Running head: Introgression in hares

Title: The legacy of recurrent introgression during the radiation of hares

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ABSTRACT

Hybridization may often be an important source of adaptive variation, but the extent and

long-term impacts of introgression have seldom been evaluated in the phylogenetic

context of a radiation. Hares (*Lepus*) represent a widespread mammalian radiation of 32

extant species characterized by striking ecological adaptations and recurrent admixture.

To understand the relevance of introgressive hybridization during the diversification of

Lepus, we analyzed whole exome sequences (61.7 Mb) from 15 species of hares (1- 4

individuals per species), spanning the global distribution of the genus, and two

outgroups. We used a coalescent framework to infer species relationships and

divergence times, despite extensive genealogical discordance. We found high levels of

allele sharing among species and show that this reflects extensive incomplete lineage

sorting and temporally layered hybridization. Our results revealed recurrent

introgression at all stages along the *Lepus* radiation, including recent gene flow between

extant species since the last glacial maximum, but also pervasive ancient introgression

occurring since near the origin of the hare lineages. We show that ancient hybridization

between northern hemisphere species has resulted in shared variation of potential

adaptive relevance to highly seasonal environments, including genes involved in

circadian rhythm regulation, pigmentation, and thermoregulation. Our results illustrate

how the genetic legacy of ancestral hybridization may persist across a radiation, leaving

a long-lasting signature of shared genetic variation that may contribute to adaptation

within and among species.

KEYWORDS

Ancient introgression; Adaptation; Hybridization; Phylogenomics; *Lepus*

Species radiations are often accompanied by extensive gene flow between nascent

lineages (e.g., Lamichhaney et al. 2015; Árnason et al. 2018; Malinsky et al. 2018; Li et

al. 2019; Barth et al. 2020). Genetic signatures of hybridization between several closely

related species could either represent recent or ongoing introgressive hybridization

(Eaton et al. 2015), or the remnants of hybridization among ancestral populations that

remain shared among contemporary species (Malinsky et al. 2018; Li et al. 2019).

Although these alternatives can be difficult to differentiate in large radiations (Eaton et

al. 2015; Malinsky et al. 2018; Vanderpool et al. 2020), both ancient and contemporary

introgression has been linked to local adaptation in several systems (e.g., Liu et al.

2015; Gittelman et al. 2016; Meier et al. 2017; Barlow et al. 2018; Giska et al. 2019;

Svardal et al. 2020). Thus, unraveling the tempo and contribution of introgression to

standing genetic variation within and among species remains a critical step in

understanding the overall importance of introgression to evolution.

Reconstructing the history of hybridization between several closely related

species requires inferring evolutionary relationships among species while considering

the two primary processes – incomplete lineage sorting and gene flow – that may cause

sharing of genetic variation among populations (Malinsky et al. 2018). The network

multispecies coalescent (NMSC) model (Than et al. 2011; Solís-Lemus et al. 2017;

Degnan 2018) offers one promising framework that appears to resolve species

relationships in the face of multiple reticulation events and rapid speciation (Kozak et

al. 2018; Edelman et al. 2019). However, the NMSC is still prohibitive for large

datasets and choosing the exact number of migration events is not straightforward (Yu

and Nakhleh 2015). Alternatively, site-based summary statistics based on tree

asymmetries (e.g., Green et al. 2010; Pease and Hahn 2015), or admixture proportions

(e.g., Reich et al. 2009; Martin et al. 2015; Malinsky et al. 2018) are simpler to

implement, but offer less power for localizing the timing and number of introgression

events when recurrent hybridization is layered across a phylogeny (Malinsky et al.

2018). A combination of methods is thus most appropriate to infer a species tree that

may have layered events of hybridization throughout time (e.g., Kozak et al. 2018;

Malinsky et al. 2018; Edelman et al. 2019; Li et al. 2019).

Hares and jackrabbits comprise a group of 32 species (genus *Lepus*; Smith et al.

2018) whose common ancestor likely originated in North America and spread

throughout most of the Northern Hemisphere and Africa presumably in the last 4-6

million years (Yamada et al. 2002; Matthee et al. 2004; Melo-Ferreira et al. 2012).

Hares are primarily associated with open grasslands, but can be found across a broad

range of biomes (e.g., desert, forest, or arctic) and elevations (e.g., from sea level to the

Himalayan or Ethiopian plateau; Smith et al., 2018). The *Lepus* radiation also provides

case studies of hybridization and introgression, since admixture has been detected

among several modern species pairs (e.g., Liu et al. 2011; Melo-Ferreira et al. 2012;

Tolesa et al. 2017; Jones et al. 2018; Seixas et al. 2018; Lado et al. 2019; Kinoshita et

al. 2019). Selection on introgressed variation has been hypothesized to have aided the

range expansion of the Iberian hare (Seixas et al. 2018), and has been directly linked to

convergent adaptive evolution of non-white winter coats in populations of two species

that change the color of their pelage seasonally (Jones et al. 2018; Giska et al. 2019;

Jones et al. 2020a). These studies suggest that the relatively recent exchange of genetic

variation among extant *Lepus* species has provided an important source of adaptive

variation. However, the phylogenetic relationships among *Lepus* species remain poorly

resolved (Halanych et al. 1999; Matthee et al. 2004; Melo-Ferreira et al. 2012; Melo-

Ferreira and Alves 2018), and the contribution of ancient gene flow to the *Lepus*

evolutionary history in a deeper phylogenetic context remains unknown.

Here, we use exome-wide data to infer the evolutionary history of 15 *Lepus*

species and show that hybridization between lineages has likely occurred since the

origin of the radiation. The combination of incomplete lineage sorting and these

temporally layered events of hybridization have resulted in extremely high levels of

shared genetic variation among extant species, including species that currently occur on

different continents. We then use the case of ancient admixture among northern latitude

species that occupy highly seasonal environments to investigate the gene content and

possible functional relevance of introgressed genomic regions. Our work demonstrates

that recurrent introgression throughout evolutionary history has made a substantial

contribution to genetic variation within and among species of this widespread

mammalian radiation.

MATERIALS AND METHODS

Taxon Sampling and Exome Sequencing

We generated new genome-wide resequencing data targeting 207,691 exonic and non-

coding regions [totaling 61.7 Megabases (Mb) total] from 14 hare species (30

individuals) and the outgroup pygmy rabbit (*Brachylagus idahoensis*; 2 individuals).

We combined these data with published whole exomes from four snowshoes hares

[*Lepus americanus*; NCBI Sequence Read Archive BioProject PRJNA420081 from

Jones et al. (2018, 2020b)] and extracted data from the reference genome of European

rabbit (*Oryctolagus cuniculus*; OryCun2.0; Carneiro *et al.*, 2014) to use as second

outgroup. Our total sample of 15 hare species (34 individuals, 1 to 4 individuals per

species) and 2 outgroup species (3 individuals) included species from all major regions

of the *Lepus* native distribution: Africa (3 species), Africa and Eurasia (1 species),

Eurasia (6 species) and North America (5 species) (Fig. 1 and Supplementary Table S1).

Exome capture experiments were performed following the procedures outlined

in Jones et al. (2018) and in the Appendix. Briefly, we obtained samples as tissue or

extracted DNA including samples from previous studies or through loans from

collaborators (Supplementary Table S1). Depending on the sample, genomic DNA was

isolated using a saline extraction method (Sambrook et al. 1989) or DNeasy Blood &

Tissue Kit (Qiagen) (Supplementary Table S1 and Appendix). We prepared Illumina

sequencing libraries for each sample following Meyer and Kircher (2010) with minor

modifications [see Appendix and Jones et al. (2018)]. Sequencing libraries were then

enriched using NimbleGen SeqCap EZ v.4.3 protocol and a custom capture design

consisting of 213,164 probes targeting ~25 Mb of protein-coding exons, ~28 Mb of

untranslated regions, and ~9 Mb of intron/intergenic regions (Jones et al. 2018).

Hybridization reactions were performed in two separate equimolar pools of indexed

libraries (31 and 29 libraries, Supplementary Table S1), together with samples used for

other studies. The target enriched pools were each sequenced across two lanes of an

Illumina HiSeq1500 sequencer (125 bp paired-end reads) at CIBIO-InBIO's New-Gen

sequencing platform, Portugal.

Read Processing and Genotyping

We trimmed adapters, low-quality bases, merged overlapping reads, and

removed PCR duplicates from raw reads using the *expHTS* pipeline (v.0.Mar112016;

https://github.com/msettles/expHTS). We then used *pseudo-it* (v1; Sarver et al. 2017) to

generate pseudo-reference exomes for each species by iteratively mapping (four

iterations and allowing ambiguity codes) cleaned single and paired-end reads from one

individual per species (Supplementary Table S1) to the rabbit reference genome

(OryCun2.0) (Carneiro et al. 2014). For snowshoe hares and black-tailed jackrabbits, we

used pseudo-references generated by Jones et al. (2018).

We then mapped data from all individuals to each species-specific pseudo-

reference using *bwa-mem* (v.0.7.12-r1039; Li 2013) with default options. Mapped reads

were sorted with *samtools* (v1.4; Li et al. 2009), assigned to read groups, filtered for

duplicates (*Picard* v1.140; http://broadinstitute.github.io/picard/), and realigned for

insertion-deletion length variation using GATK (v3.4.46; Van der Auwera et al. 2013).

We calculated coverage statistics and capture efficiency using *CalculateHSMetrics* from

Picard. For each individual, we called and filtered genotypes using the *bcftools* (v1.4;

Li 2011) mpileup, call and filter pipeline and used the curated genotypes to construct

consensus exome fasta sequences in the OryCun2.0 coordinate system (see Appendix

for more details on the filtering pipeline). Many of the steps of the bioinformatics

pipeline in our analyses benefited from parallelization provided by GNU parallel (Tange

2011).

Species Tree Inference

We used a concatenated alignment to estimate a single bifurcating phylogeny using a

maximum likelihood (ML) search and rapid bootstrapping run under the GTR+Γ model

of sequence evolution (autoMRE option) in *RAxML* (v8.2.10; Stamatakis 2018). We

then used two complementary methods to infer multispecies-coalescent trees while

accounting for local variation in phylogenetic histories along the genome. First, we

extracted alignments from the targeted regions and 200 base pairs (bp) of flanking

sequences across 50 kilobase (kb) genomic intervals, considering a balance between the

expected extent of LD in hares (10-20 kb; Jones et al. 2018) and the retention of

information for phylogenetic analysis (alignment length > 1 kb). For each window, we

filtered positions with missing data for > 30% of individuals using *TriSeq* (*TriFusion*

1.0.0; http://odiogosilva.github.io/TriFusion/), excluded windows smaller than 1 kb, and

used *RAxML* to estimate local maximum likelihood trees (GTR+Γ, 100 bootstraps) and

corresponding tree certainty scores. We then used all windows with tree certainty scores

above 5. The tree certainty score is the sum of certainty scores for all internodes of a

tree. The internode certainty score weighs the support of the bipartition represented by a

given internode against the support of the second most prevalent conflicting bipartition

(Salichos et al. 2014). In our case, the maximum theoretical value of the tree certainty

score is 31, or k – 3 with k equal to the number of taxa (Salichos et al. 2014). We

unrooted the gene trees using R package *ape* (Paradis et al. 2004) to estimate a

consensus species tree using *ASTRAL-III* (5.6.3; Zhang et al. 2018).

We also estimated coalescent species trees using only variable sites with

SVDquartets (Chifman and Kubatko 2014) implemented in *PAUP** (4a163; Swofford

2003). For the analyses based on variable sites, we recovered single nucleotide variants

(SNVs) distanced 10 kb along the genome, and excluded sites with missing information

for >30% of the individuals. For both *SVDquartets* and *ASTRAL* analyses, species trees

were estimated with and without assigning species identities and using sites/intervals

genome-wide or only from the X chromosome. The European and/or pygmy rabbits

were included for all analyses requiring outgroups. Additional details on the

phylogenetic analyses are provided in the Appendix.

Bayesian Divergence Time Inference

We performed Bayesian inference of divergence times in the inferred species tree using

an approximate maximum likelihood method and assuming an autocorrelated relaxed

molecular clock, implemented in *MCMCtree* (*PAML v.4.9*; Yang 2007) and described

in dos Reis and Yang (2011, 2019). For one individual per species (Supplementary

Table S1), we extracted the coding sequence for all genes included in our capture design

(18,798 genes in the OryCun2.0 ENSEMBLE 94 database), selecting the longest

transcript per gene using R package *biomaRt* (v2.34.2; Durinck et al. 2005, 2009) and

excluding alignments with more than 20% missing data using *AMAS* (Borowiec 2016)

(see Appendix for details). With these, we constructed a concatenated alignment with

three partitions, corresponding to the three codon positions. We assumed GTR+Γ for

the model of sequence evolution, we used the prior of 3.33 for the average substitution

rate per site per 100 million years, following Matthee et al. (2004). We discarded the

first 1,000,000 samples as burn-in and ran the program until we gathered 1,000,000

samples from the posterior, sampling every 10 iterations, and repeated the analysis

twice to ensure convergence. We checked for convergence between the two runs by

confirming a linear correlation between posterior times, trendless trace plots, and high

effective sample size (ESS) values following dos Reis and Yang (2019). Finally, we

also checked for a linear relationship between posterior times and confidence interval

widths in infinite sites plots (Inoue et al. 2010).

Lepus is poorly represented in the fossil record. The earliest hare record dates to

the early Pleistocene (2.5 million years ago; Ma) (White 1991; Lopez-Martinez 2008),

which is much more recent than molecular estimates for the genus extrapolated from

deeper fossil calibrations (4-6 Ma; Yamada et al. 2002). Therefore, we dated the species

tree either (1) using previous molecular estimates of 4-6 Ma for *Lepus* diversification

extrapolated from deep fossil record calibrations of the order Lagomorpha (Yamada et

al. 2002) and 9.7-14.5 Ma for the *Oryctolagus-Lepus* divergence (Matthee et al. 2004)

or (2) using the fossil estimates of 2.5 Ma for the lower bound of *Lepus* diversification,

and constraining this node to be no older than 4.8 Ma, which is when the fossil record

suggests that the common ancestor of Leporids (rabbits and hares) existed (Hibbard

1963; White 1991).

Ancestral Range Reconstruction

We reconstructed ancestral continental ranges for *Lepus* using the likelihood

reconstruction method (Schluter et al. 1997; Pagel 1999) implemented in *Mesquite*

(v3.51; Maddison and Maddison 2018). We generated a character matrix coding each

species as being distributed in North America (0), Eurasia (1) or Africa (2). Even

though the Cape hare *sensu lato* is distributed in Africa and Eurasia, Lado et al. (2019)

showed deep divergence and non-monophyly of African and Eurasian lineages. Because

our Cape hare samples represent the African lineage, we assigned the Cape hare

distribution to Africa. We used the Markov k-state one-parameter model (Mk1) (Lewis

2001) and the rooted topology and branch-lengths of the concatenated ML tree to infer

ancestral states of internal nodes. Nodes for which the decision threshold differed by

less than 2.0 between states were considered ambiguous.

Gene Tree Discordance and Phylogenetic Networks

To explore the amount and effect of gene tree discordance in our dataset, we performed

a series of analyses. First, we produced a split network using all gene trees previously

used as input for *ASTRAL* (tree certainty scores > 5) using *SplitsTree4* (v4.14.6; Huson

and Bryant 2006) with the option "Consensus Network with distances as means" and a

5% threshold. We also used the same set of gene trees (tree certainty scores > 5) to

produce a majority rule consensus tree with *RAxML* (-L MRE option), including

internode certainty scores (Salichos et al. 2014). We then used *DiscoVista* (Sayyari et

al. 2018) to plot *ASTRAL* quartet frequencies around nodes of interest. Finally, we

calculated Robinson-Foulds normalized distances between gene trees and the *ASTRAL*

species tree where individuals are not assigned to species, and among gene trees using

the function *RF.dist()* from the R package *phangorn* (2.4.0 ;Schliep 2011). This metric

varies between 0 (no discordance between trees) and 1 (complete discordance).

We used *PhyloNet* (v3.6.6; Yu and Nakhleh 2015) to model species

relationships under the network multispecies coalescent model, using all local

genealogies (tree certainty scores > 5). We ran *InferNetwork_MPL* (Yu and Nakhleh

2015) with 0 to 4 migration events, associating individuals to species (option -a), and

optimizing branch lengths and inheritance probabilities to compute likelihoods for each

proposed network (option -o). We used the best likelihoods per run to calculate

Bayesian Information Criteria (BIC) and Akaike information criteria corrected for small

samples sizes (AICc) following (Yu et al. 2012, 2014) to compare the resulting

networks. Networks were visualized with *IcyTree* (https://icytree.org; last accessed July

2019). We also reconstructed ancestral population graphs with *TreeMix* (v1.13, options

-global, -noss and -se; Pickrell and Pritchard 2012) based on the SNV dataset used for

SVDQuartets. We allowed 0 to 9 migration events and used the white-sided jackrabbit

(*Lepus callotis*) as the outgroup relative to all other *Lepus* species (see *Results*).

Genetic Diversity, Divergence, and Admixture

We used the *genomics general* toolkit (https://github.com/simonhmartin/; last accessed

January 14, 2019) to estimate pairwise genetic distances between species and nucleotide

diversity within species, and a custom script (available at https://github.com/evochange)

to calculate the number of heterozygous sites per individual and the subset shared

among species. All diversity estimates were based on a genome-wide concatenated

alignment, where we excluded sites with missing information for >30% of the

individuals.

We then used *genomics general* and custom scripts (available at

https://github.com/evochange) to calculate several variants of the D-statistics (Green et

al. 2010) from the informative sites in the same filtered alignment, treating the

European rabbit sequence as the ancestral state (additional details are provided in the

Appendix). Briefly, we calculated the minimum absolute value of D (D_{min}) (Malinsky et

al. 2018) for all possible species trios. We calculated z-scores for each D value using a 1

Mb block jackknife approach. After finding the minimum D per trio, D values with

Bonferroni-corrected $P \leq 0.05$ were considered significantly different from zero. We

then calculated the 'f-branch' statistic ($f_b(C)$) (Malinsky et al. 2018). The 'f-branch'

statistic measures admixture proportion between species C and branch b by calculating

admixture proportion among all possible f(A,B,C,O) combinations where A are all

descendants of branch a (sister to b), B are all descendants of branch b, and C is the

donor taxa. $f_b(C)$ is the minimum f value across all possible B and the median across all

possible A. A significant $f_b(C)$ value means that all descendants B of branch b share

alleles with C, which is more parsimoniously explained by an event of ancestral

introgression from C to b (Malinsky et al. 2018). Using the inferred species tree, we

determined all conformations (A,B;C,O) needed to calculate $f_b(C)$ for all pairs of C

species and b branches following Malinsky et al. (2018), custom scripts (available at

https://github.com/evochange) and R package *treeman* (1.1.3; Bennett et al. 2017). For

each conformation, we calculated 'admixture proportion' (f_G) as defined in Martin et al.

(2015) and Malinsky et al. (2018) and z-scores with 1 Mb block jackknife approach

following Malinsky et al. (2018). We also calculated f_{hom} (Martin et al. 2015) between

black-tailed jackrabbits (P3) and several snowshoe hare populations (as P1 and P2) to

evaluate levels of admixture estimated with $f_b(C)$ for this species pair (see *Results* and

Discussion).

We also estimated the fraction of admixture (f_d) (Martin et al. 2015) across 50 kb

genomic sliding windows ($\geq$100 sites, 5 kb steps), to localize introgression in the

genomes of northern latitude species (snowshoe hares, mountain hares, Alaskan hares,

and white-tailed jackrabbits). We performed three scans testing introgression between

snowshoe hares (P3) and Alaskan hares, mountain hares or white-tailed jackrabbits

(alternative P2), using the Iberian hare as P1. Windows of top 0.5% f_d were considered

significant. Following Liu et al. (2015), we considered that significant windows in all

three tests reflected introgression between snowshoe hares and the ancestral lineage of

white-tailed jackrabbits/mountain hares/Alaskan hares, while significant windows in

only one test result from recent introgression between the focal extant species. We

obtained the annotation of genes in these windows from the rabbit reference and

performed an enrichment analysis in *g:Profiler* (accessed September 2019; Raudvere

al. 2019) using default parameters. We also calculated d_{xy} for f_d outlier windows and the

exome-wide d_{xy} distribution between the focal pair of P2-P3 species.

RESULTS

Whole Exome Sequencing Data

We analyzed whole exome sequence data (61.7Mb) from 15 hare species and two

outgroups, combining newly generated (30 individuals from 14 hare species and 2

individuals from the outgroup pygmy rabbit) and published data (4 individuals from 1

additional hare species and the outgroup European rabbit; Carneiro et al. 2014; Jones et

al. 2018, 2020b) (Supplementary Table S1). Custom DNA captures showed high

efficiency (32.3 average fold-enrichment) and specificity (average 10% of sequenced

bases off-target; Supplementary Table S2). Mapping cleaned reads onto species-specific

pseudo-references resulted in an average target sequencing coverage of 16× (5-35× on

average per sample; Supplementary Table S2) with 57.8 million genotyped sites per

individual (Supplementary Table S2). Two lower coverage individuals (one hare

individual and one pygmy rabbit individual) and one locality duplicate (one hare) were

removed from the final dataset to maximize data quality and avoid geographic

redundancy (Supplementary Table S1). All analyses were performed on a dataset of 15

hare species (32 individuals), one pygmy rabbit, and the rabbit reference genome, unless

otherwise noted (see Supplementary Table S1).

Phylogenetic Relationships among Hares

The overall topologies of the concatenated ML phylogeny (11,949,529 positions with

no missing data) and the multispecies-coalescent species trees of *ASTRAL* (8,889 gene

trees estimated from 50 kb genomic intervals; alignment lengths between 1 kb and 29

kb) and *SVDquartets* (45,779 unlinked SNPs) were largely concordant (Supplementary

Figs. S1, S2 and S3). Branching relationships were highly supported in general

(*ASTRAL* posterior probabilities equal to 1 and *SVDquartets* bootstrap supports > 90),

except for i) the placement of European hares (*L. europaeus*) as sister to the clade of

African species, and ii) the sister relationship of the Iberian hare (*L. granatensis*) and

the clade containing the white-tailed jackrabbit (*L. townsendii*), Alaskan and mountain

hares (Fig. 1 and Supplementary Figs. S1, S2, S3 and S4). Even though the taxonomy of

some hare species has been controversial (e.g., Halanych et al. 1999; Alves et al. 2008;

Melo-Ferreira and Alves 2018; Lado et al. 2019), our analyses recovered most species

as monophyletic (Supplementary Figs. S1, S2a and S3a). Possible exceptions were the

potential paraphyly of the mountain hare (*L. timidus*) with the Alaskan hare (*L. othus*)

(suggested by *ASTRAL* and *SVDquartets*; Supplementary Figs. S2a and S3a) and of the

broom hare (*L. castroviejoi*) with the Corsican hare (*L. corsicanus*) (recovered with

ASTRAL; Supplementary Fig. S2a). Both these cases involve sister taxa that likely share

a very recent common ancestor and for which the classification as separate species has

been debated (Alves et al. 2008; Melo-Ferreira et al. 2012). Also, we consistently

estimated paraphyly of the Cape hare (*L. capensis*) with the Ethiopian hare (*L. fagani*),

with the North African Cape hare specimen sharing a more recent common ancestor

with Ethiopian hares than the South African Cape hare specimen (Supplementary Figs.

S1, S2a and S3a). This may result from the known deep intraspecific divergences and

paraphyly of Cape hare lineages (Lado et al. 2019), confirmed by our diversity and

divergence estimates (Supplementary Tables S3 and S4).

Dating the species tree using either molecular-based dates extrapolated from

deep fossil calibrations (Yamada et al. 2002; Matthee et al. 2004) or *Lepus* fossil record

calibrations (Hibbard 1963; White 1991) recovered overlapping 95% high posterior

density (HPD) intervals of divergence times for relatively recent splits (e.g.,

diversification of Eurasian and African species; Supplementary Table S5). However,

Lepus-based fossil calibrations suggested more recent ages for deeper nodes

(Supplementary Table S5 and Fig. S5). For instance, dates extrapolated from deeper

fossil calibrations outside of *Lepus* sets the base of the hare radiation at ~5.83 million

year ago (Ma) (95% HPD 6.17-5.34 Ma), whereas *Lepus* fossil calibration recovered

~4.05 Ma (95% HPD 5.00-3.18 Ma). All analyses were consistent in showing that the

deepest branching events involved North American species: white-sided jackrabbit (*L.*

callotis), black-tailed jackrabbit, and snowshoe hare (*L. americanus*) (Fig. 1b). In

accordance, ancestral reconstruction of biogeographic distributions (Fig. 1c and

Supplementary Table S6) supported an initial radiation of the genus in North America,

with subsequent colonization of Eurasia (2.85 and 1.99 Ma for deeper and *Lepus*-fossil

calibration, respectively) and Africa (1.85 and 1.33 Ma for deeper and fossil calibration,

respectively). The North America distribution of white-tailed jackrabbits and Alaskan

hares (Fig. 1a) likely results from one or two re-colonization events from Eurasia (Fig.

1c and Supplementary Table S6). These results also confirm the paraphyly of the

jackrabbit species (Halanych et al. 1999).

Fig. 1 - Hare (*Lepus* spp.) evolutionary history and biogeography. A) The

distribution of the 15 hare species studied here; **B)** Coalescent species tree estimated

with *ASTRAL;* nodes with *ASTRAL* posterior probability equal to one, *ASTRAL* quartet

score higher than 0.6, and *SVDquartets* bootstrap support higher than 90 are labeled; **C)**

Maximum-likelihood phylogeny of *Lepus* showing ancestral range reconstructions. Pie

charts show proportional likelihoods that the common ancestral was distributed in North

America (blue), Eurasia (green) or Africa (red). Asterisks depict nodes with equivocal

reconstruction of ancestor distribution.

Incomplete Lineage Sorting and Introgression

We recovered a highly supported species trees across phylogenetic methods, albeit with

extensive phylogenetic discordance among sequenced regions [average Robinson-

Foulds (RF) pairwise distance between local trees was 0.73]. No local tree completely

recovered the species tree topology (minimum RF distance between gene and species

tree was 0.13) (Supplementary Fig. S6) and the majority rule consensus tree showed

low internode certainty (Supplementary Fig. S7). Extensive phylogenetic discordance

was also apparent in the *ASTRAL* species tree with all but three branches showing

quartet scores below 0.6 (Fig. 1, Supplementary Figs. S2, S4 and Tables S7 and S8).

Splits network analysis of individual gene trees also supported many alternative

relationships (represented by cuboid structures connecting alternative topologies)

particularly involving deeper branches (Fig. 2). We estimated similar levels of

discordance between X-linked and autosomal genealogies and the inferred species trees

(Supplementary Fig. S6). Furthermore, a species tree inferred with X-linked data

differed from the genome-wide species tree and showed lower overall branch support

(Supplementary Fig. S8 and Table S9). Measures of intraspecific diversity were

relatively low within species [π between 0.13% (Corsican hare) and 0.63% (black-tailed

jackrabbit] but overlapped in range with estimates of absolute genetic divergence

between species [d_{xy} between 0.17% (broom hare and Corsican hare) and 1.11%

(snowshoe hare and white-sided jackrabbit)] (Supplementary Tables S3 and S4). Some

instances of low interspecific divergence reflect sister taxa where the species-level

designations have been debated (e.g., the Corsican-Broom hare and Mountain-Alaskan

hare complexes; Alves et al. 2008; Melo-Ferreira et al. 2012). Alternatively, high

intraspecific diversity may reflect cryptic divergence within recognized species (e.g.,

snowshoe hares, Melo-Ferreira et al. 2014; Cape hares, Lado et al. 2019).

On average, 49% of heterozygous sites in individuals of one species were shared

with individuals from at least one other species (Supplementary Table S10). Such high

levels of shared polymorphism between species coupled with extensive phylogenetic

discordance across the genome could be explained by recent rapid speciation leading to

extensive incomplete lineage sorting, secondary introgression between lineages, or a

mixture of both processes. To differentiate these alternatives, we first tested if the

proportions of shared-derived variants between species across the genome supported a

strictly bifurcating history (expected with incomplete lineage sorting) by calculating

minimum absolute D-statistics (D_{min}) (Malinsky et al. 2018) for all trios of species in

our dataset. We found that 88% of these comparisons were significantly different from

zero (Bonferroni-corrected $P < 0.05$; Supplementary Fig. S9). This suggests that the

level of shared alleles among the majority of species trios is incompatible with a single

tree (even accounting for incomplete lineage sorting), providing overwhelming support

for gene flow among species. This striking pattern of introgression could be driven by

rampant gene flow among extant species pairs, or represent signatures of introgression

between ancestral lineages that remain in modern species (Malinsky et al. 2018).

Fig. 2 - The hare (*Lepus* spp.) species tree is underlined by extensive gene tree

incongruence. Splits network constructed from 8889 gene trees (5% threshold) shows

discordance among the gene tree topologies (cuboid structures represent alternative

topologies) in deeper nodes of the species tree. Species are marked with colors

corresponding to the continents where they are distributed.

To disentangle temporally layered signatures of introgression, we combined

analyses based on the multispecies network coalescent implemented in *PhyloNet* (Yu

and Nakhleh 2015) (Supplementary Fig. S10), ancestral population graph reconstruction

with *TreeMix* (based on 30,709 biallelic SNVs; Supplementary Figs. S11 and S12;

Pickrell and Pritchard 2012), and estimates of admixture proportions among species

based on the 'f-branch' metric ($f_b(C)$) (Malinsky et al. 2018) (Fig. 3). Collectively, our

results were consistent with recurrent gene flow between species layered across the

diversification of *Lepus*. We detected introgression among extant species pairs within

all of the major geographic regions that are currently sympatric, suggesting ongoing or

recent hybridization (Fig. 3). For example, gene flow between black-tailed jackrabbits

and snowshoe hares in North America ($f_b(C)$ = 19%; P = 2.04E-169), or between

European brown hares and mountain hares from Eurasia ($f_b(C)$ = 18%; P = 1.73E-233;

Fig. 3 and Supplementary Table S11). In general, we found decreased admixture

proportions with increased genetic divergence between species, although this

correlation was only significant when considering species with non-overlapping

distributions (Fig. 4). Several species pairs with extant contact zones showed admixture

even when genetic divergence was relatively high, such as snowshoe hares and black-

tailed jackrabbits (d_{xy} = 0.97%; TMRCA ~ 4.8 Ma, Supplementary Fig. S5 and Tables

S4 and S5).

Fig. 3 – Admixture events are distributed across the hare (*Lepus* spp.) species tree.

The branch-specific statistic $f_b(C)$ represents excess allele sharing between branches b

(y-axis) and C (x-axis) of the species tree in Figure 1. The orange gradient represents

the $f_b(C)$ score, gray represents tests not consistent with the species tree and asterisks

denote block jackknifing significance at $P \leq 0.05$ (after Bonferroni correction). Tips of

the tree are colored according to their current distribution (two colors represent

distribution in both areas), and ancestral tips (dashed gray lines labeled with letters) are

colored according to the ancestral reconstruction of distribution areas from Figure 1

(two colors represent equivocal inference of the two-character states). Ancestral tips are

labeled from A to L corresponding to labels in Supplementary Table S11.

We also found evidence for introgression between ancestral populations, which

has affected deeper branches of the species tree. Network and $f_b(C)$ results suggest

ancestral events of introgression connecting major diverged clades within Eurasia (e.g.,

European hares and the ancestor of the mountain hare/Alaskan hare/white-tailed

jackrabbit clade), African and Eurasian lineages (e.g., ancestral of all African species

and the Corsican and broom hare ancestral), and Eurasian and North American lineages

(e.g., snowshoe hares and the ancestral lineage of the mountain hare/Alaskan

hare/white-tailed jackrabbit; Fig. 3, 4, Supplementary Figs. S10 and S11, and Table

S11). Finally, we found significant allele sharing among species that currently inhabit

different continents, such as North American hares and species from Africa and

Western Europe (Fig. 3 and 4). Trans-continental introgression was also suggested by a

network with two reticulations and by ancestral population graph reconstruction

(Supplementary Figs. S10c and S11). Given the inferred split times (Supplementary Fig.

S5) and biogeography (Fig. 1) of this group, these results suggest that introgression

affected the very early branches of the *Lepus* radiation, and that the genetic legacy of

these gene flow events persists in the gene pool of descendant species today (Fig. 3 and

4).

Introgression in hares

○ not significant ● significant ● allopatric ● sympatric/parapatric

Fig. 4 – Admixture proportions decrease with genetic divergence between

allopatric species. We plot $f_b(C)$ values against exome-wide divergence (d_{xy}) for extant

species pairs, coloring points when species have overlapping (sympatric/parapatric) or

non-overlapping (allopatric) distributions. The tendency line represents a linear

regression relating d_{xy} and $f_b(C)$ values calculated with function *lm()* in R.

Genes affected by Ancestral Introgression

We detected ancestral introgression involving white-tailed jackrabbits, mountain hares,

Alaskan hares, and snowshoe hares, which together have a circumpolar distribution

across northern latitude habitats (Fig. 3 and Supplementary Fig. S10). These species

inhabit the strongly seasonal boreal and/or temperate environments of North America

and Eurasia (Smith et al. 2018), and have all evolved seasonal coat color molts from

summer brown to winter white to maintain cryptic coloration with the onset of seasonal

snow cover (Mills et al. 2018). Motivated by recent work showing that introgressive

hybridization has shaped local adaptation in seasonal coat color changing species (Jones

et al. 2018; Giska et al. 2019; Jones et al. 2020a), we next examined the contribution of

gene flow between the ancestor of Alaskan hare/mountain hare/white-tailed jackrabbit

and snowshoe hares (at least 0.98 Ma from lagomorph fossil calibrations and 0.71 Ma

from *Lepus* fossil calibration; Supplementary Fig. S5 and Table S5) to standing

variation in these species.

We performed fraction of admixture (f_d) scans to detect genomic regions shared

through ancestral introgression among these species. We detected 119 putative windows

of ancient introgression across all major chromosomes, highlighting the genome-wide

impact of ancestral introgression (Fig. 5; Supplementary Table S12). The f_d outlier

windows of ancestral introgression contained 54 annotated genes (Supplementary Table

S13). This set of genes was enriched for the gene ontology term "E-Box binding" (3 of

the 54 genes; Supplementary Table S14), a DNA motif found in the promoters of many

genes, suggesting that genomic regions affected by ancient introgression may be

enriched for transcription factors involved in *trans*-regulation of gene expression.

Among these transcription factors we found the circadian clock related gene *ARNTL2*

(Sasaki et al. 2009) and pigmentation related gene *TCF4* (Furumura et al. 2001; Le Pape

et al. 2009) (Fig. 5). In addition, the list of 54 genes includes a gene involved in brown

fat differentiation (*EBF2*; Rajakumari et al. 2013), and a photoreceptor-related gene

(*PDE6H*; Kohl et al. 2012).

Fig. 5 – The impact of ancestral introgression on extant northern latitude species.

A) Events of past and recent admixture inferred in this study involving snowshoe,

Alaskan hares, mountain hares and white-tailed jackrabbits. The arrow indicates the

direction of ancestral introgression inferred by *PhyloNet*; **B)** Genomic distribution of

ancestral blocks of introgression inferred as shared outliers windows among fraction of

admixture (f_d) analysis testing for admixture among snowshoe hares and each one of the

three other northern latitude species (orange), and blocks private to the analysis with

Alaskan hares (blue) and white-tailed jackrabbits (black). Tests were run in 50 kb

genomic sliding windows, and outlier windows are in the top 0.5% of the f_d distribution.

DISCUSSION

We used whole exome data and diverse phylogenetic analyses to tease apart signatures

of stochastic lineage sorting and admixture across the evolutionary history of the *Lepus*

radiation. By accounting for these sources of phylogenetic discordance, we were able to

detect pervasive introgression between lineages that was layered across the evolution of

this recent and rapid mammalian radiation. Below we discuss the biogeographic and

evolutionary implications of our analyses, focusing on the long-term impacts of

temporally layered hybridization in shaping patterns of shared genetic variation within

and among extant species.

The Effect of Persistent Gene Flow on Phylogenetic Inference

We present a resolved genome-wide phylogeny for the genus *Lepus* despite extensive

incomplete lineage sorting and pervasive gene flow. Although we sampled just a subset

of described *Lepus* species (15 of 32 species), our genome-wide experiment covered all

major lineages across the worldwide range of hares resulting in the most extensive

phylogeny of the genus to date, substantially extending previous analyses based on more

limited genetic sampling (Halanych et al. 1999; Melo-Ferreira et al. 2012; Ge et al. 2013;

Tolesa et al. 2017). A systematic evaluation of species limits and hare taxonomy is

beyond the scope of our work as it would require genome-wide sequencing of an

expanded inter and intraspecific sample. Our sampling design is however not expected to

bias phylogenetic relationships of the sampled species and inference of discordance

among deeper lineages, and may even underestimate species diversity, thus making our

inferences of shared variation among species conservative.

To estimate species relationships, we combined species tree inferences that do not

account for gene flow with network-based inferences that explicitly consider

introgression. The resulting topologies were generally consistent between methods when

considering up to three reticulations (Fig. 1 and Supplementary Fig. S10). However, there

were clear limits to this approach. For example, the alternative placement of European,

Corsican, and broom hares, as closer to the Eurasian or African *Lepus* clades depended

on the number of reticulations considered (Supplementary Fig. S10). This uncertainty

likely reflects long-term admixture among Eurasian and African lineages, including the

European hare (Fig. 3 and Supplementary Fig. S10) whose range overlaps with species

from both continents (Fig. 1a). Another example is the alternative sister relationship of

snowshoe hares and black-tailed jackrabbits in the network with no reticulation

(Supplementary Fig. S10), which could result from ancestral introgression between these

species (see below). A network with four reticulations was less concordant than results

assuming zero to three reticulations. While an increase number of reticulation events

could better represent the widespread nature of gene flow uncovered in our work,

allowing for more than three reticulations resulted in increased branch compression

suggesting that the added instances of gene flow do not contribute to resolve gene-to-

gene incongruences (Supplementary Fig. S10) (Yu and Nakhleh 2015; Wen et al. 2016).

Interestingly, we found that gene trees constructed from X-linked loci or

autosome-linked loci showed similar levels of discordance with the whole-genome

species tree (Supplementary Fig. S6). In general, the X chromosome could be expected

to show less phylogenetic discordance due to a typically smaller effective population

size (i.e., lower levels of incomplete lineage sorting assuming equal sex ratios) and a

tendency to accumulate hybrid incompatibilities (i.e., the large X-effect) (Fontaine et al.

2015; Edelman et al. 2019; Li et al. 2019). Although there is evidence for reduced X-

linked gene flow between some hybridizing European lineages (Seixas et al. 2018) very

little is known about the genetic architecture of reproductive isolation between hare

species. Moreover, higher gene tree/species tree concordance of loci involved in

reproductive isolation may not be always expected, particularly when speciation events

are clustered in time (Wang and Hahn 2018) as is the case in *Lepus* (Supplementary Fig.

S5).

The Timing and Biogeography of the Lepus Radiation

Overall, our results suggest that the *Lepus* diversification followed major climatic shifts

that occurred during the late Miocene, Pliocene and Pleistocene, similar to other

terrestrial mammals (Simpson 1947; Ge et al. 2013). We inferred that the genus

originated in North America between 5.83 Ma (deeper fossil calibration) and 4.05 Ma

(*Lepus* fossil calibration) (in general agreement with Hibbard 1963; Halanych et al.

1999; Melo-Ferreira et al. 2012; Ge et al. 2013). This inferred origin coincides with a

global cold and dry period of the late Miocene that favored the expansion of grasslands

worldwide (Osborne and Beerling 2006; Ge et al. 2013). The colonization of Eurasia

around 2.77 Ma (deeper fossil calibration) or 1.99 Ma (*Lepus* fossil calibration) is also

coincident with faunal exchanges between North America and Eurasia during late

Pliocene and Pleistocene glacial periods that connected both continents through the

Bering land bridge (Simpson 1947; Hopkins 1959; Cook et al. 2016). After colonizing

Eurasia, *Lepus* entered Africa somewhere between the divergence of the European hare

and the African ancestral lineage (1.78 Ma or 2.48 Ma from *Lepus* or deeper fossil

calibrations respectively) and the divergence of all African species (1.33 Ma or 1.85 Ma

from *Lepus* or deeper fossil calibrations respectively) (Supplementary Fig. S5 and Table

S5).

Our biogeographic inferences confirm a secondary colonization of North

America back from Eurasia likely within the last 1 Ma (Fig. 1 and Supplementary Fig.

S5) (Halanych et al. 1999). The Eurasia-North America exchange of fauna during

colder glacial periods of the Pleistocene is thought to have involved taxa adapted to

colder environments (Simpson 1947; Hoberg et al. 2012). Consistent with this, we find

that the *Lepus* re-colonization of North America involved the ancestral lineage of

species now adapted to northern ecosystems (i.e., mountain hares, Alaskan hares, and

white-tailed jackrabbits).

617   We note that our finding of pervasive introgression (Fig. 3, Supplementary Figs.

618 S9, S10 and S11 and Table S11) may distort aspects of these biogeographic inferences

619 (Leaché et al. 2014; Solís-Lemus et al. 2016; Long and Kubatko 2018; Li et al. 2019;

620 Jiao et al. 2020). Assuming that the inferred structure of the *Lepus* phylogeny is correct,

621 as discussed above, the general biogeographic reconstructions are probably robust.

622 However, extensive ancestral gene flow is still likely to bias our estimates of species

623 divergence (Leaché et al. 2014; Li et al. 2019). The lack of varied, independent, and

624 reliable calibration points along our phylogeny hinders further evaluation of the impact

625 of introgression on our time estimates, which therefore should be interpreted with

626 caution.

628 *The Legacy of Introgression during the Rapid Radiation of Hares*

629 Extensive reticulation across the *Lepus* phylogeny suggests that lineages often

630 hybridized when they came into contact during their worldwide expansion. Some of the

631 inferred reticulation events overlap with known recent introgression, such as between

632 mountain hares and European hares (Levänen et al. 2018) or between snowshoe hares

633 and black-tailed jackrabbits (Jones et al. 2018, 2020a). However, our analysis also

634 reveals prevailing signatures of deeper hybridization between ancestral populations,

635 suggesting a persistent contribution of secondary introgression during the

636 diversification of the genus. These past hybridization events have resulted in extensive

637 shared polymorphism among extant species (Fig. 3 and Supplementary Tables S10, S11

638 and Figs. S9, S10 and S11), with significant admixture still detected among species with

639 non-overlapping distributions or inhabiting different continents (Fig. 3 and Fig. 4).

640   Our results highlight how ancient gene flow can obscure accurate detection of

641 contemporary hybridization. Similar to other systems (Malinsky et al. 2018; Li et al.

2019; Edelman et al. 2019), we estimated exceedingly high levels of introgression based

on D-statistics (e.g., 88% of D_{min} values across all possible species trios were

significant), but much less reticulation when we took the phylogeny into account (e.g.,

33% of f-branch statistics were significant). These findings suggest that some signatures

of introgression between pairs of species may actually result from earlier hybridization

between ancestral lineages. Thus, phylogenetic correlation causes non-independence of

summary-statistics and can thus lead to false positive inferences of gene flow (Eaton et

al. 2015; Malinsky et al. 2018; Li et al. 2019). Given these results, signatures of

hybridization among closely related species should be interpreted in the context of

broader phylogenetic relationships whenever possible.

We also detected some discrepancies between the magnitude of gene flow

inferred here and in previous works, which underscores the challenges of quantifying

introgression across a reticulating radiation. For instance, we inferred substantial overall

admixture proportions between black-tailed jackrabbits and snowshoe hares ($f_b(C) =$

19%, Fig. 3 and Supplementary Table S11). These estimates are one order of magnitude

higher than recent studies suggesting that ~2-3% of genomic variation in the Pacific

Northwest snowshoe hare populations descends from a pulse of gene flow from black-

tailed jackrabbits in the last ~10,000 generations (Jones et al. 2018, 2020a). Ancient

introgression persisting in all snowshoe hare populations could reconcile this

discrepancy. Indeed, we recovered admixture in the same order of magnitude (f_{hom} ~3%)

when we infer admixture proportions among different snowshoe hare populations and

black-tailed jackrabbits (Supplementary Table S15), which would then reflect recent

and geographically localized introgression. However, our quantitative estimate of

overall admixture proportions between these species (19%) also depends on the accurate

reconstruction of the species tree, which in this instance involves a short internal branch

that is not fully supported across methods, and a species (snowshoe hare) that has been

involved in multiple instances of introgression with different hare lineages (Fig. 3, 5

and Supplementary Table S11 and Fig. S10).

Introgression between species is often limited by purifying selection against

divergent alleles that are deleterious in hybrids (Schumer et al. 2018; Edelman et al.

2019), which agrees with the predominantly negative consequences of hybridization

(Mayr 1963). Nonetheless, hybridization is also expected to produce novel allelic

combinations that increase phenotypic variation (Grant and Grant 2019; Marques et al.

2019). If coincident with ecological opportunity, introgressed variation could broadly

facilitate adaptation (Grant and Grant 2019; Taylor and Larson 2019). Recent work has

shown at least two instances where introgression between *Lepus* species has driven

local adaptation (Jones et al. 2018; Giska et al. 2019), and standing variation from

introgression may have contributed even more generally to adaptation during the

radiation. We found here that allelic combinations from ancient hybridization can be

carried through several speciation events (Fig. 3 and 4), persisting in the gene pool of

consecutive species due to the rapid succession of speciation events. Much of the large

reservoir of shared variation may reflect stochastic sorting of neutral variation, while

some could be maintained by positive selection (Guerrero and Hahn 2017), facilitating

colonization of the diverse habitats currently inhabited by hare species, from desert to

arctic environments (Ge et al. 2013; Smith et al. 2018).

In this respect, introgression between the ancestor of mountain hares/Alaskan

hares/white-tailed jackrabbits and snowshoe hares (or an ancestral lineage) is

particularly intriguing. These four species have adapted to highly seasonal environments

through striking forms of phenotypic plasticity (e.g., seasonal coat color change; Mills

et al. 2013; Zimova et al. 2018) that have been at least partially shaped by adaptive

introgression at the *Agouti* pigmentation gene from non-color changing species (*ASIP*;

Jones et al. 2018; Giska et al. 2019). Here we estimated that a pulse of ancient

introgression occurred at least 0.71 Ma (*Lepus* fossil calibration; Supplementary Fig.

S5) and affected genomic regions containing genes associated with circadian rhythm

regulation (*ARNTL2*; Sasaki et al. 2009)*,* pigmentation (*TCF4*; Furumura et al. 2001; Le

Pape et al. 2009), thermoregulation (*EBF2*; Rajakumari et al. 2013) and visual

perception (*PDE6H*; Kohl et al. 2012). While *ASIP* would also be a likely candidate for

adaptive introgression between these lineages (Jones et al. 2018), our exome sequencing

coverage of this region was too sparse for detailed window-based analysis.

The functions of these introgressed genes overlap with common physiological

adaptations of northern latitude animals to seasonal conditions, such as higher metabolic

rates, regulation of body temperature and non-shivering thermogenesis (Hart et al. 1965;

Feist and Rosenmann 1975; Rogowitz 1990; Pyörnilä et al. 2008; Sheriff et al. 2009),

seasonal camouflage (Grange 1932; Hewson 1958; Hansen and Bear 1963; Mills et al.

2018; Zimova et al. 2018), and visual acuity in response to dim winter light in northern

latitudes (Stokkan et al. 2013). The functional relevance of these candidates to local

adaptation must await further testing. Substantial introgression along the rapid

diversification of a group of organisms, as we describe here, may bolster genetic

variation within species and have a greater role in local adaptation than previously

anticipated (Grant and Grant 2019; Taylor and Larson 2019). However, we also cannot

exclude the possibility that some of these shared variants have been maintained by long-

term balancing selection rather than secondary introgression (Supplementary Fig. S13;

Smith and Kronforst 2013; Liu et al. 2015; Guerrero and Hahn 2017). Regardless of

origin, the *Lepus* radiation provides an intriguing system by which to test the long-term

evolutionary importance of shared genetic variation across a rapid radiation.

DATA AVAILABILITY

719 Raw sequence reads were deposited at the Sequence Read Archive (SRA) under accession

720 numbers SRR12020579 to SRR12020510 and BioProject PRJNA639005. Other data will

721 be made available from the Dryad Digital Repository with link

722 http://dx.doi.org/10.5061/dryad.[NNNN]. The pipeline and custom scripts used for this

723 study are available at https://github.com/evochange.

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1118 **FIGURE LEGENDS**

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Fig. 1 - Hare (*Lepus* spp.) evolutionary history and biogeography. A) The

distribution of the 15 hare species studied here; **B)** Coalescent species tree estimated

with *ASTRAL;* nodes with *ASTRAL* posterior probability equal to one, *ASTRAL* quartet

score higher than 0.6, and *SVDquartets* bootstrap support higher than 90 are labeled; **C)**

Maximum-likelihood phylogeny of *Lepus* showing ancestral range reconstructions. Pie

charts show proportional likelihoods that the common ancestral was distributed in North

America (blue), Eurasia (green) or Africa (red). Asterisks depict nodes with equivocal

reconstruction of ancestor distribution.

1128

1129 **Fig. 2 - The hare (*Lepus* spp.) species tree is underlined by extensive gene tree**

1130 **incongruence.** Split network constructed from 8889 gene trees (5% threshold) shows

1131 discordance among the gene tree topologies (cuboid structures represent alternative

1132 topologies) in deeper nodes of the species tree. Species are marked with colors

1133 corresponding to the continents where they are distributed.

1134

Fig. 3 – Admixture events are distributed across the hare (*Lepus* spp.) species tree.

The branch-specific statistic $f_b(C)$ represents excess allele sharing between branches b

(y-axis) and C (x-axis) of the species tree in Figure 1. The orange gradient represents

the $f_b(C)$ score, gray represents tests not consistent with the species tree and asterisks

denote block jackknifing significance at $P \leq 0.05$ (after Bonferroni correction). Tips of

the tree are colored according to their current distribution (two colors represent

distribution in both areas), and ancestral tips (dashed gray and labeled with letters) are

colored according to the ancestral reconstruction of distribution areas from Figure 1

(two colors represent equivocal inference of the two-character states). Ancestral tips are

labeled from A to L corresponding to labels in Supplementary Table S11.

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1146   **Fig. 4 – Admixture proportions decrease with genetic divergence between**

1147   **allopatric species.** We plot $f_b(C)$ values against exome-wide divergence (d_{xy}) for extant

1148   species pairs, coloring points when species have overlapping (sympatric/parapatric) or

1149   non-overlapping (allopatric) distributions. The tendency line represents a linear

1150   regression relating d_{xy} and $f_b(C)$ values calculated with function *lm()* in R.

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Fig. 5 – The impact of ancestral introgression on extant northern latitude species.

A) Events of past and recent admixture inferred in this study involving snowshoe,

Alaskan hares, mountain hares and white-tailed jackrabbits. The arrow indicates the

direction of ancestral introgression inferred by *PhyloNet*; **B)** Genomic distribution of

ancestral blocks of introgression inferred as shared outliers windows among fraction of

admixture (f_d) analysis testing for admixture among snowshoe hares and each one of the

three other northern latitude species (orange), and blocks private to the analysis with

Alaskan hares (blue) and white-tailed jackrabbits (black). Tests were run in 50 kb

genomic sliding windows, and outlier windows are in the top 0.5% of the f_d distribution.

1161

a)

0   2000   4000 km

North America
— Alaskan hare
— Snowshoe hare
— White-tailed jackrabbit
— Black-tailed jackrabbit
— White-sided jackrabbit

Africa
— Cape hare
— Abyssinian hare
— Ethiopian hare
— Ethiopian highland hare

Eurasia
— Mountain hare
— Manchurian hare
— European hare
— Corsican hare
— Iberian hare
— Broom hare

b)

White-sided jackrabbit
Black-tailed jackrabbit
Snowshoe hare
Broom hare
Corsican hare
Manchurian hare
Iberian hare
White-tailed jackrabbit
Mountain hare
Alaskan hare
European hare
Ethiopian hare
Cape hare
Ethiopian highland hare
Abyssinian hare
European rabbit
Pygmy rabbit

0.7

○ posterior probability = 1 (ASTRAL)
▢ bootstrap support > 90 (SVDquartets)
☆ quartet score > 0.6 (ASTRAL)

c)

North America
Eurasia
Africa

White-sided jackrabbit
Black-tailed jackrabbit
Snowshoe hare
Broom hare
Corsican hare
Iberian hare
Manchurian hare
White-tailed jackrabbit
Mountain hare
Alaskan hare
European hare
Ethiopian hare
Cape hare
Ethiopian highland hare
Abyssinian hare
European rabbit
Pygmy rabbit