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RoAM: computational reconstruction of ancient methylomes and identification of differentially methylated regions

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Abstract

We present a new and considerably improved version of RoAM (Reconstruction of Ancient Methylation), a flexible tool for reconstructing ancient methylomes and identifying differentially methylated regions (DMRs) between populations. Through a series of filtering and quality control steps, RoAM produces highly reliable DNA methylation maps, making it a valuable tool for paleoepigenomics studies. We apply RoAM to pre- and post-Neolithic transition Balkan samples, and uncover DMRs in genes related to sugar metabolism. Notably, we observe post-Neolithic hypermethylation of *PTPRN2*, a regulator of insulin secretion. These results are compatible with hypoinsulinism in pre-Neolithic hunter-gatherers.

Keywords: Ancient DNA methylation, Human evolution, Ancient DNA, Neolithic transition, Dietary shifts

Background

Identifying changes in gene expression levels is a powerful tool to study evolutionary shifts and adaptations. Specifically, many efforts have been directed to identifying changes in gene regulation along the human lineage [1–3]. The rise of ancient DNA (aDNA) offers new ways to study regulatory changes that shaped modern humans and might have affected our recent evolution. Given that changes in gene regulation are difficult to read directly from the DNA sequence, and that RNA rarely survives in ancient remains, DNA methylation stands out as the best proxy of ancient gene expression levels [2, 4]. DNA methylation, which in mammals affects cytosines in the context of CpG positions, is a key epigenetic mark that is tightly associated with gene expression levels [5]. Incidentally, unlike other epigenetic marks such as histone modifications, DNA methylation is highly stable and remains on aDNA for extended periods [6].

More than a decade ago, we and others developed computational methods for reconstructing premortem DNA methylation patterns from aDNA [7, 8]. These techniques



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used the fact that deamination – the main chemical degradation of aDNA – turns methylated cytosines into thymines and unmethylated cytosines into uracils [6]. In preparing the aDNA for sequencing, a treatment with uracil-specific excision reagent (USER) is often used [6], generating an asymmetry between methylated and unmethylated cytosines, which can be used to distinguish between them by carrying out statistical analysis of the number of $C \rightarrow T$ transitions in CpG positions. A high rate of $C \rightarrow T$ indicates premortem hypermethylation, while the converse indicates hypomethylation. These pioneering methods have not only opened avenues for exploring epigenetic regulations in ancient samples, but also enabled the reconstruction of genome-wide methylation maps and the identification of differentially methylated regions (DMRs) between ancient samples. These works founded the field of paleoepigenetics, which provided significant insights into various aspects of human evolution [2, 4, 9].

Several of these aDNA reconstruction algorithms were published as tools, including RoAM [8], epiPALEOMIX [10], and its successor DAMMET [11]. However, RoAM was distributed as Matlab code, and its use was limited. DAMMET, which employs a maximum likelihood estimation to calculate methylation levels based on the $C \rightarrow T$ transition counts, is the most recent tool, but has several limitations. First, it is assumed that all four nucleotides have the same frequency of 0.25, ignoring GC content biases. Specifically, the GC content of the human genome is known to be 40.9% [12]. Second, it is assumed that there is an equal probability of 1/7 for each dinucleotide that can be read as a CpG due to a mutation, which does not align with established mutation rates in humans [13, 14]. Notably, mutations are not uniformly distributed, with $C \rightarrow T$ mutations being particularly prevalent [15]. Third, the estimation procedure includes cytosines outside of a CpG context, which are assumed to be unmethylated. However, some small levels of non-CpG methylation are known to exist [16], especially in embryonic cells and specific mature cell types such as neurons. Not much is known about the prevalence and significance of non-CpG methylation in tissues that are present in the fossil record, particularly bones and teeth. This, combined with the assumption that the deamination rate of cytosines in non-CpG context is identical to that of cytosines within CpG context, can introduce bias into the reconstructed map. Indeed, previous works showed that methylation maps provided by DAMMET show more hypomethylation than expected [17, 18].

Moreover, DAMMET generates methylation maps, but does not compare them to identify DMRs. RoAM, on the other hand, does include a method to detect DMRs, but could originally do so only between pairs of samples. As the number of published aDNA samples continues to grow, the detection of DMRs between large groups of samples has become desirable, as they have the potential to unveil DNA methylation differences between populations, within the same population across different time points, and between closely related species.

Here, we present a new python version of RoAM (Reconstruction of Ancient Methylation), which removes many of the aforementioned limitations. It is feature-rich, flexible, easy to use, and its code is freely available. It allows for the generation of premortem genome-wide aDNA methylation maps, as well as the detection of DMRs between groups of samples. The current version contains numerous improvements over the original software, including novel methods for filtering out true $C \rightarrow T$ mutations (Table 1).

Table 1 Main differences between the old and new version of RoAM

Feature	Old version (2014)	New version (2025)
DMR detection between two individual samples	✓	✗
DMR detection between two groups	✗	✓
Linear methylation reconstruction	✓	✓
Sigmoid and histogram matching methylation reconstruction	✗	✓
Can be operated from the command line	✗	✓
Code language	MATLAB	Python
Method for mutation removal	Simple thresholds	Mixture of distributions model

RoAM does not use assumptions about nucleotide frequencies, and does not rely on cytosines outside of CpG context.

To demonstrate the capabilities of RoAM, we reconstructed the methylomes of 14 samples from the Balkans and detected DMRs between pre-Neolithic specimens and post-Neolithic transition ones. As expected from the short time span separating these populations, we detected only four DMRs with modest levels of methylation change. However, the genes that are associated with these DMRs might hold clues to nutritional changes that occurred during the Neolithic transition. Notably, we provide evidence that PTPRN2, which is involved in insulin response to glucose stimulus, is overexpressed in post-Neolithic transition individuals, as expected for high carbohydrate diet. Methylation changes were also found in EIF2AK4, a sensor for amino acid deprivation that also regulates insulin, and SLC2A5, the main fructose transporter. In total, these findings may provide clues to regulatory changes that might have accompanied the major changes in diet and lifestyle that ensued following the Neolithic transition.

Results

Validations to RoAM

Given the changes introduced in the new version (Fig. 1, Table 1), we wanted to carry out validation tests, similar to those that were conducted in the original RoAM publication [8]. First, we classified all CpG position in the modern human reference genome into ten bins, based on their methylation level in present-day bone sample. Then, we computed the mean C → T ratio in each bin for an ancient sample. As expected, we observe high correlation ($R > 0.91$, Fig. 2A), corroborating the fact that C → T ratio is predictive of methylation level. Second, we produced a heatmap showing the joint histogram of a reconstructed sample and a modern bone (Fig. 2B), demonstrating high match between reconstructed and measured methylation.

Third, we used the χ^2 statistic to compare the histograms of reconstructed methylation maps produced by RoAM and DAMMET to a measured bone methylation map that was not used as a reference during the reconstruction process. Whereas $\chi^2_{RoAM} = 7 \cdot 10^5$, the same statistic for DAMMET was one order of magnitude larger, $\chi^2_{DAMMET} = 2.53 \cdot 10^6$, indicating larger distance between the histograms (Fig. 2C).

Finally, we compared the methylomes of both reconstructed and present-day bone samples to that of osteoblasts, lung, pancreas, and bladder, all taken from the methylation atlas of Loyfer et al. [19]. We focused on the top 1% of CpG positions with the

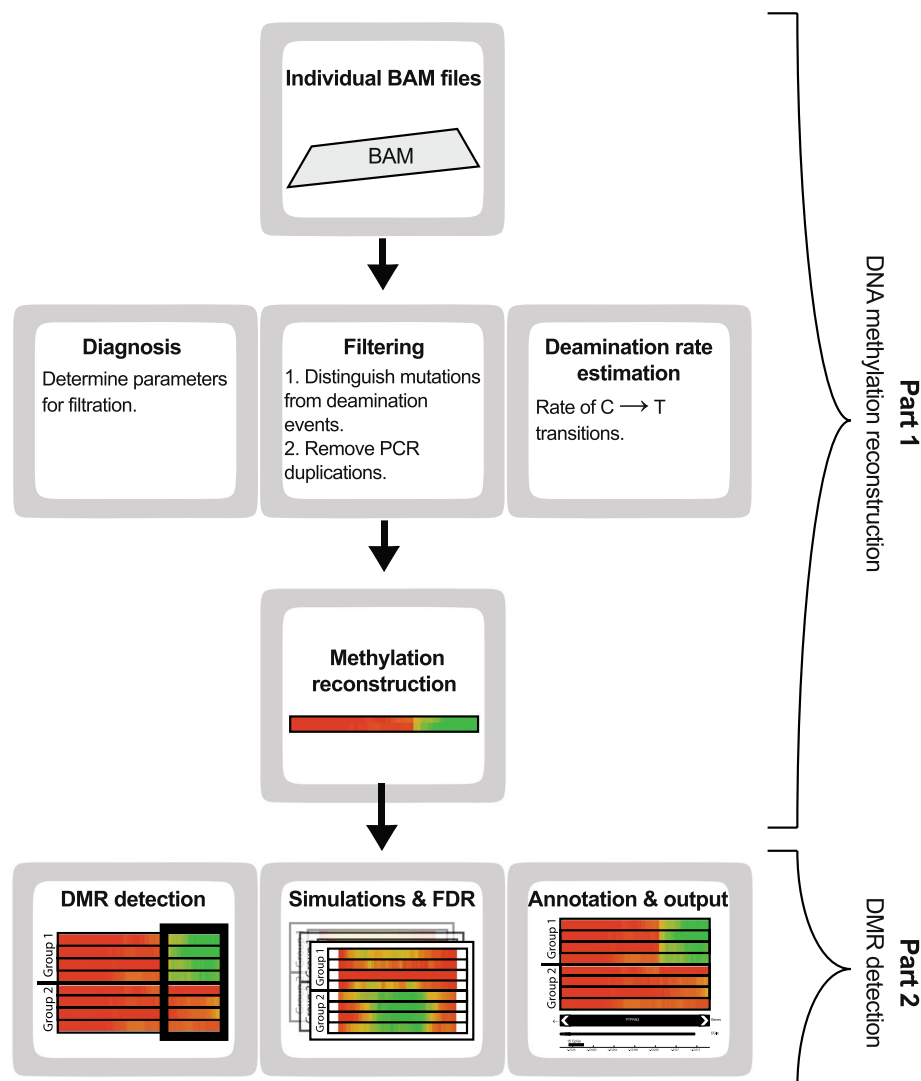


Fig. 1 The RoAM pipeline is split into two parts. In Part I, RoAM starts with BAM files of ancient genomes, and reconstructs the individual methylation maps. In Part II, RoAM detects differentially methylated regions (DMRs) distinguishing two groups of ancient samples (created with Biorender)

highest variance on chromosome 1, and calculated pairwise χ^2 distances. The resulting distance matrix, presented as a dendrogram in Fig. 2D, reveals a strong similarity between the two bone samples, which cluster closely with osteoblasts, as expected. Combined, these validations show that the reconstructed DNA methylation signal shows all the expected features of a present-day human bone methylation map.

Case study: the Neolithic transition

Epigenetics in general, and DNA methylation in particular, may respond to changes in internal or external conditions [20, 21]. Research has unveiled connections between numerous environmental factors and alterations in DNA methylation [22–25]. Consequently, even short bouts of environmental or lifestyle transitions may make epigenomic imprints that can be read.

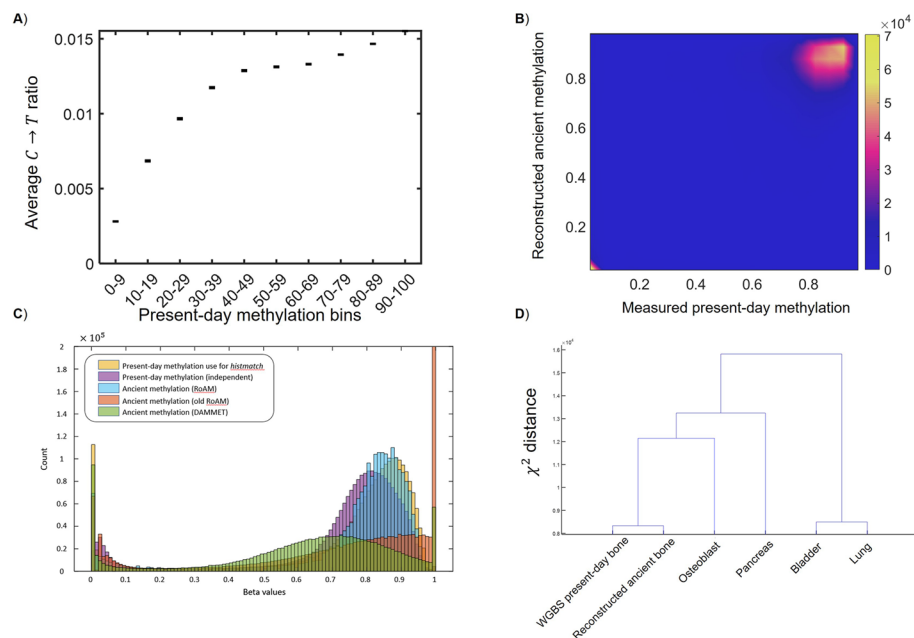


Fig. 2 **A** CpG positions were categorized into ten distinct bins based on their DNA methylation levels as directly measured in present day human bone. Average CT ratio in an ancient sample (Denisova) was calculated for each bin, and correlation was computed. **B** Methylation heatmap of an ancient sample (Denisova) compared to a smoothed modern one (data taken from chromosome 19). High concordance is observed between these samples. **C** Histograms of methylation levels of ancient samples (chromosome 1 only). Ancient sample I11116 is reconstructed by the new version of RoAM (blue), the old version (orange) and by DAMMET (green). Two modern bone samples are shown. One (yellow) was used as a reference in RoAM, and another (purple) that was not used in RoAM. **D** Dendrogram based on pairwise χ^2 distances between methylomes of reconstructed and present-day bone samples and four reference tissues (osteoblasts, lung, pancreas, bladder), using the top 1% most variable CpG sites on chromosome 1

Motivated by this, we decided to investigate potential epigenetic imprints of the Neolithic transition using RoAM. The Neolithic transition is a pivotal milestone in human history, representing a significant shift in lifestyle, from primarily that of hunting and gathering to a more sedentary one based on agriculture and animal husbandry. Pre-Neolithic humans typically lived nomadic life, relying on wild plants and animals for sustenance. The adoption of agriculture and domestication practices led to significant changes in diet, disease load, levels of physical activity and many other aspects of life. These changes might have been accompanied by biological and physiological changes (e.g. [26]). Emerging evidence suggests that DNA methylation in some genomic loci is sensitive to such lifestyle factors [27, 28]. Hence, we decided to compare the ancient epigenomes of pre-Neolithic to those of post-Neolithic transition.

Ancient individuals sequenced to high coverage are still not abundant, and tend to represent very different populations. Comparing pre- to post-Neolithic transition individuals across many different populations potentially adds confounding factors. To address this, we limited our study to 14 high-coverage individuals that come from the same region, the Balkans. Nine are pre-Neolithic individuals, and five are post-Neolithic transition ones (Table 2). Genomic data of these ancient

Table 2 List of samples used in this work. Sample IDs are according to the Allen Ancient Genome Diversity Project

Sample ID	Group	Sample age (kya)	Location	Coverage	Effective coverage	% genome reconstructed
I1507	Pre-Neolithic	7.7	Hungary (Tiszaszolos-Domaháza)	22.42	30.32	98.2
I4873	Pre-Neolithic	7.9	Serbia (Vlasak)	25.76	38.42	98.1
I4875	Pre-Neolithic	8.5	Serbia (Vlasak)	21.48	29.35	98.1
I4877	Pre-Neolithic	8.5	Serbia (Vlasak)	27.44	38.75	98.1
I4878	Pre-Neolithic	7.8	Serbia (Vlasak)	25.3	38.47	98.2
I4914	Pre-Neolithic	8.1	Serbia (Vlasak)	24.85	37.4	98.1
I5233	Pre-Neolithic	8	Serbia (Padina)	23.55	30.55	98.1
I5235	Pre-Neolithic	10.8	Serbia (Padina)	25.61	32.2	98.1
I5236	Pre-Neolithic	10	Serbia (Padina)	26.91	35.07	98.1
I1116	Post-Neolithic transition	1	Serbia (Gomolova)	26.97	41.87	98.3
I1496	Post-Neolithic transition	7	Hungary (Apc-Berekalya I)	29.96	43.25	98.2
I2520	Post-Neolithic transition	5.1	Bulgaria (Dzhulyunitsa)	24.47	40.17	98.1
I5077	Post-Neolithic transition	7	Croatia (Sopot)	27.79	41.8	98.2
I5725	Post-Neolithic transition	2.5	Croatia (Sv Kriz)	27.49	37.21	98.2

individuals were downloaded from the Allen Ancient DNA Resource (AADR) available at <https://doi.org/10.7910/DVN/FFIDCW> [29]. Notably, petrous bone was the source for DNA extraction in all 14 samples, further reducing potential effects of confounding factors.

Two samples, I1116 and I5725 are dated to a later period compared to the other post-Neolithic transition samples. While increasing the sample size, we figured their inclusion might introduce a bias to the analysis. Indeed, as will be shown below, these two samples sometimes show methylation patterns that are more distant from the other post-Neolithic transition individuals. We therefore repeated the analysis twice, with and without these two samples.

We applied RoAM to reconstruct methylation for each sample [8] and then detected DMRs between these two groups. Given the short time span separating the two groups, we expected to find only small methylation changes between them. We have therefore set the minimum methylation difference between groups (the Δ parameter, see Methods) to the very low value of 0.1.

Methylation patterns can exhibit significant variations between two distinct tissues within the same individual [30]. As a result, much of the research in the field of paleoepigenetics has concentrated on the evolutionary aspects of the skeletal system [31, 32]. The relevance of skeletal DMRs to changes in lifestyle following the Neolithic transition is debated. However, we have shown that there are loci in the genome where differential methylation in one tissue may reflect differential methylation in another tissue, as long as the methylation change occurs early during embryogenesis [4]. To help in focusing on

Table 3 DMRs separating pre- and post-Neolithic transition samples from the Balkan (ordered by Qmax—from largest to smallest)

Chrom	DMR start (hg19)	DMR end (hg19)	Qmax	# CpGs	Gene overlapped	Pre-Neolithic methylation	Post-Neolithic transition methylation	Mean methylation difference
7	157,405,128	157,407,252	406.4	145	PTPRN2	0.36	0.65	0.29
2	131,008,391	131,011,881	311.9	119		0.35	0.64	0.29
19	12,983,432	12,985,003	287.4	118	MAST1	0.50	0.77	0.27
15	40,266,418	40,269,319	255.8	79	EIF2AK4	0.40	0.74	0.34

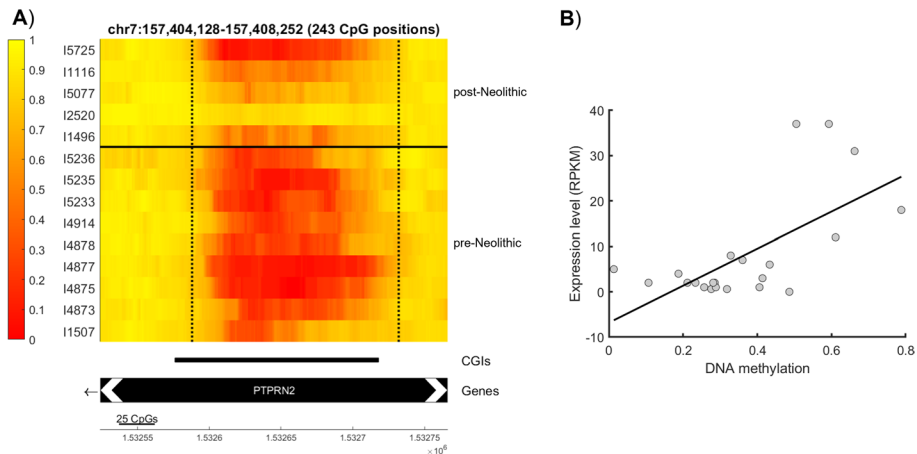


Fig. 3 The DMR within the PTPRN2 gene. **A** Reconstructed DNA methylation of the 14 Balkan samples. The DMR (dashed vertical lines) distinguishes between post-Neolithic transition samples (upper lanes) and pre-Neolithic revolution ones (lower lanes). Methylation is color coded, from low methylation in yellow to high methylation in red. Lower lanes describe the genomic locations of CpG islands (CGIs) and genes. This DMR intersects a CpG island. **B** Expression level of the PTPRN2 gene as a function of the mean methylation within the DMR, in 22 modern human tissues

differential methylation that arose during such early developmental times, we crossed our results with published methylation data derived from blood samples of modern hunter-gatherers and farmers in Africa [33], where differentially methylated sites separating these population have been identified. We only considered genes that featured methylation changes in both bone and blood.

Our conservative analysis, using all five post-Neolithic transition samples, yielded four DMRs (Table 3). Three of the DMRs are located inside gene bodies and, interestingly, all four overlap CpG islands, suggesting a possible regulatory role of these methylation changes. The DMR with the highest Q_{max} (406.4), which was the only one to be detected when the two younger post-Neolithic transition samples have been removed, was found inside the gene body of the PTPRN2 gene (also known as IA-2 β , Fig. 3A). PTPRN2 also harbors the third-highest number of differentially methylated sites in blood, separating modern African hunter-gatherers from farmers, with a total of 62 such sites. PTPRN2 is a transmembrane protein present in dense-core vesicles and represents a major auto antigen of type 1 diabetes [34]. Previous works found that PTPRN2 has a key role in insulin secretion in response to glucose stimulus, and suppression or knocking down of

this gene can impair this process [35–38]. Epigenetic regulation on PTPRN2 has been examined previously, and a DNA methylation change in a CpG site within this gene, which does not overlap with the detected DMR, has been associated with childhood obesity [39, 40].

To find the predicted effects of the DNA methylation changes on the expression level of this gene, we looked at the correlation between the methylation in this DMR and the expression of PTPRN2 across 22 tissues of present-day humans taken from the Roadmap dataset [41]. We found a significant positive correlation ($R = 0.65$, $P = 8.8 \cdot 10^{-4}$), suggesting that PTPRN2 was expressed in higher levels in post-Neolithic transition individuals (Fig. 3B). This implies lower insulin response to glucose stimulus in the pre-Neolithic individuals. No significant correlation between methylation and gene expression was found for the other DMRs.

Another DMR was detected inside EIF2AK4 (also known as GCN2, Fig. 4A), a sensor for amino acid deprivation and a regulator of lipid metabolism and gluconeogenesis [42, 43]. This kinase plays a crucial role in maintaining homeostasis during amino acid deprivation. When under a leucine-deprived diet, EIF2AK4 reduces insulin levels and increases insulin sensitivity [44, 45]. However, in mice consuming a high fat diet, the opposite effect is shown, where EIF2AK4 increases blood insulin levels and decreases insulin sensitivity [46]. Furthermore, EIF2AK4 is also implicated in diabetes, as its knockout in diabetic mice results in a decrease in serum fasting glucose and improved cardiac symptoms [47].

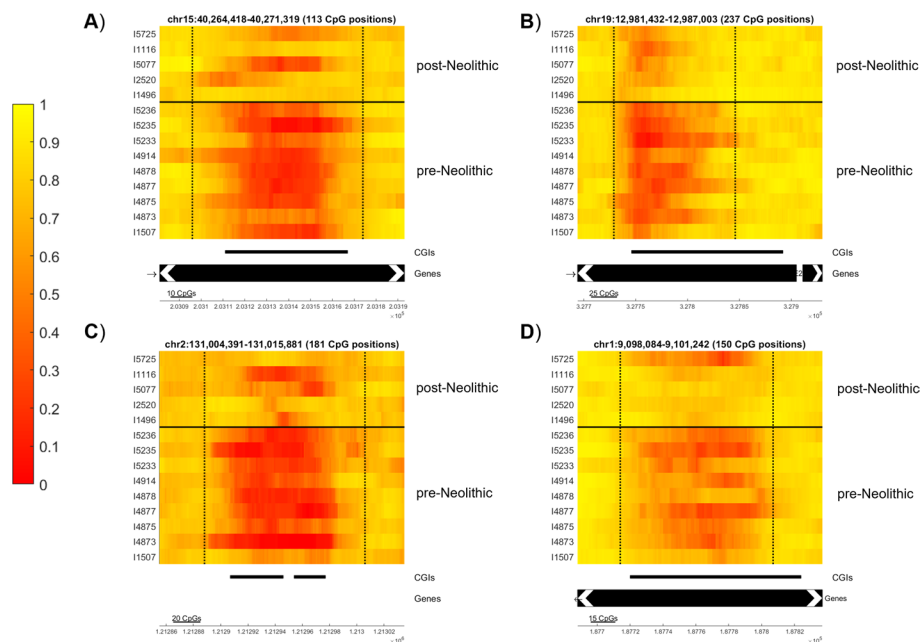


Fig. 4 Additional DMRs (bounded by dashed vertical lines) that distinguish between post-Neolithic transition samples (upper lanes) and pre-Neolithic ones (lower lanes). Methylation is color coded, from low methylation in yellow to high methylation in red. Lower lanes describe the genomic locations of CpG islands (CGIs) and genes. **A–C** The additional three DMRs detected in the analysis. **D** The DMR with the highest Q_{max} that did not pass significance threshold. All DMRs intersect CpG islands

The third DMR was detected in the gene *MAST1* (Fig. 4B), a kinase that plays a role in the central nervous system [48–50]. No relation of this gene to metabolism or any function that might be related to the Neolithic transition is currently known. The fourth DMR is not located on a promoter or a gene body (Fig. 4C).

Only four DMRs out of an original set of 155,693 had properties that meet the thresholds set by the simulations to get an FDR below 0.05 (see Methods). Although not statistically significant, we noticed an interesting DMR that was very close to crossing the required thresholds. This DMR overlaps CpG island, and is located within the *SLC2A5* gene (also known as *GLUT5*, Fig. 4D), a major fructose transporter in the gut. Many works showed that the presence of fructose stimulation can enhance, even within hours, *SLC2A5* expression in the small intestine of adult animals. The same is true of neonatal and weaning pups that do not normally consume fructose and have low levels of *SLC2A5* expression in their intestines [51–56]. *SLC2A5* is also associated with diabetes and obesity, as the gene is differentially expressed in insulin-sensitive tissues of patients with type 2 diabetes and in mouse models for diabetes and obesity, such as muscle [57] and fat tissues [58, 59].

Discussion

We introduce here RoAM, a user-friendly program designed to provide a complete analysis pipeline for computational reconstruction of ancient methylomes and the identification of DMRs that distinguish ancient populations from each other. As the significance of evolutionary epigenetics is in the rise, RoAM proves to be a valuable tool for researchers seeking to integrate paleoepigenetic insights into their studies.

An advantage of RoAM is that new features are constantly added, gradually providing it with even more power. For example, a primary limitation of the reconstruction algorithm is that it cannot work on low-coverage samples, as the counts of Cs and Ts may be too low to allow for reasonable standard error of the estimator. To overcome this, we have introduced the concept of pooling, where counts from many low-coverage samples from the same group are amalgamated to provide a methylation map that represents the entire population [60]. Pooling has already been integrated into RoAM, making it a viable tool for analyzing methylation maps in populations with low-coverage samples.

There are several limitations of the current software. First, the current implementation does not allow comparisons across more than two groups. Second, the code is limited to detecting DMRs between two groups that are exclusively composed of ancient samples. Ideally, we would like to integrate modern samples in the analyses, such that each group can potentially consist of a mixture of modern and ancient samples. Third, the algorithm exclusively performs methylation reconstruction on samples subjected to USER treatment [6]. However, this treatment is not universally performed in aDNA library preparation. Finally, it does not account for difference in deamination rates along the read [6]. We are currently actively working on developing solutions for all these limitations. RoAM will continue to be maintained and updated, with each solution promptly implemented in the code, providing RoAM with the capability to handle a growing number of samples of different types.

To showcase the algorithm, we conducted methylation reconstruction on 14 pre- and post-Neolithic transition samples from the Balkans, and identified four DMRs that distinguish between them. The genes associated with these DMRs provide insights into understanding dietary changes that were induced by the Neolithic transition. Two classic hypotheses claim that hypoinsulinism in pre-Neolithic hunter gatherers provided an adaptive advantage. The carnivore connection hypothesis [61, 62] suggests that hunter-gatherers diet was high in protein and low in carbohydrates, and that in such conditions, insulin resistance would confer an evolutionary advantage, as it allows redirection of glucose to specific requirements such as embryonic development and brain functions. The thrifty genotype hypothesis [63] suggests that hypoinsulinism was a preferred strategy for storing food in times of food scarcity due to the instability in food sources. Glucose is specifically important for fetal development and to the function of the Brain, and therefore hypoinsulinism can be a good adaptation to accommodate and supply the body needs when experiencing low or unstable glucose availability. In line with these claims, previous studies reported that hunter-gatherers from the north-western Kalahari display lower levels of blood insulin, while genetically similar communities that have adopted a sedentary lifestyle for 15 years show an increase in insulin levels during this period [64, 65]. Additional work showed that short term consumption of a paleolithic diet can decrease insulin secretion [66]. These works indicate hypoinsulinism in hunter gatherers, and specifically at lower insulin secretion. Our most pronounced DMR resides in *PTPRN2*, suggesting overexpression of this gene in post-Neolithic transition individuals compared to pre-Neolithic ones. Given its role in insulin secretion in response to glucose, this finding lends further credence to the claim that hunter-gatherers experienced hypoinsulinism. Further evidence for methylation changes in *PTPRN2* that correlate with hunting and gathering lifestyle can be found in an independent study that compared methylation levels in modern hunter gatherers and genetically related farmers in Africa [33]. In this study, *PTPRN2* stood out as the gene with the third-highest number of differentially methylated sites, amounting to a total of 62 sites.

Another DMR lies with the *EIF2AK4* gene. *EIF2AK4* regulates insulin level and sensitivity in response to varied dietary components, and specifically during amino-acid deprivation. Changes in the expression level of this gene may be linked to the dietary shift during the Neolithic transition. A plausible explanation for this change could be attributed to food scarcity, potentially resulting in the deprivation of certain amino acids for hunter-gatherers.

We also found a DMR within the *SLC2A5* gene. This DMR is filtered out because of our very strict criteria, but it was just below the threshold, so we decided to discuss it here, as it might be potentially related to the Neolithic dietary transition. *SLC2A5* is a fructose transporter, whose expression levels change when fructose consumption is increased. Our data do not allow us to determine the sign of the correlation between the methylation in this DMR and the gene expression, hence we cannot conclusively determine whether the methylation changes are associated with up or down regulation of this gene in post-Neolithic transition times. However, we do observe a notable methylation change in this gene, that warrants further experimental examination.

It should be recognized that the DMRs we detect here likely represent just a small minority of the methylated changes that accompanied the Neolithic transition. First, we

have used very strict filtering criteria, increasing precision on the expense of sensitivity. Second, we are limited by a small sample size. And third, the use of DNA methylation maps from bones means that we are able to identify only those methylation changes that occurred very early during embryogenesis, and simultaneously affect multiple tissues [4]. We will not be able to observe tissue-specific methylation changes, where the methylation change is not shared with bone. We anticipate the existence of such tissue-specific methylation changes, particularly in genes associated with immune functions and metabolism.

Methods

RoAM performs two main tasks. First, it reconstructs the methylation map of ancient samples. Then, it compares groups of samples to detect DMRs between them (Fig. 1). In the following, we thoroughly describe these two parts.

Part I. Reconstructing premortem ancient DNA methylation

The main input for this part is a BAM file of an ancient individual. Each BAM file is analyzed through five consecutive steps (Fig. 1): (1) basic processing of the BAM file; (2) diagnosis step to automatically determine filtering parameters; (3) carrying out the filtering to remove non-informative CpG positions; (4) estimating the deamination rate; and (5) reconstructing the premortem DNA methylation.

In addition to the BAM file, RoAM requires several input parameters that instruct it how to process the specific sample. Although not mandatory, it is highly recommended to provide a reference present-day DNA methylation map generated from the same tissue from which the aDNA was extracted. The reason for this is that such a reference allows for more accurate estimation of the deamination rate and methylation reconstruction. We provide such reference for present-day human bone aligned to hg19, see <https://carmelab.huji.ac.il/data.html>. A full description of all input parameters can be found in the README file in the GitHub page of RoAM, <https://github.com/swidler/roam-python> [67].

RoAM provides the user with two outputs that contain the reconstructed methylation map. One is a simple BED file, and the other is a python object that also contains all parameters that were used by the algorithm. This python object is later required for the DMR detection part.

Step I: BAM file processing

RoAM reads all relevant information from a BAM file into a python object. This object stores some descriptive characteristics of the sample, such as the sample name, species and library preparation method. In addition, it holds relevant summary statistics of the sequencing data, specifically nucleotide counts in each CpG position. At later steps, more information is stored in this object, such as the filtering parameters, the estimated deamination rate, and the reconstructed methylation values. BAM files are processed, one chromosome at a time, using Python's *pysam* module (<https://github.com/pysam-developers/pysam>). During this step, RoAM filters out low quality reads, and computes base counts for each position in a way that depends on the library preparation method. For single-strand libraries, each strand is treated independently, and only $C \rightarrow T$

transitions are relevant. For double-strand libraries, complementary $G \rightarrow A$ events along the opposite strand are counted as well.

This step is the most time-consuming part of the pipeline. However, it needs to be executed only once per sample, after which the saved object can be re-used.

Step II: diagnosis

Filtering in RoAM is aimed at removing CpG positions whose cytosine and thymine counts may be spurious or uninformative to the reconstruction process. We identify two groups of such CpG positions. First, some positions show suspiciously high coverage that suggest they are a result of PCR duplications. Second, some positions show $C \rightarrow T$ counts suggestive of premortem mutations rather than deamination.

RoAM runs a diagnostic procedure that automatically suggests parameter values that should be used during filtering. The user can manually override these suggestions.

Identifying PCR duplications

Let t_i and c_i be the counts of thymines and cytosines, respectively, in CpG position i , and let $n_i = t_i + c_i$ be the total count in this position. As a first step, we use a crude outlier removal process to remove positions with extreme values of n_i . Then, we use a more refined method to remove additional outlying positions. For the first step, we compute the 25th and 75th percentiles of all n_i values, denoted p_{25} and p_{75} , respectively. We compute the interquartile range as $r = p_{75} - p_{25}$, and remove all positions where $n_i > p_{75} + s \cdot r$. Here, s is a parameter called *span*, set by default to 5.

For the second step, let $N(c)$ be the histogram of the remaining n_i values, counting the number of CpG positions with coverage c . $N(c)$ resembles normal distribution truncated at 1, and with a heavier right-tail. To account for the truncation, we use the following method to estimate the parameters of the distribution. Let c_m be the coverage level that maximizes $N(c)$. Based on the three coverage levels $c_m - 1$, c_m , and $c_m + 1$, we estimate the parameters a , b , and c of the best-fitting binomial $N(x) = ax^2 + bx + c$. We estimate μ , the mean of the normal distribution, as the point where this function is maximized, $\hat{\mu} = -b/2a$. We call the maximum value of the function $N_0 = N(\hat{\mu}) = -b^2/4a + c$. To estimate the standard deviation, σ , we find the first bin in the histogram, b , such that b is greater than μ and for which $N(b) \leq t \cdot N_0$, where t is a parameter that is set by default to 0.1. The smaller t is, the more the approximation accounts for the heavy tail. Let $f = N(b)/N_0$ be the ratio of these two bins. Then,

$$N(b) = \frac{N}{\sigma \sqrt{2\pi}} e^{-\frac{(b-\hat{\mu})^2}{2\sigma^2}} = f \cdot N_0 = \frac{fN}{\sigma \sqrt{2\pi}},$$

where N is the total number of counts, $N = \sum_c N(c)$. Therefore, σ can be estimated using this ratio by

$$\hat{\sigma} = \frac{b - \hat{\mu}}{\sqrt{2\ln(1/f)}}.$$

Given the estimates $\hat{\mu}$ and $\hat{\sigma}$, we further remove CpG positions that might be a result of PCR duplicates by filtering out all positions whose coverage exceeds a threshold c_T ,

determined as the coverage that would yield an expected value of counts less than one. We find c_T by solving for

$$\frac{N}{\hat{\sigma}\sqrt{2\pi}} e^{-\frac{(c_T - \hat{\mu})^2}{2\hat{\sigma}^2}} < 1,$$

giving

$$C_T = \left\lceil \hat{\mu} + \hat{\sigma} \sqrt{2 \ln \left(\frac{N}{\hat{\sigma}\sqrt{2\pi}} \right)} \right\rceil.$$

Removal of PCR duplicates is done by removing all positions with

$$n_i > c_T. \quad (1)$$

Detecting $C \rightarrow T$ mutations

$C \rightarrow T$ Mutations are detected in two ways. As we detailed in past papers [8, 31], in aDNA that was sequenced using single-strand libraries, mutations can be distinguished from deamination by examining the opposite strand for $G \rightarrow A$ transitions.

Here, we developed an alternative technique, that is also applicable for aDNA sequenced using double-strand libraries. It is based on the analysis of the histogram H of t_i and removing CpG positions with $C \rightarrow T$ rates that are too high and likely represent a $C \rightarrow T$ mutation. We examine each coverage level c , independently, and therefore can now assume that we are only looking at the $N(c)$ CpG positions whose coverage is c . Denoting by p the probability of a $C \rightarrow T$ transition, we assume that H represents a mixture of three binomial distributions: (1) homozygous mutations, where $p_1 \approx 1$. (2) heterozygous mutations, where $p_2 = 0.5$. (3) non-mutated sites that went through deamination, where $p_3 \approx 0.01$; We use expectation maximization (EM) to estimate the p 's and w 's in the binomial mixture model (BMM):

$$\Pr(t_i = t) = \sum_{k=1}^3 w_k B(t|c, p_k),$$

where w_k is the weight of the k 'th distribution. The details of the EM algorithm can be found in Additional File 1, but the final threshold is given by

$$k_c = \left\lceil \frac{\ln \frac{w_2}{w_3} - c \ln 2(1 - p_3)}{\ln \frac{p_3}{1 - p_3}} \right\rceil,$$

where the $\lceil \cdot \rceil$ operator means the closest integer from above. We then remove all CpG positions whose coverage is c and where

$$t_i \geq k_c. \quad (2)$$

Step III: filtering

The filters outlined in Step II are used to determine which sites to remove. First, we clean PCR duplicates. This is done by setting a threshold c_T and removing every site where $n_i > c_T$ (see Eq. 1). Then, let i denote all the sites with a given coverage level c , where

$c = 1, \dots, c_T$. We remove all sites for which $t_i \geq k_C$ (see Eq. 2), as they are suspected as true mutations.

For single-strand libraries, we add a filter on the $G \rightarrow A$ transitions in the opposite strand. To this end, we set a maximum number of allowed A's, a_L , as well as a minimum $G \rightarrow A$ ratio, T_m (default: 0.25). Then, we remove all sites where

$$a_i > a_L \text{ and } \frac{a_i}{a_i + g_i} \geq T_m. \quad (3)$$

After filtering, a merging procedure is applied to combine information from both Cs of the same CpG position (on opposite strands). The methylation state of these two Cs should be identical [68], thus merging the counts of Cs and Ts from both strands increases the amount of information obtained from each CpG position.

Step IV: estimation of deamination rate

The deamination rate is estimated using the same technique we have previously detailed [31], and is based on the C and T counts in CpG positions whose methylation in the modern reference is above a certain threshold, m_h . This parameter should be close to one and is exactly one by default. As the maximum-likelihood estimator of the methylation in a site is

$$m_i = \frac{t_i}{\pi n_i},$$

limiting ourselves to positions where $m_i = 1$ lets us estimate the degradation rate by

$$\hat{\pi} = \frac{\sum t_i}{\sum n_i}.$$

In a case where a reference is not available, one can estimate the degradation rate by assuming knowledge of the global mean methylation in the sample, m_g . Then, we estimate the deamination rate by

$$\hat{\pi} = \frac{\sum t_i}{m_g \sum n_i},$$

where the sum is over all positions in the genomes.

This function also computes the local methylation rate for each chromosome, which allows testing for homogeneity of the deamination rate across chromosomes.

Step V: methylation reconstruction

By default, RoAM uses histogram matching to reconstruct the methylation maps by finding the non-linear transformation $m_i = f(t_i/n_i)$ that makes the histogram of m_i as close as possible to that of a reference methylation map in a modern bone [60, 69], where m_i is the estimated methylation at position i . By design, we obtain a histogram of methylation values which resembles the equivalent histogram from modern-day sample. It stands in contrast to DAMMET, which tends to show shifts towards low methylation.

Histogram matching serves as the default method for methylation reconstruction, but users may choose two other methods. One is the truncated linear transformation,

$$m_i = \max\left(0, \min\left(1, \frac{t_i}{\pi n_i}\right)\right),$$

as described and used previously [31]. To achieve a smooth truncation, RoAM also offers a third method, called the logistic transformation, where

$$m_i = \tanh\left(\frac{t_i}{\pi n_i}\right).$$

Given the typically small thymine counts in each CpG position, t_i , RoAM reconstructs methylation in windows of W consecutive CpG positions (W is always set as an odd number, and the reconstructed methylation in the window is assigned to the middle position). The user may determine the window size they wish to use, but RoAM includes two methods to automatically determine the window size.

Probability-based method

We require that the probability of observing no thymines in a window for a minimum methylation level m_0 be less than p_0 . This translates into

$$\Pr(t = 0) = (1 - \pi m_0)^n < p_0,$$

where t is the total thymine count in the window, and n is the total count of thymines and cytosines. Taking log of both sides we get

$$n \cdot \ln(1 - \pi m_0) < \ln p_0,$$

meaning that we have to have

$$n > \frac{\ln p_0}{\ln(1 - \pi m_0)}$$

in the window. If the window is covered by the average effective coverage, C , then $n = WC$. This translates into the following window size:

$$W = \lceil \frac{1}{C} \cdot \frac{\ln p_0}{\ln(1 - \pi m_0)} \rceil.$$

Relative-error-based method

We require that the relative error in estimating the methylation, when the true methylation is m_0 , be lower than $1/k$. Let the estimator for the methylation be

$$m = \frac{t}{\pi n}.$$

Then, its mean is

$$E(m) = \frac{1}{\pi} E\left(\frac{t}{n}\right) = \frac{1}{\pi} \pi m_0 = m_0,$$

and its variance is

$$s^2(m) = \frac{1}{\pi^2} V\left(\frac{t}{n}\right) = \frac{\pi m_0(1 - \pi m_0)}{\pi^2 n}.$$

We require that $s(m)/E(m) < 1/k$, hence

$$\frac{\pi m_0(1 - \pi m_0)}{\pi^2 m_0^2 n} < \frac{1}{k^2},$$

or

$$n > \frac{k^2(1 - \pi m_0)}{\pi m_0}.$$

Again, using the average effective coverage, C , this translates into

$$W = \lceil \frac{k^2(1 - \pi m_0)}{\pi m_0 C} \rceil.$$

The default that RoAM uses is the probability-based method.

Part II. DMR detection

Once reconstruction of methylation has been achieved for multiple samples, a second part of RoAM is designed to detect and statistically validate DMRs between two groups of samples. This process comprises the following steps (Fig. 1): (1) DMR detection between the two groups, (2) the use of simulations to adjust the parameters of the DMR-calling algorithm to reach a desired level of false discovery rate (FDR), and (3) annotation of the final list of DMRs. The algorithm provides a table with a list of all the DMRs, their location, annotation, and the methylation level in each of the samples, as well as the combined estimated methylation in each group.

Step I: DMR detection

Let us first examine a group of S samples. We assume that the methylation across members of the group is homogeneous, and denote the common methylation value in window j as m_j .

Let us look at sample i . We assume that the observed number of T bases in window j is binomially distributed, $t_{ij} \sim B(n_{ij}, m_j \pi_i)$, where π_i is the deamination rate of the sample and n_{ij} is the sum of the Cs and Ts in each CpG in window j in sample i . The likelihood of sample i is

$$L_{ij} = \binom{n_{ij}}{t_{ij}} (m_j \pi_i)^{t_{ij}} (1 - m_j \pi_i)^{n_{ij} - t_{ij}},$$

and the log-likelihood

$$\ell_{ij} = t_{ij} \ln(m_j \pi_i) + (n_{ij} - t_{ij}) \ln(1 - m_j \pi_i) + B_i,$$

where B_i is a term that is independent of m_j . The total log-likelihood of all S samples in the group is

$$\ell_j = \sum_{i=1}^S t_{ij} \ln(m_j \pi_i) + \sum_{i=1}^S (n_{ij} - t_{ij}) \ln(1 - m_j \pi_i) + B,$$

where $B = \sum_i B_i$ is a term independent of m_j . The score function with respect to m_j is:

$$\frac{d\ell_j}{dm_j} = \sum_{i=1}^S \frac{t_{ij}}{m_j} - \sum_{i=1}^S \frac{(n_{ij} - t_{ij}) \pi_i}{1 - m_j \pi_i} = \frac{T_j}{m_j} - \sum_{i=1}^S \frac{(n_{ij} - t_{ij}) \pi_i}{1 - m_j \pi_i},$$

where

$$T_j = \sum_{i=1}^S t_{ij}.$$

We find the maximum of the likelihood by equating the score to zero and solving using the Newton–Raphson method. Details can be found in Additional File 2.

After computing $m_{1,j}$ and $m_{2,j}$, the methylation levels in every genomic window for the two groups, we can compare the two using a similar approach to the one used in [31]. To this end, we define the two statistics

$$\ell_j^+ = \frac{m_{1,j} - m_{2,j} - \Delta}{\sqrt{V(m_{1,j}) + V(m_{2,j})}},$$

$$\ell_j^- = \frac{m_{2,j} - m_{1,j} - \Delta}{\sqrt{V(m_{1,j}) + V(m_{2,j})}}.$$

Here, Δ is a parameter of the algorithm, associated with the desired minimal methylation difference we wish to detect between the two groups. Next, we use a cumulative sum for ℓ_j^+ and ℓ_j^- to identify DMRs, as described in [31]. In brief, we define the vectors Q^+ and Q^- of the same length as ℓ_j^+ and ℓ_j^- , as

$$Q_0^+ = 0, Q_j^+ = \max(Q_{j-1}^+ + \ell_j^+, 0).$$

Q^- is defined in an analogous way.

In DMRs where group 1 is hypermethylated compared to group 2, we will obtain a sequence of positive values for ℓ_j^+ resulting in an elevation in the values of Q^+ . We define the DMR as the region $[a, b]$ between the last zero $Q_a = 0$, and the highest value $Q_b = Q_{\max}$ up to the next zero (31).

Each DMR is characterized by several properties, such as its genomic length, the number of CpG positions it harbors, and its $Q_{\max}$. This allows for further filtering of DMRs, to achieve a desired false discovery rate (FDR), as explained in the next section.

Step II: simulations and FDR

We employ simulations to filter out DMRs in such a way that we achieve a desired FDR level. The detailed procedure can be found in our previous paper [31]. In short,

we imitate the deamination process in each sample by generating Ts using a binomial process, where the coverage in each position and the deamination rate are kept constant for each sample. The methylation value in each position is determined in advance, and is kept constant across the samples from both groups, to model the null hypothesis of no methylation differences between the groups.

Subsequently, we apply the same DMR detection procedure to the simulated data and count the number of detected DMRs. This number represents the number of DMRs detected under the null hypothesis. Repeating this many times (typically 100 times), we may compute the expected fraction of false DMRs within our original list of DMRs. By default, we set an FDR threshold of 0.05, but this parameter can be adjusted by the user. Given that the simulated DMRs originate from the null hypothesis, they tend to be shorter and have smaller Q_{max} . Consequently, the algorithm applies a range of thresholds for the minimum number of CpG sites and Q_{max} , looking for a combination that would achieve the desired FDR level. If multiple sets of parameters achieve this FDR level, we select the one that filters out the fewest of the original DMRs.

Step III: annotation

The final step of this part creates annotations of the final DMR list. Two types of annotations are currently implemented: associating DMRs with gene bodies and promoters, and with CpG islands. Users are required to provide the location data (gene list and CpG island list). These files are provided in <https://carmelab.huji.ac.il/data.html> for hg19. By default, RoAM defines the promoter region of each gene as 5,000 bp upstream of the transcription start site (TSS) to 1,000 bp downstream, but this can be set by the user. In addition, annotation can be done against any list of genomic segments, inserted as a BED file.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13059-025-03702-7>.

Additional file 1: EM algorithm for detecting C → T mutations.

Additional file 2: Finding the pooled methylation using maximum likelihood.

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Authors' contributions

Y.M. and L.C. designed the algorithm, Y.M. conducted all analyses. N.R. coded the algorithm in Python. C.L. helped debugging the software. B.Y. designed the statistical test for DMR detection. E.M. and L.C. supervised the study. Y.M., E.M. and L.C. wrote the paper. All authors read and approved the final manuscript.

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Data availability

RoAM is available in Github [67] and Zenodo [70], both under the MIT License. Data used in this study were taken from the Allen Ancient DNA Resource (AADR) available at <https://doi.org/10.7910/DVN/FFIDCW> [29].

Declarations

Ethics approval and consent to participate

Not relevant for the current study.

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Competing interests

All authors declare they have no competing interests.

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