Interplay of genetic and epigenetic variation in evolutionary processes

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Statutory declaration and statement

Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt ist.

Martinsried, den 14.06.2024 Justin Merondun

Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist. Ich habe nicht versucht, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

Martinsried, den 14.06.2024 Justin Merondun

List of Papers

This thesis is based on the following chapters, which are referred to in the text by their Roman numerals.

I. Merondun J[†], Marques C[†], Andrade P, Meshcheryagina S, Galván I, Afonso S, Alves JM, Araújo PM, Bachurin G, Balacco J, Bán M, Fedrigo O, Formenti G, Fossøy F, Fülöp A, Golovatin M, Granja S, Hewson C, Honza M, Howe K, Larson G, Marton A, Moskát C, Mountcastle J, Procházka P, Red'kin Y, Sims Y, Šulc M, Tracey A, Wood JMD, Jarvis ED, Hauber ME, Carneiro M[†], Wolf JBW[†]. (2024). Evolution and genetic architecture of sex-limited polymorphism in cuckoos. *Science Advances*, 10, eadl5255. https://doi.org/10.1126/sciadv.adl5255

[†]Contributed equally

II. Merondun J, Fossøy F, Bachurin G, Golovatin M, Hewson C, Liang W, Meshcheryagina S, Procházka P, Red'kin Y, Rutila J, Stokke BG, Wolf JBW. *(In Prep)* Matrilineal capture ensures stable inheritance and phenotypic innovation in egg mimicry arms race. *Manuscript.*

III. Catalán A, **Merondun J**, Knief U, Wolf JBW (2023) Chromatin accessibility, not 5mC methylation covaries with partial dosage compensation in crows. *PLOS Genetics*. 19(9):e1010901. https://doi.org/10.1371/journal.pgen.1010901

IV. Merondun J & Wolf JBW. (*In Prep*). The contribution of epigenetic variation to evolution in crows. *Manuscript.*

I also contributed to the following articles that were submitted or published during my graduate studies but are not a part of this thesis.

Leighton, G. R. M., Bishop, J. M., O'Riain, M. J., Broadfield, J., **Meröndun, J**., Avery, G., Avery, D. M., & Serieys, L. E. K. (2020). An integrated dietary assessment increases feeding event detection in an urban carnivore. Urban Ecosystems, 23(3), 569–583. https://doi.org/10.1007/s11252-020-00946-y

White, K. S., Levi, T., Breen, J., Britt, M., **Meröndun, J.**, Martchenko, D., Shakeri, Y. N., Porter, B., & Shafer, A. B. A. (2021). Integrating Genetic Data and Demographic Modeling to Facilitate Conservation of Small, Isolated Mountain Goat Populations. The Journal of Wildlife Management, 85(2), 271–282. https://doi.org/10.1002/jwmg.21978

Meröndun, J., Kierepka, E. M., Shafer, A. B. A., & Murray, D. L. (2021). Spatial population genetics reveals competitive imbalances threatening local apex predator persistence. Biological Conservation, 256, 109062. https://doi.org/10.1016/j.biocon.2021.109062

Leighton, G. R. M., Bishop, J. M., **Merondun, J.**, Winterton, D. J., O'Riain, M. J., & Serieys, L. E. K. (2022). Hiding in plain sight: Risk mitigation by a cryptic carnivore foraging at the urban edge. Animal Conservation, 25(2), 244–258. https://doi.org/10.1111/acv.12732

Serieys, L., Leighton, G. R., **Merondun, J.**, & Bishop, J. M. (*In Review*). Denning and maternal behavior of caracals (*Caracal caracal*). Research Square. https://doi.org/10.21203/rs.3.rs-3840757/v1

Mueller, S. A., **Merondun, J.**, Lečić, S., & Wolf, J. B. W. (*In Review*). Integrating epigenetic variation into population genetic practice.

Declaration of contributions

Paper I.

Merondun J[†], Marques C[†], Andrade P, Meshcheryagina S, Galván I, Afonso S, Alves JM, Araújo PM, Bachurin G, Balacco J, Bán M, Fedrigo O, Formenti G, Fossøy F, Fülöp A, Golovatin M, Granja S, Hewson C, Honza M, Howe K, Larson G, Marton A, Moskát C, Mountcastle J, Procházka P, Red'kin Y, Sims Y, Šulc M, Tracey A, Wood JMD, Jarvis ED, Hauber ME, Carneiro M[†], Wolf JBW[†]. (2024). Evolution and genetic architecture of sex-limited polymorphism in cuckoos. *Science Advan*ces, **10**, eadl5255. 10.1126/sciadv.adl5255

[†]Contributed equally

Justin Merondun organized sample and sequencing logistics for the Munich-contributed samples, data analysis and visualization of Figures 3 & 4 and the majority of Supplementary Figures, and wrote the manuscript in combination with Cristiana Marques, Miguel Carniero, Jochen Wolf, and Mark Hauber.

Other Contributions

The core team of Justin Merondun, Cristiana Marques, Mark Hauber, Miguel Carneiro, and Jochen B. W. Wolf contributed to conceptualization, methodology, investigation, and writing.

Shared First Author:

> Cristiana Marques: conducted lab work, organized sample and sequencing logistics for the Hungarian and toepad museum samples, data analysis and visualization of Figures 1 & 2, Supplementary Figures, and wrote the manuscript in combination with Justin Merondun, Miguel Carniero, Jochen Wolf, and Mark Hauber.

Pedro Andrade assisted with the analysis of Figure 1, Ismael Galvan assisted with generating data of Figure 2. Mark E Hauber, Joel M. Alves, Sofia Granja, and Greger Larson assisted with collecting and generating toepad samples, and Sandra Afonso assisted with Hungarian sample sequencing.

Swetlana Meshcheryagina, Gennadiy Bachurin, Miklós Bán, Frode Fossøy, Attila Fülöp, Mikhail Golovatin, Chris Hewson, Marcel Honza, Attila Marton, Csaba Moskát, Petr Procházka, Yaroslav Red'kin and Michal Šulc contributed samples.

Jennifer Balacco, Olivier Fedrigo, Giulio Formenti, Kerstin Howe, Jacquelyn Mountcastle, Ying Sims, Alan Tracey, Jonathan M. D. Wood and Erich D. Jarvis assembled the genome.

Signature of the supervisor:
Signature of co-first author: Justin Merondun

Manuscript II.

Merondun J, Fossøy F, Bachurin G, Golovatin M, Hewson C, Liang W, Meshcheryagina S, Procházka P, Red'kin Y, Rutila J, Stokke BG, Wolf JBW. *(In Prep)* Matrilineal capture ensures stable inheritance and phenotypic innovation in egg mimicry arms race. *Manuscript.*

Justin Merondun performed data analyses, visualization of figures, and wrote the manuscript with input from Jochen Wolf.

Other Contributions

Frode Fossøy and Jochen B. W. Wolf organized funding acquisition and project conceptualization.

Frode Fossøy, Gennadiy Bachurin, Mikhail Golovatin, Chris Hewson, Wei Liang, Swetlana Meshcheryagina, Petr Procházka, Yaroslav Red'kin, Jarkko Rutila, and Bård G Stokke contributed samples.

Jochen Wolf

Signature of the supervisor:

Justin Merondun

Paper III.

Catalán A, **Merondun J**, Knief U, Wolf JBW (2023) Chromatin accessibility, not 5mC methylation covaries with partial dosage compensation in crows. *PLOS Genetics*. 19(9):e1010901.

Justin Merondun contributed to sequencing logistics and performed all the data analysis of DNA methylation, visualization of figures related to DNA methylation, and contributed to writing the manuscript with input from all authors.

Other Contributions

Ana Catalán coordinated the generation and analysis of chromatin accessibility data (ATACseq) and the analysis of expression data.

Jochen B. W. Wolf obtained samples and together with Ana Catalán organized project implementation, execution and wrote the first draft of the manuscript.

Ulrich Knief contributed to statistical analyses, project execution, and the writing the manuscript.

Jochen Wolf

Signature of the supervisor:

Justin Merondun

Manuscript IV.

Merondun J & Wolf JBW. (*In Prep*). The contribution of epigenetic variation to evolution in crows. *Manuscript.*

Justin Merondun contributed to project conceptualization, performed analyses, visualization of figures, and wrote the manuscript with input from Jochen B. W. Wolf.

Other Contributions

Jochen B. W. Wolf obtained samples, organized funding acquisition, provided mentorship, and led overall project execution.

Jochen Wolf

Signature of the supervisor:

Justin Merondun

Summary

Adaptation and speciation are the fundamental processes shaping the biodiversity that surrounds us. The Modern Evolutionary Synthesis, merging Darwinian theory with Mendelian principles, requires an understanding of the genetic and epigenetic contributions to population divergence across micro- and macro-evolutionary scales to explain the maintenance of biodiversity. This dissertation examines the interplay of genetic and epigenetic variation with relevance to evolution across the dimensions of wild avian populations, sexes, and species. Utilizing natural variation in two avian systems, the scope of my investigation extends to: 1) the genetic architecture sustaining female-limited polymorphism in cuckoos, the 2) evolutionary maintenance of mimetic egg phenotypes and host specialization in cuckoos, the 3) the epigenetic factors regulating dosage compensation and dosage balance in crows, and 4) the relevance of DNA methylation to speciation in crows.

In paper I, together with my colleagues I identify the genetic basis and evolutionary maintenance of a female-limited plumage polymorphism. While all male common cuckoos are grey, females are either monochromatic grey or rufous. We found that plumage polymorphism maps to the female-restricted W chromosome, and that these ancient maternal haplotypes have been maintained after descent from a common ancestor in two cuckoo sister taxa, likely through balancing selection. Our findings suggest that genetic variation residing on sex-limited chromosomes can be a key determinant in the maintenance of trait variation across species boundaries.

In paper II, I examine the genetic basis of host specialization and egg mimicry resulting from a co-evolutionary arms race. Common cuckoos are generalist obligate brood parasites exhibiting an extreme diversity of mimetic eggs which they use to exploit numerous hosts across Eurasia. I identified that matrilineal haplotypes are associated with mimetic egg phenotypes, and found that these haplotypes are maintained across the species' range from the combinatorial effects of

balancing selection and gene flow. I identify mitochondrial OXPHOS genes as the nexus of egg diversification, working in concert with nuclear and W-linked genes to provide a fast-evolving substrate to facilitate phenotypic innovation for new mimetic eggs while ensuring stable transmission of phenotypes from mothers to daughters.

In paper III, together with colleagues I shed light on the mechanisms underlying dosage balance and compensation in a female heterogametic system in Eurasian crow. While male heterogametic systems often exhibit inactivation of a female homogametic chromosome, dosage balance in avian systems is less clear. We identified a significant correlation between the upregulation of female Z-linked genes and increased chromatin accessibility, which appears to be the key driver of dosage balance between the sexes. In contrast to other systems, 5mC methylation did not covary with dosage, underlining the importance of chromatin accessibility over methylation in regulating gene dosage in crows.

In my last chapter, manuscript IV, examines the extent to which 5mC methylation contributes to nascent species divergence in Eurasian crows. Using genome and methylome sequencing data from all-black carrion crows, grey-coated hooded crows, and their hybrids, we found that taxon-related methylation divergence is restricted to intergenic space within the region of genetic differentiation responsible for plumage polymorphism. While epigenetic factors may aid in translating genetic variation to phenotype and largely coincide with the ontogenetic program, its autonomous contribution to evolution is minimal in this system.

Collectively, these studies show the complex interplay of genetic and epigenetic factors contributing to the maintenance of evolutionary patterns. These findings add to our understanding of how epigenetic and genetic mechanisms cooperate to generate and maintain evolutionarily relevant phenotypes across populations and species, and break new ground by exploring the hitherto poorly explored dynamics of sex-limited chromosomes and the contributions of epigenetic variation to evolution.

Introduction

The generation and persistence of species is intimately associated with phenotypic diversity within and across populations. This diversity forms the foundation upon which reproductive barriers can emerge, gradually leading to the creation of new species. However, quantifying the dynamics of evolutionary forces that drive the loss, creation, and maintenance of this variation over time presents a significant challenge. This difficulty largely stems from the limited perspective from observing only current phenotypic variation in wild populations. Advances in genetic and epigenetic sequencing not only offer a solution to pin down the genetic basis of currently segregating phenotypic variation, but also provide insights into the past processes shaping contemporary population variation. Through methods such as the coalescent we can reconstruct the evolutionary past, while with epigenetic data we can infer the putatively functional phenotypic landscape.

Here, I review the primary evolutionary forces shaping genetic and phenotypic variation within wild populations. I explore how these evolutionary forces contribute to the emergence of sex-limited phenotypes. Further, I discuss the evolution of distinct sexes and the development of sex chromosomes as a mechanism to balance the unique evolutionary pressures faced by each sex, and also overview the impact of these forces on epigenetic variation. Finally, I provide an introduction into the two study systems I use to examine the combined effects of these evolutionary pressures: the cuckoo and the crow.

1. Evolutionary forces shaping natural variation

Life's diversity originates fundamentally from DNA mutations (Vries, 1901). Some of these mutations confer a fitness advantage, facilitating their proliferation (Darwin, 1859). Random genetic drift purges genetic variation (S. Wright, 1931), migration introduces variation to new

populations (Dobzhansky, 1937b), and recombination shuffles variation to create new forms (Muller, 1916). These processes drive the continuous evolution of life forms on Earth, influenced by external stimuli like geoclimatic cycles and internal stimuli like biotic interactions between species (Eldredge & Gould, 1972). While some of these processes are stochastic, we can use genetic sequence data to approximate the influences of genetic drift, selection, and migration among wild populations to identify the most likely evolutionary scenarios that brought them into existence. Within the context of this dissertation, a brief overview of the various forms of selection and their corresponding genetic patterns will be useful, in addition to a discussion of the demographic scenarios which could confound the investigation.

1.1. The role of selection in shaping natural variation

Populations evolve as the fitness landscape changes, with different types of selection favoring different traits. Directional selection favors individuals at one end of the phenotypic spectrum, driving a shift in the population towards these traits (Clegg et al., 2002; Rieseberg et al., 2002). Stabilizing selection, however, favors those with intermediate traits, maintaining trait consistency (Covas et al., 2002; Hansen, 1997; Neff, 2004). Disruptive selection rewards individuals at both extremes of a trait, promoting diversity and speciation (Hendry et al., 2009; Mather, 1955). Positive selection causes beneficial alleles to become more common, reflecting an adaptive advantage (Chase et al., 2021; Prezeworski et al., 2005), whereas purifying selection purges deleterious alleles (J. Chen et al., 2017; Kutschera et al., 2020). Balancing selection preserves genetic diversity by favoring multiple alleles or phenotypes (Hedrick & Thomson, 1983; Kim et al., 2019), potentially influenced by their frequency in the population, known as frequency-dependent selection (Allen et al., 1998). Sexual selection, irrespective of other benefits such as improved survival or resource allocation, promotes traits that enhance mating success (Arnold & Wade, 1984; Cooney et al., 2019).

Furthermore, genetic regions near positively selected variants can also increase in frequency due to genetic hitchhiking (Feder et al., 2012; Kaplan et al., 1989), reducing variation in nearby sites in the form of selective sweeps (Campagna et al., 2022). Conversely, genetic variation in these regions can be reduced through linkage to deleterious mutations, a process called background

selection (D. Charlesworth et al., 1995). Overall, wild populations are subject to diverse selective pressures that leave detectable genetic signatures on their allele frequency spectra. However, these genetic patterns may also result from population's demographic histories and the influence of genetic drift. Distinguishing the impact of selection from the effects of drift in natural populations requires further investigation.

1.2. The role of demography and genetic drift in shaping natural variation

In addition to selective pressures, demographic events and genetic drift play pivotal roles in shaping the genetic diversity within natural populations. Demographic changes such as population expansions, bottlenecks, and migrations can alter allele frequencies independent of fitness advantages. Population bottlenecks can lead to a significant reduction in genetic diversity as the population size decreases (Feng et al., 2019), only to potentially increase in diversity again during expansion, albeit with a different genetic makeup than prior to the bottleneck (Carson, 1990; Sonsthagen et al., 2017; Sutton et al., 2015). This phenomenon demonstrates how demographic events can influence genetic variation and evolutionary trajectories in ways irrespective of selection (Kirkpatrick & Jarne, 2000; Nei et al., 1975).

Genetic drift, or the random fluctuation of allele frequencies from one generation to the next, becomes particularly influential in small populations. Unlike selection which acts on alleles based on their contribution to fitness, drift can lead to the fixation or loss of alleles irrespective of their fitness effects. Consequently, genetic drift can result in the loss of beneficial alleles and the fixation of deleterious alleles, contributing to the stochasticity of evolution (Kimura, 1977). Eventually, the fate of a mutation is determined by the interplay of genetic drift and selection, as is recognized in the nearly-neutral theory of evolution (Akashi et al., 2012; B. Charlesworth & Charlesworth, 2018; Kern & Hahn, 2018; Kimura, 1983; Ohta, 1992).

With relevance to this dissertation, the interplay between selection, demographic history and genetic drift can lead to complex patterns of genetic variation. For example, founder effects, a form of genetic drift, occur when a new population is established by a small number of individuals from a larger population. This can result in a population that is genetically distinct from its original population, not due to adaptive differences but due to the random sampling of alleles (A. J. Baker

& Moeed, 1987; Hawley et al., 2006), although the role of founder effects in the speciation process is still debated (Sendell-Price et al., 2021; Walsh et al., 2005; Yeung et al., 2011). Furthermore, the combined effects of demography and drift can mimic the signatures of selection, complicating the inference of evolutionary dynamics. For instance, a selective sweep can reduce genetic variation in a manner similar to a bottleneck. Distinguishing between these scenarios requires careful consideration of population history and sophisticated analysis of sequencing data, together with temporal sampling if possible (Femerling et al., 2023).

1.3. The role of coevolutionary forces in generating variation

Coevolution between hosts and their parasites often generates extreme phenotypic variation due to a continuous cycle of reciprocal selection pressures, where each species adapts in response to the other (Ebert & Fields, 2020). This interaction typically unfolds as an evolutionary arms race, exemplifying *Red Queen* dynamics where continuous adaptation is necessary for a species to maintain its relative fitness amidst coevolving species (Rabajante et al., 2016; Van Valen, 1973). This dynamic can lead to the diversification of host strategies to evade parasitism, countered by parasites developing increasingly sophisticated mechanisms to breach host defenses (Dixit et al., 2023). Obligate brood parasites, or species that depend entirely on other species for rearing their young without providing any parental care, offer prime examples to study coevolutionary dynamics (Krüger et al., 2009; Sorenson, 2002). In birds, brood parasitism has independently evolved at least seven times, resulting in several examples of extreme intraspecific phenotypic diversity (Stevens, 2013). Some bird species specialize in parasitizing a limited number of host species, while others exploit a broad range of hosts, which grants these parasites greater reproductive versatility but also presents genetic challenges in maintaining diverse parasitic traits within a population (Davies, 2000).

Two models of coevolution propose the dynamics generating, maintaining, and eliminating adaptive variation between hosts and parasites, namely balancing selection and selective sweeps (Ebert & Fields, 2020). The selective sweep model of coevolution describes the rapid rise and eventual fixation of beneficial mutations and is often invoked in coevolutionary dynamics investigating macroevolutionary diversification (Enard et al., 2016; Obbard et al., 2009). In terms

of host-parasite coevolution, both may experience sweeps at key functional loci, influencing co-evolutionary dynamics and leading to distinct genomic patterns of increased linkage disequilibrium and reduced genetic variation around the beneficial mutations (Badouin et al., 2017; Mohd-Assaad et al., 2018; Persoons et al., 2017). In contrast, the balancing selection model of coevolution explains the maintenance of high genetic diversity in host and parasite populations (Clarke, 1976). This model is based on strong interactions between specific host and parasite genotypes, driving reciprocal selection (Bento et al., 2017; Luijckx et al., 2013). According to *Red Queen* dynamics, a parasite allele increases in frequency when the corresponding host allele is prevalent, influenced by negative frequency-dependent selection that favors rare alleles (Koskella & Lively, 2009; Lively, 2018). This mechanism ensures the persistence of a diverse pool of alleles, providing an evolutionary advantage (Ejsmond et al., 2010).

2. The role of sex in evolution

Sexual reproduction, which involves the combination of genetic material from two parents, provides significant evolutionary advantages over asexual reproduction. It enhances genetic diversity and supports adaptation to changing environments (Otto, 2009), and can even help organisms resist the detrimental impacts of parasites in coevolutionary interactions (Hamilton et al., 1990). This diversity results from mechanisms like recombination and the independent assortment of gametes, providing populations with variable genetic combinations to face new selective pressures, often providing adaptive advantages (Barton & Charlesworth, 1998; Ortiz-Barrientos et al., 2016; Peñalba & Wolf, 2020; Presgraves, 2005). These concepts are relevant for the papers of this dissertation as distinct sexes have enabled populations to circumvent some constraints discussed above, specifically by selection or constraints imposed by demographic history.

2.1. The battle of the sexes

Coevolution between the sexes, driven by the interplay of competitive and cooperative reproductive strategies through sexual selection, can shape the evolutionary trajectory of species

irrespective of any survival benefits (Darwin, 1871). However, sexual conflict arises when the evolutionary interests of males and females diverge, leading to a perpetual arms race where each sex evolves counterstrategies to maximize its own reproductive success, often at the expense of the other sex (Chapman et al., 2003; Parker, 1979). Sexual conflicts can manifest through various forms, including sexual antagonism, where traits beneficial to one sex impose costs on the other. Sexual antagonism can be resolved in numerous ways, including mating preferences (Albert & Otto, 2005) or the development of sex-limited traits (Hosken et al., 2009), although correlations among many traits and sex-linked genes makes the quest for disentangling independent sex-limited genes and sex-limited traits difficult (Harano et al., 2010). Sex chromosomes, typically beginning with a sex-determining locus, play a crucial role in the evolutionary maintenance of sex and resolving sexual antagonism. Over time, these chromosomes can accumulate beneficial mutations linked to the sex-determining locus (Bull, 1983). This linkage fosters the association of sexually advantageous traits with the sex-determining locus, providing an avenue to resolve sexual antagonism (Rice, 1992; Vicoso, 2019). As recombination is gradually suppressed around this locus, it enhances the genetic linkage, limiting recombination and thereby consolidating the association between the sex-determining genes and beneficial mutations (Bachtrog et al., 2014; B. Charlesworth, 1996; Van Doorn & Kirkpatrick, 2007). Eventually, this process can lead to the degeneration of the sex-limited chromosome of the heterogametic sex, significantly diverging from its ancestral homologous counterpart due to the accumulation of mutations and reduced genetic diversity (D. Charlesworth, 2021; Lenormand et al., 2020), although recombination can still occur along the pseudo-autosomal region (Yazdi et al., 2023; Yazdi & Ellegren, 2018).

2.2. Origins of sex-limited polymorphism

The evolution of heterogametic sex determination systems (*e.g.*, XY in mammals and ZW in birds) and the evolution of sex chromosomes enriches the genetic diversity of sexual systems (Zhou et al., 2014). Such systems facilitate the emergence of sex-limited traits, where one sex – typically the heterogametic sex – exhibits unique phenotypes not found in the homogametic sex (Rice, 1984). It is theorized that traits specific to one sex would naturally occur more frequently on the heterogametic sex chromosome (Reinhold, 1999), or within genomic variation exclusive to that sex, like mtDNA or the W chromosome for traits only found in females in ZW systems (Kunte et

al., 2011). Yet, current empirical studies in wild populations reveal that female-limited polymorphisms can also be associated with autosomal structural variation, as observed in *Papilio* butterflies (Iijima et al., 2018) and *Ischnura* damselflies (Willink et al., 2023). This suggests the importance of non-recombining regions in maintaining sex-limited traits, although this genomic variation is not sex-specific. Further research is needed to determine the contributions of sex-limited genetic variation to sex-limited traits.

2.3. Sex-limited traits: basis of plumage polymorphism

In avian systems, plumage coloration can arise from a complex interplay of genetic, dietary, and physical factors (Orteu & Jiggins, 2020). These colors can be pigment-based, stemming from melanins like eumelanin and pheomelanin (Galván & Solano, 2016; Meunier et al., 2011), or they can be structural, resulting from the interaction of light with feather nanostructures (Rubenstein et al., 2021). Eumelanin produces darker shades, while pheomelanin results in lighter, reddish tones. Both of these pigments are derived from tyrosine through biochemical pathways involving enzymes such as tyrosinase (Hearing, 2011; Ito & Wakamatsu, 2011). Birds can also display colors derived from dietary carotenoids, which are absorbed and deposited into their feathers which affect coloration based on their diet (Delhey et al., 2023; Toomey et al., 2022). Additionally, unique non-carotenoid pigments such as psittacofulvins also produce colors similar to pheomelanin but have so far been identified exclusively in parrots (McGraw & Nogare, 2004; Stradi et al., 2001; Veronelli et al., 1995).

Plumage polymorphism in birds is hypothesized to be driven by different forms of selection including apostatic – a type of negative frequency-dependent selection – or disruptive or sexual selection (Galeotti et al., 2003; Roulin, 2004), often invoking survival benefits such as camouflage or detectability (Koskenpato et al., 2020). The biochemical basis of polymorphism in birds often implicates the previously mentioned pigment-based melanins (Haase et al., 1992; McGraw & Wakamatsu, 2004). In birds, the genetic basis of plumage polymorphism often involves core melanogenesis genes, such as *ASIP*, which have been recurrently documented in various species like warblers (Baiz et al., 2020, 2021), Monarchs (Campagna et al., 2022), Lonchura finches

(Stryjewski & Sorenson, 2017), fairywrens (Sin et al., 2024), wagtails (Semenov et al., 2021), and crows (Poelstra et al., 2015).

Among these polymorphisms, female-limited plumage polymorphism is observed in only 23 of the 334 bird species known for plumage variations (Galeotti et al., 2003). This form of polymorphism is thought to be driven either by apostatic selection which can enhance survival by reducing predation risks and lowering detectability in host-parasite dynamics in cuckoos (Thorogood & Davies, 2013), or by reducing sexual harassment in hummingbirds (Diamant et al., 2021). Despite its prevalence, genetic studies specifically addressing the mechanisms behind female-limited polymorphism are absent in avian systems, leaving a significant gap in understanding how these traits are genetically encoded and evolutionarily maintained.

2.4. Sex-limited traits: basis of egg polymorphism

In birds, egg coloration can result from a sophisticated interplay of genetic, environmental, and physiological factors (Cassey et al., 2012; Kilner, 2006). Similar to plumage coloration, egg colors can be pigment-based, derived from bile pigments such as biliverdin and protoporphyrin. Biliverdin results in blue and green hues, while protoporphyrin contributes to brown and speckled patterns (Kennedy & Vevers, 1976). These pigments are produced within the shell gland and deposited onto the eggshell during egg formation (Hargitai et al., 2017; R. Zhao et al., 2006). These pigments are closely associated with the heme biosynthetic pathway, in which hemoglobin is produced with contribution of the enzyme ferrochelatase inserting iron into protoporphyrin to form heme, which is then converted into biliverdin by heme oxygenase. Egg speckling and streaking also arises following the variable deposition of these pigments in the shell gland (Cheng, Ma, et al., 2023), perhaps associated with DNA methylation changes in some species during the aging process (Cheng, Li, et al., 2023).

Egg polymorphism in birds is believed to be driven by several evolutionary pressures including camouflage from predators (Gómez et al., 2018), thermal regulation (Wisocki et al., 2019), and avoidance of brood parasitism, wherein obligate brood parasites pose a risk to hosts which can fuel a coevolutionary arms race (Spottiswoode & Stevens, 2012; Stoddard & Stevens, 2011). However, despite the ecological and adaptive significance of egg color polymorphism, the specific genetic

mechanisms underpinning this diversity are not well understood. Among intraspecific egg polymorphisms, the genetic networks regulating blue eggs has a diverse autosomal basis in quails, chickens, and ducks (L. Chen et al., 2020; Ito et al., 1993; Z. Wang et al., 2013). Outside of domesticated species, the genetics of intraspecific egg polymorphism is less understood, but is hypothesized to be matrilineally restricted in some brood parasitic systems (Fossøy et al., 2016; Spottiswoode et al., 2022).

2.5. Sex chromosomes and dosage compensation

The emergence of heterogametic systems with distinct sex chromosomes, particularly as focal points in intersexual conflict, presents an evolutionary dilemma: the discrepancy in chromosome copy number between the autosomes and across the sexes (B. Charlesworth, 1996). Sex-specific gene expression has arisen as one solution to resolve this resulting aneuploidy and mitigate the endless tug of war between the sexes (Tanaka et al., 2011). Specifically, dosage compensation has evolved as a solution to the fundamental challenge posed by aneuploidy, ensuring that one sex does not suffer a disadvantage due to having only one copy of the sex chromosome (*e.g.,* females in ZW systems). Unlike well-studied X-chromosome inactivation in mammals (Borsani et al., 1991), birds exhibit a different pattern where both sexes partially balance the expression of Z-linked genes, rather than completely silencing one chromosome (Itoh et al., 2007). This partial compensation results in males and females achieving a similar expression level for only some Z-linked genes, leaving some genes with the potential for sex-limited variation related to dosage (Ellegren, 2011; J. B. Wolf & Bryk, 2011). This gene-by-gene regulation of dosage compensation in birds provides more scope for gene-specific regulation of sex-specific traits, and even postulates a role of Z-linked genes in regulating female-specific traits by reducing potentially costly antagonistic effects in males (Mank & Ellegren, 2009).

Phenotypic traits can be affected by variations in gene dosage (Henikoff, 1996). Dose-dependence is one mechanism where changes in phenotype occur only after gene expression reaches specific thresholds (Litingtung et al., 2002). Dose-dependence is well recognized in avian systems to underlie stripe color width in several birds (Haupaix et al., 2018), spotting in ducks (Xi et al., 2021), and piebalding patterns in pigeons (Maclary et al., 2023). However, in avian systems dosage

compensation is largely gene-dependent (Mank & Ellegren, 2009), and differs widely across species including a male-hypermethylated region in chickens (Höglund et al., 2024; Teranishi et al., 2001) which is not observed in white-throated sparrows (Sun et al., 2019). Further research identifying the regulatory mechanisms of dosage compensation and any dose-dependent phenotypes in diverse avian species is needed.

In both mammalian and invertebrate model systems, sex chromosome expression is often regulated through chromatin modifications. In mammals, dosage compensation is primarily achieved through mechanisms such as X-chromosome inactivation, which involves extensive chromatin remodeling including histone modifications and DNA methylation to silence one of the X chromosomes in females (Mohandas et al., 1981; Sharp et al., 2011; Wakefield et al., 1997). Similarly, in *Caenorhabditis elegans*, dosage compensation is achieved through chromatin modifications, particularly through H4K20me1 and its regulation of the dosage compensation complex (Brejc et al., 2017; Meyer, 2022; Wells et al., 2012). This mechanism initially upregulates expression from the X chromosomes in both sexes, followed by additional repression mechanisms in hermaphrodites acting on both X chromosomes to align their expression levels with the single X chromosome in males (Ercan & Lieb, 2009; Meyer, 2022; Meyer & Casson, 1986). Overall, research in *C. elegans* establishes a strong relationship between chromatin modifications and higher-order chromosome structures that are crucial for the long-range regulation of gene expression (Bian et al., 2020; Brejc et al., 2017; Liu et al., 2011). In contrast, in *Drosophila melanogaster*, dosage compensation is managed through the hyperactivation of the single male X chromosome (Cline & Meyer, 1996). This is facilitated by the dosage compensation complex, which in conjunction with the chromatin modification H4K16ac targets specific binding sites on the X chromosome, inducing both local and long-range alterations in the chromatin structure to ensure that expression levels match those of the two X chromosomes in females (Conrad & Akhtar, 2012; Gelbart & Kuroda, 2009; Malone et al., 2012). These examples emphasize the fundamental role of chromatin modifications in regulating sex chromosome dosage across diverse taxa, setting a precedent for exploring similar mechanisms in avian species, where chromatin accessibility and methylation might similarly influence gene expression and dosage compensation on the Z chromosome.

3. Non-genetic mechanisms shaping natural variation

Chromatin modifications have important functions outside of regulating dosage differences. They are also vital for cell differentiation and may, in addition, influence natural phenotypic variation beyond genetically encoded variation. It is well established that modifications, such as methylation, acetylation, and phosphorylation, are crucial during ontogeny and during development, providing the means to achieve complex multicellular forms from a single genome (Waddington, 1942). However, the role of chromatin modifications in evolution is still debated because the inheritance of accumulated somatic modifications through the germline is limited to nonexistent in vertebrates (Weismann, 1893). While interest in the inheritance of chromatin modifications remains high (Fitz-James & Cavalli, 2022; Guerrero-Bosagna et al., 2018; Hay et al., 2023; Heard & Martienssen, 2014; Perez & Lehner, 2019), the strong correlation between genetic variation and chromatin modifications suggests that their independent evolutionary contributions might be limited in vertebrates (Horsthemke, 2018; McRae et al., 2014; Villicaña & Bell, 2021). In contrast, chromatin modifications in plants potentially have broader evolutionary implications because plants do not have a strict separation between germline and somatic cells, facilitating the inheritance of chromatin modifications to the next generation (Cubas et al., 1999; Furci et al., 2019; Jacobsen & Meyerowitz, 1997; McClintock, 1984). Irrespective of transgenerational stability, the importance of inter-generational inheritance of chromatin modifications and particularly maternal influences *in utero*, is well understood (Radford et al., 2014; Rakyan et al., 2003). There is a growing recognition of the potential for rapid adaptive phenotypic changes in response to environmental pressures through chromatin modifications (Lea et al., 2017; Sepers et al., 2019), but investigations into wild populations that employ controls for genetic and developmental effects are limited in animals in general (Hu et al., 2021; Hu & Barrett, 2023; Metzger & Schulte, 2018; Rodriguez Barreto et al., 2019; Weyrich et al., 2016) and in avian contexts in particular (Sepers et al., 2023, 2024; Sun et al., 2021; Von Holdt et al., 2023).

3.1. Genetic effects on DNA methylation

DNA methylation is a chromatin modification that plays a critical role in gene regulation, particularly during early development and cellular differentiation, by silencing or activating gene

expression (Bird, 2002; Holliday & Pugh, 1975; Jones, 2012; Schübeler, 2015; Stein et al., 1982; Vardimon et al., 1982). DNA methylation is increasingly implicated in ecological and evolutionary contexts because it can be sequenced in high-throughput economically using bisulfite sequencing (Artemov et al., 2017; Bewick et al., 2016, 2019; H. Gu et al., 2011; Hu & Barrett, 2023; Lea et al., 2016, 2017; Zemach et al., 2010). However, many investigations into the effects of environmental conditions on DNA methylation are speculative as they do not control for underlying *cis*- or *trans*- acting genetic sequence (Meröndun et al., 2019; Watson et al., 2021). Spontaneous epimutations in genes that contribute to survival could be a satisfying mechanism providing rapid adaptive change in variables environments, thus circumventing the traditional constraints of random genetic mutations. Nonetheless, careful experimental design is necessary to isolate such epimutations (Sepers et al., 2019). Future research quantifying the relationship between genetic variation and DNA methylation in wild systems, ideally leveraging additional individuals raised in common environmental conditions, is needed to further isolate the independent contributory effects of chromatin modification in wild systems.

3.2. The role of epigenetic variation in speciation

Epigenetic variation, manifested as phenotypic plasticity through changes in gene expression, might play a role in vertebrate speciation by enabling rapid adaptation to changing environments (Ashe et al., 2021; Pfennig et al., 2010; Whitehead & Crawford, 2006), a prerequisite for ecological speciation (Nosil, 2008, 2012). Stable inheritance of environmentally induced epigenetic variation could mediate phenotypic plasticity promoting rapid adaptation without the need to wait for adaptive genetic mutations (Christina L. Richards & Massimo Pigliucci, 2020). While empirical studies specifically investigating the role of methylation in speciation are limited, research on species like cichlids (Vernaz et al., 2022), spiny mice (Li et al., 2020, 2020; Y. Wang et al., 2022; Y. Zhao et al., 2016), and whitefish (Laporte et al., 2019; Venney et al., 2024) suggests that epigenetic mechanisms could contribute to speciation. Theoretical work grounded in simulations indicates that epigenetic plasticity has the potential to drive speciation by reducing the fitness of migrants and hybrids, but it might also inhibit genetic adaptation by providing an alternative to typical genetic mutation processes (Greenspoon et al., 2022). Alternatively, the much higher mutation rate in methylated CpGs could introduce a novel source of genetic variation (Gorelick,

2003; Jablonka & Raz, 2009; Jones et al., 1992). Current research in vertebrates suggests that while epigenetic variation might play a role in population divergence, there is a lack of evidence linking epigenetic variation independent of genetic variation to reproductive isolation.

4. Concluding remarks and study systems

Phenotypic variation in wild populations is influenced by evolutionary forces such as mutation, selection, genetic drift, migration, and recombination. These forces operate both within and across populations and sexes, driving the cooperative and competitive dynamics that sustain diverse phenotypes in nature. Although evolutionary mechanisms like sex-limited traits and non-genetic adaptations may make an important contribution to adaptation and speciation, their impact in avian species remains underexplored. With this dissertation, I aim to contribute filling this knowledge gap by first analyzing the genetics behind female-limited plumage polymorphism and the maintenance of egg mimetic lineages in cuckoos. Subsequently, I explore the regulatory mechanisms of dosage compensation between sexes in the Eurasian crow. Finally, I leverage both genetic and epigenetic data from wild and common garden-raised crows to investigate the independent contributory effects of DNA methylation to evolution.

4.1. The common cuckoo

The common cuckoo (*Cuculus canorus*) provides a unique model to study evolutionary mechanisms due to its complex reproductive strategies and sexual dimorphism. As an obligate brood parasite, the common cuckoo builds no nest and invests no parental care into its offspring (Payne, 2005). Instead, it parasitizes a variety of host species, with up to 108 out of 160 potential hosts being parasitized within Europe alone (Moksnes & Røskaft, 1995). This parasitic strategy has the potential to initiate a coevolutionary arms race, characterized by the cuckoo's continuous enhancement of trickery to evade host detection and the hosts' concurrent evolution of refined discriminatory abilities (Davies et al., 1989). Within this dissertation, I explore two female-limited phenotypes which potentially increase the parasitic success of the common cuckoo: plumage and egg polymorphism.

Female common cuckoos exhibit sex-limited plumage polymorphism with two distinct morphs – gray and rufous – while males are monomorphic gray. The more common gray morph may mimic predatory birds like sparrowhawks, possibly mitigating aggression from host species and enhancing the cuckoo's ability to parasitize nests successfully (Davies & Welbergen, 2008; Trnka & Prokop, 2012). In contrast, the less common rufous morph may mimic kestrels (Kuroda, 1966; Voipio, 1953) and evade learned discrimination by the host of sparrowhawk and the common grey morph by the host. This suggests a potential role of negative frequency-dependent selection where the rarer morph could benefit from reduced detection by hosts or predators (Thorogood & Davies, 2012; Welbergen & Davies, 2011). However, experimental evidence suggests that many hosts do not respond to kestrels (Trnka et al., 2015), instead suggesting that plumage polymorphism may reduce sexual harassment from males (Lee et al., 2019), as is hypothesized in hummingbirds (Falk et al., 2021). Regardless of the actual mechanism, the widespread occurrence of female-limited polymorphism in common cuckoos, extending across various genera within the *Cuculinae* subfamily, indicates its potential as a balanced polymorphism that crosses species boundaries (Thorogood & Davies, 2013). Furthermore, the inability to reliably determine juvenile morphs, and the observation that male juveniles may display rufous plumage, has prompted the involvement of a dose-dependent or Z-linked genetic mechanism influencing plumage coloration (Koleček et al., 2019). However, no genetic investigations into the basis of plumage polymorphism within cuckoos currently exists.

Host specialization in cuckoos further illustrates an appealing aspect of coevolution. The parasitic nature of cuckoos has been known for millennia, wherein ‘the bird eggs, but does not build a nest. Sometimes it lays its eggs in the nest of a smaller bird after first devouring the eggs of this bird’ (4th century BC; Aristotle, 1910). This parasitic behavior captured the interest of naturalists in the 19th century (Baker, 1923; Chance, 1922, 1940; Newton, 1869), but it was not until the end of the 20th century that data emerged to assess the competing theories surrounding the maintenance of host-specific cuckoo egg morphs (Brooke & Davies, 1988; Davies & Brooke, 1988, 1989a, 1989b; Nakamura, 1990). Cuckoos have almost certainly engaged in an evolutionary arms race with various hosts, developing egg phenotypes that closely mimic those of their host species to increase the chances of successful brood parasitism (Brooke & Davies, 1988; Marchetti et al., 1998; Moksnes & Røskaft, 1995). This mimicry is critical as hosts evolve better detection and rejection methods for foreign eggs. The maintenance of these cuckoo lineages with specialized egg

phenotypes, known as *gentes*, is hypothesized to occur through strict maternal inheritance, ensuring that specific parasitic strategies are passed directly from mother to offspring (Fossøy et al., 2016; Gibbs et al., 1996, 2000; Marchetti et al., 1998). However, many *gentes* are restricted to geographic localities (J. J. Soler et al., 2009), suggesting a role for allopatric specialization of host types. Nonetheless, investigations into sympatric *gentes* indicates mimetic fidelity to hosts for numerous egg characteristics, suggesting stable maintenance even with the possibility of gene flow (Antonov et al., 2010; Fossøy et al., 2011).

Three necessary conditions must be met to sustain a mimetic egg coevolutionary arms race (Brooke & Davies, 1991). The first condition, that hosts must be discriminatory against parasitic eggs, is well established within common cuckoos across the range, with discriminatory abilities ranging from very poor in dunnocks (Brooke & Davies, 1988), moderate in thrushes (Yi et al., 2020), generally high in warblers (Davies & Brooke, 1988; Ma et al., 2022, 2024), and very high in buntings and bramblings (Vikan et al., 2010; Zhang et al., 2023), although host discrimination is spatiotemporally conditional and can be dependent on the degree of contemporary parasitism (M. Soler et al., 2012). Nevertheless, the first condition is certainly met in the cuckoo system, wherein cuckoos have been known to parasitize over 100 species which exhibit a range of discriminatory ability (Moksnes & Røskaft, 1995). The second condition is that parasitic egg phenotypes must be heritable. Female common cuckoos lay consistent egg types across their lifetimes, although they exhibit high intra-individual variation (Moksnes et al., 2008). Egg phenotype is understood to be heritable and autosomal-linked in village weaverbirds, chickens, quails, and ducks (L. Chen et al., 2020; Collias, 1993; Ito et al., 1993; Z. Wang et al., 2013), and female-linked in great tits (Gosler et al., 2000). While egg size and survival viability are linked to temperature in birds (Heming & Marini, 2015; Oguntunji & Alabi, 2010; J. M. Wang et al., 2011), and global temperatures are correlated with avian eggshell pigmentation across the avian phylogeny (Wisocki et al., 2019), there is no evidence currently to implicate environmental effects in regulating individual-level egg phenotypes in cuckoos, instead suggesting a genetic link. The third condition that must be met to fuel a coevolutionary arms race is that parasites must make the correct choice about which hosts to parasitize. This latter condition is certainly the hardest to prove in the cuckoo system, and has itself resulted in three competing sub-hypotheses (Moksnes & Røskaft, 1995) about how cuckoo egg types could be maintained, namely:

i) Host preference hypothesis (*HPH*): each *gens* specializes on a single host species.

ii) Nest site hypothesis (*NSH*): each *gens* specializes on host groups with similar eggs and nest sites and searches randomly within each group.

iii) Natal philopatry hypothesis (*NPH*): each *gens* searches for nests randomly in their natal habitat.

The Natal Philopatry Hypothesis has not been supported within European common cuckoos, instead supporting habitat imprinting (Koleček et al., 2020). Evidence supporting the remaining hypotheses is mixed, with some support for the *HPH* in Chinese common cuckoos (Yang et al., 2018) but less so in European populations (Moksnes & Røskaft, 1995). Within the Czech Republic, maternal lines, rather than paternal lines, demonstrated a preference for specific hosts, supporting the *HPH* (Skjelseth et al., 2004). This finding contrasts with additional research from Hungary and Korea, where both parents contribute to host-specificity (Fossøy et al., 2011; Lee et al., 2021). A necessary precondition of the *HPH* is that cuckoos imprint on their host, of which evidence is mixed. For instance, no imprinting was observed in British cuckoos (Brooke & Davies, 1991), with conflicting evidence in China (Yang et al., 2018). Nonetheless, continuous variation in cuckoo egg phenotypes (Drobniak et al., 2014; Stoddard & Stevens, 2011) and the imperfect match between cuckoo egg types and host eggs (Edvardsen et al., 2001; Moksnes & Røskaft, 1995) suggests a complex evolutionary trajectory for cuckoo *gentes*, likely fueled by conditional plastic responses of hosts to parasites and periods of fluctuating selection (M. Soler et al., 2012).

4.2. The Eurasian crow

The Eurasian crow is subdivided between all-black carrion crows (*Corvus (corone) corone*) and grey-coated hooded crows (*C. (c.) cornix*) which are widely distributed across Eurasia. These two lineages meet in two narrow hybrid zones across Eurasia (Haas & Brodin, 2005; Kryukov, 2019; Meise, 1928; Saino et al., 1992), where they are stable through assortative mating (Saino & Scatizzi, 1991) and social exclusion of less common phenotypes (Randler, 2007). Despite their genetic similarity, these crows exhibit a 2 Mb region of differentiation on chromosome 18 consistent as a barrier locus undergoing divergent selection (Poelstra et al., 2014). This region, which appears to be resistant to gene flow, includes multiple genes associated with melanogenesis

(Vijay et al., 2016). Structural variations (Weissensteiner et al., 2020) and epistatic interactions between this region on chromosome 18 and the *NDP* gene (Knief et al., 2019) further contribute to the differentiation between the black and grey morphs. The mapping of individual genes underlying the difference between black and grey-coated crows is rendered difficult due to near-lack of recombination in the differentiated region on chromosome 18, which includes several candidates such as *TYRP1, SLC45A2*, *HPGDS, CACNG* and *AXIN2* closely interacting with the transcription factor *MITF,* a central regulator of the melanogenesis pathway (Knief et al., 2019; Neethiraj et al., 2017; Poelstra et al., 2015; Wu et al., 2019). In this dissertation, I utilize comprehensive wild and common-garden raised crow samples encompassing the German hybrid zone to expand previous speciation genetic work in the system to explore the impact of DNA methylation. I further make use of the common garden experimental design to study dosage compensation and dosage balance, leveraging one of the genetically best studied avian systems.

The importance of chromatin modifications to the speciation process in vertebrates is currently limited to fish and mammal systems (Laporte et al., 2019; Smith et al., 2016; Vernaz et al., 2021, 2022; Y. Wang et al., 2022). Current research in avian systems has identified associations with range expansions and DNA methylation in house sparrows (Hanson et al., 2022), environmental associations with reproductive timing in tits (Lindner, Verhagen, et al., 2021; Viitaniemi et al., 2019), and chromosomal suppression in white-throated sparrows (Sun et al., 2021). However, understanding the independent contributory role of chromatin modifications to the speciation process requires sampling individuals from a continuous genotypic space after controlling for environmental effects. Further research in avian systems is needed to assess the association between genetic divergence and DNA methylation divergence. Research that controls for ontogenetic patterning of DNA methylation using common-garden raised individuals, combined with wild caught individuals encompassing a wide genotypic space, will provide a more precise understanding of how DNA methylation independently contributes to evolutionary processes in crows.

The evolutionary maintenance of dosage compensation between Z-linked genes relative to their ancestral autosomal dosage, and dosage balance between the sexes at Z-linked genes, is of crucial importance for understanding the evolution of sex chromosomes (L. Gu et al., 2017; L. Gu & Walters, 2017). Expression data from Eurasian crows shows no overall dosage compensation on

the Z chromosome, though an increase in the expression of female Z-linked genes suggests some level of partial compensation, linking sex-bias to absolute expression levels (J. B. Wolf & Bryk, 2011). However, it remains unclear how the chromatin structure of the Z chromosome contributes to this pattern. Further research is necessary to explore whether there are areas of localized compensation similar to those found in chickens (Mank & Ellegren, 2009), particularly in relation to regions that are hypermethylated in males (Höglund et al., 2024; Teranishi et al., 2001).

Results

Paper I

Evolution and genetic architecture of sex-limited polymorphism in cuckoos

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Manuscript II

Matrilineal capture ensures stable inheritance and phenotypic innovation in egg mimicry arms race

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Abstract

Coevolutionary arm races can generate phenotypic extremes, yet the maintenance of this diversity in wild populations is not well understood. The common cuckoo (Cuculus canorus) is a deceptive egg mimic that has exploited a stunning variety of hosts and harbors a diversity of counterfeit egg phenotypes. Sequencing 299 cuckoos sampled from 21 hosts and 11 egg morphologies, we reveal that cuckoo gentes—distinct lineages laying mimetic eggs—are maintained by the interplay of matrilineal inheritance and biogeographic opportunity reflected by autosomal genetic variation. Consistent with the combined forces of balancing selection and introgression, we find evidence for the stable maintenance of matrilineal haplotypes across Eurasia. Furthermore, several genes recurrently involved with differential egg morphologies are linked to oxidative phosphorylation, implicating the mitochondria in egg diversification. Our results indicate that phenotypic innovation underlying successful parasitic generalists can be maintained through sex-specific capture of a rapidly evolving locus.

Introduction

Host-parasite co-evolution often generates striking phenotypic novelty resulting from feedback loops of alternating antagonistic selection between hosts and parasites (Ebert & Fields, 2020). The resulting co-evolutionary arms race can result in *Red Queen* dynamics (Rabajante et al., 2016; Van Valen, 1973), entailing the diversification of hosts in efforts to thwart parasites, and its subsequent counterfeiting by parasites (Dixit et al., 2023). Obligate brood parasites, or animals that rely entirely on other species to raise their offspring without providing any parental care, are prime models of such coevolutionary arms races in nature (Sless et al., 2023). Within birds, brood parasitism has evolved independently at least seven times, providing some of the most outstanding examples of intraspecific phenotypic diversity (Stevens, 2013). While some avian brood parasites are highly specialized on a few hosts, others have successfully exploited dozens or even more than a hundred different hosts (Davies, 2000). While the exploitation of numerous hosts gives parasites more reproductive flexibility, preserving multiple successful parasitic traits within a single interbreeding species can be challenging because of gene flow and recombination. This necessitates either behavioral or genetic strategies to ensure the stable transmission of successful parasitic traits across generations. Within avian obligate brood parasites, a satisfying hypothesis maintains that strict maternal inheritance of counterfeit-encoding phenotypes could facilitate host specialization, irrespective of assortative mating with the 'correct' male counterpart, as observed in cuckoo finches (Spottiswoode et al., 2022).

The common cuckoo (*Cuculus canorus)* is a generalist obligate brood parasite which exploits a wide array of passerine hosts across Eurasia (Payne, 2005). A remarkable diversification of cuckoo egg phenotypes has resulted from the dynamic co-evolutionary arms race between passerine hosts capable of detecting parasitic eggs and their common cuckoo parasites that lay mimetic eggs (Moksnes & Røskaft, 1995; Stoddard & Stevens, 2011). Cuckoo *gentes*, which here we ascribe synonymously with egg type, are hypothesized to be maintained by strict maternal inheritance (Fossøy et al., 2016; Gibbs et al., 1996, 2000), although biparental inheritance and assortative mating among some hosts across the range has been suggested (Fossøy et al., 2011; J.-W. Lee et al., 2021). However, a lack of matrilineal structure within European host-types outside of the immaculate blue-egg laying cuckoos and widespread gene flow among host-types in sympatry suggests a stronger role for facultative autosomal contributions to egg diversification (Marchetti et al., 1998).

Three conditions have been proposed to explain the evolutionary maintenance of parasitic egg mimicry, namely that *i)* hosts must be discriminatory against parasitic eggs, that *ii)* egg phenotypes must be heritable, and that *iii)* parasites must make the correct choice about which hosts to parasitize (Brooke & Davies, 1991; Chance, 1940). Here, we leverage whole-genome resequencing genetic data spanning numerous cuckoo *gentes* and biogeographic localities to examine the latter two conditions, namely the geographical, autosomal, and matrilineal contributions to cuckoo host specialization and egg diversification.

Results & Discussion

Matrilineal haplotypes are maintained across the range of *C. canorus*

To determine the population genetic structure underlying cuckoo *gentes*, we performed whole genome resequencing on 299 cuckoos across the distributional range encompassing 15 countries, 21 host species, and 11 egg morphs (**Fig. S1 & Table S1**). Matrilineal phylogenies of the W chromosome and mitochondria indicated an ancient split between blue-egg laying cuckoos and their non-blue laying counterparts (95% highest posterior density (HPD) interval 2.4 – 6.5 MYA, **Table S2**), with seven qualitatively visible haplogroups (hereafter referenced as *W*), one of which corresponds to rufous plumage females within the non-blue egg cuckoos (*W4*, Merondun et al., 2024) (**Figs. 1A, S2, S3**). Population structure inferred at autosomal SNPs (N = 1,546,013) indicated a qualitative association between genetic variation and geographic distance, but not maternal haplogroup (**Figs. 1B & 1C**). Quantitatively, strong patterns of isolation-by-distance among discrete cuckoo geographic groups (N = 14, hereafter referenced as *G*), indicated significant associations with autosomal variation (Spearman's rho / mixed-model coefficient / p-value: 0.918 / 1.22 / 2.41e-18), and significant yet much weaker associations with matrilineal genetic variation (*e.g.,* W chromosome: 0.331 / 0.614 / 0.043) (**Figs. 1C, 1D & S4**). Overall, these results indicate that matrilineal haplotypes in cuckoos are maintained across large geographic distances, potentially facilitated by gene flow among populations or evolutionary forces that favor increased diversity (*i.e.,* balancing selection), or a combination thereof.

Matrilines and biogeography synergistically define cuckoo *gentes*

The observation that matrilines are maintained across great distances raises the hypothesis that these haplotypes may encode parasitically advantageous phenotypes such as egg mimicry, as observed in other brood parasitic systems (Spottiswoode et al., 2022) and hypothesized in common cuckoos (Gibbs et al., 2000), with evidence that at least the blue-egg laying *gens* is matrilineally restricted (Fossøy et al., 2016). We established the association between mimetic egg phenotypes and matrilineal variation, autosomal ancestry, and geographic location using multivariate statistics (**Fig. 2A**), and note that these results are robust to both continuous and categorical classification of covariates *(i.e.* haplogroups) and using both distance-based and classification-based logistic regression approaches (**Fig. S5**). Matrilineal distance explained the most variation in egg phenotypes (mantel $r = 0.189$) establishing the association between matrilines and mimetic egg morphs (all tests $p < 0.01$, **Fig. 2C, Table S3**). This seemingly low correlation, despite compelling qualitative mitochondrial-egg matching (**Fig. 2A**), and 100% matched egg phenotypes among maternal relatives (**Fig. 2B**), is almost certainly due to high mitochondrial distance observed among the prolific egg type *E6* – in visual appearance approximating great reed warbler eggs (*Acrocephalus arundinaceus)* – which is found in four haplogroups including the reverted eggs within the blue-egg lineage (Mantel r increases to 0.357 if *E6:W3* is encoded as a novel egg), making egg type *E6* a prime candidate as the ancestral state (**Fig. S6**). Nonetheless, geography was also predictive of egg phenotypes (Mantel $r = 0.167$), supporting the notion of synergistic matrilineal and biogeographical contributions underlying cuckoo *gentes*. In contrast, modelling cuckoo host species – instead of egg morphologies – and using similar covariates identified a primary association with geography (Mantel $r = 0.361$; **Fig. S5**), suggesting that while cuckoo host choice is constrained geographically, the breadth of diversity within cuckoo matrilines encoding numerous egg phenotypes enables them to exploit a broad range of hosts successfully. Recurrent selection on a single phenotypically relevant locus is a well-established phenomenon that maintains genetic diversity across large distances, often invoking forms of balancing selection that preserves haplotypes despite genetic homogenization (Colosimo et al., 2005; D. Lee et al., 2021). Access to a wide diversity of egg phenotypes is essential for cuckoos as hosts are a critical resource (Soler et al., 2009), so ensuring the persistence of adaptive egg morphs would be essential for exploiting new hosts, particularly during range expansions (Møller et al., 2011), and perhaps even more essential than access to typical food sources (Yom-Tov & Geffen, 2005).

Evolutionary forces maintaining matrilines

The proliferation and stability of matrilines across the species' range raises another hypothesis regarding the evolutionary forces maintaining cuckoo matrilineal diversity, namely that gene flow may facilitate the spread locally adaptive variants, as is well documented in many natural systems (Enbody et al., 2023; Rossi et al., 2024). In common cuckoos, the discovery of a relatively recent maternal ancestor common to both the blue-egg laying cuckoos of Finland which parasitize common redstarts (*Phoenicurus phoenicurus*), and those from Southeastern Asia which parasitize several hosts (Fossøy et al., 2016), raises several non-mutually exclusive hypotheses about the origin and transmission of the blue-egg phenotype (egg type *E1*). Specifically, this trait could have arisen through convergent evolution, be maintained via balancing selection through ancestral population structure post-colonization, or have been acquired via adaptive introgression among contemporary populations. While the matrilineal phylogeny makes the possibility of convergent evolution unlikely (**Fig. 1A**), we tested for introgression among cuckoo populations using autosomal SNPs between two pairs of geographically proximate populations, one pair in Europe where only one population harbors blue-egg laying cuckoos (*G4* & *G5*), and one pair in eastern Asia where both harbor cuckoos with blue eggs *(G3* & *G14,* cf. **Figs. 1B & S7***).* We use the term populations synonymously with our discrete geographic groups (*G*) to summarize contemporary breeding locations.

Despite shared demographic histories within the European and Asian populations and descent from a common ancestor around 100 Ka (**Figs. 2A & S8**), we reveal a recent pulse of gene flow between the southern Chinese population (*G3*) and the European populations between 20 – 40 Ka, a pulse not shared with the east Asian population (*G14,* **Fig. 2B**). Interestingly, subsequent analyses identified excess allele sharing among the Asian populations with the European blue-egg population (*G5*), but not with the other European population (*G4*; both positive *D*-statistics using *G4* as P1, *Z-score*: G5 ↔ G14 = 13.0, G5 ↔ G3 = 10.3, **Fig. 3C & Table S4**). Additionally, a five-population phylogenetic test indicated directional gene flow originating out of the southern Chinese population into the European blue-egg population (**Figs. 3D & S9**). Collectively, we hypothesize the most parsimonious explanation for these patterns is ancestral population substructure combined with a pulse of admixture between western (*G4, G5*) and southern (*G3*) and populations during the last glacial maxima (**Fig. 3E**). Increased contact among geographically peripheral populations during initial population separation 100 Ka, notably between the

individuals within the proto-populations of *G5* and *G14*, is a reasonable explanation for increased allele sharing between contemporary *G5* and the eastern populations compared to *G4*, although the location of ancestral breeding populations is uncertain. A second wave of contact between 20 – 40 Ka has increased allele sharing among the southern Chinese population (*G3)* with the European populations, perhaps coinciding to restricted breeding ranges observed across other birds during the last glacial maxima (Thorup et al., 2021). Gene flow among the western and southern populations is also seemingly more likely than between the western and eastern populations following contemporary migratory flyway corridors in many species (Palm et al., 2015).

Our data indicates that the persistence of adaptive matrilineal haplotypes across Eurasia could be attributed to a mix of introgression and balancing selection. Despite lacking definitive evidence for the presence of the European blue-egg (*E1*) haplotype (*W1*) in the southern population, potentially due to limited sampling, the possibility of adaptive introgression cannot be ruled out. Alternatively, negative frequency dependent selection, a form of balancing selection, could maintain diverse matrilines across the range, as is similarly observed in a female-limited color polymorphism governed by the maternal genome in *C. canorus* and *C. optatus* (Merondun et al., 2024). Balancing selection could maintain diversity in cuckoo matrilines, facilitated by behavioral adaptations of cuckoo hosts to thwart common egg morphs, as hypothesized to similarly maintain cuckoo plumage morphs (Thorogood & Davies, 2012), particularly in the context of cuckoo populations exhibiting population expansions (**Figs. 3A** & **S8**). Indeed, the autosomal coalescence of *C. canorus* populations around 100 Ka (**Fig. S8**) approximately coincides with the matrilineal coalescence of the two blue egg haplotypes (**Figs. 4A** & **S3**), suggesting maintenance in both populations by balancing selection. Therefore, while our data indicate that the European blue egg haplotypes are likely a product of ancestral population structure – or historical gene flow – currently maintained by balancing selection, our detection of high population connectivity combined with the short branch lengths of the geographically widespread *W6* and *W7* haplogroups indicate these haplotypes are prime candidates for recent adaptive introgression. Overall, these results indicate that admixture and rapid population expansions are hallmarks of contemporary cuckoo populations, facilitating the exchange of adaptive matrilineal haplotypes.

Mitochondrial complexes related to oxidative phosphorylation encode egg types

Mimetic cuckoo eggs exhibit an incredible diversity of color and spotting (Stoddard & Stevens, 2011) (**Fig. S1**). This extensive diversity, coupled with the often continuous variation observed within egg types (Drobniak et al., 2014), makes the quest for the causal variant(s) underlying egg morphs difficult. Matrilineal phylogenetic inference suggests an ancient mutation conferred the immaculate blue egg (*E1),* followed by a reversion within this lineage to other egg colors with spotting, while the non-blue haplogroups (*e.g., W4, W5, W6, W7*) diversified into other egg morphs but never an immaculate blue morph (**Fig. 4A**). We leveraged pairwise comparisons among six egg – haplotype groups (*e.g., W1:E1* vs. *W3:E6)* within three evolutionary scenarios to identify genes consistently involved in egg color variation. This approach helped pinpoint genes across different evolutionary paths rather than focusing on single causative mutations, covering scenarios of original blue egg emergence (*Ancient Blue*), blue to non-blue egg reversion (*Contemporary Reversion*), and diversification within non-blue eggs (*Contemporary Diversification*) (**Fig. 4A**).

Fixed SNPs were predominantly observed in the mitochondrial genome across all evolutionary scenarios, particularly in relation to the origin of the blue egg (54.7% of mitochondrial *Ancient Blue* SNPs were fixed), but also within the reverted egg contrast (*Contemporary Reversion* = 12.3%) and contrasting diverse eggs within the non-blue lineage (*Contemporary Diversification* = 4.48%), compared to fixed autosomal SNPs (0%, 0%, and < 0.001%, respectively) (**Fig. 4C, Table S5**). Notably, genes *ND2* and *ND4* in the mitochondria showed nonsynonymous SNPs linked to blue and reverted egg traits (**Fig. 4D**). Candidate genes containing a fixed nonsynonymous SNP were statistically over-enriched for the oxidative phosphorylation pathway (*P* = 8.76e-09) (**Fig. 4E** & **Table S6**). Further network analysis revealed connections among mitochondrial and W-linked candidate genes, including a respiratory complex I assembly factor (*NDUFAF4-L, LOC128850245* in RefSeq Assembly GCF_017976375.1), which appears to have migrated several copies onto the W chromosome (27.6-*Kb*; mean female coverage = 6.36, male coverage = 0.13) from its autosomal copy on chromosome 3 (4-*Kb*; female = 11.3, male = 11.0) (**Fig. 4E**). Mitonuclear interactions play a crucial role in reproductive compatibility (Moran et al., 2024), so our results suggest mitonuclear interactions govern cuckoo egg diversification, which are likely well established from the autosomally homogeneous populations which balance ancient matrilineal haplotypes which diverged millennia ago (**Figs. 4A**). In the non-blue lineage of cuckoo eggs, contemporary diversification is marked by the presence of autosomal or Z-linked genes with nonsynonymous SNPs. This includes two uncharacterized genes (LOC128850746 &

LOC128850426) and *TLDC2* (**Fig. 4F**), which modulates oxidative stress responses. Oxidative stress responses are indirectly linked to oxidative phosphorylation, a process that can increase the production of reactive oxygen species (ROS) in the mitochondria (Finelli & Oliver, 2017), further suggesting a role of mitonuclear interactions governing cuckoo egg diversification.

The pigmentation of avian eggshells is primarily attributed to protoporphyrin IX and biliverdin for brown and blue eggs, respectively, of which the former is a precursor of heme and the later results from the oxidative degradation of heme (Kennedy & Vevers, 1976). The enzyme producing protoporphyrin IX is known to operate within the mitochondria (Sano et al., 1959), suggesting the mitochondrion as a central network underlying cuckoo egg diversification, likely leveraging the heme biosynthetic pathway which directly interacts with the mitochondrion (Yien & Perfetto, 2022). Other avian species have co-opted numerous independent mechanisms to achieve the same result, exemplified by blue eggs conferred by SNPs upstream of efflux transporter *ABCG2* in ducks (Chen et al., 2020), an endogenous retroviral insertion upstream of anion transporter *SLCO1B3* in chickens (Z. Wang et al., 2013), and an autosomal recessive gene *ce* in quail (Ito et al., 1993). Here we reveal that cuckoos have exploited the mitochondrion and the OXPHOS system, likely leveraging the heme biosynthetic pathway, as a nexus for egg diversification.

Conclusion

Obligate brood parasites require an arsenal of tools to successfully exploit a diversity of discriminatory hosts. Here, we demonstrate that cuckoo matrilineal haplotypes are maintained by balancing selection and gene flow among populations, and that these haplotypes interact with autosomal variation to encode mimetic eggs. Leveraging the fastest-evolving genome, female cuckoos have co-opted the mitochondria to ensure the stable transmission of their adaptive phenotypes to their daughters while simultaneously maximizing their phenotypic potential.

Materials & Methods

Sample Collection & Sequencing

We sampled 299 common cuckoos encompassing their global distribution (*C. canorus*; sampling year range 1992 – 2020). Sampling was opportunistic from numerous field trips spanning 15 countries with a focus on samples where at least the host species was known (n = 223), either by direct observation of a nestling within a host species' nest (n = 197), or extrapolation if only a

single host is known to be parasitized within a locality (n = 26). A subset of cuckoos with known host also had a known egg phenotype documented before hatching (n = 157, **Fig. S1**). DNA was extracted predominantly from blood samples (n = 179), muscle tissue (n = 88), or feathers (n = 32), using the DNeasy Blood & Tissue Kit (QIAGEN). Two outgroup species were also collected and sequenced, including the Indian cuckoo (*C. micropterus;* n = 5), and the lesser cuckoo (*C. poliocephalus*, n = 2). Genomic DNA quality was assessed with a combination of NanoDrop, Qubit® 3.0 and agarose gels, followed by whole-genome resequencing library preparation with NEBNext DNA Library Prep Kits (New England Biolabs). We quantitated final libraries with either a 2200 TapeStation (Agilent Technologies) or qPCR KAPA Library Quantification Kit (Roche). Paired-end 150-bp whole-genome shotgun sequencing was conducted on a NovaSeq 6000 (Illumina; Novogene UK) or a HiSeq X (Illumina; Macrogen, South Korea) targeting 10x coverage (minimum / mean / maximum output: 11.4 / 21.8 / 86.4 *Gb*) and resulting in a total of 6.67 *Tb* output corresponding to 47.6 billion reads. All sequencing reads are available in the Sequence Read Archive under BioProject PRJNA614488. Necessary compliance with the Nagoya protocol for sampling in each country was ensured.

We agnostically assigned a *Geographic* group (G) to each common cuckoo sample (N = 299) using k-means clustering of latitude and longitude. We reduced autocorrelation from sampling bias by spatial thinning observations to 25-*Km* using spThin v0.2.0 (Aiello-Lammens et al., 2015), and chose a number of clusters (k = 14) based on changes on within-cluster variation inferred from a decrease in the gap statistic*,* corroborated with *a posteriori* data inspection (**Fig. S7**), relying on sf v1.0-4, geosphere v1.5-18, and factoextra v1.0.7 packages within R v4.3.0 for analysis (Hijmans et al., 2022; Kassambara & Mundt, n.d.; Pebesma, 2018; R Core Team, 2017).

Alignment and Variant Calling

We trimmed raw reads with BBtools v38.90 (Bushnell, 2021) using filters for minimum read length and regions failing quality filters (‘-minlen 25 -trimq 2’). Read were aligned to a soft-masked common cuckoo genome (Merondun et al., 2024; GCF_017976375.1) using the Burrows-Wheeler Aligner (BWA) v0.7.17-r1188 (aligned reads minimum / mean / maximum (million): 79.9 / 152 / 831 M) (Li & Durbin, 2009). Aligned and sorted reads in proper pairs were filtered to remove reads failing platform quality checks (‘-F 524’) using samtools v1.6 (Danecek

et al., 2021). We merged replicate sample libraries and deduplicated each binary align map (BAM) with sambamba v0.8.1 (Tarasov et al., 2015) and removed reads overhanging scaffold ends with GATK v4.2.4.0 (Auwera et al., 2013). Mean coverage was calculated in 25-*Kb* windows genome-wide with mosdepth v0.3.3 (genome-wide mean coverage across samples min / mean / max: 7.27 / 14.0 / 69.7x) (Pedersen & Quinlan, 2018).

SNP genotypes were called with bcftools v.1.16 (Danecek et al., 2021), using strict criteria in low mappability regions ('-C 50'). Genotypes were merged into chromosomal files and filtered to remove low quality sites (*QUAL* < 20, *DP* greater than twice the chromosomal average or below 307 (*i.e.,* the number of samples), *MQ* < 30, or *RPBZ* below -3.0 or above 3.0). Genotypes below 3x coverage were set to missing data and weakly heterozygous genotypes on haploid (female) Z, W, and mtDNA were assigned to the major allele (allele depth binomial test $p < 1.0e^{-5}$) using the bcftools +setGT plugin. Erroneously called remaining heterozygous sites on these haploid chromosomes were set to missing data. The Z chromosome was filtered and analyzed separately for males and females (males diploid, females haploid). Problematic regions identified using coverage discrepancies (*e.g.,* male coverage on the W chromosome) were removed for population genetic analyses, but not for the candidate gene search, as outlined previously (Merondun et al., 2024). Finally, we removed SNPs with more than 10% missing genotypes. Maternal haplotypes of mtDNA and W chromosome were reconstructed using vcf2phylip v2.7 (Ortiz, 2019) from the mtDNA and W chromosome VCFs, using a stricter mapping quality threshold *(MQ* > 40) due to the prevalence of pseudogenes and repeats on these sequences.

Identification of Relatives

We collected many samples in close proximity to one another within our field sites, resulting in a large number of siblings. We therefore identified relatives using vcftools v0.1.17 on a linkage-pruned autosomal VCF using plink v2.00a3.6LM ('--indep-pairwise 50 5 0.1 --maf 0.05') (Purcell et al., 2007) and removed any third-degree related individuals or higher with a *phi* greater than 0.0442 (n = 97) (Danecek et al., 2011). In the process of removing related individuals, we preferentially retained the female sample, and if the sex of the related samples was the same, we dropped the individual with the most missing genotypes.

We utilized our subset of related individuals to assess the transmission of certain traits (egg morph, host, habitat type) corresponding to different degrees of relatedness (*e.g.,* first-degree, second-degree) and among different maternal and paternal lines. We narrowed this analysis to only nestlings sampled within two years of one another with known egg morph, host, or habitat type (see below for habitat type designation). We assessed for each comparison if the phenotype among the two compared cuckoos was matched, and calculated the proportion of matched phenotypes across all degrees of relatedness and among maternal and paternal lines. All comparisons only considered cuckoos within the same geographic group, so that decreasing degrees of relatedness account for the local background of ancestry (*i.e.,* we only considered *unrelated* cuckoos that both occur within the same geographic group, **Fig. S7**), providing 153 individuals and 2,806 comparisons. Analyses and visualization utilized R and the tidyverse (Wickham et al., 2019).

Matrilineal Phylogenetics

We inferred maximum-likelihood phylogenies on the W chromosome (n = 68,982 SNPs) and mtDNA (n = 573 SNPs) using IQTREE v2.2.0.3 (Nguyen et al., 2015) with modelfinderplus and ascertainment bias correction. The final consensus tree was estimated using bootstrap support from 1,000 ultrafast bootstraps (Hoang et al., 2018) and 1,000 SH-like approximate likelihood ratio test bootstrap replicates (both values ≥ 95% indicated on trees). We midpoint-rooted phylogenies in R v4.1.1 (R Core Team, 2017) using phytools v1.5-1 (Revell, 2012) and visualized trees with ggtree v3.6.2 (Yu et al., 2017). For visualization, we collapsed nodes into seven arbitrary but supported higher-level haplogroups, and assigned each individual a haplogroup based on corroborated W chromosome and mtDNA variation (**Fig. S2**).

We substantiated our maximum-likelihood W chromosome phylogenies with time-calibrated Bayesian phylogenies using SNAPP v1.6.1 (Bryant et al., 2012) using 1,000 randomly sampled W chromosome SNPs, implemented with a log normal constraint on the crown of phylogeny with a mean of 5.0 and a standard deviation of 0.25, giving a 95% highest posterior density (HPD) total tree height spanning 2.71 – 7.10 million years. Two replicate SNAPP runs using different SNP subsets were assessed after at least 300K chains using Tracer to ensure convergence (**Table S2**). Final trees were extracted and annotated using Treeannotator with a 10% burn-in and 95% HPD interval heights, visualizing the output with ggtree v3.6.2 (**Fig. S3**) (Rambaut et al., 2018; Yu et

al., 2017). Additional time-calibrated phylogenies were inferred with BEAST (**Supplementary Text**, **Fig. S10**).

Following the presumption of matrilineal inheritance of egg morphologies (Fossøy et al., 2016; Gibbs et al., 2000), we reconstructed probabilities of ancestral egg morphs using the mtDNA phylogeny above including all cuckoos with known egg morph *(N* = 152). Polytomies were resolved randomly and discrete ancestral egg characters were inferred with ape v5.7-1 using default settings (*i.e.,* maximum-likelihood, equal rates of transition) and the marginal estimates were visualized with phytools v2.1-1. These results were corroborated with simulated stochastic character maps using 10,000 generations of MCMC and 100 simulations, also with equal rates of transition, implemented with phytools (**Fig. S6**).

Population Genetic Differentiation

We first assessed population genetic variation with a principal component analysis (PCA) on unlinked autosomal SNPs with a minor allele frequency of at least 5% (*N* = 1,546,013) from the unrelated sample set (*n* = 202) using plink v2.00a3.6LM (Purcell et al., 2007). PC axes 1 – 4 were visualized with symbology according to haplogroup, geographic group, and ancestry group (at *K* = 5; see below) using ggplot2 v3.4.4 (Wickham, 2016) (**Fig. S11**). Initial inspections showed a qualitative correlation between autosomal PC space and geographic distance (**Fig. S11**), so we formalized this relationship with a Procrustes analysis between latitude and longitude and the first two PC axes using vegan v2.6-4 (Dixon, 2003). We extracted the similarity statistic using a scaled transformation with the best reflection and assessed significance using a permutation test with 100,000 permutations, indicating a small optimal rotation angle (-6.21°). Based on this negligible transformation, we decided to simply show sample PC scores scaled to latitude and longitude superimposed onto a geographic map of the study extent (**Fig. S12**).

The relationship between geographic distance and genetic distance was further quantified with correlations and linear mixed models. We calculated pairwise Haversine geographic distance between the mean latitude and longitude calculated for all *Geographic* groups which had three or more female individuals (*n* = 10; **Fig. S7**) using geosphere v1.5-18 and tidyverse v2.0.0 in R v4.3.0 (Hijmans et al., 2022; R Core Team, 2017; Wickham et al., 2019). Pairwise autosomal and Z chromosomal F_{ST} was estimated in 100-*Kb* windows using vcftools v0.1.17, while pairwise Φ_{ST}

was estimated for W chromosome and mtDNA with an analysis of molecular variance (AMOVA) using the same fasta files used for phylogenetic inference, using poppr v2.94 within R (Kamvar et al., 2014). Only male and female samples were used for the Z chromosome and W chromosome estimates, respectively. We then collated geographic distance and genetic distance estimates by *Geographic* group and calculated Spearman rank correlations using cor.test, and assessed significance after Bonferroni correction. We visualized the resulting output with both log transformed F_{ST} / Φ_{ST} against log transformed geographic distance, in addition to the metric F_{ST} / $1 - F_{ST}$ (**Fig. S4**) (Rousset, 1997). A floor was added to both divergence and distance values (0.005) for log transformation of zero values. We further validated our results using linear mixed models which reduced the effects of pseudo-replication inherent in pairwise comparisons by incorporating population one and population two as random effects using lme4 v1.1-35.1 and lmerTest v3.1-3 (Bates et al., 2015; Kuznetsova et al., 2017). A separate model was fit for each genomic compartment (autosome, Z, W, mtDNA) followed by Bonferroni correction (**Table S7**).

We then assessed autosomal structure using ADMIXTURE v1.3.0 (Alexander et al., 2009) with inferred K2 – K10 using 5-fold cross validation and evaluation with evalAdmix v0.962 (Garcia-Erill & Albrechtsen, 2020). Ancestry coefficient (Q) matrices with all K and grouped by geographic groups (**Fig. S13**) and haplogroup (**Fig. S14**) were visualized in R with tidyverse v2.0.0 and viridis v0.6.4 (Garnier et al., 2023; Wickham et al., 2019). Optimal K was identified by comparing the correlation of residuals among individuals from evalAdmix and selecting the K which was closest to zero (best three: *K2, K5, K10)* while maintaining the lowest cross-validation (lower K always scored better), so we selected $K = 5$ to represent the ancestry variation for subsequent analyses requiring a discrete selection (**Figs. S15 & S16**). We visualized a spatial representation of interpolated ancestry coefficients using TESS3R v1.1.0 (Caye et al., 2016) across three K-values (**Fig. S17**).

Genetic and Geographic Associations with Egg, Host, and Habitat

We then quantified how nuclear genetic ancestry (K), geographic location (G), and maternal haplogroup (W) are related to three different cuckoo traits: mimetic egg morph, host species, and habitat type. Egg morph and host species were observed directly, while habitat type was designated from extracted habitat land class values using the MODIS collection 5 global raster (Friedl et al.,

2010) and the cuckoo sampling coordinates (**Fig. S18**), providing a subset of samples with all three traits observed among unrelated cuckoos (n = 87). Unrelated cuckoos were selected because of collinearity among predictor variables, although we note that egg morph, host, and habitat phenotypes were not completely shared among relatives outside of the matrilineal transmission of egg morphs (**Fig. S19**). First, Gower's dissimilarity matrices were calculated among unrelated cuckoos for each trait (*e.g.,* egg, host, habitat) using binarized dummy variables and the daisy function in vegan v2.6-4 (Dixon, 2003). We then calculated corresponding dissimilarity matrices of 1) nuclear genetic ancestry using the same SNP set as above (N = 1,546,013) with ape v5.7-1, 2) geographic location using latitude and longitude with geosphere v1.5-18 and sf v1.0-4, and, 3) mtDNA with ape on the haploid mtDNA sequences, providing six dissimilarity matrices encompassing the traits (*e.g.,* egg, host, habitat) and the covariates (*e.g.,* ancestry, geography, haplogroup). Distance matrices for visualized prior to analysis using nonmetric multidimensional scaling for binarized categorical response variables and classical metric multidimensional scaling for continuous explanatory variables using metaMDS and cmdscale, respectively, in vegan v2.6-4 (**Fig. S20**). We then performed cross-Mantel tests between each response variable and covariate, using the remaining two covariates as control matrices, using Spearman's rank correlation with 1,000 permutations within ecodist v2.1.3 (Goslee & Urban, 2007). We report Bonferonni-corrected p-values and the absolute value of Mantel test statistics (r) because the categorical variables lack directionality. Further analyses using both distance-based redundancy analyses incorporating a flipped analytical design, and classification-based multinomial logistic regressions corroborate results (**Supplementary Text, Fig. S5**).

Blue Egg Evolutionary History

Within common cuckoos, previous research has identified an ancient matrilineal lineage corresponding to the immaculate blue egg phenotype, which parasitizes several hosts from southeast Asia to northern Europe, notably the common redstart in Finland (*Phoenicurus phoenicurus*) (Fossøy et al., 2016; Moksnes & Røskaft, 1995). Following our result that maternal haplogroups appear resistant to the homogenizing effects of gene flow (**Fig. 1D**), and the associations with haplogroup and egg morph (**Fig. 2C**), we sought to characterize the evolutionary history of the blue egg laying cuckoos using autosomal data to determine if the blue egg (morph *E1*) is likely a product of either adaptive introgression or balancing selection. We leveraged

information between two pairs of sister populations (*Geographic* groups), one pair in Europe in which one population does not lay blue eggs and largely parasitizes great reed warblers (*Acrocephalus arundinaceus;* group *G4*) and one population which lays blue eggs and parasitizes common redstarts (*Phoenicurus phoenicurus; G5*). The second pair of sister populations from eastern Asia are both known to harbor cuckoos laying blue eggs which parasitize either grey bush chats (*Saxicola ferreus*) or ashy-throated parrotbills (*Suthora alphonsiana*) (*G14, G3)*. We minimized sampling bias by randomly subsampling an equal number of individuals from each population (n = 12), in addition to two outgroup *C. poliocephalus* samples. All of the following results are robust to subsampling with less samples and using different individuals (n = 6; **Fig. S21**).

We first established trends in historical effective population size (N_E) among the four populations using MSMC2 (Schiffels & Wang, 2020). Autosomal SNP VCFs on chromsomes 1 – 19 were re-filtered to include only the target individuals and for site-level INFO filters for mapping quality (MQ > 30), depth (DP > 150) and quality (QUAL > 20). SNPs were phased with Beagle v5.2 in windows of 40 sites with imputation. In lieu of including invariant sites, we included a sample-specific BED file corresponding to callable regions, which we defined as coverage below double and above half of the chromosomal average, calculated for each chromosome and at base-pair resolution using mosdepth v0.3.3 (Pedersen & Quinlan, 2018). Cross-coalescent analysis with MSMC2 was run using two randomly selected individuals from each population, providing eight total haplotypes for each analysis. MSMC2 input files were generated with `generate_multihetsep.py` for each chromosome, incorporating a reference sequence mappability mask created from SNPable (k = 50, r = 0.5) (Li, 2012) and the above-referenced VCFs and coverage BED files corresponding to the four samples.

We ran MSMC2 first on each population individually, followed by exhaustive haplotype comparisons for each cross-coalescent analysis (`-I 0-4,0-5,0-6,0-7,1-4,1-5,1-6,1-7,2-4,2-5,2-6,2-7,3-4,3-5,3-6,3-7`). Population-specific and cross-coalescent results were collated with `combineCrossCoal.py`. Symmetric migration among populations was inferred with MSMC-IM (K. Wang et al., 2020) using the cross-coalescent outputs, a generation time of 2.74 years (Bird et al., 2020), and the average autosomal mutation rate reported in birds ($\mu_{generation}$ = 1.01e-08) (Bergeron et al., 2023). We then repeated this entire analysis a second time with different

subsampled individuals from each population, across all six possible pairwise population contrasts for sensitivity. N_E estimates within the first four time-steps exhibited high uncertainty and were excluded, inferred from high standard deviation among replicates (**Fig. S8**). We calculated the harmonic mean of N_E across all time points, and visualized N_E through time for each population using ggplot2. Within each population, we visualized the minimum and maximum N_E observed at each time point as confidence intervals, and smoothed the estimates through time using 10,000 interpolated points. All results were analogous across the two iterations (**Fig. S8**).

The following analyses used autosomal SNP VCFs re-filtered excluding SNPs with more than 10% missing genotypes within the subset individuals (n = 50, including the two outgroup *C. poliocephalus*), and unless otherwise indicated used SNPs in linkage disequilibrium pruned using bcftools +prune with an R^2 of 0.2 in 5-*Kb* windows (n = 7,065,720 SNPs). We estimated genome-wide topological concordance from trees inferred in 50-SNP windows with a General Time Reversible (GTR) substitution model using phyml v3.3.20200621 (Guindon et al., 2010) and TWISST (Martin & Van Belleghem, 2017), with results plotted in *R* with the genomics general repository scripts (**Fig. Sr**). Pairwise excess allele sharing among populations and between populations and their internal branches was estimated using f_{branch}. We calculated *D, f4,* f_{branch}, and *Z*-scores using DSuite v0.5 (Malinsky et al., 2021), providing the most prevalent genome-wide topology inferred from TWISST as the input tree.

We next investigated the directionality of the detected admixture between the blue egg-laying population in Finland (*G5*) and eastern populations by employing a symmetric five-population phylogeny using D_{FOIL} tests. D_{FOIL} is designed to discern excess allele sharing among two pairs of in-group populations (D_{FO}, D_{IL}, D_{FI}, D_{OL}) by examining pairwise population relationships (*e.g.,* a positive D_{FO} indicates excess sharing between P1 and P3 while a negative D_{FO} indicates excess sharing between P1 and P4). A single individual from each population (*G5*, *G4*, *G14, G3*, and *C. poliocephalus*) was randomly sampled to generate D_{FOIL} input files. Genome-wide SNP-fasta files were generated using vcf2phylip v2.7 (Ortiz, 2019) from the unpruned autosomal SNP VCF, requiring no missing data among the five samples. We then divided this whole-genome SNP file into 5000-SNP windows using SeqKit v2.3.0 (Shen et al., 2016), resulting in a number of non-overlapping genomic windows (mean ± SD: 3137 ± 46.4 windows per replicate). D_{FOIL} v2017-011-25 was run with the minimum *D* denominator sites per window reduced to two, accounting

for low divergence among populations, but otherwise with default parameters. Each window was then assigned admixture events predicated on one-sided chi-squared testing as implemented with DFOIL ($P < 0.01$). Acknowledging DFOIL's sensitivity to population topology, analyses were conducted under two configurations: ((*G5,G4),(G14,G3*), *C. poliocephalus*) and *((G14,G3),(G5,G4), C. poliocephalus*), both yielding consistent patterns (**Fig. S9**). Following the specifications of DFOIL, and given the low divergence between populations *G5* and *G4* compared to *G14* and *G3* (**Fig. 1B**), the former topology is presented in the main figure. We then repeated the above analysis 20 times for each topology with different randomly sampled individuals to ensure robust population sampling (**Fig. S9**, **Table S8**). Admixture events across all replicates were visualized with default parameter boxplots in R. Asymmetric migration was statistically examined using pairwise Wilcoxon rank-sum tests between the counts of estimated symmetric admixed windows (*e.g.,* P1 → P3 vs. P3 → P1), incorporating Bonferroni correction for significance ($P < 0.05$).

Genomic Basis of Mimetic Egg Morphologies

We utilized a targeted selection of unrelated female nestlings with known egg type within our dataset to examine the genetic associations of egg morphology, requiring at least four females from each egg type for analysis ($n = 47$) (**Fig. S22**). The matrilineal phylogeny suggests an ancient mutation conferring the immaculate blue egg, followed by its reversion within this lineage back to spotted eggs (**Fig. 1A**). Egg types within the non-blue lineage have diversified into several morphs, but never an immaculate blue morph. We exploit this matrilineal system to determine the genetic basis of three egg diversification scenarios: *1)* the genomic basis of blue eggs, comparing blue eggs from both haplotypes (*W1, W2*) to eggs on the non-blue lineage (*W5, W7; Ancient Blue*); 2) the basis of reverted eggs, comparing the reverted eggs (*E6* in *W3*) to their blue-egg sisters (Contemporary *Reversion*); and 3) the genomic basis of diversification within the non-blue lineage, comparing the great-reed warbler type eggs (*E6,* present in *W5* and *W6*) to the striking eggs mimicking thick-billed warblers (*E10*). It is important to note that the following analyses on offspring offer insights into the maternal genetic factors influencing egg diversity, but paternal contributions will be obscured. The absence of direct genetic information from the mothers necessitates the assumption that the offspring share the egg phenotype of their mothers, which is likely satisfied following the observation of matrilineal inheritance in this system (Fossøy et al.,

2016), and our results indicating 100% shared egg phenotypes among sisters and along matrilines more generally (**Fig. S19**).

Genetic differentiation (F_{ST}) was estimated at base-pair resolution between the pairwise egg – haplotype comparisons from each evolutionary scenario using SNP VCFs re-filtered SNPs with less than 10% missing genotypes among subset samples, ensuring chromosomes were encoded with appropriate ploidy for females (W = 1N, mtDNA = 1N, Z = 1N). Otherwise, the same filters were applied as above (cf. *Matrilineal Phylogenetics*). We used a haploid-aware version of VCFtools to estimate F_{ST} and assigned putative functional codon annotations (e.g., *non*synonymous) to each SNP with VariantAnnotation v1.40.0 in R (Obenchain et al., 2014). We then filtered for fixed SNPs (F_{ST} = 1.0) observed in every comparison within each evolutionary scenario. For instance, if we identified a fixed SNP at position 4645 in the Ancient Blue contrast between group *E1:W1* and *E6:W5*, we also required that position to be fixed in all five other Ancient Blue contrasts. Additionally, we incorporated two control contrasts wherein the egg type was conserved but the haplotypes were diverged (*E1* across *W1* and *W2*; *E6* across *W5* and *W7*), and removed any SNPs which were fixed in those contrasts. Candidate genes were identified as having a fixed nonsynonymous SNP across all comparisons within each evolutionary scenario. W chromosome genes are underexplored in avian species and often lack robust annotations, so we assigned each gene to its closest known chicken homolog for network analysis *(e.g., LOC128850245* has a RefSeq annotation 'NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4-like', and is assigned homology to the gene *NDUFAF4).* We ensured consistency with the chicken assembly for candidate genes with a known homolog and created a network with STRING, hiding disconnected nodes and setting edges to confidence levels with otherwise default settings. We extracted the enrichment of gene ontology (GO) biological processes and KEGG pathways from this same analysis. Representative genotypes of nonsynonymous SNPS within candidate genes were visualized with ggplot2.

This analysis was supplemented with parallel all-versus-one genome-wide association analyses including all unrelated samples and egg morphs, which similarly identified a mitochondrial gene *ND*5 as a candidate involved in the greatest number of egg contrasts (**Supplementary Text**, **Figs. S23** & **S24**). However, due to the limited power of these analyses with our available samples we present the female-only analysis with higher sampling in the main text.

Primary Figures

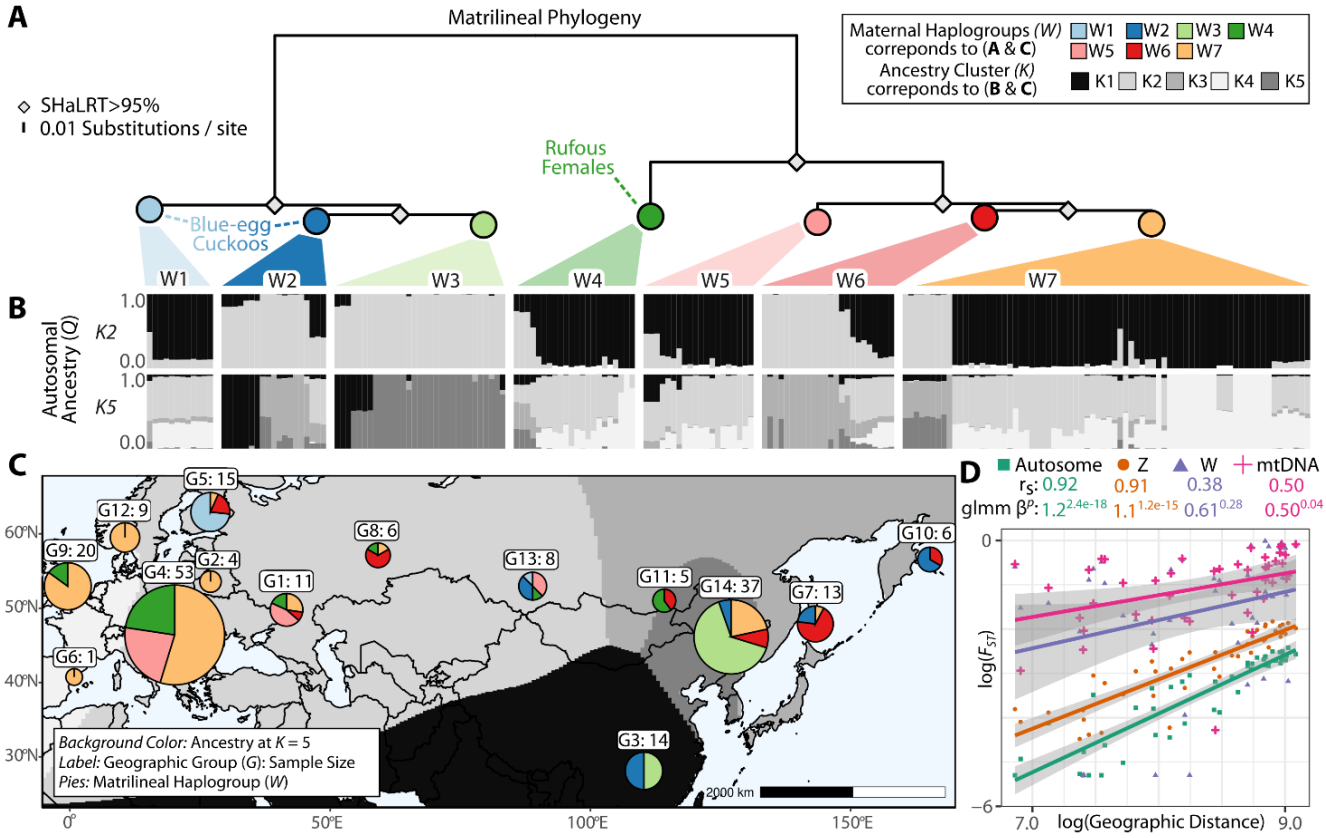

Fig. 1. Matrilineal continuity despite autosomal spatial structuring in *C. canorus*.

(**A**) Matrilineal phylogeny of the W chromosome with truncated branches identifying the 7 maternal haplogroups. (**B**) Ancestry analysis inferred from unlinked autosomal SNPs, arranged by maternal haplogroup with individuals along the *X*-axis. (**C**) Background color indicates the spatial distribution of autosomal ancestry (*K*) inferred at $K = 5$. Pie charts indicate maternal haplogroups with size corresponding to sample size. Samples were delineated into 14 discrete geographic groups with *k*-means clustering, indicated by labels and sample sizes above each pie chart. (**D**) Correlations between geographic distance (*km*) and genetic distance, summarized from autosomal ($F_{ST} - 1/ F_{ST}$) or matrilineal ($\Phi_{ST} - 1 / \Phi_{ST}$) variation across pairwise comparisons between geographic groups. Spearman's rank correlation (r_s) and linear mixed model estimates and Bonferroni-adjusted p-values (β^p) for each genomic compartment indicated above.

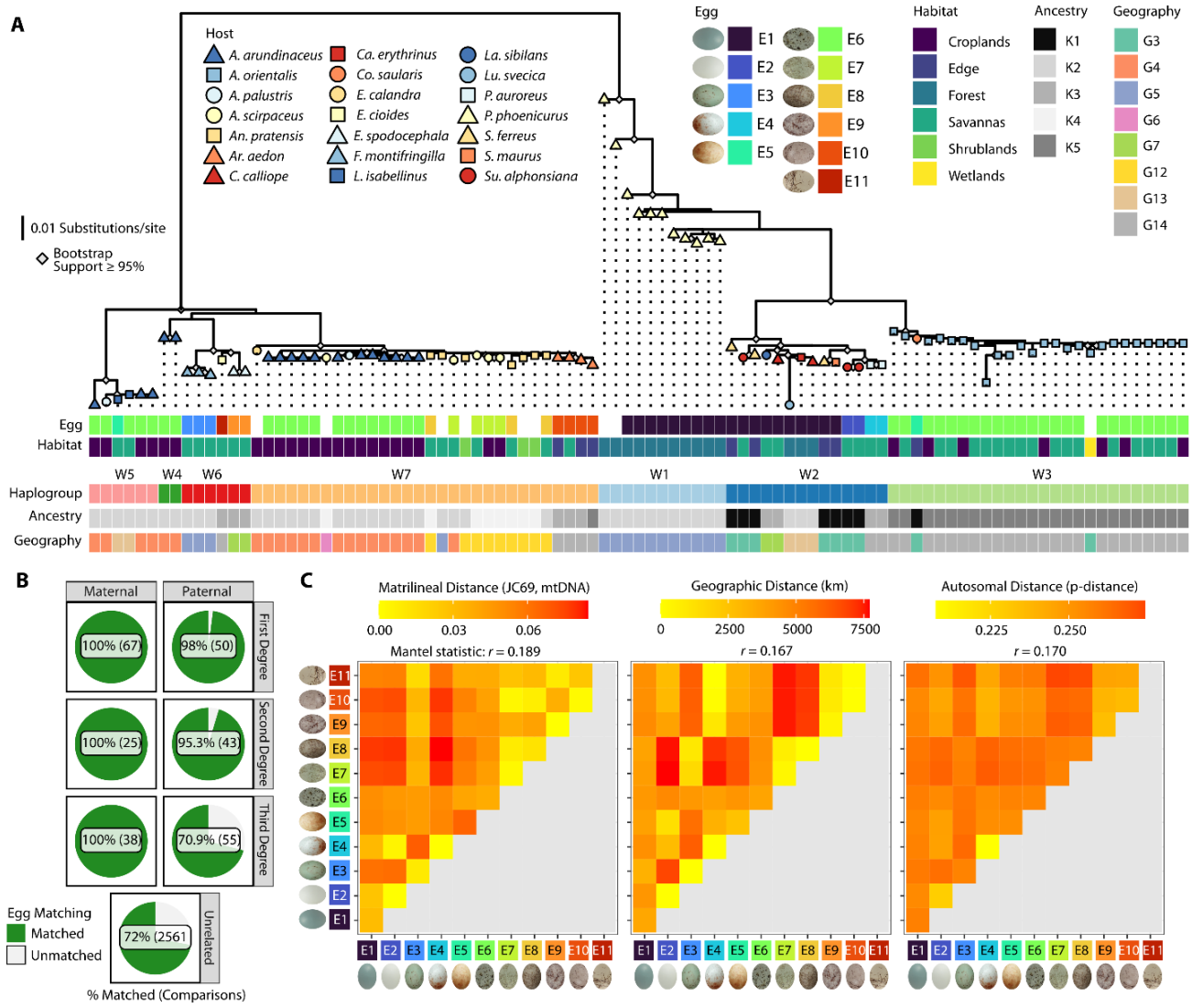

Fig. 2. Matrilineal variation associated with egg diversification in *C. canorus*.

(**A**) Midpoint-rooted mitochondrial phylogeny with tip points and upper two bar charts corresponding to discrete cuckoo traits (host species, egg morph, habitat). Lower bar charts correspond to discrete explanatory variables (maternal haplogroup (*W*), ancestry group (*K*), and geographic group (*G*)). (**B**) Analysis of egg phenotypes across different levels of autosomal genetic relatedness, restricted to comparisons within identical geographic groups (*G*) for non-related individuals. Maternal and paternal lineages were delineated using mtDNA sequence (**C**) Mean mitochondrial, geographic, and autosomal variation observed within and across egg morphs, with lower values corresponding to less variation observed within the contrast. Continuous pairwise distance matrices were summarized among all individuals for mitochondrial, geographic distance, and autosomal variation. Mantel statistics (*r*) from cross-Mantel tests are shown above each plot, quantifying the correlation between each matrix and egg type. *E11* has only a single individual so no intra-group variation could be estimated.

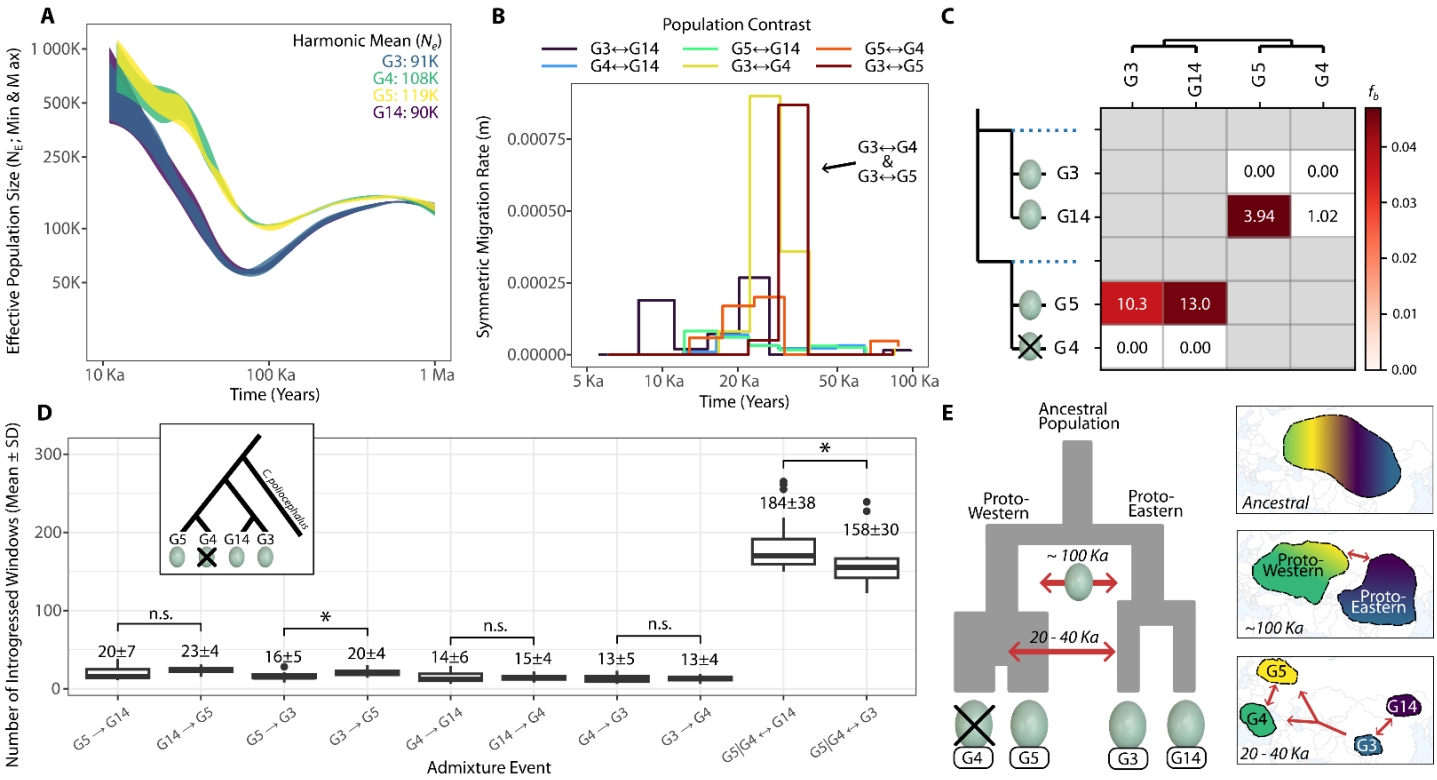

Fig. 3. High population connectivity facilitates matrilineal transfer in *C. canorus*.

(**A**) Demographic inference of two pairs of sister populations: European populations, with *G5* laying blue eggs and *G4* not, and an Asian pair, both *G3* and *G14* laying blue eggs. Minimum and maximum effective population size (N_E) across replicates is indicated by ribbons, smoothed through interpolation. Harmonic mean across all timepoints indicated. (**B**) Symmetric migration rates among population pairs derived from cross-coalescence analysis indicates a recent pulse of admixture between Southeastern China (*G3*) and European populations. (**C**) Excess allele sharing derived from f_{branch} statistics, indicating source populations along the *x*-axis and recipient populations or ancestral nodes on the *y*-axis, with Z-scores indicated within each cell. (**D**) Directionality of admixture events inferred with a five-population DFOIL test, with the mean and standard deviation of admixed windows across 20 replicates shown. Significance between symmetric contrasts is determined with a Wilcoxon rank sum test (Bonferroni-corrected $P < 0.05$). (**E**) Schematic representation of inferred population history, suggesting coalescence within *C. canorus* around 100 Ka. Excess allele sharing among *G5* and *G14* likely arise from ancestral population structure and increased contact among geographically peripheral populations during initial population divergence. A later pulse of gene flow between 20 – 40 Ka is inferred from Southeast Asia into Europe, indicating a rich history of population connectivity. Maps show approximation of contemporary breeding locations and hypothetical historical distributions.

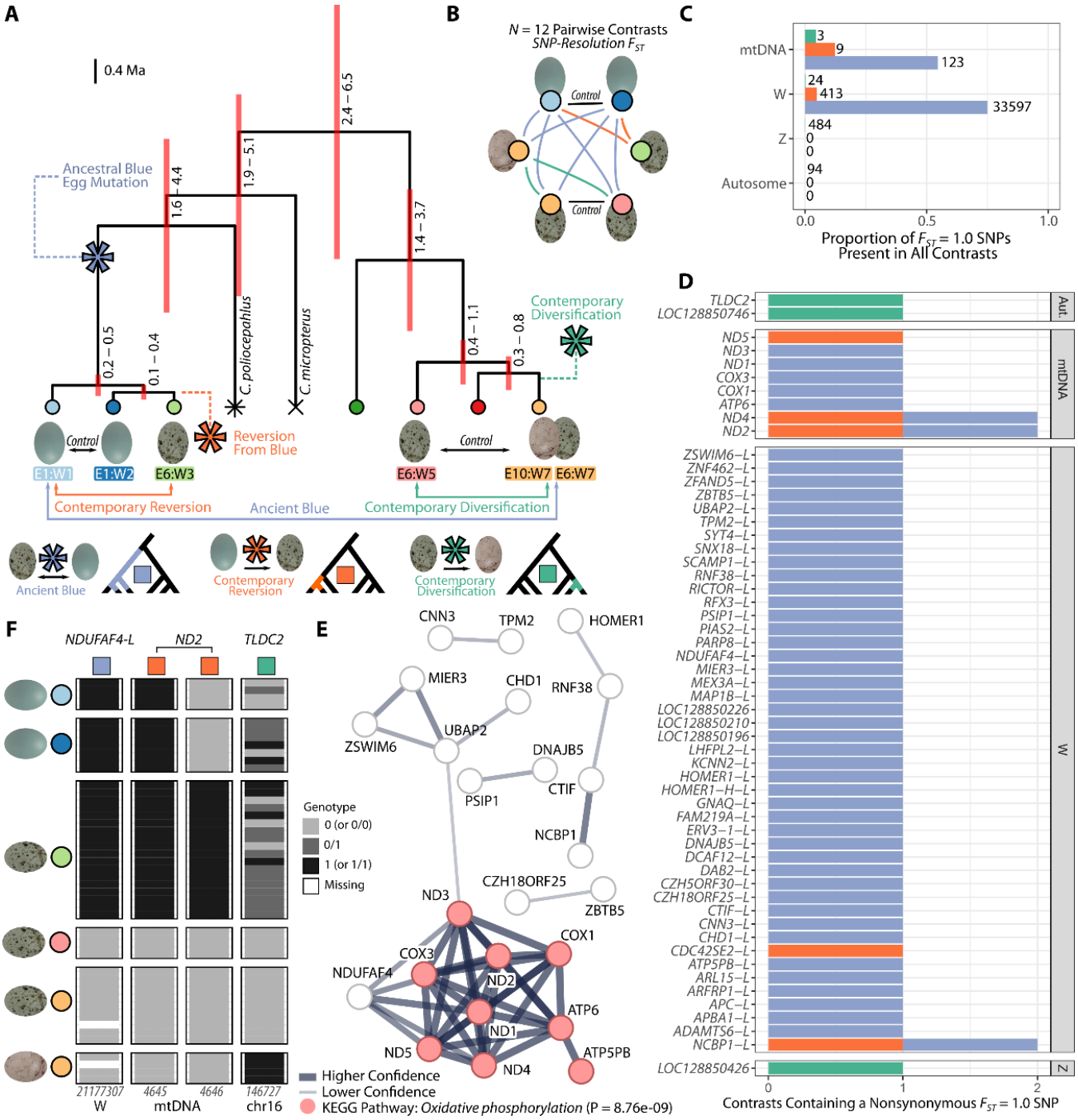
A
0.4 Ma
Ancestral Blue
Egg Mutation
2.4 – 6.5
1.9 – 5.1
1.6 – 4.4
1.4 – 3.7
0.4 – 1.1
0.3 – 0.8
0.2 – 0.5
0.1 – 0.4
C. poliocephalus
C. micropterus
Contemporary
Diversification
Control
Reversion
From Blue
E1:W1
E1:W2
E6:W3
E6:W5
E10:W7
E6:W7
Contemporary Reversion
Ancient Blue
Contemporary Diversification
B
N = 12 Pairwise Contrasts
SNP-Resolution F_ST
C
mtDNA
W
Z
Autosome
Proportion of F_ST = 1.0 SNPs
Present in All Contrasts
D
Aut.
mtDNA
W
Z
Contrasts Containing a Nonsynonymous F_ST = 1.0 SNP
E
CNN3
TPM2
HOMER1
MIER3
CHD1
RNF38
ZSWIM6
UBAP2
DNAJB5
CTIF
PSIP1
NCBP1
CZH18ORF25
ZBTB5
ND3
COX1
COX3
NDUFAF4
ND2
ATP6
ND1
ND5
ND4
ATP5PB
Higher Confidence
Lower Confidence
KEGG Pathway: Oxidative phosphorylation (P = 8.76e-09)
F
NDUFAF4-L
ND2
TLDC2
Genotype
0 (or 0/0)
0/1
1 (or 1/1)
Missing
21177307
4645
4646
146727
W
mtDNA
chr16

Fig. 4. *C. canorus* egg diversity associated with mitochondrial OXPHOS genes.

(**A**) Time-calibrated Bayesian phylogeny of the W chromosome depicting the evolutionary emergence and subsequent diversification of cuckoo egg morphs, with pivotal mutations summarized as phylogenetic contrasts indicated by asterisks: the origin of the immaculate blue egg phenotype (Ancient Blue), its subsequent reversal (Contemporary Reversion), and the diversification within non-blue lineages (Contemporary Diversification). Below, illustrative cartoons denote specific branches and mutations within the phylogeny where these evolutionary events are hypothesized to occur. (**B**) SNP-resolution F_{ST} scans between egg – haplotype groups to identify mutations associated with each phylogenetic contrast. SNPs completely fixed within each evolutionary contrast and absent in control groups were exclusively considered (*e.g.,* F_{ST} = 1.0 in all six Ancient Blue contrasts and not fixed in the controls). (**C**) Proportion of fixed SNPs within each phylogenetic contrast and across genomic compartments suggesting a mitochondrial link to egg phenotypes. (**D**) Candidate genes containing a fixed nonsynonymous SNP across phylogenetic contrasts. (**E**) Gene network associations among candidate genes indicates richment of the oxidative phosphorylation pathway. (**F**) Representative genotypes depicting the fixed nonsynonymous mutations corresponding to each phylogenetic contrast across the egg – haplotype groups. Aut. = autosome.

Supplementary Information

Supplementary information for this manuscript can be found at:

https://doi.org/10.5281/zenodo.11654765

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Paper III

Chromatin accessibility, not 5mC methylation covaries with partial dosage compensation in crows

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Manuscript IV

The contribution of epigenetic variation to evolution in crows

Merondun J & Wolf JBW

Abstract

Chromatin modifications provide a substrate for epigenetic variation with evolutionary potential. To quantify the contribution of this layer of variation to evolution we leveraged genome and methylome sequencing data from an incipient avian species: all-black carrion crows, grey-coated hooded crows and their hybrids. Combining controlled experimentation under common garden conditions and sampling of natural genetic variation across the hybrid zone we show that 5mC methylation variation was almost exclusively explained by genome properties and ontogenetic program of the organism. Evidence for an environmental contribution was minor, and all methylation variation of potential importance to speciation clustered in intergenic space within a genomic region of elevated genetic differentiation encoding the diagnostic color-contrast between taxa. We conclude that methylation variation may aid in phenotypic translation of genetic polymorphism, but provides little scope for an autonomous contribution to evolution in this system.

Introduction

Background

Mutations are the ultimate source of evolution. Prevailing evolutionary theory conceptualizes mutations as random changes to the DNA backbone filtered by selection, depleted by genetic drift, reorganized by recombination and redistributed by migration (S. Wright, 1931). The concept of epigenetic inheritance challenges this paradigm, as it introduces a second layer of potentially heritable variation that is not subject to alterations of the nucleotide sequence (Jablonka & Raz, 2009). DNA methylation is a prime candidate of a molecular epigenetic inheritance system featuring variation along the genome, within and among individuals and populations (Fitz-James & Cavalli, 2022; Heard & Martienssen, 2014). The underpinnings of variation in DNA methylation are diverse and include i) spontaneous epimutations, ii) environmental induction, iii) physiological processes establishing somatic cell fate (ontogenetic program sensu Waddington, 2008) and iv) genetic constraints imposed by genome properties (chromosomal or genomic features) or the organism's genotype (Taudt et al., 2016). While the latter couple of sources are readily incorporated into traditional evolutionary theory, the first two are not. To judge the autonomous potential of DNA methylation for evolution, it is therefore crucial to obtain a quantitative understanding of the sources shaping natural variation (Christina L. Richards & Massimo Pigliucci, 2020).

In plants, evolutionarily relevant methylation variation seems rather widespread (Zhang et al., 2018). There is evidence for spontaneous, random epimutations in DNA methylation (Hazarika et al., 2022; van der Graaf et al., 2015; Yao et al., 2021), environmentally-induced epimutations (Kawakatsu et al., 2016) and transgenerational inheritance of both (Cubas et al., 1999; Furci et al., 2019; Manning et al., 2006). In animals, with strict soma-germline separation and epigenetic reprogramming (Reik et al., 2001), variation in 5mC methylation that is independent of the genotype is expected to be more limited (Horsthemke, 2018). While animal research aiming to separate genetic, developmental, and environmental impacts on variation in DNA methylation is gaining traction (Beck et al., 2021), the evidence for stable inheritance of autonomous chromatin modifications remains scarce (Beck et al., 2021; Leroux et al., 2017; Pierron et al., 2021; Thorson et al., 2021). Numerous vertebrate studies have linked 5mC methylation variation with environmental change (Le Luyer et al., 2017; Meröndun et al., 2019; Wang et al., 2022) and/or phenotypic variation (Cossette et al., 2023), but few have done so while controlling for

confounding genetic effects (Heckwolf et al., 2020; Lindner et al., 2021; Vernaz et al., 2022)(see also (Laporte et al., 2019) for transposable elements). In birds, DNA methylation has been associated with stress resilience in tree swallows (Taff et al., 2019) and urbanization in great tits (Watson et al., 2021), while conversely a recent well-designed study employing partial cross-fostering under controlled conditions suggests a very limited role for environmental induction on methylation variation independent of the genotype (Sepers et al., 2023). Additional avian research in house sparrows suggests a role for changes in epigenetic potential (*i.e.,* the number of CpG motifs in the genome) during range expansions, while DNA methylation variation itself was inconsequential (Hanson et al., 2022).

Hybrid zones are suitable natural models to decompose heritable genetic and epigenetic variation of diverged populations. In the central part of the hybrid zone, environmental variation is limited while genetic variation is maximized in mosaic hybrid genomes that are characterized by blocks of alternating ancestry. These properties have been successfully exploited to map the genetic basis of phenotypic variation and advance our understanding of the processes governing population divergence (Barton & Hewitt, 1985; Gompert et al., 2017; Knief et al., 2019). For studies of epigenetic variation, hybrid zones confer similar advantages (Baldassarre et al., 2014). We here make use of this fact and quantified both genetic and methylation variation in a well-studied avian hybrid zone.

All-black carrion crows (*Corvus (corone) corone*) and grey-coated hooded crows (*C. (c.) cornix)* hybridize in a narrow contact zone in central Europe which is governed by assortative mating and social dynamics related to plumage pigmentation patterns (Metzler et al., 2021). Genome scans have found minimal genetic divergence across most of the genome with the notable exception of a ~2 Mb region on chromosome 18, hereafter referred to as the *focal region.* This region is subject to divergent selection, has accumulated fixed differences between parental taxa and is mainly responsible for the striking phenotypic variation (Knief et al., 2019; Poelstra et al., 2014; Vijay et al., 2016). Recombination is strongly reduced maintaining linkage disequilibrium of ancestral genetic variation (Knief et al., 2019; Weissensteiner et al., 2017). The genetic heterogeneity of the system where a largely homogenous genome-wide background is opposed to a diverged *focal region* known to be relevant for speciation provides a unique opportunity to gain insight into the determinants of DNA methylation variation within and between taxa.

Study design and multiple-experimental control

We pursued two sampling strategies. We raised wild-caught nestlings from pure parental populations in Germany and Sweden to adulthood in a common garden experiment (ComGar., **Fig.1 A, B**) and sampled additional nestlings across a hybrid zone transect in Central Europe (HybZon., **Fig. 1C**). The ComGar. was primarily designed to establish a baseline of physiological determinants of 5mC methylation (tissue, age, sex) and test for taxon differences under controlled conditions; whereas the HybZon. experiment is suited to study the effect of genetic ancestry and environmental variation under natural conditions in the wild. In conjunction with whole-genome-resequencing data, both approaches further address the effects of genome properties (chromosomal and genomic features) and genetic variation within and between taxa (D_{XY}, F_{ST}, haplotype diversity, Tajima's D, Fu & Li's D*). To quantify the intensity of 5mC methylation per CpG site, we generated reduced representation bisulfite sequencing data which allows for comparisons of orthologous sites with high read coverage between individuals (ComGar.RRBS mean and standard deviation CpG coverage: 31.2 ± 3.87x; HybZon.RRBS: 45.2 ± 4.88x; **Fig. S1**). For the ComGar. we additionally generated whole-genome bisulfite sequencing data allowing for technical validation of results across the entire genome and identification of a pervasive number of regions associated with tissue-specificity (ComGar.WGBS: 15.9 ± 1.22x) (see **Supplementary Table S1** for an overview of the dataset). After filtering, we retained a total of 699,363 (ComGar.RRBS), 820,661 (HybZon.RRBS) and 4,190,434 (ComGar.WGBS) high-quality CpG sites.

We used two approaches to assess the contributions of candidate variables (tissue, age, sex, taxon, environment) to methylation patterning: multivariate statistics examining global correlates of methylation variation (constrained dbRDA: **Fig. 1,** NMDS: **Supplementary Fig. S2**) and differential methylation analysis allowing for base-pair resolution of single differentially methylated positions (DMPs, **Fig. 3**). The multi-layered study design allowed us minimize false-positive inference of DMPs (**Fig. 2**). For example, the ComGar.RRBS experiment contained chicks, yearlings and adults allowing identification of age-related CpG sites. We require that these age-related CpGs show no conflicting evidence from any of the other experiments (DMP for different variable, *e.g.* sex in the ComGar.WGBS) and, in addition, have low variance in the experiments that only considered a single age (*e.g.* Com.Gar.WGBS: all adults, Hyb.Zon.RRBS: all chicks). The latter condition excludes conflation with additional, non-measured variables. Last,

we required a minimum effect size between groups (minimum 25% mean proportion difference). While allowing a margin for false negatives, this approach reduces spurious effects, which are rarely controlled for in studies of methylation variation (Sepers et al., 2019).

Results & Discussion

Ontogenetic program

First, we assessed the physiological determinants contributing to the organism's ontogenetic program. Tissue-specificity dominated DNA methylation variation in the ComGar.WGBS experiment (dbRDA *p*-value for tissue effects; $p_{(Tissue)} < 0.001$). Tissue explained a large proportion of overall methylation variation along with sex and taxon (overall dbRDA; $p < 0.001$, adj. $R^2 = 0.32$), each having little effect by itself ($p_{(Sex)} = 0.11$, $p_{(Taxon)}$ 0.096) (**Fig. 1A**, **Supplementary Table S2**). Within the ComGar.RRBS experiment, age class was the primary driving force. Cumulatively with sex and taxon, age stage between chick and mature crows explained moderate variation in methylation patterns (adj. $R^2 = 0.13$, all 3 variables $p < 0.001$), while there was no detectable difference between yearlings and adults ($p = 0.97$) (**Fig. 1B**). Within the HybZon.RRBS experiment, continuous proxies for taxon (hybrid index), geographic distance, and environmental variation (pre-hatch ambient temperature) were nearly uncorrelated with methylation patterning (adj. $R^2 = 0.005$), and only temperature was identified as significant ($p = 0.008$) (**Fig. 1C**). Ambient temperatures during development are known to impact global methylation patterning in birds (Sheldon et al., 2020), so our results provide some support for temperature regulation of the DNA methylation program, although the effects are slight.

At CpG resolution, DMPs largely mimicked results from the global analyses, but also provided the basis to pinpoint the genomic regions associated with taxonomic divergence after controlling for other effects of the ontogenetic program (**Supplementary Fig. S3**). By far the most well-supported DMPs were related to tissue ($n = 3{,}863$) followed by sex ($n = 6$), age ($n = 4$), taxon ($n = 4$), pre-hatch ambient temperature ($n = 3$) and geographic distance ($n = 1$) (**Supplementary Table S3**). Note that the low numbers of DMPs are a consequence of multi-experimental control minimizing false-positive inference at the cost of false negatives (**Fig. 2**). Since multi-experimental control is predicated on CpG overlap across experiments, the total number of usable CpGs was substantially reduced to 2.11% ($n = 105{,}559$) sequenced across all 3 experiments and 12.02% ($n = 601{,}516$) in at least two experiments (**Supplementary Table S4**). Within the latter category, 63,073

CpGs were classified as DMPs satisfying statistical significance from beta-binomial regressions and an effect size threshold of 25% difference in methylation (necessary condition **Fig. 2**). Of these putative DMPs, 872 (1.38%) showed conflicting classifications across experiments and were accordingly dropped (*e.g.* a taxon effect in one experiment, and an age effect in another). Of the remaining 62,201 only 6.24% (n = 3,881) survived the sufficiency criteria of multi-experimental control (**Fig. 2**, **Supplementary Table S3 & S5, Supplementary Fig. S4**).

In summary, the results on physiological determinants implicate overarching DNA methylation regulation primarily in cell fate and age-related processes, reinforcing the well-established role of DNA methylation in cell lineage differentiation (Gama-Sosa et al., 1983; Izzo et al., 2020) and ageing (De Paoli-Iseppi et al., 2019). Ancestral genetic variation separating the young taxa had a substantially less prominent signature genome-wide (**Supplementary Fig. S5**).

Methylomic and genomic interplay

The effects of genetic ancestry on methylation patterns were restricted to the focal region of genetic differentiation primarily responsible for plumage polymorphism. All four robust taxon-related DMPs were restricted to this region and showed evidence for hypomethylation of carrion crow ancestry (**Figs. 3C and 3D, Supplementary Figs. S6-S10, Supplementary Table S6**). Encoding of methylation genotypes with k-means clustering at these loci identified strong methylation linkage disequilibrium between these sites (D = 0.185, D' = 0.743) (**Supplementary Fig. S11)**, mirroring phenotypically-associated linkage blocks identified from genetic data in this region (Knief et al., 2019). The fact that these taxonomically relevant DMPs all occur in proximity to the plumage polymorphism candidates AXIN2, PRKCA, and an array of CACNG genes (Knief et al., 2019) support the notion that phenotypically relevant genetic variation and DNA methylation may indeed covary as a result of divergent selection, as has been proposed in white-throated sparrows (Sun et al., 2021) (but see Heckwolf et al., 2020). DNA methylation may thereby act as mediator translating signals of *cis*-genetic variation into differential physiological activity. Note, however, that the taxonomic DMP loci were identified using tissue (blood, spleen, liver) which is not histologically relevant to melanin production in crows (C.-C. Wu et al., 2019), and would therefore reflect a tissue-unspecific, pleiotropic signal. Moreover, none of the DMPs overlap any genes or promoters directly, and more generally, elevated methylation divergence was restricted to intergenic space while it was decreased in promoters compared to the autosomal background (p <

0.05 in all experiments; **Fig. 3E, Supplementary Table S7**). Altogether, this renders functional importance of the 5mC DMPs in the focal region less likely.

Having established the effect of local genetic divergence on 5mC methylation, we next assessed whether taxonomic variation in DNA methylation could be predicted more broadly. Using supervised machine learning regressors, methylation divergence between taxa (**Fig. 4A**) was in part predicted by genome properties (chromosome length, positioning along chromosome, functional annotation) and to a lesser extent by measures of population genetic variation (F_{ST}, D_{XY}, haplotype diversity, Tajima's D, Fu & Li's D*) (**Fig. 4B, Supplementary Table S8**). GC-content (Permutation Importance 95% CI intervals across all experiments: 19.4 – 24.0%) and promoter regions (16.9 – 27.6%) were the strongest predictors indicating decreased methylation divergence within GC-rich CpG islands near putative promoter regions (**Fig. 4C, Supplementary Tables S8 and S9**). This finding corroborates the prevalent conservation of evolutionary processes governing CpG islands in vertebrates (Bird et al., 1985; Long et al., 2016). Relative positioning along a chromosome (4.35 – 5.91%) and chromosome length (5.78 – 8.22%) were weaker predictors of taxon methylation divergence, yet lend support for methylome divergence proceeding more rapidly on micro-chromosomes or near chromosome ends where recombination is substantially higher (Kawakami et al., 2014). Further supporting the relationship between genetic variation and DNA methylation, permutation importance was highest for a measure of genetic divergence, D_{XY} (8.63 – 14.7%), followed by haplotype diversity (9.00 – 13.4%), Tajima's D (5.62 – 8.87%) and eventually genetic differentiation (F_{ST} 1.38 – 2.74%) (**Fig. 4B, Supplementary Table S9).** Our results indicate interplay between population genetic variation and DNA methylation and supports previous research identifying population-level correlates between genetic and DNA methylation variation (Carja et al., 2017; Wang et al., 2022), particularly in regions undergoing selection (Shirai et al., 2021). To compare the relative strength and obtain directionality of the interplay between DNA methylation and population genetic variation, we examined bootstrapped correlations within the focal region compared to the autosomal background. As expected, the relationship between DNA methylation and genetic variation was stronger within the focal region, particularly for D_{XY} (**Fig. 4C, Supplementary Table S10**).

In summary, genome properties and segregating genetic variation both contribute to taxonomic divergence of DNA methylation, most pronounced in the focal region undergoing divergent

selection. These results are overall consistent with a general carry-over effect of *cis*-acting genetic variation on patterns of methylation variation, as observed within *Papio* baboons (Vilgalys et al., 2018). Hitchhiking 5mC variants in linkage disequilibrium with *cis*-acting genetic variation may or may not be co-opted functionally (Hawe et al., 2022; Min et al., 2021; Taudt et al., 2016; Yagound et al., 2019) (for an example of *trans*-acting genetic variation see (Höglund et al., 2020)). Conversely, linkage disequilibrium could be reinforced by selection on DNA methylation, dragging along genetic variation. The known causal effects of DNA methylation on genetic variation are currently limited to increased deamination of methylated cytosines (Cooper & Krawczak, 1989), with these effects most pronounced in TEs (Zhou et al., 2020). While the gain or loss of genomic CpG motifs via deamination from DNA methylation could have evolutionary implications (Feinberg & Irizarry, 2010; Hanson et al., 2022), this conclusion is less parsimonious in our system where there is no evidence for divergence in TEs between taxa (Warmuth et al., 2022) and intergenic taxonomically relevant DNA methylation is restricted within the large 2mb *focal region* responsible for phenotypic divergence between taxon (Metzler et al., 2021).

Environmental Effects

Environmental inducibility of epigenetic variation has received much attention (Uller, 2019), but data under natural conditions is scarce and near-absent in animals (Heckwolf et al., 2020; Vernaz et al., 2022). The fact that environmental contrasts generally covary with genetic effects further complicates the quest (Shafer & Wolf, 2013). Our experimental setup allowed us to separate these and other confounding variables. To isolate environmental effects on DNA methylation variation, we focused on the HybZon.RRBS experiment which excludes all physiological confounding variables *a priori* (all blood from male chicks). We then isolated any conserved sites which exhibit less than a 10% range in DNA methylation in the ComGar. experiments, but show significant effects for pre-hatch temperature in the HybZon.RRBS experiment. Incubation and early developmental ambient temperatures are known to effect DNA methylation patterns in numerous tissues in broad avian taxa ranging from chickens (Corbett et al., 2020), duck (Yan et al., 2015), and zebra finch (Sheldon et al., 2020). While ambient temperature has been implicated in DNA methylation patterns related to reproductive timing in great tits (Lindner et al., 2021; Viitaniemi et al., 2019), our study supports the notion that genetic effects impart more to the epigenetic program than early rearing environment, as indicated by cross-fostering experiments (Sepers et al., 2023).

While a standard experimental approach would have identified numerous environmentally-sensitive loci (n = 61), our multi-experimental approach revealed only 3 verifiable DMPs related to ambient pre-hatch temperatures (**Fig. 3B, Supplementary Table S11**), indicating hypervariable loci or off-target effects may be responsible for numerous candidates in studies examining environmental patterns in wild populations. One of our multi-experimentally validated DMPs lies within the promoter of *LMF1*, a gene whose methylation status in humans is associated with offspring birthweight (Kheirkhah Rahimabad et al., 2021). While it lacks any functional validation in avian systems, its implication in gene-environment associations during embryonic development (Pu et al., 2020) indicates the methylation status of *LMF1* as a candidate associated with early developmental environment. The remaining two DMPs lie in the vicinity of long non-coding RNAs. Using sampling year as an additional measure for environmental variation yielded no multi-experimentally validated DMPs and recovered the same patterns associated with temperature (**Fig. S12**). While these results do not provide mechanistic insight, they do not preclude a possible role of DNA methylation in mediating environmental stimuli to regulatory gene activity irrespective of underlying genetic sequence (Caizergues et al., 2022; Lindner et al., 2021).

Overall, this study illustrates the utility and limitations of methylomic approaches and advocates a multiple, hierarchical experimental framework for addressing complexities that underlie genetic-epigenetic-environmental interactions in natural populations. While the experimental design did not allow assessment of the extent of spontaneous, putatively heritable epigenetic mutations, it provides clear evidence that natural variation in DNA methylation is firmly associated with general genome features and physiological processes orchestrating cell fate and aging. The latter conforms to the original definition of epigenetics as 'the interaction of genes and their products […] which bring the phenotype into being' (Waddington, 2008, p. 242). Taxonomically-relevant DNA methylation under controlled conditions corresponded to chromosomal features and segregating *cis*-acting genetic variation between species, tempering expectations for an independent role of epigenetic variation during nascent species divergence (Wang et al., 2022). For a small subset of sites, the study further provides evidence for environmentally induced methylation variation, though with unclear intra- (soma-to-soma) and transgenerational (soma-to-germline) heritability or functional relevance. We conclude that the main source of methylation variation in natural populations of crow is predetermined by chromosomal organization and genetic variation and

provides little scope for independent evolution at this early stage of species diversification. Whether this finding is limited to animals where the possibility of autonomous epigenetic inheritance hinges on the degree of residual epigenetic variation surviving deterministic reprogramming (Morgan et al., 1999; Reik et al., 2001) motivates further study.

Materials & Methods

Sampling – Common Garden

For an illustrative overview of the sampling design see **Fig. 1**. We designed a common garden experiment (hereafter ComGar.) to quantify DNA methylation variation corresponding to physiological factors (tissue, age, sex) and taxon differences. We first collected blood from the brachial vein of unrelated nestlings with a mean age of 22.1 days (range 14 – 26) from purebred carrion ($n = 4$) and hooded crows ($n = 4$). Carrion crows were collected in May 2014 from different nests in Konstanz in Southern Germany (47°45N′, 9°10′E) as part of a larger research program. Hooded crows were sampled around Uppsala, Sweden (59°52'N, 17°38'E) in the same month and year. These 8 individuals were transferred to Sweden by airplane and hand-raised indoors at Tovetorp field station, Sweden (58°56′N, 17°8′E). At the time when birds could feed independently, they were released into a roofed outdoor enclosure (6.5 × 4.8 × 3.5 m) and thereafter housed in single-sex groups of the same species under common garden conditions. For details of animal husbandry see (Holtmann et al., 2019). Blood sampling was repeated for these 8 individuals during the non-reproductive season at an age of 18 months (551 – 565 days) and during the reproductive season at an age of 30 months when sexual maturity is expected to have been reached in all individuals (916 – 930 days) (Blotzheim et al., 1993). For this common garden experiment, sample sizes of both carrion and hooded crows raised in captivity were matched by sex (2 males and 2 females of each taxon) which was determined molecularly (Griffith et al., 1998). In addition to these 24 blood samples (8 individuals at three time points), we collected liver and spleen tissue from 4 of these individuals at sexual maturity, representing a male and female of each taxon (hooded and carrion crow) (**Supplementary Table S1**). The 24 blood samples were subjected to RRBS (reduced representation bisulfite sequencing) to quantify sex, age, and taxon differences, while the 4 individuals with three tissue types available (blood, liver, spleen) were subjected to WGBS to quantify sex, tissue, and taxon differences (see below).

Sampling – Hybrid Zone

We designed the hybrid zone experiment (hereafter HybZon.) to isolate genetic and environmental effects on DNA methylation variation while maintaining tissue, age, and sex constant. We sampled 3 purebred male chicks of each taxon (distinct from ComGar.) in addition to 16 hybrid chicks along a transect across the German hybrid zone during field trips in May 2008, 2013 and 2014. We selected 16 hybrid individuals according to genotype information of 1,111 SNP markers spread across the genome that include two of the major loci coding for plumage colour variation, as outlined in Knief *et al.* (Knief et al., 2019), including a genetic factor on chromosome 18 (chr18) and the gene NDP on chromosome 1 with the R package *introgress v1.2.3* (Gompert & Alex Buerkle, 2010). Samples were chosen to represent the main diplotypes on chr18 (DD, DL, LL) and to encompass the full variation of genome-wide admixture (range of hybrid index: 0.0 – 1.0). With the inclusion of the purebred samples, hybrid indices thus both covered the full range from 0 (*C. c. corone*) to 1 (*C. c. cornix*) as represented by blood samples from 22 male individuals with an age range of 7 – 25 days (**Supplementary Table S1**). In addition to genetic effects this setup allows us to examine the influence of geographic distance and environmental variation. For the latter, we chose the mean temperature of the three months preceding hatch date, determined from Meteostat from the nearest local weather station (meteostat.net) (**Supplementary Table S1**). This proxy for environmental effects thus integrates the maternal environment, as well as the environment experienced during the egg stage (incubation on average 18-19 days (Blotzheim et al., 1993)).

Sequence Data Generation - Methylome

We assessed genome-wide 5mC DNA methylation with a combination of whole-genome bisulfite sequencing (WGBS) and reduced-representation bisulfite sequencing (RRBS), involving four sequencing efforts. Genomic DNA isolated from whole blood, liver, or spleen using QuickExtract kits (Epicentre, Illumina) provided input for both WGBS library preparation (150 ng; n=8 representing all tissues for each sex at parity) and RRBS library preparation (300 ng; n=24 representing blood for three age classes of each sex and taxon at parity). Common garden experiment WGBS libraries were created following the TruSeq DNA Methylation kit (Illumina Inc., EGMK91324) according to the manufacturers' protocol. RRBS libraries from the common garden experiment were created by adapter-ligating and end-repairing *MspI* digested fragments following the NEBNextUltra protocol (New England BioLabs). Bisulfite conversion was

performed with the EZ DNA Methylation Gold Kit (Zymo) and bisulfite-converted DNA was then amplified with the NEBNext Universal primers and NEBNext index primers using 12 PCR-cycles. Libraries were cleaned twice using AMPureXP beads (55 ul beads to 50 ul sample). A 0.5% spike of non-methylated lambda phage DNA was included in WGBS and RRBS libraries to confirm bisulfite-conversion efficiency. Both WGBS and RRBS libraries were evaluated using a TapeStation with the HS D1000 kit. Adapter-ligated fragments were quantified with qPCR using a library quantification kit for Illumina (KAPA Biosystems/Roche) on a CFX384Touch instrument (BioRad) prior to cluster generation and sequencing. The WGBS libraries were then sequenced paired-end on a HiSeqX with 150bp read length using v2.5 sequencing chemistry, including a 5% PhiX spike-in. RRBS libraries were sequenced on a NovaSeq 6000 using an SP Flowcell, single-end 100bp read lengths using v1 sequencing chemistry, including a 25% PhiX spike-in. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala which is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. In addition to this sequencing effort, we generated 22 RRBS libraries from the HybZon.RRBS experiment (19 surviving filtering, see below) and 4 supplemental WGBS biological samples (an additional two carrion crow tissues for each sex and supplemental sequencing for one individual from the previous WGBS effort) for the common garden experiment at a separate facility (Novogene, Co. Ltd.), bringing total sex, tissue, and taxon sampling to parity. RRBS *MspI*-fragmented libraries were created similarly to the common garden experiment detailed above, except the final libraries were sequenced paired-end on a NovaSeq 6000 with 150bp read lengths. The additional 4 WGBS libraries were generated at Novogene Co. Ltd. (Beijing, China), using the Accel-NGS® Methyl-Seq DNA library kit (Swift Biosciences) following the manufacturer's instructions. These additional WGBS libraries were sequenced on a NovaSeq 6000 paired-end with 150bp read length. For treatment of batch effects see below.

Genome Reference and Genomic Feature Annotation

All analyses were performed on the most recent chromosome-level European crow reference genome available at this time (National Centre for Biotechnology Information accession number: ASM73873v5). Analyses were carried out only on autosomal chromosomes (*i.e.,* excluding the Z and W chromosomes). Genomic features were extracted as follows. We annotated the reference genome into non-overlapping tracks of promoter regions, repeats, coding sequence (CDS),

intronic, and intergenic sequence (**Supplementary Fig. S13**). Promoter regions were identified as CpG islands located within 2kb of a gene's start coordinates. While this strategy will likely fail to identify many true promoters, particularly those with promoters not directly within the proximity of the TSS (Ioshikhes & Zhang, 2000), it will provide a conservative estimate of CpG islands with direct relevance to local transcripts. We created a *de novo* CpG island track using *makeCGI v1.3.4* (H. Wu et al., 2010), requiring a length > 200bp, a GC content > 50%, and an observed/expected CG ratio > 60%, providing 30,459 islands. Only gene elements from the RefSeq *.gff* annotation file that intersected our CpG island track with the strand-aware 2kb region upstream of the gene start were retained, using bedtools v2.29.2 (Quinlan & Hall, 2010). This resulted in 11,472 CpG-promoter islands, from a total of 17,944 genes. We identified repetitive regions with *RepeatMasker v4.1.1* (Smit et al., 2013) using the repeat library from chicken and excluding simple repeats (*-nolow*). Genic CDS and introns were extracted directly from the RefSeq annotation. A non-overlapping annotation feature track was created with *bedtools* and *R v4.1.1* (R Core Team, 2017) by prioritizing promoter regions, repeats, CDS, introns, and intergenic annotations, in that order (**Supplementary Fig. S13**). To reduce erroneous RRBS alignments, we performed an *in silico* digest of the reference genome with *MspI* using *SimRAD v0.96* (Lepais & Weir, 2014), requiring a fragment size between 40 – 350bp, providing 299,587 potential fragments with roughly 1.6M CG motifs, compared to the roughly 9.8M CG motifs in the entire reference.

Quantification of Genetic Variation

Genetic polymorphisms involving C-T and A-G transitions are problematic for bisulfite sequencing experiments because C-T and A-G mismatches between sequence and reference are used to identify DNA methylation (Barrow & Byun, 2014; Krueger & Andrews, 2011). We therefore exploited an existing population resequencing dataset from 28 male hooded and carrion crows sourced from the same allopatric populations (Uppsala, Sweden and Konstanz, Germany) analyzed in this study (14 of each taxon) to identify transition SNPs. We also used this dataset to quantify genome-wide genetic variation (F_{ST}, D_{XY}, Tajima's D, haplotype diversity, Fu & Li's D*) to assess its relationship with methylation variation. In short, 130 paired-end Illumina libraries for the 28 samples were adapter-trimmed with *BBTools v38.18* (Bushnell, 2021), aligned to the reference with *BWA v0.7.17* (Li & Durbin, 2009), merged with *samtools v1.7* (Li et al., 2009), and deduplicated with *GATK v4.1.9.0* (Auwera et al., 2013). After read trimming and filtering,

alignment to the hooded crow reference genome (accession ASM73873v5) resulted in a genome-wide mean coverage of 15.1x (range 8.03 – 30.9x), calculated in 100kb windows with *mosdepth v0.3.1* (Pedersen & Quinlan, 2018) (**Supplementary Table S12**).

An all-sites variant file was called with *bcftools v1.16* (Danecek et al., 2021). For filtering variant sites, we retained only biallelic sites passing hard filter thresholds (*QUAL > 20, DP < 2*Average DP*, or *DP > 28x*), which had scored genotypes in at least 90% of individuals (n = 25/28 with *FMT/DP >= 3*). Following hard site-level and genotype-level filters (**Supplementary Figs. S14 and S15**), we retained 9.44m variant sites and 1,010m invariant sites. Coordinates for transitions (C-T and A-G polymorphisms) were subset using *bash* scripts for methylation site masking. Population genetic metrics for hooded and carrion crows (F_{ST} and D_{XY} between taxa and Tajima's D, haplotype diversity, and Fu & Li's D* among all individuals) were calculated in windows of 5000 sites (*--windType sites --windSize 5000*), requiring a minimum of 1000 shared sites across samples (*--minSites 1000*) using the *genomics_general* repository (Martin et al., 2019) for F_{ST} and D_{XY}, and the *Theta_D_H.Est* repository for the remaining metrics (Pan et al., 2022). Principal components analysis (PCA) on LD-pruned genome-wide SNPs was performed with *SNPRelate v1.28.0* (Zheng et al., 2012), and again exclusively on the SNPs falling within the *focal region* on chromosome 18. Genetic data were visualized with *karyoploteR v1.20.0* and *ggplot2 v3.3.6* (Gel & Serra, 2017; Wickham, 2016).

Quantification of 5mC Methylation

All bisulfite sequence datasets were first trimmed with *trim_galore v0.6.7* (Krueger, 2012), ensuring the artificial filled-in positions were trimmed in the RRBS datasets (*--rrbs*), and requiring average read Phred scores of at least 20 (*--quality 20*). Bisulfite conversion was successful, measured using a non-methylated lambda phage DNA spike into each library (min,mean,max conversion rate: 98.90, 99.27, 99.74%, **Supplementary Table S13**). Reads were aligned to the European crow reference genome using *Bismark v0.23.0* with default settings (Krueger & Andrews, 2011). We extracted CpG methylation calls using *Bismark*, iteratively repeating this process after ignoring read positions exhibiting systematic methylation biases, evidenced through methylation bias plots (**Supplementary Fig. S16**). WGBS post-bisulfite adapter tagged (PBAT) libraries required additional trimming before alignment to remove artificial bases inherent to the protocol (*--clip_r1 9 --clip_r2 9 --three_prime_clip_R2 1*). We then retained methylation calls on

MspI fragments (for RRBS data only), removed any sites overlapping a C-T or A-G SNP (identified from the genetic resequencing experiment, see above) using *bedtools v2.29.2* (Quinlan & Hall, 2010) or located on a scaffold (we only analyzed sites on assigned chromosomes), and removed any sites covered by less than 10 reads in each individual for the RRBS experiments, and less than 5 reads for the WGBS experiment. We chose a lower threshold within the ComGar.WGBS experiment to maximize retained sites. RRBS reads were ensured to overlap an *in silico* digested *MspI* fragment (see above) using *bedtools* (Quinlan & Hall, 2010). Initial count inspections for RRBS data indicated a substantial increase in retained reads when overlapping *MspI* fragments at the *.bam* file level (pre-methylation extraction), as opposed to post-hoc removal of individual sites after extraction, so extraction on RRBS dataset was repeated after filtering *.bam* files for *MspI* fragments. Methylation levels (%) were consistent across read positions after trimming, indicating no systematic positional read biases, although high coverage spikes in the RRBS datasets lead us to set upper coverage limits on our datasets, for which we removed a conservative degree to alleviate any concerns with repetitive fragments that would be difficult to map with A-T rich bisulfite sequencing reads (remove the top 5% coverage outliers across all experiments). Finally, CpGs were removed from the experiment if they had missing data from any individual (ComGar.WGBS *12/12*), more than 3 individuals (ComGar.RRBS *21/24*), or 1 parental and 2 hybrids (HybZon.RRBS *16/19*) to ensure that sufficient replicates for each biological treatment existed for each site.

No batch effects associated with the two sequencing centers were apparent in the ComGar.WGBS experiment (**Supplementary Fig. S17**), identifying very repeatable measurements across sequencing centers (Spearman's rho: 0.998 for all shared CpGs above 10x coverage). Initial ordinations of the HybZon.RRBS experiment indicated three hybrid individuals (Y13, Y31, Y40) as outliers, seemingly due to either systematic hypomethylation and/or age in one sample (**Supplementary Fig. S18**). To be conservative we excluded all three individuals, as no known covariates were immediately responsible for driving the variation in all three individuals and they did not contribute to novel axes of *a priori* physiological variation (*i.e.,* the full range of hybrid indices were still retained). We replicated DNA extraction, library preparation, and sequencing for four biological samples for both RRBS and WGBS protocols, indicating higher repeatability than expected by chance (**Supplementary Fig. S19**). While correlations were lower than between technical replicate WGBS libraries (see above), these differences are likely explained by inherent differences between these protocols, sequencing coverage, and blood as a source tissue which may contain different cellular make-up in each extraction. Hatchlings were sampled from different nests to ensure unrelated individuals were sampled, and extra pair paternity in crows is low (Knief et al., 2020).

Determinants of Methylome Variation

The main goal of this study was to examine the variables associated with variation in methylation. Variables under investigation can be grouped into genome properties, genetic variation, physiological determinants, and environmental contribution (see **Fig. 1**). Depending on the variables under investigation, different approaches were used (multivariate statistics, beta-binomial regression, supervised machine learning). We examined i) the global determinants of physiological and environmental methylation variation with constrained ordinations; ii) base-pair resolution divergence associated with physiological, environmental, and genetic effects (*i.e.,* taxon, hybrid index) with beta-binomial regressions; and iii) genome property and genetic variation covariation with DNA methylation using supervised machine learning and bootstrapped correlations. Methylation data were treated according to the method requirement as a methylation proportion (ranging from 0 – 100% for distance-based redundancy analyses and machine learning regressions), or directly as methylated and non-methylated read counts (for DMP detection with beta-binomial regressions). We ensured analytical insensitivity by repeating analyses, where applicable, using multiple methylation inputs (*e.g.,* we analyzed global variation with unconstrained ordinations using both Euclidean and alternative distance measures; PCA and NMDS; analyzed the machine learning problem as a classification problem instead of a regression using binary and trinary discrete methylation response variable categories).

Physiological and Environmental Determinants

To assess the effects of tissue, age, sex, taxon, and environment on DNA methylation patterns, we used two approaches: multivariate statistics examining global correlations of methylation variation (distance-based redundancy analyses; dbRDA), and differentially methylated position (DMP) analyses allowing for ultra-fine resolution of single differentially methylated CpGs. First, for each experiment independently (ComGar.WGBS, ComGar.RRBS, HybZon.RRBS), we examined global DNA methylation variation with a dbRDA constrained ordination. Methylation input for the ordination was a matrix composed of DNA methylation proportions with columns corresponding to each individual CpG site and rows corresponding to individual samples. Optimal distance measure for dbRDA (and later NMDS) for each experiment was identified using Spearman's rank correlations within *vegan v2.5-7* using the *rankindex* function (Gower's distance:

ComGar.WGBS; Euclidean distance: HybZon.RRBS, ComGar.RRBS). A pre-processing data recipe was created with *tidyverse v1.3.1* and *tidymodels v0.1.4* (Kuhn & Wickham, 2020; Wickham et al., 2019), ensuring variables were in proper classes and that collinearity between variables was below a Spearman's rank correlation of 0.8. Scaled dbRDAs were then implemented with *vegan,* and the contributory effects of each variable were assessed with an ANOVA by term with 10,000 permutations (**Supplementary Table S2**). Adjusted R^2 and overall model significance as assessed with an ANOVA are reported (**Fig. 1**). To minimize statistical biases inherent in any single method (Anderson & Willis, 2003), we also performed an unconstrained ordination (NMDS) including an analysis of similarity (ANOSIM) on DNA methylation variation and a typical eigenvector based analysis (PCA, **Supplementary Fig. S2, Supplementary Table S2**), and observed the same patterns.

We then determined the physiological and environmental determinants of DNA methylation at CpG-resolution using DMP detection directly from methylated and non-methylated read counts. Explanatory variable effect estimates (test-statistics) and FDR-corrected *p*-values were obtained for each variable (***cf.*** **Fig. 2**) and for each experiment independently using a beta-binomial regression with an arcsine link implemented in the R package *DSS v2.42.0* (Park & Wu, 2016). Explanatory variables for ComGar.WGBS data were tissue (Blood, Liver, Spleen), sex (Male, Female), and taxon (C.C., H.C.); for ComGar.RRBS were age (Chick, Yearling, Adult), sex (Male, Female), and taxon (C.C., H.C.); for HybZon.RRBS were genetic ancestry hybrid index, geographic distance, and mean local temperature for the three months preceding hatch date (*i.e.,* a proxy for environmental effects), determined from Meteostat from the nearest local weather station (meteostat.net) (**Supplementary Table S1**). An additional variable of sampling year was also investigated, but the patterns appeared well summarized with the existing variables (**Supplementary Fig. S12**). We then assessed taxonomic differences in DNA methylation within the focal region compared to the autosomal background using bootstrapped sampling with replacement within R (R Core Team, 2017). For each DNA methylation experiment and each genomic annotation feature, we sampled a number of autosomal CpGs equal to the number of CpGs within the focal region for that subset and calculated the mean taxon effect estimate, repeating this process 1,000 times. We considered the comparisons to be non-overlapping if the 95% quantile distributions did not overlap.

Multi-experimentally Verified DMPs

Within each experiment independently, individual CpG sites were then assigned a corresponding physiological or environmental classification (*e.g.,* tissue, age, sex, taxon, temperature, distance), or were marked as conserved (less than 10% variation within entire experiment), or indeterminate (no significant variation corresponding to any of the covariates). Classifications were based on an FDR detection threshold below 10%, non-significance for other covariates (FDR > 10%), and a conservative minimum divergence between group methylation means of variables of interest of 25% (*e.g.,* absolute value of mean(male) – mean (female) > 25%). Concordance between FDR-corrected p-values and methylation divergence for each effect were checked with volcano plots (**Supplementary Fig. S20**). No absolute divergence thresholds were used for continuous covariates (hybrid index, temperature, geographic distance) as these were not factor variables and did not contain groups. Conserved sites represented a small amount of CpGs within ComGar.WGBS (10.3%, n = 432,403), and higher relative amounts in the promoter-rich reduced-representation experiments HybZon.RRBS (49.7%, n = 407,481) and ComGar.RRBS (48.5%, n = 339,410). Classified sites were then merged across experiments at base-pair resolution and subjected to multi-experimental control. A classification was considered verified for a site if i) it was inferred in at least one experiment, ii) there was no conflicting classification among all three experiments, and iii) at least one experiment serving as a negative control classified the site as conserved (***cf.*** **Fig. 2**). For instance, for a verifiable age DMP classification the site would have to be classified as age-specific in the ComGar.RRBS and be conserved in either ComGar.WGBS or HybZon.RRBS (or both), where age is controlled for. As we had no independent experiment with a negative taxon control, we required significance for hybrid index within the HybZon.RRBS experiment, as well as one corroborating taxon classification from the other experiments. While this approach relies on CpG overlap between experiments, which is of course limited by coverage and technical conditions (*i.e.,* we expect a high number of sites present in WGBS to be absent in RRBS), we preferred this strategy as it will dramatically reduce false positives by introducing multi-experimental reproducibility within a single study. Furthermore, as our primary interest is on DNA methylation divergence between taxa, integrating two metrics of taxonomic divergence (*i.e.,* parental comparisons in two experiments and hybrid index in another) will identify sites verifiably corresponding to taxonomic divergence.

We assessed sensitivity in our classification strategy outlined above by repeating the process except classifying sites based solely on the common garden experiments (*i.e.,* not requiring significance for any effects in the HybZon.RRBS). We then overlapped the HybZon.RRBS CpGs with these single-experimentally classified sites and examined the distributions of methylation variation within sites classified as *Conserved, Tissue*, or *Taxon* from the ComGar. We examined methylation variation within each hybrid group: unadmixed hooded and carrion crows, hybrids which have hybrid indices (calculated using the chromosome 18 and NDP factors) completely hooded or carrion (either 0.0 or 1.0), and hybrids intermediate to either. Permutation tests between methylation levels within the focal region and compared to the autosomal background corroborated the results found within Fig. 2D, showing hypomethylation within carrion crows and their closely-related hybrids within the island on chromosome 18, and hooded crows and their associated hybrids hypermethylated compared to the autosomal background (**Supplementary Fig. S6**). Similarly, hybrids with intermediate genetic ancestry coefficients exhibited intermediate methylation levels which did not differ from the autosomal background. No differences were observed between the focal region and the background within *Conserved* CpGs, which closely mirrored the tight distributions seen for *Tissue* windows, although we did observe constitutive hypomethylation within the focal region for tissue windows within the HybZon.RRBS (**Supplementary Fig. S6**).

Genome Properties and cis-genetic Variation

Following the observation that all (n = 4, **Supplementary Table S11**) multi-experimentally verified taxa DMPs were within a region of elevated genetic variation on chromosome 18, we sought to quantify the relationship between taxonomic DNA methylation divergence and its chromosomal substrate at genome-scale, namely using genome properties and population genetic variation. We used the taxon-related (*e.g.,* taxon for ComGar. or hybrid index for HybZon.) test-statistics from each experiment's DMP analyses as a response variable input, and intersected these with the population genetic variation (F_{ST}, D_{XY}, Tajima's D, haplotype diversity, Fu & Li's D*), and genome property information (genomic annotation, GC-content, relative position of the window along a chromosome, and chromosome length. Population genetic variation was assessed genome-wide in windows of 5kb, so we calculated mean DNA methylation divergence (taxa-related test-statistics from DMP analyses) within each window. For sensitivity, we also repeated

the entire following analysis with maximum divergence values within 5kb windows instead of the mean and arrived at similar conclusions, except that the importance for *promoter* regions was relinquished into GC-content, highlighting the interconnected nature of these covariates (**Supplementary Fig. S21**). We imputed population genetic values from the nearest 5kb windows where values did not exist (mean missing 5kb windows across experiments: n = 240, 0.78%), seemingly more common in areas of high GC-content (within missing windows = 68.0%, outside = 57.3%, **Supplementary Table S14**). We then assessed the raw distributions of our DNA methylation divergence response variable within each experiment and deemed a log transformation appropriate (**Supplementary Fig. S22**). Collinearity between explanatory variables was low across all three experiments (min,mean,max: -0.27, 0.056, 0.62, **Supplementary Table S15**).

We assessed the relationship between DNA methylation divergence and its chromosomal substrate using supervised machine learning regression (*i.e.,* random forests with *ranger v0.14.1* (M. N. Wright & Ziegler, 2017) and boosted regression trees with *XGBoost v1.7.1.1* (Chen & Guestrin, 2016)). Pre-processing and modeling used a *tidyverse v1.3.1* and *tidymodels v0.1.4* (Kuhn & Wickham, 2020; Wickham et al., 2019) framework implemented within *R v4.1.1* (R Core Team, 2017), where numerical covariates were normalized and collinearity between variables was checked (Spearman's rank correlation < 0.8). Datasets were then stratified into 75% training and 25% testing partitions to be analyzed with 5-fold cross validation. Model parameters (*trees, min_n, mtry;* and *learn rate* for XGBoost) were tuned to maximize R^2 with *finetune v1.0.1* and *caret v6.0-93* (Kuhn, 2008; Kuhn & Wickham, 2020) using a random grid search. Workflows were finalized and implemented with *tidymodels* using the tuned parameters, allowing for final extraction of covariate permutation importance using *vip v0.3.2* (Greenwell & Boehmke, 2020) and model fit (R^2 and root mean squared error, RMSE) (**Supplementary Tables S8 and S9**). Permutation importance measures explanatory variable importance by shuffling each covariate's values and measuring the impact on model fit, and is a better gauge of contributory effects than methods relying on impurity (Altmann et al., 2010). Permutation importance is therefore a useful measure to determine the importance of each covariate, but only insofar as the final models has sufficient overall explanatory power. To generate confidence intervals on covariate importance and model explanatory power, we replicated the entire process 3 times for all 3 DNA methylation experiments using different random seeds. We then scaled permutation importance within each iteration to a value of 1.0 to allow for comparisons across the two regression engines. Furthermore, to see if the

problem could be approached more parsimoniously with supervised classification techniques, we transformed our continuous DNA methylation divergence response variable into a discrete binary (and trinary) response and repeated the entire process again (**Supplementary Text, Supplementary Fig. S23**), this time assessing models with the Area Under the Receiver Operator Characteristic curve (ROC AUC) and covariate permutation importance, and observed the same patterns **(Supplementary Tables S7 and S8, Supplementary Fig. S24)**.

We complemented our supervised machine learning approach with a bootstrapped Spearman's rank correlation analysis between taxonomic DNA methylation divergence and population genetic variation, with a focus on how patterns differ between the focal region on chromosome 18 and the autosomal background. The boundaries of the focal region were established by the start and end of windows on chromosome 18 with elevated genetic differentiation ($F_{ST} > 0.3$). At this F_{ST} threshold nearly all windows were localized on chromosome 18 (99.0%, $n = 149$), and the resulting region (starting at 8.07e6 and ending at 10.07e6, 2Mb) corresponds to the length of the island identified previously, although on a different genome assembly (Knief et al., 2019; Poelstra et al., 2015). For each DNA methylation experiment, we sampled (with replacement) autosomal windows equal to the number of focal windows and calculated Spearman's rank correlation between DNA methylation divergence (absolute value of test-statistic estimates for taxon effects from DMP analyses) and population genetic measures inside and outside of the focal region. We considered the correlations to be significant if their 95% quantile distributions did not overlap zero.

Primary Figures

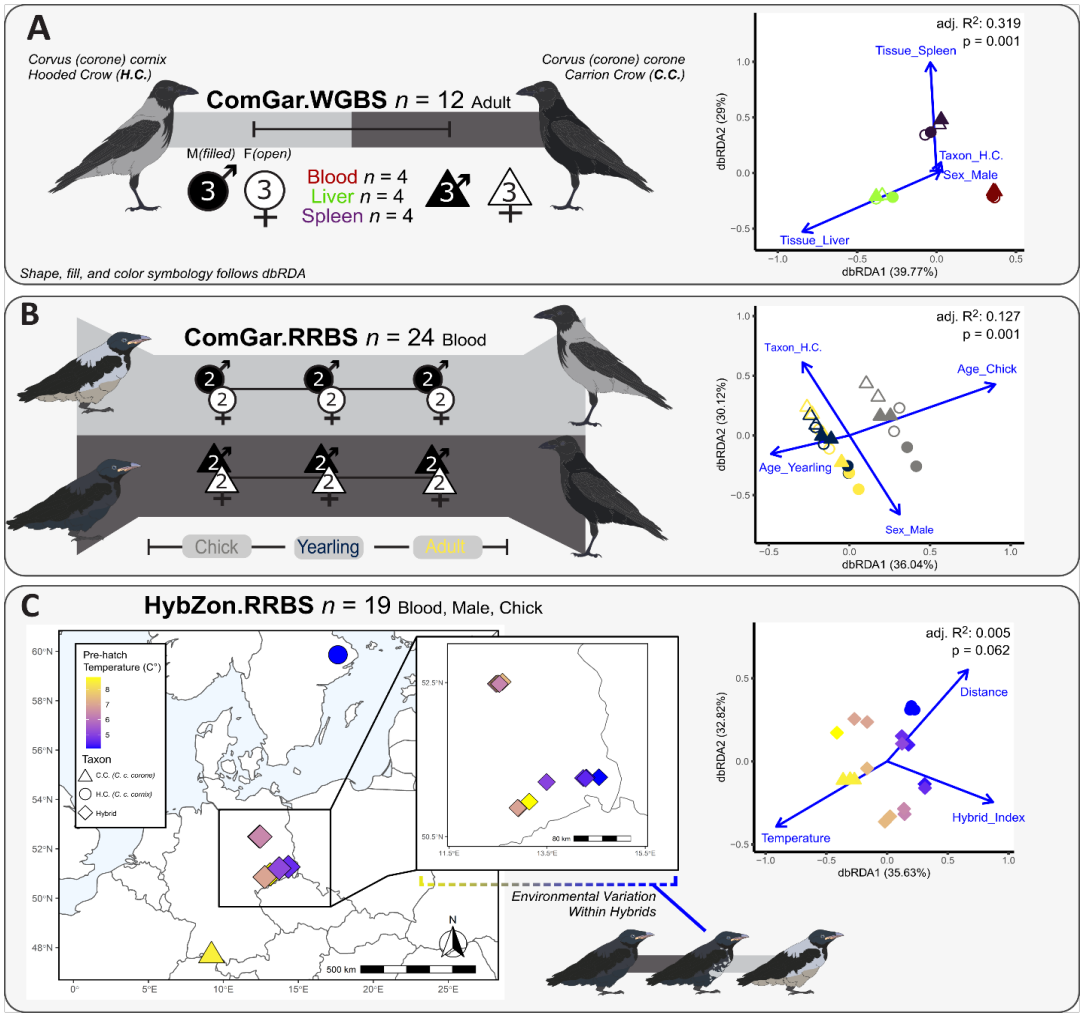

Fig. 1. Experimental design to quantify the factors associated with 5mC methylome variation in crows.

Left panels show sampling schemes for common garden (ComGar.) (**A**) WGBS and (**B**) RRBS datasets to isolate the physiological effects of tissue, age, sex, and taxon. Number of post-filtering CpG sites [min-mean-max in million]: ComGar.RRBS: 0.74-1.12-1.30; ComGar.WGBS: 3.03-8.93-15.2. (**C**) RRBS data from transect-collected blood of male nestlings across the European hybrid zone (HybZon.RRBS: 0.970-1.07-1.41) provides methylomic variation across environmental fluctuations (local pre-hatch winter temperatures) and genetic ancestry while controlling for tissue, sex and age. (**A-C**) Right panels show distance-based redundancy analyses (dbRDA) summarizing genome-wide DNA methylation variation within each experiment.

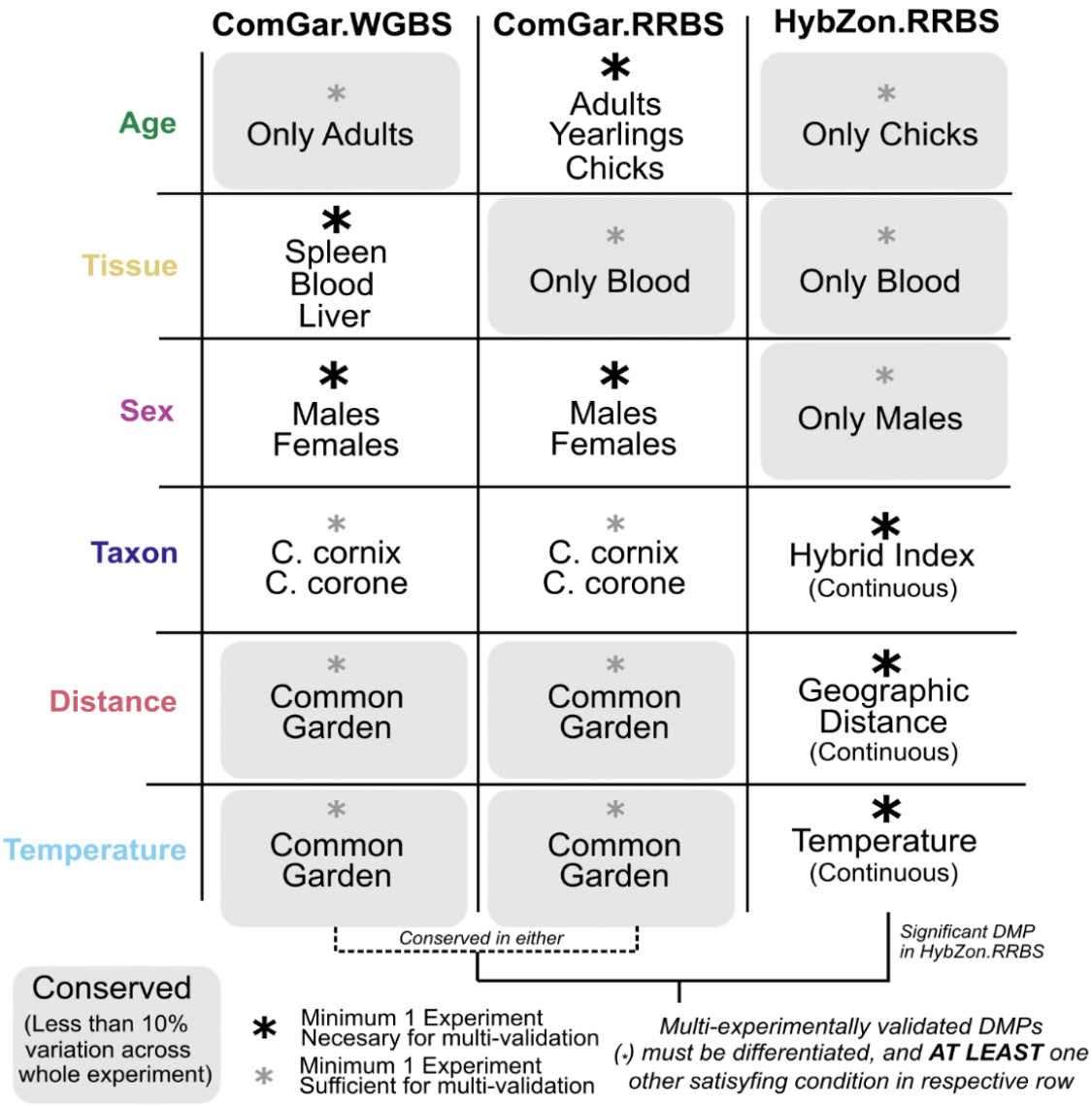

Fig. 2. Multi-experimental validation to remove spurious DMPs.

Each sequencing effort includes unique biological variation that can be exploited to control for false-positive inference (*i.e.,* multi-experimental support). Differentially methylated positions (DMP) are considered valid only if the site shows 1) statistical evidence for differential methylation in at least one of the corresponding asterisked fields in its row (*necessary condition, black asterisk*), 2) no conflicting evidence from any different variable and 3) support from *at least* one of the other classifications in that row (*sufficient condition, grey asterisk*). Sufficiency requires support for the variable of interest in another experiment (*e.g.* taxon) or low variation in an experiment where this variable has only single-state (*e.g.* age) exhibiting less than a 10% variation in methylation (*conserved, grey shading*).

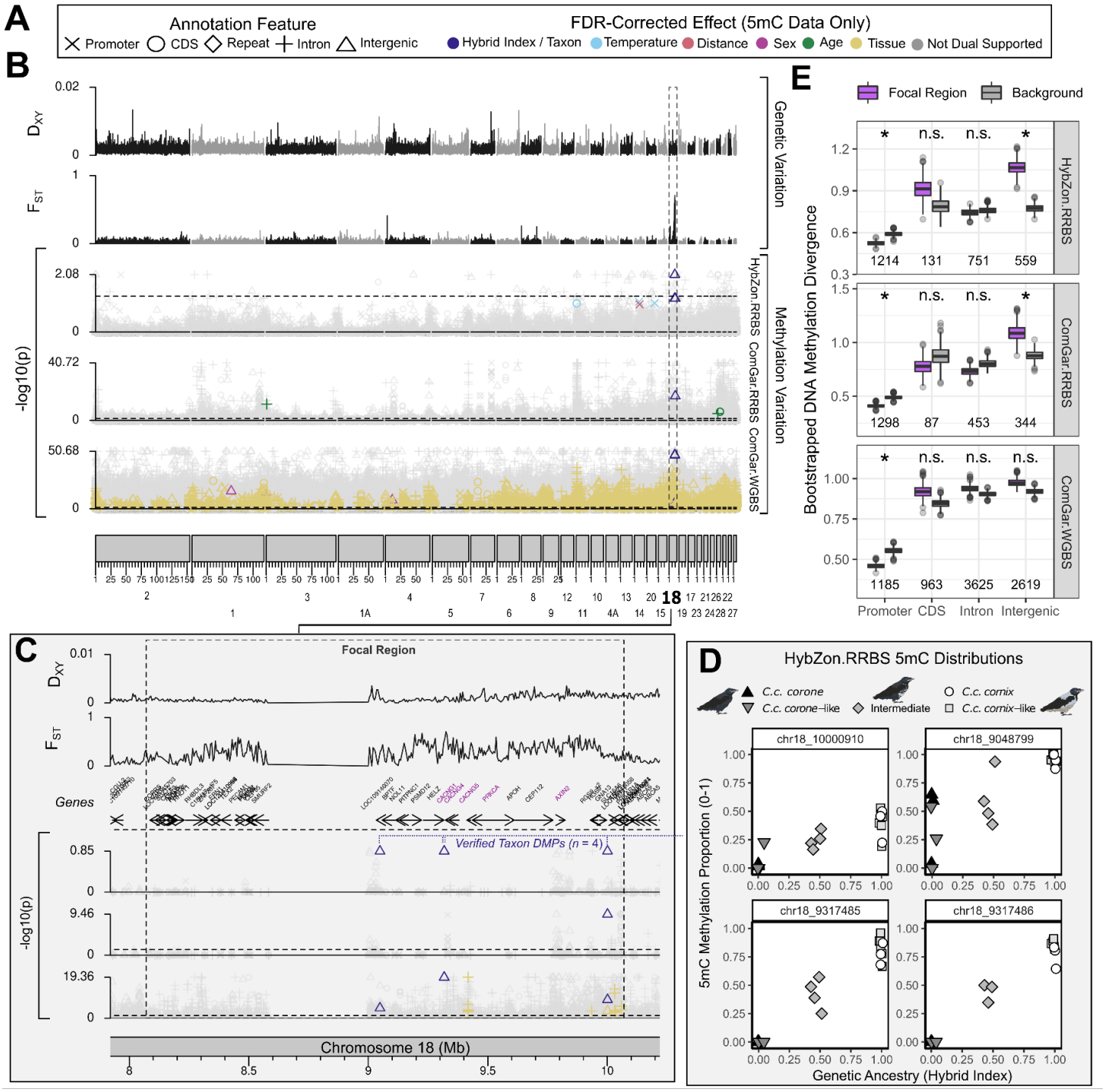
A
Annotation Feature
Promoter CDS Repeat Intron Intergenic
FDR-Corrected Effect (5mC Data Only)
Hybrid Index / Taxon Temperature Distance Sex Age Tissue Not Dual Supported
B
Genetic Variation
Methylation Variation
HybZon.RRBS ComGar.RRBS ComGar.WGBS
E
Focal Region
Background
Bootstrapped DNA Methylation Divergence
HybZon.RRBS
ComGar.RRBS
ComGar.WGBS
Promoter CDS Intron Intergenic
C
Focal Region
Genes
Verified Taxon DMPs (n = 4)
Chromosome 18 (Mb)
D
HybZon.RRBS 5mC Distributions
C.c. corone
C.c. corone–like
Intermediate
C.c. cornix
C.c. cornix–like
chr18_10000910
chr18_9048799
chr18_9317485
chr18_9317486
5mC Methylation Proportion (0-1)
Genetic Ancestry (Hybrid Index)

Fig. 3. Methylomic variation along the crow genome.

(**A**) Legend used throughout describing genomic annotation feature (symbol) and explanatory variable (colour) for each DMP. (**B**) *Genetic variation:* genetic divergence (D_{XY} [min-mean-max]: 0-0.0015-0.013) and genetic differentiation (F_{ST}: 0-0.01-0.71) across all $n = 28$ allopatric hooded and carrion crows. The region of elevated differentiation on chromosome 18 (focal region) is highlighted (99% of sites with $F_{ST} > 0.3$). *Methylation variation*: FDR-corrected base-pair level *p*-values with coloured points indicating differentially methylated positions for multi-experimentally verified DMPs. Only the lowest FDR-corrected effect was plotted for each CpG in each experiment. To aid in visualization a ceiling is imposed on minimum and maximum values within each experiment based on quantiles of the limits of visible data (*see methods*). (**C**) The focal region of high genetic differentiation ($F_{ST} = 0.298$ within vs. 0.010 outside) shows the only four multi-experimentally verified DMPs related to taxon divergence. Candidate genes underlying plumage polymorphism from Knief *et al.,* 2019 are highlighted in purple. Otherwise, tracks as in panel B. (**D**) Methylation levels (y-axis) closely follow genetic ancestry (x-axis) at the four taxonomically-related sites following the general pattern of hypomethylation of carrion crow ancestry in this region (**Supplementary Fig. S6**). (**E**) Methylation divergence for all CpGs within the focal region compared to the autosomal background. Default parameter boxplots correspond to first and third quantiles; significance indicated if either side of the 95% distribution tails do not overlap. Total CpGs used for empirical counts within focal region shown below box plots.

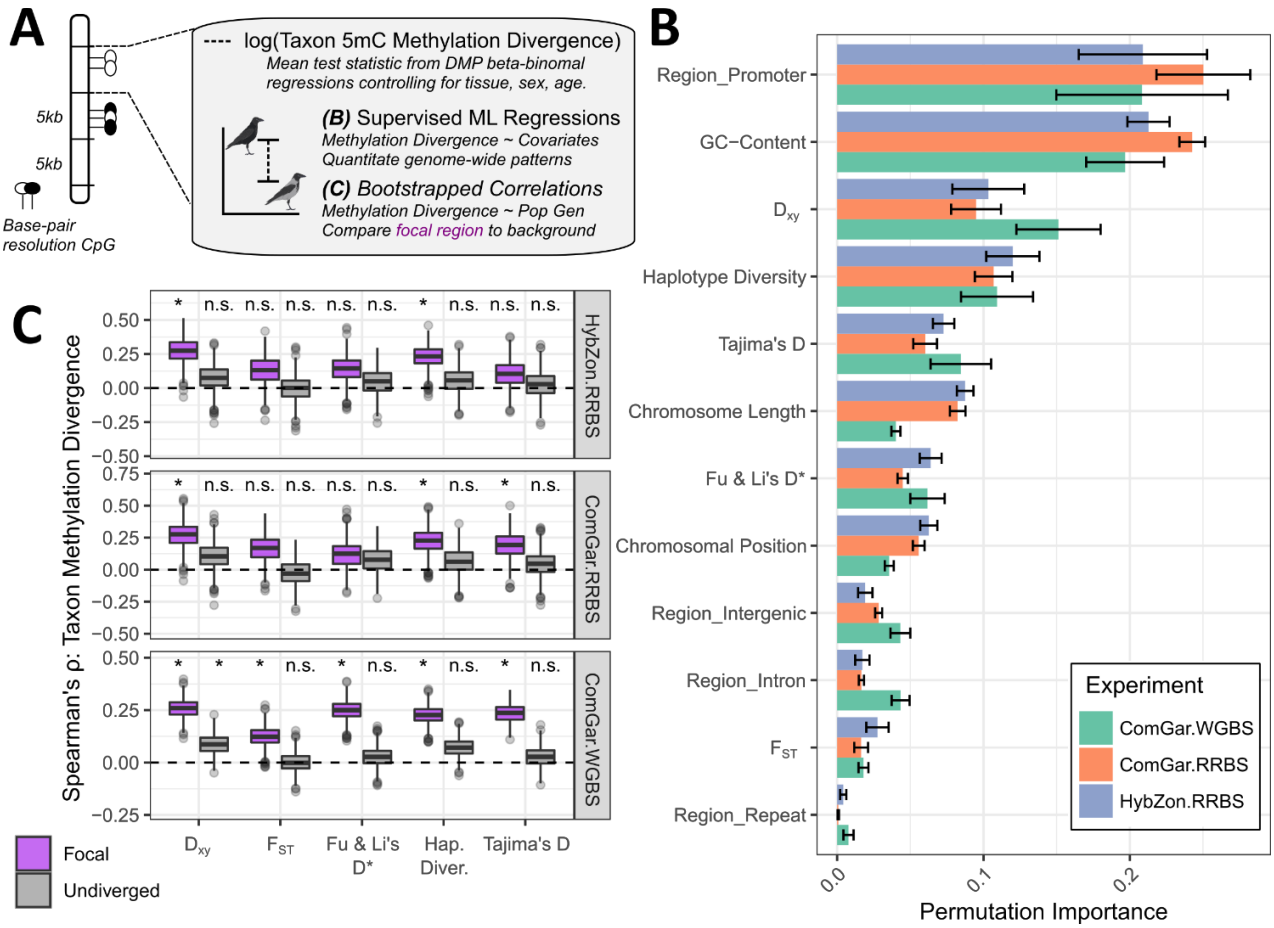

Fig. 4. Predicting 5mC methylation divergence between hooded and carrion crows.

(**A**) Cartoon depicting the two methods used to examine broad chromosomal and population genetic patterns associated with DNA methylation. (**B**) A supervised machine learning regression approach across the three methylation sequencing efforts determined the relative contributions of explanatory variables (y-axis) to taxonomic methylation divergence. Methylation divergence was summarized as the binned mean of DMP output test-statistic estimates for taxon-effects, controlling for other variables within each experiment (**Figs. 2, 3, S8**). Permutation importance measures variable importance against a decrease in model fit and indicates predictive ability. Mean R^2 across each experiment was 0.050, 0.097, 0.21. (**C**) Spearman's rank correlation between DNA methylation divergence and population genetic metrics within and outside of the focal region, indicating elevated covariation within the focal region which is characterized by a higher degree of genetic differentiation. Distributions drawn from 1,000 bootstrap sampling events; default parameter boxplots correspond to first and third quantiles; significance indicated if either side of the 95% distribution tails do not overlap zero.

Supplementary Information

Supplementary material for this manuscript can be found at:
https://www.biorxiv.org/content/10.1101/2024.05.22.595340v1

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Discussion

Deciphering the evolutionary forces generating, maintaining, and eliminating phenotypic and genetic variation in wild populations requires a careful consideration of population history, allele frequencies, and biotic interactions within and between other organisms in space and time. Coevolutionary arms races provide an exciting opportunity to examine the evolution and persistence of traits under constantly-evolving and fluctuating selection regimes, while the epigenetic landscape within nascent species offers a glimpse into the cooperative and exclusive determinants of genetic and non-genetic modifications to evolution. Below I discuss how my results from the preceding four papers fill a knowledge gap related to the maintenance of phenotypic variation and the role of epigenetic regulation during population divergence[1].

1. Overview

In paper I, I determined the mechanisms underlying the maintenance of matrilineally-restricted plumage polymorphism in *Cuculus* cuckoos. My results reveal that the mutation underlying this polymorphism arose prior to the split of two species, the common (*Cuculus canorus*) and oriental cuckoos (*C. optatus*), and was likely preserved through balancing selection. Similarly, in paper II, I assessed the genetic and biogeographical associations with host specialization within common cuckoos (*C. canorus*), demonstrating a clear link between matrilines and the adaptive phenotypes of mimetic cuckoo eggs. My identification of matrilineal egg-encoding haplotypes far surpassing the coalescence of sister taxa highlights the ancient evolutionary dynamics maintaining these trans-species matrilines across a homogeneous autosomal genetic background, particularly when considering the blue-egg laying lineage. In paper III, I demonstrate how sex-specific epigenetic regulation of chromatin accessibility against the backdrop of a conserved methylomic landscape

[1] In this discussion, I refer to the results in the possessive first person 'I' and 'my', but I emphasize that these results are the outcome of the collaborative work with my colleagues.

extends our understanding of the complexities involved in sex chromosome evolution and sex-specific variation in gene expression. Finally, in manuscript IV I demonstrate that epigenetic variation has a limited scope in the speciation process in crows. Collectively, these findings contribute to ongoing discussions about various aspects of evolutionary biology, including speciation processes in birds, the role of epigenetic variation in evolution, the influence of gene flow and selection on adaptation, the presence and preservation of balanced polymorphisms, the evolutionary role of sex chromosomes, and the development of adaptive parasitic traits in cuckoos.

2. Broad perspectives

2.1. Evolutionary processes driving speciation

What defines a species? Arguably one of the most prevalent questions asked among evolutionary biologists and the source of many lively conversations, this enigmatic question can now be perceived through the lens of genomic data (J. B. W. Wolf & Ellegren, 2017). The current understanding emphasizes that speciation occurs through the accumulation of reproductive barriers (Coyne & Orr, 1989; Dobzhansky, 1937a; Matute & Cooper, 2021). However, these barriers – either prezygotic barriers like mate choice or song, or postzygotic like hybrid incompatibilities – are not a prerequisite for divergence, as ecological specialization can contribute to speciation (Bolnick et al., 2023; McCulloch et al., 2021; Meier et al., 2018; Nosil, 2008; Papadopulos et al., 2011), even in sympatry (H. Wang et al., 2020).

My research from paper I indicates that common and oriental cuckoos constitute two distinct species and are an evolutionarily significant unit, as defined by Waples' definition that the two birds are 'substantially reproductively isolated from other conspecific population units' and that they are 'important components in the evolutionary legacy of the species' (Waples, 1991). They are substantially reproductively isolated from other conspecific populations, seemingly due to pre-zygotic assortative mating driven by song differences (Meshcheryagina & Opaev, 2021). Despite our estimate of a young evolutionary age (48 – 52K generations) the isolation appears to be sufficient for maintaining the evolutionary distinctness of these species, as evidenced by genome-wide differentiated windows ($F_{ST} = 0.17 \pm 0.15$) among co-occurring species, both of which exhibit large effective sizes (N_e).

A corollary of my results worthy of continued research is the possibility of rapid speciation perpetuated by strong prezygotic isolation, as is hypothesized in birds (Edwards et al., 2005; P. R. Grant & Grant, 1997; Hinde, 1959). There is substantial widespread evidence of prezygotic isolation through song contributing to speciation in passerines like buntings (M. C. Baker & Baker, 1990), Darwin's finches (B. R. Grant & Grant, 1996), green finches (Irwin, 2000; Irwin, Bensch, et al., 2001; Irwin, Irwin, et al., 2001), sparrows (Patten et al., 2004), greenbuls (Slabbekoorn & Smith, 2002), and antbirds (Tobias & Seddon, 2009). While this form of prezygotic isolation shows promise for explaining rapid speciation after secondary contact and can be culturally heritable (B. R. Grant & Grant, 1996; Mason et al., 2017), further studies are needed to determine if similar mechanisms operate beyond *Passeriformes*, bolstering the accumulating evidence in other bird orders (Sebastianelli et al., 2024). Furthermore, exploring whether inheritance mechanisms in cuckoos are cultural or genetic would be invaluable to contextualize the maintenance of reproductive isolation (Foote et al., 2016). Future work could use captive cuckoos, mating preference trials, controlled crosses, and direct genetic manipulation to pinpoint the mechanisms underlying prezygotic isolation in this system (Rossi et al., 2024)

2.2. Epigenetic contributions to speciation

Epigenetic variation, particularly through mechanisms like DNA methylation, is being explored for its role in speciation, focusing on how it might allow rapid adaptation to environmental changes irrespective of changes to underlying genetic sequence (Ashe et al., 2021; Greenspoon et al., 2022; Ho & Saunders, 1979). My results from manuscript IV suggest that DNA methylation is more related to developmental biology than to adaptive responses to environmental variability in crows. Contrary to studies implicating DNA methylation in speciation in various fish and mammal systems (Laporte et al., 2019; Vernaz et al., 2022; Y. Wang et al., 2022), my results indicate a limited scope for epigenetic variation to contribute to speciation in crows. My methodological approach allowed me to differentiate between ontogenetic and environmental constituents of DNA methylation, thereby controlling for swarths of false positive sites. Strikingly, the identification of taxonomic methylation divergence only within the specific differentiated region on chromosome 18 suggests a strong genetic link to DNA methylation variation (Knief et al., 2019; Poelstra et al., 2014). This finding aligns with accumulating evidence suggesting strong covariance between

genetic variation and DNA methylation (Min et al., 2021; Ord et al., 2023; Sepers et al., 2023), challenging the notion that DNA methylation is a significant driver of speciation processes.

However, the contributions of epigenetic variation to adaptive evolution, and speciation processes in particular, are likely be context-dependent. In systems where adaptive advantages are gained through phenotypic plasticity, regulated by gene expression or DNA methylation, and if these changes are heritable, they could serve as a basis for evolution (Whitehead & Crawford, 2006). Research in whitefish and arctic char has indicated that gene expression and DNA methylation changes underlie adaptive plasticity in recently diverged species (Adams & Huntingford, 2004; Horta-Lacueva et al., 2023; Laporte et al., 2019; Matlosz et al., 2022), but whether or not these epigenetic changes precede changes in genetic sequence variation requires further study in varied taxa (West-Eberhard, 2003). However, in systems with epigenetic reprogramming (Reik et al., 2001), or where phenotypic plasticity is not known to contribute to adaptive responses, the impact of epigenetic variation may be less important. The epigenetic variation observed in crows is likely more akin to that studied in baboons, which examines variations over longer time scales and more closely mirrors genetic variation (Vilgalys et al., 2018). The situation may be well different in plants, however, where epigenetic variation appears less dictated by genetic variation (Becker et al., 2011; Richards et al., 2010, 2017).

The concept of 'epigenetic potential,' defined as the presence of CpG sites, particularly upstream of genes, has also recently been hypothesized to reflect a species' capacity for adaptive response (Kilvitis et al., 2017). Yet, my findings emphasize the need for cautious interpretation of how epigenetic mechanisms, like DNA methylation, contribute to evolutionary processes. Despite the theoretical expectations, the real-world applicability of these predictions to evolutionary divergence remains largely unexplored beyond theoretical models and limited experimental setups within a single taxa (Hanson et al., 2020, 2021, 2022). My experimental design uniquely positioned me to observe the interplay between genetic and epigenetic variations within a framework of nascent speciation, suggesting that DNA methylation may play a mediating rather than a causative role in the speciation process in this system.

To further elucidate the role of DNA methylation in speciation, future research in crows could aim to integrate multi-generational epigenetic data under controlled environmental conditions leveraging controlled genetic crosses, as showcased in stickleback (Hu et al., 2021). Alternatively,

future studies could assess the stability of methylation patterns over time across the hybrid zone by exploiting ancient DNA samples to determine whether historical DNA methylation divergence precedes or follows the accumulation of genetic variants on chromosome 18 (Barouch et al., 2024). Further investigations incorporating ancient DNA and population demography could greatly enhance our understanding of the temporal dynamics of epigenetic changes in relation to speciation events.

2.3. The call for an extended evolutionary synthesis

My results from manuscript IV add to the ongoing debate about whether an extended evolutionary synthesis is necessary (K. Laland et al., 2014). One side argues that the Modern Synthesis of evolutionary biology suffices. This traditional view holds that phenotypes derive from genetic variants, most mutations are minor and lead to gradual changes, inheritance is genetic, natural selection drives adaptation, and macroevolution is simply the buildup of microevolutionary changes (Futuyma, 1998; Mayr, 1982). On the other hand, proponents of an extended evolutionary synthesis argue that evolutionary developmental biology (evo-devo), developmental plasticity, epigenetic inheritance, and niche construction theories support a more interactive nature between these processes and the causative processes of evolution (K. N. Laland et al., 2015; Pigliucci, 2007). My results from manuscript IV indicate that the principles of the Modern Synthesis are adequate to explain evolution in the crow system.

Genetic variation, driven by mutation, drift, and selection, seems to predominantly influence patterns of epigenetic variation in crows. This contrasts with more pronounced effects observed in plants and invertebrates, where DNA methylation plays a significant evolutionary role (Aagaard et al., 2022; He et al., 2018; Johnson & Kelly, 2020; Schulz et al., 2014). In crows, DNA methylation is mainly involved in ontogeny rather than in adaptive responses to environmental changes. Furthermore, my research, collectively with recent controlled experiments in tits (Sepers et al., 2023), questions the evolutionary significance of other bird studies that linked temperature changes to reproductive traits (Corbett et al., 2020; Lindner, Laine, et al., 2021; X. Yan et al., 2015). My results instead suggest context-specific relevance of epigenetic changes or a transient phenotype unlikely to impact evolutionary trajectories. This positions my work within the ongoing

discourse on the necessity and scope of an extended evolutionary synthesis, particularly its emphasis on non-genetic inheritance and developmental plasticity, which seem less applicable to crows, and likely other vertebrate species, compared to the traditional genetic frameworks of the Modern Synthesis.

2.4. The maintenance of balanced polymorphisms

What maintains polymorphisms within populations, and even across species? My results from papers I and II inform a long-standing debate between the 'classical' school, which argues that deleterious mutant alleles which deviate from the functional wild-type allele are typically present at low frequencies in a population (Muller, 1950), and the 'balance' school, which argues that balancing selection can maintain multiple alleles of a gene at intermediate frequencies, enriching genetic diversity (B. Charlesworth & Charlesworth, 2017; Dobzhansky, 1955; Lewontin, 1974). This long-standing debate between the classic and balance schools also provides the foundation for two competing models of coevolutionary pressures between hosts and parasites: the selective sweep model, which reduces genetic variation near antagonistic loci by promoting a dominant competitive allele, and the balancing selection model, which preserves diversity and favors the increased fitness of rare alleles (Ebert & Fields, 2020). My results from papers I and II indicate that the classic and balance schools of thought are not mutually exclusive and instead likely work in concert to maintain, deplete, and generate genetic variation, utilizing both the selective sweep and balancing selection model.

In paper I, my observation that balanced polymorphisms are maintained across species boundaries suggests a role for negative frequency-dependent selection in maintaining polymorphisms within and across cuckoos. This could arise because the rarer cuckoo rufous phenotypes exhibit higher fitness because hosts are less familiar with these forms, thereby reduced cuckoo detectability (Thorogood & Davies, 2012). Alternatively, multiple female plumage morphs could reduce male sexual harassment, also reducing female detectability, as has been hypothesized to maintain plumage polymorphism in common cuckoos (Lee et al., 2019). Similar mechanisms preserve trans-species female-limited polymorphisms in damselflies (Willink et al., 2023), providing a plausible ecological basis for my identification of these ancient balanced haplotypes. In contrast,

my results from manuscript II indicate that several egg-encoding matrilineal haplotypes, which are sometimes found in allopatry, can be subject to strong selection pressures which eliminate genetic variation – either purifying or divergent. This selection has resulted in the fixation of certain haplotypes that correspond to mimetic eggs, such as the immaculate blue common redstart eggs in Finland. These eggs closely resemble those of specific hosts, such as the common redstarts, which are known to be highly discriminate about unmatched eggs in their nests (Moksnes & Røskaft, 1995).

My results from manuscript II also indicate that gene flow facilitates the exchange of adaptive haplotypes among common cuckoo populations. Gene flow is a well-established mechanism which spreads alleles across the landscape (Han et al., 2017; Slatkin, 1987) and can lead to long-term maintenance of allelic variation in the metapopulation (B. Charlesworth, 2009; Gompert et al., 2021). Simultaneously, divergent selection can either drive these alleles to fixation, or balancing selection can maintain a diversity of these alleles, all of which must be understood through the lens of demographic history (Simon & Coop, 2024). In paper I, I demonstrate that contemporary interspecific gene flow between common and oriental cuckoos is not the cause of the shared plumage polymorphism, while in manuscript II, I show that the exchange of alleles is common among contemporary common cuckoo populations. My observation of diverse mitochondrial egg-encoding haplotypes maintained across disparate populations throughout the range of common cuckoos therefore suggests a combination of gene flow and balancing selection in maintaining high genetic variation.

2.5. The presence and maintenance of trans-species polymorphisms

My identification of plumage-encoded matrilineal haplotypes predating speciation in cuckoos supports the theory that these adaptive haplotypes are trans-species polymorphisms. Crucially, autosomal data indicate that these haplotypes are not the result of incomplete lineage sorting or recent adaptive introgression but are instead likely maintained by balancing selection in both species (D. Charlesworth, 2006). This highlights a key evolutionary strategy where cuckoos have co-opted female-specific variation to ensure the stable transmission of traits that increase their fitness. My results align with known examples of trans-species polymorphisms, such as those

observed in immune genes across diverse taxa including rodents, humans, corvids, and guppies (Eimes et al., 2015; Figueroa et al., 1988; Klein et al., 1993; Lighten et al., 2017), where they confer adaptive advantages that span multiple species. These findings thus not only contribute to the understanding of cuckoo-host coevolutionary dynamics, but illustrate how polymorphisms more generally can be maintained by frequency-dependent selection and coevolution across evolutionary time (Klein, 1987; Van Valen, 1973).

The idea that balanced polymorphisms could be maintained by negative frequency-dependent selection is well supported during host-parasite coevolution (Ebert & Fields, 2020; Koskella & Lively, 2009). Evidence that these balanced polymorphisms can predate speciation is mounting, particularly outside of immune genes (Kratochwil et al., 2022; Willink et al., 2023). Trans-species polymorphisms often rely on genomic regions associated with reduced recombination, such as inversion polymorphisms in ants (Brelsford et al., 2020; Z. Yan et al., 2020). However, I present the first evidence of trans-species polymorphisms that exist only within female-limited genomic variation, exemplifying the power of sex-specific genomes in maintaining adaptive variation. While autosomal variation would be subject to recombination and potential loss of phenotype – particularly if this polymorphism incurs a fitness cost to males – the evolution of female-specific traits corresponding to matrilineal variation in cuckoos facilitates the stable transmission of advantageous traits. My results therefore indicate a unique evolutionary strategy for maintaining sex-limited polymorphisms, which often rely on non-recombining inversions to ensure their stable transmission, as seen in *Drosophila* (Da Cunha, 1953; Kerr & Kerr, 1952), butterflies (Nishikawa et al., 2015), damselflies (Willink et al., 2023), and ruffs (Küpper et al., 2016).

Moving forward, plausible directions are to assess the phylogenetic extent of this shared polymorphism across other polymorphic cuckoos and to elucidate its exact genetic basis. Female-limited plumage polymorphism exists in many *Cuculiformes* species (Thorogood & Davies, 2013), and even in the closely related order *Caprimulgidae* (Galeotti et al., 2003). However, determining the exact genes involved with the plumage polymorphism will prove difficult because of the need for captive raised cuckoos and repeated feather follicle sampling for functional analyses (Poelstra et al., 2015; Wu et al., 2019). Nonetheless, other exciting avenues could strive to prove negative frequency-dependent selection as an evolutionary force maintaining plumage polymorphism. Concrete steps would be first documenting the frequency of different plumage patterns across a

range of cuckoo populations, potentially leveraging museum collections. A potentially fruitful direction could be to identify diagnostic plumage-related variants in the mitochondrial genome that could be used to infer female-limited plumage in male samples. One could then assess the frequency of morphs across entire museum collections and determine the frequency of the morph across all populations. Simultaneously, one could explore the potential for sexual antagonism by looking at how the different plumage morphs affect male and female fitness differently. This might involve experimental setups to measure the reproductive success of the two female morphs, paired with time-series data of populations to examine the relative fitness of the higher occurrence morph. The parasitic nature of cuckoos makes this no small feat, as host acceptance of the egg morph and eventual fledging would be necessary to confirm reproductive success. Overall, concretely demonstrating negative frequency-dependent selection in cuckoos would be challenging, but successful outlines in damselflies where reproductive output were measured could aid in conceptualization (Iserbyt et al., 2013).

2.6. The role of sex chromosomes in evolution

Sex chromosomes are evolutionarily labile across eukaryotes and exhibit extensive diversity even within birds (A. E. Wright et al., 2016; Zhou et al., 2014). In papers I and II, I reveal that these sex chromosomes are relevant for adaptive egg- and plumage-encoding female-limited phenotypes in cuckoos. These results support the growing appreciation for sex chromosomes in adaptive evolution (Vicoso, 2019; Zhou & Bachtrog, 2012), and highlight the need to include the heterogametic sex and its degenerated chromosome in genome assemblies. My results from paper III then provide the evolutionary framework for understanding how crows regulate the problem of aneuploidy that arises from these degenerated sex chromosomes. I demonstrate that incomplete dosage compensation on the Z chromosome, with a gene expression pattern of $Z_f < (ZZ_m = AA_f = AA_m)$, aligns with observations in other ZW chromosome systems such as snakes, birds, and *Heliconius* butterflies (Catalán et al., 2018; Itoh et al., 2010; Schield et al., 2019). This pattern indicates that despite the presence of a single copy of the Z chromosome in females, there is an upregulation of genes on this chromosome, which does not fully equalize with the gene expression levels seen in males or on autosomes. This partial compensation suggests that while some balance is achieved, it is not sufficient to completely negate the effects of gene dosage disparities between

the sexes. Overall, our results support the notion of both local gene-by-gene regulation of Z transcripts (Mank & Ellegren, 2009) and chromosome-wide chromatin remodeling, providing the genomic architecture and evolutionary opportunity for sex limited polymorphisms.

3. Synthesis and concluding remarks

Determining the contributions of mutation, drift, selection, migration and recombination to the maintenance of biodiversity is a central tenet of evolutionary biology (Rolland et al., 2023). In collaboration with my colleagues across the four chapters of this dissertation, I have examined the dynamics stabilizing adaptive phenotypes and defining species boundaries in multifaceted contexts. I have revealed compelling evidence for the importance of sex chromosomes, balanced polymorphisms, and epigenetic variation to evolutionary processes. Specifically, my research shows that cuckoos have harnessed rapidly mutating sex-specific genetic variation, capitalizing on its arrested recombination, to thrive as parasitic generalists. This evolutionary strategy is facilitated by migration between populations, allowing for the exchange of adaptive traits, while founder effects, genetic drift, and selection catalyze arms races and the refinement of phenotypes between cuckoos and their hosts in geographic isolation. Advances in whole-genome sequencing and population genetic analysis have enabled me to dissect the interplay of evolutionary forces at work, revealing ancient haplotypes that predate speciation events. Conversely, in *Corvus*, I demonstrate that epigenetic variation is largely responsible for ontogenetic and sex chromosome regulation, with limited scope for independent contributions to speciation. This dissertation sheds light on some of the evolutionary forces that create, sustain, and erode genetic and epigenetic variation, setting the stage for future inquiries into the complex evolutionary dynamics perpetuating natural systems.

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Declaration of Generative AI and AI-assisted technologies

In the preparation of this work, I used the GPT-4 language model for building and refining code to implement analyses and for improving existing sentence clarity. The model was not employed to generate new text, nor was it used to verify scientific validity or to source references. I have thoroughly reviewed and edited the content, and I am solely responsible its content.