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1
2
3 # High quality genome of the brown hare
4 (*Lepus europaeus*) with chromosome-level
5 scaffolding
6
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22 ## Abstract
23
24 We present here a high-quality genome assembly of the brown hare (*Lepus europaeus* Pallas),
25 based on a fibroblast cell line of a male specimen from Liperi, Eastern Finland. This brown hare
26 genome represents the first Finnish contribution to the European Reference Genome Atlas pilot
27 effort to generate reference genomes for European biodiversity.
28 The genome was assembled using 25X PacBio HiFi sequencing data and scaffolded
29 utilizing a Hi-C chromosome structure capture approach. After manual curation, the assembled
30 genome length was 2,930,972,003 bp with N50 of 125.8Mb. 93.16% of the assembly could be
31 assigned to 25 identified chromosomes (23 autosomes plus X and Y), matching the published
32 karyotype. The chromosomes were numbered according to size. The genome has a high degree
33 of completeness based on the BUSCO score (mammalia_odb10 database), Complete: 96.1%
34 [Single copy: 93.1%, Duplicated: 3.0%], Fragmented 0.8%, and Missing 2.9%. The mitochondrial
35 genome of the cell line was sequenced and assembled separately.
36 The brown hare genome is particularly interesting as this species readily hybridizes with
37 the mountain hare (*Lepus timidus* L.) at the species contact zone in northern Eurasia, producing
38 fertile offspring and resulting in gene flow between the two species. In addition to providing a

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useful comparison for population studies, the genome can offer insight into the chromosomal evolution among Glires in general and Lagomorpha in particular. The chromosomal assembly of the genome also demonstrates that the cell line has not acquired karyotypic changes during culture.

Keywords: Lagomorpha, reference genome, chromosome structure, phylogeny, fibroblast cell line

Introduction

The brown hare (*Lepus europaeus* Pallas), also known as the European hare, is a widespread species in the western parts of Eurasia (Bock, 2020). Besides its native range, the brown hare has been introduced to numerous regions, including the British Isles, the Falkland Islands, Canada, South America, Australia and New Zealand. Reference? In many places the brown hare is regarded as an invasive species and a threat to the local ecosystems (Stott, 2003) or native species (Reid, 2011).

The brown hare is essentially a steppe-adapted species thriving in open grasslands and avoiding forested regions. Its colonization history in Europe is complex. The species has had apparent glacial refugia during the Pleistocene in the Italian peninsula, the Balkans and Asia Minor (Fickel *et al*, 2008), with a very interesting pre-glacial diversity hotspot in the archipelago of Greece (Minoudi *et al*, 2018). After the ice age, the species has been expanding its range both naturally as well as facilitated by human-caused changes in the landscape, especially the expansion of agricultural lands and pastures. As mentioned above, the brown hare has been also frequently introduced by humans to new areas from antiquity to the present day (Petrovan, 2013), resulting in potential mixing of ancestral populations. This is true also for Finland, where the species has arrived naturally from the southeast through the Karelian isthmus, but also *via* introductions to the southwestern parts of the country in the late 19th century. Reference?

Curiously, Linné was unaware of the existence of the brown hare, despite having described the mountain hare and the South African Cape hare (*Lepus capensis* L.). In fact, the brown hare is not native to Linné's home country Sweden and was introduced there only a century after his death (Thulin, 2003). As a reflection of the early neglection of this species, Peter Simon Pallas actually never published a formal description of the brown hare (Pallas, 1778). The species authority has been attributed to him based on the inclusion of *Lepus europaeus* into his identification table of hare species of the world (Pallas, 1778: 30) (Figure 1A). Consequently, there is also no information of the type locality or any other information of the species in the original publication. The type locality of the brown hare has been retrospectively assigned – without too convincing arguments – to Burgundy, France or Poland (Holden, 2005). Identifying Poland as a possible type locality can be traced to the Russian zoologist Sergey Ognev, and is based on his interpretation of Pallas mentioning in the 1778 text the hybrid forms of *Lepus variabilis* (synonym of *L. timidus*) and *L. europaeus* from Poland and Lithuania, as well as citing later works of Pallas (Ognev, 1940). In fact, Ognev (1940: 141) boldly states that "we consider southwestern Poland as a typical terrain for the nominal breed; there are hares with the characteristics of the basic form, expressed with full clarity" (translation from Russian).

While being widespread in the temperate regions of Western and Central Europe to the Caspian steppes in historical times, the brown hare began an impressive northward expansion in

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the 19th century. In the Fennoscandian region, the species reached the St Petersburg area by 1820s (Ognev, 1940), arriving in Finland at the turn of the century (Thulin, 2003). By 1930, the species had become established throughout southern Finland, with the range expansion stagnating southwest of the N64°-N62° -line until the 1990s. Unlike its northern relative, the mountain hare (*Lepus timidus* L.), the brown hare struggles in snowy winter conditions, with 150 days of annual snow cover limiting its northward expansion (Levanen *et al*, 2018a).

Quite likely benefitting from the ongoing anthropogenic climate change, the brown hare has had another impressive range expansion during the last three decades, in some places reaching the polar circle (Levanen *et al.*, 2018a) (Figure 1B). This range expansion together with the introductions have brought the brown hare increasingly in contact with the mountain hare, especially at the northern edges of the species distribution, which constitutes a threat especially to the more temperate climate adapted populations and subspecies of the mountain hare (Reid, 2011; Reid & Montgomery, 2007; Thulin, 2003; Thulin *et al*, 2021). Besides competing, brown hares also hybridize with mountain hares, producing fertile offspring and resulting in gene flow between the species. ~~Interestingly~~, this gene flow is biased towards the brown hare (Ferreira *et al*, 2021; Levanen *et al.*, 2018a; Levanen *et al*, 2018b; Thulin & Tegelström, 2002), which obtains genetic variation from mountain hares, some of which might have adaptive significance (Pohjoismaki *et al*, 2021). In contrast to the brown hare's expansion towards north, many of the Central European brown hare populations are contracting in range and numbers, likely driven by changes in agricultural practices and land use (Schai-Braun *et al*, 2013; Smith *et al*, 2005).

Overall, the brown hare's features as an invasive species outside Europe and expansive species in the north, its hybridization tendency and complex population ancestries make the species highly interesting not only to study population genetics and adaptation mechanisms, but also for the understanding the genomic makeup of species boundaries (Gaertner *et al*, 2022). These studies would be greatly facilitated by the availability of high quality, chromosomally scaffolded reference genomes (Blaxter *et al*, 2022).

Over the last decade we have witnessed a boom in sequencing technologies. This has led to many sequencing initiatives, such as the European Reference Genome Atlas (ERGA) and the Darwin Tree of Life (DToL), that aim to produce high quality reference genomes for a variety of organisms (Blaxter *et al.*, 2022). One recent technological advance is third generation sequencing or high-throughput sequencing of long molecules, providing the core of many of these assemblies. With read length N50's > 19 kbp, many genomes are being released with high contiguity compared to the early 2000's that were based on second generation short read sequencing technologies. Similarly, Hi-C techniques have further propelled these genomes into the realm of chromosome-scale assemblies, a level previously only held by model organisms. Coupled with these advances is also a decrease in price per base pair, making sequencing available to many laboratories and research groups. Combining the technologies of third generation and Hi-C sequencing, it is feasible to assemble reference genomes also for non-model organisms (Lawniczak *et al*, 2022). Here we apply these two technologies to produce a high-quality reference genome of the brown hare (*Lepus europaeus*).

While there is no existing genome assembly for the brown hare, a mountain hare assembly (GCA_009760805) exists, representing the Irish subspecies *L. t. hibernicus* and is based on a female specimen, thus lacking the Y-chromosome. This assembly is a so-called "pseudoreference", scaffolded using the rabbit (*Oryctolagus cuniculus* L.) reference genome

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126 (Marques *et al*, 2020). The original rabbit reference genome had been established using whole-
127 genome shotgun sequencing of females representing the inbred Thorbecke New Zealand White
128 line and was quite fragmented (Carneiro *et al*, 2014), updated only recently with long-read
129 sequencing data (Bai *et al*, 2021). In addition, the diploid chromosome number in domestic
130 rabbits is 2n = 44 (Korstanje *et al*, 1999), whereas it is 48 in brown hare and mountain hare
131 (Gustavsson, 1972).
132 To obtain a reference genome conforming to current standards, we used a fibroblast cell
133 line (LE1) of a male specimen from Liperi, Eastern Finland (Gaertner *et al.*, 2022), as a source of
134 high-molecular weight (HMW) DNA and fresh RNA. Compared to solid tissues, fibroblasts are
135 optimal for Hi-C, as the method was originally developed for cells growing in a monolayer
136 (Lieberman-Aiden *et al*, 2009).
137 We assembled the genome of *L. europaeus* using 25X genome coverage of PacBio HiFi
138 read data and then further scaffolded the assembly with Hi-C sequencing data. The assembled
139 genome is 2.96 Gbp in length with a contig N50 of 58 Mbp and a scaffold N90 of 25. The genome
140 has a high degree of completeness based on the BUSCO score, Complete: 96.1% [Single copy:
141 93.1%, Duplicated: 3.0%] Fragmented 0.8%, and Missing 2.9% based on the mammalia_odb10
142 database. As the scaffold N90 is 25, the genome assembly is near-chromosome scale as there is
143 an expected 23+X+Y karyotype for this species. By using RNA-seq data as extrinsic evidence for
144 the genome annotation, we were able to produce 19,906 gene models for this assembly. We trust
145 that the produced genome will prove to be valuable resource for future studies with hares, as
146 well as provide interesting insight into the genome evolution among Glires.

Much of the last paragraph of the intro repeats the abstract, and contains details on methods and results
I suggest condensing this to the bare minimum (aims), and consolidating the other info into abstract, methods, results and discussion.

147 Methods

148

149 Sampling and confirming of the species identity

150 A young male brown hare was hunted in October 2018 in Kuorinka, Liperi, Finland
151 (62.6207 N, 29.4478 E, Figure 1B). The collection location is agricultural area with a mosaic of
152 fields, shrubs and mixed forest with a strong brown hare population and only occasional mountain
153 hares. In Finland, brown hares are highly dependent on similar man-made environments
154 (Levanen *et al.*, 2018a; Levänen *et al*, 2019). The sampled population is sympatric with mountain
155 hares, as is the case for almost all Finnish brown hares. However, the population represents a
156 geographic continuum through Russian Karelia and the Baltic states to Poland, making our
157 specimen more representative of the suggested type population sampled by Pallas, compared to
158 western European brown hares. Although a trivial point, it is recommendable that the species
159 reference genome would represent or be closely related to the type locality population
160 (Lawniczak *et al.*, 2022). As pointed out earlier, Pallas was also aware of hybrids between
161 mountain hares and brown hares in Poland, making it unlikely to find eastern examples of brown
162 hares devoid of ancestral admixture with mountain hares.
163 The sampling had minimal impact on the local brown hare population and no impact on
164 the habitat. As brown hares are legal game animals in Finland and the hunting followed the
165 regional hunting seasons and legislation (Metsästyslaki [Hunting law] 1993/615/5§), the sampling
166 adheres to the ARRIVE guidelines and no ethical assessment for the sampling was required. The
167 sampling also did not involve International Trade in Endangered Species of Wild Fauna and Flora
168 (CITES) or other export of specimens, as defined by the Convention on Biological Diversity (CBD).

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The species identity was confirmed at site based on the morphological features distinguishing the brown hare from the mountain hare, the only other hare species in northern Europe. Further analysis of the coding part of the genome and mitochondrial DNA haplotyping showed minimal ancestral admixture with mountain hares (Gaertner *et al.*, 2022). In fact, unlike many other brown hares in the region, showing adaptive introgression of mountain hare specific UCP1 alleles, the specimen is homozygous for the common ancestral brown hare allele, UCP02 (Pohjoismaki *et al.*, 2021).

clarify even more explicitly that/whether these two cited studies investigated the exactly same specimen (DNA) that is here sequenced for the whole genome.

Generation and vouchering of the cell line

The fibroblast cell line (LE1) was isolated from the specimen as described earlier (Gaertner *et al.*, 2022) and is deposited as cryopreserved living cells under voucher number ZFMK-TIS-69747 into the biobank of the Stiftung Leibniz-Institut zur Analyse des Biodiversitätswandels, Zoological Research Museum Alexander König (ZFMK), Bonn, Germany. Additional identifiers for the sample are mLepEur2 and ERGA specimen ID ERGA_FI_3610_002 (COPO portal, https://copo-project.org).

HMW DNA extraction and PacBio HiFi sequencing

A salting out-method was used to extract high molecular weight DNA from cells grown to confluency on a 10 cm cell culture dish. Briefly, the cells were detached from the dish using trypsin, followed by centrifugation and two washes with PBS. The salting out-method then followed the 10X Genomics “Salting Out Method for DNA extraction from cells” protocol. Reference? The high molecular weight DNA was quantified using the a Qubit 3.0, followed by qualification with an 0.8 % Agarose gel. The DNA was analyzed by DNA Sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki on the PacBio Sequel II. Sequence data from two flow cells was produced.

‘sequenced’

this section is missing relevant info on how the extracted DNA was prepped prior to Pacbio seuqnecing; info on e.g. size selection, library prep etc should be added here

Mitochondrial DNA (mtDNA) sequencing

The mitochondrial genomes of our hare cell lines (Gaertner *et al.*, 2022) have been sequenced for the purposes of another study using approximately 2 kb overlapping PCR-amplified fragments of mtDNA. The primers used to amplify the mountain hare mtDNA were as follows:

Le93F: TTGTTTTGTAGCAAGTTTACACATGC
Le184R: GCTTAATACCTGCTCCTCTTGATCTA
Le1580F: TTAAACCCATAGTTGGCCTAAAAGC
Le1635R: TTGAGCTTTAACGCTTTCTTAATTGA
Le3045F: AGGCGTATTATTTATCCTAGCAACCT
Le3175R: CCTCATAAGAAATGGTCTGTGCGA
Le3921F: CCCCCTAATCTTTTCCATCATCCTAT
Le4482R: TCATCCTATATGGGCAATTGAGGAAT
Le4689F: AGGCTTTATTCCAAAGTGAATTATTATTCA
Le5417R: AGGCTCCAAATAAAAGGTAGAGAGTT
Le6696F: ATACCGTCTCATCAATAGGCTCCTTC
Le6756R: ATAAAGATTATTACTATTACAGCGGTTAGA
Le8603F: AGCCTATATCTACATGATAATACTTAATGA
Le8698R: CGGATAAGGCCCCGGTAAGTGG

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213 Le10552F: TTGAAGCAACACTAATCCCTACACTA
214 Le10613R: TCGTTCTGTTTGATTACCTCATCGT
215 Le11301F: ACCATTAACCTTCTAGGAGAGCTTCT
216 Le11807R: AGGATAATGATTGAGACGGCTATTGA
217 Le12407F: GTCTAATCCTAGCTGCTACAGGTAAG
218 Le12791R: GAGCATAAAAAGAGTATAGCTTTGAA
219 Le14204F: ATTGTTAACCACTCTCTAATCGACCT
220 Le14514R: CCAATGTTTCAGGTTTCTAGGTAAGT
221 Lt16056F: TGGGGTATGCTTGGACTCAAC
222 Le16119R: TCGTCTACAATAAGTGCACCGG
223
224 In total, 12 separate reactions were prepared to cover the mitochondria genome:
225 1. Lt16056F + Le184R: 1871 bp
226 2. Le93F + Le1635R: 1543 bp
227 3. Le1580F + Le3175R: 1596 bp
228 4. Le3045F + Le4482R: 1438 bp
229 5. Le3921F + Le5417R: 1497 bp
230 6. Le4689F + Le6756R: 2068 bp
231 7. Le6696F + Le8698R: 2003 bp
232 8. Le8603F + Le10613R: 2011 bp
233 9. Le10552F + Le11807R: 1256 bp
234 10. Le11301F + Le12791R: 1491 bp
235 11. Le12407F + Le14514R: 2108 bp
236 12. Le14204F + Le16119R: 1916 bp
237 (Expected fragment size based on the published *Lepus europaeus* mtDNA sequence from Sweden
238 [NC_004028.1]).
239
240 The fragments were amplified from total DNA preparations using a PCR program with a 1 min
241 94 °C denaturing step, followed by 35 cycles of 94 °C for 15 s, 56 °C for 15 s and 72 °C for 2 min
242 and a final 3 min elongation step at 72 °C. The obtained products were gel purified using the
243 GeneJET gel extraction kit (Thermo Scientific) and sent for sequencing using Illumina MiniSeq at
244 the Genome Center of Eastern Finland.
245
246 The sequence of the non-coding region-containing PCR fragment (Lt16056F + Lt184R) was further
247 validated by Sanger sequencing, applying also the following additional primer:
248 Le101F: TATAAATTCCTGCCAAACCCCAAAAA
249

how? together with which other primer?

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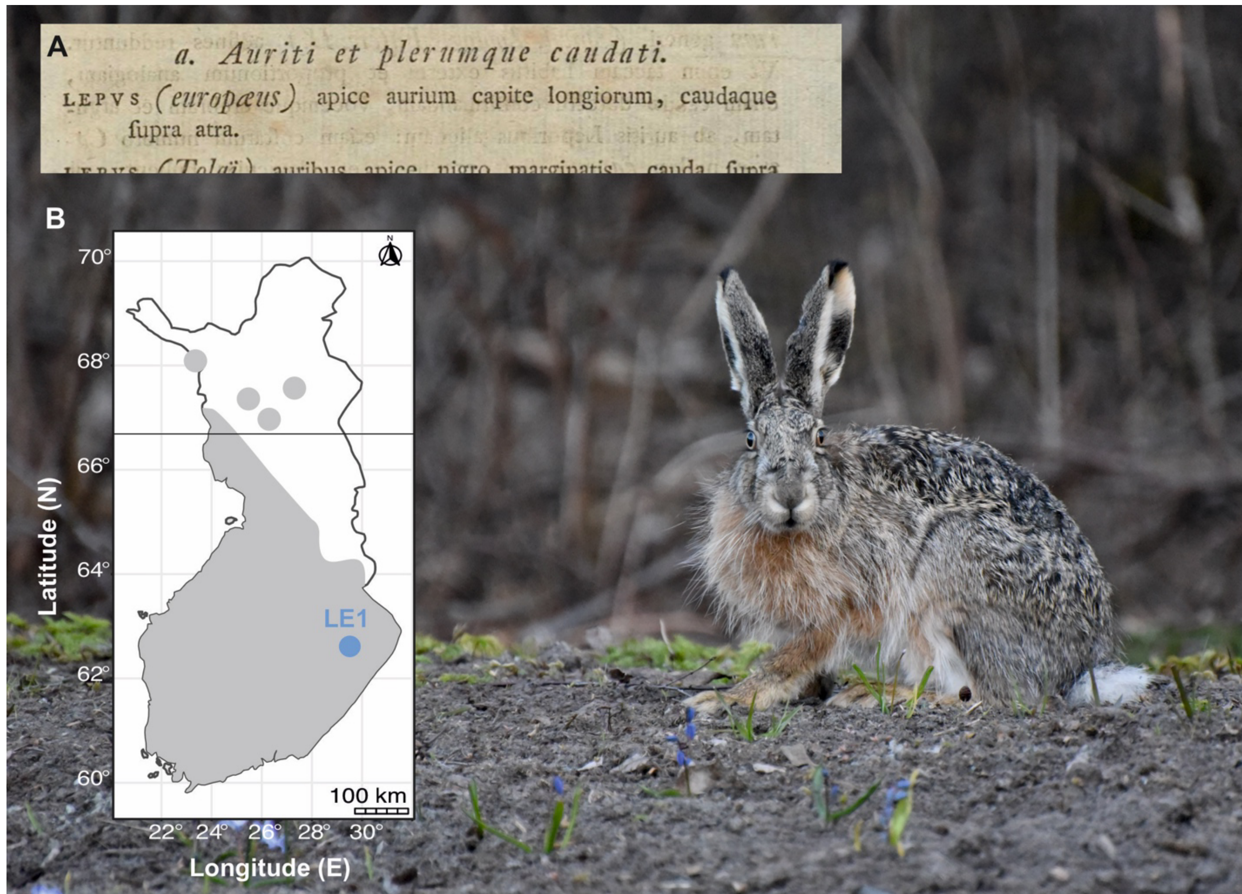
250


251 **Figure 1**. The brown hare. (A) An excerpt from the identification table by Pallas (1778: 30), which
252 has been recognized as the species description for *Lepus europaeus*. “Tips of the ears longer than
253 the head, tail black above.” (translation from Latin). (B) The geographic location of the LE1 sample
254 used in this study. The grey-shaded area shows the approximate current distribution of the
255 brown hare in Finland, based on the data in the Finnish Biodiversity Information Facility (FinBIF,
256 https://laji.fi/en). Individual records from the north are from settlemens or towns. The arctic
257 circle (black line) runs along the 66.6 ° parallel. Background photo shows a typical Finnish male
258 brown hare. Photo taken in Joensuu, 20 km East from the LE1 sampling site by Dr. Mervi
259 Kunnasranta.
260
261 **Hi-C library preparation**
262 Hi-C sequencing libraries were prepared following the protocol of (Belaghzal *et al*, 2017)
263 with the following changes: 1.) To prepare a diverse sequencing library, we performed the Hi-C
264 protocol in triplicates, 2.) Size fractionation was performed using the NEBNext Ultra II FS DNA
265 module, 3.) Library preparation was performed using the NEBNext Ultra II library preparation kit
266 for Illumina and 4.) Library enrichment was performed using triplicate PCR reactions with six
267 cycles of PCR. The PCR reactions were then purified using Ampure XP beads at a ratio of 0.9X. The
268 final clean libraries were quantified using Qbit, followed by agarose gel electrophoresis to confirm
269 the fragment size. The sequencing was performed on a single lane of the Illumina NovaSeq 6000
270 using the SP flowcell with paired-end chemistry 2 x 150bp.
271

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272 **Genome assembly**

273 HiFiasm version 0.18.7 (Cheng *et al*, 2021) was used to assemble the 25X (rq >0.99) PacBio
274 HiFi reads using the arguments *-l3 –h1 and –h2 –primary* to integrate the Hi-C read data and
275 produce a primary assembly. We then continued with the scaffolding of the primary assembly
276 and annotation. To process the HiC data, we first mapped the Hi-C data to the primary genome
277 assembly using BWA-mem version 0.7.17 (Li & Durbin, 2009) with the arguments *-SP*. The mapped
278 reads were then parsed and filtered using pairtools version 1.0.2 (Open2C *et al*, 2023). To parse
279 the aligned Hi-C reads, we used the options *–no-flip –dd-columns mapq –walks-policy mask*. The
280 parsed pair data was then sorted and deduplicated using default arguments. Finally, we selected
281 unique-unique and unique-rescued pairs and split these into the pairs file and bam file for input
282 in YaHS version 1.1 (Zhou *et al*, 2023). YAHS was run using the default parameters with the primary
283 contig assembly and the filtered Hi-C bam file. Contiguity and general genome statistics were
284 calculated using QUAST version 5.2.0 (Mikheenko *et al*, 2018). We assessed the completeness of
285 the genome by calculating the number of complete single copy orthologs with BUSCO version
286 5.1.2 (Manni *et al*, 2021), using the mammalia_odb10 database as well as the more lineage
287 specific glires_odb10 database.
288
289 **Genome annotation**

290 Repeat annotation of the genome was performed with EDTA version 2.1.0 (Ou *et al*, 2019),
291 a *de novo* repeat identification pipeline. Using the repeat library produced by EDTA, we masked
292 the scaffolded genome using RepeatMasker version 4.1.1 (Smit *et al*, 2013-2015). RNA-seq data
293 from the same cell line (SRA accession number: SRR18740842) as well as RNA-seq data from other
294 *L. timidus* libraries (SRA accession number: SRR10020054, SRR10020055, SRR10020060,
295 SRR10491719, SRR18740839, SRR18740840, and SRR18740841) was collected from the sequence
296 read archive (SRA). The RNA-seq data was trimmed using fastp version 0.23.2 (Chen *et al*, 2018)
297 and mapped against the masked genome using HISAT2 version 2.1.0 (Kim *et al*, 2019) with the
298 default parameters. Furthermore, we included the protein sequences of the assembled
299 transcripts from this cell line as further evidence for the genome annotation. These lines of gene
300 evidence were included in the annotation using BRAKER3 version 3.02 (Bruna *et al*, 2021).
301 Telomeric sequences [AACCCT]n were identified using a Telomere Identification toolkit (tidk)
302 version 0.2.31. The telomeric sequence copy number was then calculated in windows of 200kb
303 for visualization using Circos (Krzywinski *et al*, 2009).
304
305 **Mitochondrial DNA assembly and annotation**

306 Mitochondrial DNA was assembled from the PCR-amplified and Illumina sequenced
307 mtDNA using the MitoZ pipeline (Meng *et al*, 2019). After comparing the results of the pipeline's
308 outputs with different kmer options, we selected the best assembly as final. Run options used in
309 the final assembly were --clade Chordata –fastq_read_length 150, --requiring_taxa Chordata --
310 genetic_code 2 --kmers_megahit 21 29 39 59 79 99 119 141. The tools invoked by the pipeline
311 included fastp (Chen *et al.*, 2018) for cleaning the raw data, MEGAHIT (Li *et al*, 2015) for assembly,
312 after which sequences were filtered to ensure the correct taxa by HMMER (Wheeler & Eddy, 2013)
313 and further filtered for completeness of protein-coding genes. Annotation steps were done using
314 TBLASTN (Gertz *et al*, 2006), GeneWise (Birney *et al*, 2004) and MiTFi (Juhling *et al*, 2012). Final
315 manual curation, the annotation of the non-coding region (NCR) as well as the illustration of the

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316 mitochondrial genome was done using Geneious® 10.2.6. The functional loci on the NCR were
317 identified based on the similarity with the human (NC_012920) and mouse (FJ374652) NCR
318 sequences.
319

320 **Manual curation**

321 The assembled and annotated genome was manually curated to further improve its
322 quality as described in (Howe *et al*, 2021).
323

324 **Comparison with previous assembly**

325 We performed a comparison of our scaffolded assembly to the current *L. timidus* genome
326 assembly (NCBI Accession number: GCA_009760805). Mapping to the genome was performed
327 using minimap2 version 2.21 (Li, 2018) with the arguments *-asm5*. A dot plot of the alignment
328 was created using the R script pafCoordsDotPlotly.R (https://github.com/tpoorten/dotPlotly).
329

330 # Results

331

332 The genome assemblies can be accessed via BioSample accession SAMN33984520 as well as
333 BioProject accession PRJNA1009711 for the primary assembly and PRJNA1009710 for the
334 alternative assembly.
335

336 **Genome assembly**

337 The expected haploid genome size of the *L. europaeus* is similar to *L. timidus*, which has
338 been published to be 3.25 pg (3,178,500,000 bp) (Vinogradov, 1998), containing 23 autosomal
339 chromosomes and two sex chromosomes (Gustavsson, 1972). PacBio HiFi sequencing with two
340 flow cells resulted in 25 X coverage of the expected genome size. The sequence N50 of the HiFi
341 data was 19.97 Kb. Based on the PacBio HiFi data, the resulting genome size using k-mer (K = 21)
342 coverage is 2.4 Gbp (Figure 2A). The illumina sequencing of the Hi-C data produced 494,285,585
343 paired reads, representing about 51 X coverage of the genome. The duplication rate of the Hi-C
344 data was 17 %. Assembly with HiFiasm yielded a contig assembly of 2.96 Gbp made up of 671
345 contigs with a contig N50 of 58 Mbp. The longest contig was 149 Mbp. Using the uniquely mapped
346 Hi-C data, we were able to scaffold the contigs and fix misassembled contigs. The Hi-C scaffolded
347 assembly (Figure 2B) was 2.96 Gbp in size, similar to the contig assembly, while the largest scaffold
348 was 149 Mbp. The scaffold N50 was 124 Mbp (N=10) and the scaffold N90 was 21 Mbp (N=25).
349 The BUSCO scores of the *L. europaeus* assembly suggest it to be near-complete, with the following
350 results: Complete: 96.1% [Single copy: 93.1 %, Duplicated: 3.0 %] Fragmented 0.8 %, and Missing
351 2.9 % based on the mammalia_odb10 database.
352 To further improve the assembly, manual curation was performed using Hi-C maps
353 (Table 1), resulting in 76 scaffold breaks, 81 joins and removal of 62 erroneously duplicated
354 contigs. These interventions led to an increase in scaffold N50 of 1.69 % from 123.7 Mb to
355 125.8 Mb, and a reduction in scaffold count of 9.21 % from 716 to 650. Of the finalized assembly,
356 93.16 % could be assigned to 25 identified chromosomes (23 autosomes plus X and Y), matching
357 the published karyotype. Chromosomes were numbered according to size. The curated genome
358 length was 2,930,972,003 bp.

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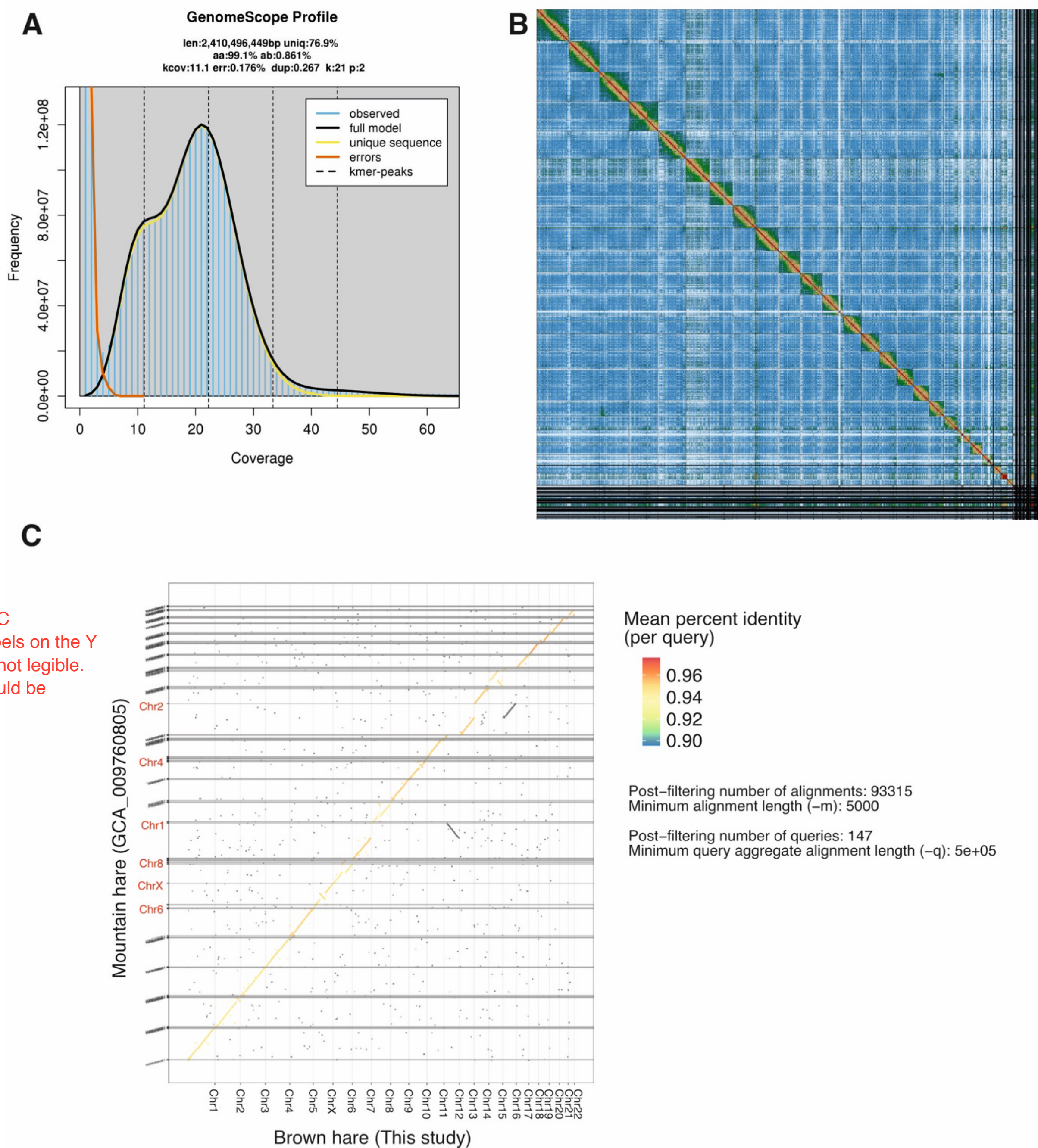
in panel C
some labels on the Y
axis are not legible.
This should be
clarified.



359
360 **Figure 2.** A) Genomescope2 profile of the PacBio HiFi data. B) Pretext map of Hi-C
361 scaffolding after manual curation. C) Dot plot comparison with the previous assembly.
362 Some examples of the previous chromosome assemblies are highlighted.
363 can you clarify better what is shown vs what isn't? Why are some highlighted, some not? See comment above about the Y axis labels.

364 **Table 1.** The brown hare reference genome chromosome assignment and assembly statistics

Chromosome assignment	
Chr length	2,730,543,680
Chr length %	93.16
Autosomes	23

Table 1 might be confusing to some reader
what is the chr length % (what is divided by what?
and the different fields should have the units included, when
possible, e.g. bp for basepairs.

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X	1
Y	1
Unlocalised	19

Statistics for primary assembly after manual curation	
# scaffolds	650
Total scaffold length	2,930,972,003
Average scaffold length	4,509,187.70
Scaffold N50	125,776,599
Scaffold auN	117,151,857.99
Scaffold L50	10
Largest scaffold	184,151,585
Smallest scaffold	1000
# contigs	1068
Total contig length	2,930,889,045
Average contig length	2,744,278.13
Contig N50	43,409,466
Contig auN	47,572,960.95
Contig L50	21
Largest contig	141,329,000
Smallest contig	1000
# gaps in scaffolds	418
Total gap length in scaffolds	82,958
Average gap length in scaffolds	198.46
Gap N50 in scaffolds	200
Gap auN in scaffolds	199.70
Gap L50 in scaffolds	208
Largest gap in scaffolds	200
Smallest gap in scaffolds	31
Base composition	
A	821,587,687
C	645,897,412
G	644,604,632
T	818,799,314
GC content	44.03 %
# soft-masked bases	0
# segments	1,068
Total segment length	2,930,889,045

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Average segment length	2,744,278.13
# gaps	418
# paths	650

Genome annotation

EDTA produced a curated custom repeat library of 7,045 repetitive elements. ~~Interestingly,~~ the proportion of the genome masked as repetitive elements was higher than expected at 46.8 % of the genome. In the previous genome assembly, only 28 % of the genome was masked, and similarly the *k*-mer based estimate of the repetitive elements was 26.7 %. To annotate the genome, we mapped ~1.6 billion RNA-seq reads from previously published RNA-seq libraries, including this cell line. The average mapping rate of all libraries was 89.95 %, which we consider a good indicator of the quality of this genome. Using BRAKER3, we were able to annotate 19,906 gene models in the genome. We then ran BUSCO on the predicted genes and the results show a good level of completeness: Complete: 81.1 % [Single copy: 63.3 %, Duplicate: 17.8 %], Fragmented: 1.1 %, Missing: 17.8 %, Total: 9226 when compared to the mammalia_odb10 database. Telomeric sequences on both ends of the chromosome were detected on 13 chromosomes (Figure 3A). Furthermore, telomeric sequences were found in high copy number throughout the chromosome length, a feature previously noted using FISH in mountain hares (Forsyth *et al*, 2005).

Comparison to the mountain hare assembly

Minimap2 was able to align 97.3 % of the contig sequences from the existing mountain hare genome assembly (GCA_009760805) to this genome (Figure 2C). As pointed out earlier, the mountain hare genome assembly has been scaffolded against the rabbit reference genome (Giska *et al*, 2022), which has a different chromosome count, among other differences. Despite these chromosome assembly and species differences, we have a high level of sequence similarity with the mountain hare genome assembly as well as a high degree of synteny. However, there a few minor notable inversions and structural differences between the two genomes (Figure 2C). These differences are mostly explained by the fact that the previous genome assembly retains the chromosomal arrangement of the rabbit genome (Beklemisheva *et al*, 2011). For example, in assembly presented in this study, chr7 and chr 12 have a good degree of synteny with the Chr1 (Chr1 - Rabbit) of the mountain hare assembly, demonstrating that the rabbit chromosome 1 is a fusion of two ancestral lagomorph chromosomes. Similarly, also the mountain hare assembly Chr2 – Rabbit is a fused chromosome and has good synteny and orientation with chr13 and chr16 in this genome. This exemplifies the importance of high-quality reference genomes to analyze chromosome evolution across lineages and opens doors for future studies of chromosomal evolution among mammals.

preferably provide a quantitative measure for both aspects

Mitochondrial genome

While it is possible to recover the entire mitochondrial genome from the HiFi sequencing data (Uliano-Silva *et al*, 2023), we had already previously sequenced the mitochondrial genomes of our hare cell lines for the purposes of another study. The assembly of the Illumina data

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405 provided a 16,836 bp mtDNA sequence, which was complete and circular, with a 142 bp non-
406 repetitive overlap between the 3´ and 5´ ends of the original assembly removed from the final
407 version. All the expected vertebrate mitochondrial genes, 37 in total, were found (Figure 3B).
408 Interestingly, the primary assembly was notably smaller than expected based on the
409 original PCR amplicons, with a notable difference between the expected and obtained non-coding
410 region (NCR) length. Sanger sequencing of the PCR-fragment containing the NCR (Lt16056F +
411 Le184R) revealed that it contained a sequence run consisting of six head-to-tail repeats of a
412 140 bp element between OriH and tRNA-Phe, explaining the incorrect assembly of the NCR
413 sequence from the short-read Illumina data. With the addition of the repeat elements, the final
414 mitochondrial genome size of our specimen is 17,540 bp.
415 Based on the sequence similarity with human and mouse mitochondrial genomes, we
416 were able to tentatively identify the termination associated sequence (TAS, 2 nt difference to
417 human), required for the generation of the 7S DNA and thus the displacement loop (D-loop)
418 structure on the NCR (Crews *et al*, 1979). Interestingly, assuming that the 7S DNA is roughly the
419 same size as in other mammals (600-650 nt), its 5'-end would map to the beginning of another
420 highly repetitive 240 bp region, likely to have structural significance. This is a notably longer
421 sequence run than the tRNA-like sequence reported at the replication origin of *L. timidus* mtDNA
422 (Melo-Ferreira *et al*, 2014). We have preliminary assigned this structure to correspond to the
423 heavy-strand origin of replication (OriH), supported by the fact that a similar, tRNA sequence-
424 derived hairpin structure is required for the priming of the origin of the light-strand replication
425 (OriL) (Fuste *et al*, 2010). Experimental validation of the proposed OriH is required.
426
427

428
429 **Figure 3.** A reference genome for the brown hare. A) A size map of 23 autosomes as well as X and
430 Y sex chromosomes of *Lepus europaeus*. Telomeric sequences and their relative lengths are
431 indicated on the inner circle. B) A schematic illustration of the mountain hare mitochondrial
432 genome. Genes encoded by the H-strand are placed inside and the L-strand genes outside of the
433 circle. NCR = non-coding region.

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434 **Discussion**

435

436 Given the challenges of assembling a large mammalian genome, we have produced a high-quality
437 genome of the brown hare. The genome has a high degree of contiguity (Contig N50 43 Mbp) and
438 completeness (BUSCO complete 96.1 %). Interestingly, the genome contains a high number of
439 repetitive elements, a fact that warrants further investigation to elucidate their identity. The
440 genome is in chromosome scale, and all 23 autosomes as well as X and Y chromosomes could be
441 scaffolded. Compared with the previously published mountain hare genome, the brown hare
442 genome is much more contiguous and also resolves chromosome structure including telomeric
443 repeats on both ends of several chromosomes (Figure 3A).

444 Together, this genome and the mountain hare genome will provide a solid base for future
445 studies. Having a high-quality genome for both species has important implications for the fields
446 of evolutionary and conservation biology. Due to the high contiguity of the genome, it will be
447 possible to perform accurate analyses of linkage disequilibrium as well as the identification of
448 runs of homozygosity (ROH). Accurate ROH analysis is useful for identifying and understanding
449 the impact of inbreeding burden on the genome of increasingly threatened and isolated species,
450 as quantitative traits can be associated with ROH burden (Ceballos *et al*, 2018). Furthermore, this
451 information might provide important insights into the hybridization of the brown hare with the
452 mountain hare, as well as help to pinpoint genomic regions that might be helping the species to
453 adapt to local environmental conditions during the ongoing range expansion (Pohjoismaki *et al.*,
454 2021).

455 The obtained 17,540 bp long mitochondrial genome also provides some interesting
456 aspects of mtDNA sequence variation within mammalian species. The size difference to human
457 (16,569 bp) and mouse (16,298 bp) mitochondrial genomes is caused by a rather long non-coding
458 region (2,102 bp vs. 1,123 bp and 879 bp in humans and mice, respectively), longer rRNA and
459 tRNA genes, as well as additional non-coding nucleotides between genes. Interestingly, the
460 mitochondrial genome of our specimen is slightly smaller than the 17,734 bp long previous NCBI
461 Reference Sequence for brown hare (NC_004028.1) from Sweden. The main size difference can
462 be attributed to a copy number difference in the repeat elements within the non-coding region,
463 with seven in the previous reference mtDNA vs. six in our specimen. Indels elsewhere in the NCR
464 explain why the length difference does not add up to simple multiplies of 140 bp repeat element.
465 Repeat length polymorphisms seem to be a common theme in hare mtDNA and we have noted
466 similar within-species size variation in the NCR also among Finnish mountain hares, with some
467 evidence of these variants occurring in multiple heteroplasmy within an individual (a manuscript
468 in preparation). To our understanding, such within-population and within-individual length
469 variation in the non-coding region is unusual and warrants for further investigation.

widespread in vertebrates. OK to study, but not key to mention here

470 The main technical challenge for highly continuous, good quality reference genomes Is the
471 availability of relatively large quantities of intact HMW DNA for HiFi sequencing. This typically
472 requires snap freezing of tissue samples and assuring an intact cold chain of below -80 °C for their
473 sending, handling, and long-term storage. While development in sequencing technologies can
474 reduce the required DNA amounts, obtaining high sequencing coverage over a large vertebrate
475 genome is easier when material is plentiful. Similarly, RNA is very sensitive to degradation in post-
476 mortem tissue samples, also requiring immediate preparation or snap-freezing to be suitable for

Line 465: this requires a reference regarding hares, but it needs to acknowledge that length polymorphism between and also within individuals (heteroplasmy) is common in vertebrates for the tandem repeat of the control region
see e.g. https://doi.org/10.1093/oxfordjournals.molbev.a025818; https://doi.org/10.1046/j.1365-294x.1998.00495.x

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477 RNA-seq. Although transcript data is not obligatory, it is highly useful for the purpose of genome
478 annotation. The requirement of large chunks of fresh tissue for -omics purposes complicates the
479 sampling of most vertebrates species. For example, in Finland all land vertebrates are protected
480 by law, with very few exceptions, such as game animals and pest species. Capturing and lethal
481 sampling is especially problematic for endangered species. Not only will the sampling require
482 extensive permits, but it is also difficult to justify and conduct ethically.
483 Although the source specimen for the cell line utilized in this study was hunted, fibroblasts
484 can be isolated from relatively small skin biopsies, such as ear clippings (Seluanov *et al*, 2010),
485 with minimally invasive sampling and harm to the individual. These cells can be expanded in
486 tissue culture and, when immortalized, provide a highly scalable source of fresh DNA and RNA.
487 Furthermore, living cells can be stored as frozen stocks in biobanks for decades (Fazekas *et al*,
488 2017). Tissue sampling can be done directly into the growth medium in ambient temperature,
489 allowing several days for the transport to the laboratory without a need for dry ice or N_2-
490 cryocontainers, which greatly simplifies collection and logistics. The same applies to the isolated
491 cells. As an example, we sent living cell suspensions from Joensuu, Finland to the ZFMK biobank
492 in Bonn, Germany, over regular airmail in ambient temperature. The receiving laboratory can then
493 amplify the cells by culturing and cryopreserve them in sufficient replicate stocks. It is worth to
494 point out that the obtained genome scaffolded to 23 autosomes, X and Y chromosomes, as
495 expected for the species, shows no evidence of karyotypic drift caused by cell culture, dispelling
496 possible concerns for the use of cell lines for such work (Gaertner *et al.*, 2022).
497 The brown hare is an iconic European mammal, locally familiar to a wider public of nature-
498 goers, farmers and hunters, while being at the same time an invasive species on many other
499 continents. It has populations with complex ancestral makeup due to isolated Pleistocene refugia,
500 frequent introductions and cross-species hybridization, making it an exceptionally interesting
501 mammal for genetic studies. Furthermore, it can be both an endangered, as well as a rapidly
502 expanding species, depending on the local environmental context, prompting opportunities for
503 both conservation as well as invasion genetics studies. A high-quality reference genome will allow
504 us also to peer deeper into genome structure and chromosome evolution, which will be
505 particularly interesting from the viewpoint of genetic introgression (Juric *et al*, 2016) and
506 maintenance of species boundaries. Any issues of genetic compatibility between species should
507 leave their fingerprint in the genomes (Skov *et al*, 2023), providing interesting insight into the
508 speciation mechanisms (Wolf *et al*, 2010). Other *Lepus* reference genomes available soon will
509 help to complete this picture.

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521 Data, scripts, code, and supplementary information availability

522 The genome assembly is available from the NCBI database under BioProject ID PRJNA950335. The
523 cell line is available through the corresponding author as well as from the ZFMK biobank.
524

525 Conflict of interest disclosure

526 The authors declare that they comply with the PCI rule of having no financial conflicts of interest
527 in relation to the content of the article.
528

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532

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