Spring 2023 – Epigenetics and Systems Biology
Discussion Session (Evolutionary Biology)
Michael K. Skinner – Biol 476/576
Week 15 (April 20)

Epigenetics and Evolutionary Biology

Primary Papers

1. Skinner, et al. (2014) Genome Biology and Evolution 6:1972-1989. (PMID: 25062919)
2. Anastasiadi D, et al. (2021) Trends Ecol Evol. 36(12):1124-1140. (PMID: 34489118)
3. Sadler KC. (2022) Bioessays. 20:e2200036. (PMID: 36403219)

Discussion

Student 37 – Ref #1 above

- What was the model system and experimental design?
- What epigenetic observations were provided and how might environmental epigenetics impact evolution?
- Is this a Lamarckian contribution to evolution?

Student 38 – Ref #2 above

- What was the role of epigenetics in phenotypic variation?
- What epigenetic differences were observed between the species?
- What is the integration of genetics, epigenetics and evolution suggested?

Student 1 – Ref #3 above

- What are the model systems and experimental data considered?
- What phylogeny associations were observed?
- How could epigenetics be involved in the potential adaptive response?

Epigenetics and the Evolution of Darwin's Finches

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Data deposition: All DMR and CNV genomic data obtained in this study have been deposited in the NCBI public GEO database under the accession (GEO #: GSE58334).

Abstract

The prevailing theory for the molecular basis of evolution involves genetic mutations that ultimately generate the heritable phenotypic variation on which natural selection acts. However, epigenetic transgenerational inheritance of phenotypic variation may also play an important role in evolutionary change. A growing number of studies have demonstrated the presence of epigenetic inheritance in a variety of different organisms that can persist for hundreds of generations. The possibility that epigenetic changes can accumulate over longer periods of evolutionary time has seldom been tested empirically. This study was designed to compare epigenetic changes among several closely related species of Darwin's finches, a well-known example of adaptive radiation. Erythrocyte DNA was obtained from five species of sympatric Darwin's finches that vary in phylogenetic relatedness. Genome-wide alterations in genetic mutations using copy number variation (CNV) were compared with epigenetic alterations associated with differential DNA methylation regions (epimutations). Epimutations were more common than genetic CNV mutations among the five species; furthermore, the number of epimutations increased monotonically with phylogenetic distance. Interestingly, the number of genetic CNV mutations did not consistently increase with phylogenetic distance. The number, chromosomal locations, regional clustering, and lack of overlap of epimutations and genetic mutations suggest that epigenetic changes are distinct and that they correlate with the evolutionary history of Darwin's finches. The potential functional significance of the epimutations was explored by comparing their locations on the genome to the location of evolutionarily important genes and cellular pathways in birds. Specific epimutations were associated with genes related to the bone morphogenic protein, toll receptor, and melanogenesis signaling pathways. Species-specific epimutations were significantly overrepresented in these pathways. As environmental factors are known to result in heritable changes in the epigenome, it is possible that epigenetic changes contribute to the molecular basis of the evolution of Darwin's finches.

Key words: epimutations, DNA methylation, copy number variation, phylogeny, adaptive radiation, BMP, toll, melanogenesis.

Introduction

Epigenetic change has been postulated to play a role in the ecology and evolution of natural populations (Richards et al. 2010; Holeski et al. 2012; Liebl et al. 2013). Epigenetic changes are broadly defined as "molecular processes around DNA that regulate genome activity independent of DNA sequence and are mitotically stable" (Skinner et al. 2010). Some epigenetic processes are also meiotically stable and are transmitted through the germline (Anway et al. 2005; Jirtle and Skinner 2007). These epigenetic mechanisms, such as DNA methylation, can become programmed (e.g., imprinted) and inherited over generations with potential evolutionary impacts. Environmental factors have been shown to promote the epigenetic transgenerational inheritance of phenotypic variants (Skinner et al. 2010). In recent years, the importance of environmental cues in the induction of such variation has been widely acknowledged (Bonduriansky 2012). Thus, like genetic change (Greenspan 2009), epigenetic change may also play an important role in evolution (Guerrero-Bosagna et al. 2005; Day and Bonduriansky 2011; Geoghegan and Spencer 2012, 2013a, 2013b, 2013c; Klironomos et al. 2013).

In order for inherited epigenetic changes to play a significant role in microevolution, they must persist for tens of generations, or longer (Slatkin 2009). It is conceivable that epigenetic changes may also accumulate over longer periods of evolutionary time, contributing to processes such as adaptive radiation (Rebollo et al. 2010; Flatscher et al. 2012). This hypothesis assumes that epigenetic changes persist over thousands of generations. An initial step in testing this hypothesis would be to compare epigenetic differences among closely related species, and whether such changes accumulate over short spans of macroevolutionary time. For example, do epigenetic changes accumulate with phylogenetic distance? Addressing this question was the primary goal of this study.

The study was designed to explore the relationship between epigenetic changes and the evolutionary history of several species of Darwin's finches in the Galapagos Islands. This group of birds has been central to work on a variety of important topics in evolutionary biology, including adaptive radiation, character displacement, rapid evolution, hybridization between species, evolutionary developmental mechanisms, and the effect of invasive pathogens and parasites (Grant and Grant 2008; Huber et al. 2010; Donohue 2011). The adaptive radiation of Darwin's finches over a period of 2–3 Myr resulted in 14 extant species that fill distinct ecological niches. These species show striking variation in body size and the size and shape of their beaks (Grant and Grant 2008). Darwin's finches were selected for study because they are a well-studied example of the evolution of closely related species into different ecological niches (Grant and Grant 2008; Donohue 2011).

Natural selection is a process in which environmental factors influence the survival and reproductive success of individuals bearing different phenotypes. Only selection on phenotypic traits with a heritable basis can lead to evolutionary change (Endler 1986). Observations indicate that epigenetic mechanisms have a role in influencing genomic variability (Huttley 2004; Ying and Huttley 2011). As epigenetic changes are also influenced by environmental factors, and can be heritable across generations (Skinner et al. 2010), they provide another molecular mechanism that can influence evolutionary change. Although Lamarck (1802) proposed that environmental factors can influence inheritance directly, his mechanism has not been widely recognized as a component of modern evolutionary theory (Day and Bonduriansky 2011). Recent work in epigenetics shows that epigenetic changes can, in fact, increase the heritable phenotypic variation available to natural selection (Holeski et al. 2012; Liebl et al. 2013). Thus, epigenetics appears to provide a molecular mechanism that can increase phenotypic variation on which selection acts (Skinner 2011). The integration of genetic and epigenetic mechanisms has the potential to significantly expand our understanding of the origins of phenotypic variation and how environment can influence evolution.

For example, Crews et al. (2007) investigated the ability of an environmental factor (toxicant) to promote the epigenetic transgenerational inheritance of alterations in the mate preferences of rats, with consequences for sexual selection. An F0 generation gestating female rat was exposed to the agricultural fungicide vinclozolin transiently. A dramatic alteration in the mate preferences of the F3 generation was observed (Crews et al. 2007) along with epigenetic alterations (termed epimutations) in the germline (sperm) (Guerrero-Bosagna et al. 2010). Transgenerational transcriptome changes in brain regions correlated with these alterations in mate preference behavior were also observed (Skinner et al. 2008, 2014). Thus, an environmental factor that altered mate preference was found to promote a transgenerational alteration in the sperm epigenome in an imprinted-like manner that was inherited for multiple generations (Crews et al. 2007; Skinner et al. 2010). Studies such as these suggest that environmental epigenetics may play a role in evolutionary changes through processes, such as sexual selection.

Recent reviews suggest a pervasive role for epigenetics in evolution (Rebollo et al. 2010; Day and Bonduriansky 2011; Kuzawa and Thayer 2011; Flatscher et al. 2012; Klironomos et al. 2013). The primary goal of this study was to test whether epigenetic changes accumulate over the long periods of evolutionary time required for speciation with adaptive radiation. Genome wide analyses were used to investigate changes in genetic and epigenetic variation among five species of Darwin's finches. The measure of genetic variation was copy number variation (CNV), which has been shown to provide useful and stable genetic markers with potentially more phenotypic functional links than point mutations such as single nucleotide polymorphisms (SNPs) (Lupski 2007; Sudmant et al. 2013). CNVs involve an increase or decrease in the number of copies of a repeat element at a specific genomic location. Recently, CNV changes in primates and other species have been shown to be very useful genetic measures for comparing evolutionary events (Nozawa et al. 2007; Gazave et al. 2011; Poptsova et al. 2013). CNV changes are involved in gene duplication and deletion phenomena, as well as repeat element phenomenon such as translocation events and can be influenced by DNA methylation (Skinner et al. 2010; Macia et al. 2011; Tang et al. 2012). The measure of epigenetic variation used was differential DNA methylation sites, which are known to be stable and heritable (Skinner et al. 2010). Comparing data for both genetic mutations (i.e., CNV) and epimutations (i.e., DNA methylation) allowed the relative magnitudes of these sources of variation to be compared across the five species included in the study.

Materials and Methods

Finch Field Work and Collection of Blood

Blood samples were collected from birds captured January–April 2009 at El Garrapatero, a lowland arid site on Santa Cruz Island, Galapagos Archipelago, Ecuador (Koop et al. 2011).

Birds were captured with mist nests and banded with numbered Monel bands to track recaptures. Birds were identified, aged, and sexed using size and plumage characteristics. A small blood sample (90 μl) from each bird was collected in a microcapillary tube through brachial venipuncture. Samples were stored on wet ice in the field, then erythrocytes purified by centrifugation and cells stored in a −20 °C freezer at a field station. Following the field season, samples were placed in a −80 °C freezer for longer term storage. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #07-08004) and by the Galápagos National Park (PC-04-10: #0054411).

DNA Processing

Erythrocyte DNA was isolated with DNAeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and then stored at −80 °C prior to analysis. DNA was sonicated following a previously described protocol (without protease inhibitors) (Tateno et al. 2000) and then purified using a series of washes and centrifugations (Ward et al. 1999) from variable number of animals per species analyzed. The same concentrations of DNA from individual blood samples were then used to produce pools of DNA material. Two DNA pools were produced in total per species, each one containing the same amount of DNA from different animals. The number of individuals used per pool is shown in supplementary table S6, Supplementary Material online. These DNA pools were then used for chromosomal genomic hybridization (CGH) arrays or chromatin immunoprecipitation of methylated DNA fragments (MeDIP).

CNV Analysis

The array used for the CNV analysis was a CGH custom design by Roche Nimblegen that consisted of a whole-genome tiling array of zebra finch (*Taeniopygia guttata*) with 720,000 probes per array. The probe size ranged from 50 to 75 mer in length with median probe spacing of 1,395 bp. Two different comparative (CNV vs. CNV) hybridization experiments were performed (two subarrays) for each species in query (*Geospiza fuliginosa* [FUL], *G. scandens* [SCA], *Camarhynchus parvulus* [PAR], and *Platyspiza crassirostris* [CRA]) versus control *G. fortis* (FOR), with each subarray including hybridizations from DNA pools from these different species. Two DNA pools were built for each species (supplementary table S6, Supplementary Material online). For one subarray of each species, DNA samples from the experimental groups were labeled with Cy5 and DNA samples from the control lineage were labeled with Cy3. For the other subarray of each species, a dye swap was performed so that DNA samples from the experimental groups were labeled with Cy3 and DNA samples from the control lineage were labeled with Cy5.

For the CNV experiment raw data from the Cy3 and Cy5 channels were imported into R (R Development Core Team 2010), checked for quality, and converted to *MA* values ($M = \text{Cy5} - \text{Cy3}$; $A = [\text{Cy5} + \text{Cy3}]/2$). Within array and between array normalizations were performed as previously described (Manikkam et al. 2012). Following normalization, the average value of each probe was calculated and three different CNV algorithms were used on each of these probes including circular binary segmentation from the DNA copy (Olshen et al. 2004), CGHseg (Picard et al. 2005) and cghFlasso (Tibshirani and Wang 2008). These three algorithms were used with the default parameters. The average values from the output of these algorithms were obtained. A threshold of 0.04 as a cutoff was used on the summary (average of the log-ratio from the three algorithms) where gains are probes above the positive threshold and losses are probes below the negative threshold. Consecutive probes (≥ 3) of gains and losses were used to identify separate CNV regions. A cutoff of three-probe minimum was used and those regions were considered a valid CNV. The statistically significant CNVs were identified and *P* values associated with each region presented. A cutoff of $P < 10^{-5}$ was used to select the final regions of gains and losses.

Differential DNA Methylation Regions Analysis

MeDIP was performed as previously described (Guerrero-Bosagna et al. 2010) as follows: 6 μg of genomic DNA was subjected to series of three 20-pulse sonications at 20% amplitude and the appropriate fragment size (200–1,000 ng) was verified through 2% agarose gels; the sonicated genomic DNA was resuspended in 350 μl TE buffer and denatured for 10 min at 95 °C and then immediately placed on ice for 5 min; 100 μl of 5× IP buffer (50 mM Na-phosphate pH 7, 700 mM NaCl (PBS), 0.25% Triton X-100) was added to the sonicated and denatured DNA. An overnight incubation of the DNA was performed with 5 μg of antibody anti-5-methylCytidine monoclonal from Diagenode (Denville, NJ) at 4 °C on a rotating platform. Protein A/G beads from Santa Cruz were prewashed on PBS–BSA (bovine serum albumin) 0.1% and resuspended in 40 μl 1× IP (immunoprecipitation) buffer. Beads were then added to the DNA-antibody complex and incubated 2 h at 4 °C on a rotating platform. Beads bound to DNA-antibody complex were washed three times with 1 ml 1× IP buffer; washes included incubation for 5 min at 4 °C on a rotating platform and then centrifugation at 6,000 rpm for 2 min. Beads DNA-antibody complex were then resuspended in 250 μl digestion buffer (50 mM Tris–HCl pH 8, 10 mM ethylenediaminetetraacetic acid, 0.5% SDS (sodium dodecyl sulfate) and 3.5 μl of proteinase K (20 mg/ml) was added to each sample and then incubated overnight at 55 °C on a rotating platform. DNA purification was performed first with phenol and then with chloroform:isoamyl alcohol. Two washes were then performed with 70% ethanol, 1 M NaCl, and glycogen. MeDIP-selected DNA was then resuspended in 30 μl TE buffer.

The array used for the differential methylation analysis was a DNA-methylated custom array by Roche Nimblegen that

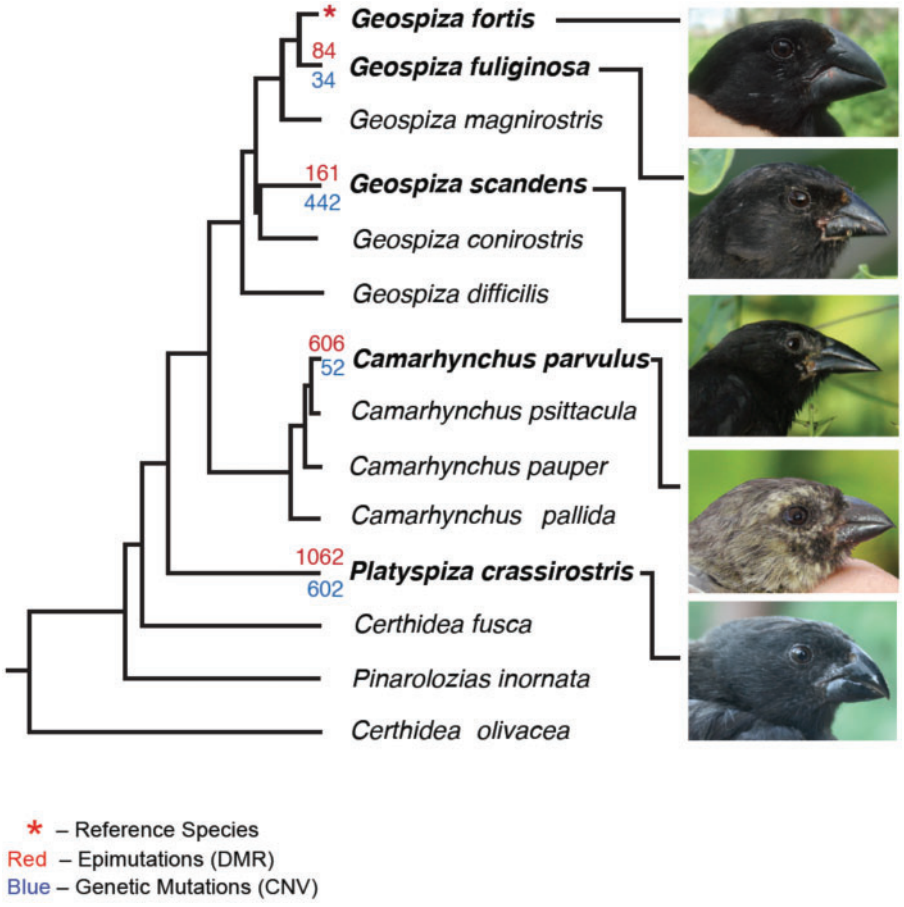


Fig. 1.—Number of epimutations and genetic mutations in relation to the phylogenetic relationships of five species of Darwin's finches. Photographs (by J.A.H.K. or S.A.K.) show variation in bill size and shape. Numbers on branches are the number of differences (three or more probes; table 1) in epimutations (DMR; in red) .and genetic mutations (CNV; in blue) for each of four species, compared with a single reference species FOR (asterisk). The phylogram is based on allele length variation at 16 polymorphic microsatellite loci (from Petren et al. 1999). The topology of the tree is similar to that proposed by Lack (1947) on the basis of morphological traits.

consisted of a whole-genome tiling array of zebra finch (*Taeniopygia guttata*) made of four 2.1M and one 3x720k array with 8,539,570 probes per array. Probe sizes were 50–75 mer in length and median probe spacing was 200 bp. Two different comparative (MeDIP vs. MeDIP) hybridization experiments were performed (two subarrays) for each experimental species (FUL, SCA, PAR, CRA) versus control FOR, with each subarray including hybridizations from MeDIP DNA from DNA pools from these different species (supplementary table S6, Supplementary Material online). For one subarray of each species, MeDIP DNA samples from the experimental groups were labeled with Cy5 and MeDIP DNA samples from the control lineage were labeled with Cy3. For the other subarray of each species, a dye swap was performed so that MeDIP DNA samples from the experimental groups were labeled with Cy3 and MeDIP DNA samples from the control lineage were labeled with Cy5.

For each comparative hybridization experiment, raw data from both the Cy3 and Cy5 channels were imported into R, checked for quality, and converted into *MA* values. The normalization procedure is as previously described (Guerrero-Bosagna et al. 2010). Following normalization each adjacent $\geq$3 probe set value represents the median intensity difference between FUL, SCA, PAR and CRA and control FOR of a 600-bp window. Significance was assigned to probe differences between experimental species samples and reference FOR samples by calculating the median value of the intensity differences as compared with a normal distribution scaled to the experimental mean and standard deviation of the normalized data. A *Z* score and *P* value were computed for each probe from that distribution. The statistically significant differential DNA methylation regions (DMR) were identified and *P* values associated with each region represented, as previously described (Guerrero-Bosagna et al. 2010).

A

Differential DNA Methylation Regions (DMR) (Epimutations)							
All probes ($p<10^{-5}$)				3 or more probes ($p<10^{-5}$)			
	(Up)	(Down)	Total		(Up)	(Down)	Total
FUL	116	398	514	FUL	76	8	84
SCA	211	679	890	SCA	17	144	161
PAR	191	1438	1629	PAR	28	578	606
CRA	361	2406	2767	CRA	61	1001	1062
Total	**Up**	**Down**	**Total Sites**	**Total**	**Up**	**Down**	**Total Sites**
	879	4921	5800		182	1731	1913

Copy Number Variation (CNV)							
All probes ($p<10^{-5}$)				3 or more probes ($p<10^{-5}$)			
	Gains	Loss	Total		Gains	Loss	Total
FUL	59	12	71	FUL	28	6	34
SCA	567	22	589	SCA	440	2	442
PAR	78	217	295	PAR	15	37	52
CRA	621	194	815	CRA	541	61	602
Total	**Gains**	**Loss**	**Total Sites**	**Total**	**Gains**	**Loss**	**Total Sites**
	1325	445	1770		1024	106	1130

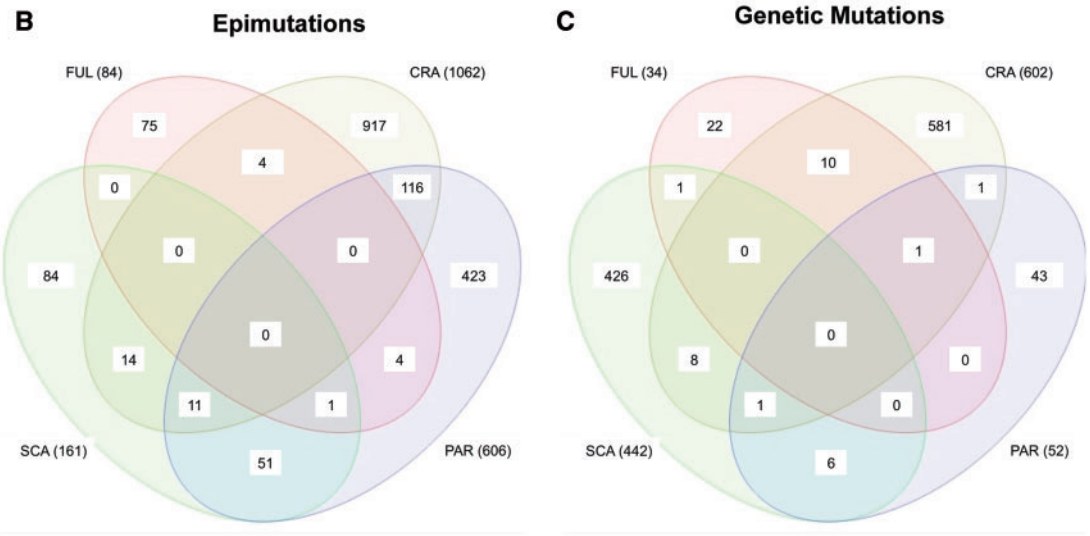


Fig. 2.—Number of epimutations and genetic mutations associated with Darwin's finches. The number of differential DMR epimutations and CNV genetic mutations (*A*). DMR and CNV that differ significantly ($P < 10^{-5}$) from the reference species (FOR) are presented for all oligonucleotide probes, compared with peaks of three or more adjacent probes. The epimutations with an increase (Up) or decrease (Down) in DNA methylation are indicated. Those genetic mutations with an increase (Gain) or decrease (Loss) in CNV are indicated. Venn diagrams for epimutations (*B*) and genetic mutations (*C*) show overlaps between epimutations (DMR) and genetic mutations (CNV) among species. The species and total number of sites compared are listed on the outside of each colored elliptical.

Additional Bioinformatics and Statistics

The July 2008 assembly of the zebra finch genome (taeGut1, WUSTL v3.2.4) produced by the Genome Sequencing Center at the Washington University in St Louis (WUSTL) School of Medicine was retrieved (WUSTL 2008). A seed file was constructed and a BSgenome package was forged for using the Finch DNA sequence in the R code (Herve Pages BSgenome: Infrastructure for Biostrings-based genome data packages. R

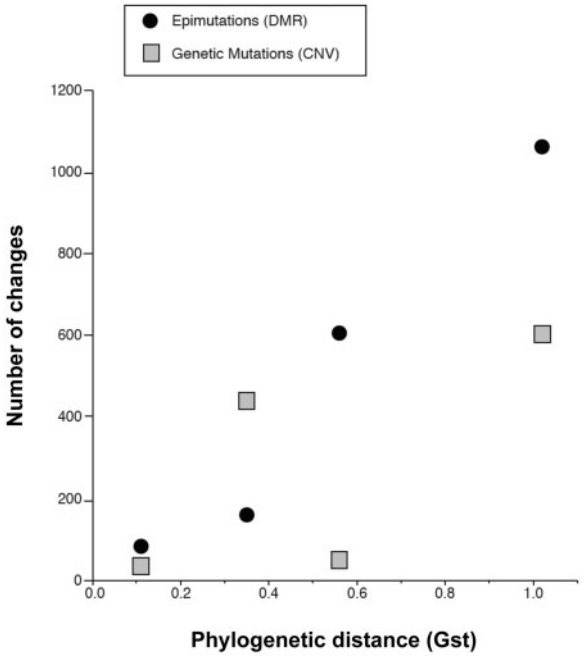


Fig. 3.—Phylogenetic distance is correlated with epigenetic changes, but not genetic changes. Branch lengths in figure 1 were used as measures of phylogenetic distance. The number of epimutations increased with phylogenetic distance (Spearman Rho = 1.0, $P < 0.0001$). In contrast, the number of genetic mutations did not increase with phylogenetic distance (Spearman Rho = 0.8, $P = 0.2$).

package version 1.24.0). This sequence was used to design the custom tiling arrays and to perform the bioinformatics.

The chromosomal location of CNV and DMR clusters used an R-code developed to find chromosomal locations of clusters (Skinner et al. 2012). A 2-Mb sliding window with 50,000 base intervals was used to find the associated CNV and DMR in each window. A *Z*-test statistical analysis with $P < 0.05$ was used on these windows to find the ones with overrepresented CNV and DMR were merged together to form clusters. A typical cluster region averaged approximately 3 Mb in size.

The DMR and CNV association with specific zebra finch genes and genome locations used the Gene NCBI database for zebra finch gene locations and correlated the epimutations associated (overlapped) with the genes. The three adjacent probes constituted approximately a 200-bp homology search. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway associations were identified as previously described (Skinner et al. 2012). Statistically significant overrepresentation uses a Fisher's exact analysis.

Spearman Rank correlation coefficients were used to test for a relationship between phylogenetic distance and epigenetic and genetic changes (Whitlock and Schluter 2009).

Results

Phylogenetic relationships of the five finch species in this study are shown in figure 1. The taxa chosen for this study included: Two species of ground finches, FOR and FUL, which have crushing beaks with relatively deep bases; the cactus finch SCA, which has a long thin beak used for probing flowers; the small tree finch PAR, which has curved mandibles used for applying force at the tips; and the vegetarian finch CRA, which has a relatively short stubby bill used for crushing food along its entire length (Grant and Grant 2008; Donohue 2011; Rands et al. 2013). FOR was selected as a reference species for comparing genetic and epigenetic alterations among the remaining four species. Branch lengths in figure 1 were used as measures of phylogenetic distance.

The experimental design used purified erythrocytes from the different species. Although DNA sequences are the same for all cell types of an organism, the epigenome is distinct for each cell type, providing a molecular mechanism for the genome activity and functions that differ among different cell types (Skinner et al. 2010). Therefore, to investigate the overall epigenome requires a purified cell type. As birds have erythrocytes (red blood cells) that contain nuclei, samples of purified erythrocytes were collected from each of the Darwin's finch species to obtain DNA for molecular analysis.

The epigenetic alterations termed epimutations were assessed through the identification of differential DMR. The DMR were identified with the use of MeDIP with a methyl cytosine antibody, followed by a genome wide tiling array (Chip) for an MeDIP-Chip protocol (Guerrero-Bosagna et al. 2010). Although other epigenetic processes such as histone modifications, chromatin structure, and noncoding RNA are also important, DNA methylation is the best known epigenetic process associated with germline-mediated heritability and environmental manipulations (Skinner et al. 2010). Genetic variation was assessed using CNVs (i.e., amplifications and deletions of repeat elements) in the DNA using a CGH protocol (Pinkel and Albertson 2005; Gazave et al. 2011).

The reference genome used for the analysis was that of the zebra finch (*Taeniopygia guttata*) (Clayton et al. 2009), which had a preliminary estimate of greater than 83% similarity with a partial shotgun sequence of a Darwin's finch genome (Rands et al. 2013). This study actually suggests a much higher degree of identity. The zebra finch genome was tiled in a genome wide array with a 200-bp resolution and for a CGH array with a 1,500-bp resolution. These arrays were used in a competitive hybridization protocol between FOR (reference species) and the other four species (Guerrero-Bosagna et al. 2010). Differential hybridization using two different fluorescent DNA labeling tags identified the CNV with CGH using genomic DNA and the epimutation DMR with a MeDIP-Chip protocol. A statistical significance threshold of $P < 10^{-5}$ was set for the CNV or epimutation to be identified as a gain or loss compared with the reference species (fig. 2 and supplementary tables S1 and S2, Supplementary Material online). The data for all probes (oligonucleotides on the arrays) are presented. However, the criteria used to identify the CNV and DMR required the involvement of three or more adjacent

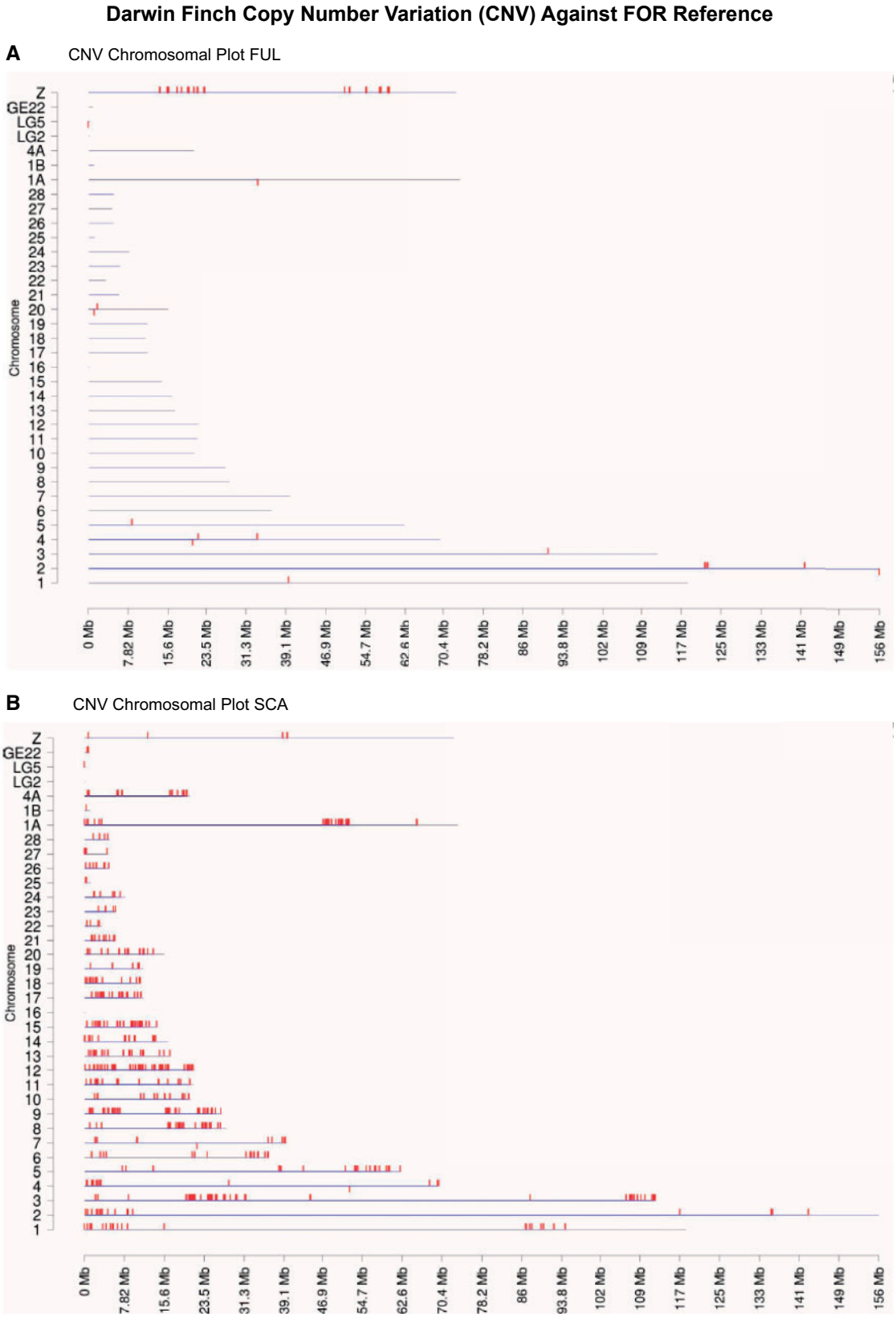


Fig. 4.—Chromosomal locations of the CNVs for each species. The chromosome number and size are presented in reference to the zebra finch genome. The chromosomal location of each CNV is marked with a red tick for FUL (*A*), SCA (*B*), PAR (*C*), and CRA (*D*).

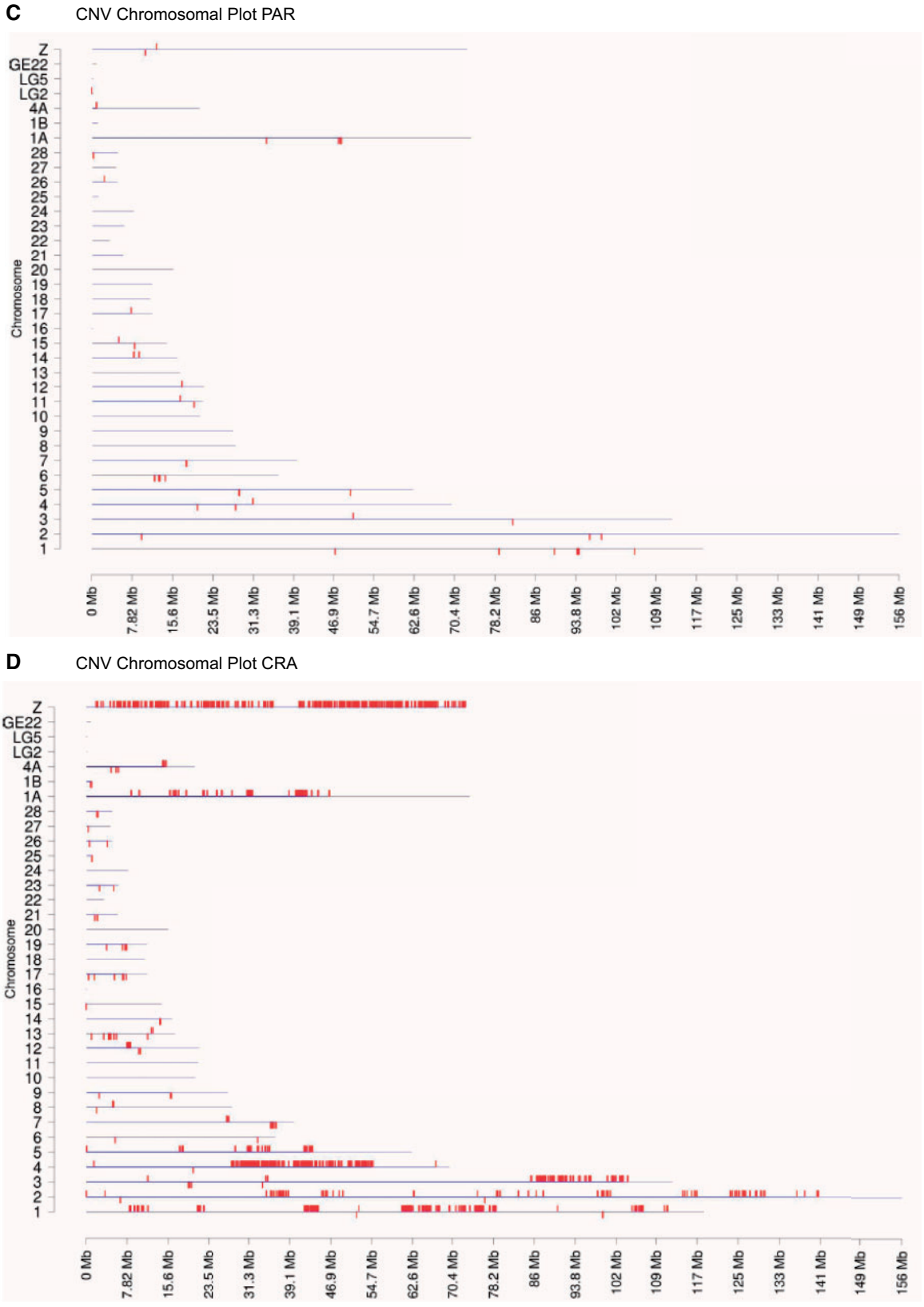


Fig. 4.—Continued.

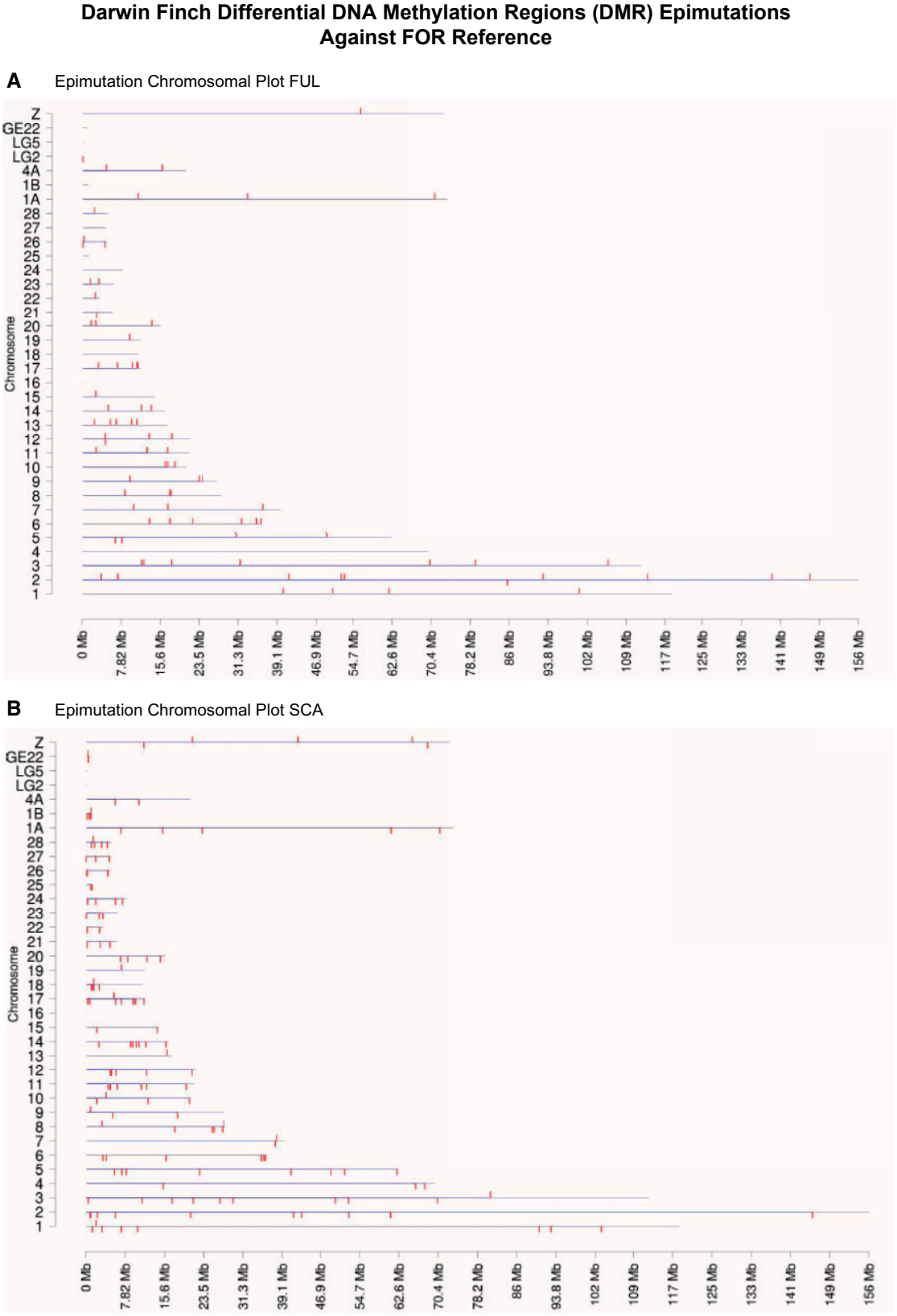
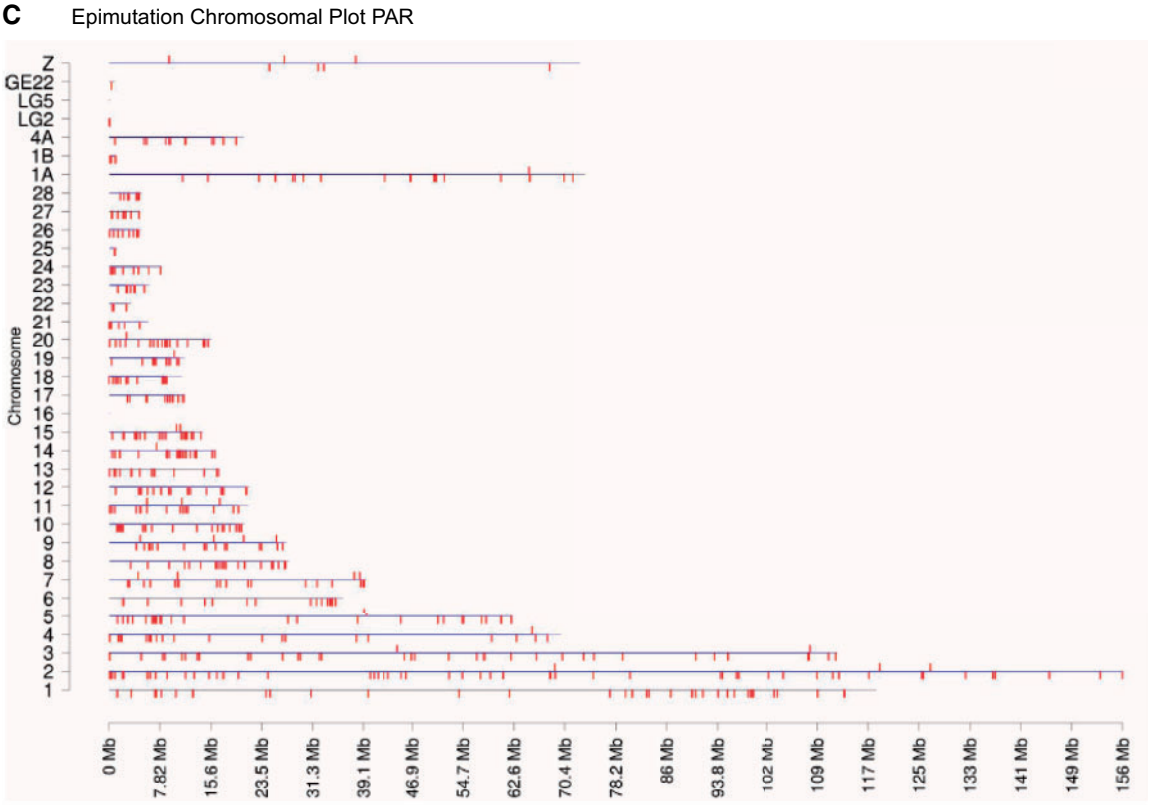


Fig. 5.—Chromosomal locations of the epimutations for each species. The chromosome number and size are presented in reference to the zebra finch genome. The chromosomal location of each DMR is marked with a red tick for FUL (*A*), SCA (*B*), PAR (*C*), and CRA (*D*).



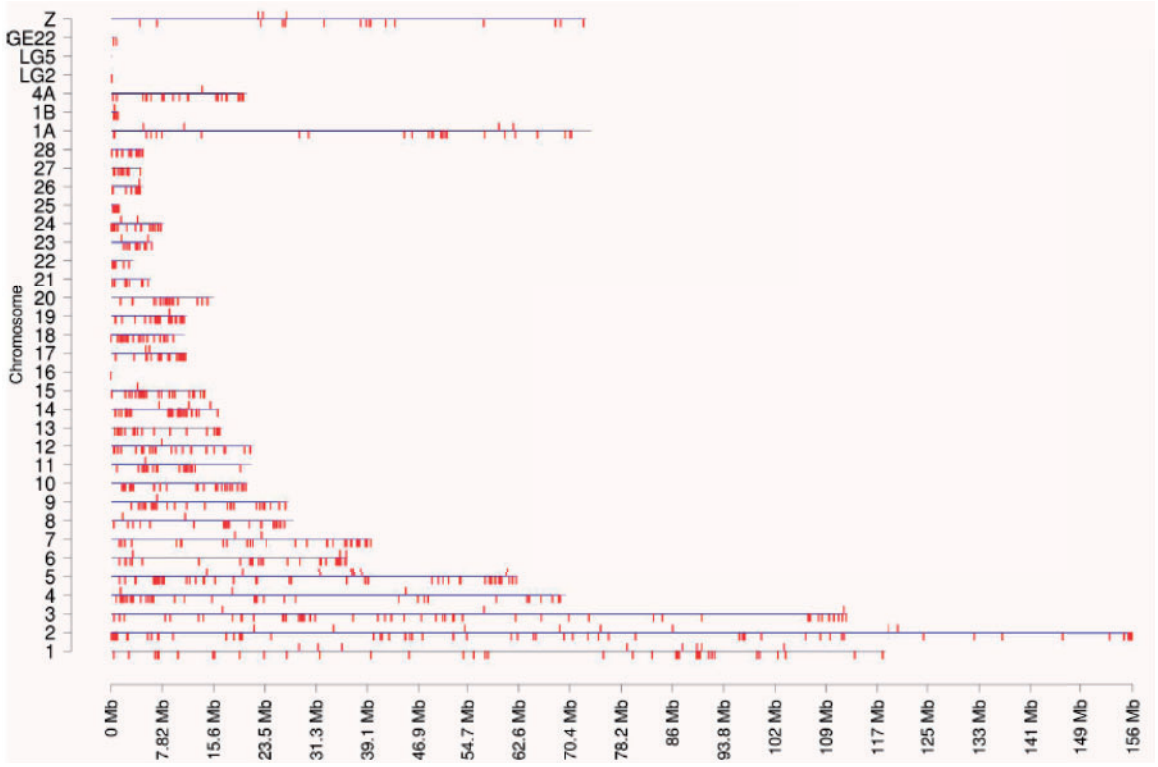


Fig. 5.—Continued.

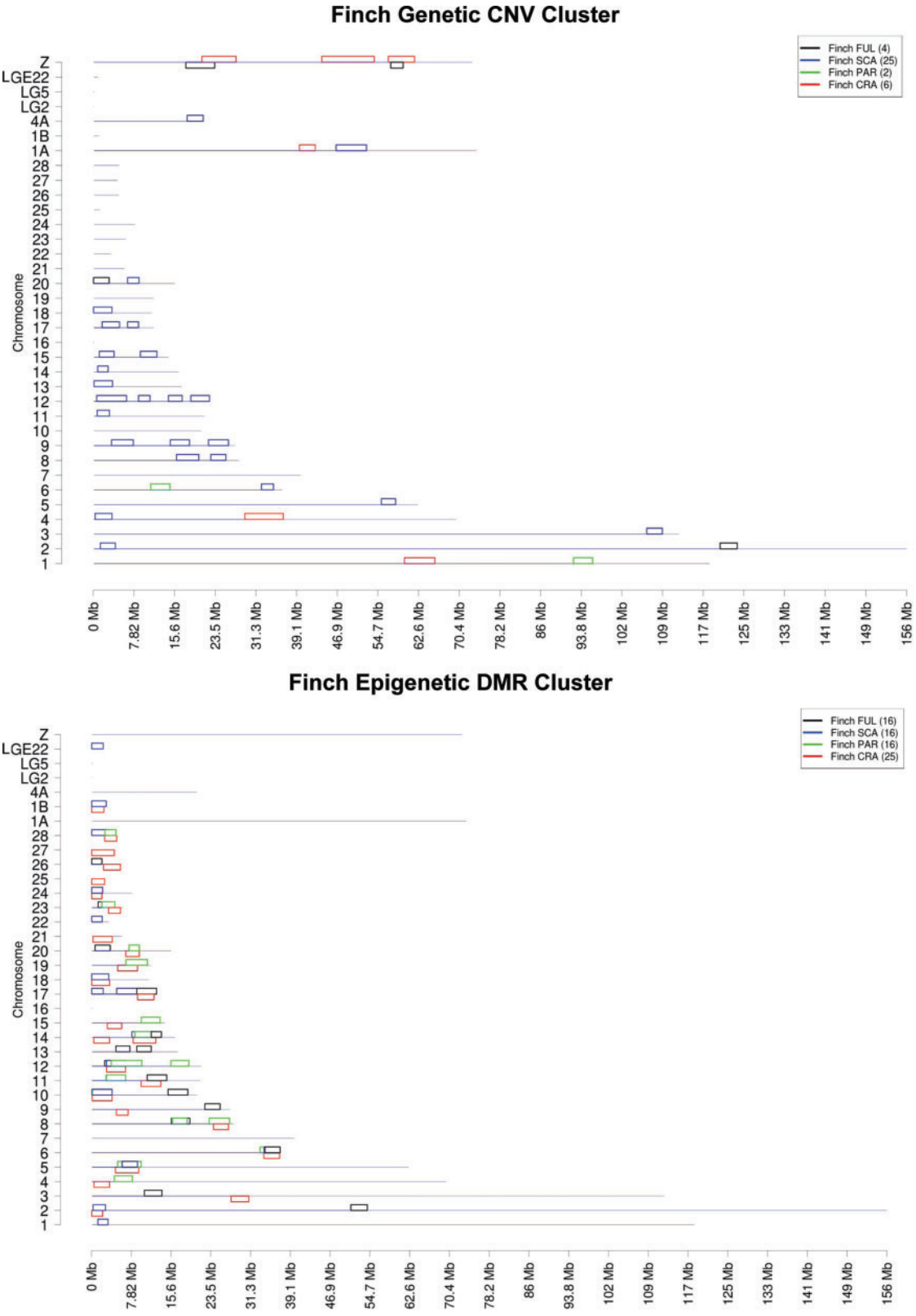


Fig. 6.—Chromosomal locations for clusters of CNV and DMR. The chromosome number and size are presented in reference to the zebra finch genome. The chromosomal location of statistically significant ($P < 10^{-5}$) overrepresented clusters of CNV (*A*) and DMR (*B*). The legend shows species and total number of clusters.

probes in the genome sequence having significant differential hybridization. These selection criteria reduce the number of false positives and provide a more reliable comparison (fig. 2). Therefore, the data presented used stringent criteria and represent the most reproducible epimutations and genetic CNV mutations among all three different experiments.

The increases or decreases in DNA methylation for the DMR are presented, along with the total number of epimutations in figure 2. The majority of epimutations for all species but FUL involves a decrease in DNA methylation (fig. 2*A*). The gains or losses in CNV are also presented, along with the total number of genetic alterations. The majority of genetic mutations for all species but PAR involves an increase in CNV number. Interestingly, the number of epimutations observed was generally higher, using the criteria selected, than the number of genetic alterations (fig. 2). However, the overall magnitude of epigenetic change was comparable to that of genetic change. Data for the five different species are shown in figure 1 for both epimutations (red) and genetic alterations (blue). The number of epimutations was significantly correlated with phylogenetic distance, whereas the number of genetic mutations was not (fig. 3).

The chromosomal locations of the CNV for the different finch species are shown in figure 4. CNVs were found on most chromosomes, with FUL having the least and CRA having the most. The chromosomal locations of the DMR epimutations for the different finch species are shown in figure 5. All chromosomes were found to have epimutations, with CRA having the highest number. These chromosomal plots suggested that some of the species might have clusters of CNV and/or DMR on some of the chromosomes (figs. 3 and 4). Therefore, a cluster analysis previously described (Skinner et al. 2012) was used to examine 50-kb regions throughout the genome to test for statistically significant ($P < 10^{-5}$) overrepresentation of CNV or DMR (fig. 6). Clusters, which have an average size of 3 Mb, are shown as species-specific boxes for CNV (fig. 6*A*) and for DMR (fig. 6*B*). Cluster characteristics and overlap are presented in supplementary table S3, Supplementary Material online. Clusters were obtained for all species, with a higher number of DMR clusters than CNV clusters. The highest number of CNV clusters was in SCA, with more than a 4-fold increase over CRA (fig. 6). Therefore, in addition to having more CNV than expected (assuming an increasing number with phylogenetic distance), SCA showed more CNV clusters than other species (fig. 2). Genome instability in these cluster regions may influence the increased numbers of CNV in SCA, which increases the presence of CNV clusters. In contrast, SCA did not show more DMR numbers or clusters than expected, assuming an increasing number with phylogenetic distance. Epimutation cluster overlap was more common among species (fig. 6 and table 1), suggesting that specific regions of the chromosomes were more susceptible to epigenetic alterations. Altered DNA methylation states have been experimentally shown to be stable for hundreds of generations (Cubas et al. 1999; Akimoto et al. 2007; Skinner et al. 2010).

Table 1
Cluster Overlap between Species

CNVs				
	CNV			
	FUL	**SCA**	**PAR**	**CRA**
FUL	4	0	0	2
SCA	0	25	0	0
PAR	0	0	2	0
CRA	2	0	0	6
Epimutations				
	DMR			
	FUL	**SCA**	**PAR**	**CRA**
FUL	16	5	6	7
SCA	5	16	8	11
PAR	6	8	16	11
CRA	7	11	11	25

NOTE.—The overlap of CNV or DMR clusters between species is presented for the CNVs and epimutations.

The potential overlaps in specific CNV or DMR sites among species were examined. The overlap in genetic mutations among the four species is shown in a Venn diagram in figure 2*C*, whereas the overlap in epimutations is shown in figure 2*B*. No overlap in specific CNV or DMR sites was observed among all species, and less than 10% overlap was generally observed between any two species. Interestingly, the CNV overlap between FUL and CRA was higher than for the other species (fig. 2*C*). Generally, genetic and epigenetic alterations were distinct between species, with the majority being species specific. The epimutations showed more overlap between species than the genetic CNV mutations (fig. 2*B* and table 1). In considering within species overlap between the CNV and epimutations, less than 3% had common genomic locations. Therefore, the epimutations do not appear to be linked to the genetic CNV mutations, but are distinct.

The final analysis examined the potential functional significance of the epimutations by examining DMR and genes known to be associated with avian evolution. Several gene families and cellular signaling pathways have previously been shown to be involved in bird evolution, including the bone morphogenic protein (BMP) family and pathway (Abzhanov et al. 2004; Badyaev et al. 2008), the toll receptor family and signaling pathway (Alcaide and Edwards 2011), and the melanins family and pathway (Mundy 2005). All the genes associated with these signaling pathways were localized on the finch genome and compared with the genomic locations of the epimutations and CNV. Epimutation-associated genes within the BMP pathway (fig. 7), toll pathway (fig. 8), and

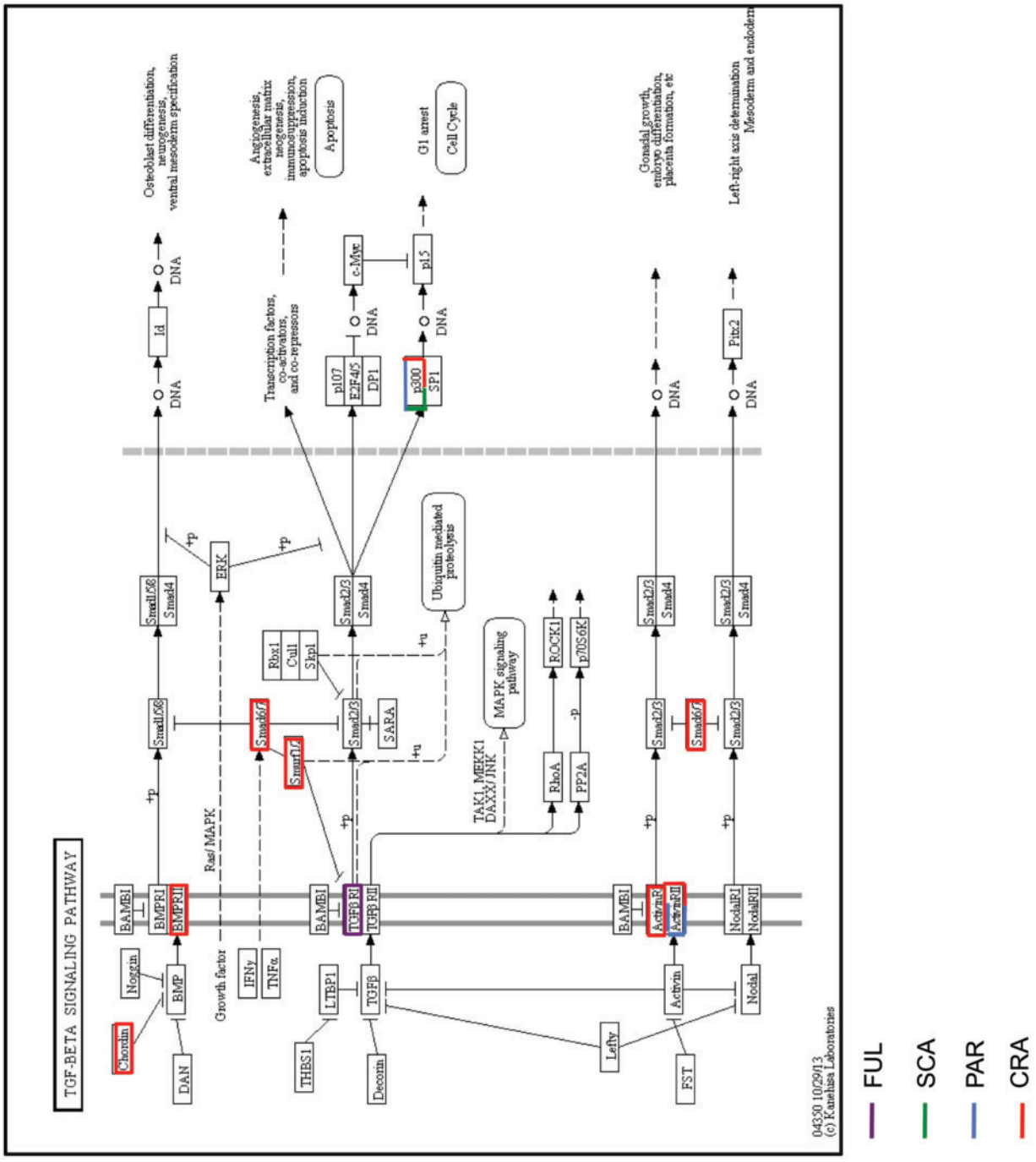


Fig. 7.—Epimutation-associated genes and correlated BMP pathway. The genes having associated epimutations in the signaling pathway presented for the different species are identified as FUL (purple), SCA (green), PAR (blue), and CRA (red) colored boxed genes.

melanin's pathway (fig. 9) are shown. Epimutations were overrepresented in all of these pathways (Fisher's exact test: BMP/TGFbeta (transforming growth factor) pathway, $P < 1 \times 10^{-6}$; toll pathway, $P < 5.7 \times 10^{-4}$; melanogenesis pathway, $P < 2.5 \times 10^{-13}$). Interestingly, the BMP pathway involved in beak development and shape had a statistically significant overrepresentation of CRA-associated epimutations when examined independently ($P < 2.7 \times 10^{-5}$) (fig. 7). In addition, the toll receptor pathway involved in immune response had a statistically significant overrepresentation of PAR-associated epimutations when examined independently ($P < 7.7 \times 10^{-4}$) (fig. 8). The melanogenesis pathway involved in color had a mixture of epimutations from most of the species when examined independently ($P < 7 \times 10^{-5}$) (fig. 9).

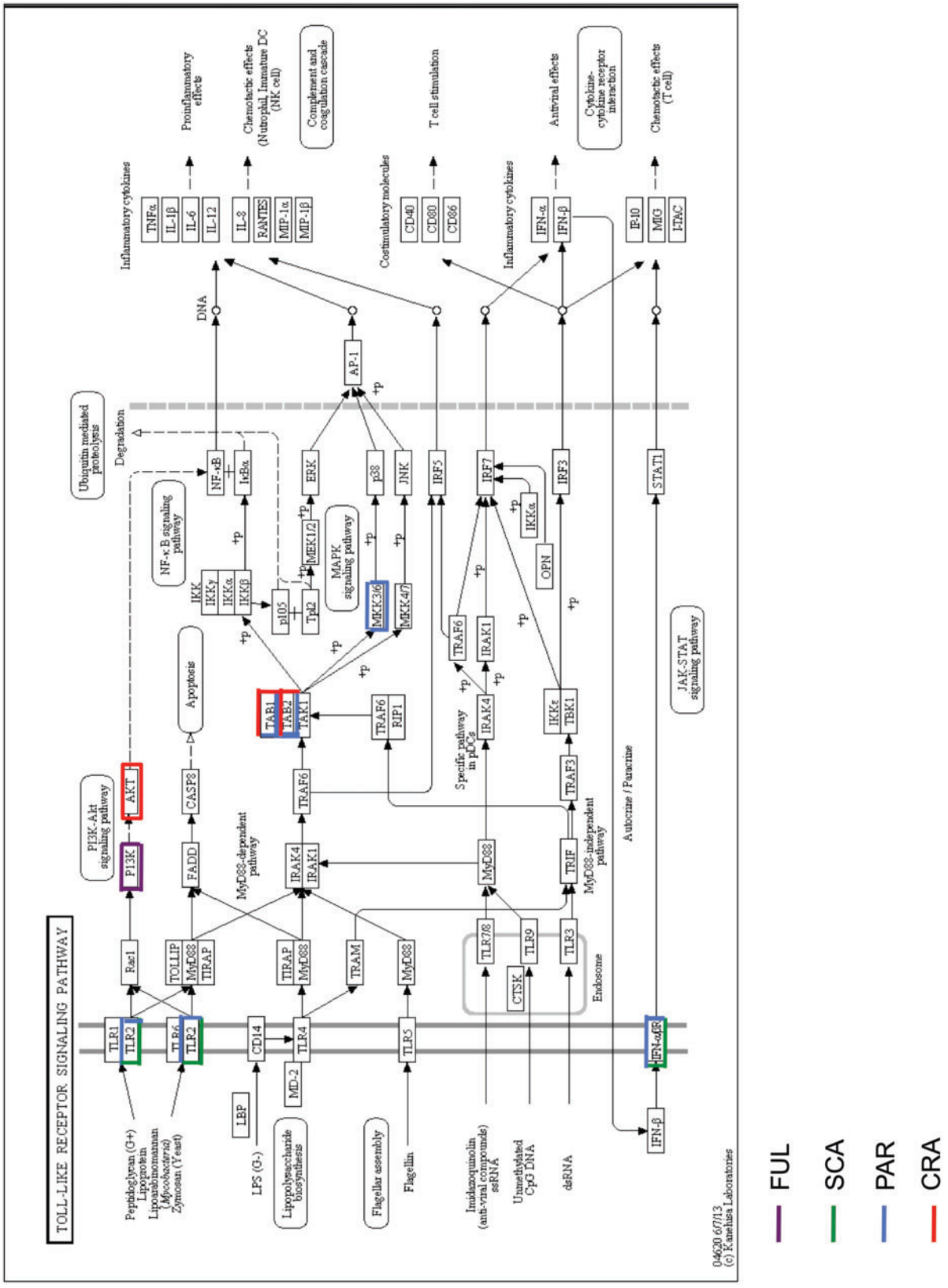


Fig. 8.—Epimutation-associated genes and correlated toll receptor pathway. The genes having associated epimutations in the signaling pathway presented for the different species are identified as FUL (purple), SCA (green), PAR (blue), and CRA (red) colored boxed genes.

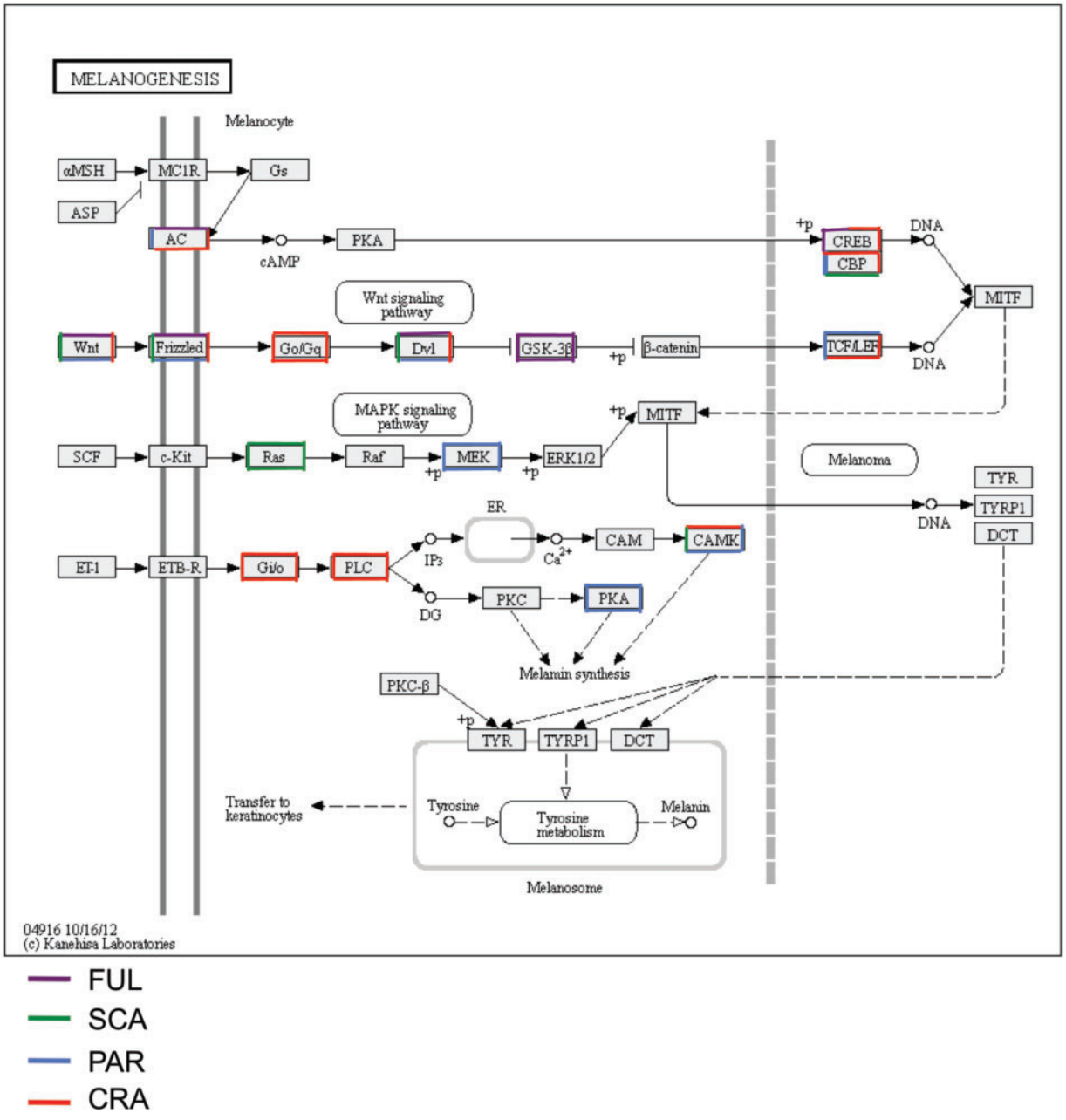


Fig. 9.—Epimutation-associated genes and correlated melanogenesis pathway. The genes having associated epimutations in the signaling pathway presented for the different species are identified as FUL (purple), SCA (green), PAR (blue), and CRA (red) colored boxed genes.

In addition to the pathway-specific genes, the total number of epimutations and CNV associated with genes are presented in table 2, with full lists in supplementary tables S4 and S5, Supplementary Material online. The epimutations and CNV for single probe and ≥3 probe identification are presented in table 2. Observations indicate that approximately half of the epimutations and CNV identified were associated with genes. Therefore, a high percentage of the epimutations and CNV identified were associated with genes and were statistically overrepresented in several gene pathways previously shown to be involved in particular aspects of avian evolution. Although this gene association analysis demonstrates that epimutations correlate with genes and important pathways, the functional or causal link to specific evolutionary processes remains to be investigated.

Discussion

This study provides one of the first genome-wide comparisons of genetic and epigenetic mutations among related species of

Table 2
Epimutation and CNV Gene Associations

CNVs				
	Total CNV 1+ Probes	Total CNV 3+ Probes	CNV Association with 14K Genes 1+ Probes	CNV Association with 14K Genes 3+ Probes
FUL	71	34	40	24
SCA	589	442	363	350
PAR	295	52	136	37
CRA	815	602	437	345
Epimutations				
	Total Epimutations 1+ Probes	Total Epimutations 3+ Probes	Epimutation Association with 14K Genes 1+ Probes	Epimutation Association with 14K Genes 3+ Probes
FUL	514	84	295	48
SCA	890	161	558	115
PAR	1,629	606	996	407
CRA	2,767	1,062	1,611	639

NOTE.—The 14,000 zebra finch genes annotated having epimutation or CNV associations are presented for the total number of associations (overlaps) for both regions identified with single (1+ probes) and adjacent (3+ probes) data sets.

organisms. There were relatively more epimutations than genetic CNV mutations among the five species of Darwin's finches, which suggests that epimutations are a major component of genome variation during evolutionary change. There was also a statistically significant correlation between the number of epigenetic differences and phylogenetic distance between finches (figs. 1 and 3), indicating that the number of epigenetic changes continues to accumulate over long periods of evolutionary time (2–3 Myr). In contrast, there was no significant relationship between the number of genetic CNV changes and phylogenetic distance.

The zebra finch genome was used as a reference for this study because a complete Darwin's finch genome is not yet available. The zebra finch genome showed hybridization with all probes on the array for each of the Darwin's finch species, suggesting that the genomes appear to be extremely similar. Loss of heterozygosity (absence of genomic regions, resulting in lack of probe hybridization) was not identified in any of the analyses. This suggests a high level of conservation and identity between the species' genomes. In the event the Darwin's finch genome has additional DNA sequence that is not present in the zebra finch genome, we would not have detected this DNA. Therefore, our data may be an underestimate of the Darwin's finch genome. Another technical limitation of our study was that we only considered genetic CNV (amplifications and deletions of repeat elements), but not other genetic variants such as point mutations or translocations. Although CNV frequency is higher than other mutations (e.g., SNPs) and stable in the genome (Gazave et al. 2011), this study's focus on CNV should kept in mind. The epimutations examined are differential DMR that have previously been shown to be frequent and transgenerationally stable (Anway et al. 2005; Guerrero-Bosagna et al. 2010; Skinner et al. 2010). Although other epigenetic processes such as histone modification, altered chromatin structure, and noncoding RNA may also be important, DNA methylation is the most established heritable epigenetic mark. This aspect of the experimental design should be kept in mind.

Among the five species of finches there were fewer genetic mutations (CNV) than epigenetic mutations. However, the cactus finch SCA showed a surprisingly large number of genetic CNV mutations than expected when compared with the reference species (FOR). The SCA mutations also clustered to similar locations on the genome to a greater extent than in the other species (fig. 6*A*). The reason for the disproportionately large number of CNV in the SCA comparison is unclear.

In contrast to the genetic mutation (CNV) analysis, the number of epimutations increased monotonically with phylogenetic distance (figs. 1 and 3). Overlap of specific epigenetic sites among species was minimal, including those for SCA (fig. 2*B*). An interesting possibility is that the epigenome may alter genome stability and generate genetic variation within species. A similar phenomenon has been shown for cancer, in which epigenetic alterations may precede genetic changes and alter genomic stability (Feinberg 2004). A decrease in the DNA methylation of specific repeat elements has previously been shown to correlate with an increase in CNV (Macia et al. 2011; Tang et al. 2012). Therefore, environmentally induced abnormal epigenetic shifts may influence genetic

mutations, such that a combination of epigenetics and genetics promotes phenotypic variation. Our observations demonstrate a relationship between the number of epigenetic changes and phylogenetic distance.

A comparison of the positions of epimutations and known gene families was also carried out. These gene families included those involved in the BMP pathway, which is related to beak shape (Badyaev et al. 2008), the toll receptor pathway, which is involved in immunological function (Alcaide and Edwards 2011), and the melanogenesis pathway, which affects color (Mundy 2005). Genes in all three of these families and signaling pathways were found to have species-specific epimutations (figs. 7–9). Future studies should focus on the causal relationship between epigenetic alterations and phenotypic traits.

Genetic mutations are postulated to provide much of the variation upon which natural selection acts (Gazave et al. 2011; Stoltzfus 2012). However, genetic changes alone are limited in their ability to explain phenomena ranging from the molecular basis of disease etiology to aspects of evolution (Skinner et al. 2010; Day and Bonduriansky 2011; Longo et al. 2012; Klironomos et al. 2013). Therefore, genetic mutations may not be the only molecular factors to consider (Richards 2006, 2009). Indeed, epigenetic and genetic changes may jointly regulate genome activity and evolution, as recent evolutionary biology modeling suggests (Day and Bonduriansky 2011; Klironomos et al. 2013). This integration of genetics and epigenetics may improve our understanding of the molecular control of many aspects of biology, including evolution.

Supplementary Material

Supplementary tables S1–S6 are available at *Genome Biology and Evolution* online (http://www.gbe. oxfordjournals.org/).

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Trends in
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Review

Epigenetic inheritance and reproductive mode in plants and animals

Dafni Anastasiadi [1,4], Clare J. Venney [2,4], Louis Bernatchez [2], and Maren Wellenreuther [1,3,*]

Epigenetic inheritance is another piece of the puzzle of nongenetic inheritance, although the prevalence, sources, persistence, and phenotypic consequences of heritable epigenetic marks across taxa remain unclear. We systematically reviewed over 500 studies from the past 5 years to identify trends in the frequency of epigenetic inheritance due to differences in reproductive mode and germline development. Genetic, intrinsic (e.g., disease), and extrinsic (e.g., environmental) factors were identified as sources of epigenetic inheritance, with impacts on phenotype and adaptation depending on environmental predictability. Our review shows that multigenerational persistence of epigenomic patterns is common in both plants and animals, but also highlights many knowledge gaps that remain to be filled. We provide a framework to guide future studies towards understanding the generational persistence and eco-evolutionary significance of epigenomic patterns.

Highlights

Epigenetic mechanisms can alter gene expression and allow species to respond rapidly to their environments by modifying their phenotypes.

Reproductive mode (i.e., sexual versus asexual, oviparity versus viviparity in animals) and germline development commonly predict the persistence of epigenetic marks.

The consequences of persistent epigenomic variation vary depending on the sources (intrinsic, genetic, extrinsic).

Environmental predictability is a key factor for determining the consequences of epigenetic inheritance on phenotype and fitness.

We provide a roadmap for future studies to further our understanding of the extent and evolutionary importance of epigenetic inheritance by quantifying: (i) persistence across generations, (ii) contributions to phenotype and fitness, and (iii) cross-taxa comparisons.

The eco-evolutionary significance of epigenomic variation

The inheritance of acquired traits has long fascinated biologists and led to intense debate. In 1956, Conrad Waddington demonstrated that the inheritance of environmentally induced traits was possible [1], while also coining the term '**epigenetics**' (see Glossary). Since then, the meaning of the term epigenetics has changed in different fields; we define it as 'genome-associated mechanisms of non-DNA sequence-based inheritance' [2,3]. The molecular mechanisms mediating the inheritance of acquired traits have been described in several landmark studies [4–6] and the field has rapidly advanced during the last decade (for an historical context, see [7]). In this review, we focus on the three most widely studied epigenetic mechanisms [3]: DNA methylation, histone modifications, and noncoding RNA (ncRNA) expression (Box 1). The roles of these processes in the establishment, maintenance, and regulation of gene expression can significantly affect the eco-evolutionary dynamics of species (recently reviewed in [8–10]).

Epigenomic variation is nearly ubiquitous in plants and animals and can change at a considerably faster rate than genomic variation [11,12] (i.e., within a single generation [13–17]). Epigenetic inheritance, a source of nongenetic inheritance, occurs when epigenetic modifications (Box 1) are passed on through reproduction to the next generation. The persistence of **epigenomic** variation across generations has been heavily debated, partly because underlying mechanisms were not understood [18] and early research in mammals suggested complete epigenome erasure between generations [19,20]. Unlike the genome, the epigenome is tissue-specific and patterns between soma and germline likely differ. Consequently, the germline is the predominant source of epigenetic inheritance in many species, although some species develop gametes from somatic tissue, while others establish distinct germline tissue early in development. Therefore, the mode of epigenetic inheritance is expected to differ depending on reproductive mode and life history.

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The reproductive strategy (sexual vs. asexual), as well as the timing and nature of events leading to germline formation, are expected to influence epigenetic inheritance. For epigenetic inheritance to occur in gametic reproduction, environmentally or intrinsically induced epigenetic changes must be incorporated into the germline [21]. While it was once accepted that the **Weismann barrier** prevented somatic cells from altering the germline after cell differentiation, this idea has been disproven through research on epigenetic inheritance [21,22]. Soma-to-germline communication may be possible through extracellular RNA [23,24]; however, it is unclear to what extent the germline absorbs somatic epigenetic changes after segregation. Therefore, the timing of germline segregation may influence epigenetic inheritance due to the potential for whole-epigenome inheritance upon germline formation, which is unlikely to occur after segregation. In animals where the germline segregates and differentiates early in development, the timing of gametogenesis and mode of reproduction (**oviparity** vs. **viviparity**) are expected to impact epigenetic inheritance. In viviparous mammals, two rounds of extensive erasure of epigenetic patterns occur (during gametogenesis and embryogenesis), resulting in the resetting of most epigenetic marks, although a small number remain intact [19,20]. In other animals, erasure of epigenetic patterns during gametogenesis and embryogenesis is either absent or understudied [25], thus germline-to-soma transmission is expected to be more prevalent. Late segregation of the germline, common in plants but also found in metazoans such as snails, sea urchins, sponges, and cnidarians [26], results in a long period during which environmentally induced epigenetic changes can be incorporated [16]. DNA methylation and histone modifications are maintained during sexual reproduction in plants, although some reprogramming occurs [27,28]. Thus, late germline segregation should increase the potential for epigenetic inheritance. While germline-to-soma transmission is common, there is variation in the frequency of epigenetic inheritance among species.

Here, we systematically reviewed over 500 studies from the past 5 years on the **multigenerational inheritance** of epigenetic marks in plants and animals (see Supplementary File 1 for search criteria and Table S1 for a full list of studies, in the supplemental information online). Our goals were to: (i) assess the frequency of epigenetic inheritance depending on reproductive mode and germline development; (ii) assess the sources, persistence, and consequences of epigenetic inheritance; and (iii) provide a roadmap with guidelines for future studies to answer outstanding questions and challenges.

Epigenetic inheritance through sexual reproduction

Early germline differentiation reduces potential for epigenetic inheritance

Viviparity

Epigenetic inheritance has been extensively studied in viviparous species (77.5% of 570 reviewed studies; Figures 1 and 2A,B; Table 1; reviewed in [18,20,29]). Viviparity is mostly restricted to mammals, with numerous studies in humans (*Homo sapiens*, $n = 230$), mice (*Mus musculus*, $n = 98$), and rats (*Rattus norvegicus*, $n = 87$), although other domesticated and model mammals (e.g., guinea pigs, *Cavia* spp.) were also represented ($n = 25$). For viviparous species, epigenetic inheritance is limited to gametogenesis for paternal effects, while maternal epigenetic inheritance was thought to occur from gametogenesis to **gonadal sex determination** of the offspring [29]. However, several studies identified maternal epigenetic inheritance due to exposures shortly before parturition (i.e., after offspring gonadal development [30–33]).

Transgenerational inheritance is complicated by viviparity since intrauterine development implies the simultaneous presence of three generations via the female germline: the gestating mother (F_0), the embryo (F_1), and the germline of the embryo (F_2) [34]. Thus, while epigenetic inheritance in viviparous species is only considered 'truly' transgenerational when transmitted to the unexposed

Glossary

Agamogenesis: type of asexual reproduction where only female gametes are produced (i.e., no male gamete is involved).
Apomixis: asexual reproduction in plants where fertilization is absent (i.e., the female gamete develops without fertilization).
Copy number variation (CNV): variation in the number of copies of a nucleotide sequence between individuals.
CpG: a cytosine adjacent to a guanine residue in the DNA sequence. The main site of DNA methylation in animal genomes.
Diversified bet-hedging: phenotypic variability of individuals with the same genotype increases, resulting in higher variance of fitness, which can buffer survival of the genotype in unpredictable environments.
Epigenator signals: transient environmental cues and downstream intracellular signaling pathways that trigger epigenetic changes.
Epigenetic buffering: epigenomic changes contributing to phenotypic resilience of a population facing fluctuating environments.
Epigenetics: genome-associated mechanisms of heritable changes not dependent on changes to DNA sequence.
Epigenetic trap: an intrinsically or extrinsically induced epigenetic change that is maladaptive and does not contribute to diversified bet-hedging strategies.
Epigenomics: epigenetic changes across the whole genome.
Epigenomic variation: interindividual variation in the molecular epigenetic marks.
Facilitated epigenetic variation: epigenetic variation that is induced by environmental stimuli in the context of a specific genotype.
Genetic assimilation: a phenotype shifts from being environmentally induced to genetically encoded when the environment/trigger is stable.
Gonadal sex determination: development of the bipotential gonad into testis or ovary.
H3K9me2: dimethylation of histone H3 lysine 9, a repressive histone modification that condenses the DNA.
H3K9me3: trimethylation of histone H3 lysine 9, a repressive histone modification that condenses the DNA.
Intergenerational inheritance: persistence of effects from parent to offspring.

F_3 generation, increased capacity for maternal epigenetic inheritance in the F_1 and even directly to the F_2 generations exists due to *in utero* development. The abundance of mammalian studies has clarified the optimal timing of parental exposure for epigenetic inheritance to occur, allowing the informed design of studies that maximize the potential for inheritance. This, coupled with the increased potential for epigenetic inheritance due to intrauterine development, explains why examples of viviparous epigenetic inheritance are abundant in the literature. Noteworthy examples include transgenerational studies on maternal exposure to environmental chemicals on complete germline epigenetic inheritance (DNA methylation, ncRNA, and histone modifications) in F_1 through to F_3 sperm in rats [35–37].

Oviparity

Studies in oviparous organisms detected epigenetic inheritance despite their under-representation in the literature (9.82% of 570 reviewed studies), although at a lower frequency (86.1%) than viviparous organisms (91.4%) (Figures 1 and 2C; Table 1). Oviparous, sexually reproducing animals were represented in our review by birds (n = 9 studies), fishes (n = 25), insects (n = 9), crustaceans (n = 2), echinoderms (n = 1), molluscs (n = 3), and one nematode (n = 7). For oviparous reproduction, germline epigenetic changes must be incorporated before the release of gametes, thus there is a strict cut-off for transmission. This was thought to be limited to the short period of gamete maturation in animals, although a recent study in zebrafish (*Danio rerio*) exposed to the pesticide chlorpyrifos-oxon 4 hours to 5 days postfertilization identified differences in DNA methylation that persisted to F_2 [38].

We identified considerable **parental effects** on the offspring epigenome, although few studies discriminated between maternal and paternal effects in oviparous animals. Paternal epigenetic inheritance was less studied in animals (n = 4), but research in Atlantic salmon (*Salmo salar*) [39], European sea bass (*Dicentrarchus labrax*) [40], and Pacific oyster (*Crassostrea gigas*) [41] identified paternal effects on DNA methylation. Maternal epigenetic inheritance was more frequently studied (n = 6), with maternal inheritance of ncRNA expression reported in chicken (*Gallus gallus domesticus*) [42] and annual killifish (*Austrofundulus limnaeus*) [43], as well as maternally-inherited DNA methylation in chicken [44,45] and Chinook salmon (*Oncorhynchus tshawytscha*) [46]. Thus, due to the lack of intrauterine development (i.e., increased maternal influence over offspring epigenetics in viviparous organisms), there is a greater capacity for paternal epigenetic inheritance in oviparous organisms, although maternal effects are more common and frequently studied due to higher maternal investment into gametes.

Late germline differentiation increases the critical window for inheritance

Species with late germline segregation, including plants (n = 46) and one echinoderm, showed high capacity for epigenetic inheritance (Figure 2C). These organisms have an extended time window for epigenetic inheritance due to the creation of germline cells from somatic tissue, hypothetically leading to increased potential for epigenetic inheritance. Consistent with this, there were few studies in plants where epigenetic marks were not transmitted to F_1 and F_2 generations (Figure 1). Parental dominance effects in plants influenced DNA methylation [47,48] and ncRNA expression [48], depending on whether a genotype was used as mother or father. Maternal environment affected DNA methylation in the offspring of purple sea urchin (*Strongylocentrotus purpuratus*) [49].

Self-pollination

Many plants are capable of both self- and cross-pollination [34] and several studies considered the effects of self-pollination on the offspring epigenome (n = 9; Table 1). Studies that involved self-pollination showed long-term persistence of epigenetic inheritance. Cross-pollination between species or lines to induce hybridization followed by self-pollination to produce genetically

Multigenerational inheritance: persistence of effects across generations regardless of exposure to the initial trigger.
Obligatory epigenetic variation: epigenetic variation that is completely dependent on the underlying genetic variation.
Oviparity: a sexual reproductive mode where oocyte and sperm combine to produce offspring, either internally or externally, but egg development occurs outside the body.
Parental effects: effects of parental genotype or environment on offspring phenotype or function that are not due to genetic inheritance.
Parthenogenesis: an asexual reproductive mode where an unfertilized oocyte develops into a viable offspring.
Pure epigenetic variation: epigenetic variation that arises due to developmental stochasticity.
Transgenerational inheritance: persistence of effects up to the first generation completely unexposed, even as germline cells, to the initial trigger.
Vegetative reproduction: an asexual reproductive mode where offspring develops directly from a segment of parental tissue, without the use of gametes.
Viviparity: a sexual reproductive mode where oocyte and sperm combine to produce an embryo that develops inside the parent.
Weismann barrier: concept that the germline is separate from and cannot be influenced by somatic cells.

uniform descendants (n = 12) resulted in inheritance of ncRNA expression until F_{12} in rice (*Oryza sativa*) [50] and of DNA methylation until F_6 in brown mustard (*Brassica juncea*) [51]. Thus, self-pollination can lead to increased similarity in the epigenetic marks carried by parent and offspring compared with cross-pollination between different individuals, with potential long-term effects on the offspring epigenome.

Epigenetic inheritance in asexual organisms

Epigenetic inheritance in agamogenesis

Epigenetic inheritance could be particularly beneficial to asexual organisms, allowing them to cope with environmental stress in the absence of generational genetic variation, resulting in epigenetic mechanisms expanding the range of phenotypes encoded by their genome (Box 2) [52–54]. Despite the potential importance of epigenetic inheritance for asexual organisms, we found only three studies in **parthenogenetic** animals and two in **apomictic** plants. Similar to sexually reproducing organisms, gamete-producing asexual organisms would need to incorporate changes before gamete maturation, although they have the potential for increased control over the offspring epigenome due to uniparental inheritance of epigenetic marks. Asexual organisms that can switch between sexual reproduction and parthenogenesis, such as Cape honey bee (*Apis mellifera capensis*), transmit different methylation patterns, depending on the reproductive strategy used [55].

Despite the dearth of studies in organisms reproducing through **agamogenesis**, epigenetic inheritance can have important implications for offspring survival. A study in the parthenogenetic brown citrus aphid (*Aphis citricidus*) found that maternal crowding decreased offspring aci-miR-9b miRNA expression, resulting in winged offspring that could escape crowded habitats [56]. In apomictic dandelions (*Taraxacum* spp.), altered DNA methylation and ncRNA expression induced by drought were inherited for two to three generations in unexposed offspring [57,58], highlighting the potential for long-term epigenetic inheritance in organisms reproducing asexually without fertilization. Asexual organisms can make use of both plasticity and epigenetically

Box 1. Epigenetic mechanisms

Useful concepts introduced recently, such as 'nongenetic interpretive machinery' [116] and 'inherited gene regulation' [77], encompass various nongenetic molecular mechanisms, but there are three widely accepted epigenetic mechanisms [3].

DNA methylation commonly refers to the addition of a methyl group ($–CH_3$) to the 5′ carbon of cytosine nucleotides, although there are other forms such as 5-hydroxymethylation, the oxidized derivative of cytosine methylation [126]. DNA methylation primarily occurs in a **CpG** context in animals, although CpHpG and CpHpH contexts (where H is an A, T, or C) are common in plants [127]. DNA methylation generally results in the suppression of transcription in a nonlinear, time- and context-dependent manner, but can also be associated with active transcription [128,129].

Histone modifications (including acetylation, phosphorylation, and methylation) occur on specific amino acids of histone proteins, influencing chromatin structure and the transcriptional activity of proximal genes [130,131]. Histone acetylation and phosphorylation reduce chromatin compaction due to their slight negative charge reducing the strength of electrostatic effects between histones and DNA, thus allowing transcriptional machinery to access and transcribe the DNA [130]. Histone methylation can result in either transcriptional activation or repression, depending on where it occurs. For example, **H3K9me3** results in transcriptional activation, while H3K9me2 is associated with transcriptional repression [130]. In animal sperm, histones are usually replaced by protamines, however, part of the histones with their associated modifications may be retained (histone retention) [132].

Noncoding RNAs (ncRNA), including small RNAs and long ncRNAs, do not code for proteins, but instead post-transcriptionally regulate gene expression [133,134], often by binding and silencing complementary RNA molecules [134].

Epigenetic variation is induced by **epigenator signals** from environmental cues [135]. This triggers intracellular pathways that translate signals into chromatin changes via the epigenetic initiators (e.g., ncRNA or DNA-binding molecules) [135,136]. These changes can be converted to permanent states via epigenetic maintainers (e.g., DNA methylation and histone modifications) [135,136]. DNA methylation and histone modifications are altered (either deposited or removed) through enzymatic mechanisms that also function to preserve DNA methylation and histone modifications through cell division and beyond [134]. Richards [78] proposed that epigenetic variation can arise due to genetic effects (obligatory), stochastic environmental or developmental effects regardless of genotype (pure), or a stochastic effect that can occur due to an individual's genotype (facilitated; Figure I). Obligatory and pure represent the two extremes of dependency between epigenetic and genetic variation.

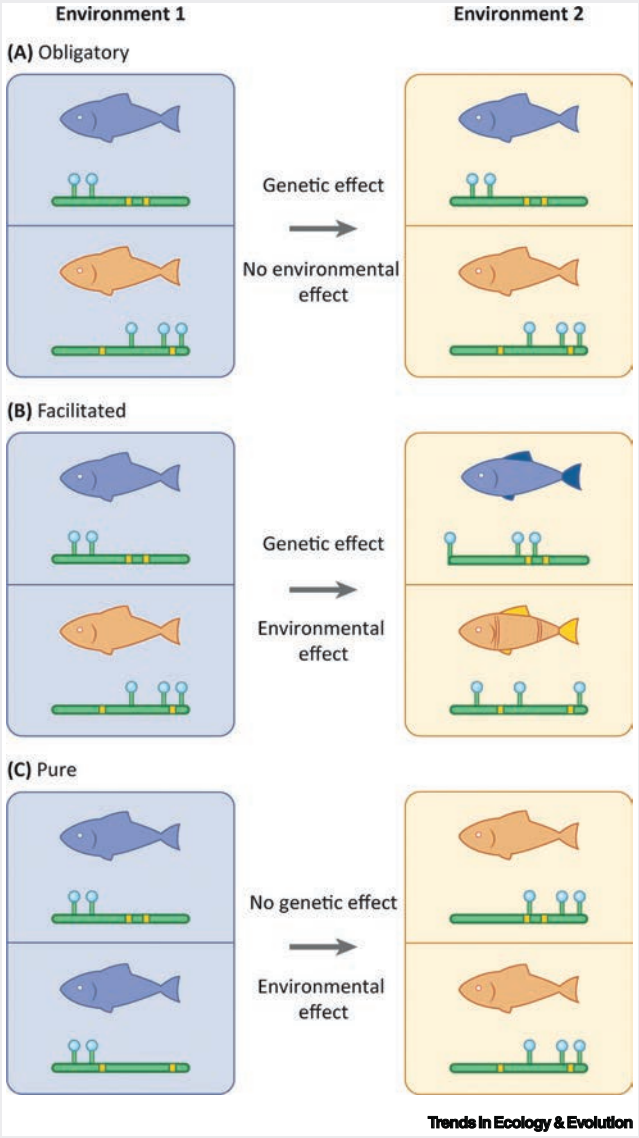

Figure I. Obligatory, facilitated, and **pure epigenomic variants** can arise depending on the relative importance of genetic variation in determining epigenetic marks, with implications for phenotype. Lollipops represent epigenetic modifications on top of the DNA sequence, while yellow bars represent genetic variants. Two different environments are shown by red and blue backgrounds. Novel phenotypes are indicated by altered fish color. (A) Obligatory epigenetic variants are entirely due to genotype, thus, result in the same phenotype regardless of the difference between environments. (B) Different genotypes allow the induction of unique facilitated epigenotypes associated with different phenotypes in contrasting environments (i.e., different genotypes develop different epigenotypes in response to the same environmental shift). (C) Pure epigenetic variants are not associated with the genotype and thus result in plastic phenotypic changes that are common in different environments.

inherited **diversified bet-hedging** in response to the same stressor (Box 2). Interestingly in dandelions, ncRNA expression showed intergenerational plasticity [57], while variation in DNA methylation among offspring increased [58], suggesting that closely related organisms can make use of both strategies in response to the same stressor.

Epigenetic inheritance in vegetative reproduction

Organisms utilizing **vegetative reproduction** should have the greatest propensity for epigenetic inheritance. There is no distinct germline in vegetative organisms; offspring arise as a fragment of the parent, with any somatic epigenetic changes passed on to offspring. Studies involving vegetative reproduction were rare in plants ($n = 6$) and animals ($n = 2$). These studies showed high fidelity of epigenetic inheritance. Relative to sexually produced offspring, vegetative offspring had either

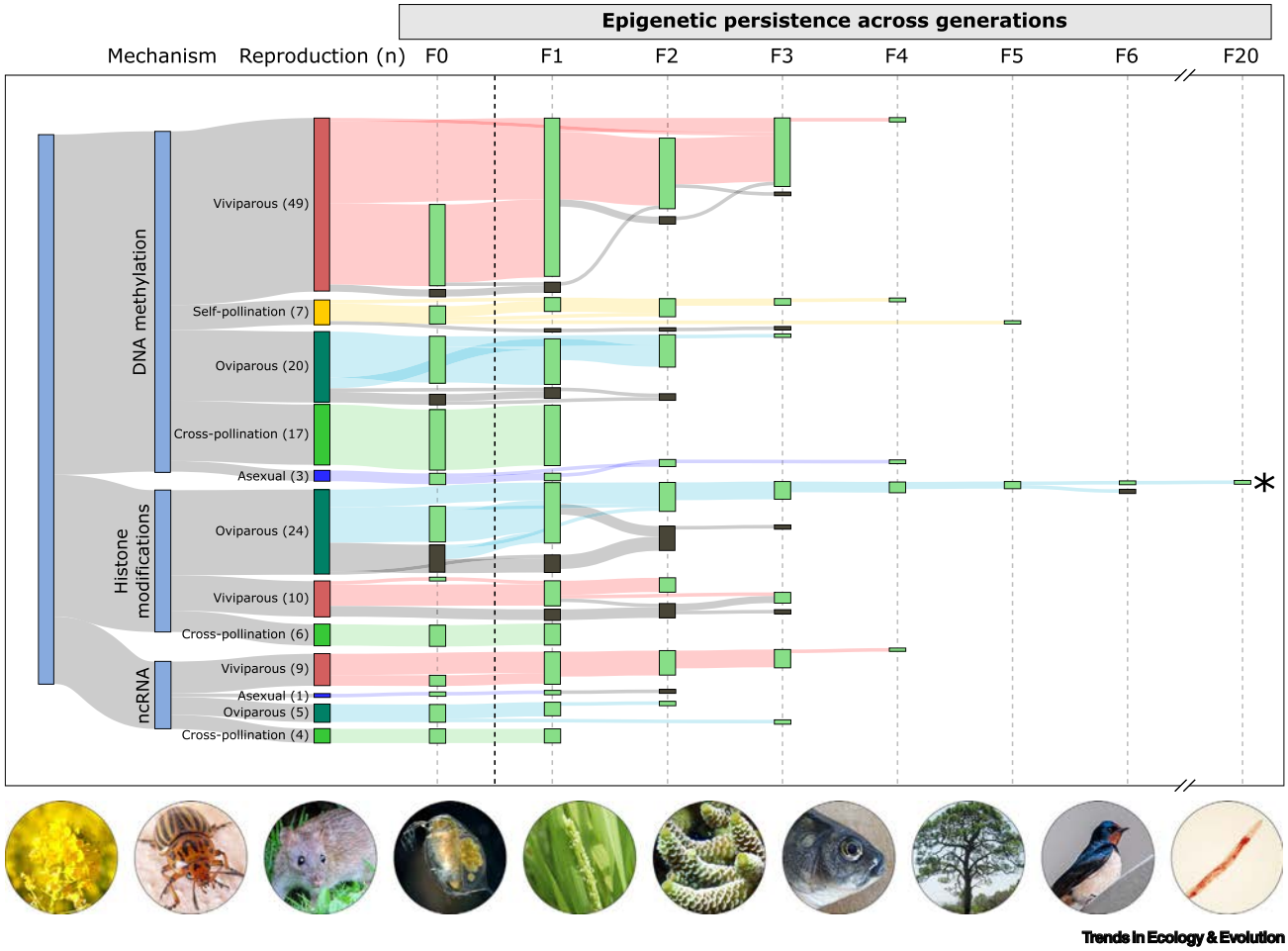

Figure 1. Frequency and persistence of epigenetic inheritance across generations based on the analysis of different epigenetic mechanisms (DNA methylation, noncoding RNA expression, and histone modifications) and reproductive modes performed on multigenerational studies across a diversity of plant and animal taxa. Analyses involved 155 tests of epigenetic inheritance based on 127 unique multigenerational studies, while studies using multiple reproductive modes within a lineage were excluded. See Table 1 for taxon-specific information. Flow width is proportional to the number of studies at each node. The number of individual tests of epigenetic inheritance for each reproductive mode is given in brackets next to the reproductive mode and subsequent flows are color-coded by reproductive mode. Green bars indicate confirmed epigenetic inheritance while gray bars indicate lack of epigenetic inheritance. The black dotted line indicates inheritance from F_0 (germline or soma) tissue to descendants. One study on histone modifications in *Caenorhabditis elegans*, marked with an asterisk at the center right of the figure, found evidence for epigenetic inheritance through F_1–F_{20} generations (F_7–F_{19} omitted for brevity). Round photos underneath the graph display some of the study species that were included in this review.

equal (potato, *Solanum tuberosum*) [59,60] or increased (apple, *Malus domestica*) [61] fidelity of epigenetic inheritance, resulting in increased parental control and heritability of epigenetic marks among generations. Studies on vegetative organisms, including reef-building corals (*Acropora millepora*) and green algae (*Chlamydomonas reinhardtii*), support plasticity rather than diversified bet-hedging and suggest that epigenetic inheritance can improve offspring fitness [62,63]. However, we cannot rule out diversified bet-hedging due to the small number of relevant studies.

Sources, persistence, and consequences of epigenetic inheritance

Intrinsic and extrinsic sources

Epigenetic variation is influenced by intrinsic and extrinsic effects. Intrinsic effects, such as health and physiological status of parents, can have considerable effects on the offspring epigenome.

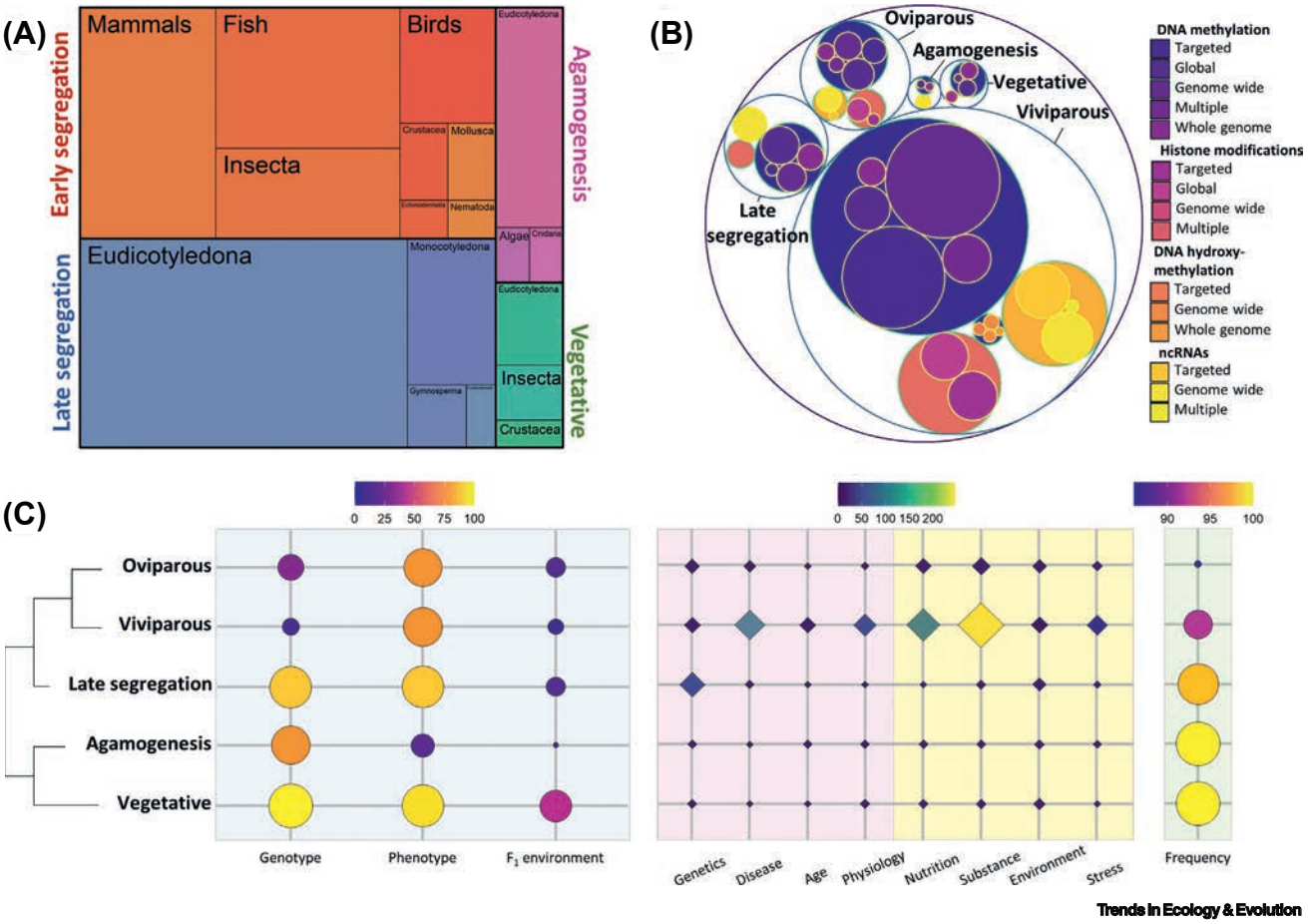

Figure 2. Summary of the reviewed literature by reproductive mode. Sexual reproduction is divided into early and late germline segregation, with early germline segregation further divided into oviparous and viviparous reproduction. Asexual reproduction includes agamogenesis (gamete-producing organisms) and vegetative reproduction. See Table 1 for detailed numbers. (A) Overview of the number of species (not studies) represented in the literature review, colored by reproductive mode. (B) Epigenetic mechanisms and methods used to study them based on reproductive mode represented by open circles. Early segregation mode is divided in oviparous and viviparous. The filled circles within each mode represent epigenetic mechanisms and within each of them, colors represent specific methods used for each epigenetic mechanism, as shown in the legend. Methods are grouped as global (low resolution), targeted, genome wide, whole genome, or multiple apporaches used in combination. (C) Frequency of assessment of genetic effects, phenotypic consequences, offspring fitness in matched–mismatched environments, intrinsic and extrinsic drivers of epigenetic inheritance, as well as the frequency of epigenetic inheritance depending on reproductive mode. Early segregation mode is divided in oviparous and viviparous. Bubble sizes are proportional to frequency (0–100%) and rhomboid sizes are proportional to number of studies and colored as shown in the legends. Background colors in the middle panel correspond to intrinsic (pink) or extrinsic (yellow) sources of epigenetic inheritance.

Intrinsic parental effects are often associated with maladaptive phenotypes and lead to **epigenetic traps** [64]. Studies identified epigenetic inheritance due to age (e.g., [65–67]), obesity (e.g., [68,69]), and, in mammals, maternal and gestational diseases (e.g., [70–72]), which typically have negative effects [64].

Despite extrinsic factors such as environmental exposures often being transient, they can have long-lasting effects. Well-known examples of altered DNA methylation patterns in humans persisted for decades after parturition, such as starvation during the Dutch Famine of World War II [73] and maternal smoking [74]. Exposure of Colorado potato beetle (*Leptinotarsa decemlineata*) to insecticides [75], dandelions to salicylic acid [58], and rice to heavy metals [76] resulted in epigenetic changes in F_2 progeny.

Table 1. Summary of literature review per taxa[a]

Reproduction mode	Taxa (species)	Studies	Epigenetic mechanism and method[b]	Effect	Genotype	Phenotype	F_1 environment	Frequency
1. Sexual reproduction (615/672)								
1.1 Early germline segregation (550/606)	Nematoda (1)	7	Histone modifications (G, 1; CG, 1; GW, 2)	Genetics (I, 1), environment (E, 1), nutrition (E, 1), substance exposure (E, 1)	Y (1), N (3)	Y (3), N (2)	N (4)	4/7
			ncRNA (GW, 3)	Genetics (I, 1), disease (I, 1), substance exposure (E, 1)	N (3)	Y (2), N (1)	N (3)	3/3
	Crustacea (2)	2	DNA methylation (G)	Substance exposure (E)	N	Y	Y	1/1
			Histone modifications (G)	Disease (I)	N	Y	N	2/3
	Mollusca (2)	3	DNA methylation (G, 2; MA, 1)	Substance exposure (E, 3)	Y (1), N (2)	Y (3)	N (3)	2/3
	Echinodermata (1)	1	ncRNAs (GW)	Genetics (I)	Y	Y	N	1/1
	Insecta (9)	9	DNA methylation (G, 3; GW, 1; WG, 1)	Genetics (I, 1), disease (I, 1), stress (E, 1), substance exposure (E, 2)	Y (2), N (3)	Y (3), N (2)	N (5)	4/5
			Histone modifications (G, 3; GW, 1)	Genetics (I, 1), disease (I, 1), nutrition (E, 1), substance exposure (E, 1)	Y (2), N (2)	Y (3), N (1)	Y (2), N (2)	5/5
			ncRNA (GW)	Genetics (I)	Y	Y	N	1/1
	Fish (14)	25	DNA methylation (G, 6; CG, 4; GW, 8; WG,2; MA, 2)	Genetics (I, 3), physiological status (I, 1), environment (E, 6), nutrition (E, 3), substance exposure (E, 9)	Y (9), N (13)	Y (17), N (5)	Y (3), N (19)	20/22
			histone modifications (G, 2; CG, 1)	Substance exposure (E, 3)	N (3)	Y (2), N (1)	N (3)	8/9
			ncRNAs (CG, 1; GW, 1)	genetics (I, 1), substance exposure (E, 1)	N (2)	Y (1), N (1)	N (2)	3/3
	Birds (6)	9	DNA methylation (G, 1; CG, 4; GW, 1; WG, 1)	Genetics (I, 1), disease (I, 1), stress (E, 1), environment (E, 1), substance exposure (E, 2)	Y (1), N (6)	Y (4), N (3)	Y (1), N (6)	6/7
			ncRNAs (GW, 2)	nutrition (E, 2), environment (E, 1)	N (2)	Y (2)	N (2)	2/2
	Mammals (17)	441	DNA hydroxymethylation (G, 3; CG, 2; GW, 2; WG, 1)	Physiological status (I, 2), nutrition (E, 2), substance exposure (E, 5)	Y (1), N (7)	Y (7), N (1)	Y (1), N (7)	8/8

(continued on next page)

Table 1. (continued)

Reproduction mode	Taxa (species)	Studies	Epigenetic mechanism and method[b]	Effect	Genotype	Phenotype	F_1 environment	Frequency
			DNA methylation (G, 26; CG, 134; GW, 167; WG, 10; MA, 30)	Age (14), genetics (15), disease (59), physiological status (35), stress (31), environment (16), nutrition (76), substance exposure (157)	Y (37), N (330)	Y (260), N (107)	Y (18), N (349)	341/367
			Histone modifications (G, 29; CG, 31; GW, 26; MA, 1)	Disease (18), physiological status (1), stress (2), environment (3), nutrition (15), substance exposure (53)	Y (1), N (86)	Y (76), N (11)	Y (7), N (80)	71/87
			ncRNAs (CG, 37; GW, 33; MA, 2)	Age (2), genetics (1), disease (16), physiological status (8), stress (3), nutrition (16), substance exposure (31)	Y (4), N (68)	Y (51), N (21)	Y (7), N (65)	68/72
1.2 Late germline segregation (65/66)	Echinodermata (1)	1	DNA methylation (GW)	Environment (E)	Y	Y	Y	1/1
	Gymnosperma (2)	1	DNA methylation (G)	Genetics (I)	N	Y	N	1/1
	Monocotyledona (7)	10	DNA methylation (G, 2; CG, 1; WG, 1)	Genetics (I, 2), environment (E, 1), substance exposure (E, 1)	Y (3), N (1)	Y (4)	Y (1), N (3)	4/4
			Histone modification (GW, 2)	Genetics (I, 2)	Y (2)	Y (2)	N (2)	2/2
			ncRNA (GW, 5)	Genetics (I, 4), environment (E, 1)	Y (5)	Y (3), N (2)	N (5)	5/5
	Eudicotidae (37)	45	DNA methylation (G, 16; CG, 1; GW, 10; WG, 8)	Genetics (I, 27), disease (I, 1), stress (E, 1), environment (E, 4), substance exposure (E, 2)	Y (32), N (3)	Y (33), N (2)	Y (7), N (28)	34/35
			Histone modifications (GW, 8)	Genetics (I, 8)	Y (8)	Y (8)	N (8)	8/8
			ncRNAs (GW, 10)	Genetics (I, 9), environment (E, 1)	Y (9), N (1)	Y (7), N (3)	N (10)	10/10
2. Asexual reproduction (16/16)								
2.1 Gamete-producing (5/5)	Crustacea (1)	1	ncRNAs (GW)	Age (I), nutrition (E)	Y	N	N	1/1
	Insecta (2)	2	DNA methylation (WG)	Physiological status (I)	Y	N	N	1/1
			ncRNAs (GW)	Stress (E)	N	Y	N	1/1
	Eudicotidae (3)	2	DNA methylation (G)	Genetics (I),	Y	N	N	1/1

Table 1. (continued)

Reproduction mode	Taxa (species)	Studies	Epigenetic mechanism and method[b]	Effect	Genotype	Phenotype	F_1 environment	Frequency
				substance exposure (E), environment (E)				
			ncRNAs (GW)	Substance exposure (E), environment (E)	Y	N	N	1/1
2.2 Vegetative (11/11)	Algae (1)	1	DNA methylation (WG)	Environment (E)	Y	Y	Y	1/1
	Cnidaria (1)	1	DNA methylation (GW)	Environment (E)	Y	Y	Y	1/1
	Eudicotidae (8)	9	DNA methylation (G, 4; WG, 3)	Genetics (I, 2), physiological status (I, 2), environment (E, 3), substance exposure (E, 1)	Y (7)	Y (6), N (1)	Y (1), N (6)	7/7
			Histone modifications (CG, 2)	Substance exposure (E, 2)	Y (2)	Y (2)	Y (2)	2/2

[a]Numbers in parentheses represent instances unless otherwise stated. Studies that assessed inheritance of more than one mechanism have multiple entries. For full list of references, see Table S1 in the supplemental information online.
[b]CG, candidate gene; E, extrinsic; G, global (low resolution); GW, genome wide; I, intrinsic; MA, multiple approaches; N, no; WG, whole genome; Y, yes.

Genetic effects

Epigenetic variation can be linked to genetic variation, which should thus be considered in multigenerational studies. This link is a continuum ranging from complete dependence, where epigenetic variation is strictly genetically encoded and associated with predictable phenotypes, to independence, where epigenetic variation may be unpredictable since it arises due to developmental stochasticity regardless of genotype (Box 1) [16,77,78]. When inheritance is partially or fully genetically encoded, epigenetic inheritance should occur regardless of reproductive mode. We found 126 studies that considered genotype (Figure 2C), generally without investigating interdependence of genetic and epigenetic variation. There is some evidence for genetic variation driving epigenetic inheritance, including a study in *Caenorhabditis elegans* that identified genetically driven increases in **H3K9me2** levels until F_{20} [79]. Extensive research has characterized polyploidization and/or hybridization effects on **intergenerational inheritance** in plants (e.g., [80–84]), including a study in rice hybrids showing parental dominance in ncRNA expression in F_{12} [50]. A few studies have also characterized polyploidization and/or hybridization effects in insects [85,86] and fish [87,88]. However, epigenetic variation arises rapidly compared with genetic variation [89,90]. A landmark study in thale cress (*Arabidopsis thaliana*) showed the rate of epimutations was sufficient to uncouple genetic and epigenetic variation [89]. Thus, the relative influence of genotype on epigenetic marks (**obligatory epigenetic variation** vs. **facilitated epigenetic variation**) and the permanency of these effects across generations are likely system-dependent.

Persistence of epigenetic inheritance

While adaptive phenotypes can be epigenetically induced within a single generation, transgenerational epigenetic inheritance is expected to be important for evolution since it can persist for many generations and thus be subject to selection. However, the processes associated with germline segregation likely affect the persistence of epigenetic inheritance. Exceptional

Box 2. Epigenetic plasticity, diversified heritable bet-hedging, and genetic assimilation

Epigenetic variation can increase the phenotypic range encoded by a single genome [52]. Plastic phenotypic responses via a targeted increase or decrease in DNA methylation, ncRNA expression, or specific histone modifications in offspring can be facilitated by epigenetic inheritance from parental generation(s). However, this leads to a uniform response among siblings, which can be maladaptive if the parent incorrectly predicts the environment of the offspring, creating a mismatch between the two environments. An alternative strategy is diversified bet-hedging, which increases epigenetic and phenotypic variation in offspring and their chances to cope with the environment [16,64,137]. Plasticity and diversified bet-hedging are hypothesized to be of particular importance for asexual organisms [52] or genetically impoverished populations (e.g., invasive species), as mechanisms to increase phenotypic diversity despite the lack of genetic variation among full-sibling progeny [52,108]. However, it is expected that diversified bet-hedging would be more important than plasticity for organisms living in highly stochastic, unpredictable environments [108,137]. Epigenetics provide mechanisms for phenotypes induced by bet-hedging strategies to be heritable, thus leading to heritable bet-hedging [64]. These strategies may lead to epigenetic buffering and allow populations to persist in rapidly changing and unpredictable environments [16,64].

When environmental conditions persist and epigenetic variation is maintained across generations, genetic assimilation may occur wherein a plastic phenotype becomes genetically encoded. Epigenetically facilitated genetic assimilation can occur due to cytosine methylation and other DNA modifications becoming spontaneously deaminated, commonly resulting in a point mutation from C to T [11,138], although mutations to A and G are also possible [138], with frequency of mutation 10–50 times more than unmethylated cytosines [139]. Histone modifications and ncRNA expression also contribute to increased mutagenesis [11]. Genetic assimilation may occur via increased mutagenesis, but also through differential marking of transposable element machinery or through promoting differential silencing and activation of **copy number variations (CNVs)** [108,119,121]. Either of these processes may lead to genetic assimilation of phenotypic variants (Figure I), resulting in transgenerational epigenetic inheritance becoming stably genetically encoded (Figure I) [11]. Thus, epigenetic mechanisms can, over generational time, contribute to the genetic evolution of organisms.

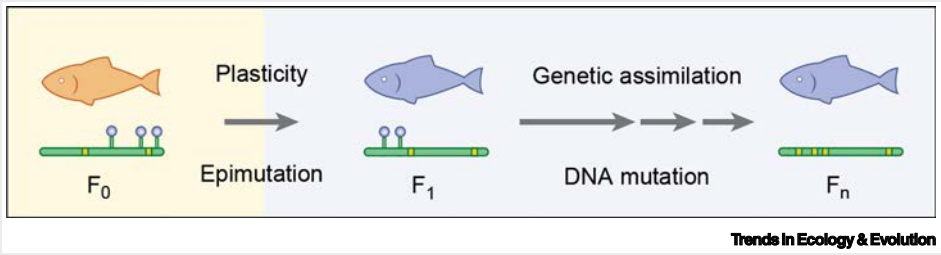

Figure I. Genetic assimilation of plastic epigenetic changes after prolonged environmental conditions. Environmental change induces epigenetic differences, which, after a sufficiently long period of environmental stability, result in point mutations and genetic assimilation. Pure and facilitated epigenetic variants in F_1 may result in genetic variants in F_n. Lollipops represent epigenetic modifications, while yellow bars represent genetic variation. Two different environments are shown by red and blue backgrounds. Novel phenotypes are indicated by altered fish color.

cases of epigenetic inheritance were reported in plants, likely due to late germline segregation and the lack of epigenetic resetting both favoring long-term inheritance. After five generations of selection in *A. thaliana*, novel phenotypes induced in the F_6 were linked to epigenomic patterns stably inherited for two generations, contributing to rapid adaptation (Figure 1) [91]. Expression of ncRNA induced by a polyploidization event persisted for six to 12 generations in hybrids between Asian rice (*O. sativa)* and perennial wild rice (*Oryza longistaminata)* (Figure 1) [50,92]. Other exceptional examples of multigenerational inheritance found persistence of DNA methylation in F_4 [80,93], F_5 [94], and F_6 [51], and ncRNA expression in F_6 [92] and F_{12} [95,96]. These exceptional instances of inheritance often involve a genetic basis underlying epigenetic variation.

While long-term epigenetic inheritance is less likely in organisms with early germline segregation and some extent of germline epigenome reprogramming, the persistence of epigenetic marks in some oviparous organisms rivals that of plants. For instance, studies in *C. elegans*,

a hermaphroditic metazoan with early germline segregation and frequent self-fertilization [97], detected inheritance up to F_4 [98], F_5 [99], and F_{20} [79]. Epigenetic inheritance in viviparous and other oviparous species was often significant to F_3 or F_4 but rarely assessed beyond these generations.

Phenotypic consequences

Persistent epigenetic effects can impact offspring phenotype and fitness, which was assessed in 418 studies (Figure 2C). While some of these studies show that the interplay between epigenetic variation, genetic variation, and gene expression is dynamic [77], epigenetic effects on RNA and downstream molecular phenotypes were only assessed in 128 and 106 studies, respectively. Other studies evaluated effects on morphology ($n = 140$), function ($n = 93$), behavior ($n = 39$), performance (e.g., growth, yield; $n = 11$), and health ($n = 38$). Epigenetic inheritance has been associated with behavior [100–102], longevity [79], and growth and survival [41]. In the agricultural context, epigenetic inheritance can influence phenotypes relevant to crop domestication by improving performance traits such as growth [94,103] and pathogen resistance [104].

Environmental predictability

Regardless of reproductive mode, multigenerational inheritance can be adaptive when parents accurately 'predict' the future offspring environment but are likely maladaptive otherwise [64,105–108]. Offspring fitness in matched versus mismatched environments is understudied ($n = 45$), yet evidence indicates that correct parental prediction of the offspring environment increases offspring fitness. For instance, reciprocally transplanted vegetative reef building corals that modified DNA methylation to resemble local, established corals had higher fitness [62]. In predictable chronic stress experiments, altered DNA methylation was reported for 200 generations of asexual unicellular green alga [63]. Disruption of epigenetic inheritance reduced algal adaptability, highlighting the importance of environmental predictability on the adaptive value of epigenetic inheritance [63]. This suggests that intergenerational inheritance can be maladaptive when environments are incorrectly predicted and offspring are unable to override parental effects. Environmental predictability may be related to a species' lifespan rather than reproductive mode, with short-lived species having higher environmental similarity between generations than long-lived species. However, multigenerational studies in long-lived species pose considerable logistical issues.

A framework for understanding the eco-evolutionary significance of epigenomic variation

Our review shows that multigenerational persistence of epigenomic patterns is common, but also highlights many knowledge gaps that remain to be filled. Most of the current literature focuses on DNA methylation, likely due to the straightforward methods associated with methylation analysis, and the stability of this mark. There are multitudes of studies on model mammals (mouse, rat, human) due to the biomedical field pioneering the study of epigenetic inheritance. This has led to the repeated confirmation that epigenetic inheritance is common in viviparous animals, although highly diverse oviparous taxa (e.g., fishes, insects) are understudied. Here, we propose a roadmap as a potential guide for future research to better understand the persistence and evolutionary significance of epigenomic patterns across generations via three independent but interconnected steps (Figure 3).

In Step 1, we suggest further research on the identification, characterization, and phenotypic consequences of epigenomic variation, which is the focus of most current studies. Quantitative epigenomic studies assessing the relative importance of environmental versus genetic sources

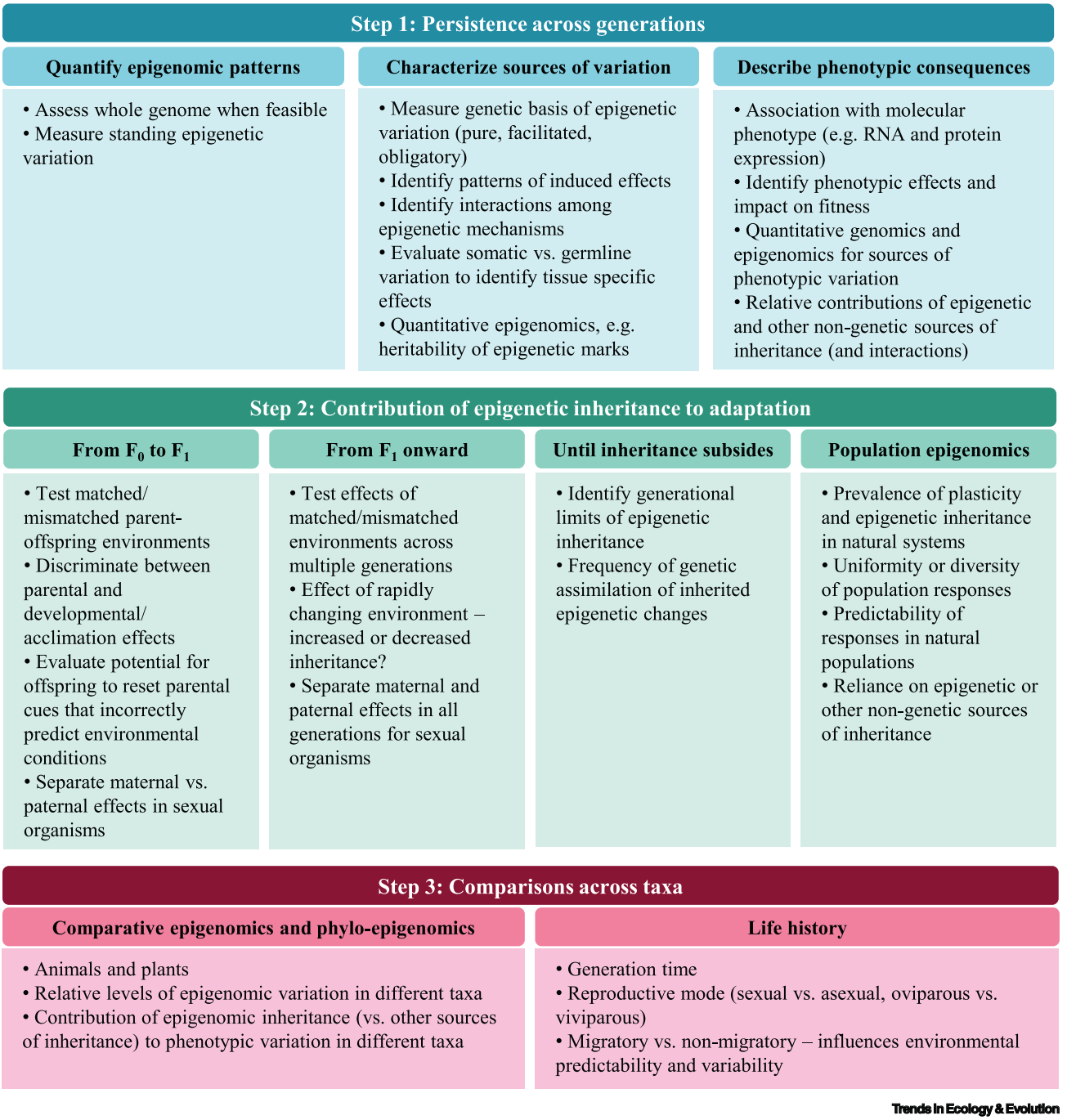

Figure 3. Research roadmap to study the persistence and eco-evolutionary significance of epigenomic patterns over generational time.

of epigenetic variation (e.g., [109–111]), as well as studies linking phenotypic variation to an epigenetic basis, will inform our understanding of the sources and heritability of epigenetic variation. Studies should assess epigenetic inheritance and phenotypic outcomes until inheritance subsides (in some cases, for tens to hundreds of generations) to understand the long-term impacts

of epigenetic inheritance with different reproductive modes and timing of germline segregation, as outlined in Step 1 (Figure 3).

Step 2 focuses on studying the adaptive potential of epigenetic inheritance to clarify its role in the persistence of organisms. Novel phenotypes can be rapidly induced in response to environmental change [16,112] via epigenetic mechanisms and, when inherited, the offspring is primed for an environment predicted based on parental experience [12,113,114]. Thus, even in a single generation, epigenetically induced phenotypes can be adaptive in the face of environmental change through **epigenetic buffering**. However, multigenerational epigenomic patterns are expected to play a more important role for adaptation. Epigenetic inheritance can result in intergenerational inheritance of phenotypes and cellular states, although an organism's epigenetic state can also be subject to selection (for reviews see [115–117]). Theory predicts that epigenetic inheritance will accelerate adaptation if epigenetic changes are stable and have a small effect, while they will slow adaptation if they have the same fitness effects as genetic variation [118]. Organisms with phenotype switching can have an 'epigenetic advantage' in rapidly changing or temporally complex environments, contributing to population adaptability: environmentally induced epigenetic phenotypes can arise simultaneously in many individuals to cope with transient environments and, unlike mutations, can be reversed [10,112]. However, long-term multigenerational studies of natural populations are rare due to the effort and resources required to quantify epigenetic inheritance in such settings. Multigenerational epigenetic changes may also be genetically assimilated to form stable genetic variants (Box 2) [64,108,119]. There is evidence for DNA mutations arising and becoming assimilated in the genome due to DNA methylation [120,121], histone modifications [122,123], and ncRNAs [124]. Thus, epigenetic mechanisms can result in short-term modifications to phenotype and function. They can also create permanent genetic variation when **genetic assimilation** occurs. The importance of epigenetic inheritance in adaptation and the creation of novel genetic mutations can be clarified through proposed research in Step 2 (Figure 3).

Comparisons of patterns and outcomes of epigenomic variation will determine the role of epigenetics in the eco-evolutionary history of species, as outlined in Step 3 (Figure 3). Phylo-epigenetic trees of 176 mammalian species followed evolutionary distances of genetic phylogenetic trees and showed that epigenetic marks relate to life history traits such as age and lifespan [125]. Thus, epigenetic mechanisms likely contribute to evolution and align with genetic measures of evolution, potentially through partial or complete genetic control over the epigenome. However, other sources of nongenetic inheritance should be considered in tandem with epigenetic mechanisms to understand the broad molecular basis of inheritance and adaptation. Representation of species with diverse life history traits (e.g., generation time, migratory behavior) that affect environmental predictability across generations will help to disentangle the relative importance of epigenetic inheritance in response to changing environments. Wide representation of all reproductive modes across taxa is necessary to evaluate the realized significance of epigenetic inheritance in eco-evolutionary potential across the tree of life.

Concluding remarks

Studying the sources and consequences of epigenetic inheritance is critical to understanding nongenetic inheritance, phenotype, and the adaptive potential of populations and species. Our synthesis suggests that reproductive mode and germline development influence the prevalence and persistence of epigenetic inheritance, although many questions remain (see Outstanding questions). It is of utmost importance that the sources, sensitive windows, persistence, fitness consequences, and life history implications of epigenetic inheritance are quantified to better understand their contribution to adaptation and evolution, particularly in the context of rapid environmental change.

Outstanding questions

Do reproductive mode and germline segregation timing affect the genomic extent of epigenome inheritance intergenerationally and transgenerationally? Is there variation in the relative inheritance of different epigenetic marks (histones, ncRNA expression, and DNA methylation)?

What extent of epigenetic changes are communicated between soma and germline once germline segregation is complete?

How do reproductive mode and germline development affect the generation at which epigenetic inheritance subsides? How does this differ among epigenetic mechanisms? Among sexes?

Do the links between epigenetic and genetic variation vary according to reproductive mode? What fraction of epigenetic inheritance is due to parental genotype?

To what extent do reproductive mode and timing of germline segregation influence the contribution of epigenetic variation to nongenetic phenotypic inheritance?

What is the relative importance of epigenetic variation versus other sources of genetic and nongenetic inheritance (e.g., hormones, microbiomes, nutrient provisioning, behavior, habitat choice), and are there interactions among different inheritance mechanisms?

What are the consequences of epigenetic inheritance when parents correctly or incorrectly predict offspring environment? Can offspring modify maladaptive inherited epigenetic marks? Can epigenetic inheritance result in parent–offspring conflict?

Do taxa with different reproductive modes differ with respect to levels of epigenetic variation and inheritance? Does the contribution of epigenetic inheritance to phenotype differ among taxa based on life history?

How does epigenetic inheritance contribute to the persistence of natural populations reproducing sexually and asexually?

Do populations of a species differ in their capacity for epigenetic inheritance (e.g., due to genetic and environmental differences)?

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Declaration of interests

No interests are declared.

Supplemental information

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PROBLEMS & PARADIGMS

Prospects & Overviews

Epigenetics across the evolutionary tree: New paradigms from non-model animals

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Abstract

All animals have evolved solutions to manage their genomes, enabling the efficient organization of meters of DNA strands in the nucleus and allowing for nuanced regulation of gene expression while keeping transposable elements suppressed. Epigenetic modifications are central to accomplishing all these. Recent advances in sequencing technologies and the development of techniques that profile epigenetic marks and chromatin accessibility using reagents that can be used in any species has catapulted epigenomic studies in diverse animal species, shedding light on the multitude of epigenomic mechanisms utilized across the evolutionary tree. Now, comparative epigenomics is a rapidly growing field that is uncovering mechanistic aspects of epigenetic modifications and chromatin organization in non-model invertebrates, ranging from octopus to sponges. This review puts recent discoveries in the epigenetics of non-model invertebrates in historical context, and describes new insight into the patterning and functions of DNA methylation and other highly conserved epigenetic modifications.

KEYWORDS
cephalopods, DNA methylation, epigenetics, epigenomics, non-model organisms, transposable elements

INTRODUCTION

Ever since DNA was discovered as the genetic material over a century ago, scientists have been intent on deciphering how it serves as the organismal blueprint and how it is packaged in the nucleus. Much of the pioneering work in genetics and epigenetics utilized diverse animals and plants, but the scope narrowed to a few model organisms during the molecular biology and genomics era. A recent flurry of papers using non-model animals has updated our understanding of how epigenetic modifications play diverse roles in organizing the genome, regulating gene expression and controlling transposable element (TE) mobilization. DNA methylation is the best studied epigenetic modification in non-model species, and is therefore covered in the most detail here. Since the extensive work done in insect epigenetics has been reviewed recently,[1,2] we focus on non-model invertebrates, including sponges, tunicates, and on exciting new studies in cephalopods, where epigenetic data complements centuries of work by naturalists, neuroscientists, developmental, regenerative, and reproductive biologists.

Abbreviations: piRNA, piwi interacting RNA; TE, transposable element; TSS, transcription start site; WGBS, whole genome bisulfite sequencing.

THE NON-MODEL ORGANISM RENAISSANCE IN (EPI)GENOMICS

In the pioneering days of genetics research, organisms of choice were those in close reach and amenable to experimentation; Mendel's peas, Morgan's fruit flies, and McClintock's maize are famous examples. Innumerable foundational discoveries in animal genetics and chromatin biology were enabled by the unique features of species that were easy to obtain: the large chromosomes of salamander embryos used by

Flemming provided the first description of chromatin, the prominent nuclei and fast divisions in roundworm, grasshopper, and sea urchin embryos enabled Boveri and Sutton to show that chromosomes contain genetic material and the rapid meiotic divisions of starfish, clam, and frog oocytes enabled Ruderman and Hunt to identify mechanisms regulating chromosome segregation.

In subsequent years, however, the focus has narrowed, with mice, zebrafish, fruit flies, and nematode worms becoming widely used model organisms in genetic and epigenetic studies, the latter two species often serving as the only examples of invertebrates. In the past decade, fast and affordable genome sequencing and epigenomic profiling technologies have been developed, enabling comparative epigenomics research to branch across the evolutionary tree. Publicly available and annotated genome sequences generated through efforts like the Darwin Tree of Life Programme,[3] which aims to sequence most of the 41 000 animals in Britain and Ireland, and the Zoonomia Consortium, which has recently assembled genomes for 131 animal species.[4] In parallel, affordable approaches to carry out epigenomic studies are being developed and applied broadly. BioProject – a central repository for genomic datasets generated by collaborative groups – has cataloged nearly 900 epigenomic datasets from invertebrates, although fewer than 20% of these are from non-model animals, and strikingly an entire phyla – Annealida – has yet to have a dataset included in this repository (Figure 1). A recent preprint describing the DNA methylation landscape in 580 non-model animals – many of which do not have a reference genome[5] – represents a significant advance for comparative epigenomics, although only a quarter of the datasets in this effort were generated from invertebrate species. Thus, while further diversification of the species used for epigenomic studies is needed, this gap is being filled quickly by researchers who are gathering samples of animals from around the world for performing epigenomic analysis, uncovering conserved epigenetic marks on heterochromatin and enhancers as well as unique features of the epigenomic landscape.

GENOME MANAGEMENT

All animal cells have evolved diverse epigenetic mechanisms to manage gene regulation, transposon suppression, and genome organization. Genes serve as the instruction manual for the construction and function of all cells, and nuanced and highly responsive gene expression profiles that shape cell identity and enable cells to respond to stimuli are largely dictated by epigenetic modifications. In many animals, half of the genome is made up of repetitive sequences, that is, simple repeats, satellites, and TEs. Having a layered approach to control of these "jumping genes" is important as mobilization can wreak havoc on genome integrity and gene regulatory mechanisms.[9,10,11] Finally, as the DNA strand is long and unwieldy, it needs to be wrapped, packaged, and organized in the nucleus. Topological genome organization is, in part, dictated by epigenetic modifications that enable long range connections in the genome and also tether regions of the genome to structures in the nucleus. Epigenetics is key to managing each of these processes, with marks that keep the chromatin open as critical to allow gene activation while heterochromatin represses gene expression, keeps TEs in check, and tethers regions of the genome to the nuclear lamina, nucleolus, and other nuclear structures.

Importantly, genome management functions can be performed by more than one tool in the epigenetic toolbox, allowing for compensation in cases when an epigenetic mark becomes depleted or repatterned. For instance, in embryonic stem cells engineered to be devoid of DNA methylation, other repressive epigenetic marks – H3 lysine 9 trimethylation (H3K9me3) or H3K27me3 – compensate to silence different populations of TEs.[12] We made a similar discovery in mouse livers engineered to have DNA hypomethylation in hepatocytes. We found that, although TEs were hypomethylated, they were not expressed, which we attributed to repatterning of another repressive mark, H3K27me3 which became enriched on hypomethylated transposons.[13] These studies suggest plasticity of epigenetic modifications, raising the possibility that some epigenetic marks serve different roles in different species.

Finally, combining model and non-model organisms is emerging as a powerful approach to carry out functional epigenomic studies. This is exemplified by a recent study that tested the hypothesis that there are conserved regulatory relationship between pairs of genes positioned side by side in the genomes of organisms separated by millions of years of evolution (i.e., microsyntenies).[14] They asked whether regulatory regions for one gene (the target) are located in the body of the other (the bystander) by identifying microsyntenies conserved in human, mouse, zebrafish, *Drosophila melanogaster*, and the sponge, *Amphimedon*. They then tested whether a microsyntenic locus indeed encapsulated both the regulatory and the target regions by focusing on the conserved microsynty containing the genes *Islet* (target) and *Scaper* (bystander). Remarkably, when the sponge locus was inserted in the zebrafish and mouse genome, the target gene was expressed in specific regions of the developing brain, fin, and ear in zebrafish and the neural tube limb bud, otic vesicle, and optic cup in the mouse, all structures that are lacking in the sponge body plan. This pattern was generated despite a lack of sequence homology across species, indicating that transcription factor binding motif was an unlikely mechanism for this regulatory relationship. Instead, this data suggests that the sponge enhancer – and likely the associated and conserved epigenetic marks – could confer regulatory capacity in the context of zebrafish chromatin. This illustrates that gene regulatory mechanisms relying on epigenetic or chromatin structure may function across diverse animals.

THE EPIGENETIC TOOLBOX

In all animals studied to date, DNA is wound around histones creating the nucleosome as a unit of chromatin. The configuration of open or closed chromatin is regulated by a set of epigenetic modifications on histones or DNA. Components of the epigenetic toolbox include methylation of DNA, where 5-methylcytosine (5mC) in the CpG context is most common, although adenine methylation has also been reported.[15,16] Other tools that cells deploy to organize the genome and regulate gene expression are plentiful and diverse

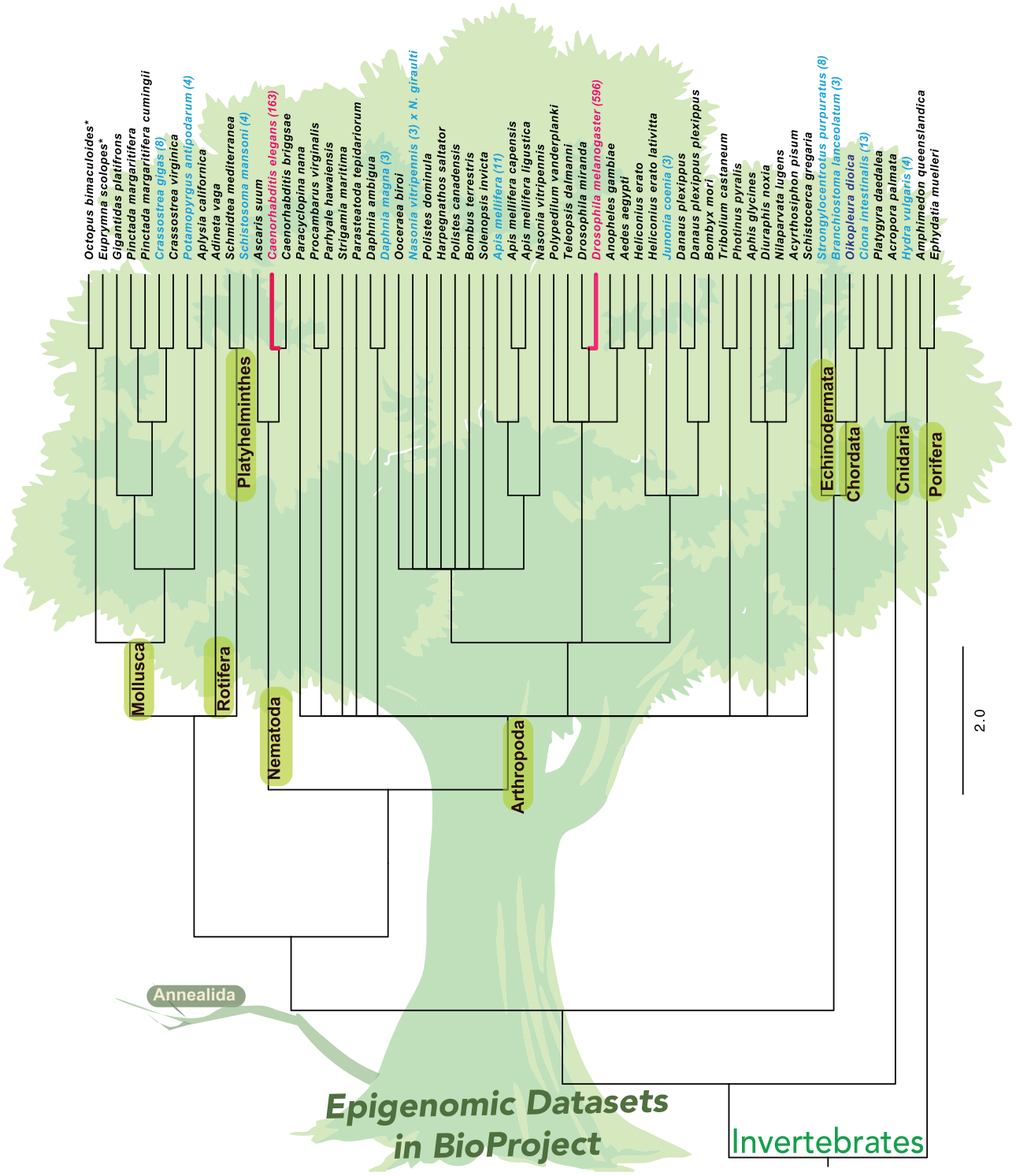

FIGURE 1 The biodiversity of epigenomic datasets for invertebrate animals cataloged in BioProject (NCBI). A tree of the invertebrate animals for which epigenomic datasets have been deposited in BioProject as of June 2022. All species with more than two datasets present have the number indicated after their name and are indicated with blue or pink font, with pink representing model organisms (*C. elegans* and *D. melanogaster*). Species with only one or two datasets are written in black. *Indicates two species of cephalopods – *Octopus bimaculoides Euprymna scolopes* – who have had some epigenomic analysis by our group[6] and others.[7,8] Despite the abundance of animals in the annelid phylum, no datasets from an annelid have deposited to date in BioProject. Scale bar indicates 20% genetic variation.

Box 1. The Epigenetic Toolkit

ATAC-seq: Assay for Transposase-Accessible Chromatin with high-throughput sequencing allows researchers to profile open and closed chromatin from cells of any animal without the use of species-specific reagents.

Chromatin Conformation Capture: Molecular approaches that enable identification of the regions of the genome which come into contact with each other, defining the structural domains of the genome. These approaches include techniques like circular chromosome conformation capture (4C), which enables the investigation of all the regions of the genome which interact with one locus, and the powerful genome wide interaction maps generated by Hi-C, which relies on capturing the physical proximity between loci in a 3D space followed by high throughput sequencing.

Chromatin States: Binning the regions of the genome based on shared combinations of epigenetic marks, similar to how the Decimal System organizes books in a library. Loci that have the same epigenetic marks are thought to have the same capacity for being expressed.

DNA methylation: Methyl group added to cytosine residues in the CpG context, referred to as 5-Methyl Cytosine (5mC). Cytosine methylation in other contexts (i.e. CpH where H is any base other than guanine) is also detected in some animals, mostly vertebrates as well as in some plants and prokaryotes. In some scenarios (i.e. CpG islands), 5mC is repressive, whereas in others (i.e. gene body methylation), high levels of DNA methylation is associated with gene expression.

DNA methyltransferases (DNMTs): Enzymes that transfer a methyl group to a residue in the DNA backbone, with 5-methylcytosine (5mC) the most common in animals. DNMT1 is the maintenance methyltransferase which copies DNA methylation patterns from parent to daughter strands during DNA replication. Homologs of DNMT3a and 3b are the de novo methyltransferases that deposit 5mC to new loci.

Histone Post-Translational Modifications (PTMs): The residues of histone tails – i.e. the portion of histone proteins that are positioned outside of the wrap around the nucleosome – can be modified, with the most common modifications being mono-, di- or tri-methylation, acetylation, phosphorylation, and ubiquitination. Different modifications on the same residue can have opposing effects.

Histone H3 lysine 9 di or tri methylation (H3K9me2/3): A canonical marker of constitutive heterochromatin.

Histone H3 lysine 27 modification: H3K27ac is a marker of active enhancers and actively transcribed genes, whereas H23K27me3 is a marker on promoters of repressed genes which are highly regulated.

Histone acetylation: Unmodified histones are positively charged, facilitating interaction with negatively charged DNA. Addition of acetyl groups neutralizes the positive charge on unmodified histones, and thereby decreases interaction between histones and DNA, resulting in chromatin opening. Acetylation is mediated by the histone acetyltransferases (HAT) and are removed by deacetylases (HDAC). The most commonly acetylated residues are H3K27ac, H3K9ac, H4K5ac and H4K8ac.

Non-coding RNAs (ncRNA): There are several classes of RNA molecules that do not code for proteins, and of these, microRNAs (miRNA), piwi interacting RNAs (piRNAs) and long-noncoding RNAs (lncRNAs) are the most well studied ncRNAs that can interact directly with the DNA to regulate the open or closed state of the chromatin.

Nucleosome remodeling and deacetylase (NuRD) Complex: A complex of 7 subunits that include histone deacetylase enzymes and methyl binding proteins, thereby linking histone modifications and DNA methylation.

Ten eleven translocation (TET) enzymes: Carry out oxidation of 5mC converting it to 5-hydroxymethylcytosine (5hmC) and responsible for active removal of DNA methylation.

post-translational modifications (PTM) on histone tails, such as methylation, acetylation, phosphorylation, and ubiquitination on distinct residues of histone tails, non-coding RNAs, histone variants, and structural proteins like actin[17,18] (Box 1).

With the advent of whole genome sequencing, epigenomics arose as a field using experimental and computational approaches that enable researchers to extract information about epigenetic modifications and chromatin organization across an entire genome (Box 1). Many epigenomic techniques commonly used in model organisms rely on antibodies that recognize specific histone modifications or the enzymes that write epigenetic modifications or those that bind chromatin. Issues with antibody crossreactivity can limit utility in other animals. Such studies in non-model organisms rely on techniques that assess chromatin and epigenetic landscape using reagents that can be used across species coupled with next-generation sequencing approaches to obtain genome wide data. DNA methylation can be profiled using only genomic DNA treated with bisulfite which can chemically convert any unmodified cytosine, and the chromatin landscape can be assessed using Assay for Transposase-Accessible Chromatin (ATAC-seq) which relies on a transposase inserting a tag that can then be used in sequencing. Chromosome conformation capture techniques like Hi-C can be used to define genome organization and regulatory relationships in any cell type, as only molecular biology reagents and high throughput sequencing are required. Despite these advances, the mechanisms of genome organization and epigenome patterning remain unknown for most animals. Current efforts at broadening the scope of animals

included in epigenomic studies and the application of approaches to manipulate the epigenome in non-model organisms are important for understanding these fundamental principles of genome regulation.

When sequencing technologies were new, a pragmatic approach was taken to prioritize which species had their genomes sequenced, that is, those with small genomes or those holding great importance to the biomedical research field were selected as the pioneers. Technological advances and declining costs have massively expanded the number of animals amenable to genomic and epigenomic studies, with the genomes of over a thousand animals now available.

Many of the histone PMTs that create heterochromatin, mark enhancers, and regulate transcription are well conserved across eukaryotes, however, little is known about how these are used in combination or what potential novel epigenetic modifications may be present in little studied animals. Comparative epigenomics can elucidate which mechanisms are distinct to some animals, and which are universal. The recently discovery of N4-methylcytosine – a modification previously only found in bacteria – present on active TEs and tandem repeats in a rotifer[19] exemplifies the power of comparative epigenomics to find novel mechanisms of genome management.

HETEROCHROMATIN, EUCHROMATIN, AND CHROMATIN STATES

Animal genomes are broadly divided into open (euchromatic) and closed (heterochromatic) configurations. Euchromatic regions are packed with genes, replicated during early S-phase, and decorated with epigenetic modifications such as acetylated histones that push histones away from each other. This allows access of transcription factors and other proteins to interact with and regulate DNA. Regulatory marks such as those that decorate enhancers (H3K27ac) or the acetylation of multiple lysine residues in H3 and H4 are enriched in euchromatin. Actively transcribed genes are typically marked by H3K4me3 surrounding the transcription start site (TSS), but the pattern of this distribution varies: in human and sea anemones, H3K4me3 is enriched after the TSS but in human genes, there is also a peak before the TSS that is missing in anemones.[20] This difference was attributed to an interplay with DNA methylation in anemone, which is enriched on gene bodies of actively transcribed genes and can function to restrict H3K4me3 occupation.

Heterochromatin is typified by tightly packed nucleosomes.[21] Constitutive heterochromatin is defined as the regions that are locked down, such as repetitive elements, regions associated with structural features of chromosomes, the rim of the nucleolus, and the nuclear lamina. In model organisms, H3K9me2/3 marks heterochromatin, and, in vertebrate genomes, it co-occurs with DNA methylation. Facultative – or flexible – heterochromatin can switch between closed and open, so that in some cellular states transcription factor access is restricted, but in other states it is permitted. The repressive mark H3K27me3 is characteristic of facultative heterochromatin and is deposited by the polycomb repressive complex.[21] The pattering and function of these marks in model organisms has been reviewed extensively.[22,23,1,24,21] These marks are highly conserved in animals as diverse as insects,[1] anemone,[20] flatworms,[24] cephalopods,[6] and sponges,[26] indicating that these are common and efficient mechanisms for creating heterochromatin.

The combinatorial nature of the epigenetic code means that any genomic locus can have multiple marks. A powerful machine learning approach that combines multiple epigenetic profiling datasets to predict the likelihood of marks co-occurring at the same locus and as well as those that do not co-occur defines chromatin states.[27,28] These states are inferred to have functional properties. For instance, H3K4me3/2 and H3K36me3 frequently co-occur on actively transcribed genes, regions which exclude H3K9me3, and thus are highly permissive for transcription factor binding and transcriptional complex access. Distinct chromatin states can predict cell identity even before a cell fully differentiates as the atlas of chromatin states across mouse development showed.[29] Chromatin states can also indicate regions that repress TEs or confer structural properties to chromatin, such as the static states that were described across human tissues.[30]

The sponge, *Amphimedon queenslandica*, which had its genome sequenced over a decade ago,[31] is one of the few non-model invertebrates where chromatin states have been defined. Profiles of RNA polymerase and histone PTMs known to be involved in gene activation (H3K4me3), facultative chromatin (H3K27me3), and marks of enhancers (H3K4me1 and H3K27ac) were integrated to define states predicting enhancers, gene expression profiles. This showed that H3K4me3 marks genes that were highly expressed compared to those marked with H3K27me3 which were silenced[26] indicating a high conservation of this gene regulatory code. Sponges occupy a unique position at the base of the metazoan branch and thus these findings uncover ancient mechanisms of genome organization and gene regulation.

DNA METHYLATION: PATTERN AND FUNCTION

Over half a century ago, the "epi-cytosine" present in bovine DNA was described,[32] and one of the first comparative epigenomic studies followed soon after, describing variable levels of 5mC in fish, cow, sheep, insects, and plants, and absent from bacteria and viruses.[33–34] 5mC was then found as a central component of constitutive heterochromatin in mammals, and the first function of DNA methylation was discovered to be in X-inactivation,[35] imprinting,[36] and transposon silencing.[37,10,38] Later, DNA methylation was shown to contribute silencing some genes in a cell type specific fashion[24] and in cancer.

Biochemical techniques can measure bulk 5mC levels without requiring a sequenced genome while sequencing based approaches are required for determining methylation patterns at base pair resolution. Analysis of CpG distribution in sequenced genomes is a computational approach to infer methylation levels. This is based on the hypermutability of methylated cytosines, whereby 5mC is deaminated to thymidine, creating C-G mismatches. Over time, genomes with CpG methylation have fewer cytosines than expected so that the observed:expected (O:E) ratio of CpGs serves as a proxy for DNA methylation: an O:E

ratio well below 1, like in zebrafish and mice, means that CpGs were hypermutated and thus likely methylated, whereas a CpG ratio close to 1 indicates no or low methylation, like in *D. melanogaster* and *C. elegans*. The genomes of octopus and sea squirt have o:e ratios little less than,1[7,39,6] consistent with the low level of methylation in these species. This feature of hypermutability indicates that retaining DNA methylation on protein coding regions comes at a cost, while it can be advantageous on TEs where mutations reduce mobilization capacity.

Compared to profiling histone modifications where specific antibodies and tissue processing protocols are required, generating methylome datasets only relies on extracting genomic DNA, a high throughput sequencing facility and alignment to a reference genome (although one recent study profiled the methylome using a reference genome independent approach[5]). Common approaches for base-pair resolution of DNA methylation rely on whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS). These techniques have shown that 5mC is found primarily in the CpG context,[40,7,41,42] with a low level of non-CpG methylation[7] in vertebrates, where CpG methylation is enriched in repetitive DNA – mostly transposons – pericentromeric regions and telomeres. Additionally, some gene bodies have high methylation levels in both vertebrates and invertebrates.

The essential role of DNA methylation in TEs suppression is reflected in a static pattern of 5mC across vertebrate cell types, with high levels in repetitive DNA and absence from CpG islands present in promoters.[43,44,45,46] A compelling theory is that TE suppression was the first function of DNA methylation, and then other silencing functions evolved later.[38] Early investigators in the DNA methylation field entertained the idea that it functioned as a broad mechanism of gene silencing. This was supported by findings that methylation of regulatory elements, either when introduced by experimental manipulation[47,48] or by pathological changes, such as cancer,[49] correlated with gene silencing. This paradigm has infiltrated textbooks and prominent review articles, but there is scant evidence supporting the notion that DNA methylation plays anything but a backstage role in regulating gene expression under physiological conditions. An illustrative example is provided by a recent study using WGBS to profile DNA methylation and ATAC-seq in specific cell types isolated during zebrafish fin regeneration. Profiling samples collected following amputation revealed no significant changes in DNA methylation patterns, despite widespread transcriptomic and chromatin accessibility changes.[50] Thus, even during a physiological process that is characterized by dramatic changes in cell identity and behavior, the DNA methylation pattern remained unchanged. Another example provided by our studies of zebrafish mutants with DNA hypomethylation showed widespread gene expression changes, but none could be attributed to loss of promoter methylation.[20,52,53] Instead, we concluded that the observed gene expression changes was due to transposon de-repression as a consequence of DNA methylation loss which then triggered activation of immune response genes.[20,53–54] Finally, the report that fewer than 100 genes changed expression when DNA methylation was introduced into a budding yeast, which lacks endogenous DNA methylation, further validates the idea that DNA methylation plays only a minor role, if at all, in regulating gene expression.[55] However, whether DNA methylation regulates genes in other species remains to be investigated.

THE HIGHS AND LOWS OF DNA METHYLATION IN DIVERSE ANIMAL SPECIES

Early studies on DNA methylation included mammals, fish, sea squirts,[40] sea urchins,[56] and insects. Further research on the function of DNA methylation in animals has largely focused on vertebrate animals and plants, as the most commonly used invertebrate model organisms – *D. melanogaster* and Caenorhabditis elegans – lack DNA methylation entirely.

In recent years, the tide has turned, with methylome maps available from a wide range of organisms, with a remarkable 580 methylomes reported in a single study this year.[5] This facilitates comparative epigenomic studies which have revealed three broad categories of DNA methylation patterns (Figure 2): (1) hypermethylated genomes like vertebrates, over 50% of all CpGs are methylated; (2) hypomethylated species, such as sea squirts, lancet, many mollusks, several insects, and most sponges,[57,7,41,42] 20% or fewer of all CpGs are methylated; and (3) unmethylated species like *D. melanogaster*, *C. elegans*, and other nematodes (Figures 2–3). Within these broad categories, subcategories are emerging as more animals are analyzed. For instance, *Ciona intestinalis* has an overall higher level of methylation than honeybee or octopus.[7,6,58,42] Other clear exceptions are of great interest: while some sponges have low methylation patterns like *C. intestinalis*,[23,7,41,58,42] *A. queenslandica* has a hypermethylation pattern that resembles vertebrates.[57] The surprising finding of a vertebrate methylation pattern in a sponge was attributed to convergent evolution.

In species with hypomethylated genomes, 5mC is clustered on a subset of gene bodies, but is largely absent from TEs and intergenic regions. A common feature of both hyper- and hypomethylated genomes is that promoters are devoid of methylation[59] (Figure 2), potentially protecting them from being shunted to the constitutive, methylated heterochromatin which would permanently silence those genes. Both hyper and hypomethylated species also have DNA methylation on a subset of gene bodies where it is correlated with higher levels of transcription in all species examined.[60,41,61,6,62,63,58,42] This correlation may be set via the interplay between the methylation machinery and histone PTMs, with multiple examples of crosstalk between epigenetic marks. For example, DNMTs have a domain that recognizes H3K36me3, which is found on actively transcribed genes.[64] Insight into how the gene body methylation pattern is set in hypomethylated species came from studies in anemone, tunicates, and silk moth, where it was found that orthologs of genes marked by H3K36me3 in *Drosophila* are those that are decorated with 5mC in these species.[62] Since H3K36me3 is a mark of actively transcribed genes, this correlation implicates H3K36me3 as an epigenetic modification that activates expression of these genes, with DNA methylation layered on as an additional feature.

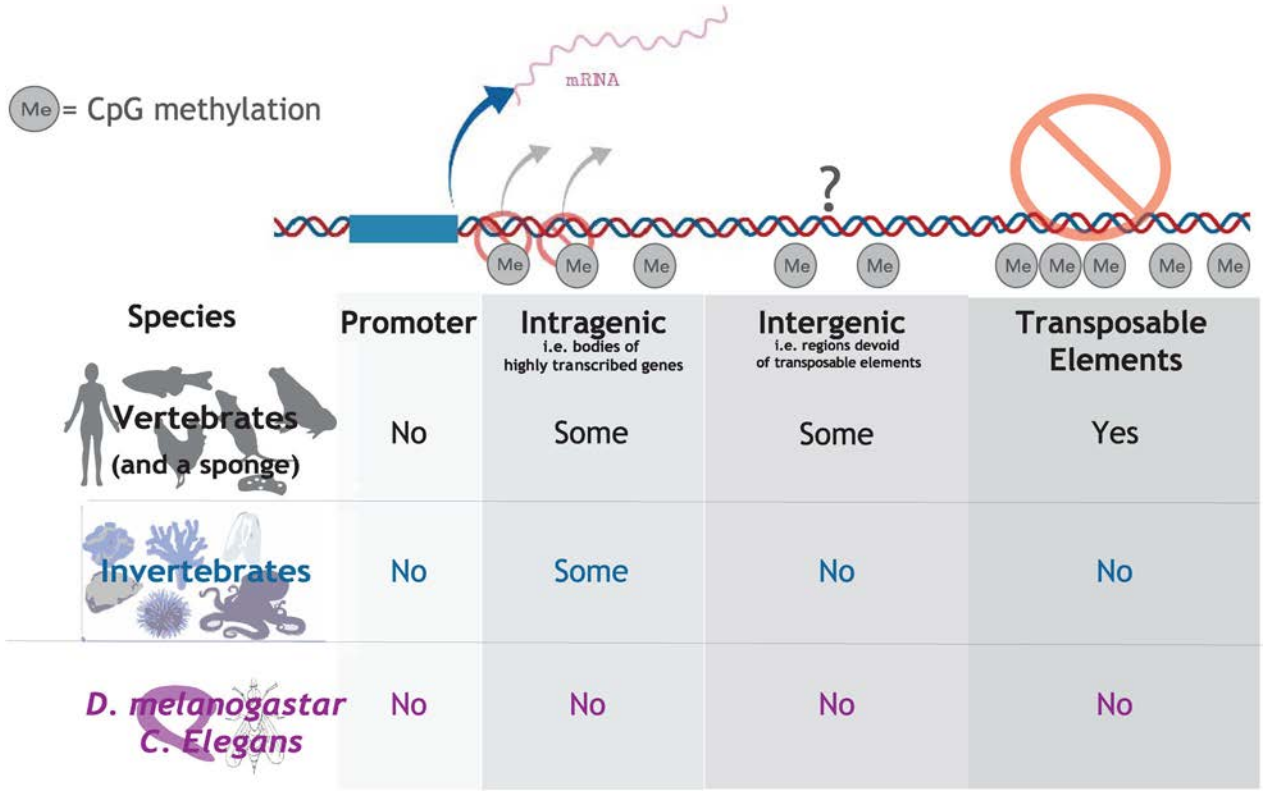

FIGURE 2 Patterns and functions of DNA methylation in animals. 5-Methylcytosine (5mC, indicated by Me) is absent from promoters of all species, prevents spurious transcription by decorating gene bodies, and blocks transposon activation in vertebrates.

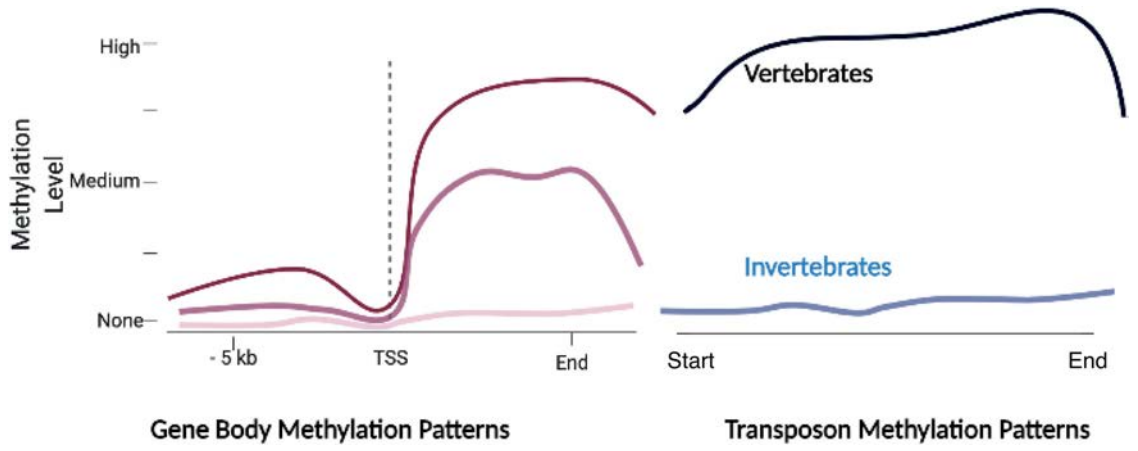

FIGURE 3 Patterns of DNA methylation levels on genes and transposons. Species with hyper and hypomethylated genomes have different methylome patterns. Some genes have no DNA methylation on gene bodies, while others have high levels of methylation throughout the gene body, and others have intermediate level of methylation. Most transposons are highly methylated in species with hypermethylated genomes while species with hypomethylated genomes have low to no methylation.

A recent study examined WGBS data from the brain of 12 distantly related animal species including analysis of human, zebrafish, and species that are not well studied, such as finch, lamprey, lancet, octopus, and anemone.[7] This verified the hypermethylation of vertebrate genomes but showed that lamprey and two bird species had slightly different patterns, suggesting that genome hypermethylation evolved after the branching between lampreys and jawed vertebrates. This indicated that, in animals, CpH methylation evolved in vertebrates, but it remains unclear what the function of CpH methylation is in the animals that have it. We extended CpGs methylation analysis by dissecting the methylation pattern in *Octopus bimaculoides*, revealing that the gene bodies of 1/3 of all genes are highly methylated, while the vast

majority of genes had low to no methylation. The methylation pattern was directly correlated with a high level of expression across tissue types,[6] suggesting that gene body methylation serves to maintain static expression of housekeeping genes.

Functional analysis of DNA methylation in whole animals requires experimental systems that permit manipulation of methylation patterns. We used genetics in zebrafish to show that loss of either *dnmt1* or *uhrf1* results in multisystemic developmental defects, nuclear morphological defects, cell cycle arrest, and embryonic lethality.[20,52,53–54] While many non-model animals are not yet amenable to genetic approaches, using aquatic animals for such studies is straightforward, since DNMT inhibitors can be added to the culture water. Such experiments in oyster,[65] and sea urchin embryos,[66] which have hypomethylated genomes, resulted in embryonic defects and death. In the annelid worm, *Platynereis dumerilii*, which have a relatively high level of bulk DNA methylation, DNMT1 inhibitors caused defects in body patterning, embryo growth and survival, and impaired regeneration of amputated posterior segments.[67] This is important, as it shows that DNA methylation serves an essential function in both hyper and hypomethylated species. In vertebrates, widespread TE activation has been implicated as a primary cause of cell death and embryonic lethality due to DNA methylation loss. However, in mollusks, where CpGs in TEs are not methylated,[6,68,69] it may play a regulatory function on genes that are important for cell survival. Alternatively, loss of DNA methylation in these species may active a cell cycle checkpoint, as we found in zebrafish.[52,70] Functional studies to delineate the mechanisms of DNA methylation patterning and consequences of methylome perturbation in non-model organisms will elucidate specific roles that methylation plays in gene regulation and development.

WRITING DNA METHYLATION: CONSERVED MACHINERY, DIFFERENT METHYLOMES

5mC is written by the DNA methyltransferase (DNMT) enzymes.[71] These are categorized as maintenance DNMTs that copy DNA methylation patterns from the parental to daughter strands during DNA replication (DNMT1) and *de novo* DNMTs (homologs of mammalian DNMT3a and 3b), which deposit 5mC at previously unmethylated loci. Demethylation is largely carried out by – ten eleven translocation (TET) enzymes. Interestingly, many studies have shown that the DNMTs are highly conserved across animals, even in those with low levels of DNA methylation,[72] and roles in addition to maintenance methylation have been reported for DNMT1.[73,67]

Maintenance methylation requires the epigenetic regulator ubiquitin PhD and RING finger containing protein 1 (UHRF1) to recruit DNMT1 to hemi-methylated DNA during S-phase.[74,75] UHRF1 was initially described as a vertebrate specific factor because it was not found in the *D. melanogaster* and *C. elegans* genomes. More recent studies have identified UHRF1, and many of the other genes that encode factors involved in DNA methylation recognition or deposition to be highly conserved, including sponges,[57] mollusks,[6,76,77] and other invertebrates.[23,7,67] Nearly all research into UHRF1 function has utilized vertebrate models, where depletion leads global DNA hypomethylation.[74,41,52,75,78] Interestingly, a study of one fungus that retains DNA methylation showed that loss of UHRF1 did not lead to DNA hypomethylation,[79] suggesting that in some species, it may have lost its essential function in maintenance DNA methylation. UHRF1 also promotes other heterochromatin marks by reading histone modifications characteristic of heterochromatic and transcriptionally inactive regions, including H3K9me3 and unmodified H3R2.[80,81] The combined activity of UHRF1 as an epigenetic reader and as a recruiter of enzymes that write repressive marks ensures that the elements of the repressive epigenome co-occupy regions of the genome packaged in heterochromatin. It is possible that UHRF1 plays a primary role in DNA methylation in some species, while in others it may be predominantly involved in histone modification.

Genetic approaches in vertebrate models have demonstrated that DNMT1 and UHRF1 are essential for DNA methylation, cell viability, and embryonic development.[74,20,52,70,82,54,75,78] By surveying 50 representative genomes covering the span of animal evolution, we found *uhrf1* to be highly conserved with the noted finding that those animals that lacked *uhrf1* also lacked *dnmt1*.[6] This extends work by others showing that DNMTs, UHRF1, and factors that participate in the Nucleosome Remodeling and Deacetylase (NuRD) complex, which functions to link histone acetylation and DNA methylation, are conserved in most animals, with the noted absence in some insects, flatworms, and nematodes. This raises the question of whether the functions ascribed to the vertebrate homologs of the genes that are required for writing, reading, and erasing the methylome are retained in other animals.

What sets the pattern of DNA methylation? One possibility is that intrinsic properties of the DNA methylation machinery target it to specific loci. Alternatively, other epigenetic elements could direct the methylation machinery. A recent study took an innovative approach to address this question by expressing DNMTs from mouse in budding yeast, which lacks DNA methylation.[55] While this only resulted in 5%–25% of all CpGs becoming methylated at levels exceeding 50%, it was remarkable that the pattern of DNA methylation in these methylated yeast genomes resembled mouse genomes: depleted from TSSs and enriched at the 3′ ends of genes. Interestingly, this was anti-correlated, albeit modestly, with highly expressed genes and with a histone PTM marking actively transcribed genes. This suggests that DNMTs have an intrinsic feature that influences DNA methylation patterning.

An alternative model to explain methylome patterning relies on epigenetic elements serving as placeholders to set the DNA methylation pattern. The co-occurrence of H3K9me3 and DNA methylation in vertebrate genomes could facilitate this, perhaps due to the function of UHRF1, the NuRD complex, or by DNMTs themselves, which can link these modifications, suggesting these features are co-deposited. This is supported by experiments examining zygotic methylome establishment where the histone variant H2afz, and H3K4me1 serve as "placeholders," occupying unmethylated regions of the paternal genome so that after the parental methylome is erased, the same pattern can be written on the zygote genome.[83] Experiments which broadened H2afz distribution in sperm resulted in a compensatory exclusion of

DNA methylation from these regions. An interesting finding from this study is that the maternal methylome and H2afz pattern is reconfigured to match the embryonic pattern in maternal haploids, where sperm are not required,[83] indicating that maternal genomes do not require a paternal template for instruction. While these and other studies suggest both intrinsic and placeholder mechanisms participate in methylome patterning, nothing is known about how the canonical invertebrate DNA methylation pattern is set (Figure 3). Functional genomic approaches will advance this, as genome editing is now possible in non-model animals like squid.[51,84]

MANAGING TRANSPOSON THREAT: THE EPIGENOME AND BEYOND

McClintock's discovery of mobile DNA elements upended the scientific community, changing the paradigm that DNA sequences were fixed. From this, we know that the "dark matter" of most genomes are remnants of ancient viruses, which if mobilized, can rearrange the genome and both create novel gene regulatory networks and disrupt gene expression.[85] While such hypermutability mediated by TE mobilization can provide evolutionary advantages, uncontrolled TE activation poses a threat to cell and organismal survival, necessitating effective and redundant mechanisms for suppressing them.

In vertebrate models where functional analysis of DNA methylation has been carried out, it is clear that loss of DNA methylation derepresses some – but not all – TEs. Accordingly, other repressive epigenetic marks have been found to control distinct TE families,[86,87,88,89,90,53,12,91] and compensation for DNA methylation loss on TEs by other repressive marks has been shown in several species,[12,13,92] including one rotifer in which horizontal gene transfer from bacteria enables incorporation of N-4 methylcytosine on TEs.[19]

In some non-methylated organisms, TE suppression is mediated by non-coding RNAs termed piwi interacting RNAs (piRNAs). These compact the chromatin surrounding TEs and degrade TE transcripts.[93] Mammals also have piRNAs, but they are not the primary defense against transposons, except in specific scenarios, such as in germ cells. Interestingly, in mollusks, the PIWI/piRNA pathway appears to be active in both somatic and germ cells.[94] piRNAs are extremely abundant and diverse in sponges and cnidarians,[95] suggesting they may function in these species to suppress transposons or have other regulatory functions.

Other mechanisms for controlling transposons will undoubtedly be discovered by expanding the diversity of animals investigated. The benefit of expanding from mammals, which have a largely homogenous TE population, is that the TE populations in the genomes of other species are more diverse, allowing investigation into how different TE families are regulated. For instance, teleost have the highest number of TE vertebrate superfamilies[96,97,98] and cephalopods have a remarkable diversity in TE demographics as well as high divergence in these populations in closely related species.[99,100] This suggests that TE repression was loosened during the evolution of these animals, facilitating mobilization and expansion of some families.

This could explain some of the novelties of the octopus genome. There is a trend for genome size to directly correlate with transposon burden and it has been proposed that DNA methylation allows this to occur as it serves to keep TEs in check.[101] The *O. bimaculoides* weighs in at over 2.7 gigabases, nearly half of which are repetitive sequences,[99,102] but what makes this unusual is that TEs are not methylated[6] and they are differentially expressed in octopus tissues.[102,103] Thus, TEs may not be dormant viral remnants in octopus, but could allow for genome rearrangements that could generate the unique anatomical and behavioral features of octopus. Cephalopods have a very active RNA editing pathway,[104] which a recent study showed targets repetitive sequences.[99] Thus, editing transposon RNA could act as a "mutagen," perhaps in concert with RNA interference or piRNA strategies. Unraveling the mechanisms by which octopus and other cephalopods mitigate transposon threat will shed light on how these organisms may tolerate – or perhaps even benefit from – controlled release of TEs.

CONCLUSION

Genetics, genomics, and epigenetics research has benefited from the resources, information, and community of researchers that use model organisms. These features were ones that led me to pivot from studying frogs and starfish where the tools were scant, to focusing on zebrafish and mice. However, the rapid pace of epigenomics tools and sequenced genomes is reducing the barrier to studying genomics and epigenetics in animals that fascinate us is now lowered. We now join the growing group of researchers exploring epigenetics across the evolutionary tree to investigate the diverse mechanisms of how the genome is regulated, packaged, and protected from transposons.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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