bioinformatics* **1**:
910 6–8.
911 Peña L, Garcia P, Jiménez MA, Benito A, Pérez-Alenza MD, Sánchez B (2006).
912 Histopathological and immunohistochemical findings in lymphoid tissues
913 of the endangered Iberian lynx (Lynx pardinus). *Comp Immunol Microbiol*
914 *Infect Dis* **29**: 114–26.
915 Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, *et al.* (2014).
916 The biodiversity of species and their rates of extinction, distribution, and
917 protection. *Science* **344**: 1246752.
918 Pritchard DJ, Fa JE, Oldfield S, Harrop SR (2012). Bring the captive closer to
919 the wild: redefining the role of ex situ conservation. *Oryx* **46**: 18–23.
920 Rahbek C (1993). Captive breeding – a useful tool in the preservation of
921 biodiversity? *Biodivers Conserv* **2**: 426–37.
922 Redford KH, Jensen DB, Breheny JJ (2012). Integrating the captive and the
923 wild. *Science* **338**: 1157–8.
924 Rodríguez A, Calzada J (2015). Lynx pardinus. The IUCN Red List of
925 Threatened Species. *http://dx.doi.org/102305/IUCNUK2015-*
926 *2RLTST12520A50655794.en*.
927 Rodríguez A, Delibes M (1992). Current range and status of the Iberian lynx

928 Felis pardina Temminck, 1824 in Spain. *Biol Conserv* **61**: 189–96.
929 Rodríguez A, Delibes M (2002). Internal structure and patterns of contraction
930 in the geographic range of the Iberian lynx. *Ecography (Cop)* **25**: 314–28.
931 Ruiz-López MJ, Gañán N, Godoy JA, Del Olmo A, Garde J, Espeso G, *et al.* (2012).
932 Heterozygosity-fitness correlations and inbreeding depression in two
933 Critically Endangered mammals. *Conserv Biol* **26**: 1121–9.
934 Russello MA, Amato G (2007). On the horns of a dilemma: molecular
935 approaches refine ex situ conservation in crisis. *Mol Ecol* **16**: 2405–6.
936 Ryder O, Miller W, Ralls K, Ballou JD, Steiner CC, Mitelberg A, *et al.* (2016).
937 Whole genome sequencing of California condors is now utilized for
938 guiding genetic management. In: *International Plant and Animal Genome*
939 *XXIV Conference, 8-13 January 2016*, San Diego, CA, p .
940 Sambrook J, Russell DW (2006). Purification of nucleic acids by extraction
941 with phenol:chloroform. *Cold Spring Harb Protoc* **2006**: pdb.prot4455.
942 Schäfer F, Reiners TE (2017). Long term vs short term impact of founder
943 relatedness on gene diversity and inbreeding within the European
944 Endangered Species Programme (EEP) for the Nepalese red panda
945 (Ailurus f. fulgens). *J Zoo Aquarium Res* **5**: 86–91.
946 Seal US, Foose TJ, Ellis S (1994). Conservation Assessment and Management
947 Plans (CAMPs) and Global Captive Action Plans (GCAPs). In: Olney PJ,
948 Mace G, Feistner A (eds) *Creative conservation: interactive management*
949 *of wild and captive animals*, Springer Netherlands: Dordrecht, pp 312–25.
950 Simón MA, Gil-Sánchez JM, Ruiz G, Garrote G, Mccain EB, Fernández L, *et al.*
951 (2012). Reverse of the decline of the Endangered Iberian lynx. *Conserv*
952 *Biol* **26**: 731–6.
953 Snyder NFR, Derrickson SR, Beissinger SR, Wiley JW, Smith TB, Toone WD, *et*
954 *al.* (1996). Limitations of captive breeding in endangered species
955 recovery. *Conserv Biol* **10**: 338–48.
956 Templeton A (1986). Coadaptation and outbreeding depression. In: Soule M
957 (ed) *Conservation biology. The science of scarcity and diversity*, Sinauer
958 Assoc.: Sunderland, MA, pp 105–21.
959 The R Core Team (2015). R: a language and environment for statistical
960 computing. R Foundation for Statistical Computing, Vienna.
961 The SSC Re-introduction Specialist Group (1995). IUCN/SSC guidelines for re-
962 introductions. In: *Forty-first meeting of the IUCN Council*, Gland, p .
963 Tokarska M, Marshall T, Kowalczyk R, Wójcik JM, Pertoldi C, Kristensen TN, *et*
964 *al.* (2009). Effectiveness of microsatellite and SNP markers for parentage
965 and identity analysis in species with low genetic diversity: the case of
966 European bison. *Heredity (Edinb)* **103**: 326–32.
967 Toro MA, Caballero A (2005). Characterization and conservation of genetic
968 diversity in subdivided populations. *Philos Trans R Soc B Biol Sci* **360**:
969 1367–78.
970 Vargas A, Breitenmoser C, Breitenmoser U (2009). *Iberian lynx ex situ*
971 *conservation: an interdisciplinary approach.* Fundación Biodiversidad in
972 collaboration with: International Union for Conservation of Nature,
973 Species Survival Commission (IUCN /SSC) Cat Specialist Group: Madrid.
974 Vargas A, Rivas A, Sánchez I, Martínez F, Godoy JA, Roldán E, *et al.* (2009).

Iberian lynx conservation breeding program - update. *Cat News* **50**: 17.
Vargas A, Sánchez I, Martínez F, Rivas A, Godoy JA, Roldán E, *et al.* (2008). The Iberian lynx Lynx pardinus conservation breeding program. *Int Zoo Yearb* **42**: 190–8.
Wahlund S (1928). Zusammensetzung von populationen und korrelationserscheinungen vom standpunkt der vererbungslehre aus betrachtet. *Hereditas* **11**: 65–106.
Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–70.
Wilson EO (1992). *The diversity of life*. Belknap Press: Cambridge.
Witzenberger KA, Hochkirch A (2011). Ex situ conservation genetics: a review of molecular studies on the genetic consequences of captive breeding programmes for endangered animal species. *Biodivers Conserv* **20**: 1843–61.

Figure Legends

Fig. 1. Comparison of the number of alleles (in parentheses: number of alleles after removing singletons) between each captive wild-born set (CAP-WB) and its respective source population (FRL-WB). Left: Doñana population (DON); right: Andújar population (AND). Four admixed CAP-WB-DON individuals were excluded.

Fig. 2. Relatedness between pairs of CAP-WB individuals. Different shades of grey depict comparison within the AND subset, within the DON subset, and between DON and AND individuals. CAP-WB: captive wild-born individuals; DON: Doñana; AND: Andújar.

Fig. 3. Evolution of diversity and inbreeding statistics over time for each year's CAP-CB births (cohorts approach). a: observed heterozygosity (H_O) and expected heterozygosity (H_E); dashed lines represent the theoretical maximum H_E (OPT), and each FRL-WB population's H_E. b: fixation index (F_{IS}); the dashed line indicates $F_{IS} = 0$. DON: Doñana; AND: Andújar.

Fig. 4. Evolution of diversity statistics over time for each year's total population (census approach). a: observed heterozygosity (H_O); dashed lines represent the theoretical maximum H_E (OPT) and each FRL-WB population's H_E. b: expected heterozygosity (H_E), with the same dashed lines as in a. c: fixation index (F); the dashed line indicates $F_{IS} = 0$. CAP: captive population; CAP-CB: captive-born individuals; CAP-WB: captive wild-born individuals; FRL-WB: free-living wild-born individuals; DON: Doñana; AND: Andújar.

Fig. 5. Evolution of pedigree-based diversity and inbreeding statistics over time when enabling (ON) or not enabling (OFF) the empirical kinship matrix. a: genetic diversity (GD); b: inbreeding coefficient (F).

Supplementary Figure Legends

Supplementary Fig. S1. Evolution of the captive population size (N). a: yearly CAP-WB recruitments grouped by ancestry: pure AND, pure DON and backcross DON (DON x F1). b: yearly CAP-CB births broken down into three categories: dead (individuals that died before the 1st of November of the year in which they were born), released (individuals alive on the 1st of November that were eventually released from captivity), and alive (individuals alive on the 1st of November that were not later released). c: total number of CAP alive and still in captivity on the 1st of November of each year. CAP: captive individuals; CAP-CB: captive-born individuals; CAP-WB: captive wild-born individuals.

Supplementary Fig. S2. Map of the Southern Iberian Peninsula showing the distribution area of the two remnant populations (AND & DON, in dark grey) as well as the location of the main breeding facilities (black dots). AND: Andújar; DON: Doñana.

Supplementary Fig. S3. Theoretical distribution of H_E with varying contributions of AND (relative to DON). The theoretical maximum H_E value of 0.54 (dashed horizontal line) is attained when AND:DON proportions are 0.64:0.36 (dashed vertical line). H_E: expected heterozygosity; AND: Andújar; DON: Doñana.

Supplementary Fig. S4. Evolution of the proportions of captive wild-born individuals from Andújar (CAP-WB-AND; straight line) and actual contributing founders from AND (dashed line). CAP-WB: captive wild-born individuals; AND: Andújar; AND OPT: optimal proportion of AND founders. Proportions for captive wild-born individuals from Doñana (CAP-WB-DON) are complementary to these.

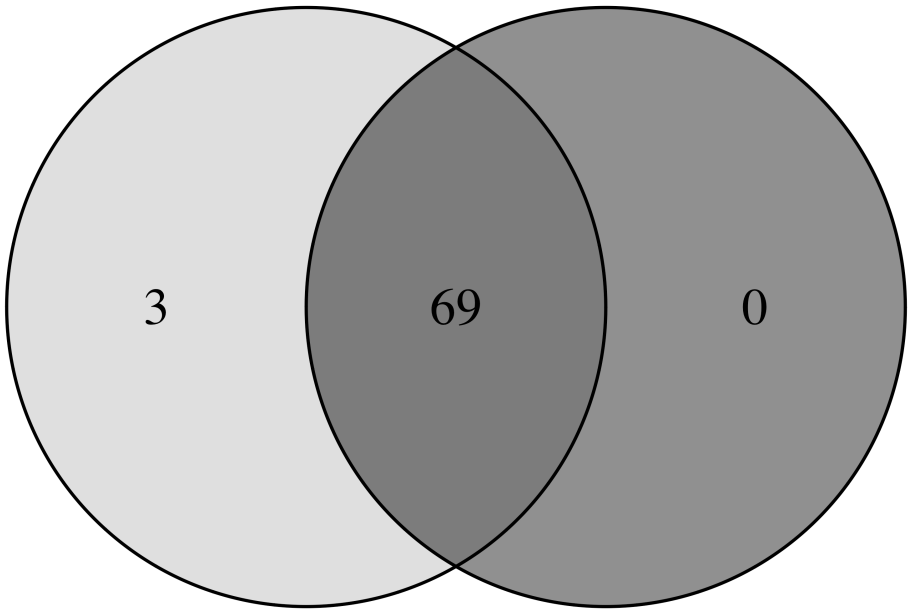
Supplementary Fig. S5. Percentage of relationships between pairs of CAP-WB individuals that fell within the following categories: related (classified by ML-RELATE as any combination of HS, FS and PO), unrelated (classified by ML-RELATE as U), and ambiguous (both U and any other kinship category included in the 95% confidence set). Left: pure CAP-WB-DON; right: CAP-WB-AND. CAP-WB: captive wild-born individuals; DON: Doñana; AND: Andújar.

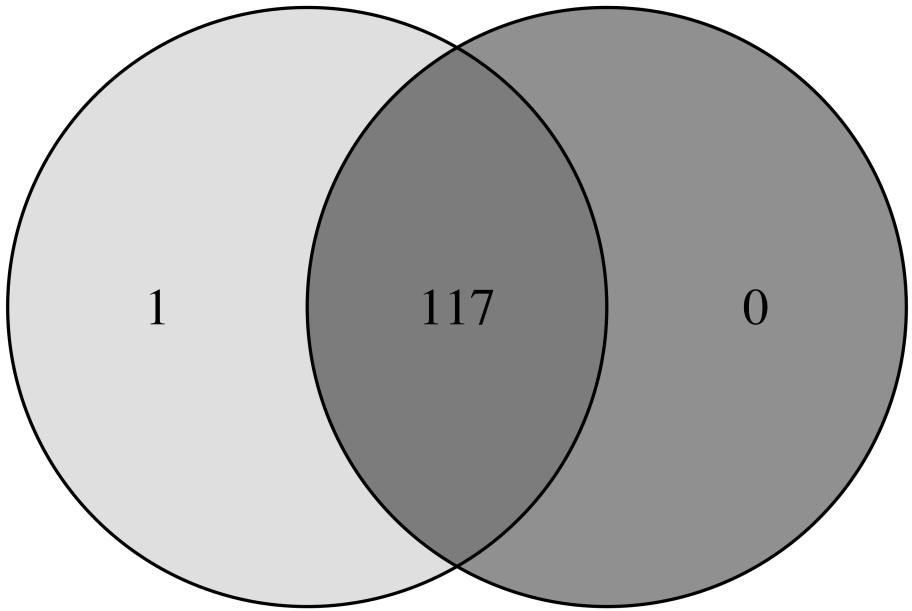
1045

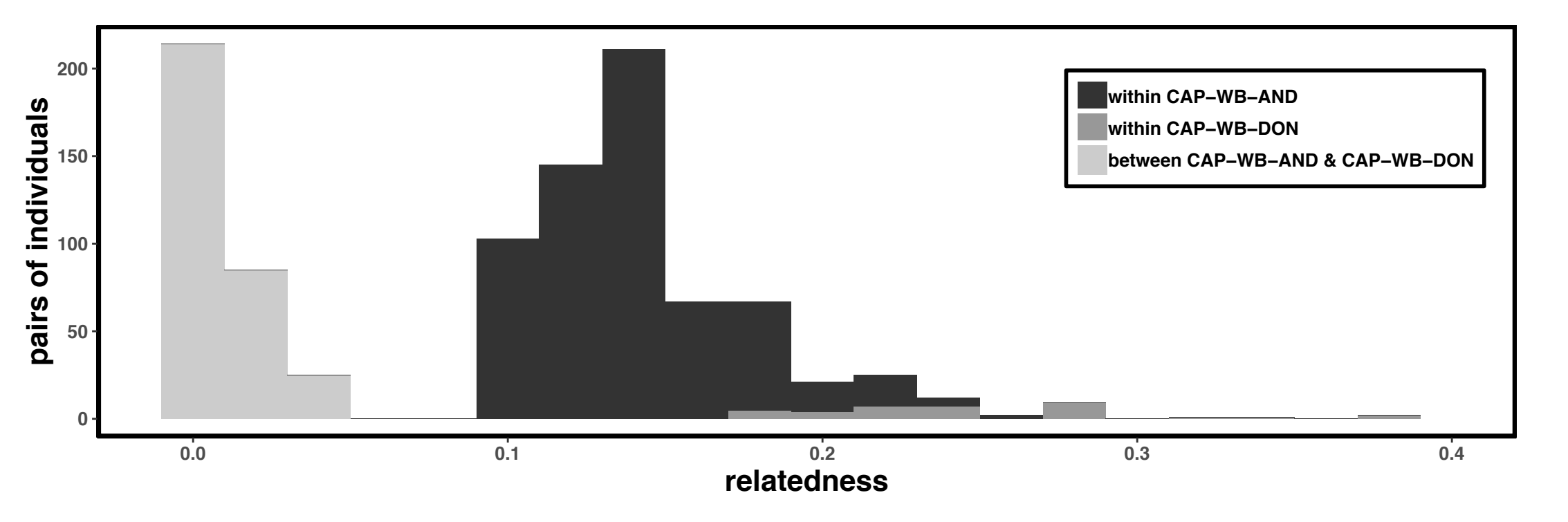
1046 Supplementary Fig. S6. Diagram of the genetic structure among the main breeding facilities that comprise

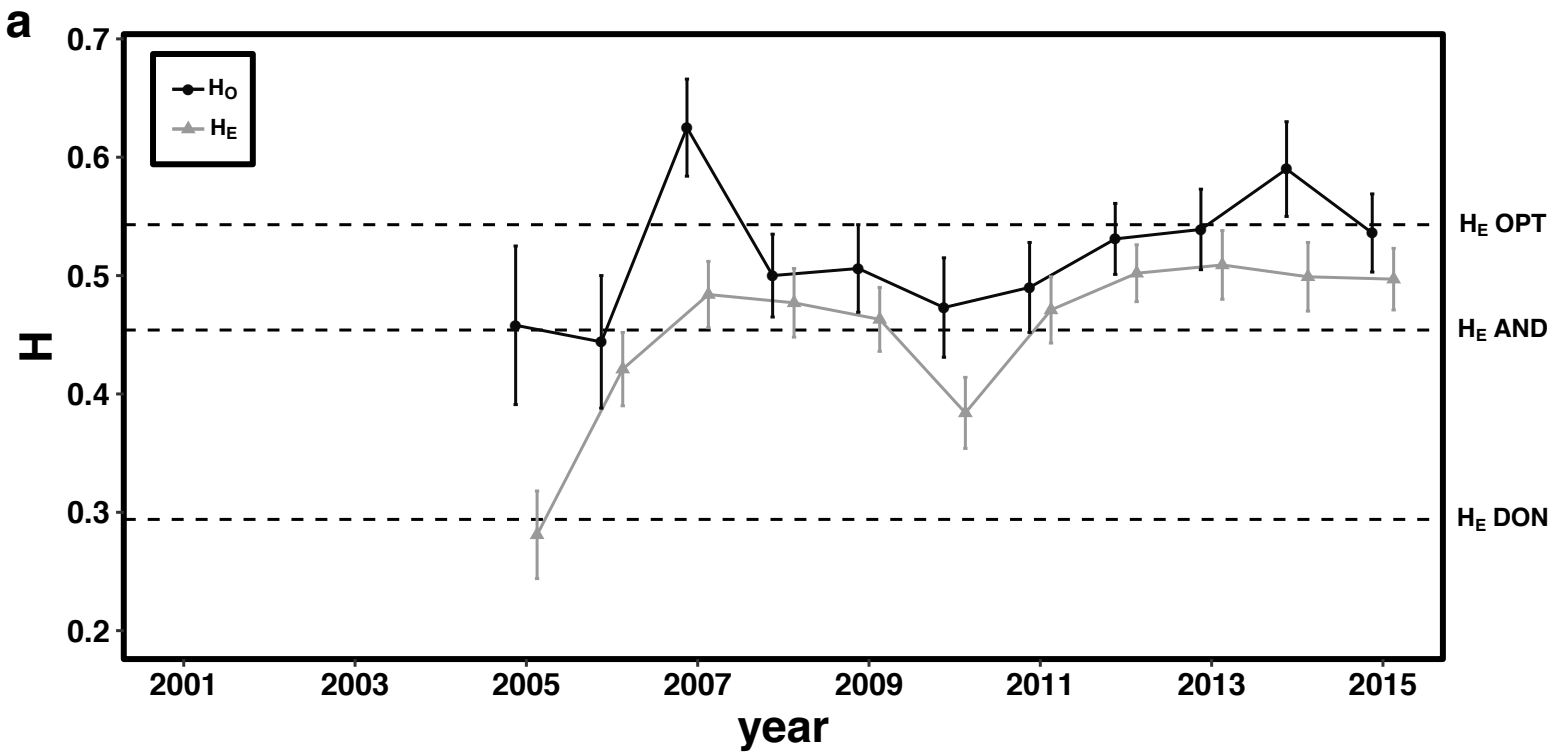
1047 the Iberian lynx *ex situ* programme, measured as FST values [99% CI].

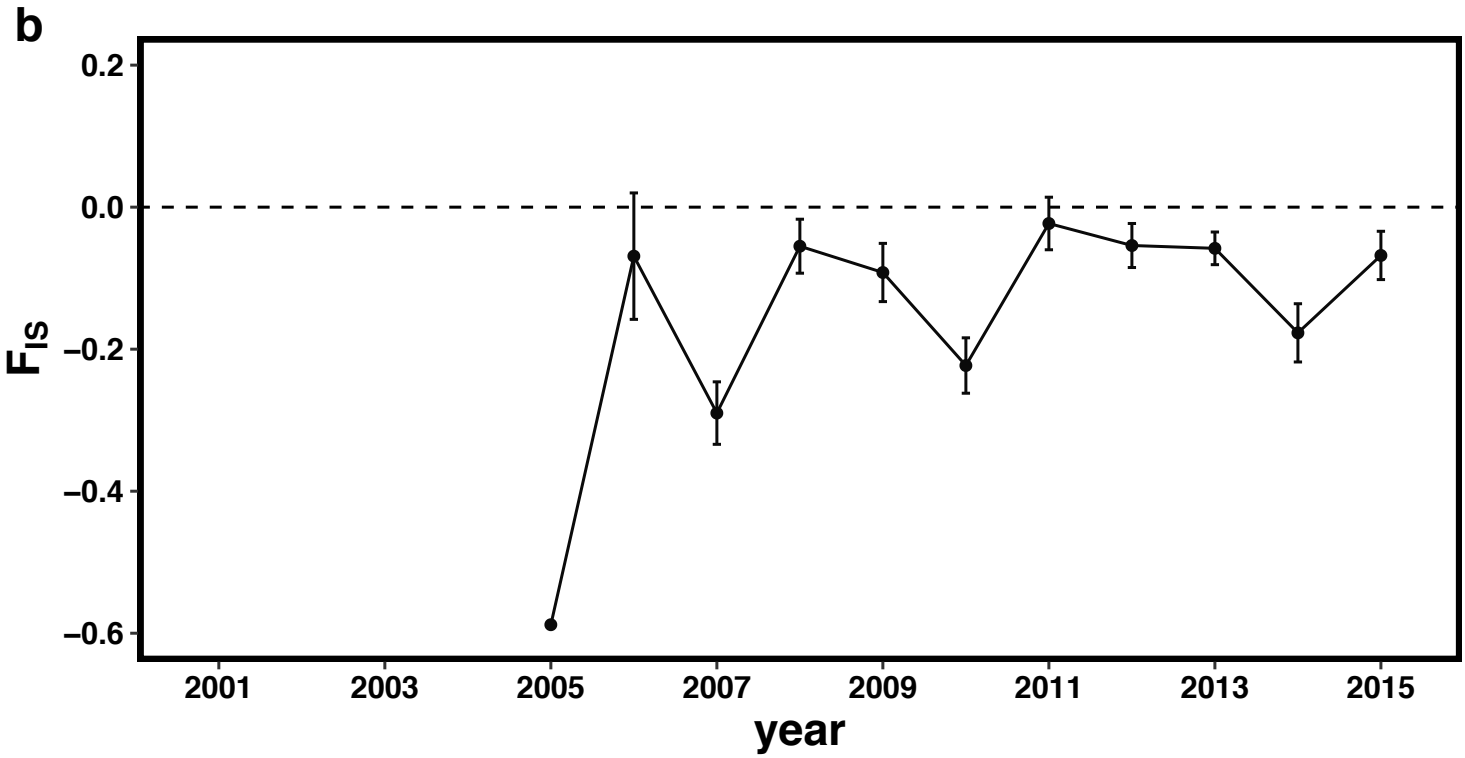
1048

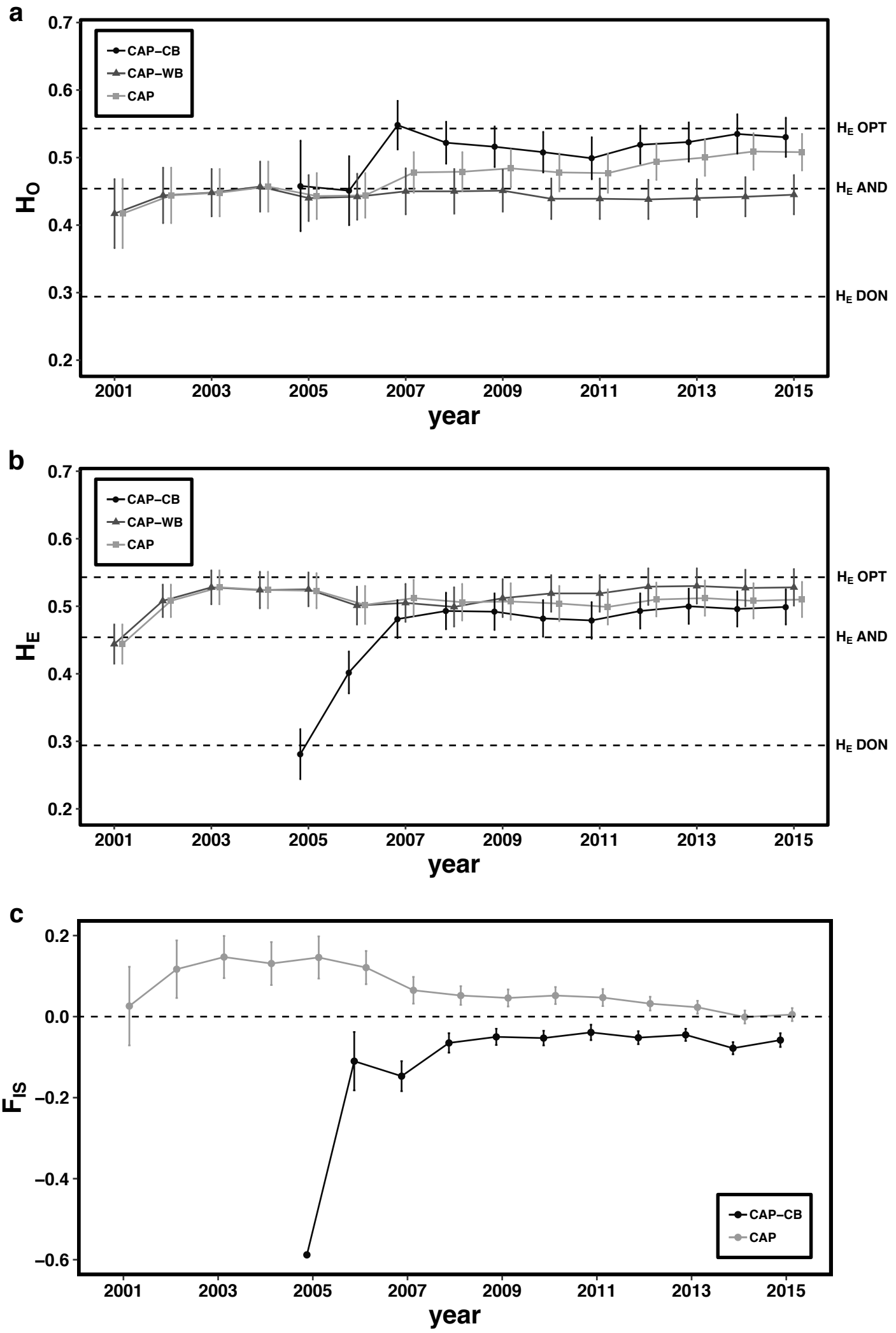

3
69
0
FRL-WB-DON
CAP-CB-DON (pure only)

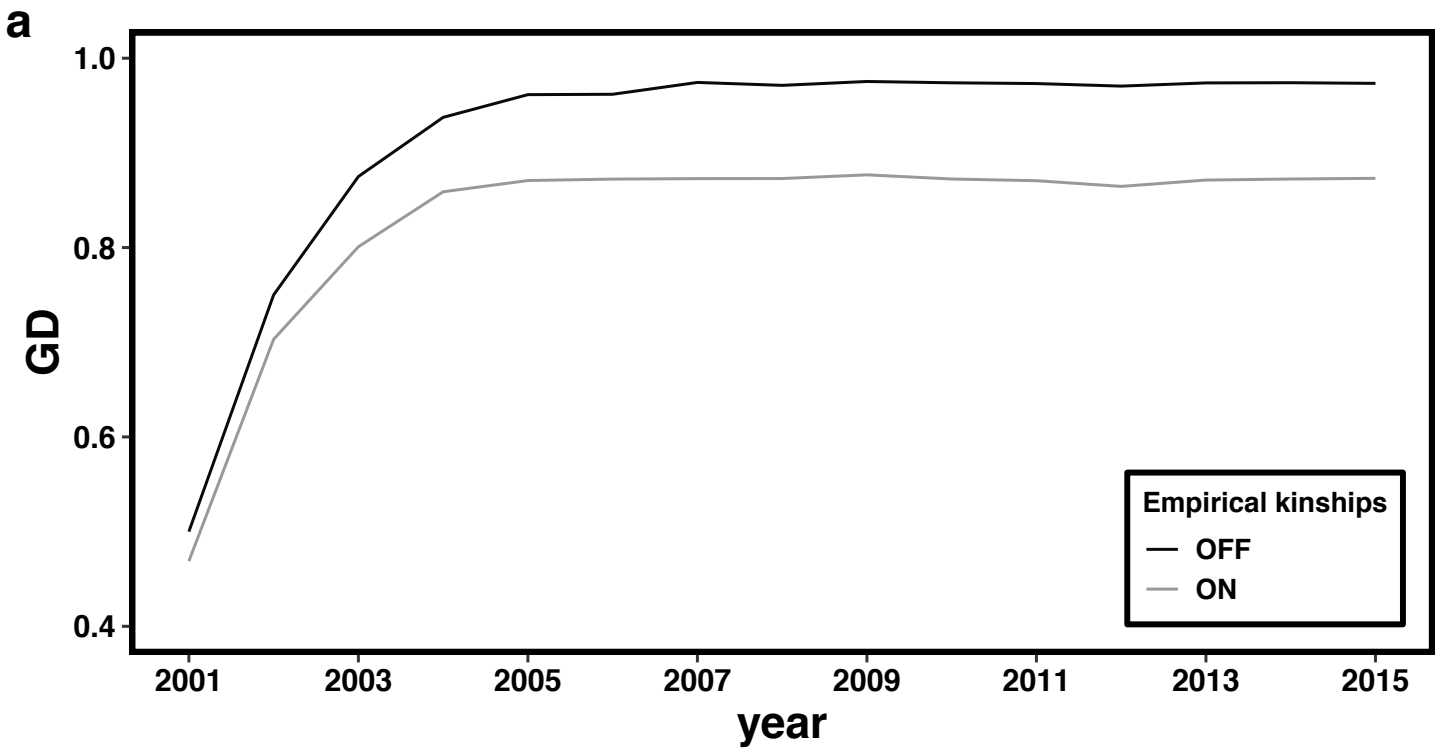

1
117
0
FRL-WB-AND
CAP-CB-AND

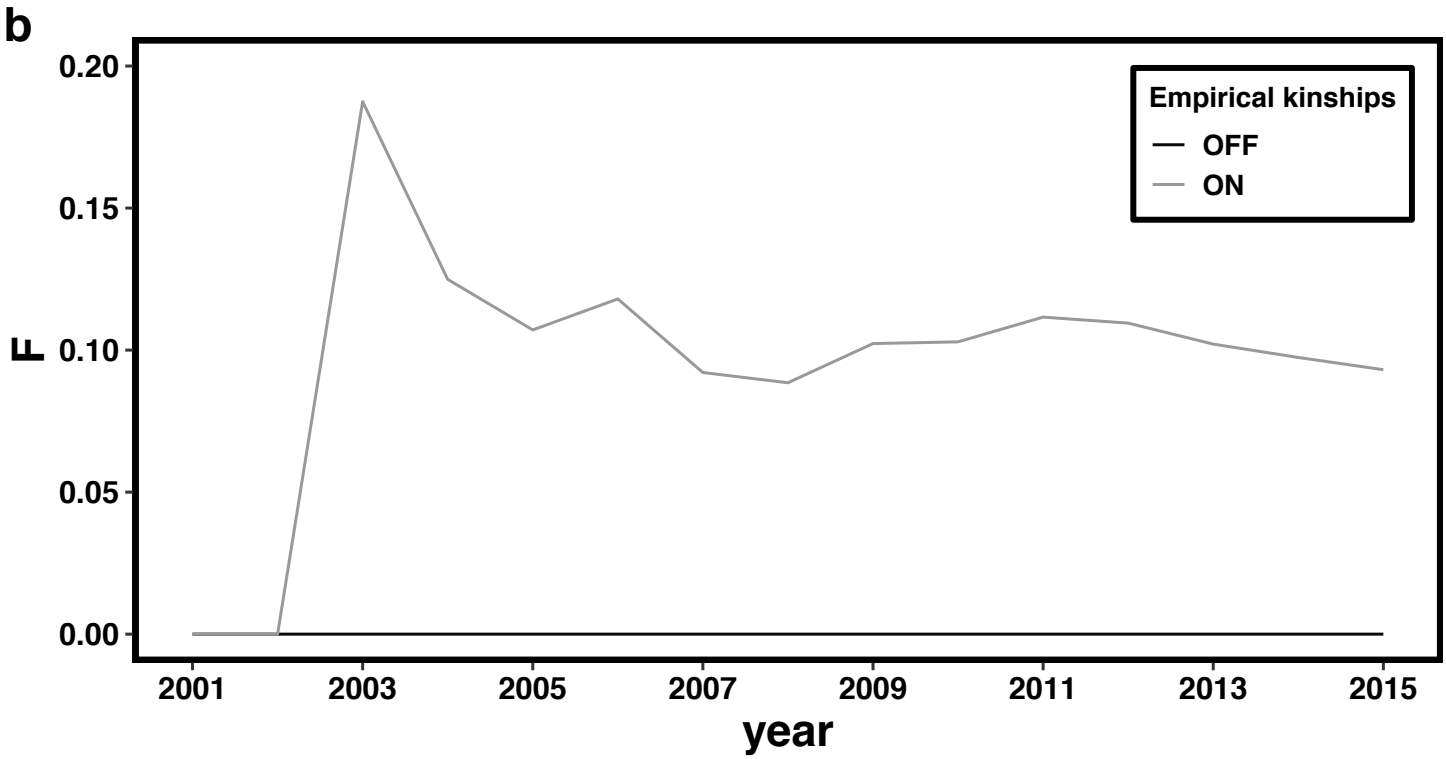

pairs of individuals
200
150
100
50
0
0.0
0.1
0.2
0.3
0.4
relatedness
within CAP–WB–AND
within CAP–WB–DON
between CAP–WB–AND & CAP–WB–DON

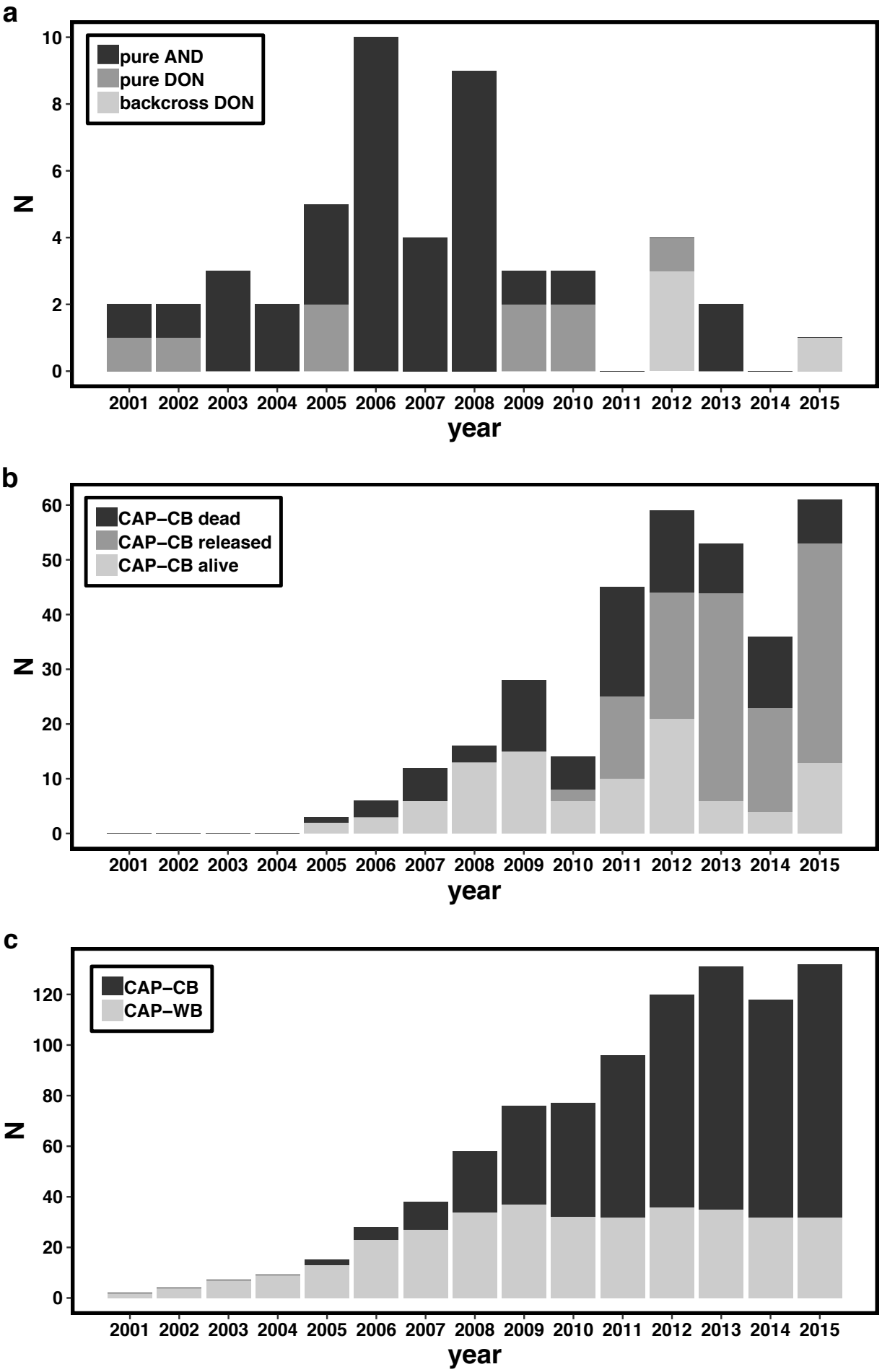

a
0.7
0.6
0.5
0.4
0.3
0.2
H
H_O
H_E
H_E OPT
H_E AND
H_E DON
2001
2003
2005
2007
2009
2011
2013
2015
year


b
0.2
0.0
−0.2
−0.4
−0.6
F_{IS}
2001
2003
2005
2007
2009
2011
2013
2015
year

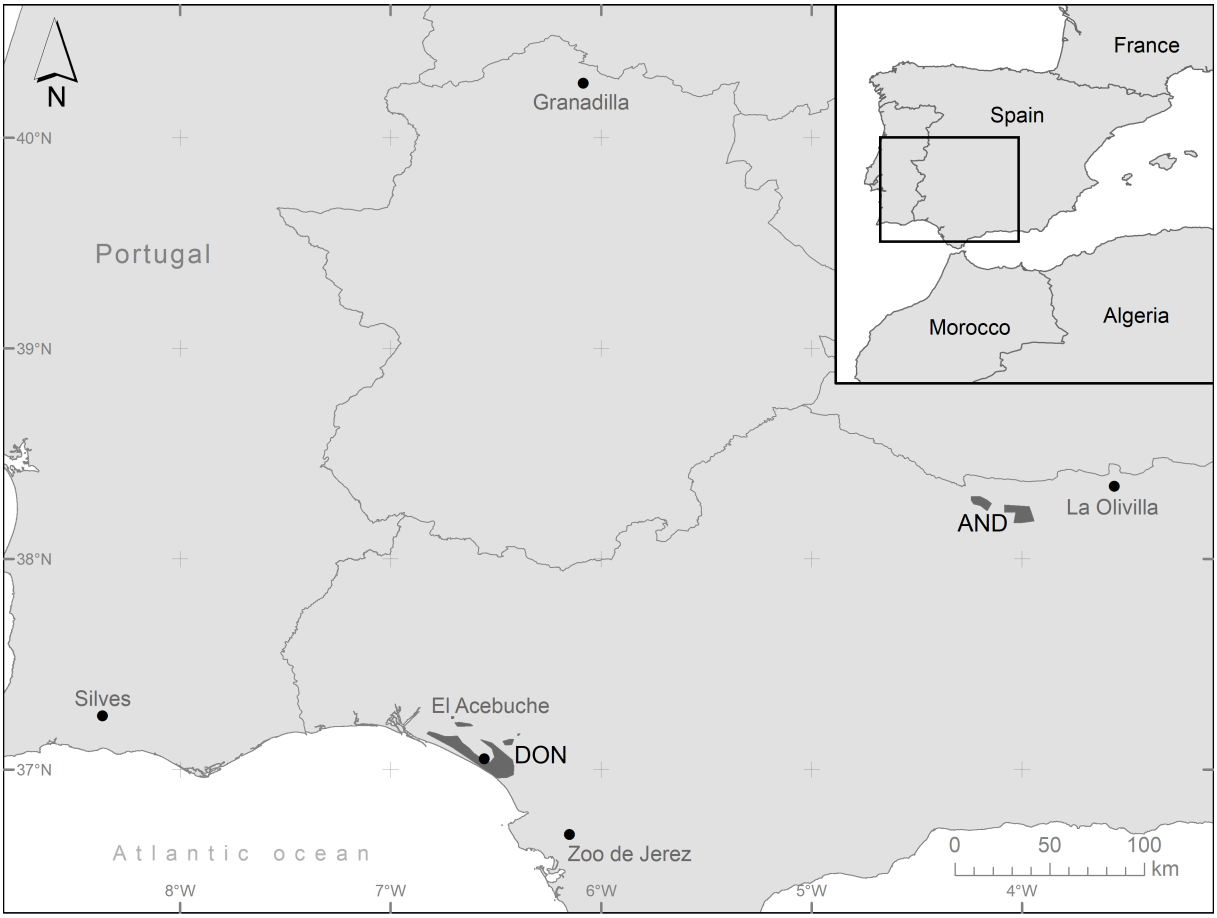

a
CAP–CB
CAP–WB
CAP
H_O
H_E OPT
H_E AND
H_E DON
year
b
CAP–CB
CAP–WB
CAP
H_E
H_E OPT
H_E AND
H_E DON
year
c
F_{IS}
CAP–CB
CAP
year


a
1.0
0.8
0.6
0.4
GD
2001
2003
2005
2007
2009
2011
2013
2015
year
Empirical kinships
OFF
ON

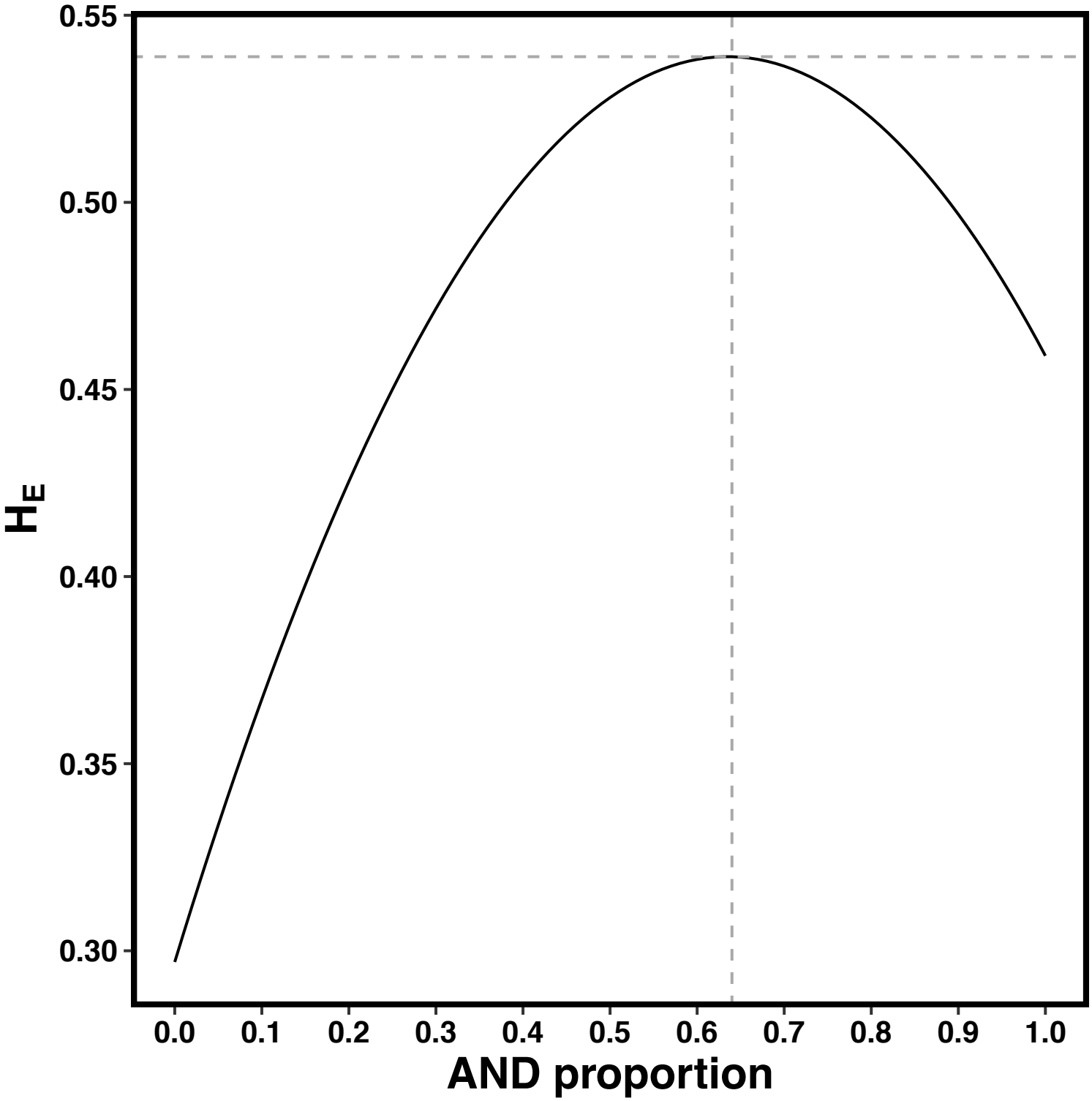

b
0.20
0.15
0.10
0.05
0.00
F
2001
2003
2005
2007
2009
2011
2013
2015
year
Empirical kinships
OFF
ON



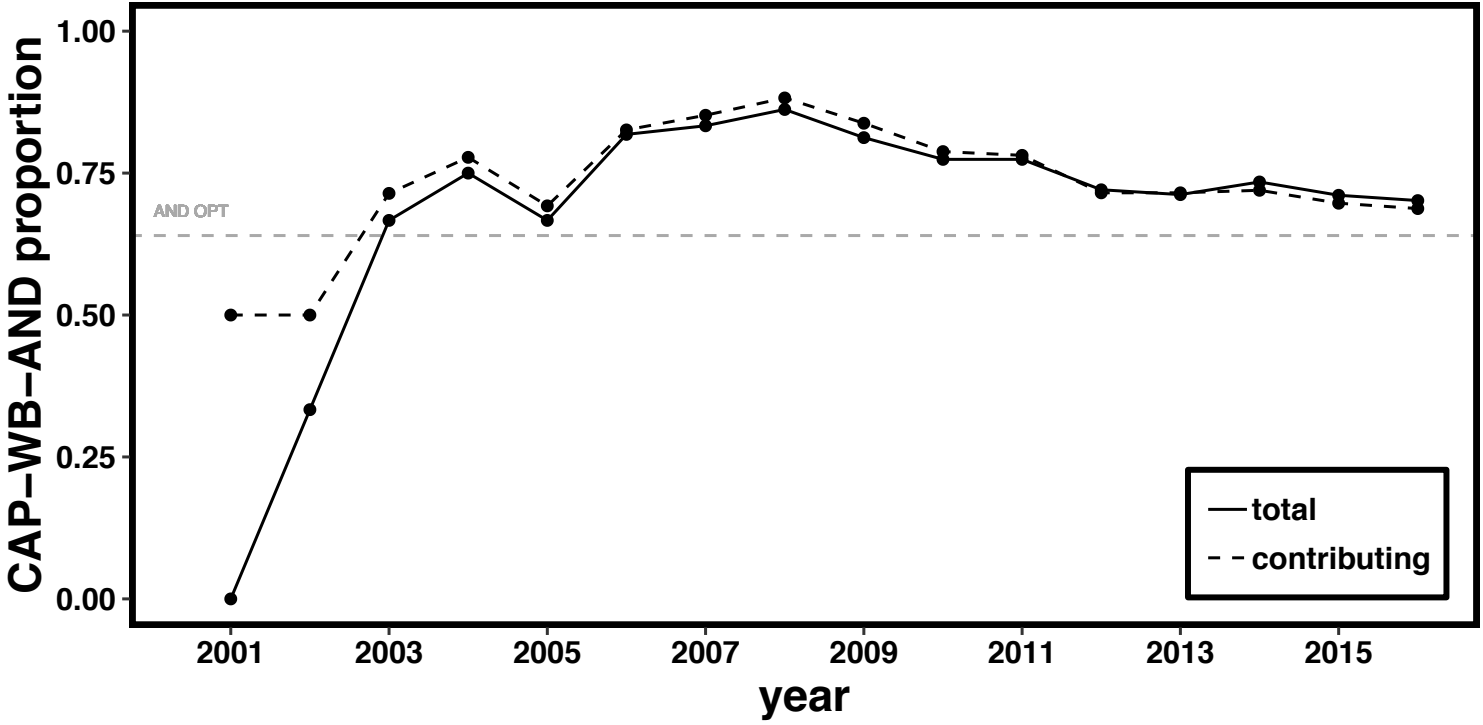
Supplementary Fig. S1. Evolution of the captive population size (N). a: yearly CAP-WB recruitments grouped by ancestry: pure AND, pure DON and backcross DON (DON x F1). b: yearly CAP-CB births broken down into three categories: dead (individuals that died before the 1st of November of the year in which they were born), released (individuals alive on the 1st of November that were eventually released from captivity), and alive (individuals alive on the 1st of November that were not released later). c: total number of CAP alive and still in captivity on the 1st of November of each year. CAP: captive individuals; CAP-CB: captive-born individuals; CAP-WB: captive wild-born individuals.



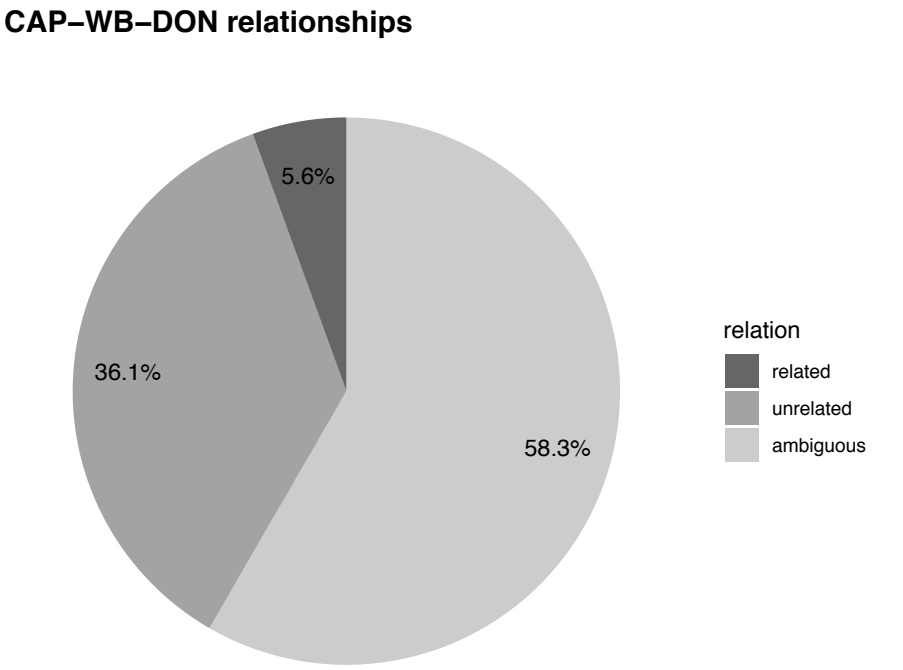
Supplementary Fig. S2. Map of the Southern Iberian Peninsula showing the distribution area of the two remnant populations (AND & DON, in dark grey) as well as the location of the main breeding facilities (black dots). AND: Andújar; DON: Doñana.

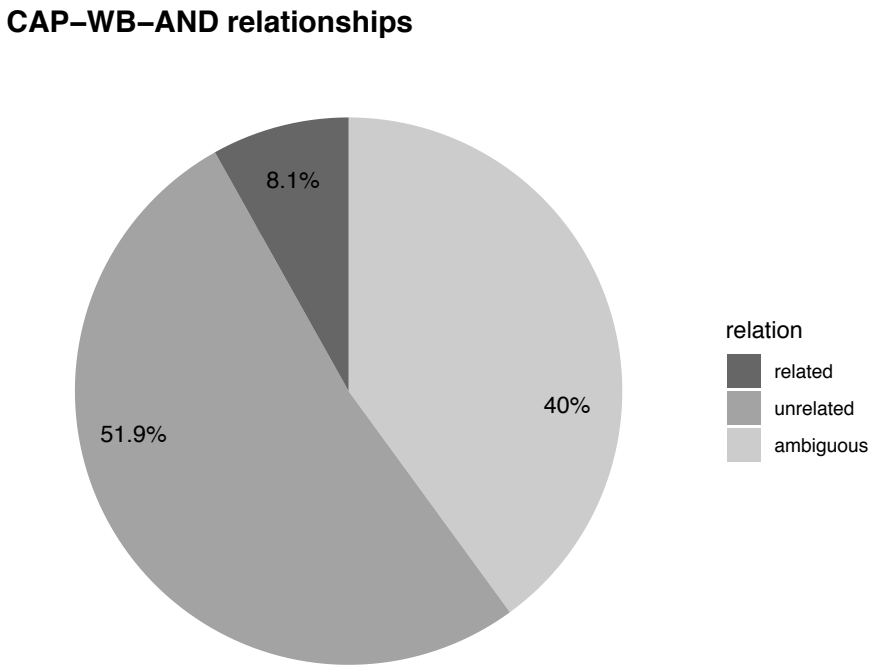


Supplementary Fig. S3. Theoretical distribution of H_E with varying contributions of AND (relative to DON). The theoretical maximum H_E value of 0.54 (dashed horizontal line) is attained when AND:DON proportions are 0.64:0.36 (dashed vertical line). H_E: expected heterozygosity; AND: Andújar; DON: Doñana.

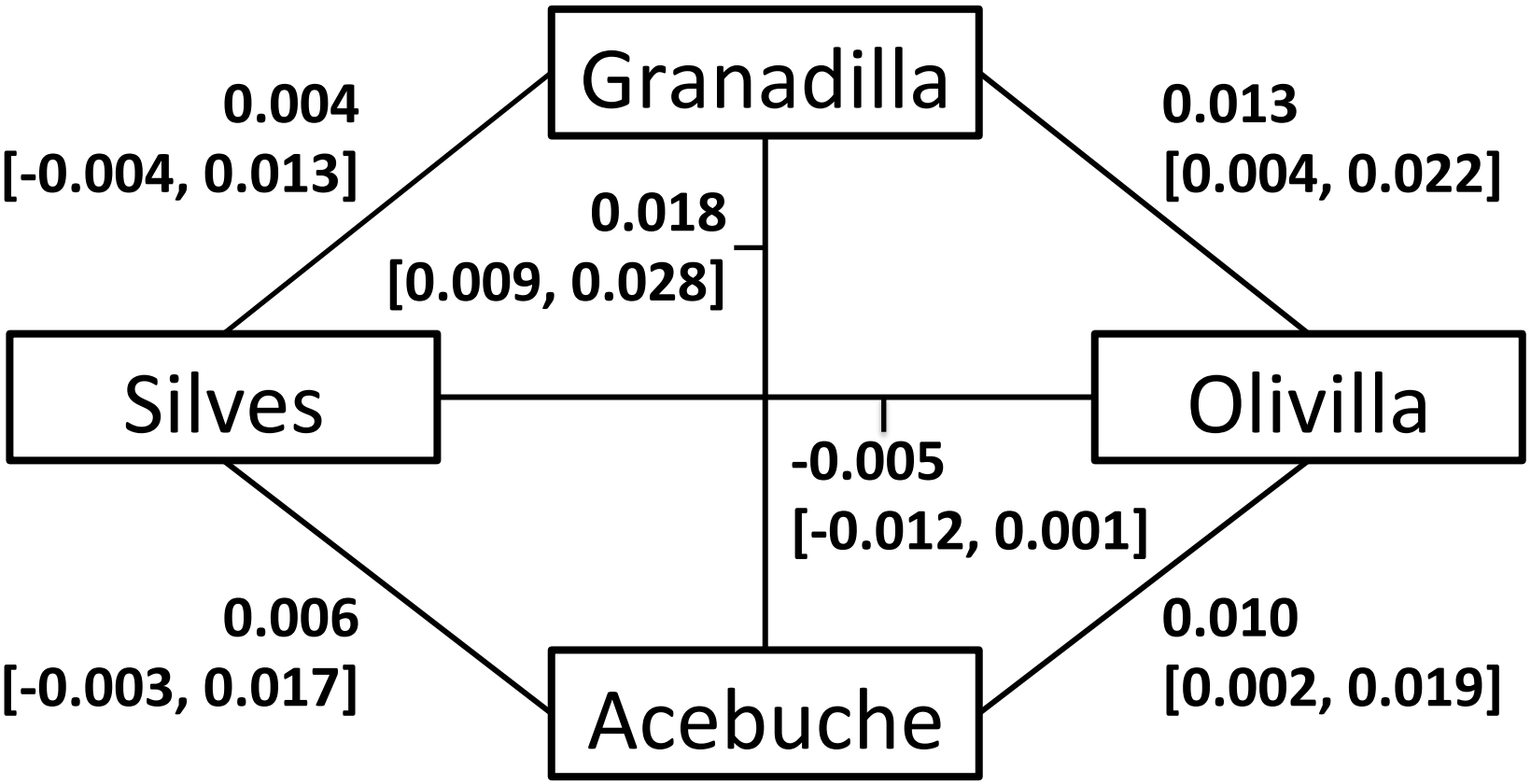


Supplementary Fig. S4. Evolution of the proportions of total captive wild-born individuals from Andújar (CAP-WB-AND; straight line) and actual contributing founders from AND (dashed line). CAP-WB: captive wild-born individuals; AND: Andújar; AND OPT: optimal proportion of AND founders. Proportions for captive wild-born individuals from Doñana (CAP-WB-DON) are complementary to these.





Supplementary Fig. S5. Percentage of relationships between pairs of CAP-WB individuals that fell within the following categories: related (classified by ML-RELATE as any combination of HS, FS and PO), unrelated (classified by ML-RELATE as U), and ambiguous (both U and any other kinship category included in the 95% confidence set). Left: pure CAP-WB-DON; right: CAP-WB-AND. CAP-WB: captive wild-born individuals; DON: Doñana; AND: Andújar.



Supplementary Fig. S6. Diagram of the genetic structure among the main breeding facilities that comprise the Iberian lynx ex situ programme, measured as FST values [99% CI].