Purging of deleterious burden in the endangered Iberian lynx

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Supporting Methods

Species and population demography
Current knowledge of the demographic history of the Eurasian and Iberian lynx mostly arises from previous inferences of effective population size through time with different methods. The two species were conjunctly analyzed using pairwise sequentially Markovian coalescent (PSMC) by Abascal et al. (1), who inferred a drastic reduction in population size in the two species from ca. 60,000 to ca. 12,000 (Iberian) or 14,000 (Eurasian) in the period 700–100 kya (see Fig. 1b in [1]), which may be related to their initial separation, followed by a transient increase to a maximum of 17,000 (Iberian) and 27,000 (Eurasian) before declining 10 kya to 6,000 (Iberian) and 17,000 (Eurasian). The same study investigated the more recent demography of the Iberian lynx through site frequency spectra (SFS) based methods, which inferred several serial bottlenecks throughout the species history: the first one, around 30,000 ya, reduced its effective size from ca. 27,000 to 2,500; the second one further lowered its size to 277 around 300 ya; the final one was invoked to account for $N_e \approx 30$ estimated for the period 2000-2007. The Eurasian lynx demography was later investigated by Lucena-Perez et al. (2), who confirmed the trajectory inferred previously for the $10^4 - 10^6$ ya period while observing a steeper decline of western than eastern populations since 100,000 ya. The more recent demography was reconstructed using Skyline plots and the linkage disequilibrium (LD) based SNeP software. Both methods suggested a continuous decline across all populations from a maximum of 60,000 to around 1,000 individuals in the last 10,000 years, with some variation in rate and numbers among populations. Therefore, the Eurasian lynx has maintained consistently higher (two to three-fold) effective sizes than the Iberian lynx throughout their histories, and although both species may have declined during the Holocene, the Eurasian lynx has undergone a more gradual decay, in contrast with the Iberian lynx whose recent demography seems to be marked by serial drastic bottlenecks which resulted in much smaller population sizes.

Regarding the Eurasian lynx populations analyzed here, Kirov (KIR) has apparently maintained effective sizes over 1,000 at least until very recently, whereas Poland (POL) and Norway (NOR), according to written records, went through population bottlenecks and became isolated during the last few centuries ([2] and references therein), as is reflected in their genetic diversity being, respectively, 80% and 74% of that found in KIR.

On the other hand, the two Iberian lynx populations analyzed here, which in the past were part of a likely panmictic population, are now the only remnants of a steep process of fragmentation, decline and extirpation spanning the last two centuries (3). Whereas Andújar (AND) became
isolated around 1950 and has since decreased in size to a minimum of ~21 estimated for the year 2002, Doñana (DON) became isolated ca. 200 ya (~40 generations ago) and has maintained effective sizes around 20 ever since, before reaching a new low of ~12.5 in 2002. This is reflected in their low overall genomic diversity, particularly in DON where it amounts to 52% of that found in AND.

Genomic datasets
We arranged samples in datasets (Table S7) for depth filtering purposes. Our analyses were carried out on 48 samples re-sequenced at a depth of ~5.75x (range: 5 to 7x) using Illumina HiSeq2000 v3 and v4. Of these, 20 samples (12 from AND and eight from DON) formed the Iberian lynx main (ILM) dataset, while the remaining 28 samples (12 from KIR, eight from NOR and eight from POL) composed the Eurasian lynx main (ELM) dataset. For validation purposes, we also incorporated one sample from KIR previously sequenced at a depth of 22x using Illumina HiSeq X-10 (4), which comprised the Eurasian lynx secondary (ELS) dataset. Lastly, we also included an Iberian lynx secondary (ILS) dataset, which consisted of a total of six AND and four DON individuals previously re-sequenced at a depth of ~26x (range: 24.5 to 28.5x) using Illumina HiSeq2000 v1.5 (1), and an additional individual from AND, the one providing the reference genome, which was subsampled to a depth in the range of the re-sequenced individuals (1). Although we did not use this older ILS dataset in the comparative analyses due to the presence of a large batch effect between it and the rest of the datasets (please refer to “Dataset comparisons” within the “Supporting Results and Discussion” section), we included it in the variant calling for the sake of enhancing the database of putatively deleterious mutations of the Iberian lynx. Finally, we also included one bobcat individual previously re-sequenced at the whole-genome level to assist in the polarization of mutations (4).

DNA extraction, library preparation, and sequencing
DNA was extracted from good quality tissue or blood samples (which were digested overnight using proteinase K) using NucleoMag B-beads (NucleoMag DNA from tissue kit) in the LEM-EBD (Seville, Spain). For each sample, 2.0 micrograms of genomic DNA were sheared on a Covaris™ E210, and then end-repaired, adenylated, and ligated to Illumina specific indexed paired-end adaptors using the NO-PCR protocol, TruSeq™ DNA Sample Preparation Kit v2 (Illumina Inc.), and the KAPA Library Preparation kit (Kapa Biosystems). Then, AMPure XP beads (Agencourt, Beckman Coulter) were used to select fragments with an insert size of 220-550bp. The Library Quantification Kit (Kapa Biosystems) was used to quantify the final
libraries, which were sequenced at the Centro Nacional de Análisis Genómico (CNAG-CRG; Barcelona, Spain) in a fraction of a sequencing lane of HiSeq2000 flowcell v3 (Illumina Inc.), in paired-end mode 2x101bp, and using TruSeq SBS Kit v3-HS (Illumina Inc.). Sequencing, primary data and image analysis, base calling, and quality scoring of the runs all followed standard Illumina procedures.

**Read processing and mapping**

Read processing and mapping were mostly performed as described elsewhere (2). Following the evaluation of the quality of the data using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc), SeqPrep (https://github.com/jstjohn/SeqPrep) was used to perform adapter trimming. Trimmed reads were mapped to the Iberian lynx reference genome using BWA-MEM (5) with default parameters. We then used picard-tools (https://broadinstitute.github.io/picard/) to add read groups to each bam file, and samtools merge (6) to merge all bam files from the same individual. Duplicates were marked with MarkDuplicates from Picard (https://broadinstitute.github.io/picard/) v2.4.1. In order to conduct a Base Quality Score Recalibration (BQSR), which is important to detect and correct systematic errors made by the sequencer when estimating the quality score of each base call, a set of known variants is necessary. GATK v3.4 (7) was used following GATK recommendations (https://gatkforums.broadinstitute.org/gatk/discussion/44/base-quality-score-recalibration-bqsr) to perform a local realignment and a preliminary round of variant calling on the unrecalibrated data. The resulting variants that fulfilled the following criteria: QD > 2 & MQ > 40 & FS < 100, were used to mask polymorphic sites during the BQSR. To minimize the risk of mismapping lineage-specific genomic elements and identifying potential contaminant variants as endogenous, we used the bamtools filter (8) to filter out reads with an edit distance (NM) higher than 2 (NM ≥ 3) and 3 (NM ≥ 4) in Iberian lynx and Eurasian lynx samples, respectively. These slightly different thresholds were adopted to account for the greater divergence between the Eurasian lynx and the Iberian lynx reference genome; however, results from all analyses were nearly identical if the less stringent NM = 3 threshold was universally applied to all samples. These points indicate altogether that the downstream detection of false positive variant calls supported by Eurasian-line specific genomic elements should be rare, but we further minimized any potential bias by using a combination of filters both prior to the variant calling (i.e., the exclusion of low mappability sites among other problematic regions, as described below in the “Variant filtering” section) as well as on the called set. In the latter case, variants found in sites that were genotyped in less than 85% of all individuals were discarded from further analyses, as
were those with low overall depth (DP < 200) or high depth (above the average plus three times the standard deviation) within any of the four datasets of samples (see below).

**Variant polarization**

We developed a multi-step pipeline with the purpose of distinguishing between the ancestral and derived state of the identified variants using information from the outgroup species of the *Lynx* genus, the bobcat (*Lynx rufus*), as well as two additional outgroups: the domestic cat (*Felis catus*), and the tiger (*Panthera tigris*). Briefly, we first retrieved the haploidized whole-genome sequence of the bobcat individual and used this information to polarize our initial set of variants. Then, for our final set of filtered variants, we: i) retrieved the cat and tiger information from the available Iberian lynx - cat - tiger synteny (1), ii) applied outgroup parsimony criteria to infer the common ancestral state of the two focal lynx species, and iii) fine-tuned the polarization accordingly.

In detail, as a first step we retrieved the haploid sequence (in fasta format) of the bobcat individual from its reads aligned to the Iberian lynx reference genome using the combination of: i) samtools mpileup (6), with the options –s –q30 to disregard sites with mapping quality lower than 30, and ii) pu2fa from Chrom-Compare (https://github.com/Paleogenomics/Chrom-Compare). By default, this procedure resolves haploidization at polymorphic sites by picking a random allele, and assigns an N to unmapped or low-quality sites. This way, the ancestral state was inferred for 97% of the bases in the reference genome. We then employed fill-aa from VCFtools v0.1.13 (9) to import the ancestral state into the INFO/AA (ancestral allele) field of the vcf file as a stepping-stone to the polarization of variants into ancestral and derived alleles (rather than reference and alternate state). Such rearrangement of the vcf file was carried out using VcfFilterJdk (10).

Next, we intersected our final curated dataset of variants with the available Iberian lynx - cat - tiger synteny (1) to retrieve the cat and tiger states for all these positions. We then applied outgroup analysis based on simple parsimony criteria (11) to infer the ancestral state at the innermost outgroup node of the phylogenetic tree. For those variants for which no information was available in tiger, the ancestral state was inferred based on the remaining two outgroup states. For those variants for which the synteny was not available (~10% of those in our dataset, and enriched in intergenic sites), we maintained the bobcat-based polarization. To carry out this second round of polarization, we used a custom script to edit the INFO/AA field of the vcf file, which was subsequently rearranged using VcfFilterJdk as before. Finally, the ancestral state
haploid sequence in fasta format was generated by editing that of the bobcat individual using a custom script. This ancestral state fasta was then used to build the proper new annotation database for SnpEff (12).

Consistency between the bobcat- and the parsimony-based polarization methods was very high, particularly within coding categories. For example, only 25 missense deleterious variants (0.29% of the total syntenic missense deleterious variants in the final dataset) showed differences between both methods, with all 25 resulting in unresolvable (rather than inverse) polarization under the parsimony-based method. In any case, misinterpreting an ancestral allele as derived due to a polarization error is more likely to happen in a species with more ancestral alleles, i.e., in a species where natural selection has been more efficient. Thus, polarization errors are in principle expected to mask the consequences of selection by overestimating the deleterious burden, producing a downward bias for the reduction of burden due to additive deleterious alleles (in the larger populations) and due to purging of recessive deleterious alleles (in the smaller ones). In other words, mispolarization is expected to reduce the experimental power to detect both purging and non-purging selection.

**Variant filtering**

A stepwise filtering framework was designed to keep only high confidence biallelic SNPs. First, INDELs and multiallelic SNPs (with three or more alleles) were removed from the dataset using GATK v3.7 SelectVariants (7). Second, monomorphic positions (i.e., those with the same allele fixed in both species), as well as variants that could not be polarized (i.e., those lacking the ancestral allele information), were filtered out using BCFtools (13). Third, GATK v3.7 SelectVariants was again used to apply hard-filters following the GATK best practices manual (14). Standard thresholds were accepted for all filtering statistics after inspection of their genome-wide distributions; in detail, variants were discarded if they met any of the following criteria: QUAL < 30.0, QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, and ReadPosRankSum < -8.0. Fourth, to exclude sites that were covered unequally across the different sequencing technologies or the two species, and those that were poorly covered in general, the command filter -e from BCFtools was applied with the flags F_MISSING > 0.15 & DP < 200. By doing so, only variants found in sites that were genotyped in at least 85% of all individuals and showed high overall depth were kept. Finally, all polymorphic sites with a depth exceeding the average plus three times the standard deviation in any of the four datasets of samples (i.e., ILM, ILS, ELM, ELS) were filtered out from the global dataset to discard false
variants caused by collapsed paralogs. For this purpose, depth distribution data was computed using ANGSD (15).

Additionally, we used GATK v3.7 SelectVariants to extract for each species the set of locally fixed variants, which were eventually used for fixation rate analyses, and the set of segregating variants, used to build population unfolded site frequency spectra (uSFS).

**Variant annotation**

We used SnpEff v4.3i (12) to annotate variants based on an annotation file that included only the principal isoforms, as identified in the Iberian lynx genome project (1). This file was subsequently modified to incorporate intergenic and intronic regions (which were delimited by exclusion criteria), as well as UCNEs, whose annotation was carried from the human genome assembly hg19 (16) to cat coordinates (assembly felCat5) using liftOver (http://rohsdb.cmb.usc.edu/GBshape/cgi-bin/hgLiftOver), and then to the Iberian lynx reference genome positions by using the available lynx-to-cat synteny (1). In the end, this custom annotation resource enabled the classification of variants into intergenic, intronic, CDS, and UCNE categories. The ability of SnpEff to predict the functional effects of variants allowed us to define three further mutation categories within CDS: synonymous, missense (i.e., non-synonymous), and LoF. The latter group comprises variants whose predicted impact on function is classified by SnpEff as high effect, including stop-gained, start-gained, start-lost, splice-acceptor, and splice-donor variants. Because LoF mutations are usually enriched in errors relative to other mutation types (17), we also identified high-confidence mutations to validate our results for this category (see next section). Given that some annotation categories constitute a wide amalgam of gene models and selection coefficients, we further broke these down into subclasses, as detailed below.

- Missense tolerated vs. deleterious:

  We classified missense variants into tolerated and deleterious based on their degree of conservation, the biochemical distance between the encoded aminoacids, and the sequence context, as inferred by Provean v1.1.5 (18). First, the fasta sequences of all genes (N = 21,243) in the bobcat genome were mined using bedtools getfasta (19). The conversion to the reverse complementary sequence (for genes in the reverse strand) and the removal of surplus nucleotides (to account for the reading frame in the case of partial genes) were performed with a custom linux-based script. Second, nucleotide sequences were translated to amino acids via the Translate web tool from the ExPASy Bioinformatics Resources Portal (20). Third, the ancestral and derived amino acids for
each missense variant were extracted from the vcf file. Fourth, the Provean
deletersiousness score was computed for each variant using Provean v1.1.5 with default
parameters. Fifth, missense mutations with a Provean score higher than -2.5 were
classified as “missense tolerated”, whereas those with a score equal to or lower than -2.5
were classified as “missense deleterious”.

· UCNE moderate-effect vs. UCNE high-effect:

Variants in regions annotated as UCNE were categorized into two groups according to
their presumed evolutionary constraint, estimated through Genomic Evolutionary Rate
Profiling (GERP) scores (21) that were remapped from human to lynx. First, we
downloaded the fasta sequences of the reference human genome, assembly
hg19/GRCh37, from the University of California Santa Cruz (UCSC) portal
(http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/chromFa.tar.gz) and used the
command makeblastdb from BLAST v2.6.0+ (22) to build the human BLAST database.
Second, we ran the command blastn to align all lynx UCNE sequences against the human
genome. Only the best hit was kept for each queried sequence. Third, the software
blastn2snp (http://lindenb.github.io/jvarkit/BlastNToSnp.html) with the minimum gap
size option set to 1 was used to retrieve the coordinates of gaps from both the query and
the hit sequences. By feeding this information to a custom linux-based script we were
then able to retrieve the exact nucleotide-level equivalence between the reference lynx
and human coordinates. Fourth, nucleotide-resolution GERP scores for these human
regions were downloaded from the UCSC portal (https://genome.ucsc.edu/cgi-
bin/hgTables). Fifth, GERP scores were remapped from human to lynx coordinates using
a custom linux-based script that incorporated the previously generated lynx-human
coordinates equivalence data. Finally, UCNE variants were classified into two groups
based on their GERP score: those with $2 < \text{GERP} \leq 5$ were grouped into “UCNE
moderate-effect”, while those with GERP $> 5$ were classified as “UCNE high-effect”.
Variants with GERP $\leq 2$ were discarded from further analyses on account of their low
number and their presumed low constraint.

Additional validation of LoF mutations

In order to validate our derived count results for the LoF category, which is usually enriched in
errors relative to other mutation categories (17), we conducted the following additional checks:
i) we computed the distribution of the number of different LoF mutations (i.e. LoF entries in the
vcf file) per gene, and ii) we computed the derived count (as described in the Main text) for two
different subsets of LoF mutations from which we excluded, respectively, those in genes at the
upper end of the mutation rate distribution (possibly pseudogenes), and those in genes which showed annotation problems. To obtain the former subset, we first counted the number of different mutations (i.e. entries in the vcf file) laying in coding sequences (CDS) per gene divided by the sum of the length of its CDS, and then discarded all LoF mutations occurring in the 570 genes for which this statistic was higher than 0.01. To generate the second subset, we simply filtered out all LoF mutations for which SnpEff (12) issued any annotation warning, including incomplete transcript warnings.

**Derived count in gene subsets**

We obtained the number of derived alleles per individual for the missense deleterious and LoF categories for two subsets of (presumably) functionally relevant genes: i) the one-to-one orthologs (N = 10,155), which were extracted from the available Iberian lynx-to-human orthologs database (1); and ii) genes intolerant to variation, defined as those with negative Residual Variation Intolerance Score (RVIS), which is a gene-based score of the deficit of standing functional variation (and therefore of the intensity of purifying selection) computed for human sequence data (23). To map RVIS values to genes in our annotation file, we first built a database of lynx genes for which their UniProt IDs were available. These included: i) genes in the aforementioned lynx-to-human orthologs database, from which we discarded all ‘many-to-many’ entries (N = 15,292 remained), and ii) multi-species one-to-one orthologs (N = 3,884) which had been used to annotate pathways in a previous study (1). Next, we downloaded the RVIS version of the data built on the Exome Aggregation Consortium (ExAC) v2 release 2.0 (http://genic-intolerance.org/data/RVIS_Unpublished_ExACv2_March2017.txt). In order to be able to intersect it with the orthologs database we converted gene names in the RVIS database to UniProtKB codes through the Retrieve/ID mapping tool (24) from the UniProt website (https://www.uniprot.org/uploadlists/), and filtered in only the reviewed results after selecting *Homo sapiens* as the species. A total of 17,564 unique UniProtKB IDs were retrieved, of which 12,976 had a match to the orthologs database, and 6,848 fulfilled the RVIS < 0 criterion.

**Derived count in genome compartments**

We also obtained the number of derived alleles per individual separately for the autosomal subtelomeric and pericentromeric regions. These were defined at the contig level by including those with at least 1,000 bp annotated previously as subtelomeric or pericentromeric (by taking 10 Mb from the chromosome end or around the centromere, respectively), based on the synteny with cat (1). We then calculated for each compartment the population arithmetic mean of the
individual counts, and the species average of the corresponding population means weighted by their corresponding sampling sizes.

Similarly, we counted the number of derived alleles per individual for the X chromosome, defined at the scaffold level based on the syntenic with cat (1), after discarding all bases assigned as subtelomeric in order to exclude the pseudoautosomal region. For this chromosome, to compensate for the fact that GATK (7) can only deal with one ploidy at a time, we divided the number of derived alleles by two in the case of males before obtaining its haploid population and weighted species averages.

**Per site estimates**
Average per site estimates for each category require an estimation of the total number of sites in the category following the filtering steps. Under the assumption that the effect of our filters on variant sites is proportional to that on monomorphic sites, we calculated the proportion of variants that passed the filters for the intergenic, intronic, coding, and UCNE categories, and then applied it to the callable number of sites in the respective category. This number was calculated as the sum of the lengths of all corresponding tracks in the annotation file after subtracting repetitive and low mappability regions, as these had previously been excluded from the variant calling. In the case of synonymous, missense, and LoF mutations, which are potential states of coding mutations rather than classes of sites, we used the following densities relative to the CDS category: 28.5%, 68.1%, and 3.4%, respectively, which were estimated by Yamaguchi-Kabata et al. (25) for the human genome. Finally, to compute the per-site estimates of heterozygosity, the number of filtered sites in the category was used as the denominator for the number of heterozygous derived alleles in the category.

**Statistical error and significance testing**
In order to estimate the statistical errors of the population and species averages for the different genomic variables, which allow testing whether the observed differences between them are significant, we need to consider two sources of variation. One is the sampling variance within each population or species, which produces a sampling error due to the limited number of individuals that have been sequenced. However, even if this sampling error was negligible (e.g., if a very high number of sequences from each population were available), populations that are evolving under identical selective pressures and demographic scenarios are expected to be different due to the stochastic nature of the evolutionary process (i.e., the random nature of
breeding events, recombination, segregation, mutation, etc.), and thus observed differences between the means of the populations or the two species may not be ascribed to differences in the forces governing evolution. In other words, evolutionary stochasticity must be considered as the second source of variation.

For each variable analyzed, the variance within (\(V_w\)) was estimated as the sample variance (for populations) or as the weighted average of the within population variances (for species). The sampling error of the average was then estimated as \(SE = \sqrt{V_w/T}\), where \(T\) is the total number of individuals available for that population or species. The stochastic evolutionary variance of each mean (\(V_s\)) was obtained by randomly resampling a set of genomic blocks 1,000 times to simulate additional sampling data (as detailed in the next section), a procedure referred to as bootstrapping in Simons and Sella (26). We then obtained the overall errors of the average of each population or species (\(M\)) as the square root of the corresponding overall variance, computed as \(\sigma(M) = \sqrt{SE^2 + V_s}\).

Finally, for each of the following five categories: synonymous, missense tolerated, missense deleterious, LoF, and UCNE, we used this \(\sigma(M)\) estimate for the average number of derived alleles per individual (uncorrected, and also corrected by the intronic and synonymous categories) to assess the significance of the differences between species using a Z-test. We controlled the overall type-I error of the five categories using the sequential Bonferroni test, and we also identified the contrasts that are significant after controlling for the FDR (27).

Error estimates and significance for tests focused on subtelomeric and pericentromeric regions were estimated analogously to those of the whole genome, although only blocks that had at least 85% of their sites annotated as subtelomeric or pericentromeric (respectively) were considered for the resampling simulation procedure (see next section). In addition, we used the Z-test to evaluate the significance of the differences between the two species for the variable \(D_{tel-cen}\), which results from subtracting the pericentromeric from the subtelomeric species average for the derived count. We plotted all aforementioned population and species averages, together with the estimated error bars, using the R (28) package ggplot2 (29).

**Resampling simulation procedure**

The stochastic evolutionary variance of each population or species mean (\(V_s\)) is obtained using a resampling simulation procedure, referred to as bootstrapping in Simons & Sella (26). For this purpose we split the ~2.4Gb genome into 2,400 blocks of ~1Mb (more specifically 1,005,504b)
of length each, and in order to generate a synthetic simulated genome of the same size as the
original one we resampled with replacement 2,400 blocks from the pool of 1,201 autosomal
blocks that lay in scaffolds of length above the required threshold of ~1Mb. We then retrieved
from this synthetic genome the individual counts for all the genomic variables and categories
(see Main text), and averaged them by population. This resampling procedure was repeated
1,000 times. Then for each original population average we computed the stochastic evolutionary
variance over the 1,000 simulated averages. Since the blocks over which we were able to
resample aren’t representative of the whole genome due to the fragmented nature of the
reference genome assembly, we adjusted these variances by the squared ratio between the
original empirical mean and the resampled overall mean. Finally, we averaged these adjusted
stochastic evolutionary variances \(V_s\) over populations weighing by the corresponding sample
sizes to obtain for each species an estimate of the stochastic evolutionary variance of the species
average. This estimate is conservatively large, as the evolutionary stochasticity can be larger at
the population than at the species level.

In the case of the telomeric (centromeric) regions, we first selected out of the aforementioned
1,201 autosomal blocks the pool of 141 (162) blocks that had at least 85% of their sites
annotated as telomeric (centromeric), and from these we resampled with replacement a total of
282 (324) blocks, which is the expected number for the whole genome. We then obtained the
individual number of derived alleles from these synthetic telomeric (centromeric) regions.
Mirroring the genome-wide procedure, we performed a total of 1,000 simulations, and estimated
the error rates as described before.

\textbf{R}_{X/Y} \textit{ratio}

In addition to counting the number of derived alleles per individual, we computed the \(R_{X/Y}\)
statistic, which compares two populations in terms of the number of derived alleles found at
sites within a particular genomic category in one population rather than the other (30, 31). To
compute this statistic, first we define

\[
L_{X,Y}(C) = \frac{\sum_{i \in C} f^X_i (1-f^Y_i)}{\sum_{i \in C} f^Y_i (1-f^X_i)},
\]

where \(C\) is a particular category within coding, \(i\) refers to each site in \(C\), and \(f^X_i\) is the derived
allele frequency in population \(X\) calculated as the number of derived alleles divided by the total
number of alleles found in the population. Similarly, \(f^Y_i\) is the derived allele frequency in
population \(Y\). Then, if \(N\) is the intronic category, we define
\[
R_{X/Y} = \frac{L_{X/Y}(C)}{L_{X/Y}(N)},
\]

normalizing the previous \(L_{X/Y}(C)\) ratio by its value over putatively neutral intronic sites, with the aim to mitigate any potential technical biases (e.g.: reference, calling or filtering biases) or population-specific differences in overall mutation rate.

We calculated the \(R_{X/Y}\) statistic for all combinations of population pairs within each species, and for KIR and AND (the less eroded population from each species) as a between-species comparison. Estimates of the variance in \(R_{X/Y}\) were obtained using 100 block jackknifes on the set of sites in \(C\).

\textbf{\(\pi_N/\pi_S\) and \(P_N/P_S\) ratios}

We calculated the missense (i.e. non-synonymous) and synonymous nucleotide diversity (\(\pi_N\) and \(\pi_S\), respectively) for each population on a site-by-site basis using VCFtools v0.1.13, and then averaged them across sites and obtained the \(\pi_N/\pi_S\) ratio, which is often interpreted as a measure of the efficacy of selection (e.g.: 32). A related statistic, the ratio of the number of missense to synonymous segregating sites, i.e. the \(P_N/P_S\) ratio (33), was calculated for each individual and then averaged across populations. For this purpose, segregating sites were defined at the species level.

\textbf{Theoretical predictions of the derived count}

Here we consider the model developed by García-Dorado (34, 35) to predict the genetic architecture of fitness and its evolution after a reduction in population size, including inbreeding load and inbreeding depression, and we extend it to obtain predictions for the expected value and evolution of the derived count. The derived count is defined as the overall number of derived alleles (segregating plus fixed) per individual or per gamete (i.e., the diploid or haploid derived count, respectively), and is synonymous with the deleterious burden in the case of (putatively) deleterious categories. For simplicity, in this theoretical context we use the haploid derived count. To better understand this approach, we will first assume an ancestral population with a very large effective size (\(N_0\)) that is close to the mutation-selection (MS) balance and has a haploid derived count of \(\Sigma q\), where \(q\) is the frequency of derived alleles; for the ancestral population this sum is only over segregating sites. At this MS equilibrium, the per generation increase in \(\Sigma q\) due to new mutation, which is just the haploid
rate of deleterious mutation ($\lambda$), is cancelled out by the change caused by selection [$\Delta(\Sigma q_{sel})$]. According to eq. 8 in (34):

$$\Delta(\Sigma q_{sel}) = -s \Sigma q \{h - K (1 - 2h)\}$$

(1)

where $s$ is the selection coefficient against the deleterious allele in homozygous genotypes, $h$ is the fraction of $s$ that is expressed in heterozygotes and $K = \Sigma q^2/\Sigma q$ is the proportion of deleterious copies that undergo selection in homozygosis (eq. 10 in 34). Therefore, at this MS balance, $\lambda - s \Sigma q \{h - K (1 - 2h)\} = 0$, so that the derived count in the ancestral population is

$$\Sigma q_0 = \frac{\lambda}{hs + K_0 s(1 - 2h)}$$

(2)

where the subscript 0 denotes the ancestral population. After the effective size reduces to $N_i$, the population will approach a mutation-selection-drift (MSD) balance where, assuming $N_i s > 1$, the derived count can be analogously approximated as

$$\Sigma q_1 = \frac{\lambda}{hs + K_1 s(1 - 2h)}$$

(3)

As the drift term is not included in this expression (see Eq. 11 in 34), this includes the fraction of the burden corresponding to deleterious alleles which, even though fixed in our population due to genetic drift, would be segregating at a population with infinite size that is undergoing exactly the same selective pressure (same $K_1$ value). Since the ratio of alleles in homozygosis is now larger ($K_1 > K_0$), for recessive deleterious alleles ($h < 1/2$) this burden will be smaller than in the ancestral population.

The transition from the ancestral to the new balance is described in (35) for the inbreeding load (eq. 11). Here we adapt that prediction to obtain the corresponding transition for the deleterious burden. This requires introducing the continuous increase in the deleterious burden ascribed to the limited efficiency of natural selection against the continuous mutation caused by genetic drift. This is approximated by the per generation rate of deleterious fixation at the new MSD balance, computed as $\lambda \cdot 2N_i U$, where $U$ is the fixation probability for a new mutation (eq. 3 in 36). Therefore, adding this continuous accumulation of deleterious mutations to the derived count deduced from the transition between the ancestral (eq. 2) and the new (eq. 3) balance, we obtain the following expression for the overall haploid deleterious derived count through generations ($t$):

$$L_t = \frac{\theta_t}{f_t} \Sigma q_0 (1 - f_t) + \Sigma q_1 \left[1 - \frac{\theta_t}{f_t} (1 - f_t)\right] + t(\lambda \cdot 2N_i U)$$

(4)
where $f_i$ and $g_i$ are the standard Wright inbreeding coefficient and the purged inbreeding coefficient, respectively (35). Predictions are separately obtained for each period of constant size, where the corresponding initial derived count is adjusted for the final derived count of the previous period.

This approach involves several approximations, including the assumption that deleterious alleles are initially at low frequency, and the absence of linkage disequilibrium, but it provides very illustrative predictions. To improve our understanding of the contribution of deleterious mutations of different effects to the evolution of the derived count of the different putatively deleterious categories, we computed these predictions for a set of representative $s,h$ values representing mutations of different severity ($s = 0.001, 0.01, 0.1$) and dominance. Since $h \approx 0$ can lead to huge amounts of hidden burden for very large populations and, therefore, to very large contributions of purging to the evolution of the overall derived count, we considered the more conservative $h = 0.05$ value and, for the sake of symmetry, $h = 0.25$ and 0.45.
Supporting Results and Discussion

Dataset comparisons
The Iberian lynx secondary (ILS) dataset, reported in a previous study (1), was sequenced with an older version of the Illumina HiSeq technology relative to the rest of samples, and showed several signs of comparatively worse quality, including a lower percentage of mapped reads among total reads, a considerably lower percentage of uniquely mapped reads among total reads (which came with a proportion of duplicate reads that was two orders of magnitude higher) and also, according to the fastQC ([https://www.bioinformatics.babraham.ac.uk/projects/fastqc](https://www.bioinformatics.babraham.ac.uk/projects/fastqc)) checks, good enough but lower median sequencing qualities with a notably higher variance, and often a slightly shifted distribution of the %GC when compared with samples from the other three datasets. In connection with this, there is a batch effect between the ILS dataset and the rest which subsampling procedures cannot correct nor soften, and which seems to be related to library preparation and sequencing technology biases. Even so, the ILS samples still showed consistently less derived alleles in the two key deleterious categories (missense deleterious and LoF) than the single Eurasian lynx secondary (ELS) sample with similar depth of coverage, but again, the presence of such a pervasive confounding factor entails that these comparisons are not really informative, and thus, we report comparisons between the Iberian lynx main (ILM) and Eurasian lynx main (ELM) datasets.

Notwithstanding, the ILS samples are still valuable at the variant discovery and classification (i.e. segregating vs. fixed) stages and, most importantly, during our stringent depth and missingness filtering steps, which take into account all datasets. In addition, these samples contribute lots of variants to the whole-genome database of potentially deleterious mutations that we report here for the first time, which is expected to be of great use for future lynx research and conservation efforts.

Finally, we observed almost identical patterns of derived counts between the ELM and ELS datasets across all annotation categories, both before and after subsampling the ELS sample to an average depth target in the range of the ELM samples. Considering that no batch effect was appreciated between these two datasets, these results suggest that our derived count estimates for the lower coverage samples are highly accurate.
Additional validation of LoF mutations

As part of the additional validation of the LoF mutations in our dataset we first examined the number of different LoF mutations (i.e. LoF entries in the vcf file) per gene. The highest number that we found was 3, and was recorded for two genes. Over 96% of all genes in our dataset with at least one LoF mutation had only one such mutation. Thus, no gene showed evident signs of abnormally high LoF mutation rates. Furthermore, the relative derived count difference between the two species for the LoF mutations after discarding those occurring in the 508 genes at the upper end of the mutation rate distribution was virtually identical to that for the unfiltered dataset. These results suggest that pseudogenes and highly mutated genes are excluded for the most part from our curated version of the Iberian lynx reference annotation, and importantly, they do not shape the derived count patterns of LoF mutations. For the second subset (from which sites with annotation issues were filtered out), and in spite of the high fraction of discarded mutations (~20%), the relative derived count differences between the two species did not vary much at all either (they became larger, although only very slightly so). This suggests that the patterns of LoF mutations in potentially wrongly annotated genes are very similar to patterns of LoF mutations in high confidence genes (see Fig. S5). Altogether, these checks strengthen the reliability of our findings on deleterious burden for the LoF category.

Derived count in gene subsets

For the two subsets of (presumably) functionally relevant genes analyzed here (i.e., one-to-one lynx-to-human orthologs, and genes with negative RVIS score), the relative differences between populations for their average number of derived alleles were very similar to our main results, with the Eurasian lynx populations consistently showing higher levels of putatively deleterious derived mutations than the Iberian lynx ones (Fig. S6). LoF mutations in the one-to-one orthologs comprised the sole exception, as they did not conform to any clear pattern of differences between populations or species, perhaps suggesting that those highly deleterious mutations which are purged faster in smaller than in larger populations tend to accumulate in (partially) redundant genes in the first place.

$R_{XY}$ ratio

The alternative approach based on the $R_{XY}$ ratio, a statistic that compares two populations in terms of the number of derived alleles found in one population rather than the other, led to results consistent with those based on the individual number of derived alleles; differences were
significant only for the between-species comparison, with AND showing a deficit of missense deleterious and LoF derived alleles when compared to KIR (Fig. S9).

Note that in the definition of this ratio the burden contributed by each derived allele is given a higher weight if the allele is less frequent in the other population, resulting in a measurement of the idiosyncratic burden of each population. Therefore, $R_{XY}$ depends on the ratio of the idiosyncratic burden of population $X$ to the idiosyncratic burden of population $Y$. Using idiosyncratic burden has the advantage of providing a scaling that enhances the differences in burden between the two populations, as if we were removing the burden that is common to both. It has the drawback of comparing a fraction of the burden that is defined only by reference to an alternative specific population, and is less intuitive to interpret in evolutionary terms. We argue that comparing this idiosyncratic burden instead of the overall burden is not expected to increase the power to detect differences between populations.

**$P_N/P_S$ and $\pi_N/\pi_S$ ratios**

Changes in genetic load in bottlenecked populations have often been interpreted through the analysis of patterns of polymorphism within coding sequences. More specifically, higher values for the $\pi_N/\pi_S$ and the $P_N/P_S$ ratios have been usually interpreted as a sign of the relaxation of purifying selection, and thus to an increased genetic load (e.g.: 37–39). Here we found similarly high values of $P_N/P_S$ among all five populations, ranging between 0.736 and 0.763, with the two Iberian lynx populations yielding the two lowest values (Fig. S8). The $\pi_N/\pi_S$ ratio showed an even smaller range between 0.321 and 0.330 (Fig. S8). While these values are comparable to those obtained for other threatened populations in previous studies, it’s important to remark that these comparisons may be hindered by technical biases related to differences in sequencing technology, coverage, sample size, variant calling and filtering, and calculation methods among others. Most importantly, they are affected by demography in complex ways, their behavior depends on the distribution of the selection coefficients and allele frequencies, and higher ratios may be caused by the quicker approach to equilibrium values of selected sites (26). These ratios have thus been considered poor estimators of the deleterious burden when compared to the individual-based values reported in this study.
Figure S1: average heterozygosity (with error bars) per annotation category and population relative to the Kirov population values. Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations. syn.: synonymous; m.: missense; tol.: tolerated; del.: deleterious; LoF: loss-of-function; UCNE: ultra-conserved non-coding elements; mod.: moderate; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S2: average derived homozygosity (with error bars) per annotation category and population relative to the Kirov population values. Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations. syn.: synonymous; m.: missense; tol.: tolerated; del.: deleterious; LoF: loss-of-function; UCNE: ultra-conserved non-coding elements; mod.: moderate; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S3: number of total (segregating and fixed) sites in homozygosis for the derived allele per annotation category and individual (each represented by one point). Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations. syn.: synonymous; m.: missense; tol.: tolerated; del.: deleterious; LoF: loss-of-function; UCNE: ultra-conserved non-coding elements; mod.: moderate; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S4: unfolded site frequency spectrum (uSFS) of segregating sites (defined at the species level) for each population (in columns) and annotation category (in rows). To construct the uSFS only sites with no missing data were considered. The rightmost bar of each panel corresponds to sites fixed at the population level. Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations; syn.: synonymous; m.: missense; tol.: tolerated; del.: deleterious; LoF: loss-of-function; UCNE: ultra-conserved non-coding elements; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S5: average number of derived alleles per population for different sets of LoF mutations: “all” includes all LoF mutations; “no_high” excludes LoF mutations in genes with the highest mutation rate; “no_warn” excludes LoF mutations in genes with annotation warnings (see text for details). Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations. LoF: loss-of-function; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S6: average number of derived alleles per population for different subsets of (purportedly) functionally relevant genes: “all” includes all genes; “1to1” includes only one-to-one lynx-to-human orthologs; “rvis<0” includes genes with negative RVIS score, i.e., genes intolerant to variation (see text for details). Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations. m.: missense; LoF: loss-of-function; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S7: average number of derived missense deleterious alleles (with error bars) per genomic region and species relative to the Eurasian lynx values. Error bars could not be calculated for the X chromosome, but the averages do not support the hypothesis of purging in the Iberian lynx X chromosome. Blue: Eurasian lynx; red: Iberian lynx; m.: missense; X chr.: X chromosome; EL: Eurasian lynx; IL: Iberian lynx.

Figure S8: average $\pi_N/\pi_S$ (left panel) and $P_N/P_S$ (right panel) values per population. Blue: Eurasian lynx populations; red: Iberian lynx populations; dark tones: largest population of each species; light tones: smaller populations. KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
Figure S9: relative number of derived alleles that are frequent in one population and not another ($R_{X/Y}$), i.e., the idiosyncratic burden, across different coding mutation categories. Error bars represent ±2SD. m.: missense; LoF: loss-of-function; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.

Figure S10: demography of the two populations for which predictions of the genomic load are represented in Fig. 4. This model is based on the known demography of the two lynx species analyzed here, with the thin line representing Iberian lynx demography, and the thick line a simplification of Eurasian lynx demography.
**Supporting Tables:**

Table S1: Average derived allele frequency of segregating sites (defined at the species level) for each population (in columns starting from the second) and annotation category (in rows).

| Category      | EL KIR | EL POL | EL NOR | IL AND | IL DON |
|---------------|--------|--------|--------|--------|--------|
| Intergenic    | 0.406  | 0.506  | 0.519  | 0.457  | 0.65   |
| Introns       | 0.402  | 0.505  | 0.519  | 0.449  | 0.649  |
| Synonymous    | 0.361  | 0.481  | 0.483  | 0.402  | 0.586  |
| Missense      | 0.356  | 0.47   | 0.476  | 0.395  | 0.595  |
| M. tolerated  | 0.37   | 0.483  | 0.493  | 0.41   | 0.605  |
| M. deleterious| 0.335  | 0.45   | 0.451  | 0.373  | 0.579  |
| LoF           | 0.328  | 0.425  | 0.442  | 0.362  | 0.544  |
| UCNE          | 0.283  | 0.386  | 0.391  | 0.343  | 0.506  |

m.: missense; LoF: loss-of-function; UCNE: ultra-conserved non-coding elements; EL: Eurasian lynx; IL: Iberian lynx; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.

Table S2: Summary of the statistical analysis of derived count differences between the Eurasian and the Iberian lynx, corrected by the intronic category. The table shows for the five tested genomic categories the mean derived count of Iberian lynx relative to Eurasian lynx and corrected by the intronic category, the value of the Z-test performed using the overall error of the species mean \( \sigma(M) \), and the resulting two-tailed p-value.

| Category          | Mean derived count of Iberian relative to Eurasian lynx (intronic-corrected) | Z-value | p-value (two-tailed) |
|-------------------|--------------------------------------------------------------------------------|---------|---------------------|
| Synonymous        | 0.974                                                                         | 0.865   | 0.387               |
| Missense tolerated| 1.006                                                                         | -0.171  | 0.865               |
| #Missense deleterious| 0.899                                                                       | 2.446   | 0.014               |
| # LoF             | 0.715                                                                         | 2.604   | 0.009               |
| UCNE              | 1.036                                                                         | -0.278  | 0.781               |

#The hypothesis of equal derived count for both species would be rejected in favor of the hypothesis that loads are different even after a 5%-significance sequential Bonferroni multitest adjustment or using a 5% False Discovery Rate (FDR); LoF: loss-of-function.
Table S3: Summary of the statistical analysis of derived count differences between the Eurasian and the Iberian lynx, corrected by the synonymous category. The table shows for the four tested genomic categories the mean derived count of Iberian lynx relative to Eurasian lynx and corrected by the synonymous category, the value of the Z-test performed using the overall error of the species mean $\sigma(M)$, and the resulting one-tailed p-value.

| Category            | Mean derived count of Iberian relative to Eurasian lynx (synon.-corrected) | Z-value | p-value (one-tailed) |
|---------------------|------------------------------------------------------------------------------|---------|----------------------|
| Synonymous          | 1                                                                            | 0       | 0.5                  |
| Missense tolerated  | 1.034                                                                       | -0.901  | 0.816                |
| Missense deleterious| 0.924                                                                       | 1.83    | 0.034                |
| * LoF               | 0.734                                                                       | 2.397   | 0.008                |

*The hypothesis of equal derived count for both species would be rejected in favor of the corresponding alternative purging hypothesis that load is smaller in Iberian lynx even after a 5%-significance sequential Bonferroni multitest adjustment or using a 5% False Discovery Rate (FDR); synon.: synonymous; LoF: loss-of-function.

Table S4: Summary of the statistical analysis of derived count differences between the Eurasian and the Iberian lynx in telomeric regions. The table shows for the five tested genomic categories the mean derived count of Iberian lynx relative to Eurasian lynx, the value of the Z-test performed using the overall error of the species mean $\sigma(M)$, and the resulting two-tailed p-value.

| Category            | Mean telomeric derived count of Iberian relative to Eurasian lynx | Z-value | p-value (two-tailed) |
|---------------------|------------------------------------------------------------------|---------|----------------------|
| Synonymous          | 0.946                                                            | 0.659   | 0.51                 |
| Missense tolerated  | 0.973                                                            | 0.3     | 0.768                |
| Missense deleterious| 0.792                                                            | 2.153   | 0.031                |
| LoF                 | 1.001                                                            | -0.004  | 0.997                |
| UCNE                | 0.738                                                            | 0.801   | 0.423                |

LoF: loss-of-function; UCNE: ultra-conserved non-coding elements.
Table S5: Summary of the statistical analysis of derived count differences between the Eurasian and the Iberian lynx in centromeric regions. The table shows for the five tested genomic categories the mean derived count of Iberian lynx relative to Eurasian lynx, the value of the Z-test performed using the overall error of the species mean $\sigma(M)$, and the resulting two-tailed p-value.

| Category       | Mean centromeric derived count of Iberian relative to Eurasian lynx | Z-value | p-value (two-tailed) |
|----------------|---------------------------------------------------------------------|--------|----------------------|
| Synonymous     | 0.967                                                               | 0.436  | 0.663                |
| Missense tolerated | 1.009                                                             | -0.095 | 0.925               |
| Missense deleterious | 1.046                                                             | -0.381 | 0.703               |
| LoF            | 0.715                                                               | 1.074  | 0.283               |
| UCNE           | 0.853                                                               | 0.385  | 0.7                  |

LoF: loss-of-function; UCNE: ultra-conserved non-coding elements.

Table S6: Summary of the statistical analysis of derived count differences between the Eurasian and the Iberian lynx for $D_{tel-cen}$, which results from subtracting the centromeric from the telomeric species averages. The table shows for the five tested genomic categories the mean derived count of Iberian lynx relative to Eurasian lynx, the value of the Z-test performed using the overall error of the species mean $\sigma(M)$, and the resulting two-tailed p-value.

| Category       | Mean $D_{tel-cen}$ derived count of Iberian relative to Eurasian lynx | Z-value | p-value (two-tailed) |
|----------------|---------------------------------------------------------------------|--------|----------------------|
| Synonymous     | 0.836                                                               | 0.251  | 0.401                |
| Missense tolerated | 0.871                                                              | 0.29   | 0.386               |
| Missense deleterious | 0.163                                                              | 1.868  | 0.031               |
| LoF            | -0.511                                                              | 0.711  | 0.239               |
| UCNE           | 0.461                                                               | 0.375  | 0.354               |

LoF: loss-of-function; UCNE: ultra-conserved non-coding elements.
Table S7: Summary of the studied samples, including the species and dataset which they belong to, their population of origin, their original report when applicable, and basic sequencing information.

| Sample       | Species     | Dataset | Population       | Original report                        | Sequencing platform | WG depth |
|--------------|-------------|---------|------------------|----------------------------------------|---------------------|----------|
| c_hr_zz_0001 | Lynx rufus  | -       | -                | -                                      | HiSeq X-10          | 25.66    |
| c_ll_ki_0090 | Lynx lynx   | ELS     | KIR (Kirov region)| Present study*                         | HiSeq X-10          | 21.89    |
| c_ll_ki_0091 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.11     |
| c_ll_ki_0092 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 5.32     |
| c_ll_ki_0093 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 5.29     |
| c_ll_ki_0094 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 5.85     |
| c_ll_ki_0095 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 5.29     |
| c_ll_ki_0096 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.12     |
| c_ll_ki_0097 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.03     |
| c_ll_ki_0098 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 5.78     |
| c_ll_ki_0099 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.01     |
| c_ll_ki_0100 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.17     |
| c_ll_ki_0101 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.30     |
| c_ll_ki_0102 | Lynx lynx   | ELM     | KIR (Kirov region)| Present study*                         | HiSeq2000,v3        | 6.07     |
| c_ll_no_0075 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.43     |
| c_ll_no_0076 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.80     |
| c_ll_no_0077 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.14     |
| c_ll_no_0078 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.25     |
| c_ll_no_0079 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.09     |
| c_ll_no_0080 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.22     |
| c_ll_no_0081 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.54     |
| c_ll_no_0082 | Lynx lynx   | ELM     | NOR (Norway)     | Present study*                         | HiSeq2000,v3        | 5.36     |
| c_ll_po_0001 | Lynx lynx   | ELM     | POL (Białowieża Primeval Forest) | Present study* | HiSeq2000,v4 | 6.52    |
| c_ll_po_0002 | Lynx lynx   | ELM     | POL (Białowieża Primeval Forest) | Present study* | HiSeq2000,v4 | 6.61    |
| c_ll_po_0003 | Lynx lynx   | ELM     | POL (Białowieża Primeval Forest) | Present study* | HiSeq2000,v4 | 6.44    |
| c_ll_po_0011 | Lynx lynx   | ELM     | POL (Białowieża Primeval Forest) | Present study* | HiSeq2000,v4 | 6.45    |
| c_ll_po_0014 | Lynx lynx   | ELM     | POL (Białowieża Primeval Forest) | Present study* | HiSeq2000,v4 | 6.39    |
| c_ll_po_0019 | Lynx lynx   | ELM     | POL (Białowieża Primeval Forest) | Present study* | HiSeq2000,v4 | 6.38    |
| c_ll_po_0015 | Lynx lynx   | ELM     | POL (Knyszyn Primeval Forests) | Present study* | HiSeq2000,v4 | 6.06    |
| c_ll_po_0016 | Lynx lynx   | ELM     | POL (Knyszyn Primeval Forests) | Present study* | HiSeq2000,v4 | 6.11    |
| c_lp_do_0007 | Lynx pardinus | ILS     | DON (Doñana-Aljarafe) | Abascal et al. 2016 | HiSeq2000,v1.5 | 24.81    |
| c_lp_do_0153 | Lynx pardinus | ILS     | DON (Doñana-Aljarafe) | Abascal et al. 2016 | HiSeq2000,v1.5 | 24.98    |
| c_lp_do_0173 | Lynx pardinus | ILS     | DON (Doñana-Aljarafe) | Abascal et al. 2016 | HiSeq2000,v1.5 | 26.14    |
| c_lp_do_0443 | Lynx pardinus | ILS     | DON (Doñana-Aljarafe) | Abascal et al. 2016 | HiSeq2000,v1.5 | 26.51    |
| c_lp_sm_0138 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | HiSeq2000,v1.5 | 26.20    |
| c_lp_sm_0140 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | HiSeq2000,v1.5 | 26.38    |
| c_lp_sm_0185 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | HiSeq2000,v1.5 | 24.64    |
| c_lp_sm_0186 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | HiSeq2000,v1.5 | 26.21    |
| c_lp_sm_0221 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | GAIIx + HiSeq2000 | 27.78    |
| c_lp_sm_0298 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | HiSeq2000,v1.5 | 28.42    |
| c_lp_sm_0359 | Lynx pardinus | ILS     | AND (Andújar-Cardeña) | Abascal et al. 2016 | HiSeq2000,v1.5 | 28.33    |
| c_lp_do_0141 | Lynx pardinus | ILM     | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 4.99     |
| c_lp_do_0144 | Lynx pardinus | ILM     | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 6.03     |
| c_lp do 0162 | Lynx pardinus | ILM | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 5.84 |
|---------------|----------------|-----|-----------------------|--------------|-------------|-----|
| c_lp do 0163 | Lynx pardinus | ILM | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 5.57 |
| c_lp do 0300 | Lynx pardinus | ILM | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 5.61 |
| c_lp do 0333 | Lynx pardinus | ILM | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 6.14 |
| c_lp do 0335 | Lynx pardinus | ILM | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 5.19 |
| c_lp do 0444 | Lynx pardinus | ILM | DON (Doñana-Aljarafe) | Present study | HiSeq2000,v3 | 5.27 |
| c_lp sm 0134 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.89 |
| c_lp sm 0155 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.50 |
| c_lp sm 0156 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 6.02 |
| c_lp sm 0161 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.26 |
| c_lp sm 0206 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.66 |
| c_lp sm 0208 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.32 |
| c_lp sm 0213 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.46 |
| c_lp sm 0226 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.59 |
| c_lp sm 0276 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.55 |
| c_lp sm 0320 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.57 |
| c_lp sm 0325 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.40 |
| c_lp sm 0450 | Lynx pardinus | ILM | AND (Andújar-Cardeña) | Present study | HiSeq2000,v3 | 5.48 |

* The intergenic sequences of these samples were previously reported in Lucena-Perez et al. (2020).

ELS: Eurasian lynx secondary dataset; ELM: Eurasian lynx main dataset; ILS: Iberian lynx secondary dataset; ILM: Iberian lynx main dataset; KIR: Kirov; POL: Poland; NOR: Norway; AND: Andújar; DON: Doñana.
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