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CONSECUTIVE EVENTS OF CLIMATE- AND NICHE ADAPTATION PROGRESSIVELY DEPRIVE GENETIC DIVERSITY FROM A LARGE POPULATION OF AN ICE-AGE ADAPTED RODENT

THE GENOME OF ALPINE MARMOT

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There are several cases in evolutionary history, in which species of large population size get suddenly extinct. While low genetic diversity is considered a general risk factor, the sudden disappearance of a species has often followed global changes in climate, such as the disappearance of the Pleistocene cold-steppe, commonly known as the great ice age. Upon assembling a reference genome and re-sequencing individuals from representative populations, we reconstructed the genetic past of the Alpine Marmot *(Marmota marmota)*, a rodent remnant of this glacial epoch that persists in large numbers in the high altitude Alpine meadow. Unexpectedly, despite a large consensus population size, we called the so far lowest level of intra-individual genetic diversity of any wild animal, and discover that the marmot is compromised in purifying selection of deleterious mutations. We can trace this situation to a life history that is characterized by consecutive events of climate and niche adaptation. By acting together, these events disentangle population size from genetic diversity. First, a successful metabolic adaptation to an ice-age climate altered Marmot's fatty acid metabolism, and consistent with a life history of an increase in body size, triggered a progressive, long-term decline from an effective population size of more than 200,000 to only tens of thousands. Upon disappearance of the cold steppe a colonisation of the high Altitude habitat, in which temperatures remained in their favourable range, created local bottlenecks. In the warmer Neocene, the new habitat eventually insularized the Marmot sub-populations, effectively preventing their genetic diversity to recover. The case of the Alpine marmot reveals that upon a global changes in climate, a large population size does not necessarily ensure purifying selection of deleterious variants, and predispose a species for the progressive loss of its genetic fitness.

INTRODUCTION

With the threat of global climate change, it is increasingly important to understand how populations respond to rapidly changing environments. Past events have shown that while some species respond successfully to major changes in climate (Kumar et al., 2015; Robson et al., 2015), others fail to adapt and get extinct (Nogues-Bravo et al., 2008). Mechanisms that define the successful re-adaptation are barely understood, and at present, it is hence difficult to predict the outcome for a given species in the context of contemporary or even future changes in climate.

A major change in the prehistoric climate was characterized by the disappearance of the cold steppe of the Pleistocene, an extensive biome during the Last Glacial Maximum, spanning Eurasia to North America, and from the Arctic islands southwards to China. This habitat was Abundant for approximately 100,000 years, but disappeared about 12,000 years ago. With the decline of the cold steppe, came a decline of its fauna, with several uniquely adapted species, like the woolly mammoth (Mammuthus primigenius), and woolly rhinoceros (Coelodonta antiquitatis), becoming extinct, while others, like the muskox (Ovibos moschatus) and Arctic fox (Dicrostonyx torquatus) persisted in the arctic, where temperatures remained in the favourable range (Alvarez-Lao and Garcia, 2011; Stewart et al., 2010).

A third group of ice-age adapted animals persisted in high-altitude mountain habitats. This group contains a ground-dwelling squirrel, known as the Alpine marmot (Marmota marmota), a close relative of the American groundhog. Widely distributed across the European Steppe during the cold period of the early Quaternary (Besson, 1971; Couturier, 1955), the Alpine marmot now inhabits the high altitude meadows of the Alps and Tatra

Mountains. Here it persists with a cold-adapted physiology and lifestyle, including large body size, an extensive period of winter hibernation, and a high degree of sociality, including a form of cooperative breeding, where adult subordinates warm juveniles during hibernation (Allaine and Theuriau, 2004; Arnold, 1988; Zimina and Gerasimov, 1973).

With tens of thousands of animals found in a wide range of populations across the Alps, according to the current IUCN classification, the conservation status of the Alpine marmot is considered of least concern. However, recent surveys revealed that Alpine marmots are affected by contemporary climate warming over the last 25 years. Body mass, litter size, and pup winter survival are all negatively impacted by increases in winter temperature (Canale et al., 2016; Rezouki et al., 2016; Tafani et al., 2013). Paradoxically, this is explained by a decrease in the temperature of their winter burrows, caused by thinning of the snow cover (Canale et al., 2016; Rezouki et al., 2016; Tafani et al., 2013). A direct sensitivity to the most recent changes in climate is specific to the *Alpine marmot*, and not observed in the 13 other species of marmots, and consistent with their tight niche adaptation. (Bichet et al., 2016; Tafani etal., 2013).

Here, we addressed the genetic basis of the Alpine marmot's physiology and adaptation, by sequencing, assembling and analysing its complete genome, extended by re-sequencing individuals from different populations, that enabled a combined demographic, phylogenetic and physiological analysis of this species in unprecedented detail. We find, to our surprise, that despite clear evidence for effective niche adaptation and large census population size, the Alpine marmot represents one of the most extreme cases of low genetic diversity among mammalia. For instance, we observe lower levels of heterozygosity than extremely isolated or endangered species such as the Iberian Lynx, and comparable levels to artificially inbred laboratory mice. As a consequence, - at present - purifying selection appears ineffective. We find an explanation to this situation in a very slow recovery from past losses in genetic diversity, including that caused by the range contraction at the end of the Pleistocene. We show further that this slow recovery is a consequence of the marmot's climate-adapted life history. The case of the Alpine marmot, revealing a species that does not recover its genetic diversity due to its climate-adapted life history, provides a plausible explanation why a large population size might not protect a species from ineffective natural selection, or even sudden extinction (Murray et al., 2017).

METHODS

Sample collection

Four animals (two males, two females) each were obtained from three wild Alpine marmot populations in the Central Alps near Mauls (I, at 2367 m.a.s.l. at Mt Senges 46°52'40.55"N 11°34'56.12"E (Suppl Figure 1), around St Martin, Gsies, (I) (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 Sassiere (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 (Supplementary Table 12).

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 protein-

ase 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 I) 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, Truseq version2 kit), mate pair ("gelfree" library (MP3000) and 5kbp, lOkbp and 20kbp 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. For genome re-sequencing of the other individuals we constructed paired end libraries with insert sizes of 300-500 bp using the Illumina Truseq version2 kit. Sequence data were generated by either Hiseq2500 (2 x 100 bp) or Nextseq500 sequencers (2 x 150 bp).

RESULTS

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 (Raiser et al., 2012), genomic DNA was sequenced by two complementary sequencing technologies (Illumina, Roche/454) and different types of library protocols for illumina sequencing. 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 contigN_{50} size of ~44 Kbp, scaffold N_{50} size of 5.6 Mbp and superscaffold N50 size of 31.3 Mbp. The large superscaffold N_{50} size was achieved by collinearity analyses based on the genome of the thirteen-lined ground squirrel *(Ictidomys tridecemlineatus)*, the closest Alpine marmot relative for which a genome was available), and the house mouse (*Mus musculus*, Supplementary Table 2). The draft genome assemblies of thirteen-lined ground squirrel (scaff N_{50}=8.2 Mbp) and Alpine marmot (scaff N_{50}=5.6 Mbp) were highly complementary during the collinearity scaffolding process. Thus a future genome assembly version of the thirteen-lined ground squirrel could likewise take advantage from the *M marmota* genome assembly. The Alpine marmot genome was then annotated upon the inclusion of mRNA expression data, generated by mRNA sequencing from spleen and liver tissues, employing the MAKER pipeline (Cantarel et al., 2008), expanded by comparative approaches as well as manual curation. Eventually, we yielded a reference set of 22,349 protein coding genes. Of this gene set, -19,000 genes could be annotated with gene symbols and -14,700 associated to functional pathways.

Slow rates of evolution in the nuclear genome of the Alpine marmot.

A phylogenomic analysis of the Alpine marmot genome confirmed its relationships to other mammalian and rodent species (Blanga-Kanfi et al., 2009; Fabre et al., 2012) (Figure 1B-D). Consistent results were obtained from collinearity analyses of conserved regions (Figure 1B), and from phylogenetic inference, whether using large alignments of nuclear genome (Figure 1C), or whole mitochondrial genomes (Figure 1D), or the well-sampled Cytochrome B gene.

We also identified an integration of the mitochondrial genome into the nuclear genome (a nuclear mitochondrial DNA segment or NUMT (Hazkani-Covo et al., 2010). The

marmot NUMT is unusually large (comprising 91% of the mitochondrial genome), and well conserved (with 84% similarity). This conservation is unlikely to be due to purifying selection, because the NUMT carries a high number of stop codons (due to differences between the nuclear and mitochondrial genetic codes), and had no detectable mRNA expression. Furthermore, the conservation cannot be attributed to a very recent date of insertion. The same NUMT is found in Ictidomys (NW_004936830.1), and phylogenetic analysis suggests that the insertion occurred before the common ancestor of Marmota, Ictidomys and Cynomys. Over the same evolutionary time period, substitutions occurred at most synonymous sites in their mitochondrial genomes (mitochondrial KS estimates: Ictidomys-Marmota 0.48; Tamias-Marmota 1.11). As such, the high conservation of the NUMT suggests a slow rate of evolution in the nuclear genome of the Alpine marmot, particularly when compared to rodent species such as the mouse and rat. This is also consistent with the high levels of conservation observed between the marmot and human genome assemblies (Figure 1B). and the short branch leading to the marmot in (Figure 1C). This slow rate is one way that the life history of the marmot can affect its genome evolution.

DISCUSSION

When environments change, some populations respond successfully, while others go extinct. The reasons for this variation remain unclear, and particular uncertainty surrounds the role - if any - of low genetic variation. This is especially true of notorious cases in which a very large population has suddenly become extinct. The passenger pigeon is one such case, and a recent genomic analysis has shown, intriguingly, that this species was characterized by low variation (Murray et al., 2017). But this cannot tell us whether the low variation contributed to the extinction. Indeed, some evidence against a causal role comes from the work of Romiguier et al. (Romiguier et al., 2014), who showed, across a wide range of animal species, that genome-wide variation was well predicted by their life history, and not at all by their conservation status (Romiguier et al., 2014).

Our analyses of the genomic history of the Alpine marmot has a special relevance to these debates. We have shown that the Alpine marmot is extremely well adapted to its current niche, with an adaptive life history that is matched by physiological changes in fatty acid biosynthesis and storage, and with parasite clearance, which occurs before hibernation. This high level of adaptive fit is reflected in the species' current abundance in its Alpine meadow habitat. However, we have also shown that natural selection against deleterious mutations is now largely ineffective in this species. This is because, despite its large population size, the Alpine marmot has very low levels of genetic variation. Indeed, the levels observed have previously been associated to species associated with serious conservation concerns, or extreme isolation, such as the the extremely isolated California island fox, Iberian lynx or Polar Bear, and are much lower as the values obtained for Mountain Gorilla or Naked Mole rat, for instance (Figure 3A).

We have shown that a reduction in genetic variation occurred during a past event of climate change: the retreat of the cold steppe at the end of the last glacial cycle. But despite this signature of contingent environmental change, we have also shown that the low diversity of the Alpine marmot was explained from its life history, both before and after the range contraction. We have argued that these two facts are intimately connected. The recovery of genetic variation, following the end-Pleistocene event, has been retarded by the marmot's adaptive life history. Put another way, the long-lasting effects of occasional

environmental changes have led to the predictably low levels of genetic variation we observe today.

Our results have two, contrasting implications 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 low levels of genetic variation, for tens of thousands of years. Conversely, however, there is no cause for complacency. If adaptation to future environmental 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. If low genetic variation is a contributory factor to extinction risk, then large populations might be at risk, as well as small ones.

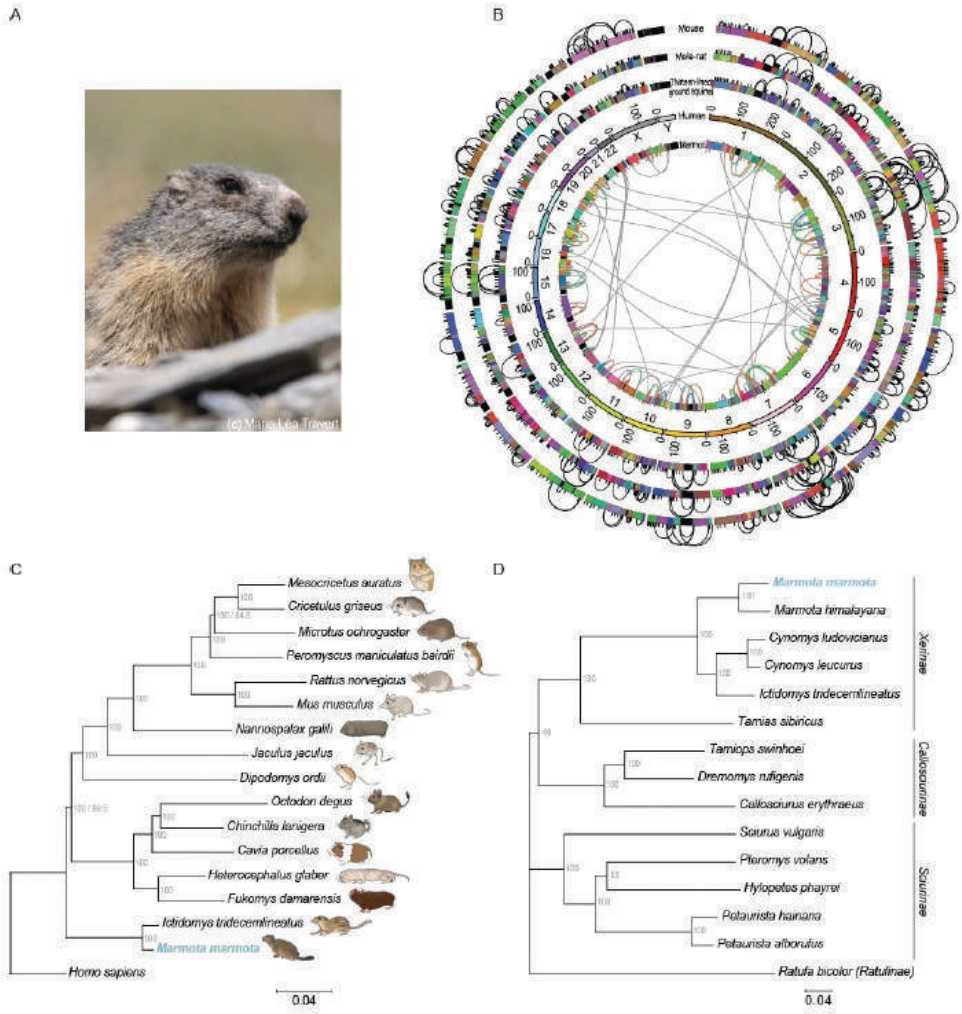

Figure 1. *A slow rate of genomic evolution and the phylogenetic relationship of the Alpine marmot as revealed by its nuclear and mitochondrial reference genome.*

A. *Marmota marmota* is a large, ground-dwelling, highly social rodent, which has colonized high-altitude meadows across the Alps since the end of the last glaciation in the Quaternary.

B. Collinearity of the *M. marmota marmota* genome with its close relatives, and that of other rodents. The *M marmota* genome aligns to a higher fraction of the human genome (outgroup) than to its fellow rodents (i.e. mouse and mole), one of several indicators of a slower rate of genomic evolution. Here collinear blocks in the human chromosomes are colored by assigning random colors for each scaffold/chromosome in the aligned species genome assemblies. Thus small blocks with many colors depict lower N_{50} scaffold length of genome assemblies. Connections indicate collinearity breaks / block rearrangements compared to thehuman genome (intra-chromosomal only for the plotted rodents, except for *M marmota* where interchromosomal rearrangements are plotted inside of the graph). Connections observed in *M. marmota* that are conserved across the rodents shown (green; //=72); in all except *M. musculus* (blue, n=13); conserved between I. tridecemlineatus and *M. marmota* (purple; //=57); or specific to *M marmota* (orange; n=148/

C. Reconstruction of the phylogenetic tree of Rodentia. The tree is derived from multiple whole-genome alignments of protein coding and non-coding sequences from avilable rodent genomes (about 94 Mbp alignment per species). Humans are included as an outgroup. The short branch length of the Alpine marmot since the split from the LCA of primates and rodents agrees with the higher fraction of alignable genomic sequence between the Alpine marmot and human compared to Alpine marmot and Mouse/Mole-rat (Supp. Table 6).

D. A phylogenetic tree for Sciuridae based on their mitochondrial genomes, of the subfamilies Xerinae (including Marmotini), Sciurinae (Squirrels), Callosciurinae, and Ratufmae.

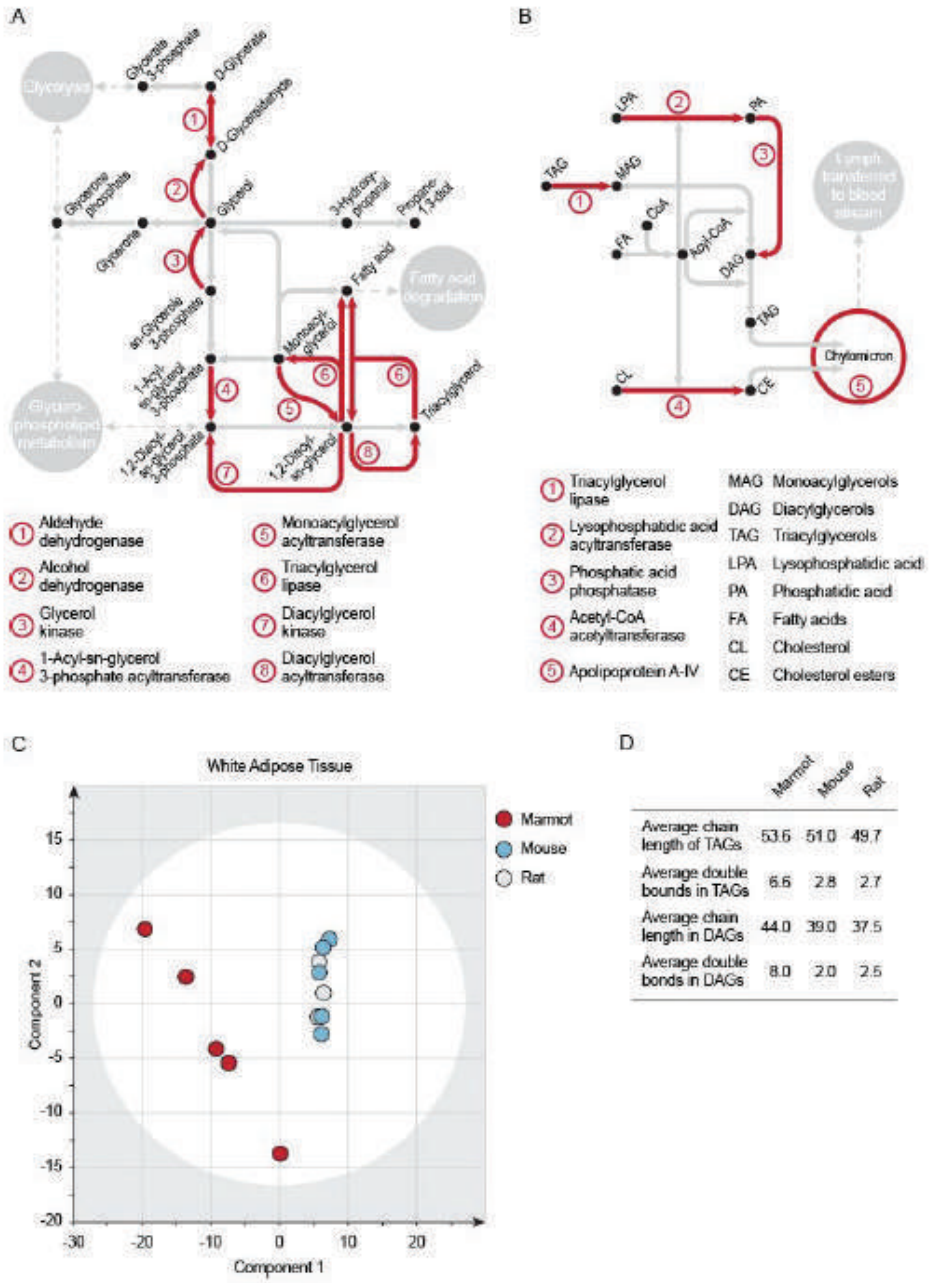

Figure 2. *Genomic signatures of cold-climate adaptation affect metabolism in the Alpine marmot*

A. Phylogenetic Analysis by Maximum Likelihood (PAML) followed by tests for functional enrichment identifies biological processes that underwent increased selection in Alpine marmot The hibernating rodents, marmot and thirteen-lined ground squirrel,

show significant enrichment of adaptive substitutions in the metabolic pathways required for diacylglyceride (DAG) and triacylglyceride (TAG) biosynthesis. Enzymes encoded by genes under positive selection are highlighted in red.

B. Alpine marmot shows specific and significant enrichment of adaptive substitutions in genes required for fatty acid storage, when compared to the thirteen-lined ground squirrel. Enzyme encoding genes positively selected are highlighted in red.

C. Partial least squares-discriminatory analysis (PLS-DA), of the white adipose tissue (WAT) lipid composition, as determined by liquid chromatography - tandem mass spectrometry, comparing mouse, rat, and Alpine marmot WAT. The Alpine marmot WAT clearly distinguishes from that of rat and mouse.

D. Higher degree of unsaturation, and longer chain lengths, in Alpine marmot WAT DAGs and TAGs, as determined by liquid chromatography - tandem mass spectrometry.

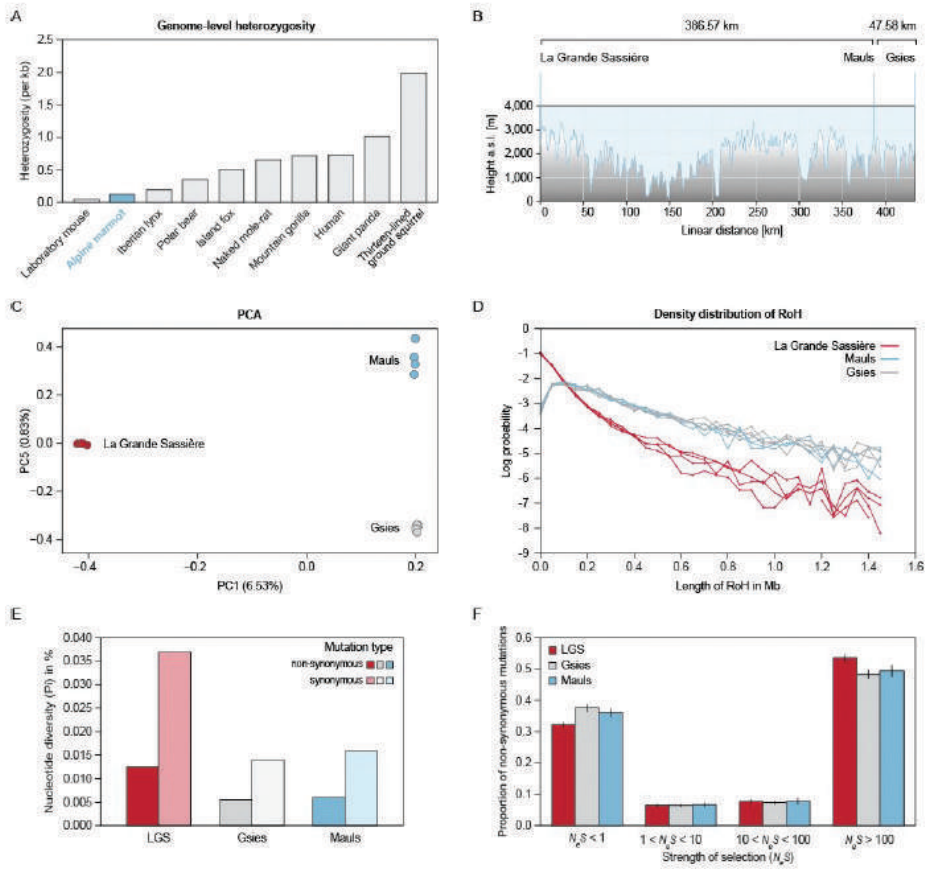

Figure 3. *Extremely low levels of genetic diversity and impaired purifying selection characterize different Alpine marmot populations*

A. The Alpine marmot genome is characterized by low heterozygosity at the genome level. The heterozygosity for the other mammalian genomes has been determined by re-mapping the original sequence reads used to assemble their reference genomes, using identical software parameters (Supplementary Table 11).

B. Alpine marmot populations located in Mauls (I, Supplementary Figure 1), Gsies (I) and La Grande Sassiere (LGS), shown as geographical distances as well as height profiles between these.

C. Principal component analysis (PCA) of whole genome genetic diversity (single nucleotide polymorphisms, including singletons) of animals from Mauls, Gsies, and La Grande Sassiere. PCI distinguishes the Mauls and Gsies from the La Grande Sassiere marmots, while PC5 separates all three populations. Axes 2 to 4 mainly describe genetic diversity within the "La Grande Sassiere" (LGS) population, which has comparable diversity to the combined sample.

D. Logarithmic density distributions of runs of homozygosity for individuals of the three populations. Distributions are very similar for the Mauls and Gsies populations but different for LGS, well explained by their differences in local breeding sizes. There is little evidence of consanguineous mating, nor of a recent bottleneck recovery.

E. Differences in the coding diversity (synonymous and nonsynonymous sites) among the three marmot populations. La Grande Sassiere (LGS) individuals are around three times more diverse that the inner Alpine populations. (Right panel)

F. Distribution of fitness effects of nonsynonymous mutations suggests that more than 30% of nonsynonymous mutations within the Alpine marmot populations are effectively neutral, with a further 5-10% in the nearly neutral range. There is little variation of fitness effects across populations.

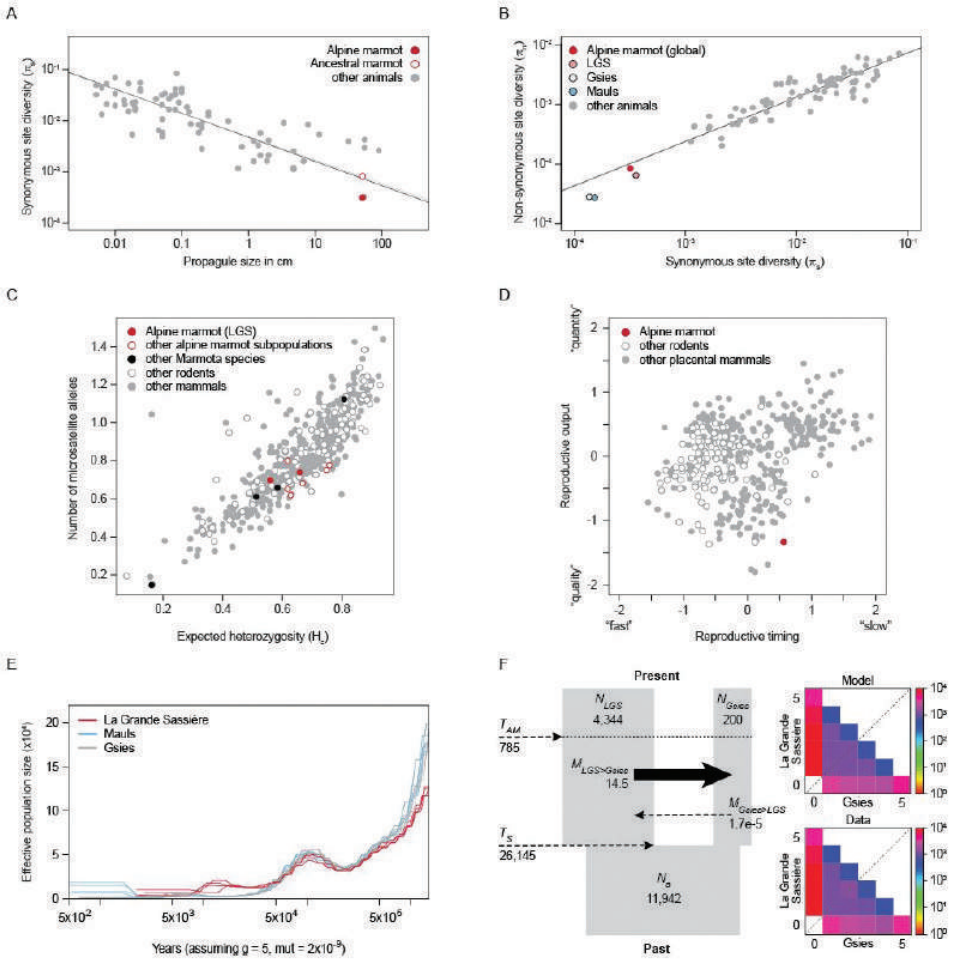

Figure 4. *The low genomic diversity in the Alpine marmot is explained by its life history and a lack of recovery from a past bottleneck*

A. The genetic diversity of the Alpine marmot is explained from its Life History. Species-wide synonymous site diversity from a wide range of animal species (Romiguier et al., 2014), plotted against their "propagule size" (i.e., the size in centimetres of the dispersing life stage). The delayed dispersal of the alpine marmot, and its large adult body size, yields a very large propagule size (Ge et al., 2013), consistent with the observed low diversity (filled red point). The fit is even closer when we consider the diversity we have inferred for the ancestral marmot population at the end of the Pleistocene (empty red point; Figure 4F). 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 is consistent with their effective population size, and the pattern observed across diverse animal species. Data from the Alpine marmot (colored points), have been added to the data

of (Romiguier et al., 2014). 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 empty red bordered circles. Other species in the genus Marmota are shown as black filled circles, including the threatened M. Vancouver ensis, which appears at the bottom left of the graph.

D. Life history of the Alpine marmot (red point) in comparison to other Eutherian mammals (data from (Jones et al., 2009)). After correcting for body mass, much of the variance in mammalian life histories can be captured by two factors (Bielby et al., 2007): "reproductive output" (in which species vary according to their investment in offspring "quality" versus "quantity") and "reproductive timing" (in which species vary on a "fast-slow" continuum). The Alpine marmot appears as an extreme outlier.

E. Pairwise sequential Markovian coalescent (PSMC) analysis reveals details of the genetic past of the Alpine marmot. Evident is a decline in the LGS population after the last glacial maximum. Earlier events might suggest a longer-term decline, but might also indicate partial isolation between breeding populations.

F. The ancient migration {AM) is the most likely demographic scenario for the Gsies and La Grande Sassiere (LGS) populations inferred from the joint site frequency spectrum (SFS). This model predicts that a large ancestral population split up ~26,145ya into two smaller daughter populations. Gene flow between these two populations ceased ~785ya and was strongly asymmetrical with most migrants going from the large LGS population to the very small Gsies population (right upper panel). The joint SFS for the LGS and Gsies populations was obtained from 178,098 SNPs (right lower panel).

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