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Concepts, mechanisms and implications of long-term epigenetic inheritance

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Many modes and mechanisms of epigenetic inheritance have been elucidated in eukaryotes. Most of them are relatively short-term, generally not exceeding one or a few organismal generations. However, emerging evidence indicates that one mechanism, cytosine DNA methylation, can mediate epigenetic inheritance over much longer timescales, which are mostly or completely inaccessible in the laboratory. Here we discuss the evidence for, and mechanisms and implications of, such long-term epigenetic inheritance. We argue that compelling evidence supports the long-term epigenetic inheritance of gene body methylation, at least in the model angiosperm *Arabidopsis thaliana*, and that variation in such methylation can therefore serve as an epigenetic basis for phenotypic variation in natural populations.

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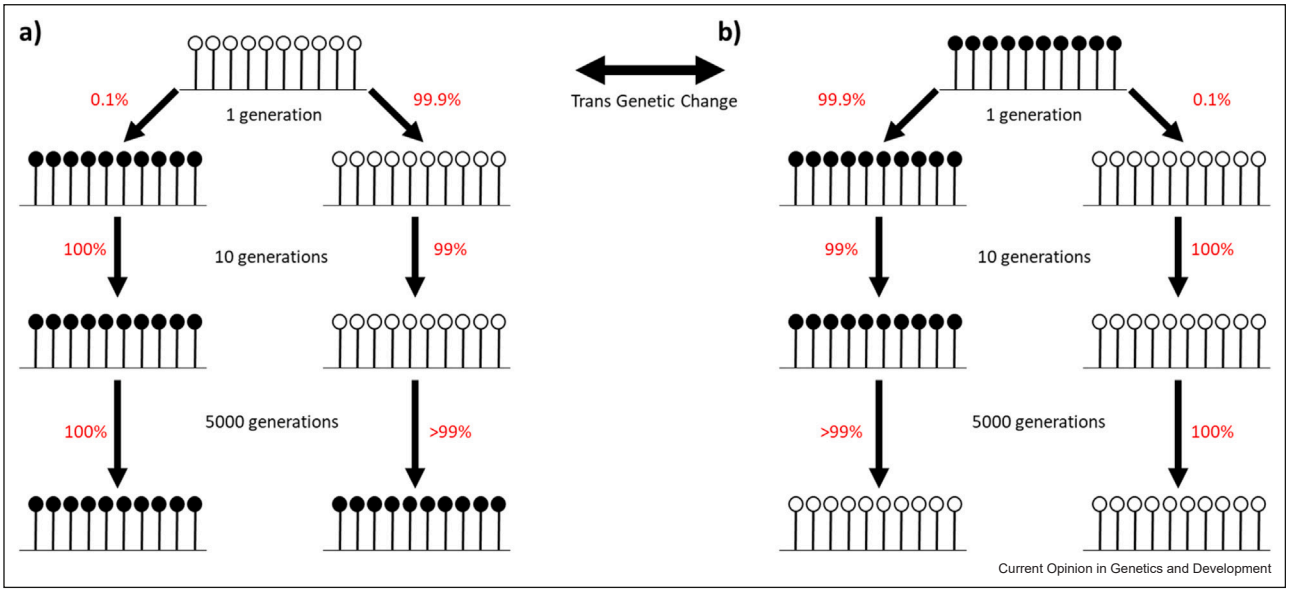
Introduction

Epigenetics is the study of inheritance modes that do not encode information directly in the DNA sequence. Many forms and mechanisms of epigenetic inheritance have been described, most of which are relatively short-term [1,2]. For the purpose of this article, we define 'short-term' as timescales that are easily accessible in the laboratory. This can be a single cell division, one multicellular organismal generation (for example, genomic imprinting [3]), a few generations (for example, stress priming [4]), up to the tens of generations (~1000 cell divisions) involved, for example, in experiments that quantify the stability of epigenetic inheritance [5,6]. In contrast, we define 'long-term' epigenetic inheritance — the subject of this review — as occurring over timescales that are generally not accessible in the laboratory: thousands of generations that cover tens of thousands to millions of cell cycles. The only molecular mechanism that has been plausibly proposed to mediate long-term epigenetic inheritance is cytosine DNA methylation [7,8], and we will therefore restrict ourselves to this form of epigenetics.

The study of long-term epigenetic inheritance poses special challenges. If DNA methylation at a locus, such as a transposable element (TE), is epigenetically inherited in the laboratory, and the TE is universally methylated in a natural population that spans over 100,000 generations, one may be tempted to assume that methylation of the TE has been epigenetically inherited over this timescale. However, this assumption is not warranted, because TE sequences can and do attract methylation [9]. To appreciate this issue, consider a locus that has an effectively perfectly stable methylated state, but a slightly unstable unmethylated state so that the unmethylated epiallele has a 0.1% chance per generation of becoming methylated (Figure 1a). In the laboratory, the inheritance of both states will be almost purely epigenetic. Put differently, DNA methylation will be an epigenetic genotype that cannot be predicted from the DNA sequence. However, in the absence of selection, > 99% of alleles will be methylated after 5000 generations (Figure 1a). Hence, over tens of thousands of generations, DNA methylation will effectively be a phenotype that can be almost perfectly predicted from the DNA sequence. Therefore, whether methylation states are dominated by epigenetic or genetic inheritance is dependent on the timescale under consideration, with the timescale of epigenetic inheritance determined by its stability (Figure 1a).

A related challenge is that mutations in factors that mediate DNA methylation could cause the stabilities of the two states of our locus to flip, so that the methylated state becomes slightly unstable while the unmethylated state becomes effectively perfectly stable, and hence the unmethylated state will dominate after thousands of generations (Figure 1b). Natural methylation variation at

Figure 1



Epigenetic inheritance has a characteristic timescale. **(a)** An unmethylated locus has a 0.1% probability of gaining methylation due to the interaction of its sequence with the global methylation machinery. The methylated state is epigenetically inherited with 100% stability. Over 10 generations, 99% (0.999^{10}) of unmethylated epialleles will remain unmethylated, and 100% of methylated epialleles will remain methylated. Hence, methylation variation at the locus will be almost completely epigenetic, as the methylation state cannot be predicted from genetic information. However, after 5000 generations, > 99% ($1–0.999^{5000}$) of unmethylated alleles will become methylated. Over this timescale, the methylation state of the locus can be almost perfectly predicted from the genetic features of the locus and the methylation system and hence is genetically determined. **(b)** *Trans* genetic changes (occurring elsewhere in the genome), for example, mutations in factors that mediate methylation, destabilize the methylated state and stabilize the unmethylated state. Over 5000 generations, > 99% of the methylated epialleles become unmethylated. This genetic process can recur multiple times, generating subpopulations with genetically determined methylated and unmethylated alleles without any local genetic polymorphism. Each circle indicates a cytosine, with white circles representing unmethylated cytosines and black circles indicating methylated cytosines.

the locus would then be driven by genetic variation elsewhere in the genome, and effects of this kind have indeed been described [10–13]. This means that even if a locus shows the epigenetic inheritance of DNA methylation in the laboratory, and no local sequence variation, an epigenetic basis for natural methylation variation cannot be assumed.

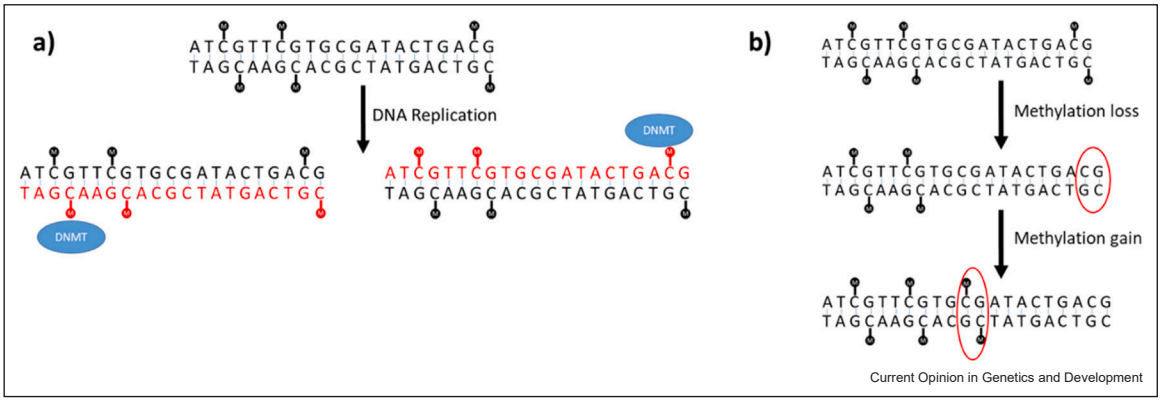
Several approaches have been used to elucidate long-term epigenetic inheritance. First, the stability of epigenetic inheritance can be quantified in the laboratory, and these measurements can be correlated with the patterns of methylation variation in nature [14,15]. Correlations suggest that the natural variation may have at least a partial epigenetic basis [7,16]. Second, the patterns of natural methylation and DNA sequence variation can be associated to discover whether genetic variation can account for the methylation variation [11,17–22]. Finally, mathematical models can be used to simulate epigenetic inheritance over any timescale, and the results can be compared with natural variation patterns to determine if these can be explained by epigenetics [23–30]. Such studies have led to several epigenetic inheritance paradigms, which we will describe below.

Semiconservative epigenetic inheritance of CG methylation and its limitations

Cytosine methylation in plants, animals, fungi and several other eukaryotic lineages predominantly occurs within CG dinucleotides [31]. The core paradigm for the epigenetic inheritance of CG methylation (mCG) was proposed over 40 years ago and relies on the semiconservative nature of DNA replication and the symmetry of the CG dinucleotide [32,33]. Within a CG dinucleotide, there are two methylated cytosines, one on each strand. After DNA replication, each original strand will still contain a single methylated cytosine, whereas the cytosine on the newly synthesized strand will be unmethylated. This hemimethylated CG site recruits a DNA methyltransferase that restores methylation on the newly synthesized DNA strand. Thus, mCG inheritance follows the same semiconservative principle as DNA replication (Figure 2a).

Nevertheless, this process, much like DNA replication itself, makes mistakes [34]. These occur in either direction: loss of an mCG site or gain of an untemplated mCG site (Figure 2b). The absolute and relative rates of these epimutations, and their spatial distributions,

Figure 2



Semiconservative epigenetic inheritance of mCG. **(a)** After DNA replication, each new DNA double helix has one copy of the original strand (black), which retains DNA methylation. Dnmt1/5 binds these hemimethylated CG sites and restores methylation on the newly synthesized strand (red). Thus, the symmetry of CG sites allows semiconservative epigenetic inheritance of mCG. **(b)** Losses of mCG or gains of untemplated mCG (circled) produce epigenetic inheritance errors.

determine the overall mCG epigenetic dynamics and steady state.

Cryptococcus neoformans: low loss and very low gain

An epigenetic model that closely approximates pure semiconservative inheritance has been proposed for the fungus *Cryptococcus neoformans* (Figure 3) [8]. *C. neoformans* has a single methyltransferase, Dnmt5 [35], which uses adenosine triphosphate (ATP) hydrolysis to achieve high specificity for hemimethylated CG sites [36,37]. A low rate of mCG loss (approximately 10^{-4} per site per cell cycle), and an even lower rate of apparently random gain (5×10^{-6}), have been reported *in vivo* [8]. For a locus with half of its CGs methylated, these rates correspond to an error rate of 5×10^{-5} per cell cycle (the average of the two rates; Table 1). This high fidelity has been proposed to enable the epigenetic inheritance of mCG over millions of years [8].

A unique feature of the *C. neoformans* paradigm is that the system is not at a steady state as the rates of loss and gain imply an mCG half-life of just 130 years [8]. Hence, natural selection has been proposed to counteract mCG loss (Figure 3) [8]. A mathematical model of such epigenetic inheritance would have to integrate natural selection, but this has not yet been attempted. Therefore, whether plausibly strong natural selection could stabilize epigenetic inheritance over the proposed timescales (or even much shorter timescales) is unknown.

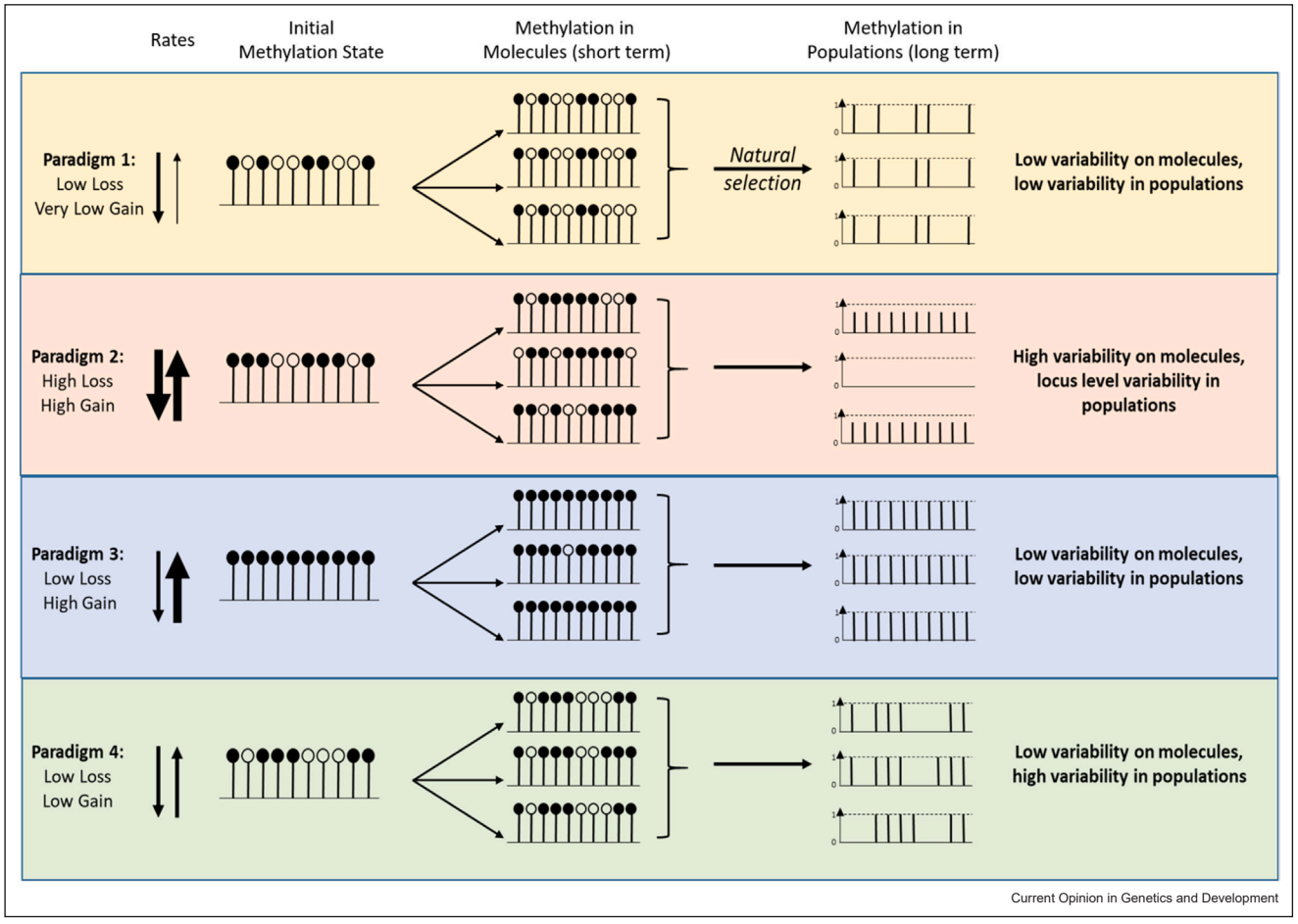
Mammals: high loss and high gain

Mammalian mCG epimutation rates are much higher than in *C. neoformans*, with both loss and gain rates (and hence the error rate) approximately 5×10^{-2} (5%) per cell cycle (although epimutation rates at some CG sites may be substantially lower) [30,38]. The epimutation rates were measured in mouse cell lines in which all DNA demethylases and DNA methyltransferases except Dnmt1 — the key mammalian CG methyltransferase — were removed, indicating that mammalian Dnmt1 has much lower fidelity than *C. neoformans* Dnmt5, at least at a substantial fraction of genomic CG sites (Table 1). Dnmt1-catalyzed mCG gains are not random but are targeted towards methylated regions [30,39].

Mathematical models show that given sufficiently strong feedback — for example, mCG favoring nearby gain and unmethylated CG sites favoring nearby loss — such high epimutation rates are nevertheless compatible with bistable locus-level epigenetic inheritance of mCG. This means that either an overall methylated or unmethylated state of a locus can be robustly inherited, with locus-level methylation variability generated by stochastic epimutations that occasionally flip the overall methylation state [26–29]. Thus, the mammalian high-gain, high-loss paradigm (Figure 3) can, in principle, mediate long-term epigenetic inheritance and create mCG variation independently of genetic variation. However, mammalian mCG is generally thought not to be epigenetically heritable beyond one or a few generations due to extensive reprogramming during embryogenesis and germline development [40], so the prospect of long-term epigenetic inheritance remains theoretical.

Curiously, mammalian-like epimutation rates can be induced in *Arabidopsis thaliana* plants, which also use Dnmt1 (called MET1 in plants) for mCG [41], by genetically inactivating linker histone H1 and the nucleosome remodeler DDM1 [42]. Unlike for fungi or mammalian cells grown in culture, plant epimutation rates must initially be calculated per generation, and

Figure 3



Four paradigms for the epigenetic inheritance of mCG. Different paradigms produce distinct patterns of methylation variation between DNA molecules and within populations. Variability as a result of stochastic epimutations at individual CG sites is shown, whereas locus-level methylation reprogramming events, such as those that occur during mammalian development [40], are presumed to be absent. Natural selection in the first paradigm reduces long-term variability. The initial methylation state is shown within an example locus for each paradigm, followed by how the methylation pattern might vary between individual DNA molecules within a closely related population of cells (e.g. the cells of a single individual). Finally, variability in the population (over long-term timescales) is shown at the same example locus for each paradigm, with each individual pattern representing the average methylation of a multicellular individual (or a closely related group of cells for a unicellular species). The upward arrows indicate methylation gain; the downward arrows indicate methylation loss. The dotted line indicates 100% fractional methylation at a given cytosine.

then divided by the estimated number of cell cycles per generation (34 in *Arabidopsis* [43]). The per-cell cycle gain and loss rates on the order of 10^{-2} are observed within TEs of the mutant plants, where they result in intermediate mCG levels that are stable over multiple generations [42].

Table 1

Rates of methylation loss (per mCG, per cell cycle) and gain (per unmethylated CG, per cell cycle) within each paradigm.

	Loss (per cell cycle)	Gain (per cell cycle)	Error Rate	Enzyme
C. neoformans: low loss, v. low gain	1×10^{-4}	5×10^{-6}	5×10^{-5}	Dnmt5
Mammals: high loss, high gain	5×10^{-2}	5×10^{-2}	5×10^{-2}	Dnmt1
Plant TEs: low loss, high gain	$>4\times10^{-7}$	1×10^{-5} - 1×10^{-2}	?	Dnmt1*
Plant genes: low loss, low gain	3×10^{-5}	3×10^{-5}	3×10^{-5}	Dnmt1

The error rate indicates the per-cell-cycle change that would be seen *in vivo* within a locus that is 50% methylated. The enzyme is the methyltransferase that catalyzes mCG in the experimental system used to calculate the rates. *Other methyltransferases contribute substantially to the plant TE mCG gain rate, for which published estimates vary over at least three orders of magnitude [42,45–47].

Plant transposable elements: low loss and high gain

The epimutation rates in *Arabidopsis* TEs discussed above are artificial because mCG losses are greatly enhanced by inactivating DDM1 [44]. TE epimutation rates have also been extensively studied in wild-type *Arabidopsis*. Initially, very low epimutation rates were reported: approximately 4×10^{-7} per cell cycle for loss and 10^{-5} for gain (Table 1) [45,46], with even lower rates reported for some TE subsets [47]. The loss rate is substantially lower (250-fold) than even that reported for *C. neoformans*, with the key distinction that the gain rate for *Arabidopsis* TEs is at least 25 times higher than the loss rate (Table 1). The bias toward gain is expected to produce uniformly high mCG at TEs (Figure 3) [45], which is indeed observed [48,49].

We have recently proposed that gain rates in *Arabidopsis* TEs are actually much higher, on the order of 10^{-2} [42], and thus similar to mammalian gain rates (Table 1). We believe that the measurement of gain rates at loci with uniformly high mCG is inherently challenging, because gain rates can, by definition, only be measured at unmethylated CG sites, and these will be few and unrepresentative in a high gain regime. High gain rates might also lead to the underestimation of loss rates, as losses are converted to gains before they can be measured. Still, the low-loss, high-gain paradigm we propose for plant TEs (Figure 3) retains the core feature of gain rates that are much higher than loss rates.

Whether a low-loss, high-gain paradigm is compatible with long-term epigenetic inheritance is unclear. The main challenge is restricting gains to already methylated loci. In the absence of strong feedback that maintains the unmethylated state, it will be very unstable, and all loci that can in principle support methylation will be rapidly (re-)methylated. It is perhaps not a coincidence that the low-loss, high-gain regime operates at TEs, which are silenced by mCG [50], and where the epigenetic system might therefore be expected to favor methylation over bistability. Put differently, unmethylated TEs might reasonably be regarded as aberrations. Consistently, the variation of TE or TE-like methylation in natural populations has been repeatedly linked with local genetic variation, as well as genetic variation in the factors that mediate TE methylation [10–13,18,19,22]. This is not to say that long-term epigenetic inheritance of TE methylation isn't possible or doesn't exist, but this may be an exception rather than the rule [51]. Hence, natural selection may primarily act on the DNA sequences of TEs and TE-like elements, including the sequence-determined propensity to attract and maintain DNA methylation at various levels of stability, instead of on their epigenetically inherited methylated and unmethylated states [51].

Plant genes: low loss and low gain

The final paradigm we will discuss applies to methylation within genes (gene body methylation, or gbM). This has been extensively explored in *Arabidopsis*, and involves loss and gain rates of about 3×10^{-5} per cell cycle when measured within the methylated regions [5,6,23,45–47]. Unlike with TEs, there is little reason to doubt these rates, which — analogously to recent mammalian studies [30,38] — were in one case measured in plants that lack all non-Dnmt1 methyltransferases [23]. The corresponding error rate (3×10^{-5}) is about the same as in *C. neoformans* (Table 1), indicating that *Arabidopsis* Dnmt1 and *C. neoformans* Dnmt5 have approximately equal fidelity *in vivo*. Because the gain and loss rates are low, the methylated and unmethylated states of each CG are stable over one or a few generations. As the rates are also balanced, a multicellular individual or a population of closely related cells will have a characteristic mosaic pattern of interspersed methylated and unmethylated sites (Figure 3) [5]. This methylation pattern is seen in the genes of many invertebrates [17,52], and therefore the overall paradigm may also apply there.

Recently, we discovered that *Arabidopsis* gbM favors nearby Dnmt1-catalyzed gains and disfavors losses [23], as has been proposed for mammalian mCG [30]. Based on this, we created a mathematical model that recapitulates empirical gbM dynamics and predicts steady-state gbM [23]. The model shows that gbM inheritance is almost purely epigenetic in the short term due to the low epimutation rates, but in the long term gbM features are genetically determined by the local sequence and global factors that include the histone variant H2A.Z and the DNA demethylase ROS1 [23]. However, stochastic epimutations can accumulate to generate major epigenetic gbM fluctuations that can last for thousands of generations [23]. These fluctuations can account for the majority of gbM variation in natural populations, indicating that natural gbM variation has an epigenetic basis [23]. This is consistent with the observations that epigenetic gbM variation in the laboratory is well-correlated with that in nature [7], and that local gbM variation is largely independent of genetic variation [11,22]. Thus, all three methods — epimutation measurements, association studies, and mathematical modeling — point to epigenetic inheritance of gbM patterns over thousands of generations.

Implications for natural phenotypic variation

Understanding long-term epigenetic inheritance is challenging due to the timescales involved. Nonetheless, much progress has been made in recent years through a combination of studies that quantify epigenetic inheritance dynamics, analyses of natural variation, and

mathematical modeling that can access any timescale. These studies have greatly improved our conceptual understanding of epigenetic inheritance, and have revealed that long-term epigenetic inheritance is not only possible, but – at least in the case of gbM – highly plausible.

The implications of long-term epigenetic inheritance are potentially profound [51]. The core paradigm of evolutionary biology, known as the Modern Synthesis, holds that heritable phenotypic variation is caused by DNA sequence variation [53]. There is extensive evidence across many species that DNA methylation is associated with and can cause variation in gene expression and phenotype [51,54–56]. If the methylation patterns can be epigenetically inherited over thousands of generations, they can form a durable epigenetic basis for phenotypic variation within populations [57]. Such effects would need to be combined with genetic inheritance and natural selection to understand how populations evolve. The coming years promise the exciting possibility of integrating epigenetic inheritance into the modern evolutionary framework.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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- • of special interest
- •• of outstanding interest

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