Mountain- and brown hare genetic polymorphisms to survey local adaptations and conservation status of the heath hare (*Lepus timidus sylvaticus*, Nilsson 1831)

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We provide the first whole genome sequences from three specimens of the mountain hare subspecies the heath hare (*Lepus timidus sylvaticus*), along with samples from two mountain hares (*Lepus timidus timidus*) and two brown hares (*Lepus europaeus*) from Sweden. The heath hare has a unique grey winter pelage as compared to other mountain hares (white) and brown hares (mostly brown), and face regional extinction, likely due to competitive exclusion from the non-native brown hare. Whole genome resequencing from the seven hare specimens were mapped to the *Lepus timidus* pseudoreference genome and used for detection of 11,363,883 polymorphic nucleotide positions. The data presented here could be useful for addressing local adaptations and conservation status of mountain hares and brown hares in Sweden, including unique subspecies.

Background & Summary

Two morphologically distinct mountain hare subspecies are present in Sweden, the nominal subspecies (*Lepus timidus timidus* Linnaeus, 1758), with extensive arctic/subarctic Eurasian distribution, and the endemic heath hare (*Lepus timidus sylvaticus* Nilsson, 1831)1, a more temperate adapted subspecies that occur in southern Scandinavia and, presumably, west Estonia2. The most conspicuous difference is the colour of their winter pelage, which is white in the nominal mountain hare subspecies and grey in the heath hare.

The geographic distribution of the nominal subspecies has gradually receded northwards in Sweden. One possible reason for this is competition from the introduced brown hare (*Lepus europaeus* Pallas, 1778) in the mid-19th century3, but the retreat may also be driven by climate change induced camouflage mismatch or other habitat changes4–6. While the heath hare is better camouflaged in areas void of snow, it too might face local extinction due to habitat alteration and competition from brown hare, as indicated from a persistent decline in hunting harvest data7.

Earlier research has also shown interspecific gene flow (i.e., introgression) between the two species, *Lepus timidus* and *Lepus europaeus*8. Introgression may facilitate adaptation of the non-native brown hare to boreal prerequisites (e.g., seasonal shift to white winter pelage,9 cold climate and/or pathogen resistance9). This two-species complex thus offers an excellent model system for exploring how rapid, human-induced ecological change affects species including, their behavioural, demographic and evolutionary interactions over time. In addition to the conservation challenges mentioned, both mountain hares and heath hares are culturally important game species in Sweden and declining populations cause concern.

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The genomic data reported here can be used for addressing complex adaptive patterns, assess species interactions and as a resource for developing monitoring tools for threatened populations. They also provide valuable insight into the subspecies differences and evolutionary histories. We have described 11,363,883 polymorphisms in whole genome resequencing data in order to detect differentiating markers and provide an example analysis screening of pigmentation genes that may influence winter pelage colour\(^{15}\). In addition to assessment of local adaptations of the two hare species, conservation status of the heath hare and developing management tools, the genomic data reported may also be used for added value to the understanding of the nature of interactions between mountain hares and brown hare in other contacts zones throughout their distribution\(^{17,18}\). In combination with harvest data, pellet inventories and camera trapping, these genetic data with potential additions could be used to develop integrative monitoring tools for mountain hares and brown hares.

### Methods

**Sample collection and DNA extraction.** Muscle tissue samples were obtained from seven hares shot from 2005–2017 during the regular hunting season in Sweden. The regular hunting season stretches from September 1 to February 15 for mountain hares in southern Sweden and to the last day of February in northern Sweden. The regular hunting season for brown hares is from September 1 to the last day of February across all of Sweden. The samples are representing two hare species and one hare sub-species (Table 1) in Sweden (Fig. 1A): two individuals of *Lepus europaeus*, two *Lepus timidus timidus* and three of *Lepus timidus sylvaticus*. All three *L. t. sylvaticus* (Le911, Le918 and Le919) samples were collected during January and February, when blue/light grey winter pelage confirm subspecies status (Table 1; Angerbjörn & Flux 1995). In addition, samples from one *L. t. timidus* (Le921) in sympathy with *L. europaeus* was collected in January, when the white winter pelage of this subspecies enable easy species determination. Samples from the final three specimens, one *L. t. timidus* (Le922) and two *L. europaeus* (LeA01 and LeV01), were collected in areas where the respective species occur in allopatri (details available in Table 1). Template DNA was extracted using the QiAmpSymphony DNA Mini Kit following the manufacturer’s instructions.

**Library preparation and sequencing.** The seven samples were sequenced over four lanes on a single flowcell on the Illumina HiSeq X. A single Illumina TruSeq PCR free library (Illumina, USA) was prepared for each hare sample at the SciLifeLab in Stockholm, Sweden (National Genomics Infrastructure Stockholm, Sweden). The insert size of the libraries was 350 bp. The samples were then pooled in equimolar ratios and sequenced over 4 lanes on the Illumina HiSeqX platform using the 2 × 151 Hiseq X SBS chemistry. The Bcl to FastQ conversion was performed using bcl2fastq_v2.19.1.403 of the Illumina software package CASAVA.

**Bioinformatics.** Quality of the raw sequencing reads\(^{14}\) was assessed using FastQC version 0.11.9\(^{15}\) (Fig. 1C). Adapter sequences were then removed from the raw sequencing reads using cutadapt version 2.10\(^{16}\) followed by further read quality checks. The trimmed reads were then mapped to the *Lepus timidus* genome\(^{11}\) (NCBI accession: GCA_009760805.1) with the Burrows Wheeler Aligner, BWA version 0.7.17\(^{17}\) using default the settings for the bwa mem algorithm. Each individual was sequenced over different lanes of sequencing, therefore all files for each individual were used as input during the Picard version 2.21.4 markduplicates\(^{18}\) step resulting in a single bam file per individual with PCR and optical duplicates removed. Variants were called across the genome in 100,000 bp regions using freebayes version 1.3.1\(^{18}\). VCFTools version 0.1.16\(^{21}\) was used to apply four rounds of variant filtering to the raw variant file. First, we allowed a maximum missing genotype rate of 50%, a minor allele count 3 and, a minimum variant phred quality score 30. Secondly, we then applied a minor Allele Frequency cut-off of 0.05 and a minimum mean depth of 20. Thirdly, we filtered variants with an allele Balance Frequency cut-off of 0.25 & AB < 0.75 | AB < 0.01. Finally, a hard filter allowing a maximum mean depth of 30 was applied. This filtering resulted in a variant file with high quality calls. A detailed list of commands and tools can be found in Table 2. Phasing of the genotypes in the final VCF file was achieved using beagle version 4.1\(^{22}\).

Details of the final VCF file\(^{23}\) were calculated using vt. To calculate the number of shared and unique SNPs, the VCF file was partitioned based on the sample species or subspecies. Then the variable statistics were recalculated using vcfixup version 1.0.0 and filtered to keep only variable sites (AC > 2 and AF < 1)\(^{23}\). Finally, the private VCF files were converted to bed files and the intersects of the bed files were calculated using intervenc\(^{24}\). Population differentiation was estimated in R version 4.0.4 (2021-02-15)\(^{25}\) using the glpca function contained in the package adegenet version 2.1.5\(^{26,27}\).

### Table 1. Metadata of the tissue samples (muscle) collected from hares shot during the regular hunting season. Occurrence defines whether the mountain hares and brown hares are sympatric or allopatic in the specific area. Sample Sequence Read Accessions (SRA) are associated with BioProject accession number PRJNA803958.

| ID     | Date collected | Species                  | Occurrence | Locality       | Country | Longitude | Latitude | Sex | SRA accession |
|--------|----------------|--------------------------|------------|----------------|---------|-----------|----------|-----|---------------|
| Le911  | 14.1.2017      | *Lepus timidus* (sylvaticus) | Sympatric  | Hallands väderö | Sweden  | 12.5872   | 56.443   | M   | SAMN25691261  |
| Le918  | 19.1.2017      | *Lepus timidus* (sylvaticus) | Sympatric  | Köberg          | Sweden  | 12.4126   | 58.1631  | n.a.| SAMN25691262  |
| Le919  | 12.2.2017      | *Lepus timidus* (sylvaticus) | Sympatric  | Hällö           | Sweden  | 11.2276   | 58.3368  | n.a.| SAMN25691263  |
| Le921  | 10.1.2005      | *Lepus timidus* (timidus)  | Sympatric  | Grimsmo         | Sweden  | 15.4725   | 59.7287  | F   | SAMN25691264  |
| Le922  | 29.9.2007      | *Lepus timidus* (timidus)  | Allopatic  | Svartnäset      | Sweden  | 14.81     | 62.6502  | n.a.| SAMN25691265  |
| LeA01  | 21.10.2011     | *Lepus europaeus*         | Allopatic  | Alnarp          | Sweden  | 13.0844   | 55.661   | M   | SAMN25691266  |
| LeV01  | 30.10.2011     | *Lepus europaeus*         | Allopatic  | Ven             | Sweden  | 12.7482   | 55.8995  | F   | SAMN25691267  |
To generate a mitochondrial phylogenetic tree with other hare species, we assembled the mitochondrial genomes of each individual. The mitochondrial genomes were assembled using NovoPlasty version 4.3. NovoPlasty requires a seed sequence with which to build the assembly from, thus we used the whole mitochondrial genome of *Lepus timidus* (Genbank accession NC024040) as the seed sequence. The assembled mitochondrial genomes were then aligned with those of the *Oryctolagus cuniculus* (accession number AJ001588.1), *L. europaeus*, and *L. timidus sylvaticus*.

**Fig. 1** (A) Map of sample locations for brown hares (*L. europaeus*; red), heath hares (*L. timidus sylvaticus*; green) and mountain hares (*L. t. timidus*; blue). (B) Principal Component Analysis of variants. Overlapping points are outlined in black. (C) Phred quality scores across all base for each individual sequencing library. Note, a phred score of Q30 is equivalent to 1 incorrect base in 1000 or 99.9% accuracy in the base call. (D) Venn diagram of shared and unique variants by species.
capensis (accession number GU937113.1), L. americanus (accession numbers NC_024043.1 and KJ397613.1), L. townsendii (accession numbers NC_024041.1 and KJ397609.1), L. coreanus (accession number KF040450.1), L. granatensis (accession number NC_024042.1 and KJ397610.1), L. toloi (accession number KM609214.1), L. yar-kandensis (accession numbers MN450151.1 and MG279351.1), L. sinensis (accession number KM362831.1), L. hainanus (accession number JQ219462.1), L. capensis (accession number GU937113.1), L. capensis (accession number GU937113.1), L. capensis (accession number LC073697.1), L. timidus (accession numbers NC_024040.1, KJ397605.1, KR030072.1, KR030070.1, KR030069.1, KR013248.1 and KR019013.1) using MAFFT v 7.49029. The phylogenetic tree was then built using the IQ-tree web service 30, with the best model determined by ModelFinder31 and using 200 ultrafast bootstrap replicates. A second phylogenetic tree was generated using the genome wide variants identified. This phylogenetic tree was estimated RaXML32 version 8.2.12 with a GTR $+\Gamma$+ ASC model and 200 rapid bootstrapping replicates. The whole genome ML tree was then ultrametrised using the chronos function in Ape version 5.433 to the estimated divergence time of Lepus europaeus and Lepus timidus.

**Validation.** Technical validation of the sequencing quality was performed using FastQC, followed by compilation of the data with MultiQCT24. To validate the origin of the datasets we used two methods, firstly the confirmation of the winter phenotype and second confirmation of the phylogenetic relationship and their ancestry coefficients. The phylogenetic relationship was assessed based on the mitochondrial genome as described above including other hare species and then using the genome wide variants from the closed analysis. The ancestry coefficients were determined using Poppr version 2.9.335 based on the DAPC method.

**Usage example.** To further illustrate a potential use of the dataset, we performed an example analysis of SNPs in 59 known pigmentation genes10, which could be used to understand the genetics of pelage color (Fig. 3). First, rabbit or mouse reference sequences were downloaded for each gene contained in Table 2 of Hoekstra, (2006) from the NCBI database. These genes were then mapped onto the pseudodereference Lepus timidus genome using the minimap226 splice aware transcript algorithm. We then counted the number of SNPs overlapping the pelage color gene regions identified based on the mapping with BEDTools17 and present this data(Fig. 3).
Sequencing data. The sequencing was successful and resulted in a total of 3.24 billion reads. The sequencing read quality was high with an average phred value greater than Q30 (Fig. 1C). The average mapping rate of the reads to the pseudoreference *Lepus timidus* genome is 93.59% with an average of 79.9% of reads mapping as proper pairs (Table 3). The raw sequencing data can be accessed from the NCBI short read archive under project accession and individual accession numbers can be found in Table 1.

### Table 3. Sequencing and mapping statistics. These statistics are based on the combined number of reads over all lanes of sequencing. * Note, this is assuming a genome size of 2.7Gbp².³

| ID    | Read length and type | % Duplicate reads | Error rate % | Reads Mapped (Million) | X Coverage of genome⁴ | % Reads mapped | % Mapped and proper pairs | MapQ 0 | Total sequences (Million) |
|-------|----------------------|-------------------|--------------|------------------------|-----------------------|-----------------|---------------------------|--------|--------------------------|
| LE911 | 150 bp Paired        | 13.08%            | 1.70%        | 463                    | 24                    | 93.40%          | 79.80%                    | 9.00%  | 495.7                    |
| LE918 | 150 bp Paired        | 13.68%            | 1.68%        | 441.1                  | 23                    | 93.60%          | 80.30%                    | 8.90%  | 471.3                    |
| LE919 | 150 bp Paired        | 14.17%            | 1.73%        | 321.1                  | 17                    | 93.40%          | 80.50%                    | 8.60%  | 343.7                    |
| LE921 | 150 bp Paired        | 12.56%            | 1.61%        | 409                    | 22                    | 93.70%          | 80.50%                    | 8.90%  | 436.3                    |
| LE922 | 150 bp Paired        | 13.18%            | 1.65%        | 453.6                  | 23                    | 93.40%          | 80.40%                    | 8.80%  | 466.5                    |
| LEA01 | 150 bp Paired        | 13.13%            | 2.54%        | 459.5                  | 24                    | 93.70%          | 79.00%                    | 9.30%  | 490.6                    |
| LEV01 | 150 bp Paired        | 13.66%            | 2.58%        | 502.7                  | 26                    | 93.90%          | 78.50%                    | 9.60%  | 535.6                    |

**Table 3.** Sequencing and mapping statistics. These statistics are based on the combined number of reads over all lanes of sequencing. * Note, this is assuming a genome size of 2.7Gbp².³

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**Variant calling and filtering.** Variant calling was performed using a parallel version of the freebayes software on the CSC Puhti server (Table 2) (CSC – IT Center for Science, Finland). Freebayes is a haplotype-aware variant caller and identified a total of 78,968,182 variants across the genome. After the application of various...
filters (Table 2) we recovered 11,363,883 high quality variants (Table 4) spread across the entire pseudoreference genome.

**Mitochondrial genome assemblies.** We assembled near complete mitochondrial genome sequences for each individual. The mitochondrial genome assemblies range from 16,648 bp to 17,612 bps in length, with four individuals producing circular contigs (LeA01, LE911, LE919 and LE921). All mitochondrial genes are present for each individual. The alignment of the mitochondrial genomes produced in this study are included as part of the Dryad dataset in the phylip format23.

**Technical Validation**

The PCA analysis (Fig. 1B) and complot (Fig. 2C) of the filtered SNP dataset further confirms the different species groupings. A similar grouping pattern has been observed in *Lepus* species sampled throughout Finland38, where the genetic diversity of the *L. europaeus* is small, likely due to a founder effect resulting in tight clustering of these samples, with more broad clustering of the *L. timidus* samples (Fig. 1B). Interspecific introgression may also influence the observed patterns38. Of the 11,363,883 high quality variants, 1,230,035 were unique to the *L. europaeus* and 596,291 to *L. timidus* (Fig. 1C).

To confirm the relationship of the hare samples, we generated a maximum likelihood (ML) phylogenetic tree based on the whole genome SNPs (Fig. 2A). The phylogenetic clustering of these samples confirms the origin of the samples, as the first branch separates the *Lepus europaeus* and *Lepus timidus*, followed by the separation of the *L. timidus* and the subspecies *L. timidus sylvaticus*. The sub-species creates a further split between the sample from the mainland and the two collected on the islands in the southwest of Sweden. Similarly, the phylogeny based on the mitochondrial genomes (Fig. 2B), first shows a split between the *L. europaeus* and *L. timidus*. Then the *L. timidus timidus* and *L. timidus sylvaticus* samples were further split. Interestingly, with the inclusion of other *L. timidus* genomes from the far East, the *L. timidus sylvaticus* clustered with these samples. The observed differences between whole genome and mitochondrial genome data may be influenced by introgressive patterns as well as different means of inheritance.

**Usage Notes**

The genomic data described could be used to assess adaptations of hares in, for example, pigmentation, cold durability and pathogen resistance in local, regional or a wide range of distributions. A particular value is to develop an integrated monitoring tool for assessing the conservation status of the heath hare in southern and central Sweden. This could be done by integrating genetic data, harvest data, pellet inventories and camera

| Filter applied | Number of variants in | Number of variants out |
|----------------|----------------------|------------------------|
| Maximum missing genotype 50% -- Minor allele count 3 -- Minimum quality score 30. | 78268182 | 19643329 |
| Minor Allele Frequency 0.05 -- Minimum mean depth of 20 | 19643329 | 12813212 |
| Filtering based on Allele Balance (AB): AB > 0.25 & AB < 0.75 | 12813212 | 12260751 |
| max mean depth of 30 | 12260751 | 11363883 |

Table 4. Number of variants called and filtered.
trapping. In addition to general conservation, the long-term preservation of the heath hare, as well as understanding of the nature of interactions between mountain hares and brown hares, is important from a resource perspective as hares are a game species in Sweden and elsewhere and, as such, a source of meat and recreation. Despite the small sample set, the data indicates a notable number of private alleles in the two mountain hare subspecies (Fig. 1D). The resulting divergence between the two clades (Fig. 2A,B) is clearly visible in the phylogenies and in ancestry complot analysis (Fig. 2C), which support that the subspecies represent biological entities beyond a single gene winter pelage colour difference. Furthermore, these private alleles could be used to examine the diversification of hares and their subspecies through the use of SNP panels. The subspecies split in Fig. 2A may have persisted in sympatry since the end of the last glacial maximum, although the timing needs further validation. The mechanisms maintaining the genetic divergence warrant further studies and raise an interesting question about the bona fide subspecies status of the heath hare.

To illustrate an example of how this genomic data could be integrated with future analyses we counted the number of variants found in known pigmentation genes. Although, the sample size for this analysis is too small in itself to provide any conclusions, we were able to identify a number of variants within and around genes (Fig. 3) with known effects on pigmentation. If integrated with a larger dataset, we trust that the genomic data provided here will provide a valuable resource for population genetics, conservation biology and evolutionary studies on hares.

**Code availability**

All programs and commands used to present this data are contained in Table 2.

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Competing interests
The authors declare no competing interests.

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