Ice-Age Climate Adaptations Trap the Alpine Marmot in a State of Low Genetic Diversity

Highlights

- The Alpine marmot is among the least genomically diverse animal species
- Its diversity was lost during consecutive ice-age climate-related events
- An extreme lifestyle hampered the subsequent recovery of genetic variation
- Alpine marmots show why large populations can coexist with very low genetic variation

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In Brief

Despite being highly abundant and well adapted, Gossmann et al. report that the Alpine marmot is among the least genetically diverse animal species. The low diversity is found to be the consequence of consecutive, climate-related events, including long-term extreme niche adaptation, that also greatly retarded the recovery of its genetic diversity.
**SUMMARY**

Some species responded successfully to prehistoric changes in climate [1, 2], while others failed to adapt and became extinct [3]. The factors that determine successful climate adaptation remain poorly understood. We constructed a reference genome and studied physiological adaptations in the Alpine marmot (Marmota marmota), a large ground-dwelling squirrel exquisitely adapted to the “ice-age” climate of the Pleistocene steppe [4, 5]. Since the disappearance of this habitat, the rodent persists in large numbers in the high-altitude Alpine meadow [6, 7]. Genome and metabolome showed evidence of adaptation consistent with cold climate, affecting white adipose tissue. Conversely, however, we found that the Alpine marmot has levels of genetic variation that are among the lowest for mammals, such that deleterious mutations are less effectively purged. Our data rule out typical explanations for low diversity, such as high levels of consanguineous mating, or a very recent bottleneck. Instead, ancient demographic reconstruction revealed that genetic diversity was lost during the climate shifts of the Pleistocene and has not recovered, despite the current high population size. We attribute this slow recovery to the marmot’s adaptive life history. The case of the Alpine marmot reveals a complicated relationship between climatic changes, genetic diversity, and conservation status. It shows that species of extremely low genetic diversity can be very successful and persist over thousands of years, but also that climate-adapted life history can trap a species in a persistent state of low genetic diversity.

**RESULTS AND DISCUSSION**

We sequenced, assembled, and annotated a reference genome for the Alpine marmot (Figure 1A) on the basis of a wild-living male selected from a typical, central Alpine habitat (Mauls...
region, North Italy; Data S1; STAR Methods). Phylogenomic and phylogenetic analyses confirmed the Alpine marmot’s relationships to other mammals, rodents, squirrels, and marmots, including the groundhog (Marmota monax) [8, 9] (Figures 1B–1D; Data S1; Figure S1). We also identified an unusually large integration of mitochondrial genome into the nuclear genome (nuclear mitochondrial DNA segment, NUMT [10]), which comprises 91% of the mitochondrial genome. The NUMT is well conserved (with 84% similarity to the mtDNA), despite no evidence of functional constraint (no expression on the mRNA level, and many premature stop codons). The nuclear insertion occurred before the common ancestor of Marmota, Ictidomys, and Cynomys (Figures 1D and S2), during which time substitutions occurred at most synonymous sites in the mitochondrial genomes (mitochondrial Ks estimates: Ictidomys-Marmota 0.48; Tamias-Marmota 1.11). This is suggestive of a low rate of nuclear genome evolution, and this was confirmed by a comparison of genome-wide rates in other rodents (median synonymous substitutions per codon/year: Marmota:0.0017 versus Ictidomys:0.0020, Wilcoxon test p = 1.6 × 10^{-20}; Marmota/Ictidomys:0.0029 versus Mus/Rattus:0.0042, p = 6.1 × 10^{-106}) and by a collinearity analysis (Figure 1B). The Alpine marmot is therefore characterized by an overall low rate of genomic evolution.

We next searched for genes undergoing exceptional rates of protein evolution specific to hibernating rodents (Figure S3A). Among a group of 1,571 differentially evolving genes, there was specific enrichment for genes related to photoreception (Data S1), and for the metabolic pathway of glycerolipid metabolism, which is essential for the synthesis of triacylglycerols (TAGs), the precursor of fatty acids (Figure 2A). Furthermore, genes in the pathway of fat digestion and absorption, which is essential for the utilization of stored fats, have undergone diversifying rates of evolution within the marmot lineage after the split with the thirteen-lined ground squirrel (Ictidomys tridecemlineatus; Figures 2B and S3B).

While the specific enrichment for photoreception was unexpected, the adaptation of the lipidome is plausibly associated with cold temperature adaptation. For physical reasons, a higher degree of unsaturation increases membrane fluidity at low temperature. This adaptation is particularly evident in the white adipose tissue (WAT) that serves hibernating animals for energy
storage [11], where the levels of polyunsaturated fatty acids are positively correlated with survival during winter hibernation [11–13]. We therefore used mass spectrometry and recorded a lipidome of WAT obtained from Alpine marmots and compared it to that of two non-hibernating rodents: rats (Wistar line) and mice (C57Bl6 line). WAT (Figure 2C; Table S1) TAG and diacylglycerol (DAG) lipids were highly discriminatory, and in the Alpine marmot characterized by greater acyl chain length and unsaturation. Indeed, some changes were substantial: detected up to 4-fold higher level of unsaturation in TAGs and DAGs, the main energy storage lipids that need to be accessed at low temperature (Figure 2D).

A further known adaptation of the Alpine marmot is complete parasite clearance prior to hibernation [14]. While we found no enrichment at the pathway level, four genes involved in anti-parasite defense exhibit significantly elevated molecular substitution rates in comparison to the thirteen-lined ground squirrel. The fastest gene was Interleukin 4 \( (dN/dS \text{ of } 2.3072, \text{ top 1\% in a phylogenetic analysis by maximum likelihood (PAML) analysis) (Data S1).} \) The cytokine-cytokine receptor pathway may therefore have undergone adaptive evolution, suggesting that parasite clearance before hibernation might be more than a passive process caused by starvation of the parasites.

We next studied the genome-level diversity of the Alpine marmot. Unexpectedly, the within-individual diversity was found to be remarkably low, with a heterozygosity of 0.12 per kilobase (Figure 3A). To place this result in context, we performed the same analysis on a panel of other mammalian genomes. As well as humans, and close relatives of the marmot, we chose species known for very low heterozygosity, often associated with conservation risk, habitat loss, extreme isolation, or artificial inbreeding [15] (Data S1). Although it is not considered a conservation concern, and despite its high abundance and large geographic range, the Alpine marmot is the least heterozygous among the panel of wild-living animals, including the extreme case of low diversity for a wild-living animal, the Iberian Lynx (Figure 3A). Lower heterozygosity was found only in the lab mouse (129P2/OlaHsd), artificially backcrossed for decades (0.05/kb; Data S1). The Alpine marmot also remains extreme among a large number of species for which heterozygosity values are available in the literature [15].
An individual may have low levels of genetic diversity for three different reasons: either there is low diversity in their species as a whole or in their local breeding population, or they might have resulted from close inbreeding (i.e., consanguineous mating) within an otherwise diverse population [16]. The latter two explanations were strong possibilities in the Alpine marmot, where breeding takes place in extended family groups, and inbreeding depression has been observed [17–20]. To distinguish between these possibilities, we resequenced a further 11 Alpine marmot individuals (Data S1), both from the reference population, and two additional possibilities, we resequenced a further 11 Alpine marmot individuals—LGS individuals had higher intra-individual diversity (Figures 3E and S4C), and—consistent with this—LGS individuals had higher intra-individual diversity (Figures S4 and 3D). In particular, the Mauls and Gsies marmots had heterozygosity of ~0.1–0.13/kb, similar to the reference animal (Figure 3A), while estimates from LGS marmots were over twice as high (0.29–0.34/kb; Figure S4C), although these values are still extremely low compared to other mammals (Figure 3A).

The data suggest that the smaller local populations (Gsies and Mauls) contain a high proportion of close relatives (Figure S4A), but there was no evidence of consanguineous mating, whose signature is well explained by their differences in local breeding sizes. There is little evidence of consanguineous mating, nor of a recent bottleneck recovery.

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empty red bordered circles. Other species in the genus *Marmota* are shown as black filled circles, including the threatened *M. vancouverensis*, which appears at the bottom left of the graph. In difference to the genome-wide diversity, for which the Marmot is an extreme (A and B), it has a typical diversity in these sites that are characterized by a higher spontaneous mutation rate.

(D) Life history of the Alpine marmot (red point) in comparison to other Eutherian mammals (data from [26]). After correcting for body mass, much of the variance in character is due to variations among species that have substantially different life histories. In particular, the levels of effective selective constraint, which are a result of both its large adult body size and the delayed dispersal of the Alpine marmot, and its large adult body size, yields a very large propagule size [25], consistent with the observed low diversity (filled red point). The diversity inferred for the ancestral marmot population at the end of the Pleistocene (empty red point) fits this prediction even more closely. The correlations observed are very similar whether the marmot data are excluded (gray lines) or included (black lines).

(B) The strength of purifying selection on amino acid variation in Alpine marmots corresponds to their low effective population size revealed from a pattern consistent across diverse animal species. Data from the Alpine marmot (colored points) have been added to the data of [24]. The correlations are very similar whether the marmot data are excluded (gray lines) or included (black lines).

(C) Microsatellite diversity in different Alpine marmot populations compared to many other species of mammals, including other marmot and rodent species. The number of microsatellite alleles (y axis) is plotted against the expected heterozygosity (x axis). Populations of the Alpine marmot from LGS are shown as red points, and estimates from other subpopulations of the same species, also from the French alps, are shown in as

When the effective population size is low, natural selection can become less effective. This situation was evident in the Alpine marmot genome. First, ratios of amino acid changing to synonymous polymorphism are high ($\tau_{\text{AC}}/\tau_{\text{SN}}$: LGS 33.7%; Mauls 37.5%; Gsies 39.0%; combined sample: 34.6%; Figure 3E). Second, the distribution of fitness effects [23] suggests that 30%–40% of amino acid variants are under ineffective purifying selection, and a further 5%–10% in the “slightly deleterious” range (1 < $N_{\mu}S < 10$), where selection might become ineffective, following any further drop in $N_{\mu}$ (Figure 3F).

Given the fact that the Alpine marmot is well adapted and highly abundant, these results initially appeared surprising. To explain the low diversity, we next considered the marmot’s unusual life history, which differs, in part substantially, from that of typical (Alpine) mammals. Previous work has shown that species-wide diversity across a broad range of animal species is well predicted by their “propagule size,” i.e., the size in centimeters of the dispersing life stage. The delayed dispersal of the Alpine marmot, and its large adult body size, yields a very large propagule size [25], consistent with the observed low diversity (filled red point). The diversity inferred for the ancestral marmot population at the end of the Pleistocene (empty red point; F) fits this prediction even more closely. The correlations observed are very similar whether the marmot data are excluded (gray lines) or included (black lines).
its delayed, adult dispersal, consistent with its form of proto-
cooperative breeding. Even after correcting for body size, the
Alpine marmot is extreme among mammals, and especially ro-
dents, in the extent to which it invests in a small number of
"high-quality" offspring (Figure 4D). These traits are plausibly
adaptations to cold-climate habitation [25, 26].

While correlations of genetic diversity with life history are well
established, it remains unclear exactly why they hold. One pos-
sibility is that a species’ life history has a major influence on its
response to demographic perturbations, such as major changes
in climate [24]. Such events are historical contingencies, but
different species might respond in predictably different ways,
with predictable consequences for their genetic variation. The
Alpine marmot is a useful case study here, because its fossil re-
cord provides clear evidence of a major demographic perturbation,
associated with climate change. In particular, the species
underwent a large range contraction toward the end of the
Pleistocene, after the last glacial maximum [28]. The shift from
the steppe to Alpine habitats might also have brought increasing
isolation, exacerbated by the expansion of forests that replaced
the cold steppe of the Pleistocene, and that are incompatible
with the Alpine marmot’s lifestyle. To shed light on the demo-
graphic history and its effects on genetic variation, we recon-
structed the effective population size over time, using the pair-
wise sequentially Markovian coalescent (PSMC). Toward the
end of the Pleistocene (left-hand side of the plot in Figure 4E)
the PSMCs confirmed the signature of the known range contrac-
tion, with a dip in the LGS population size between the last glacial
maximum (~20 ka), and the start of the Holocene (11.65 ka). This
signature is messy, but this is as expected in a structured popu-
lation [29, 30]. To investigate the more recent demographic
events, we analyzed the genome-wide site frequency spectrum of
the two least connected populations (LGS and Gsies; Figure 4F; Table S3). After comparing several different demographic models, we inferred that these populations descended from a
single ancestral population, that was roughly three times larger
than the current populations (11,942 versus 4,544 breeding indi-
viduals). The population split and decline is dated at 26 kybp,
with confidence intervals overlapping the last glacial maximum.
We also infer strong and asymmetrical gene flow, continuing
long after the split. Our findings are consistent with a post-glaci-
ation colonization hypothesis progressing from the West to East
Alps that matches the fossil record [21, 22].

By combining the population size estimates (Figure 4F), and
our measure of current diversity, \( \pi_G \), we can estimate the genetic
diversity of this ancestral marmot population (empty red point,
Figure 4A). The inferred ancestral diversity is remarkably close
to the value that would be predicted from the marmot propagule
size (inferred ancestral, \( \pi_G = 8.6 \times 10^{-4} \); predicted from propa-
gule size, \( \pi_G = 7.7 \times 10^{-4} \)).

If the low genetic diversity of the Alpine marmot is due to a
slow recovery from past demographic events, then we might
predict to see signs of an ongoing recovery in the data. No
such evidence was found in the genome-wide data: neither
RoH, nor the site frequency spectrum showed signs of recovery
from a bottleneck (Figure 3D; Tajima’s D at synonymous
sites = 0.45). However, in regions of the genome with typical mu-
utation rates, the recovery of diversity might be glacially slow.
In this case, a recovery would leave its signature only in rare re-
gions with very high mutation rates, such as microsatellite loci
[24, 31]. Indeed, in stark contrast to their low genome-wide diver-
sity, the microsatellite diversity of Alpine marmots was found to
be typical, of mammals as a whole, of rodents, and of the genus
Marmota (Figure 4C). Levels of microsatellite diversity in this
genus are conspicuously lower only for Marmota vancouver-
ensis, which lives only in the limited habitat of Vancouver island,
and is the sole marmot species under threat of extinction [32].

Taken as a whole, these results have two contrasting implic-
ations for our understanding of extinction risk. First, it is clear that
low levels of genome-wide variation, on their own, need not
imply an imminent threat of extinction. The Alpine marmot has
persisted successfully, with remarkably low levels of genetic
variation, for tens of thousands of years. Conversely, however,
there is no cause for complacency. If adaptation to future envi-
ronmental change does require abundant genomic variation,
then populations may be unable to respond, even if they are
characterized by high levels of microsatellite diversity and large
population size. All species may undergo occasional demo-
graphic fluctuations, but factors such as low fecundity, long gen-
eration time, and a slow rate of genome evolution would cause
some species to take much longer to replenish their genetic di-
versity after these events. All of these factors are characteristic
of the Alpine marmot, very plausibly due to its niche adaptation
(Figures 1B, 1C, and 4D), and our data suggest that even their
large population size was not sufficient to regenerate diversity
over thousands of years. Hence, if low genetic variation is a
contributory factor to extinction risk, not only small but also large
populations can be at risk, if their life history traps them perma-
nently in a state of low genetic diversity.

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AUTHOR CONTRIBUTIONS


DEVELOPMENT AND SOFTWARE AVAILABILITY

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CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for further information should be directed to and will be fulfilled by the Lead Contact, Markus Ralser (markus.ralser@crick.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sample collection
Four animals (two males, two females) each were obtained from three wild Alpine marmot populations in the Central Alps near Mauls (Italy, at 2367 m.a.s.l. at Mt Senges 46°52'40.55"N 11°34'56.12"E (including the reference individual), around St Martin, Gsies, (Italy) (at > 2,000 m.a.s.l., 46°49'44.2"N 12°12'15.5"E), and in the nature reserve of La Grande Sassière (at 2,340 m a.s.l., French Alps, 45°29’N, 65°90’E, animals 1426, 1442, 1467 and 1508). All animals were from different families. The animals’ sex was confirmed by genome analysis (Data S1). Italian Alpine marmot samples were obtained from the Forestry and Hunting Authorities South Tyrol according to national guidelines. The fieldwork involving the French Alpine marmot samples was undertaken after deliverance of the permit number AP n82010/121 by the Préfecture de la Savoie. A.C. is authorized for experimentation with animals (diploma n8R45GRETAF110). The protocol has been approved by the ethical committee of the University of Claude Bernard Lyon 1 (n8BH2012-92 V1). All procedures involving rats and mice lipidomics analysis were carried out in accordance with UK Home Office protocols by a personal license holder.

METHOD DETAILS

General approach
To sequence, assemble and annotate a reference genome for the Alpine marmot (Figure 1A) including both sex chromosomes, we selected a wild-living male, in a typical habitat: a high altitude valley of the Central Alps that is largely free of artificial barriers due to tourism or industrial agriculture (mount Senges, near ‘Mauls’ village, Bolzano province, Italy, 46°52’40.5”N 11°34’56.1”E, 2367 above sea level). In order to minimize potential technology biases in low-frequency variant calling [33], genomic DNA was sequenced by two complementary sequencing technologies (Illumina and Roche/454) and different types of library protocols for illumina sequencing (Data S1). Using a hybrid assembly approach, to make the best use of short- and long-read data we assembled a genome consensus sequence of 2.51 Gbp, with a contig N50 size of ~44 Kbp, scaffold N50 size of 5.6 Mbp and superscaffold N50 size of 31.3 Mbp (Data S1). The large superscaffold N50 size was achieved by collinearity analyses based on the genome of the thirteen-lined ground squirrel
DNA extraction, genomic sequencing and resequencing

Genomic DNA was extracted from spleen, liver, bone and hair tissues by the QIAamp DNA Mini-Kit (QIAGEN) according to the manufacturer’s instructions (including proteinase K digest to obtain high molecular weight DNA). To create the Alpine marmot reference genome, we sequenced an animal from the most centrally located population (Mauls Island) using Illumina Hiseq 2500 short read and Roche / 454 long read sequencing technologies. We constructed paired end (500 bp and 800 bp gel selected fragment size, TrueSeq version2 kit), mate pair (“gelfree” library (MP3000) and 5kb, 10kb and 20kb gel selected fragment size, Nextera Mate Pair Kit) and Roche/454 single read libraries. We produced a high sequencing coverage based on the paired end libraries and supplementary lower coverage using the mate-pair libraries and the 454 technology (sequencing statistics are given in Data S1). For genome re-sequencing of the other individuals we constructed paired end libraries with insert sizes of 300-500 bp using the Illumina TrueSeq version2 kit. Sequence data were generated by either Hiseq2500 (2 × 100 bp) or Nextseq500 sequencers (2 × 150 bp) (Data S1).

Assembly of a reference genome for the Alpine marmot

Prior to assembly we filtered high quality non-duplicate Illumina reads and removed adaptor sequences from the paired end reads. Mate pair reads were filtered using the Nextclip tool [35]. Next, we kept the largest region of a read that had no PhredQ value below 11 and was exceeding 32 bp in length for genome assembly. Processing of Roche/454 reads was included in the Newbler version 3 step of the genome assembly.

The sequencing reads were assembled in a hybrid approach using the IDBA assembler followed by the Newbler assembler (v3.0, similar as in [36]; IDBA version 1.1 was used to assemble all short reads into contigs and locally re-assembled contigs using iterative kmers with sizes 33,63,93,123 and 124). Contigs and locally re-assembled contigs from IDBA were split into 29kbp fragments with 4000bp overlaps to meet maximum read length of Newbler, so that 454 data could be added to the assembly. Mate pair data were added to allow for scaffolding. We applied all filtered reads of the 5,000bp, 10,000bp and 20,000bp libraries for scaffolding. To reduce computational time of the Newbler assembly, we included only 15,000,000 read pairs of the gelfree mate pair library (MP3000) to the Newbler assembly (corresponding to ~15 X fragment or physical coverage of the genome).

The assembly short range continuity (contig N50) was improved by the GapCloser v1.12. We used Illumina libraries with high sequencing coverage in this regard (PE500, PE800 and MP3000 library). Long range continuity (scaffold to superscaffold N50) was improved by comparison with the thirteen-lined ground squirrel (Ictidomys tridecemlineatus) and the house mouse (Mus musculus) MM10 genomes. We used whole genome alignments which were done using the LAST aligner [37] to infer links by putative genome collinearity between our Alpine marmot scaffolds, which were then applied by SSPACE2 to arrange the scaffolds into superscaffolds (as described in [38]). We assigned MM10 chromosomal IDs to the superscaffolds.

Finally, we identified additional overlaps between neighboring contigs in superscaffolds by BLASTn [39] (min. identity 95%/min. length 43) and then joined these contigs (contigA end overlaps contigB start).

For genome collinearity analysis, we aligned the genome assemblies of Marmota marmota, Ictidomys tridecemlineatus, Heterocephalus glaber and Mus musculus against the assembled human chromosomes (GRCh38) using the LAST aligner [37]. Filtering for ortholog alignments was done by single_cov2. Blocks of shared collinearity were calculated by converting MAF format to the satsuma tabular format and then running the BlockDisplaySatsuma script from the Satsuma v1.17 package [40]. The BlockDisplay-Satsuma script was run a second time after removal of smaller collinear blocks (~ 6,000bp). The removal of these spurious blocks after round 1 resulted in larger blocks after round 2. Collinear blocks along the 22+XY human chromosomes were plotted using CIRCOS [41]. Additionally, we plotted links between collinear blocks to determine the phylogenetic position of the rearrangements.

Phylogenomic tree for rodent species

The Alpine marmot genome was aligned to whole genomes of 15 other rodent species and the human genome as outgroup. Genome assemblies were downloaded from the public NCBI assembly repository (as of January 2015). The genomes were aligned to the Alpine marmot genome using LAST [37]. The output was screened for ortholog matches using single_cov2 from the MultiZ package [42]. Pairwise alignments were combined into a multiple alignment using MultiZ. The multiple alignment file (MAF) was screened for blocks aligned in all species. All alignment blocks were concatenated into a multi fasta alignment (length 94 Mbp). We found that 500kbp fragments of the total alignment were sufficient to produce a stable tree topology using the FastTree [43] method with the
GTR model of evolution. We split the whole alignment into 188 independent segments of 500kbp and calculated trees for each segment. We compared these 188 trees to the consensus tree using CompareToBootstrap.pl from the FastTree website: http://meta.microbesonline.org/fasttree.

**Spleen RNA extraction and RNASeq**

For coding gene annotation RNaseq library from spleen we used QIAGEN RNAeasy kit for RNA isolation followed by the Illumina True-seq v2 RNA kit for library construction. The RNaseq libraries were sequenced by the Illumina HiSeq2500 using a paired-end protocol with read lengths of 50bp or 100bp.

**Repeat annotation**

In addition to repeat libraries from RepBase, Custom repeat libraries were created using RepeatModeler version open-1.0.8, RepeatScout version 1.0.5, RECON version 1.08, and Tandem Repeats Finder (TRF). RepeatMasker version 4.0.5 was used to predict repeats in the marmot genome assembly marMar2.1 from the repeat libraries.

**Gene model prediction**

To avoid spurious matches to the genome, low-complexity repeat regions were masked from the genome assembly marMar2.1 using RepeatMasker. The paired-end RNASeq reads were aligned to marMar2.1 using TopHat v2.0.9 [44]. The transcripts were assembled using Cufflinks and merged with Cuffmerge [45]. The predicted proteomes of human, mouse, rat and thirteen-lined ground squirrel were obtained from Ensembl [46] and UniProt [47] and the protein sequences of naked mole-rat was obtained from NCBI [48] and UniProt respectively. The gene models were predicted with MAKER [34] genome annotation pipeline in three iterations (genome browser track: “Maker”). The predicted proteomes downloaded from UniProt [47] were used for the homology search. The assembled transcripts from RNaseq reads were included as experimental evidence in the pipeline. In the first iteration, ab initio prediction was made using Augustus [49] with human as the training species model. In the second and final iterations, gene models that were obtained as output from the previous iteration were utilized for training SNAP [50] for ab initio prediction. Maximum annotation edit distance threshold of 0.75 and minimum protein size of 50 amino acids were used as thresholds for filtering of gene models.

The gene models were also predicted from a custom annotation pipeline in which gene models were predicted from homology search using SPALN aligner v2.1.2 [51], where the predicted proteins for the above mentioned species from Ensembl [46] and NCBI [48] were utilized (see genome browser track “Aligned Proteins”). We chose the best-scoring protein in a cluster based on exact exon–exon matches in a first iteration, and overlapping exons in a second iteration (track “Best Proteins match”). Second, the CDS models from SPALN were combined with spliced transcripts assembled from RNaseq using Cuffmerge [45] This resulted in a high number of possible transcript models, whose open reading frames were annotated by the Transdecoder tool (https://transdecoder.github.io). The transcript models were weighted with scores assigned for the different models based on their origin (highest rank: RNaseq only, lowest rank: SPALN only) and the open reading frames length. In addition, only gene models of at least 50 amino acids in length were retained.

The two sets of gene models were manually inspected, and the consensus gene models were chosen as the reference gene models (see track “protein coding genes”). If the gene models from the two sets were different, individual sources of evidence were utilized in choosing reference gene models.

**Functional annotation**

BLASTP [39] was used for Alpine marmot coding sequences against the predicted proteomes of above mentioned species obtained from Ensembl [46] and NCBI [48] databases. The functional annotation was inferred for Alpine marmot proteins from their best BLAST [39] matches. Alpine marmot proteins were also associated to gene symbols from their homologous proteins with functional annotation. The Gene Ontology (GO) terms [52] were assigned for Alpine marmot predicted proteins by identifying shared signatures with proteins of known function using InterProScan v5.17-56.0 [53]. Alpine marmot protein-coding genes were annotated to their metabolic and cellular pathways by KEGG [54] Automated Annotation Server (KAAS). This provides KEGG Orthology annotation to each gene and corresponding pathway annotations.

For orthology annotation (COG/ eggnOG, KO annotation), the predicted protein sequences were compared to the eggnOG 4.1 Release 77 database, using diamond aligner with options “blastp -k 3 -e 0.0001–sensitive.” Results were post filtered using custom Perl scripts, filtering for the best hit with an alignment length of at least 50% of the reference sequence and an e-value cutoff of 1e-10. NOG categories were assigned by linking the relevant COG (http://eggnogdb.embl.de/download/eggnog_4.1/data/NOG/NOG.members.tsv.gz).

**Non-coding RNA annotation**

The genome assembly that was masked with RepeatMasker was also used for tRNA annotation in order to avoid spurious matches to low complexity regions. tRNA genes were annotated from the repeat masked genome with tRNAscan-SE-1.23 [56].

**Mitochondrial genome annotation**

Gene models for the mitochondrial genome were predicted using Open Reading Frame Finder (ORFfinder, http://www.ncbi.nlm.nih.gov/orffinder/). The functional annotations were transferred to predicted ORFs from protein coding genes of known functions from
the NCBI non-redundant sequence database [48] through BLASTP [39]. Similarly, mitochondrial tRNAs were predicted with tRNAscan-SE-1.23 [56].

**Sciuridae phylogeny based on mtDNA conservation**

Complete mitochondrial genomes of members of Sciuridae were downloaded from GenBank (accessed 15th Feb 2016, although excluding the genome identified as the Daurian ground squirrel (Spermophilus dauricus [57], because the phylogenetic placement of this genome suggests misidentification, or introgression between distantly-related species). The complete genomes were aligned with MUSCLE v. 3.8.31 [58] and manually corrected. Because highly variable regions cannot be aligned between sciurid subfamilies, we then extracted non-overlapping coding sequences, according to the annotation of Pallas’ squirrel (Callosciurus erythraeus, GenBank: NC_025550), and made a concatenated alignment of 3,786 translatable codons. Phylogeny was estimated via maximum likelihood using RAxML v. 8.2.4 [59], using its GTR+G model and 1,000 rapid bootstraps. The phylogeny shown fits standard taxonomy [60, 61]; [62], and an identical topology was obtained when we repeated the analysis after excluding the rapidly-evolving third codon positions.

**Protein coding sequence alignment across species**

For all predicted marmot protein-coding genes, we obtained DNA and protein sequences of potential orthologs from nine mammals species. Seven genomes were from other rodents plus the rabbit (Oryctolagus cuniculus), and a human genome. Sequence annotations were obtained from the NCBI database [48] for human (Homo sapiens, GenBank: GCF_000001405.29), mouse (Mus musculus domesticus, GenBank: GCF_000001635.24), rat (Rattus norvegicus, GenBank: GCF_000001895.5), rabbit (GenBank: GCF_000003625.3), Upper Galilee mountains blind mole-rat (Nannospalax galili, GenBank: GCF_000622305.1), chinese hamster (Cricetulus griseus, GenBank: GCF_000419365.1), naked mole-rat (GenBank: GCF_000247695.1), thirteen-lined ground squirrel GenBank: GCF_000236235.1) and damaraland mole-rat (Fukomys damarensis, GenBank: GCF_000743615.1). Orthologs of the predicted Alpine marmot proteins were identified using best protein BLAST [39] hits of each refseq-annotated genome using an expect value (E) threshold of 0.01 and a minimum percent identity of 65%. Protein sequences were then aligned using MUSCLE [58]. Alignment quality at each individual position was measured using the probabilistic framework of ZORRO [63] and inconsistent positions (positional score < 9) were removed from the alignment. The filtered protein alignments were then prepared along with their respective coding DNA sequences with PAL2NAL [64] to produce codon-based alignments as input for the substitution rate analysis.

**Inferring positive natural selection on protein coding genes**

We used PAML [65] v4.8a to calculate the rate of substitution at nonsynonymous (amino-acid changing) and synonymous sites in protein coding genes. The ratio of these quantities is denoted by \(\frac{dN}{dS} = \omega\). Estimated \(\omega\ values < 1, = 1, and > 1 indicate purifying selection, neutral evolution, and diversifying (positive) selection, respectively. Pairwise estimates of \(dN\) and \(dS\) of two protein coding sequences were obtained using the pairwise maximum-likelihood approach implemented in PAML (runmode = -2). We also used two branch models taking the underlying phylogeny into account. First, we tested for differences in substitution rates between the Marmota+Ictidomys clade (Figure S3A), and the remaining species using a two branch model. Second, we tested for further heterogeneity within the Marmota+Ictidomys clade, with a four branch model (Figure S3B). The two branch model was compared to a single ratio model, and the four branch model was compared to a two branch model. Significant differences between the models were assessed by likelihood-ratio tests (LRTs) which assume that 2\(\ln L\) is approximately \(\chi^2\) distributed, with the degrees of freedom equal to the number of free parameters. \(P\)-values were corrected for multiple testing using the false discovery rate (FDR), according to the procedure of [66].

**Gene set enrichment analysis**

Genes with an FDR-adjusted \(p\)-value \(< 0.05 in ‘between branches’ category, and FDR-adjusted \(p\)-value \(\geq 0.05 in ‘within branch’ category, were categorized as being rapidly evolving between the two clades of rodents (i.e., the clade that contains the Alpine marmot and thirteen-lined ground squirrel, and the clade that contains the other sequenced rodents). The genes that had FDR-adjusted \(p\) \(= 0.05 in ‘between branches’ category and FDR-adjusted \(p\) \(< 0.05 in ‘within branch’ category were categorized as being rapidly evolving within the hibernating rodent branch, but not between the two rodent branches. In addition, other genes exhibiting rapid evolution (falling in the top 10% or 1% of \(dN/dS\) values) in a series of pairwise comparisons (Alpine marmot - thirteen-lined ground squirrel; Alpine marmot - human; and Alpine marmot - mouse) were also filtered for further analysis. Gene set enrichment analysis and pathway enrichment analysis was carried out on these datasets using hypergeometric testing with WebGestalt toolkit [67]. The multiple testing correction used FDR \(< 0.01 as the threshold for significant enrichment. In addition, gene sets involved in functions of interest, namely anti-parasite defense and fatty acid desaturation were prepared. Regardless of enrichment at pathway or gene family level, we also checked all rapidly evolving genes in “marmot - thirteen-lined ground squirrel” comparison.

**Variant impact analysis between Alpine marmot and thirteen-lined ground squirrel**

The marmota genome (as single_cov2 treated MAF file) was converted to sam format using maf-convert a tool which is provided with LAST aligner [37], the thirteen-lined ground squirrel genome was used as reference sequence. The sam file was converted to a bam
Heterozygosity analysis across species and SNP calling of Alpine marmot individuals

Complementing the Alpine marmot data, the paired-end read data, genome assembly data and annotation data of other mammalian species were downloaded from their respective sources (Data S1). Reads were aligned to the genome assembly with bwa -mem v0.7.17 [71, 72]. Duplicate fragments introduced by PCR based library preparation were removed using Picard tools’ MarkDuplicates (version 2.12.1-SNAPSHOT; http://broadinstitute.github.io/picard). For detecting variation in Alpine marmot samples the Genome Analysis Toolkit’s (GATK version 3.6) HaplotypeCaller was used in gvcf mode [73]. Individual gvcf files were used for joint genotyping with GATK’s GenotypeGVCFs tool to build a single variant file containing every Alpine marmot sample. For comparative analyses of the genic regions between marmot and other mammals the mapped read files were analyzed for variation using GATK’s HaplotypeCaller (version nightly-2017-07-11) restricted to regions listed in the respective species’ gff file (Data S1). Further filtering was based on base-wise coverages that were determined for these regions with bedtools coverage (v2.24.0; doi: 10.1093/bioinformatics/btq033). The “vfutils” script from SAMtools were used to further filter the SNPs. 20% of mean coverage and 200% of mean coverage were chosen as minimum and maximum coverage for variant filtering. We also required to have at least 6 supporting reads for a genotype and that heterozygous allele read are in balance, i.e., the ratio of reference allele and alternative allele is between 0.23 and 0.76 [15]. In addition, minimum RMS mapping quality (Q) of 20 was used for filtering SNPs. VCFTools v0.1.11 [74] was used for all post-filtering steps including INDEL removal, removal of homozygous SNPs and calculation of relatedness and inbreeding coefficients (–relatedness2 and –het options). Site quality value of 20 was also used as a threshold for filtering high quality SNPs. Runs of homozygosity (RoH) were calculated for each re-sequenced individual for autosomes only, using bcftools v1.7 roh [75] implemented with the -O r option, and results are shown for RoH > 2MB, which would be indicative of recent inbreeding.

Dendrogram-based Alpine marmot population analysis

SNP calling and filtering was carried out for all 12 sequenced Alpine marmot individuals as described above. Genetic distances were calculated from these matrices and cluster dendrograms were then produced from these distances. The depth of coverage of mitochondrial genomes from the 12 sequenced individuals were determined from BAM alignments using ‘genomeCoverageBed’ function of BEDTools [76]. The SNPs that mapped to mitochondrial genome were filtered using VCFTools [74]. A population-level variant matrix was created and the ‘co-phylogenetic correlation’ function was used to calculate the correlation between hierarchical clusters that were obtained from nuclear genome SNPs and mitochondrial SNPs respectively. The hierarchical clustering and co-phylogenetic correlation was carried out with R (v.3.4.3).

Demographic inference with PSMC

Each of the 12 Alpine marmot genomes was analyzed using pairwise sequential Markovian coalescent analysis (PSMC) [30]. Using heterozygous positions, PSMC infers rates of coalescence over time. To convert relative to absolute timescales, we assumed an average generation time, g, of 5 years [77], and a mutation rate of 2 × 10^{-9} per year per site. This estimate was obtained from the median divergence at synonymous sites in the nuclear genome (dS = 0.04) between the Alpine marmot and thirteen lined ground squirrel sequence, and assuming a split at 8.5Mya. Under the most straightforward interpretation of these plots, population sizes were much larger in the earlier Pleistocene (1myr and before), and underwent a steady decline (Figure 4E). However, this interpretation ignores the strong possibility of population subdivision, and in this case, the older events are determined by migration rate between local breeding populations, and not the species-wide effective population size [29, 30]. We therefore focused on inference of recent events.

Diffusion Approximation for Demographic Inference (DADI) and PCA

SNP calling and filtering was carried out for all 12 sequenced Alpine marmot individuals as described above. We filtered the raw SNP dataset by removing non bi-allelic and low quality SNPs (average DP < 10 or > 50, QUAL < 30). We then detected false positive SNPs (FP-SNPs) by using the two independent sequencing datasets of the reference individual. Since both datasets were from the same individual, we reasoned that any position differing by homozygous genotypes was a false positive SNP (mostly due to mapping errors in low complexity and/or duplicated regions). We thus computed the density of homozygous SNPs in 5Kb windows and removed from our dataset any window with more than 1 FP-SNP. Doing so, we discarded 96% of the detected FP-SNPs by removing 10% of the genome only. To filter out the last undetected FP-SNPs, we applied hard filters according to the GATK Best Practices recommendations [78, 79]. Hard filter values were defined by checking the distribution of the following statistics for the detected FP-SNPs: QD > 2, SOR < 3, MQ > 50, MQRankSum < -2.4, ReadPosRankSum > 0.6, ReadPosRankSum < -2.2, ReadPosRankSum > 2.4. Finally, we masked genotypes with GQ < 10. After cleaning, 2,357,482 SNPs remained. PCA was computed with Plink v1.90b3.44 including singletons.
We then kept one SNP per 20kb-windows as a requirement for independence among loci. Such a thinning has led to a total of 178,098 SNPs left for analysis. Joint folded SFS for La Grande Sassière and Gsies populations respectively were estimated using the program aâli [8]. Thus joint SFS ranges from 0 to 4 allele counts in both samples.

We used the power of composite likelihood diffusion approximation implemented in aâli to infer demographic history of La Grande Sassière and Gsies populations. We tested a first set of 4 models including Strict Isolation (SI), Isolation with Migration (IM), Ancient Migration (AM) and Secondary Contacts (SC) [81]. In the four DADI models, an ancestral population of effective size Na splits into two daughter populations (N1 and N2, respectively) at time Ts. The two daughter populations may either not exchange migrants at all (Strict Isolation (SI), 4 parameters), or undergo continuous bidirectional gene flow (Isolation with Migration (IM), 6 parameters), or bidirectional gene flow ceasing at time Ta after the split (Ancestral Migration (AM), 7 parameters) or bidirectional gene flow starting at time Tsc after the split (Secondary Contact (SC), 7 parameters). These models were evaluated and fitted with the observed joint SFS using 50 replicate runs per model. Models were ranked according to their log likelihoods. For nested models, comparison was performed using likelihood ratio tests. For non-nested models, we used Akaike Information Criterion (AIC) (see Table S3). Parameter estimation used a non-thinned dataset including 1,780,734 SNPs and the best-fitting model.

Coding diversity analysis
Genic diversity for coding regions was obtained for the 11 re-sequenced individuals to avoid reference bias, based on SNP calling for the aâli analysis prior to thinning, as described above. We focused on bi-allelic SNP variation and created the folded site frequency spectra for synonymous and nonsynonymous sites on a gene by gene basis using the python egglib package [82]. Statistics (θ, π and Tajima’s D) were calculated on the summed site frequency spectra across all genes. Because of the evidence of population structure, we obtained population genetic statistics for each population separately as well as jointly for all 11 individuals. To estimate the distribution of fitness effects (DFE) of new nonsynonymous mutations we used a method that controls for segregation of slightly deleterious mutations [23], with the site frequency spectra for synonymous mutations as the neutral reference. Here, the strength of selection is measured by the selection coefficient s, and the efficacy of selection, by the product of the selection coefficient and the effective population size (N_e). Low levels of N_e illustrate less effective (e.g., low) selection against deleterious mutations. Population genetic estimates (e.g., π_N/π_e) for populations from other animal species were obtained from [83] and [24].

Microsatellite diversity across the mammals
To compare the diversity at microsatellite loci of the Alpine marmot to other mammal species, we plotted the number of microsatellite alleles against the expected heterozygosity in a wide range of published datasets (Figure 4C). We show populations of the Alpine marmot from LGS, and estimates from other subpopulations, also from the French Alps. We included other species in the genus Marmota, such as the threatened M. vancouverensis and other rodent species. The Alpine marmot data come from individual published sources [18, 84], while the data from all other species were retrieved from the compilation of microsatellite data in the VarVer database [85].

Life history of the Alpine marmot in comparison to other Eutherian mammals
To compare the life history of the Alpine marmot to other Eutherian mammals (Figure 4D), we followed the approach of Bielby and coauthors [27]. These authors showed that, after correcting for body mass, much of the variance in mammalian life histories could be captured by two factors, i.e., weighted sums of multiple life history variables. One factor included contributions from neonatal mass (g), litter size, and gestation length (days), and can be considered as a measure of “reproductive output,” in which species vary according to their investment in offspring “quality” versus “quantity.” The other factor includes contributions from interbirth interval, weaning age, and age at sexual maturity (all measured in days), and can be considered as a measure of “reproductive timing,” in which species vary on a “fast-slow” continuum. Figure 4D uses all records from placental mammals in the PanTheria database [26], which includes high quality measures of all seven quantities (the six life history variables and adult body mass). All quantities were log transformed, and then we calculated the residuals of the regressions of each variable onto body mass. We then calculated a weighted sum of these residuals using the loadings for Eutheria reported in Table 1 of reference [27].

Lipidomics
Male rats (Wistar, 6 weeks old) and male mice (C57Bl6, 6 weeks old) (Charles River Laboratories) were housed in conventional cages at room temperature with a 12-h light/dark photoperiod. All procedures were carried out in accordance with UK Home Office protocols by a personal license holder.

Lipids were extracted from 50mg of Alpine marmot, rat or mouse white adipose tissue as previously described [86]. Samples were reconstituted in 500 μL 2:1:1 isopropyl alcohol:acetonitrile:water and were analyzed in positive ion mode using a Waters Xevo G2 quadrupole time of flight (Q-ToF) mass spectrometer combined with an Ultra Performance Liquid Chromatography (UPLC) unit (Acquity, Waters Corporation, Manchester, UK). 1μL of the sample was injected onto an Acquity UPLC Charged Surface Hybrid (CSHOT) C18 column (1.7μm x 2.1mm x 100mm) (Waters Corporation) held at 55°C. The binary solvent system (flow rate 0.4ml/min) consisted of solvent A containing HPLC grade acetonitrile-water (60:40) with 10mM ammonium formate and solvent B consisting of LC-MS grade acetonitrile-isopropanol (10:90) and 10mM ammonium formate. The gradient started from 60% A / 40% B, reached 99% B in 18min, then returned back to the starting condition, and remained there for the next 2min. The data was collected over the mass range of m/z 105-1800 with a scan duration of 0.2 s. The source temperature was set at 120°C and nitrogen was used as the desolvation gas (900 L/h).
The voltages of the sampling cone, extraction cone and capillary were 30kV, 3.5kV and 2kV respectively, with a collision energy of 6V for each single scan, and a collision ramp from 20 to 40V for the fragmentation function. As lockmass, a solution of 2ng/l acetonitrile-water (50:50) leucine enkephaline (m/z 556.2771) with 0.1% formic acid was infused into the instrument every 30 s.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were conducted with appropriate packages in R and Python.

DATA AND SOFTWARE AVAILABILITY

The Alpine marmot genome is made available at NCBI [48] and ENA [87] genome archives (marMar2.1). The accession number for the Alpine marmot genome and sequence reads of the 11 re-sequenced individuals reported in this paper is GenBank: GCF_001458135 and ENA: GCF_001458135. For visualization, we have also made it accessible via the UCSC genome browser [88] including gene and repeat annotations, a BLAT [89] server for alignment searches and possibilities to upload and view custom data. The browser is available at http://public-genomes-ngs.molgen.mpg.de.