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Title: Adaptive Introgression Underlies Polymorphic Seasonal Camouflage in Snowshoe Hares

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Abstract: Snowshoe hares (*Lepus americanus*) maintain seasonal camouflage by molting to a white winter coat, but in regions with low snow cover some hares remain brown in the winter. We show that *cis*-regulatory variation controlling seasonal expression of the *Agouti* gene underlies this adaptive winter camouflage polymorphism. Genetic variation at *Agouti* clustered by winter coat color across multiple hare and jackrabbit species, revealing a history of recurrent interspecific gene flow. Brown winter coats in snowshoe hares originated from an introgressed black-tailed jackrabbit allele that has swept to high frequency in mild winter environments. These discoveries show that exchange of genetic variants underlying key ecological traits through hybridization can seed past and ongoing adaptation to rapidly changing environments.

One Sentence Summary: Introgression at the pigmentation gene *Agouti* underlies local adaptation in seasonal camouflage of snowshoe hares.

Main Text: Many species undergo reversible changes in morphology, physiology, and behavior to cope with the challenges of seasonal environments. These critical components of phenotypic plasticity often track the environment through the photoperiod-dependent release of hormones (*1*). However, circannual rhythms can become desynchronized when abiotic conditions change rapidly (*2*), leading to declines in population fitness (*3*). The capacity of species to adapt to rapidly changing environments will depend in part on the proximate and ultimate causes of variation underlying seasonal traits (*4*, *5*), which remain poorly understood at the molecular level (*1*, *2*).

At least 21 bird and mammal species undergo autumn molts from brown to white coats (*6*, *7*) as part of a suite of plastic trait responses to seasonal environments. We used natural variation in seasonal camouflage of the snowshoe hare (*Lepus americanus*) to understand the genetic basis of this critical seasonal trait. Autumn molts to white winter coats are cued by photoperiod (*8*) and generally track seasonal snow cover. Direct estimates of hare survival have shown that mismatch between coat color and snow cover increases predation (*3*). White winter coats predominate across the snowshoe hare range, but some populations molt into brown winter coats (Fig. 1). In the Pacific Northwest (PNW), shifts in the probability of white coats coincide with a gradient in snow cover from warmer coastal to colder inland environments, consistent with local selection for seasonal camouflage with color morphs co-occurring across a broad polymorphic zone (Fig. 1C) (*7*).

To dissect the genetic basis of polymorphic seasonal camouflage, we used whole genome sequences for a winter-white hare from Montana (MT, 33× coverage) (*9*, *10*) and a winter-brown hare from Washington (WA, 22× coverage) and constructed a reference through iterative mapping (*11*) to the rabbit genome (*9*, *12*). We then sequenced 80 whole exomes (62 Mb, 21× ±

7.6 per individual) from two regions in the PNW polymorphic zone (WA: *n*=26, Oregon hereafter OR: *n*=26, each region 50% winter-white), a monomorphic winter-white locality in MT (*n*=14), and a monomorphic winter-brown locality in British Columbia (BC: *n*=14; table S1). If the polymorphic zone represents admixture between previously isolated populations, then genetic structure could obscure genotype-phenotype associations (*13*). Analysis of 38,694 unlinked single nucleotide polymorphisms (SNPs) revealed geographic structure (Fig. 1C), but genome-wide genetic differentiation (F_{ST}) between winter-brown and winter-white individuals was ~0 within polymorphic localities (table S2). The polymorphic zone also showed no evidence of admixture based on patterns of linkage disequilibrium (fig. S1) or allele sharing with other populations (table S3) (*14*). Thus, geographic variation for winter coat color in the PNW likely reflects primary intergradation across a gradient in snow cover.

We tested 513,812 SNPs for coat color associations across polymorphic populations and identified a single outlier region on chromosome 4 in perfect association with winter coat color (P=4.24×10^{-10}; Fig. 2A, fig. S2, Additional Data table S1) (*12*). We then augmented exome data with low-coverage whole genome resequencing of polymorphic zone hares (~20× per color morph). Coat color associations based on genotype likelihoods (15,173,804 SNPs) (*15*) confirmed a single outlier region (fig. S3) localized to a ~225 kilobase (kb) interval of elevated F_{ST} between color morphs centered on the pigmentation gene *Agouti* and two flanking genes, *Ahcy* and *Eif2s2* (Fig. 2B). Winter-brown hares were homozygous (*n*=26) for brown-associated alleles (hereafter, *a*), while winter-white hares were either heterozygous (*n*=24) or homozygous (*n*=2) for the alternative allele (hereafter, *A*; Fig. 2C). We then induced autumn molts in 18 captive wild-caught hares (WA: *n*=11, MT: *n*=7) and found perfect concordance between *Agouti* genotypes and winter coat colors (Fig. 2C, table S4). This experiment included a heterozygous

(*Aa*) wild-caught pregnant winter-white female from WA that gave birth in captivity to both winter-white and winter-brown offspring (Fig. 2D). Therefore, winter coat color segregates as a dominant locus in both wild and captive animals.

The agouti signaling protein (ASIP) antagonizes the melanocortin-1 receptor (MC1R) in follicular melanocytes, shifting melanogenesis towards lighter pheomelanin pigments or inhibiting pigment production (*16*). MC1R mutations suppress expression of winter-white coats in dark or blue color morphs of arctic foxes, suggesting that ASIP-MC1R interactions are involved in the development of seasonal color molts (*17*). *Agouti* is typically expressed as ventral or hair cycle-specific isoforms distinguished by alternative 5' untranslated regions (UTRs, Fig. 2B) (*18*). Both isoforms have been associated with lighter dorsal pelage (*19*, *20*). We hypothesized that the development of winter-white coats, which mostly lack pigments (*8*), is controlled by isoform-specific upregulation of *Agouti* during the autumn molt. To test this, we quantified allele-specific expression of both isoforms and the closely linked *Ahcy* locus in dorsal skin biopsies from three captive heterozygous hares (*Aa*) undergoing brown-to-white molts. Quantitative PCR verified expression of *Ahcy* and the *Agouti* hair-cycle isoform while expression of the ventral isoform was negligible (Fig. 3A, table S5, table S6). Targeted pyrosequencing revealed highly skewed expression toward the white (*A*) allele of the hair-cycle *Agouti* isoform ($P<0.0001$, Student's t-test), indicative of *cis*-regulatory variation, while *Ahcy* showed equal allelic expression (Fig. 3B, table S7). These data suggest that winter-white coats develop as a consequence of increased expression of *Agouti* during the autumn molt, which fits with our observed dominance relationships and previous studies on the evolution of lighter pelage in deer mice (*19*, *20*). Our findings directly link *Agouti* expression and the evolution of seasonal camouflage in snowshoe hares and suggest that *cis*-regulatory evolution plays an important role in the origin of novel seasonal traits.

Comparison of winter-white (MT) and winter-brown genomes (WA) revealed extraordinarily elevated levels of absolute genetic divergence across *Agouti* (*Agouti* d_{XY} = 1.6%; genome-wide d_{XY}=0.41%; bootstrapped P<0.0001; Fig. 4A, fig. S4), indicating that the color polymorphism did not arise from a recent *de novo* mutation. Alternatively, elevated divergence could reflect either the long-term maintenance of polymorphism or introgression from another species (*21*, *22*). Six of the 32 species of hares and jackrabbits (genus *Lepus*) have winter-white molts but evolutionary relationships within this rapid radiation are poorly resolved (*23*). To examine the origins of winter coat color variants, we combined whole genome sequences of two additional winter-white snowshoe hares from Pennsylvania (PA) and Utah (UT), two winter-brown black-tailed jackrabbits (*L. californicus*) from Nevada, and a previously sequenced winter-white mountain hare (*L. timidus*) from Europe (*10*). Phylogenetic analyses (*24*) predicted an exceptionally rare topology at *Agouti* that clustered individuals by winter coat color (Fig. 4B, fig. S5B). Pairwise divergence between all winter-brown and white individuals was significantly elevated across a known *cis*-regulatory region of *Agouti* (*25*, *26*) ~40 kb upstream of the transcription start site of the hair-cycle isoform (P<0.001; Fig. 4A, fig. S4). Divergence peaked across a ~20 kb interval (d_{XY}= 2.2-2.4%) that included a 1,033 base pair insertion on the winter-white haplotype and a ~4.3 kb deletion on the winter-brown haplotype (fig. S4). Additional functional data are needed to determine if either of these candidate mutations underlie the observed *cis*-regulatory differences in *Agouti* expression (Fig. 3B).

The elevated interspecific divergence between color groups suggests that the winter-white and brown *Agouti* alleles may have arisen relatively early in *Lepus* (*21*). In contrast, divergence within color groups was strongly reduced across a larger interval encompassing *Agouti* (Fig. 4A, fig. S6), indicating that winter coat color alleles may have been shared through hybridization. In support of this hypothesis, we found low but significant levels of genome-wide introgression (*27*) between snowshoe hares and both black-tailed jackrabbits and mountain hares (table S8). Window-based analyses of absolute divergence and derived allele sharing (*28*) identified *Agouti* among the strongest genome-wide signatures of introgression in both winter-brown and winter-white clusters (fig. S7).

Previous studies demonstrated mitochondrial DNA introgression from black-tailed jackrabbits, a western North American prairie-scrub species, into PNW snowshoe hares and speculated that hybridization may have contributed to the evolution of brown winter coats in snowshoe hares (*29*, *30*). Consistent with this, winter-brown snowshoe hares unambiguously nested within black-tailed jackrabbit variation at *Agouti* (Fig. 4B, fig. S5B) resulting in a 174 kb interval of significantly reduced divergence between species (d_{XY}=0.42% versus 1.2% genome-wide) embedded within a 236 kb interval of significantly elevated admixture proportions ($\hat{f}_{hom}$=0.71; Fig. 4A). Strong selection at a locus in the ancestral population can reduce divergence between species (*31*), resulting in false inferences of admixture (*28*); however, coalescent simulations of shared polymorphism with and without selection in the ancestral population indicate that such a long interval of shallow divergence is highly unlikely in the absence of interspecific gene flow (Fig. 4C, fig. S7, fig. S8). We also detected introgression within the winter-white *Agouti* group (fig. S7, fig. S8). Resolving the origin and functional relevance of these signatures awaits further investigation given that three other North American *Lepus* species undergo some degree of seasonal coat color change (*7*).

To link introgression with local adaptation we tested for selective sweeps based on allele frequency skews (*32*) while controlling for demographic history (fig. S9, table S9). We detected a hard sweep overlapping *Agouti* in winter-brown individuals from the polymorphic zone, but no evidence for a sweep in winter-white individuals (fig. S10, fig. S11). We estimate that the sweep of the winter-brown allele in the PNW occurred 3-15 kya, following the retreat of the Cordilleran ice sheet (*33*). High inferred selection coefficients (s) on the introgressed winter-brown *Agouti* background ($\bar{s}_{WA}$=0.024, $\bar{s}_{OR}$=0.015; fig. S11C) and fixation of alternative *Agouti* alleles between monomorphic winter-brown (BC) and winter-white (MT) localities (Fig. 4D), despite high gene flow (table S9), indicate that seasonal camouflage is maintained under strong local selection.

Despite widespread evidence of hybridization between animal species, introgression has rarely been directly linked to ecological adaptation (*34–36*). We have shown that introgression has shaped locally adaptive seasonal camouflage in snowshoe hares. Recurrent introgression of coat color variants could facilitate evolutionary responses to environmental change within populations as well as the long-term maintenance of adaptive variation among species, similar to adaptive polymorphisms in beak morphology across the radiation of Darwin's finches (*22, 34*). Introgression of winter-brown coats into snowshoe hares may have enabled their persistence in environments with more ephemeral seasonal snow following the end of the last glacial maximum. Temperate snow cover duration is predicted to dramatically decrease over the next century under most models of climate change (*37*), which may further intensify directional selection for winter-brown camouflage (*3*, *6*). Thus, the recent establishment of this dynamic color polymorphism through introgression is likely to be a critical component of ongoing adaptation to rapidly changing seasonal environments (*7*) in this iconic ecological model.

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helped develop the exome capture experiments. M.R.J. performed data analyses under the guidance of J.M.G., J.M.F., and J.D.J. M.R.J. and J.M.G. wrote the paper with input from the other authors. All authors approved the manuscript before submission. **Competing interests**: None declared. **Data and materials availability**: Original sequence data are available in the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA420081 (accession numbers SAMN08146448- SAMN08146534). Previously generated whole genome sequence data of snowshoe hare (SAMN02782769, SAMN07526959) and mountain hare (SAMN07526960) are also available in the Sequence Read Archive.

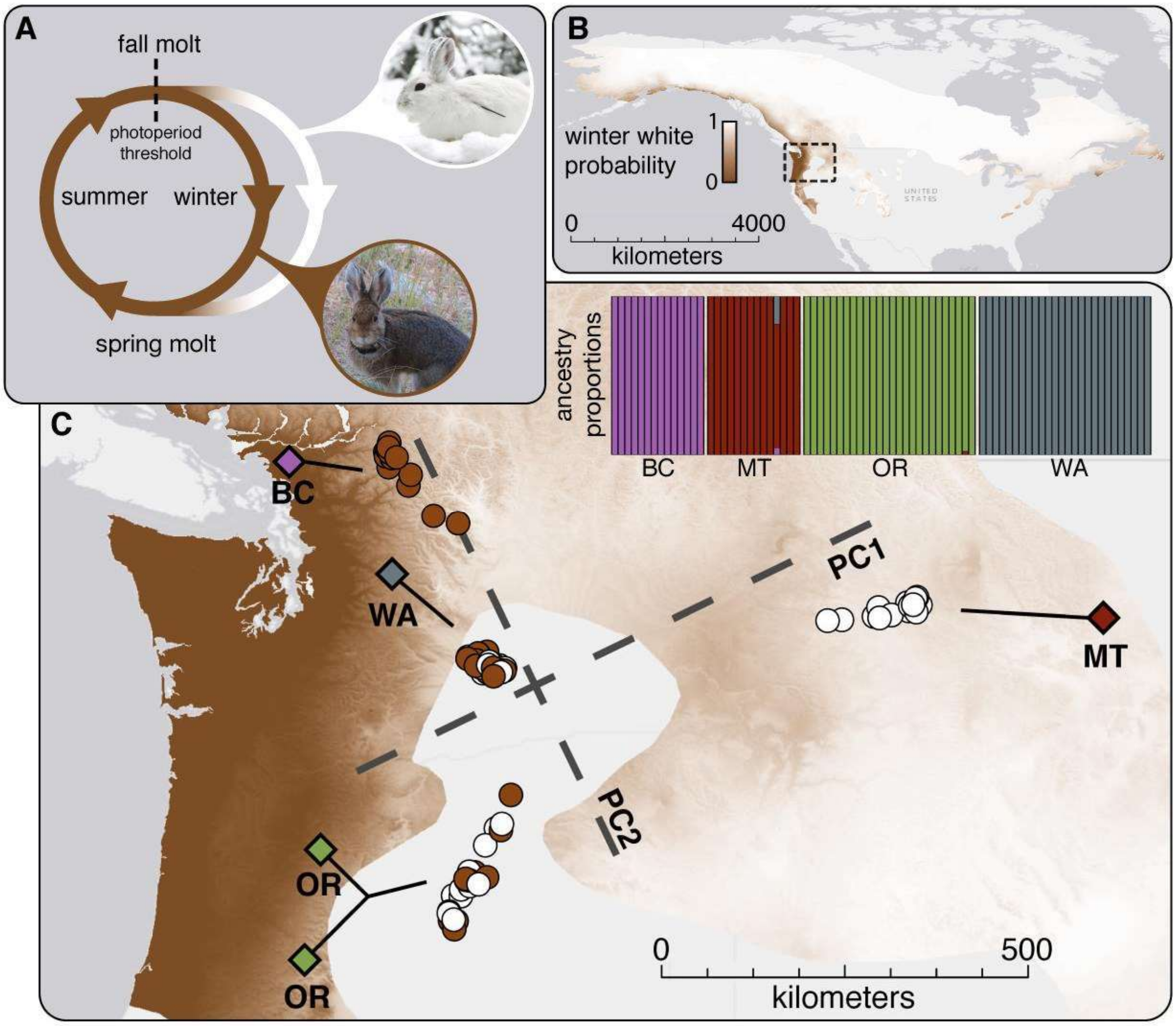

Fig. 1. Winter coat color polymorphism and population structure in snowshoe hares. (**A**) Alternative winter color morphs in snowshoe hares. (**B**) The modeled range-wide probability of winter-white coats, adapted from (*7*). (**C**) Principle components (PC1 – 7.42%, PC2 – 5.27%; coat color represented as brown/white circles) and population ancestry plots of 38,694 unlinked SNPs derived from 80 exomes sampled from five localities (colored diamonds) overlaid on the probability of winter-white coats in the Pacific Northwest.

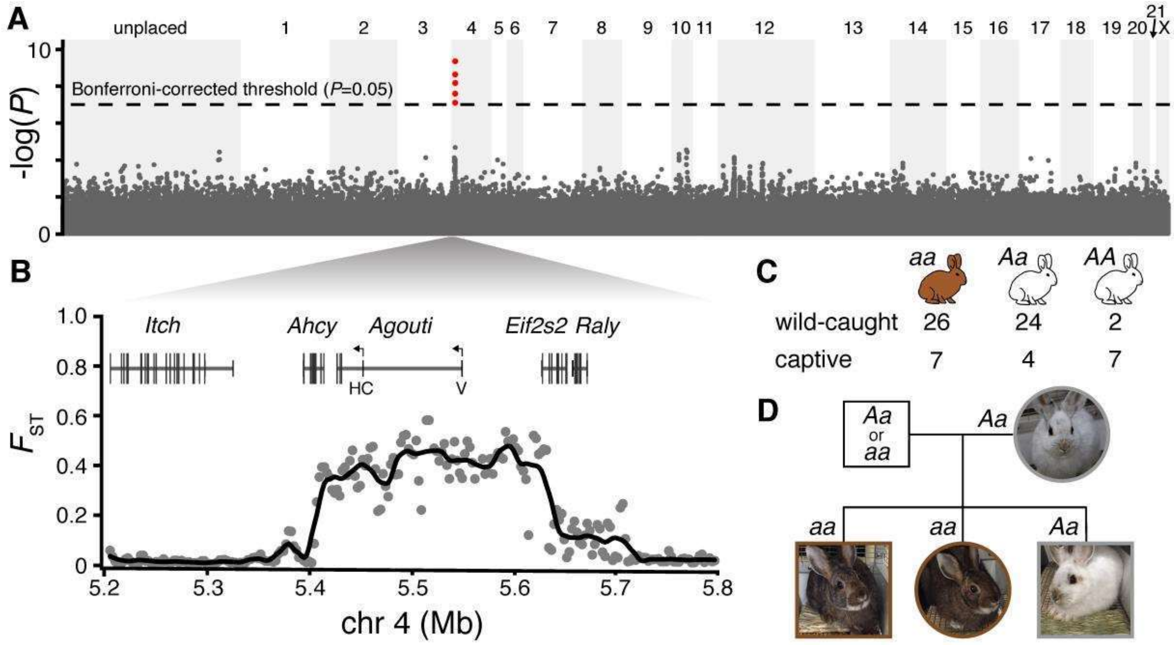

Fig. 2. The genetic basis of winter coat color polymorphism. (**A**) Exome SNP associations ($-\log_{10}$ of P-values, assuming dominant minor allele, 513,812 SNPs) for polymorphic zone individuals. Red points above dashed line exceed the Bonferroni-corrected threshold of P=0.05. (**B**) Gene structure across the associated interval and alternative *Agouti* transcription start sites (arrows) corresponding to hair-cycle (HC) and ventral (V) 5' UTRs. Sliding window averages of F_{ST} (5 kb with 2.5 kb step) between winter-white and winter-brown individuals with low-coverage whole genomes (15,173,804 SNPs). (**C**) Dominance of winter coat color inferred from *Agouti* genotypes of wild (OR and WA; Hardy-Weinberg χ^2 =1.6, P=0.21) and captive (WA and MT) hares. (**D**) Pedigree and genotypes of a mixed phenotype family (paternal genotype is unknown, but inferred to carry the *a* allele).

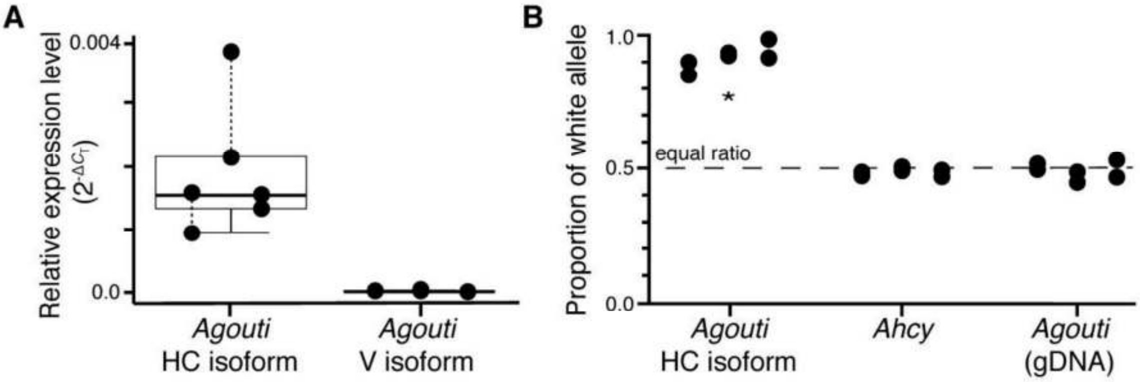

Fig. 3. *Agouti* expression in snowshoe hares during autumn molts. (**A**) The relative expression level ($2^{-\Delta C_T}$, normalized to *Gapdh*) of hair-cycle (HC) and ventral (V) *Agouti* isoforms in molting skin of winter-white (*Aa*) snowshoe hares. (**B**) Relative abundance of the winter-white allele in the same skin samples for *Agouti* hair-cycle transcripts, *Ahcy* transcripts, and *Agouti* genomic DNA. White allele proportions were significantly elevated in *Agouti* transcripts compared to *Ahcy* transcripts and *Agouti* genomic DNA (P<0.00001, Student's t-test). Pairs of points represent technical replicates.

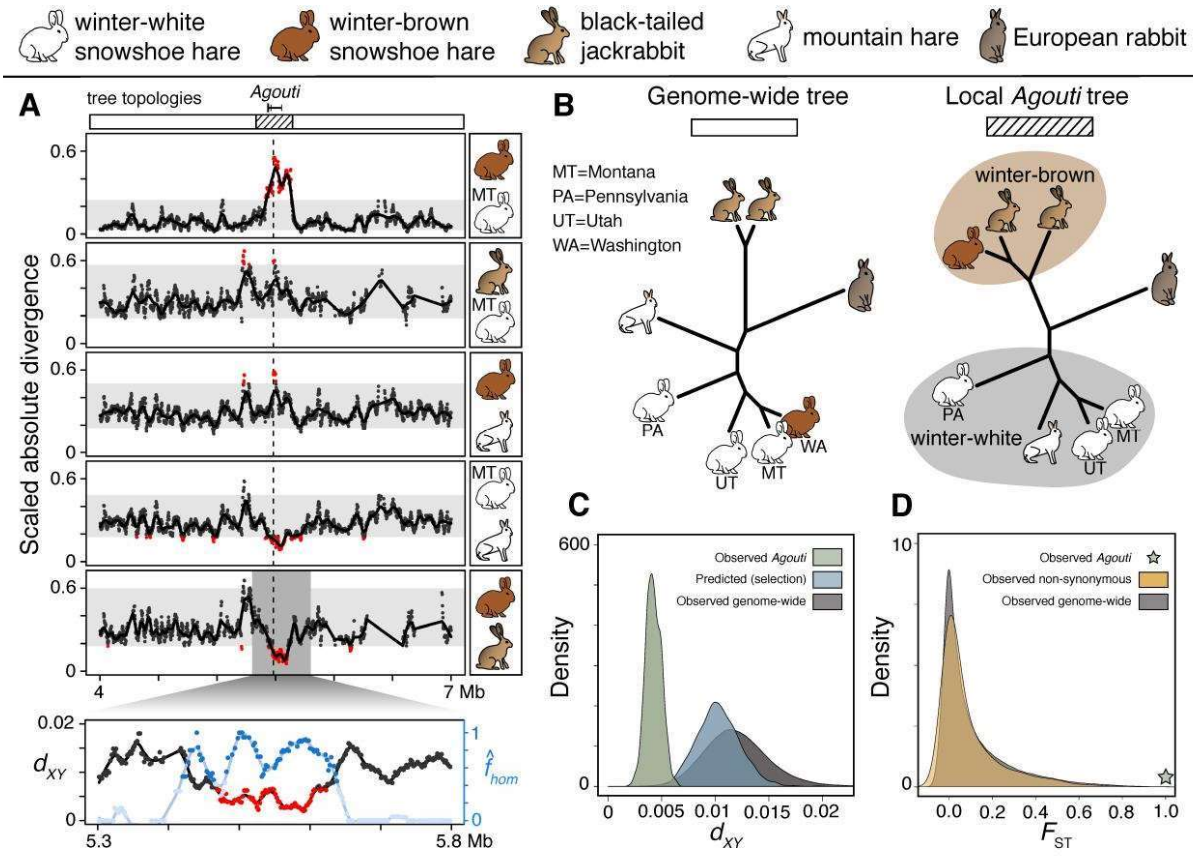

Fig. 4. The evolution of winter coat color alleles in hares and jackrabbits. (**A**) Estimated tree topologies across the *Agouti* region (top, see 4B). Mutation-scaled absolute genetic divergence in 20 kb sliding windows for pairs of individuals (dashed line indicates location of candidate insertion-deletion mutations). Gray rectangles represent 99.8% bootstrap quantiles and red points are windows with one-tailed $P<0.001$. Bottom plot shows a finer scale of absolute divergence in black (d_{XY}, red points with one-tailed $P<0.001$) and the fraction of introgression in blue ($\hat{f}_{hom}$, dark blue points with z-score>4) between black-tailed jackrabbits and the WA winter-brown snowshoe hare. (**B**) The most common genome-wide topology (white) and the local *Agouti* topology (hatched; rabbit outgroup). (**C**) Distributions of d_{XY} between the winter-brown snowshoe hare and black-tailed jackrabbits genome-wide (gray), at *Agouti* (green), and under simulations of strong ancestral selection (blue). (**D**) Distributions of SNP F_{ST} values between BC

(monomorphic winter-brown) and MT (monomorphic winter-white) hares genome-wide (gray) and for non-synonymous SNPs (yellow). $F_{ST}=1$ at a diagnostic *Agouti* SNP, indicated with a green star.