

Christina L. Richards, Massimo Pigliucci

# Epigenetic Inheritance. A Decade into the Extended Evolutionary Synthesis

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CHRISTINA L. RICHARDS, MASSIMO PIGLIUCCI

# Epigenetic Inheritance

## A Decade into the Extended Evolutionary Synthesis

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A decade ago, a landmark edited collection of essays made official the ongoing quest for an Extended Evolutionary Synthesis (EES), beyond the classic Modern Synthesis that took shape in the 1930s and '40s. When *Evolution – The Extended Synthesis* was published, research on epigenetic inheritance was at its onset, with the theory being far ahead of the empirical results. An important book, *Evolution in Four Dimensions* by Eva Jablonka and Marion Lamb, articulated the notion of multiple channels of inheritance, including genetic, epigenetic, behavioral, and symbolic. These multiple modes of inheriting information within the biosphere joined a number of other empirical findings and conceptual advances to articulate the early version of the EES. In the intervening decade, our empirical understanding of epigenetic inheritance has improved, so it seems that a reevaluation of where we are and where we may possibly be going, is in order. Progress is constrained by the fact that understanding systems of inheritance requires time-consuming experimental designs that incorporate multiple generations. Further, technological limitations have yet to be overcome and most of our understanding of genome level processes is still confined to model species. In this paper we discuss what we have learned so far about epigenetic inheritance and what developments are needed to make further progress. We conclude with a discussion of the current role of epigenetic inheritance in the EES, and how it has changed in the intervening decade.

**Keywords:** Ecological Epigenetics, Epigenetic Inheritance, Experimental Design, Extended Evolutionary Synthesis, Model Species, Modern Synthesis.

### 1. A brief history of evolutionary theory

The notion that the universe in general, and biological systems in particular, may have changed over time is a very old one. The pre-Socratic philosopher Anaximander (610-546 BCE) thought that the first life forms on earth had come from the planet's wetter environments and that human

*Christina L. Richards, University of Tübingen Plant Evolutionary Ecology Group, Auf der Morgenstelle 5 D-72076 Tübingen, Germany; University of South Florida, Department of Integrative Biology, 4202 East Fowler Avenue, Tampa, FL, USA, [clr@usf.edu](mailto:clr@usf.edu)*

*Massimo Pigliucci, The City College of New York, Department of Philosophy, 160 Convent Avenue, NY, USA, [mpigliucci@ccny.cuny.edu](mailto:mpigliucci@ccny.cuny.edu)*

beings came from fish-like organisms. About a century later, Empedocles (494-434 BCE) articulated an early version of a theory of evolution, suggesting that a range of creatures that had existed in the past had been eliminated over time because they were not well adapted to survive. However, he did not envision a cumulative process that could actually create new forms, only a negative filter acting against the unfit (Adamson, 2016).

Jumping forward to the XIX century, James Hutton, in his *Investigation of the Principles of Knowledge* (1794) is credited with having articulated one of the early modern versions of the principle of natural selection, though as a speculative notion, not backed up by Darwin's famous 'one long argument' or by systematic empirical evidence. Hutton wrote:

If an organised body is not in the situation and circumstances best adapted to its sustenance and propagation, then, in conceiving an indefinite variety among the individuals of that species, we must be assured, that, on the one hand, those which depart most from the best adapted constitution, will be most liable to perish, while, on the other hand, those organised bodies, which most approach to the best constitution for the present circumstances, will be best adapted to continue, in preserving themselves and multiplying the individuals of their race (Hutton 1794; quoted in Pearson, 2003).

In 1794, Erasmus Darwin – Charles' grandfather – published his *Zoonomia*, in which he proposed that all warm-blooded animals descended from a single ancestor, and that plants preceded animals in the course of the history of life. He even suggested that the most fit males would be the ones leaving more offspring, thus assuring the survival of the species. More or less at the same time, Jean-Baptiste de Lamarck (1744-1829) was the first to propose a coherent, if incorrect, theory of evolution that came with an account of inheritance, a component that even Charles Darwin's own theory was famously going to lack. Lamarck thought that the use and disuse of parts would make it so that environmentally-triggered positive responses by an organism would be transmitted to the following generation, as in his famous example of the gradual elongation of the giraffe's neck.

By the time we get to the landmark paper jointly presented by Charles Darwin and Alfred R. Wallace (1858) to the Linnaean Society, and to the publication of *On the Origin of Species* the following year, a number of scientists had proposed the concept of common descent of all living forms. But early theories lacked both a mechanism and the sort of systematic evidence that Darwin and Wallace finally provided. Of course, the Darwin-Wallace theory represented only a new beginning for the long history of the notion of evolutionary change. What we refer to as neo-Darwinism appeared shortly thereafter, before the end of the 19th century, courtesy of the very same Wallace and of developmental biologist August Weismann. The latter had demonstrated the separation between somatic and germ lines that finally excluded Lamarckism, a notion with which Darwin himself had

flirted. Neo-Darwinism then went through a period of “eclipse” (Bowler, 1983) during which it was doubted especially by palaeontologists, with a good number of researchers entertaining the idea of orthogenetic (i.e., directed) evolution over long time spans, initially proposed by Wilhelm Haacke in 1893 (Gould, 2002).

The eclipse became a full-blown crisis at the turn of the XX century, with the rediscovery of Mendel’s work (Mendel and Bateson, 2009). The problem was that Mendelian inheritance is particulate, while Darwin’s natural selection needed gradually varying, quantitative traits. Or so it appeared to be the case until Ronald Fisher, J.B.S. Haldane, and Sewall Wright established the modern field of statistical genetics, demonstrated how a large number of particulate factors (genes) can generate continuous phenotypic distributions in organismal traits, and laid the theoretical foundations for what became the Modern Synthesis (MS: Fisher, 1930; Haldane, 1932; Wright, 1932).

The MS then underwent a second phase, catalysed by the work of Dobzhansky (1937), Huxley (1942), Mayr (1942), Simpson (1944), and Stebbins (1950). These authors applied the insights of the statistical geneticists to a number of areas of inquiry, from population genetics to systematics, from palaeontology to botany. The current ‘standard model’ in evolutionary biology was born asserting that genes are the sole source of heredity, variants segregate according to Mendelian rules and inheritance of environmentally induced non-genetic variation is impossible (Keller, 2014; Bonduriansky and Day, 2018; Müller, 2017; Stoltzfus, 2017).

## 2. Epigenetic inheritance and why it matters

Up to this point in the story, the concept of epigenetic inheritance is pretty much nowhere to be seen, unless one counts Lamarck’s fuzzy ideas, expressed in pre-Darwinian and pre-Mendelian terms. But after its complete dominance of the intellectual landscape, the MS began to show cracks due to its relatively narrow scope, as well as the fact that it famously left out entire fields (e.g. developmental biology: Gilbert, Opitz and Raff, 1996) and ignored widespread biological phenomena, treating them as ‘noise’ (e.g. phenotypic plasticity: Pigliucci, 2001).

Calls began for an Extended Evolutionary Synthesis (EES: Pigliucci and Müller, 2010), conceived not as a rejection of either Darwinism or inheritance based on DNA sequence as articulated in the MS, but as an expansion to include several new fields of investigation (e.g. evo-devo: Hall, 2003), to re-evaluate the role of other fields (e.g. palaeontology: Gould, 1977), and to reconsider a by now well established array of biological phenomena that were not accounted for by the MS, including, but not limited to, epigenetic inheritance.

As far as the latter is concerned, the turning point was Jablonka and Lamb's *Evolution in Four Dimensions* (2005), where the authors pointed out the existence of not one, but four channels of inheritance within the biological world: the classical genetic one, epigenetic (non-DNA based cellular transmission), behavioural (copying), and symbolic (language). Jablonka and Lamb took a broad view of what they characterized as 'the epigenetic turn' and its impact on the (evolving) structure of evolutionary theory:

There are three main types of epigenetic research that are having an impact on evolutionary thinking. The first was pioneered more than 60 years ago by Waddington in Great Britain and Schmalhausen in the Soviet Union, both of whom took a view of evolution that was centered on the complementary aspects of developmental canalizations and phenotypic plasticity. [...] The second epigenetic approach to evolutionary change emphasizes the mechanisms that give rise to phenotypic plasticity. [...] The third type of epigenetic research [...] focuses on cell memory and cell heredity (Jablonka and Lamb, 2010, pp. 140-141).

It is the latter that the present paper is concerned with. Four types of mechanisms were suggested in this context by Jablonka and Lamb: 1) systems based on self-sustaining regularity loops; 2) systems involving structural templating; 3) chromatin marking systems; and 4) RNA-mediated inheritance. In practice, and for the purposes of this paper, epigenetic research typically refers to chemical modifications of DNA or chromatin that can influence gene activity without changes in DNA sequence, and the majority of research has been focused on DNA methylation (Jablonka and Raz, 2009; Kilvitis *et al.*, 2014; Banta and Richards, 2018). The crucial questions that needed to be addressed, then, were: a) what sort of evidence do we have for the occurrence of the types of epigenetic inheritance mentioned above? And b) what conceptual work is required to integrate this kind of epigenetic inheritance within the logical structure of the EES? We will provide updates to both questions in the sections that follow, but the baseline is provided again by Jablonka and collaborators.

In answer to question (a), Jablonka and Raz (2009) published an extensive review of the evidence available up to the end of the first decade of the 21st century. Their Table 1 (pp. 140-152) lists 101 documented cases, providing for each: taxon, involved trait, locus or cellular system, degree of stability over time, inducing conditions, type of epigenetic inheritance, and references. They concluded that evidence of epigenetic inheritance is most common in fungi and plants, though it is found across most major taxa. Not surprisingly, perhaps, they found that the relative importance of different kinds of epigenetic inheritance systems varies across taxa. For instance, budding yeast simply lacks RNAi mechanisms, while some animal groups appear to have lost DNA methylation. Moreover, transgenerational

structural inheritance and inheritance via self-sustaining loops is more common in unicellular organisms and fungi.

Concerning question (b), Jablonka and Lamb (2010) put forth arguments to the effect that epigenetic inheritance may affect the following: adaptation, genetic assimilation (Pigliucci and Murren, 2003), reproductive isolation, evolution of development, macroevolutionary change, and major transitions in evolution (Maynard Smith and Szathmáry, 1998). They conclude that:

The Modern Synthesis denied the possibility of soft inheritance, and insisted that evolution is usually gradual. However, the mechanisms of epigenetic inheritance that we have discussed are simultaneously involved in the regulation of gene expression and production of phenotypes, as well as in the transmission of information between cells and organisms; they therefore enable soft inheritance. [...] A broader notion of heredity, based on the mechanisms of epigenetic inheritance at all levels of biological organization, could help to unite the different developmental approaches and transform our understanding of evolution (pp. 168-169).

While we appreciate the importance of this message, Jablonka and Lamb's frequent use of terms like 'soft inheritance', and especially 'neo-Lamarckism', are vague and unnecessarily controversial. We also disagree with Jablonka and Lamb's contention that transgenerational epigenetic inheritance somehow undermines Darwinism, or even the MS (Jablonka and Lamb, 2010). As they themselves put it, what we are after is "a broader notion of heredity", not a substitutive one. Epigenetic inheritance, together with other non-genetic inheritance mechanisms more generally and other conceptual and empirical components of the EES, constitutes a further enlargement of the original Darwinism, building on the Modern Synthesis, not replacing it. There have, after all, been no paradigm shifts (*sensu* Kuhn) in the entire history of biology since Darwin (Pigliucci, 2012).

### **3. How has the concept of epigenetic inheritance been operationalized empirically?**

When *Evolution: The Extended Synthesis* was published, there was already growing interest among evolutionary ecologists in understanding the role of epigenetic processes in ecology and evolution (e.g. Bossdorf, Richards and Pigliucci, 2008; Johannes, Colot and Jansen, 2008; Richards, Bossdorf and Pigliucci, 2010). While there had been a handful of relevant studies investigating epigenetic mechanisms, there were two additional important publications in 2005 which discussed how the application of molecular approaches could help to investigate the potential importance of epigenetic inheritance.

Salmon, Ainouche and Wendel (2005) published one of the first studies

in non-model species using a modification of the Amplified Fragment Length Polymorphism (AFLP) molecular markers that were commonly used in a variety of applications to measure genetic variation (Reyna-Lopez, Simpson and Ruiz-Herrera, 1987; Cervera, Ruiz-Garcia and Martinez-Zapater, 2002). The modified protocol allowed for detection of anonymous methylation polymorphisms by taking advantage of methylation sensitive enzymes in the standard AFLP protocol (methylation sensitive AFLP). Salmon, Ainouche and Wendel (2005) used this approach to demonstrate that hybridization, and to a lesser extent polyploidization, triggered DNA methylation changes in *Spartina* species. The study suggested that the morphological plasticity and larger ecological amplitude of the highly invasive *Spartina anglica* could be partly due to methylation changes that weren't explained by genetic ones.

The methylation sensitive AFLP protocol could be applied to virtually any species and became increasingly popular among scientists asking a diversity of questions in evolutionary ecology (Schrey *et al.*, 2013). A search on Web of Science in June 2020 revealed that 130 studies have now used this approach in ecology and evolution research (in the fields of Genetics Heredity (83), Ecology (37), Evolutionary Biology (33) or Environmental Science (8)). In addition to this important study, Rapp and Wendel (2005) outlined ideas about how epigenetic mechanisms might contribute to morphological variation and population genetics, with implications for understanding response to environmental challenges and “genomic shock” (*sensu* McClintock, 1984) in one of the first attempts to discuss how molecular epigenetics studies could shed light on evolutionary processes.

By 2010, several studies had already established that some epigenetic markers are not reset each generation, but are faithfully transmitted across generations (Jablonka and Raz, 2009), that natural variation exists at the epigenetic level (Cervera, Ruiz-Garcia and Martinez-Zapater, 2002; Vaughn *et al.*, 2007), and that epigenetic variation alone can cause significant heritable variation in phenotypic traits (Johannes *et al.*, 2009; reviewed in Richards, Verhoeven and Bossdorf, 2012; Kilvitis *et al.*, 2014).

However, most information about epigenetic mechanisms had been obtained from very few agricultural crops and model species such as mouse, *Arabidopsis thaliana*, and human diseases, frequently under artificial conditions. Despite a lot of lab-based work, the data were difficult to evaluate in terms of the importance of epigenetic processes in natural populations. Several authors argued for expanding research efforts into ecologically relevant circumstances across model and non-model organisms and outlined experimental and statistical approaches that would facilitate the merging of molecular based insight with sound evolutionary ecology (Bossdorf, Richards and Pigliucci, 2008; Johannes, Colot and Jansen, 2008; Richards, Bossdorf and Pigliucci, 2010; Richards, Bossdorf and Verhoeven, 2010).

In the last decade, many studies have finely dissected the epigenomics of the model plant *A. thaliana*, and several evolutionary ecologists have embraced the opportunity to work with *A. thaliana* and take advantage of the tremendous amount of genomics information available for this species. In addition, a number of comparative studies of crop plants with their wild relatives have taken advantage of artificial selection as a process similar to natural selection to reveal the molecular underpinnings associated with the response to selection more generally. At the same time, genomics resources and approaches for human and non-model organisms have also developed and we now have information about a diversity of plant, animal and fungal species.

Together these approaches have taken three main paths that reveal components of epigenetic inheritance and shed light on its importance: 1) documenting the amount and distribution of epigenetic variation in natural populations; 2) exploring the causes of epigenetic variation; and 3) understanding the consequences of epigenetic variation (Kilvitis *et al.*, 2014; Richards *et al.*, 2017). Here, we summarize some of the salient findings in model species, domesticated species, and a variety of non-model species that have been discovered in the last 10 years.

### 3.1. Amount and distribution of epigenetic variation

The first step many researchers have taken to understand the role of epigenetic inheritance is to quantify how much epigenetic variation exists in nature, how it compares to levels of genetic variation, and how it is distributed. When the EES was published, we had very little information on the amount of epigenetic variation within and among any species.

One of the earliest studies of natural variation in DNA methylation in *A. thaliana* evaluated only 10 accessions using the methylation sensitive AFLP protocol, and showed that methylation patterns among plants from the same inbred accession varied by only 1%, but in pairwise comparisons among plants from different accessions by 24-34% (Cervera, Ruiz-Garcia and Martinez-Zapater, 2002). Vaughn *et al.* (2007), provided more resolution on variation in DNA methylation by showing how transposable elements are almost always methylated, and methylation is highly polymorphic within genes (Vaughn *et al.*, 2007). That study examined only two distinct accessions (Columbia and Landsberg) of *A. thaliana*, and was limited to a ‘tiling array’ of only one of the five chromosomes (n. 4) of this species. The authors also used 18 genic loci to evaluate variation among 96 accessions based on coarse information from digestion with an enzyme (McrBC) that cleaves DNA containing methylcytosine on one or both strands (Vaughn *et al.*, 2007).



Studies of variation within other plant species or animals and fungi were limited – and moreover, model species such as *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* lack methylation almost entirely and so provided no meaningful insight (Schmitz, Lewis and Goll, 2019; de Mendoza, Lister and Bogdanovic, 2020). In addition to *A. thaliana* studies, a few early papers used methylation sensitive AFLP to document variation in DNA methylation within and among populations in several other plant species, and in only one study of animals (Schrey *et al.*, 2013).

While the methylation sensitive AFLP are anonymous markers and are limited in application to understanding the functional significance of DNA methylation (Schrey *et al.*, 2013; Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019), these studies provided the proof of concept for calculating population genetic measures of diversity within and among wild populations. They also provided early evidence that variation was often significantly higher for methylation-sensitive markers than for normal (methylation-insensitive) genetic markers, and that epigenetic diversity was correlated to different environmental conditions (Richards, Bossdorf and Verhoeven, 2010; Richards, Verhoeven and Bossdorf, 2012).

These results supported the contention that selection may have shaped epigenetic variation and resulted in associations between heritable epigenetic differences and habitat characteristics, and that epigenetic variation could play a role in adaptation (*ibid.*). However, most studies that found correlations between epigenetic diversity and different habitats were performed on field collected material and could not differentiate environmentally induced epigenetic effects from persistent ones, so it was unclear how much of these methylation polymorphisms reflected epigenetic inheritance instead of plasticity induced within a single generation (Richards, Bossdorf and Pigliucci, 2010; Richards, Bossdorf and Verhoeven, 2010).

The quest for ever more precise information about levels of DNA methylation was emboldened in 2008 with the application of bisulfite treatment of DNA. Bisulfite deaminates unmethylated cytosines and allows for identification of methylation at single cytosine resolution when comparing bisulfite treated DNA to a reference (Cokus *et al.*, 2008; Lister *et al.*, 2008, 2009). The Whole Genome Bisulfite Sequencing (WGBS) approach was quickly recognized as the ‘gold standard’ for examining DNA methylation, but was not applied to studies of diversity until well after the publication of the EES.

WGBS studies in *A. thaliana* (Schmitz *et al.*, 2013; Kawakatsu *et al.*, 2016; Meng *et al.*, 2016), several crops (Li *et al.*, 2020), and humans (Fraser *et al.*, 2012; Heyn *et al.*, 2013; Carja *et al.*, 2017) supported the early findings of variation in DNA methylation among different lines or genotypes within species. The single base pair resolution of sequencing also revealed that the amount and function of DNA methylation depends on the sequence

context (i.e. CG, CHG, or CHH where H is A, C or T) and the type of genomic region (gene promoters, gene bodies, transposable elements: Niederhuth and Schmitz, 2017; Richards *et al.*, 2017; de Mendoza, Lister and Bogdanovic, 2020).

Studies across a diversity of taxa have shown that cytosine methylation and the machinery that maintains methylation vary widely from completely missing in *D. melanogaster*, *C. elegans*, *S. cerevisiae* and *Schizosaccharomyces pombe* to vertebrate genomes where more than 80% of the cytosines in the CG context are methylated (Schmitz, Lewis and Goll, 2019; de Mendoza, Lister and Bogdanovic, 2020). In mammals, for example, most cytosines in the CG context are methylated except for those in promoters, and promoters can be methylated in association with genomic imprinting (de Mendoza, Lister and Bogdanovic, 2020). By contrast, insects generally have a much lower level of methylation (15% of CG) and methylation has been lost independently in several lineages (Schmitz, Lewis and Goll, 2019). Even prokaryotes use methylation, although primarily as a defense mechanism. Ecologically relevant epigenetic mechanisms in microbes are virtually unexplored, but several authors have suggested that methylation could be important in microbial adaptation (Casadesús *et al.*, 2013; Phillips *et al.*, 2019).

While there are six main types of DNA methyltransferases, the evolutionary relationships across taxa show few universally conserved functions with lineage specific losses and additions (reviewed in Schmitz, Lewis and Goll, 2019; de Mendoza *et al.*, 2020). Some researchers have postulated that the differences among lineages could partly be due to the fact that deamination of 5mC can result in mutation of cytosine into thymine, potentially resulting in loss of function (de Mendoza, Lister and Bogdanovic, 2020). Similarly, targeting of transposable elements varies across taxa: TE's are almost uniformly methylated in plants while most TEs that are methylated in invertebrate genomes are found within gene bodies and repetitive elements in intergenic regions remain unmethylated (Schmitz, Lewis and Goll, 2019; de Mendoza, Lister and Bogdanovic, 2020).

Although WGBS data are now available for more than 150 eukaryotic genomes (Schmitz, Lewis and Goll, 2019), this approach has not yet been widely applied to populations of species to address the importance of epigenetic diversity and inheritance mostly because of the cost associated with the large numbers of samples required for these studies. Instead, several Reduced-Representation Bisulfite Sequencing methods have been developed and have been used to document DNA methylation variation in plant and animal species (Richards *et al.*, 2017; Boquete *et al.*, 2020; Gawehns *et al.*, 2020; e.g. van Moorsel *et al.*, 2019; Alvarez *et al.*, 2020; Johnson and Kelly, 2020; Robertson *et al.*, 2020).

In the meantime, researchers have continued to use methylation sensitive AFLP to compare genetic and epigenetic structures of populations

and their environmental correlates (reviewed in Richards *et al.*, 2017), with a notable increase of studies in animals (Schrey *et al.*, 2013, 2016; Sheldon *et al.*, 2018). Following the tradition of ecological genomics, studies of non-model species have applied sophisticated multi-variate statistics to explore the relationships between epigenetic variation, genetic variation, environmental variation, and phenotypic variation (Herrera, Medrano and Bazaga, 2017; Wang *et al.*, 2020).

Particularly in studies of natural populations *in situ* when experimental manipulations are not possible, ordination techniques allow for modeling the relationships between these different types of variation simultaneously (Foust *et al.*, 2016; Keller, Lasky and Yi, 2016; Schrey *et al.*, 2016; Herrera, Medrano and Bazaga, 2017; Wang *et al.*, 2020; Alvarez *et al.*, 2020; Robertson *et al.*, 2020). Generally, across model and non-model species, variation in DNA methylation exceeds variation in DNA sequence (but see Foust *et al.*, 2016; Gáspár, Bossdorf and Durka, 2019; Chen *et al.*, 2020), is often correlated with ecological factors, and some of these relationships are not completely explained by patterns of genetic relatedness (Schrey *et al.*, 2016; Herrera, Medrano and Bazaga, 2017; Sheldon *et al.*, 2018; Gáspár, Bossdorf and Durka, 2019; Medrano *et al.*, 2020; reviewed in Richards *et al.*, 2017).

### 3.2. What are the causes of epigenetic variation?

Several characteristics of epigenetic data challenge our ability to understand its importance for inheritance. Considering that we are interested in how epigenetic mechanisms may explain inheritance above and beyond what is already explained by genetic variation, one of the major lines of inquiry has been to understand the causes of epigenetic variation.

Epigenetic variation can arise stochastically, result from genetic differences, and can be induced by environmental variation or the genomic ‘shock’ of hybridization and genome doubling in polyploids (Parisod *et al.*, 2010; Kilvitis *et al.*, 2014; Richards *et al.*, 2017; e.g. Salmon, Ainouche and Wendel, 2005; Parisod *et al.*, 2009; Schmid *et al.*, 2018). In addition, a large portion of epigenetic variation may not be functionally relevant or may be reset for the next generation and hence have no independent effect on evolutionary processes that is not explained by genetic variation. In order to isolate causes and consequences of epigenetic variation, it can be treated as either a dependent or independent factor in analyses. Evaluating DNA methylation as a dependent factor or phenotype has allowed for screening for associations of DNA methylation with genetic markers (e.g. Dubin *et al.*, 2015) and environmental conditions (e.g. Verhoeven *et al.*, 2010; Richards, Schrey and Pigliucci, 2012; Alvarez *et al.*, 2020; Robertson *et al.*, 2020). By contrast, methylation variation can be treated the same way as conventional

genetic markers in mapping approaches to explain phenotypic variation, as a consequence of epigenetic variation.

While the origin of epigenetic variation was largely unexplored before 2010, early studies had suggested that stochastic epimutation is probably higher than sequence mutation, and heritable epigenetic modifications can be triggered by exposure to different environmental conditions, hybridization, or the activity of TEs (e.g. Morgan *et al.*, 1999; Salmon, Ainouche and Wendel, 2005; Parisod *et al.*, 2009; Verhoeven *et al.*, 2010; Groszman *et al.*, 2011).

In the last 10 years, studies in the *A. thaliana* mutation accumulation lines have confirmed that epimutations occur much more frequently than genetic mutations, they do not occur randomly across the genome, and they occur more often in genic regions than in TEs (reviewed in Richards *et al.*, 2017). Another study in maize showed that the forward epimutation rate was about 10 times larger than the backward epimutation rate, and two orders of magnitude larger than that of DNA mutation rate (Xu *et al.*, 2020). In humans, the epimutation rate appears to be lower than in *A. thaliana*, but was also estimated to be over two orders of magnitude greater than the germline genetic mutation rate (Carja *et al.*, 2017). We know of no studies in non-model species with no genomics resources that have estimated epimutation rates, but Verhoeven *et al.* (2010) showed that DNA methylation differences develop between individual plants even in a common environment, and that most of these changes are inherited by clonal offspring. A recent whole genome survey of *Populus trichocarpa* showed mutation and epimutation rates were very similar to *A. thaliana* (Hofmeister *et al.*, 2019).

Despite quite some effort in the last 10 years, we have very little data in any system that can address to what extent functional epigenetic variation can arise completely independently of genetic variation. On the contrary, several studies in *A. thaliana* have found that a large portion of epigenetic differences among accessions is explained by single mutations in the methylation machinery that dramatically impact the methylome.

Taking advantage of mutation accumulation over 30 generations in *A. thaliana* lines, one study found that a mutation in the methyltransferase MEE57 led to a 40% increase in differences between one line compared to others (Becker *et al.*, 2011). Similarly, alternative alleles of the DNA methyltransferase CMT2 were associated with differences in CHH methylation in response to temperature in 150 Swedish accessions and in the 1001 genomes (Dubin *et al.*, 2015; Kawakatsu *et al.*, 2016; Sasaki *et al.*, 2019). Further investigation on methylation in 303 TE families across the genome revealed the importance of variation in NRPE1 for methylation of TEs targeted by the RNA-directed DNA methylation pathway (Sasaki *et al.*, 2019). In rice (e.g. Li *et al.*, 2012) and humans (Carja *et al.*, 2017) bisulfite

sequencing studies have also suggested that functional DNA methylation is largely correlated with genetic variation (but see Heyn *et al.*, 2013).

In non-model species, many studies have concluded that DNA methylation cannot simply be predicted from patterns of genetic variation, which supports a role for epigenetics that is at least partly independent of genetics (Kilvitis *et al.*, 2014; Richards *et al.*, 2017; Sheldon *et al.*, 2018). In particular, researchers have adopted ordination approaches like redundancy analysis (RDA) to evaluate how well overall similarities in DNA methylation profiles among individuals can be predicted from their DNA sequence similarities (e.g. Foust *et al.*, 2016; Gáspár, Bossdorf and Durka, 2019; Alvarez *et al.*, 2020; Chen *et al.*, 2020).

Some studies show that RDA detects significant epigenetic differences that are associated with environmental conditions after accounting for genetic variation (e.g. Foust *et al.*, 2016; Schrey *et al.*, 2016), while others find that methylation is explained by genetic patterns (e.g. Alvarez *et al.*, 2020; Robertson *et al.*, 2020). While these studies have attempted to disentangle epigenetic variation from genetic control, they have used low-resolution molecular markers that could not rule out the possibility that methylation patterns could be explained by genetic polymorphisms that were not detected (Richards *et al.*, 2017). Even in *A. thaliana*, understanding the source of methylation differences is limited by the power to detect individual changes, particularly since rare insertions of TEs could have important effects on methylation patterns (Sasaki *et al.*, 2019). So far, the low genomic resolution of studies of most organisms precludes pinpointing the causality of epigenetic effects (Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019; Sasaki *et al.*, 2019).

Many studies have shown that epigenetic variation can be induced, but the inheritance of induced changes is still not universally supported (Kilvitis *et al.*, 2014; Richards *et al.*, 2017; Bošković and Rando, 2018; Bonduriansky and Day, 2018; Eirin-Lopez and Putnam, 2019). Several studies in *A. thaliana* have shown that DNA methylation reacts to environmental changes such as abiotic and biotic stress and that these epigenetic changes are sometimes associated with changes in gene expression throughout the genome, but other studies indicate that inheritance of stress-induced DNA methylation changes is limited (Richards *et al.*, 2017; Lu, Zhou and Zhao, 2020).

One study in *A. thaliana* reported no inheritance of induced phenotypic, gene expression or methylation responses to mild drought stress (Van Dooren *et al.*, 2020). Another found limited inheritance of induced phenotypic responses that were associated with ancestral drought stress, but could not associate the response with causative changes in DNA methylation (Ganguly *et al.*, 2017). Schmid *et al.* (2018) reported that experimental populations of recombinant inbred lines (or RILs, created by crossing the Landsberg and Cvi accessions of *A. thaliana*) had accumulated

methylation differences that were correlated with different environments and heritable phenotypic differences. But their analysis suggested that the environment-associated methylation polymorphisms could have been initiated during the original hybridization of the two accessions to create the RIL population (Groszmann *et al.*, 2011). This interpretation is reminiscent of Barbara McClintock's (1984) ideas about how 'genomic shock' that occurs during hybridization of two genomes could induce epigenetic variation (Salmon, Ainouche and Wendel, 2005; Rapp and Wendel, 2005; Parisod *et al.*, 2009; Groszmann *et al.*, 2011). Schmid *et al.* (2018) found a reduction in epigenetic diversity, which could indicate selection for specific epigenetic variants during the course of the experiment. However, the study could not discriminate whether the epigenetic variation was already present at low frequency in the population prior to the experiment or induced by the novel environments during the experiment (Schmid *et al.*, 2018).

Several studies in non-model plant and animal species have found that heritable changes in DNA methylation can be induced in response to experimental application of stress (e.g. Weyrich *et al.*, 2016, 2018; Richards *et al.*, 2017; Eirin-Lopez and Putnam, 2019). Others argue that epigenetic responses could be particularly important for species that are clonal or have low genetic diversity (Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015; Richards *et al.*, 2017). In particular, some insight has been gained by taking advantage of the natural experiment of invasion of novel habitat, which usually involves reduced genetic possibilities (reviewed in Liebl *et al.*, 2015; e.g. Sheldon *et al.*, 2018). However, studies from invasive populations may also reflect natural selection acting on variation in DNA sequence or created by spontaneous epimutations that may not have been induced by the environment. Further, the invasion process may reveal novel genome level dynamics dependent on genetic architecture, cryptic variation or new genomic level interactions (Dlugosch *et al.*, 2015; Stapley, Santure and Dennis, 2015). These studies also require growth in common environments to evaluate the inheritance of the epigenetic differences (Richards, Schrey and Pigliucci, 2012; Xie *et al.*, 2015), which is difficult to accomplish in some plant and most animal species.

Large scale studies of inheritance of epigenetic mechanisms other than DNA methylation are uncommon, but these mechanisms could be particularly important for organisms like *Drosophila* which lack DNA methylation but have a complex machinery of histone modifications (Hennig and Weyrich, 2013). Histone modifications and small RNAs have also been implicated in the process that allows for some loci to avoid the assumed 'global' erasure of cytosine methylation that occurs during gametogenesis and fertilization (Bošković and Rando, 2018). Across various taxa, researchers now understand that gametes of both sexes can carry functional small RNAs or histone modifications, but the implications for

these types of mechanisms have not been well explored at the population level. Laboratory studies indicate that epigenetic silencing is impaired by heat shock through heterochromatin modifications which persist for several generations in *D. melanogaster* and *C. elegans* (Bošković and Rando, 2018). Studies of starvation in *C. elegans*, trauma and diet stress in mice, drought stress and salicylic acid exposure in apomictic dandelion, and hybridization in *A. thaliana* confirmed the inheritance of stress or genome merger induced small RNA changes (Groszmann *et al.*, 2011; Rechavi *et al.*, 2014; Morgado *et al.*, 2017; Bošković and Rando, 2018; Bonduriansky and Day, 2018). These studies suggest the potential importance of small RNA and chromatin changes but also indicate the necessity of further studies incorporating relevant ecological experimental design.

### 3.3. What are the consequences of epigenetic variation?

Before the publication of the EES, there were a few compelling examples of clear epigenetic contribution to phenotypes that could be evolutionarily important in plants, animals and some fungi – including inheritance of floral symmetry, maternal behavior induced changes in offspring, and environmentally-sensitive disease susceptibility and progression (Jablonka and Raz, 2009; Feinberg and Irizarry, 2010; Richards, Verhoeven and Bossdorf, 2012; Ledón-Rettig, Richards and Martin, 2013; Kilvitis *et al.*, 2014). Many of these examples relied on the assumption that changes in DNA methylation were involved directly in the regulation of gene expression, particularly since methylation of gene promoters has been associated with gene silencing (Paun, Verhoeven and Richards, 2019).

However, evidence from studies in the last 10 years does not universally support that methylation regulates gene expression, and repression of transcription by methylation mechanisms might not be as widespread as previously thought (de Mendoza, Lister and Bogdanovic, 2020). In fact, many transcription factors show increased DNA binding affinity for methylated DNA, and gene body methylation can be associated with high expression (Schmitz, Lewis and Goll, 2019; de Mendoza, Lister and Bogdanovic, 2020).

In addition, changes in gene expression may cause variation in patterns of DNA methylation (Secco *et al.*, 2015; Meng *et al.*, 2016; Niederhuth and Schmitz, 2017). In plants, DNA methylation of the 5-prime end of genes has been correlated with gene silencing, but outside of this region the functional relevance of gene body methylation varies by context and across taxa, and it is often not correlated or only weakly correlated to gene expression (Niederhuth *et al.*, 2016; Niederhuth and Schmitz, 2017).

Similarly, early studies suggested that methylation was important for

cast determination in eusocial species such as ants or honeybees, but this apparently does not hold across all social insect species (de de Mendoza, Lister and Bogdanovic, 2020). Insects generally have lower levels of methylation than other animals, and methylation as well as the associated methylation machinery has been lost independently several times within insect lineages (Schmitz, Lewis and Goll, 2019). In general, patterns of DNA methylation appear to vary tremendously across taxa and do not simply predict gene expression or phenotypic variation.

Several important genomic resources have been developed in *A. thaliana* that have confirmed the potential importance of epigenetic inheritance. From well before the publication of the EES book, lines that carry mutations in genes required for proper functioning of the epigenetic machinery were isolated, e.g. genes that code for enzymes involved in creation or maintenance of epigenetic variation. These mutations are typically isolated in otherwise genetically uniform backgrounds, and have been used in a variety of experiments to identify which phenotypes are influenced by epigenetic mechanisms, how they affect response to environmental challenges, and how inheritance is altered in the mutant (Richards, Verhoeven and Bossdorf, 2012; Richards *et al.*, 2017).

One of the most powerful tools that has provided direct evidence for a link between epigenetic variation and heritable phenotypic variation are the epigenetic recombinant inbred lines (epiRILs) that were created from the cross between the wild type of the Columbia accession and two lines that originated from the Columbia accession but have mutations that reduce DNA methylation (i.e. *ddm1* or *met1*; Richards, Verhoeven and Bossdorf, 2012; Richards *et al.*, 2017). The epiRILs from within each cross are nearly identical in DNA sequence genome-wide, but differ from each other in DNA methylation (Johannes *et al.*, 2009; Reinders *et al.*, 2009; Cortijo *et al.*, 2014). Linkage mapping experiments with epiRILs demonstrate that DNA methylation explained heritable phenotypic effects (Cortijo *et al.*, 2014), while experimental studies showed heritable variation in phenotypic plasticity (Zhang *et al.*, 2013), and that epigenetic diversity contributed to productivity (Latzel *et al.*, 2013; Puy *et al.*, 2020). Even though these lines originate from a single genotype background, a significant fraction of the causal methylation polymorphisms are also variable in natural *A. thaliana* populations, and could be functionally important in the wild (Cortijo *et al.*, 2014).

Further, while phenotypic differences were greatest among natural accessions, heritable variation within epiRILs was equivalent to that within natural accessions and within normal RILs (i.e., those created by hybridizing two different accessions) (Zhang *et al.*, 2018). A study comparing inheritance of epigenetic effects in these lines to that of natural accessions confirmed that epigenetic diversity can provide as much phenotypic diversity



as genetically diverse lines and that this diversity ameliorates competition, potentially contributing to increased productivity (Puy *et al.*, 2020).

While the epiRIL studies are powerful as a proof of concept of how methylation variation can code for phenotypic variation, they are limited to a single original genotype, artificially created from crossings with methylation mutants in *A. thaliana* (*ddm1* or *met1*). Wild populations of plants and animals typically harbour high levels of genetic variation, and are difficult to manipulate as such.

Another approach has been to take advantage of invasive species with low genetic variation, or investigate patterns based on historical knowledge about the age of populations (Liebl *et al.*, 2015; Richards *et al.*, 2017). For example, methylation patterns were associated with different habitats in Japanese knotweeds (Richards, Schrey and Pigliucci, 2012; Robertson *et al.*, 2020), and northern expansion of Crofton weed (*Ageratina adenophora*) in China (Xie *et al.*, 2015), even after the plants were grown in a common environment. Several studies in house sparrows took advantage of knowledge about the movement of an invasion across the landscape and indicate that DNA methylation may play an important role in the ability of genetically depauperate populations to adapt to novel environments (Schrey *et al.*, 2012; Liebl *et al.*, 2013, 2015).

In particular, Liebl *et al.* (2013) evaluated seven populations from a single founding event and uncovered a negative relationship between epigenetic and genetic diversity, suggesting that epigenetic diversity could provide an important source of phenotypic variation when genetic diversity is reduced in the initial stages of invasion. Another study of house sparrows, from populations that had been established for longer periods of time, suggested that this relationship had dissipated (Sheldon *et al.*, 2018). Still, these studies cannot separate potential genetic effects from epigenetic ones, since the epigenetic patterns could be attributed to the response of specific genotypes rather than to epigenetic inheritance per se.

Several studies in plants have been able to isolate inheritance of such plasticity in experiments that replicate genotypes in different environments in one generation and then assess inheritance of the induced effects (e.g. Herman and Sultan, 2016; Puy *et al.*, 2019; Alvarez, Bleich and Donohue, 2020; Gáspár, Bossdorf and Durka, 2019). Importantly, these studies can only isolate the epigenetic effect of DNA methylation from non-genetic effects more generally, if they also manipulate genome wide DNA methylation with chemicals like 5-azacytidine or zebularine (Richards *et al.*, 2017; Puy *et al.*, 2018; e.g. Herman and Sultan, 2016; Puy *et al.*, 2020), or evaluate methylation sensitive molecular markers (e.g. Gáspár, Bossdorf and Durka, 2019). Several such studies have found support for the inheritance of induced responses, that these responses could contribute to adaptation, and that the magnitude of these effects appears to vary by genotype (Herman

and Sultan, 2016; Puy *et al.*, 2020). However, once again, isolating truly independent epigenetic variation will require much more detailed information about genomic mechanisms in these species.

In addition to ecological experiments, one approach with a long history for understanding evolution of phenotypes has been to take advantage of insights provided by artificial selection, e.g. through domestication events (Darwin, 1868; Purugganan and Fuller, 2009; Fuller *et al.*, 2011). Crop plants become differentiated across their cultivated range due to different human preferences and selection on phenotypes in new environments in a process similar to natural selection, and despite the fact that cultivated plants are presumed to have undergone genetic bottlenecks (Hyten *et al.*, 2006; Berger *et al.*, 2012; Meyer and Purugganan, 2013).

Several studies in crops have identified DNA sequence differences associated with domestication (Hufford *et al.*, 2012; Meyer and Purugganan, 2013; Lin *et al.*, 2014; Zhou *et al.*, 2015), but how epigenetic variation could be involved in cultivation remains poorly understood (Piperno, 2017; Lu, Zhou and Zhao, 2020). Recent work has shown that heritable phenotypic diversity of crops can be epigenetically based and can respond to selection (Hauben *et al.*, 2009; Ji, Neumann and Schmitz, 2015; Gallusci *et al.*, 2017; Shen *et al.*, 2018). Studies comparing domesticated species to their wild progenitors have found that epigenetic processes are involved in the rewiring of the regulatory machinery that underlies gene expression differences between crops and their progenitors (Li *et al.*, 2012; Sauvage *et al.*, 2017; Xu *et al.*, 2020).

For example, Shen *et al.* (2018) found 5,412 differentially methylated regions associated with soybean domestication and improvement. While these studies relied on comparison of lineages that have diverged from their wild relatives, one study used AFLP in a non-model species to examine how molecular variation changed through the recent domestication of a wild plant, and identified novel patterns of epigenetic diversity that did not conform to patterns of genetic diversity in domesticated compared to wild populations of the same species (Chen *et al.*, 2020).

Similar in concept to studies of domestication, comparative studies of modern to ancient humans and non-human primates are beginning to reveal the potential importance of epigenetic mechanisms in human evolution. Notably, comparisons of DNA methylation across species but within the same tissue type reflect the primate phylogeny, while tissue specific differences are well conserved among these species (Mathov *et al.*, 2020). Human specific differences in DNA methylation appear to be enriched at genes associated with a range of diseases and neurological disorders (Gokhman *et al.*, 2019; Mathov *et al.*, 2020). While this work has been constrained by technological limitations and the degradation of ancient DNA, recent developments have provided exciting insights into

how epigenetic modifications are highly correlated to the evolution of anatomical adaptations in ancient and modern human lineages (Gokhman *et al.*, 2014, 2019; Mathov *et al.*, 2020).

On a more contemporary and rapid time scale, studies of human cancers have suggested that interactions between disruption of epigenetic regulation and genetic mutation can lead to rapid evolution of cancer cell populations by contributing to increased phenotypic variance that selection within the tumor environment can act on (Timp and Feinberg, 2013; Feinberg, Koldobskiy and Göndör, 2016). As was found in studies of *A. thaliana*, cancer studies have found that a wide variety of mutations in different components of the epigenetic machinery can have critical roles in cancer initiation (Feinberg, Koldobskiy and Göndör, 2016). Considering the complex interactions between genetic and epigenetic variation in cancer progression, disentangling genetic and epigenetic sources of variation continues to be a fundamental challenge.

Before the EES, several theoretical models already had been proposed to describe the evolutionary consequences of epigenetic variation in natural populations (Richards, Verhoeven and Bossdorf, 2012). These early models were limited by a lack of information on the behavior of epigenetic markers, but they showed how epigenetic mechanisms, which are more dynamic and reversible than DNA sequence, could add adaptive flexibility, contribute to buffering against environmental change, or preserve advantageous phenotypes in a canalization or genetic assimilation type process, and create the potential for novel evolutionary outcomes in the absence of genetic variation.

Conversely, quantitative genetic models did not explicitly incorporate epigenetic effects. Several authors suggested that epigenetic effects could be modeled as additional parameters in an analysis of variance-type approach, which typically models phenotypic variance due to genotype, environment, and genotype-by-environment interactions (e.g. Richards, Bossdorf and Pigliucci, 2010). Yet, we cautioned that adding epigenotype as a main effect in such models would also result in the need for more complex interactions like epigenotype-by-genotype and epigenotype-by-environment to be incorporated into the model, which would make experimental design largely intractable (Richards, Bossdorf and Pigliucci, 2010, Richards *et al.*, 2017).

In the last 10 years, several authors have made attempts to tackle this problem (Richards *et al.*, 2017; Bonduriansky and Day, 2018). In one case, Banta and Richards (2018) argue that instead of adding a separate main effect of ‘epigenotype’ and the interactions involved therein, epigenetic effects could be assumed to influence each parameter of the phenotypic variance formula:  $VP$  (total phenotypic variance) =  $VG$  (genetic variance) +  $VE$  (environmental variance) +  $VG \times E$  (genotype-by-environment interaction) +  $2COVGE$  (the genotype – environment covariance) +  $V\epsilon$  (residual

variance), requiring careful examination of each parameter to identify how they are contributing to phenotype. Regardless of the approach, isolating epigenetic from genetic effects and other forms of non-genetic effects will be an enduring challenge (Bonduriansky and Day, 2018).

#### 4. What technological developments are needed to make further progress?

In spite of the progress that has been made in investigating the molecular machinery involved in epigenetic processes, a priority for understanding epigenetic inheritance in the coming years must be to develop tools that enable higher resolution probing of genomes in more realistic scenarios, and in a wider diversity of systems (Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019). This continues to be a priority because one important lesson we have learned, thanks to detailed studies in *A. thaliana* and in human cancers, is that epigenetic variation can be dramatically shaped by genetic variants: we know that single nucleotide polymorphisms can have a remarkable impact on the methylome (Becker *et al.*, 2011; Timp and Feinberg, 2013; Dubin *et al.*, 2015; Feinberg, Koldobskiy and Göndör, 2016; Sasaki *et al.*, 2019). Even in clonal plants, where compelling arguments are made for the importance of epigenetic effects (Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015; Richards *et al.*, 2017), the low level of genetic variation that arises from somatic mutations in natural clonal lineages cannot be excluded since several studies have reported that high rates of somatic mutation may allow asexual species to maintain abundant genetic variation and adapt to changing environmental conditions (reviewed in Schoen and Schulz, 2019; see also discussions in Chen *et al.*, 2020; Robertson *et al.*, 2020).

However, all the progress made in *A. thaliana* notwithstanding, including the sequencing of over 1001 epigenomes at single base pair resolution, we still don't completely understand how the different characteristics of the genome function to create phenotype or respond to environment, and we are limited in our ability to capture the rare events that might shape evolutionary trajectories (Sasaki *et al.*, 2019).

The most recent annotation of the *A. thaliana* genome remains incomplete – reporting some type of information for 91.8% of the predicted protein coding genes, with many of the annotations being only hypothetical (Cheng *et al.*, 2017). Further, while *A. thaliana* has been an excellent model species for identifying molecular mechanisms that are relevant across all plant and non-plant taxa (Robertson *et al.*, 2015; Provart *et al.*, 2016), the genome is smaller than most plant genomes, has a high level of selfing and homozygosity, and has far fewer transposable elements, which likely influence the dynamics of epigenetic processes (reviewed in Parisod *et*

*al.*, 2010; Richards *et al.*, 2017). In addition, sequencing-based techniques provide the potential to identify functional genomic regions, but correct annotations rely on genomic resources in a closely related species.

In polyploid species, the number of duplicated genes and the potential for neo-functionalization among them creates additional uncertainty for annotations (Primmer *et al.*, 2013). For example, a large body of work in *Spartina alterniflora* shows that gene retention, small RNA variation, and expression of different copies of genes vary across the genome (Ainouche *et al.*, 2009; Ferreira de Carvalho, 2013, 2017; Boutte *et al.*, 2016; Cavé-Radet *et al.*, 2019), which could partly be due to gene diversification and sub-functionalization (Salmon and Ainouche, 2015; Shimizu-Inatsugi *et al.*, 2017). In addition to the impact of polyploidy and gene duplication, the role of epigenetic mechanisms more generally has already been shown to vary across taxa, and with different genomic features (Ainouche *et al.*, 2009; Niederhuth *et al.*, 2016; Schmitz, Lewis and Goll, 2019; de Mendoza, Lister and Bogdanovic, 2020). This mirrors the findings that selective advantages of phenotypic plasticity and transgenerational effects differ among species, depending on their ecology and life history characteristics (Herman and Sultan, 2011; Herman *et al.*, 2014; Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015; Sultan, 2015).

Despite the lack of complete genome information, marker-based approaches and RRBS methods have successfully detected DNA methylation variation and how it is correlated to evolutionary processes in several species. Combining these methods with appropriate experiments will continue to identify DNA methylation variants that impact performance and associate with heritable phenotypes, although there may always be undetected genetic variants that control observed DNA methylation patterns.

That said, for large plant genomes in particular, WGBS and RRBS approaches still face significant challenges of correctly identifying duplication, heterozygosity, ploidy and repetitive sequences (Salmon and Ainouche, 2015; Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019). Sequencing approaches generally will require more sequencing depth to identify all copies of polyploid loci, particularly for those with high degrees of heterozygosity (Salmon and Ainouche, 2015). Polyploid organisms will typically have multiple copies of each locus, and it is difficult to differentiate polymorphisms that lie among different copies at a given locus from polymorphisms that define different locations within the genome, especially when based on short reads (100's of basepairs) like Illumina data (Salmon and Ainouche, 2015; Paun, Verhoeven and Richards, 2019).

Furthermore, while we know that methylation of the promoter region is highly correlated with gene silencing, without a well-annotated reference genome of the target species or a close relative, it is virtually impossible to identify those RRBS fragments that overlap promoter regions (Alvarez *et al.*,

2020; Robertson *et al.*, 2020). Mapping the genomic landscape at this level of detail is still limited to very few species, but will be critical for dissecting the relative contributions of sequence level and methylation variation to evolutionary change. Combining methylation surveys with surveys of gene expression and phenotypes, and generating a draft reference genome will be important for associating DNA methylation with gene function (Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019).

While several authors have reported an eclipse of the use of the anonymous methylation sensitive AFLP markers in favor of sequencing approaches (Schrey *et al.*, 2013; Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019), it is unclear whether this is in fact the case. So far, only one study compared findings from RRBS to those of MS-AFLP on the same samples (Alvarez *et al.*, 2020), with the expectation that the substantial increase in markers (92,999 compared to 39 polymorphic methylation loci, respectively) would lend greater resolution to detect patterns of DNA methylation variation that were not found in AFLP. The epiGBS survey detected significant differentiation in both genetic variation and DNA methylation, but the study found only 240 differentially methylated positions, which did not overlap with differentially expressed genes. This study shows how the RRBS approach suffers from surveying only a small fraction of the genome, and without a reference genome, researchers cannot assess if fragments overlap with promoter regions (see also van Moorsel *et al.*, 2019; Robertson *et al.*, 2020). Therefore, while studies with RRBS techniques offer increased power to detect broad, genome-wide patterns of variation that may be correlated to ecology, they are still limited in terms of detecting specific gene functions. Continued reduction in sequencing cost and advances in long-read sequencing methods will make it possible for more and more transcriptomes and genomes to become available, and we can look forward to a more sophisticated understanding of epigenomics in non-model systems.

Aside from the tremendous effort already invested and further work needed to understand the dynamics of DNA methylation in natural populations, evidence suggests that maintenance of epigenetic information is dependent on interactions among epigenetic mechanisms, but the details of how this works are currently unknown (Bošković and Rando, 2018). Much more investigation is required to document the dynamics of small RNA and chromatin structure in natural populations, as well as the causes and consequences of these types of variation. A full rendering of inheritance of epigenetic mechanisms and how they interact is not yet within reach for any species. It will be even more difficult to work out how these mechanisms coordinate with genetic and other non-genetic mechanisms and truly understand how the genome translates into phenotypes (Keller, 2014; Bonduriansky and Day, 2018).

In addition to sequencing and other molecular level efforts, researchers in epigenetic inheritance need to develop appropriate data analysis methods. Studies designed to understand epigenetic inheritance require complex ecological experimental designs that include multiple generations and random effects like population and genotype. In addition, RRBS and WGBS data are complex and conceptually different from data generated through more established approaches based on genetic markers, microarrays or RNAseq. One telling example is that single nucleotide methylation polymorphisms have turned out to be less likely to be linked to function than regions of the genome that are differentially methylated (DMRs). Defining the best way to characterize DMRs has not been straightforward, and addressing the complexity of epigenetic data generally is an ongoing challenge (Richards *et al.*, 2017; Paun, Verhoeven and Richards, 2019; Sasaki *et al.*, 2019).

Perhaps as critical as genomics resource development, many of the arguments we made before the publication of the EES book about the importance of design still apply to future studies (Richards, Bossdorf and Pigliucci, 2010; Richards, Bossdorf and Verhoeven, 2010). We have emphasized that DNA methylation can mediate plasticity by e.g. modulating expression, but phenotypic plasticity and epigenetic response are not interchangeable terms: there are plastic responses that are not epigenetic (including provisioning and biochemical function: Herman and Sultan, 2011; Sultan, 2015; Banta and Richards, 2018).

Many studies have found that the capacity to adjust epigenetic modifications, and the potential for inheritance of these modifications might have different adaptive benefits depending on genotypes (e.g. Alvarez, Bleich and Donohue, 2020). In this respect, the capacity for methylation is partly a property of the genotype, and genotype-specificity in epigenetic or transgenerational effects may be common (Herman and Sultan, 2016; Alvarez, Bleich and Donohue, 2020). Phenotypic plasticity is heritable and a genotype specific property, but critically distinct from the concept of the inheritance of an induced response (so called transgenerational plasticity). Multivariate statistics can explore how much of the overall epigenetic variation can be predicted from genetic relatedness, and identify differences in genetic and epigenetic patterns. However, more detailed experiments are required to identify the independence, importance and inheritance of the observed epigenetic effects. Follow-up experiments can take advantage of targeted bisulfite sequencing and expression of candidate loci across genetic backgrounds, knockouts or transgenic organisms, which will be useful for validation of findings based on correlation. Importantly, many studies have found that some expression patterns are only exposed under complex natural stimuli, which is likely to hold for epigenetic mechanisms as well (Alvarez, Schrey and Richards, 2015; Richards *et al.*, 2017).

## 5. Epigenetic inheritance, the EES, and what the future looks like

We are now in a much better position to assess the second question we posed at the beginning: what, in light of the current state of the art in the field, may the role of epigenetic inheritance be within the Extended Evolutionary Synthesis? When the volume on the EES edited by Pigliucci and Müller came out in 2010 the Extended Synthesis was in a relatively early stage of its formulation – though there had been movement in that direction for a while even by then (e.g. Schlichting and Pigliucci, 1998). Two of the contributors to the 2010 volume were Eva Jablonka and Marion Lamb, who put forth the case that epigenetic inheritance was a major pillar of the EES.

A more recent comprehensive attempt at articulating the EES is the one published by Laland *et al.* (2015). In that paper, the authors present a number of one-on-one comparisons between the MS and the EES, particularly in terms of the respective assumptions (Table 1 in Laland *et al.*, 2015), alternative interpretations of core concepts (such as developmental bias, phenotypic plasticity, inheritance, and niche construction; Table 2), views of development (Figure 1), and empirical predictions (Table 3). They also provide a detailed graphic (Figure 2) of the conceptual structure of the EES. The paper – of which Jablonka was a co-author – thus affords us a highly structured point of reference for the question at hand.

Let us start with the core assumptions. In terms of inheritance, for the MS “genes constitute the only general inheritance system”. By contrast, for the EES “inheritance extends beyond genes to encompass transgenerational epigenetic inheritance, physiological inheritance, ecological inheritance, social (behavioural) transmission and cultural inheritance”. Notice that epigenetic inheritance is only one of a number of channels of extended inheritance contemplated within the EES framework. In the text, Laland *et al.* are more explicit:

Parent-offspring similarity occurs not only because of transmission of DNA, but because parents transfer a variety of developmental resources that enable reconstruction of developmental niches. These include components of the egg and post-fertilization resources, behavioural interactions between parents and offspring, parental modification of other components of the biotic and abiotic environment, and inheritance of symbionts directly through the mother’s germ cells or by infection. In addition, recent research reveals that vertical and horizontal social transmission is widespread in both vertebrates and invertebrates, and can both initiate population divergence and trigger speciation. [...] There is also increasing evidence for more stable transgenerational epigenetic inheritance, or the transmission across generations of cellular states without modification of the DNA sequence, which demonstrates that adaptive evolution may proceed by selection on epigenetic variants as well as variation in DNA sequence (Laland *et al.*, 2015, p. 1022).



Again, then, epigenetic inheritance is certainly mentioned, but as one of a number of components of the EES when it comes to the issue of inheritance, which is itself one of a number of areas in which the EES differs from the MS (the other ones being the role of natural selection in evolution, the origin of genetic variation, whether evolution is gradual or not, gene-vs-organism-centred views, and the contrast between micro- and macro-evolution).

Contra the MS, the EES treats heredity as an inclusive category of disparate phenomena, where cultural inheritance, maternal effects, and so forth, are not considered special, exceptional, or minor, but as constitutive of a panoply of inheritance mechanisms exerting evolutionary effects. Crucially, phenotypes are not inherited, but rather developmentally constructed by way of a number of resources, including, but certainly not limited to, epigenetic variants. Importantly, non-genetic inheritance (again, a broader category than epigenetic as discussed in this paper) is thought to facilitate the origin and spread of phenotypic novelties, an old stumbling block for the MS and its restrictive framework based on population genetics theory.

All in all, contrasting the 2010 book, the *Laland et al.*'s paper, and the state of the field as we have assessed it in the current paper, we conclude that epigenetic inheritance is here to stay as one type of non-genetic inheritance and a component of the Extended Evolutionary Synthesis, but also that its role in isolation from other non-genetic forms of inheritance may not be as pivotal as was initially suggested. Of course, ultimately, this is a matter for further empirical research to settle.

In particular, in natural populations and through ecological experiments, we have a respectable level of information about one epigenetic mechanism, namely DNA methylation. However, most of our knowledge about this mechanism is fairly coarse grained and lacking critical fine-scale genomic context. In addition, better understanding of the other molecular epigenetic mechanisms, like the action of small RNAs, chromatin modifications, and cellular location is required to truly flesh out how epigenetic mechanisms interact with each other to contribute to heredity along with genetic and other non-genetic mechanisms that ultimately translate into organismal performance. A better understanding of these issues will not come just from studying a few model organisms in the lab, but will further require difficult experiments on multiple organisms in real complex environmental settings. Even with more resources, the chance of catching the molecular level events that can shape evolutionary trajectories will require much patience and fortitude.

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