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The Genetics of Speciation and Colouration in Carrion and Hooded Crows

JELMER POELSTRA

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Abstract
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A fundamental goal in biological research is to gain an understanding of the evolutionary processes and genetic elements that drive speciation. Genes responsible for reproductive isolation in young divergent lineages are particularly poorly known. In this thesis, the speciation genetics of carrion (*Corvus* (*corone*) *corone*) and hooded (*C.* (*corone*) *cornix*) crows were studied. These taxa differ strikingly in colouration and meet in a narrow hybrid zone in Europe, yet appear to be very similar genetically. A major component of reproductive isolation is social selection on colour differences.

First, we investigated the genetic basis of plumage divergence between carrion and hooded crows using a candidate gene approach. Nucleotide divergence was confirmed to be low, while there was no evidence for any of the sequenced genes to be associated with colour differences.

Second, we performed a simulation study to assess the performance of RNA-seq, a relatively novel approach that we later employed ourselves. We asked how variation in transcriptome complexity and bioinformatic workflow affected the accuracy of gene expression profiling. We generally found reassuring robustness and made a number of specific recommendations.

Third, we compared the corticosterone stress response of carrion and hooded crows. In accordance with the hypothesis that the degree of melanization and physiological traits are correlated due to pleiotropy, we found a higher stress response in hooded crows, and detected possibly associated gene expression in pituitary.

Fourth, we investigated genomic divergence by assembling a hooded crow reference genome followed by whole-genome resequencing of four European population samples. Northern European carrion crows were more similar to hooded crows than to Spanish carrion crows, pointing towards rampant introgression far beyond the hybrid zone. Nevertheless, several narrow genomic regions harboured high between-taxon divergence and were potentially associated with phenotypic traits.

Fifth, we compared whole-transcriptome gene expression profiles between crows, focusing on skin with developing feathers. We used a design that allowed to differentiate between taxon-specific, colour-specific and body patterning effects. Widespread underexpression of genes in the melanogenesis pathway was associated with grey colour, and we detected several genes that may contribute to colour divergence in this system.

Keywords: evolutionary genetics, genomics, birds, next-generation sequencing, pigmentation, pigmentation genetics, eumelanin, social selection, gene expression, population genomics

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List of Papers

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

I Poelstra, J.W., Ellegren, H., Wolf, J.B.W. (2013) An extensive candidate gene approach to speciation: diversity, divergence and linkage disequilibrium in candidate pigmentation genes across the European crow hybrid zone. *Heredity,* advance online publication 24 July 2013

II Vijay, N.*, Poelstra, J.W.*, Künstner, A., Wolf, J.B.W. (2013) Challenges and strategies in transcriptome assembly and differential gene expression quantification. A comprehensive *in silico* assessment of RNA-seq experiments. *Molecular Ecology,* 22:620–634

III Poelstra, J.W., Müller, I., Vijay, N., Baglione, V., Wolf, J.B.W. (2013) Covariance between colouration, corticosterone response and gene expression patterns suggests pleiotropy in the melanocortin system in carrion and hooded crows. *Manuscript*

IV Poelstra, J.W.*, Vijay, N.*, Bossu, C.M.*, Lantz, H., Müller, I., Baglione, V., Unneberg, P., Wikelski, M., Grabherr, M., Wolf, J.B.W. (2013) The architecture of genomic and phenotypic divergence across the European crow hybrid zone. *Manuscript*

V Poelstra, J.W., Vijay, N., Müller, I., Ryll, B., Baglione, V., Wikelski, M., Wolf, J.B.W. (2013) The genetic basis of colouration patterning and divergence in carrion and hooded crows as inferred from transcriptome-wide gene expression profiles. *Manuscript*

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Abbreviations

AFLP	Amplified fragment length polymorphism
cDNA	Complementary DNA (deoxyribonucleic acid)
DNA	Deoxyribonucleic acid
LD	Linkage disequilibrium
mRNA	Messenger RNA (ribonucleic acid)
QTL	Quantitative trait locus
RAD	Restriction site associated DNA (deoxyribonucleic acid)
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing (using next-generation sequencing)

Introduction

1.1 Speciation

1.1.1 Introduction

The process of speciation concerns the splitting of one species into two: it is the fundamental historical process underlying the biodiversity that we observe on earth. Scientific interest in how speciation works has been considerable ever since it was coined the "mystery of mysteries" in the early 19th century by the philosopher John Herschel (1836). A few years later, Charles Darwin (1859) recognized that species are subject to gradual evolutionary change. Darwin also noticed that hybrids between different species had lower fitness due to reduced fertility or survival, but could not reconcile this with his view of gradual change by natural selection. The evolution of reproductive isolation –resulting in for example reduced hybrid fitness– is perhaps the most important part of speciation, and one of the major challenges in current speciation research is to obtain a detailed understanding of this process. Reproductive isolation is the force that reduces or prevents gene flow between (incipient) species when they occur in the same geographical area, and underlies what has been argued to be the main "species problem": why do sexually reproducing organisms in sympatry appear to fall into discrete clusters (Coyne & Orr 2004)?

Central issues in the study of reproductive isolation include the relative importance of different evolutionary forces contributing to its evolution (such as gene flow, natural and sexual selection, genetic drift, recombination, and mutation) and the importance of different types of isolating barriers. These barriers are most often separated into prezygotic and postzygotic components, i.e. those that prevent mating and conception, and those that act after conception, such as partial or complete hybrid infertility or inviability. Reproductive isolation of any kind is expected to result from genetically based differences between populations or species. Therefore, further examples of key questions in speciation research are: (1) what kind of genetic changes promote reproductive isolation (e.g. coding or regulatory mutations), (2) how are they embedded in the genome (e.g. genomic distribution and pleiotropic effects), and (3) which evolutionary processes

have led to their fixation? These questions have motivated the field of speci-speciation genetics, which is further discussed in section 1.2.

1.1.2 Hybrid zones and geography of speciation

Hybrid zones between divergent taxa have long received much attention in the study of speciation, and are commonly characterized as "natural laboratories of evolution" or "windows on the evolutionary process" (Barton & Hewitt 1985; Hewitt 1988; Harrison 1990). Hybrid zones can capture a range of intermediate stages of speciation, in which reproductive isolation is present, but incomplete. These are arguably the most informative stages of speciation, because once it is complete, one can only catalogue differences between species without knowing which of these differences were instrumental in generating reproductive isolation. Conversely, divergent populations often do not overlap in their geographic ranges (especially during early stages of speciation), prohibiting the study of reproductive isolation under natural conditions. Thus, hybrid zones provide a uniquely suitable framework for the study of reproductive isolation in natural systems.

Hybrid zones are often the result of secondary contact between previously isolated populations (Hewitt 2000). Pleistocene climate fluctuations caused cycles of range contraction and expansion in a wide variety of organisms (Hewitt 2001). During cold periods in the western Palearctic, many species retreated to isolated refugial areas including the Iberian Peninsula, Italy, and the Balkans. Upon northward population expansion during interglacial periods, these previously isolated populations came into secondary contact, resulting in several potential outcomes. In the case of complete reproductive isolation, diverged populations, which can then be viewed as full species, may come to co-occur in sympatry, or instead competitively exclude each other. In the case of incomplete reproductive isolation, hybridization could lead to a collapse of divergence between populations, or to the formation of a hybrid zone.

Geographical movement of hybrid zones is commonly observed, even across short time scales (i.e. years to decades, Buggs 2007). Other hybrid zones may appear to be stable, but even these are unlikely to remain static over geological time scales. Besides the occurrence of new epochs of isolation due to climatic fluctuations, reproductive isolation might gradually break down, or instead strengthen and thereby complete speciation (a process known as reinforcement, Servedio & Noor 2003).

Generally, periods of geographic isolation are thought to be an important driving factor in the majority of speciation events (Coyne & Orr 2004), especially in birds (Price 2008). Nevertheless, recent molecular results, theoretical studies, and studies focusing on speciation driven by adaptation to divergent ecological niches (ecological speciation) all suggest a larger role of gene flow during speciation than previously appreciated (Pinho & Hey

2010; Nosil 2012; Feder *et al.* 2012), and there is now convincing evidence for fully sympatric speciation in a number of cases (reviewed in Bolnick & Fitzpatrick 2007).

1.1.3 Social selection, colouration and speciation

Divergence among geographically separated populations is often most striking in traits under sexual selection and other forms of social selection, such as colouration, ornamentation, and song (Darwin 1871; Ritchie 2007). The argument has often been made that social selection may promote speciation (West-Eberhard 1983; Hochberg *et al.* 2003), especially those components associated with sexual interaction (Kraaijeveld *et al.* 2011; Maan & Seehausen 2011). Empirical studies have supported the idea that social selection can drive trait divergence at particularly high rates (Masta & Maddison 2002; Arnegard *et al.* 2010), as was also suggested by a recent comparative study across 23 bird families (Seddon *et al.* 2013). Moreover, socially selected traits may diverge in any arbitrary direction, whereas natural selection often leads to convergent and parallel adaptations (Schluter & Nagel 1995; Price 1998; Stern 2013).

An important question remains: does divergence in phenotype, such as colour, by itself cause reproductive isolation? This would be expected when mating is commonly assortative by phenotype, such as due to co-evolution between traits and preferences (as suggested by Lande 1981, 1982). However, mating preferences need not always be highest for current or local trait values, and individuals may prefer signals that have evolved in populations other than their own (e.g. Ryan & Wagner 1987).

A recent paper demonstrated that speciation rates among colour-polymorphic birds are higher than in monomorphic species (Hugall & Stuart-Fox 2012). This suggests that colouration differences among individuals may directly promote speciation events (Gray & McKinnon 2007). In birds, widespread sexual imprinting on parental phenotypes (Immelmann 1972; Ten Cate *et al.* 1992) represents an additional mechanism promoting assortative mating by phenotype (Irwin & Price 1999; Verzijden *et al.* 2012). Moreover, environmentally mediated effects of colour differences can also contribute to reproductive isolation. First, fitness of colour variants may differ among habitats (Roulin 2004; McKinnon & Pierotti 2010). Second, colouration differences may have pleiotropic effects on physiology and behaviour and thereby contribute to ecological or behavioural isolation (see section 1.3.3).

Taken together, it is expected, though certainly not inevitable, that divergence of socially selected traits such as colour represents an important prezygotic reproductive barrier.

1.2 Speciation genetics

1.2.1 Introduction

Studying the genetics of speciation is crucial for elucidating the evolutionary mechanisms and processes that promote speciation. Until recently, speciation genetic research was confined to model organisms such as *Drosophila*, where hybridization and genetic mapping experiments have predominantly focused on the genetics of post-zygotic isolation (Coyne & Orr 2004; Presgraves 2010). Recent progress in sequencing techniques has allowed the field to emerge beyond laboratory organisms, and central questions in speciation genetics can now also be addressed in natural populations (Wolf *et al.* 2010b).

1.2.2 Speciation genes

An obvious avenue of research in speciation genetics is to identify specific genetic changes that contribute to speciation. A "speciation gene" can be any functional genomic element that conveys some degree of reproductive isolation (Orr *et al.* 2004). The cataloguing of such genes simultaneously addresses fundamental questions in evolutionary genetics, such as the relative importance of regulatory versus coding mutations, and the role of specific classes of genes (e.g. with low pleiotropy, Stern & Orgogozo 2009).

A surprising result from research on "speciation genes" thus far is that many of the implicated sequences appear to be involved in molecular arms races associated with selfish genes, genetic conflict and pathogens (Presgraves 2010). However, this research has been almost entirely restricted to postzygotic reproductive isolation, such as hybrid sterility, and has probed only a small set of species, mostly *Drosophila*. Moreover, many of the investigated species pairs are strongly reproductively isolated, with a variety of types of barriers involved. Therefore, while the pinpointed genes may currently contribute to postzygotic isolation in the lab, it is unclear whether they were relevant in initially establishing reproductive isolation. Research across diverse lineages and earlier stages of the speciation process is now emerging (Chan *et al.* 2009; The Heliconius Genome Consortium 2012), and is needed to capture the genetic basis of speciation at its onset. While prezygotic barriers are thought to be important components of reproductive isolation, due to the simple fact that they act *before* postzygotic barriers (Coyne & Orr 2004), surprisingly little is known about genes involved in generating prezygotic isolation (Michalak & Noor 2006).

In papers I, IV, and V of this thesis, I have investigated the genetic basis of colouration differences between carrion and hooded crows, a potential "speciation phenotype". In the next sections, I will first discuss some

strategies to relate phenotypes to genes, and will briefly discuss the genetics underlying melanin-based colouration.

1.2.3 Relating (speciation) phenotypes to genes

Several methodological approaches are commonly used to study the genetics underlying trait differences among species, populations, or individuals. The most common method in the context of speciation phenotypes is to cross large numbers of individuals with divergent phenotypes. Segregation or blending patterns of the phenotype can then be related to (often genetic) markers with known locations. These types of studies are generally restricted to organisms that can be easily bred in laboratories, and for which extensive genetic resources are available. Techniques more easily employed in wild populations require either naturally occurring within-population variation in the trait of interest or phenotypic differentiation across shallow genetic population structure, and are thus suitable for speciation studies only when lineages have recently diverged and/or significant gene flow occurs.

When variation in the trait of interest naturally segregates (including through natural hybridization in hybrid zones) and a population pedigree is known, quantitative traits may be mapped through QTL mapping. Genomic resolution is often limited by the number of meioses in the pedigree, typically leading to the identification of fairly broad genomic regions associated with a trait (e.g., Slate *et al.* 2009; Tarka *et al.* 2010). A second mapping approach is association mapping wherein natural genetic variation is simply correlated with trait values. Association mapping relies on linkage disequilibrium (LD) to generate correlations between genotyped markers and causal sites, unless causal sites are probed. This is only likely when entire genomes or specific candidate genes –*a priori* suspected to be associated with a trait– are sequenced.

Another approach to home in on the genetic basis of phenotypic variation uses information from gene expression patterns. This was until recently restricted to model species, because the technology required to quantify gene expression profiles (e.g. microarrays) relied on extensive *a priori* knowledge of the transcriptome of the species in question. However, high throughput transcriptome sequencing (RNA-seq) has now made it possible to sequence entire transcriptomes of virtually any organism (Wang *et al.* 2009; Ekblom & Galindo 2011; Wolf 2013). Because of the much smaller size of the transcriptome compared to the entire genome, this can relatively easily be performed at high coverage for multiple individuals. As an intermediate step between genotype and phenotype, gene expression patterns can be particularly informative, especially in situations where regulatory changes underlie phenotypic variation. Differences in gene expression may be easier to detect than gene sequence differences because a change in the expression

of one gene cascades across several genes in a pathway. This may, however, simultaneously hamper direct identification of causal elements.

In paper II of this thesis, I have performed an investigation into the performance of high-throughput transcriptome sequencing for non-model organisms, and how this performance varies with bioinformatic procedures and transcriptome complexity of the study species. In papers III, IV, and V, I have employed RNA-seq to study gene expression divergence between carrion and hooded crows, and to investigate the genetic basis of colouration differences between them.

1.2.4 Speciation genomics

An aspect of speciation genetics that is rapidly gaining prominence –again largely due to advances in sequencing techniques– is the study of the genomic architecture of speciation. Coalescent analyses of sequence data across an ever-increasing array of organisms have in many cases provided evidence for gene flow during speciation (although the prevalence of gene flow remains controversial, Pinho & Hey 2010; Via 2012). Characterizing how the landscape of genomic divergence builds up or remains distinct in the face of homogenizing gene flow is an important area of research.

A little over a decade ago, the "genic view of speciation" was proposed by Chung-I Wu (2001). This idea predicts heterogeneous landscapes of genomic divergence where areas of high divergence (called "divergence islands" or "speciation islands") stand out against a background of low divergence. As time progresses, divergence islands are expected to increase or collapse, depending on the relative strength of, for example, recombination and divergent selection (Abbott *et al.* 2013). Structural genomic mutations causing inversions, and other processes reducing recombination, are of immediate importance acting as barriers to gene flow (Hoffmann & Rieseberg 2008). At the same time, localized genomic divergence may facilitate the eventual build-up of divergence globally across the genome. Ecological speciation models distinguish between genome hitchhiking, that is overall genome divergence as a result of reduced gene flow (Shafer & Wolf 2013), and divergence hitchhiking, creating more localized "genomic islands of divergence" as a result of linkage to speciation genes (Feder *et al.* 2012).

In the study of genetic divergence and its distribution across the genome, the last few years have seen a transition from AFLP, microsatellite typing and Sanger sequencing, utilizing between tens and up to a few hundreds of loci, to reduced representation libraries (such as through RAD sequencing, Baird *et al.* 2008) with thousands of loci and even whole-genome approaches. In paper IV of this thesis, I have investigated the genomic landscape of divergence between carrion and hooded crows by whole-genome (re)sequencing of several population samples.

1.3 Genetics of colouration

1.3.1 Introduction

For reasons discussed above, colouration is a candidate phenotype for promoting speciation. In bird plumage, colouration is produced using feather micro-structure and by integrating pigments into feathers. Structural colouration produces blue and (ultra)violet colours and iridescent colouration, by scattering or refracting incoming light using feather barbule layering and air in between these layers (Prum *et al.* 1998; Doucet *et al.* 2006). The two main types of pigments in bird plumage are carotenoids and melanins. Carotenoid pigments produce colours such as yellows, greens, and bright reds. Birds cannot synthesize carotenoids *de novo* and instead obtain them as a part of their diet, after which they may be deposited in feathers. Some species do have the ability to metabolically transform carotenoids and thereby alter their colouration (McGraw 2006). The most common type of pigment is melanin, which can in turn be divided in two main types: pheomelanins, producing reddish-brown colouration, and eumelanin, resulting in dull browns, greys, and blacks.

While very little is known about the genetics of structural colouration and the deposition and modification of carotenoids, melanin-based colouration has for many decades been a key research area in evolutionary genetics (Hoekstra 2006). The biochemical and genetic pathways underlying the production of melanin are fairly well understood in model species such as mouse (Bennett & Lamoreux 2003; Slominski *et al.* 2004; Mills & Patterson 2009; Manceau *et al.* 2010). Since the general mechanisms of melanin-based colouration seem to be largely conserved among vertebrates (Hubbard *et al.* 2010), it is possible to study the genetics of melanin-based colouration in non-model organisms.

Across vertebrates, pheo- and eumelanins are produced by specialized cells, melanocytes, which are located in the skin. Within melanocytes, membrane-bound organelles called melanosomes synthesize melanin, and are then transported out of the melanocytes and into keratinocytes. Several processes, such as melanocyte development and proliferation, melanosome biogenesis and transport, and the amount and type of melanin produced within melanosomes, are all involved in producing visible pigmentation in skin or feathers. Mutations in genes involved in any of these processes have been linked to variation in pigmentation in mice, as well as mild to very severe negative pleiotropic effects and associated diseases (Bennett & Lamoreux 2003; Hoekstra 2006; Montolio *et al.* 2013). Mutations that affect the rate of melanogenesis within melanosomes are generally least likely to have negative pleiotropic effects.

One of the key genes in the pathway is *MC1R*, which is activated by αMSH and in turn activates the central transcription factor *MITF* via the cAMP-pathway. *MITF* subsequently controls transcription of many genes, including several that are directly involved in eumelanin synthesis. Within melanosomes, L-tyrosine is in several steps transformed to either pheo- or eumelanin, through reactions promoted by *TYRP1* and *TYRP2* (Hearing 1999). When *MC1R* activity is low due to binding of its antagonist *ASIP*, eumelanin synthesis is reduced and pheomelanin synthesis may be increased.

1.3.2 Genes underlying colour variation in birds

In birds, mutations causing variation in colouration have mostly been identified in domesticated species, using both candidate gene and genetic mapping approaches (reviewed in Roulin & Ducrest 2013). In Japanese quail, mutations with large effects on melanin-based plumage variation have been detected in and near *ASIP* (two mutations), *EDNRB2*, *MC1R*, *MITF*, *MLPH*, *SLC45A2*, and *TYRP1*. For chicken, implicated genes are *MC1R*, *MLPH*, *PMEL17*, *SLC45A2*, *SOX10*, and *TYR*. These mutations include deletions (eight cases), a retrovirus insertion (one case), and non-synonymous (mostly missense) nucleotide substitutions (six cases). Finally, *MC1R* mutations affecting colouration have also been found in domestic ducks (a nucleotide substitution), domestic turkey (a nucleotide substitution) and domestic guinea fowl (a deletion). A comprehensive list of genes found to be associated with melanin colouration in birds is provided by Roulin & Ducrest (2013).

In wild birds, striking colour polymorphisms have primarily been investigated using candidate gene approaches, since genetic mapping approaches are often not feasible (for reasons discussed above). In most cases, sequencing has been limited to the *MC1R* gene, since it is one of the most obvious candidates and an easily sequenced gene due to its small size. This has led to the identification of non-synonymous *MC1R* mutations associated with colour in snow goose (Mundy *et al.* 2004), red-footed booby (Baião & Parker 2012), Tahiti reed warbler (Cibois *et al.* 2012), Eleanora's falcon (Gangoso *et al.* 2011), gyr falcon (Johnson *et al.* 2012; Zhan *et al.* 2012), arctic skua (Mundy *et al.* 2004), bananaquit (Theron *et al.* 2001), and monarch flycatchers (Uy *et al.* 2009). Colour variation in these species concerns within-population polymorphisms, except in the monarch flycatchers, where all-black and chestnut-bellied subspecies occur on separate islands. In most of these cases, colour variation further involves discrete morphs with one of them entirely melanic (dark brown or black).

In paper I of this thesis, I have employed a candidate gene approach to investigate the genetic basis of colour differences between carrion and hooded crows. In papers IV and V, I returned to this topic using genome and transcriptome sequencing.

1.3.3 Pleiotropic effects of melanogenesis

The production of eumelanin is generally initiated when αMSH binds to the MC1R receptor. αMSH is one of several melanocortins, which are post-translational products of *POMC*. Melanocortins not only bind to MC1R but also to four other melanocortin receptors (MC2R - MC5R). The activation of these receptors affects a range of physiological and behavioural traits unrelated to pigmentation, such as the HPA (hypothalamus-pituitary-adrenal axis) stress response (MC2R), energy expenditure (MC3R and MC4R), sexual activity (MC4R), and aggressiveness (MC5R) (reviewed in Ducrest *et al.* 2008; Roulin & Ducrest 2011). If eumelanin-based colour differences are due to variation in melanocortin levels, and levels of the different melanocortins co-vary with each other and across tissues, a correlation between the degree of colour variation and behavioural and physiological traits is expected. Specifically, more melanic animals are predicted to display higher resistance to stress, higher sexual activity, higher levels of aggression, better immune responses, and higher metabolic rates.

A literature review by Ducrest et al. (2008) suggested that a relationship between eumelanin-based colouration, behaviour and physiology is widespread across animals, and follows the predictions outlined above. Most studies on wild animals were conducted on birds, where associations in the expected direction were found between eumelanin-based colouration and, among others: sexual behaviour (7 out of 7 cases), aggressiveness (17 out of 24 cases), and stress response (6 out of 7 cases). This relationship can have important evolutionary consequences. It may contribute to geographical patterns of colour variation, the occurrence of colour polymorphisms, and may precipitate reproductive isolation. Colouration can evolve along for the ride when sexual or natural selection is primarily acting on physiological traits. Conversely, colour divergence between populations due to habitat adaptation or sexual selection may also result in behavioural and physiological divergence thereby further contributing to reproductive isolation. Finally, phenotypically convergent evolution is not necessarily convergent at the molecular level (Stern 2013), which may affect evolutionary trajectories. Two populations of beach mice that have independently adapted to pale sandy habitats by evolving pale colouration have done so using mutations in genes that differ in pleiotropic effects (Steiner *et al.* 2009; Roulin & Ducrest 2011).

In paper III of this thesis, I have investigated whether crows that differ dramatically in the amount of eumelanin-based colouration also differ in an important physiological trait, the corticosterone response, as predicted by the pleiotropy hypothesis discussed above.

1.4 Study system: carrion and hooded crows

1.4.1 The genus Corvus

Corvus is a genus of large passerine birds in the family Corvidae that contains 40-44 currently recognized species. Most species are colloquially referred to as crows, while some of the larger species are commonly known as ravens. The genus likely originated in the Palearctic, and now inhabits all continents except Antarctica (Jønsson *et al.* 2012). Crows have throughout folklore been considered smart birds, and research has confirmed that crows are large-brained and more intelligent than most other birds (Emery & Clayton 2004). Studies have shown natural and experimental tool use in New Caledonian crows (*Corvus moneduloides*) (Rutz *et al.* 2010), experimental problem solving in rooks (*C. frugilegus*) (Bird & Emery 2009), and social learning in American crows (*C. brachyrhynchos*) (Cornell *et al.* 2012). Crows are highly social birds (Clayton & Emery 2007), which may be one of the factors underlying the evolution of their intelligence (Emery & Clayton 2003; Byrne & Bates 2007).

Morphologically, crows are a rather uniform group: most species are similar in shape and proportions, and most are all-black, often with iridescent colouration especially on the wings (Madge & Burn 1994). The most striking phenotypic variation is the occurrence of extensive white or grey plumage in seven species: Daurian jackdaw (*C. dauuricus*), collared crow (*C. torquatus*), house crow (*Corvus splendens*), piping crow (*C. typicus*), grey crow (*C. tristis*), pied crow (*C. albus*), and hooded crow (*C. (corone) cornix*). In the grey crow, only immature individuals are pale (and across the entire bird). In the remaining six species, the grey to white plumage is similarly distributed across the torso, differing mainly in degree of paleness and size of the area it covers (Figure 1). Interestingly, these species are not each others' closest relatives – instead, most have an all black sister taxon (species or subspecies), suggesting independent evolution of the pied plumage. Three of these taxon pairs with divergent colouration are known to hybridize: western (*C. monedula*) and Daurian jackdaws (occasional hybridization, Madge & Burn 1994), dwarf ravens (C. *edithae*) and pied crows (regular hybridization possibly in hybrid zones, Londei 2008), and carrion (*C. (corone) corone*) and hooded crows (well-defined hybrid zones).

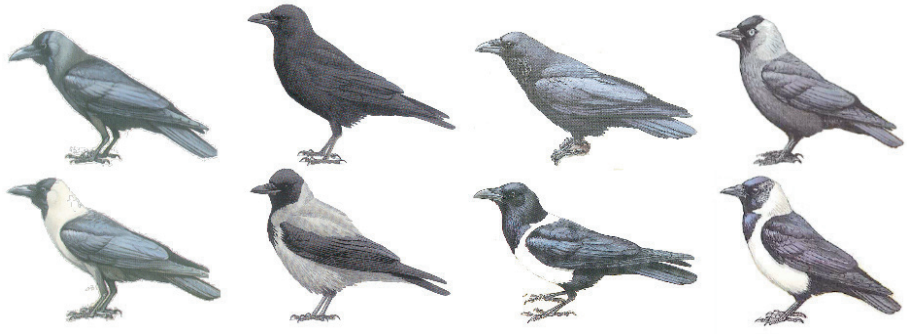

Figure 1. Colour variation among crow species. Four pairs of sister taxa with divergent colouration are shown, with increasing levels of reproductive isolation (between the two members of the pair) to the right. All-black taxa are on the top row and "pied" taxa on the bottom row. From left to right: house crow (subspecies *zugmayeri* top, subspecies *insolens* bottom), carrion (top) and hooded crow, dwarf raven (top) and pied crow, western (top) and Daurian jackdaw. Note the similar distribution of pale parts across the torso in the four pied species, although the precise extent is unique in every species and is most extensive in hooded crows. While pale parts are grey in house crows and hooded crows, they are pure white in pied crows and Daurian jackdaws.

Among other *Corvus* species, regular hybridization in putative hybrid zones has been reported for American and northwestern crows (Johnston 1961), and between northern raven and three of its allospecies: brown-necked raven (*C. ruficollis*), large-billed crow (*C. macrorhynchos*), and jungle crow (*C.* (*macrorhynchos*) *levaillantii*) (McCarthy 2006 and references therein). It should be noted, though, that hybridization among very similar looking species is hard to establish. For example, much controversy exists over the existence of the northwestern crow as a separate taxon, and it can only be identified by range (Sibley 2000), which nonetheless overlaps with American crow. Clearly, if the parent taxa cannot be separated morphologically, hybrids between them cannot be identified either.

The taxon pairs in *Corvus* with one all-black and one "pied" species are interesting in the context of speciation, especially as they differ in the degree of reproductive isolation: near-complete among jackdaws, potentially intermediate between dwarf ravens and pied crows, and with widespread hybridization in hybrid zones between carrion and hooded crows. Moreover, the house crow exhibits colouration variation among subspecies (see Figure 1), possibly representing yet a lower level of reproductive isolation.

1.4.2 A textbook example of a hybrid zone

In this thesis, I have investigated genetic divergence between carrion and hooded crows. They are the most studied of the aforementioned taxon pairs of crows. The hybrid zone between them has long attracted the attention of

ornithologists (Meise 1928) and was used as a prime example of speciation and hybrid zones by Ernst Mayr (1942, 1963) and subsequently in textbooks on evolution (Stearns & Hoekstra 2005; Ridley 2007). In the following sections, I will describe the central features of the system that are of relevance for the research in this thesis.

Linnaeus (1758) originally described carrion and hooded crows as separate species. In the 20th century, carrion and hooded crows were mostly considered subspecies of the same species (Cramp & Perrins 1994; Madge & Burn 1994), yet they have currently once again obtained species status on many lists (Parkin *et al.* 2003; Clements 2007; Gill & Donsker 2013). The taxonomic status of carrion and hooded crows is not important for this thesis – the existence of controversy surrounding it is merely a good illustration of the fact that speciation in this system is at an intermediate stage. I will refer to them as separate "taxa" and denote their scientific names as *Corvus* (*corone*) *corone* and *Corvus* (*corone*) *cornix*.

Carrion and hooded crows have a curious distributional pattern known as a "leapfrog" distribution (Remsen 1984): one taxon occurs in two separate areas (in this case carrion crow, occurring in western Europe as well as eastern Asia), while the second taxon occupies the region in between (in this case hooded crow, occurring roughly in eastern Europe and western Asia) (Figure 2). The simplest explanation for the origin of this distributional pattern is that an all-black ancestor of the taxa became isolated in at least three refugial areas, likely during range contractions associated with Pleistocene climate fluctuations. Subsequently, the central population –the ancestors of hooded crows– underwent a marked phenotypic change, followed by post-Pleistocene range expansions with the three populations forming hybrid zones upon secondary contact. Alternatively, ancestral populations may have had a phenotype similar to hooded crows with two populations independently evolving all-black plumage, or a widespread polymorphism may have occurred followed by differential fixation among populations. Sister to carrion and hooded crows is the collared crow (Jønsson *et al.* 2012), which has a plumage pattern more similar to hooded crow (although with pure white instead of grey across a smaller area of the torso). Sister to the superspecies group containing carrion, hooded, and collared crows are the American and northwestern crows (Jønsson *et al.* 2012), both of which are all-black.

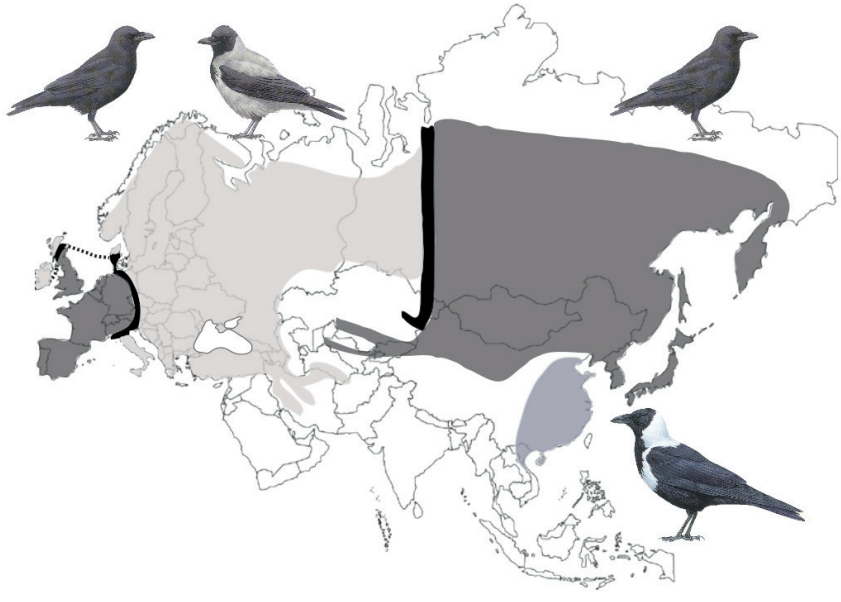

Figure 2. Distribution of carrion crows (dark grey, both in western Europe and eastern Asia) and hooded crows (pale grey, Europe to western Asia), and their sister species, collared crow (grey, mainly eastern China). The thick black lines indicate the locations of the hybrid zones between carrion and hooded crows. Map redrawn after Haring *et al.* (2012) by C. Bossu. Carrion and hooded crow drawings after Mullarney *et al.* (1999), collared crow drawing after Madge & Burn (1994).

Carrion and hooded crows form well-defined, narrow hybrid zones in the areas where their ranges meet: across central Europe (Meise 1928) and Siberia (Kryukov & Blinov 1994) (see Figure 2). Across most of Europe, the hybrid zone location aligns approximately with those of contact or hybrid zones of several other species of birds and mammals (Hewitt 2000). Such areas where contact zones of diverse organisms coincide have been dubbed "suture zones" (Remington 1968) and are a result of parallel movement of populations from Pleistocene refugia.

1.4.3 Divergence between carrion and hooded crows

Several previous studies have investigated genetic structure between carrion and hooded crows, using mitochondrial DNA (Kryukov & Suzuki 2000; Haring *et al.* 2007; Kryukov *et al.* 2012), microsatellites (Haas *et al.* 2009), RFLPs (Kryukov *et al.* 1992), allozymes (Saino *et al.* 1992), and nuclear introns (Wolf *et al.* 2010a). All have revealed very low levels of genetic differentiation, which is surprising in light of the narrow hybrid zone and

clear phenotypic differentiation. There are several possible and non-mutually-exclusive explanations for this pattern.

First, divergence might have occurred in the absence of geographic isolation, with gene flow never ceasing entirely. This appears less plausible, as building up initial divergence in the face of gene flow usually requires strong ecological selection (often in concert with sexual selection, Coyne 2007; van Doorn *et al.* 2009; Bird *et al.* 2012). Carrion and hooded crows do not generally differ in ecology: both use a variety of similar habitats in their respective and vast geographical distributions (Madge & Burn 1994). Madge & Burn (1994) noted that hooded crows may, at least in parts of their range, be more closely linked to human habitation and be more sociable than carrion crows. However, variation in social behaviour is also found within the taxa: cooperative breeding is common in Spanish but not in more northerly populations of carrion crows (Baglione *et al.* 2002). Detailed studies within the hybrid zone have detected subtle and contradictory differences in habitat use (Rolando & Laiolo 1992; Saino 1992) to effectively no differences (Haas *et al.* 2010). Further, ecological speciation appears to be particularly rare in birds, and potential cases all involve seed-eating species such as crossbills (Smith *et al.* 2012). This is in line with theoretical predictions that strongly bi- or multi-modal resource distributions are important to promote divergence with gene flow (e.g., Thibert-Plante & Hendry 2011).

Another potential explanation for the lack of genetic differentiation between carrion and hooded crows is that divergence is of very recent origin, or similarly, that multiple but short periods of isolation have taken place, e.g. only at ice age peak(s). Because crows thrive in open habitats (Madge & Burn 1994; Haring *et al.* 2007), their ranges might have extended further northwards during glacial periods than those of organisms dependent on ample vegetation or forests. This scenario would imply that phenotypic divergence has occurred very rapidly and suggests the action of strong, presumably social, selection (see Londei 2013). The occurrence of rapid phenotypic divergence in socially selected traits has been well documented, especially in birds (Ödeen & Björklund 2003; Milá *et al.* 2007b; a; Gay *et al.* 2009; e.g. Campagna *et al.* 2012), but also other animals (e.g. Stapley *et al.* 2010; Oh & Shaw 2013).

A third explanation is that genetic isolation and divergence might have been more extensive initially but subsequent admixture after range expansion homogenized allele frequencies. This would predict higher divergence between refugial populations, which in Europe was likely the Iberian Peninsula for the ancestors of carrion crows, and Italy, the Balkans and/or the Middle East for those of hooded crows. Crows used for previous genetic analyses were sampled north of potential refugia, so this possibility remains untested. Some support for this explanation comes from the observation that a strongly divergent mitochondrial haplotype segregates

within populations of eastern carrion crows (*C. c. orientalis*) (Haring *et al.* 2007; Kryukov *et al.* 2012), suggesting that admixture of formerly divergent populations has occurred. To test this hypothesis, I have included sampling from a Spanish population in genomic sequencing in paper IV.

Wolf *et al.* (2010a) conducted a transcriptome sequencing study on brain samples from wild-caught carrion crows from Germany and hooded crows from Ireland. They found differentiation in overall gene expression profiles from carrion and hooded crows, suggesting that in this system, divergence at the gene expression level may have been rapid and more obvious than at the nucleotide level. However, because sampling conditions were not standardized, the observed differences may have also been due to phenotypic plasticity as an effect of environmental differences (Gibson 2008). To distinguish between these possibilities, I have investigated gene expression divergence under common garden conditions in papers III, IV, and V.

1.4.4 Reproductive isolation between carrion and hooded crows

The width, stability, movement, and composition of hybrid zones may help reveal which evolutionary processes are at work. At equilibrium, hybrid zone width is governed by a balance between reproductive isolation and dispersal into the hybrid zone (Barton & Hewitt 1985). If reproductive isolation is very weak or absent (the "neutral diffusion" model), differentiation will decay, resulting in panmixia. With increasing reproductive isolation (the "tension zone" model), equilibrium hybrid zone width will be narrower, and the distribution of parental types and hybrids within the zone is expected to become bimodal (that is, with a predominance of parental types) (Jiggins & Mallet 2000). Finally, asymmetric isolation and dominance relationships, and differences in dispersal, result in the movement of hybrid zones.

The European hybrid zone between carrion and hooded crows has been studied in some detail in Germany and Denmark (Meise 1928; Haas & Brodin 2005), Scotland (Cook 1975), and Italy (e.g. Saino & Villa 1992). Hybrid zone width varies between around 50 and 150 kilometres, which is rather narrow: carrion and hooded crows represent a clear outlier when plotting hybrid zone width against genetic divergence (Price 2008).

The hybrid zone has been largely stable, though by comparing its present-day location with that reported by Meise (Meise 1928), locally varying movement was detected in Denmark (Haas & Brodin 2005) and Scotland (Cook 1975). In both of these cases, local hybrid zone movement was northwards, at the expense of hooded crows. No changes in hybrid zone width were found. With respect to hybrid zone composition, within the centre of the hybrid zone, a fifth to a third of the population consists of hybrids (Kryukov & Blinov 1989; Saino & Villa 1992; Haas & Brodin

2005). The distribution of phenotypes in the hybrid zone is thus between bimodal and "flat".

Taken together, the characteristics of the hybrid zones between carrion and hooded crows suggest the presence of fairly strong reproductive isolation. The question remains: what are the reproductive barriers involved? First, there are indications for (albeit weak) intrinsic postzygotic isolation from the Italian hybrid zone. Saino (1990) found an effect of female (but not male) phenotype, with hybrid females fledging less chicks than phenotypically pure individuals. Saino & Bolzern (1992) reported that hybrids laid smaller eggs than pure parental forms (with eggs of carrion crows also being larger than those of hooded crows). However, Saino & Villa (1992) found no effect on reproductive success in hybrid phenotypes and an asymmetry in reproductive success outside of the hybrid zone with carrion crows performing better.

Second, there is strong evidence for assortative mating from several areas in the European hybrid zone (Saino & Villa 1992; Rolando 1993; Risch & Andersen 1998; Randler 2007; Haas *et al.* 2010), and the Siberian hybrid zone (Kryukov & Blinov 1989). Theoretical studies specifically parameterized for the crow system (Brodin & Haas 2006, 2007) moreover indicate that assortative mating due to sexual imprinting is likely to reduce interspecific matings and that lowered hybrid fitness can result from inefficient imprinting on hybrid plumage.

Third, social selection more broadly defined may contribute to reproductive isolation. Crows are highly social birds and have been shown to be able to distinguish group members from non-group members (Kondo *et al.* 2012). Londei (2013) suggested that a "reject-the-unusual model of segregation" through social interactions may be an important aspect of isolation between carrion and hooded crows. Moreover, a hypothesized preference for sharp plumage contrasts (indeed, all *Corvus* taxa that are not entirely melanic have highly contrasting patches) would disadvantage the often mottled and irregularly patterned hybrids. In combination, these mechanisms may lead to strong positive-frequency dependent selection keeping colour variants differentially fixed everywhere except centrally in the hybrid zone.

In summary, plumage differences by itself appear to be the most important factor generating partial reproductive isolation between crows. I have investigated the currently unknown genetics that underlie this "speciation phenotype" in papers I, IV, and V. The hypothesis of pleiotropic effects in the melanocortin system predicts physiological differences between carrion and hooded crows, which I have tested in paper III. Finally, the intriguing observation of very low genetic differentiation begs the question what the precise genomic architecture of divergence between these taxa is, and whether or not differentiation is higher in potentially refugial populations. These questions have been addressed in paper IV.

2. Research Aims

2.1 General research aims

The general goal of my doctoral thesis was to conduct an investigation of the evolutionary processes underlying the marked phenotypic but shallow genome-wide divergence between two closely related species of corvids, carrion and hooded crows. My focus was on characterizing patterns of genomic divergence (at both the nucleotide and the gene expression level) and establishing a link to phenotypic differences.

2.2 Specific research aims

Paper I – To test whether genetic variation in a set of candidate melanin pigmentation genes could be related to the plumage colour differences between carrion and hooded crows. To study levels of genetic diversity within the taxa, and genetic differentiation between them. To quantify patterns of linkage disequilibrium, since these affect the power to detect genotype-phenotype associations.

Paper II – To investigate the performance of transcriptome assembly, gene expression quantification, and inference of differential expression for RNA-seq data in the face of variation in transcriptome complexity, sequencing error, and bioinformatic workflow.

Paper III – To test whether hooded crows showed a stronger corticosterone stress response than carrion crows, as predicted by the hypothesis that melanin-based pigmentation and several behavioural and physiological traits are correlated due to pleiotropy in the melanocortin system. To explore the genetic basis of these potential pleiotropic effects.

Paper IV – To sequence, assemble and annotate a crow reference genome to be used as a backbone for population genomic and transcriptomic analyses. To investigate patterns of whole-genome DNA sequence diversity and divergence, and link these with gene expression and colour differentiation between carrion and hooded crow populations from Europe.

Paper V – To understand colouration differences between and within carrion and hooded crows using patterns of gene expression in several tissues, focusing on skin with active feather follicles.

3. Summaries of Papers

Paper I: Pigmentation candidate genes

Patterns of animal colouration are of great evolutionary interest, since colouration is an important component of intra- and inter-species communication. Evolutionary change in colouration patterns can be the result of adaptation to a changing or novel habitat. It could also be due to fixation of arbitrary novel phenotypes that confer a social or sexual advantage. Upon secondary contact, divergent colouration may prevent or reduce mating between populations and as a result contribute to speciation. In the carrion and hooded crow system, colouration differences play an important role in speciation. All black carrion crows occur in western Europe and meet grey-coated hooded crows in a narrow hybrid zone running through central Europe. Surprisingly, previous studies have demonstrated very low levels of neutral genetic differentiation between them. While there is some evidence for limited postzygotic reproductive barriers, the marked phenotypic difference appears to be maintained mainly by assortative mating. The genetic basis of the colour divergence, which is caused by differences in eumelanin pigmentation, is currently not known. Haas *et al.* (2009) sequenced the *MC1R* gene in carrion and hooded crows, but did not detect any causal mutations.

Here, we selected 37 of the most promising candidates pigmentation genes characterized in mouse, and designed primers from zebra finch – chicken exon alignments. Successful amplification of the majority of primers in a selection of bird species across the avian phylogeny suggested that these primers can serve as a future resource for basically any bird species. We sequenced 107 amplicons (on average three amplicons per gene, in total 60 kb) for 23 carrion and 23 hooded crows and quantified genetic variation within and between the taxa. Genes were partially sequenced, with a focus on introns, using the rationale that linkage disequilibrium between causal (likely not sequenced) and nearby (sequenced) sites elevates divergence even in these nearby sites. The base level of divergence therefore influences the statistical power to detect colour associated genetic variants, as does the genetic architecture of the trait. The expected low background levels of genetic differentiation between carrion and hooded crows and the presumed mono- or oligogenic basis of the colour differences make this approach

feasible in this system, and render it comparable to association studies within a polymorphic population.

Genetic diversity was similar in carrion and hooded crows (with an average π of 0.0013), and levels of genetic differentiation between the taxa were confirmed to be low: F_{ST} values varied between 0.00 and 0.12 with an average of 0.02. Outlier detection methods did not identify any amplicons with differentiation that was higher than expected under neutrality. Statistics such as Tajima's D, Fu's F_S, DHEW, and the HKA test revealed several amplicons that deviated from neutrality. However, the absence of congruence among tests for individual amplicons, and a lack of elevated differentiation for potentially non-neutral amplicons indicated that non-neutral behaviour is likely caused by demographic changes. The inferred population recombination rate was high ($4N_er \sim 0.03$), and levels of linkage disequilibrium dropped strongly within the order of few hundreds of base pairs,

In conclusion, colour divergence in this system may be mediated by gene(s) outside of the set of candidate genes screened in this study. Alternatively, the observed low levels of linkage disequilibrium may have compromised power to detect causal loci with nearby markers, especially for large genes and for potential causal mutations in regulatory regions.

Paper II: Methodological considerations on transcriptome sequencing

Until recently, gene expression quantification was mostly performed using hybridization of cDNA to oligonucleotide probes, such as in microarrays. These methods rely on extensive *a priori* knowledge of the transcriptome of the studied species in question and have therefore been mostly restricted to model species. With recent advances in sequencing technology, it has become possible to simultaneously characterize a transcriptome and quantify gene expression by simply performing high-coverage shotgun sequencing of whole RNA-derived cDNA. This technique, known as RNA-seq, is now widely used for both model species and non-model species. Reasons for the popularity of RNA-seq include the opportunity to discover novel transcriptome features and the reported higher accuracy and greater dynamic range of RNA-seq in quantifying mRNA concentrations compared to microarrays.

However, there has been a lack of systematic investigations into how variation in critical factors such as levels of transcriptome complexity and bioinformatic workflow may affect and potentially bias inference of read counts and differential expression. For example, what are the effects of high levels of polymorphism, especially in combination with high error rates? At

which level of divergence are *de novo* assemblies preferable to mapping against a known transcriptome of a related species?

We investigated the effects of variation in the following parameters: mode of assembly (three *de novo* assemblers vs. mapping assembly on divergent reference genomes), RNA library normalization (uniform vs. strongly skewed gene expression profile), software for the inference of differential expression (*edgeR* vs. *baySeq*), sequencing error ε (0 vs. 0.01), polymorphism π (0 vs. 0.001 vs. 0.01), transcriptome complexity (~4,400 unique genes vs. ~17,400 genes with paralogs of different age, presence or absence of alternative splicing), and annotation quality of the reference genome. Metrics by which we evaluated performance were measures describing transcriptome assembly quality, and the accuracy of the inference of absolute expression levels and of differential expression.

In general, we found that transcriptome assembly and gene expression profiling was accurate and robust to variation in most parameters. With respect to bioinformatic strategies, mapping assemblies generally outperformed *de novo* assemblies, library normalization lead did not improve the assembly, and the *edgeR* software slightly outperformed *baySeq*. Regarding transcriptome characteristics, increased transcriptome complexity (size, paralogs, alternative splicing) had a negative and considerable effect on assembly and the inference of differential expression. In contrast, the effects of polymorphism (and sequencing error) were almost negligible. Gene name assignment for *de novo* assemblies proved challenging. Blast-type approaches were more sensitive than suffix-tree based methods, but also generated more false positives.

These results provide guidance in the design and analysis of RNA-seq experiments. It is reassuring that the accuracy of inferred (differential) gene expression levels was generally high and that we found very limited negative effects of high levels of polymorphism and sequencing error.

Paper III: Pleiotropy in the melanocortin system

In many species of animals, the amount of melanin-based colouration is correlated with several physiological and behavioural traits. For example, more strongly melanized individuals are generally more resistant to stress, more aggressive, and differ in energy homeostasis. This correlation may arise due to pleiotropic effects of genes involved in melanogenesis. Specifically, the propriomelanocortin (*POMC*) gene produces several so-called melanocortins, which bind to and activate five different melanocortin receptors including MC1R. Higher activity of MC1R will primarily result in increased melanin production, whereas the activation of other melanocortin receptors may influence traits such as the hypothalamus-pituitary-axis (HPA) stress response, energy expenditure, sexual activity, and

aggressiveness. The relationship between melanin-based colouration, behav-behaviour, and physiology can have important consequences for the evolutionary dynamics of natural populations.

Most studies on this relationship have been performed with quantitative or discrete colour variation occurring within populations, since widespread divergence between populations might compromise inferences in a between-population context. However, carrion and hooded crows are strongly divergent in melanin-based colouration yet genetically very similar, and are thus an interesting case study. Consistent with their more melanic colouration, carrion crows have been shown to be more aggressive than and dominant over hooded crows and hybrids, but it is currently unknown whether any of the predicted physiological differences also exist. A physiological response that is particularly interesting in this context is the HPA stress response: the release of glucocorticoids (corticosterone, in the case of birds) that alters physiology and behaviour to effectively deal with incoming stressors.

Here, we raised carrion crows from Germany and Spain and hooded crows from Poland and Sweden in a common garden environment in Germany. We measured levels of corticosterone in blood plasma for a low stress (blood taken less than three minutes after onset of stress) and a high stress (blood taken at least twenty minutes after onset of stress) treatment. Corticosterone is produced in the adrenal gland in response to hormonal stimulation by ACTH, a posttranslationally modified product of the *POMC* gene, which is expressed at high levels in the pituitary gland. We quantified gene expression levels by RNA-seq in forebrain for 19 crows and in the pituitary gland for a single carrion crow and a single hooded crow.

Hooded crows showed a stronger stress response than carrion crows: corticosterone levels increased to a higher level at stress in hooded crows, while base levels were similar between the taxa. In the forebrain, gene expression patterns were nearly identical in carrion and hooded crows. This suggests that a previous finding of overall gene expression differences between the taxa may have been due to phenotypic plasticity, since gene expression profiles of those samples were directly assayed from wild-caught birds. *POMC* was among the three most highly expressed genes in the pituitary gland. It was expressed to an almost two-fold higher level of *POMC* expression in the carrion crow, although we couldn't support this statistically in the absence of replication.

This study identified a difference in the HPA axis response between carrion and hooded crows that is consistent with the idea that the melanocortin system not only regulates plumage colouration in crows, but also has pleiotropic effects on behaviour and physiology. Gene expression in pituitary suggested that *POMC* may be differentially regulated between taxa, but larger sample sizes will be needed to explore this hypothesis further.

Paper IV: The genomic architecture of divergence

A central subject in evolutionary genetics is the process of genome divergence during speciation. It is currently unclear how gene flow influences the genomic landscape of this divergence: do we see localized peaks of divergence across a background of low differentiation, or does divergence build up more gradually across the genome? If high divergence peaks occur, what are the evolutionary forces responsible for their establishment and maintenance, and how are they linked to phenotypic divergence and reproductive isolation?

Base levels of genetic divergence between carrion and hooded crows have been shown to be very low. However, geographic sampling has not included potential refugial areas, and sequencing has been limited to a very small proportion of the genome. More extensive geographic and genomic sampling is thus needed to provide insights into the processes underlying divergence and to study phenotype-genotype relationships relevant for speciation.

Here, we first sequenced the genome of a Swedish hooded crow at high coverage, which was then assembled and annotated. Next, we performed whole-genome resequencing of 60 crows (30 hooded crows from Sweden and Poland, and 30 carrion crows from Germany and Spain) at on average 12x sequence coverage. Consistent with previous studies, we found a background of low genome-wide differentiation (F_{ST} = 0.02). Interestingly, the Spanish carrion crow population was the most divergent population, such that German carrion crows were more similar to hooded crow populations than to Spanish carrion crows. This is consistent with the idea outlined in section 1.4.3 that carrion and hooded may have been isolated for more extensive periods of time than what is suggested by genetic differentiation in northern areas, and that northern populations may be highly admixed (whereas Spain likely represents a refugial population).

We next asked whether despite rampant gene flow, any genomic regions with high differentiation remain. We scanned the genome for such regions using two main methods. First, we calculated F_{ST} between carrion and hooded crows for 50kb windows across the genome. Second, we used a method implemented in the software *Saguaro*, which calculates distance matrices between individuals, producing phylogenetic hypotheses between all individuals, so-called "cacti", across the genome. This method is especially useful as an approach complementary to window-based F_{ST} because genomic regions of any size can get assigned to a certain cactus.

We found evidence for only five narrow divergence peaks, which were associated with low nucleotide diversity most likely due to reduced recombination rates. The largest and most extreme peak was a 2 Mb region that harboured 81 out of 83 confidently scored fixed differences between carrion and hooded crows. This region potentially contained an inversion

and showed signs of selection and an overrepresentation of derived alleles in hooded crows.

Finally, using RNA-seq, we quantified gene expression divergence between carrion and hooded crows in forebrain, gonads, liver, skin from torso, and skin from head. Overall levels of gene expression divergence between carrion and hooded crows were very low, while in skin from torso (where carrion crows are black and hooded grey), widespread underexpression of genes related to melanogenesis was detected. We did not find an overall overrepresentation of differentially expressed genes (nor of genes associated with melanogenesis) among regions with high F_{ST} and divergent cacti, yet several genes (most notably *HPGDS* and *NDP)* were highlighted when combining information from the melanogenesis pathway, population genomics and gene expression data.

This study has provided evidence for local genomic differentiation in the face of widespread gene flow between carrion and hooded crows, and has identified genes potentially responsible for phenotypic divergence.

Paper V: Gene expression and colouration patterning and divergence

In paper I, the genetic basis of colouration differences between carrion and hooded crows was investigated using a candidate gene approach. Some of the limitations of this approach included an *a priori* restriction to a small set of the most promising candidate genes, reliance on linkage disequilibrium of the investigated region to causal variants, and accordingly low power for detecting cis-regulatory mutations. Transcriptome-wide quantification of gene expression in focal tissues and during a developmental stage in which phenotypes are established provides a promising alternative approach. As introduced in paper II, recent advances in sequencing technologies have made it possible to conduct such studies in non-model organisms.

In this paper, we analysed whole-transcriptome sequencing (RNA-seq) data from tissue samples of both carrion and hooded crows that were raised under common garden conditions (see paper III and IV). Skin samples were obtained during a period of intense feather regrowth from areas of the crown and throat (pooled as “skin from head”) and belly and mantle (pooled as “skin from torso”). Three of these pooled samples (skin from head in both taxa, and skin from torso in carrion crows) produced black feathers, whereas skin from torso in hooded crow produced grey feathers. Hence, with this design we were able to contrast grey with black skin in two ways: between the species for the same skin region, and within hooded crow for different skin regions. We could therefore separate colour-specific expression from

effects associated with body patterning (torso vs. head in both taxa) and taxon-specificity (carrion vs. hooded crow in both skin types).

We identified many genes (n = 472) with differential expression associated with patterning (head vs. torso) in both taxa. Among these, a set of homeobox (Hox) genes showed the strongest signal. While patterning in terms of gene expression differences was thus visible among black head and torso regions in carrion crows, it was more pronounced in hooded crows, where head and torso differ in colouration. In combination with the near-absence of between-taxon expression differences in both skin types (n = 4 genes), this suggests that colour divergence may (in part) result from underlying differences in genetic elements controlling body patterning.

Genes with colour-related expression patterns (n = 93) were mostly down-regulated in skin producing grey feathers, and were strongly enriched for pigmentation related genes. This widespread downregulation in the melanogenesis pathway for grey skin appeared to be mediated by the *MITF* transcription factor, directly downstream of which expression differences were most pronounced. *MITF* was therefore identified as a central candidate to cause colour differences by regulatory divergence, along with several genes underexpressed in grey that (also) act upstream of *MITF*: *HPGDS*, *NDP*, *RAS*-*GRF1*, and *MC1R*. The latter is regulated by *POMC*, which was also implicated by overexpression of genes regulated by its products in the gonads in carrion crow. Moreover, a role for *POMC* is also consistent with results from paper III, demonstrating the presence a stronger stress response in hooded crows and potentially higher *POMC* expression in the pituitary of carrion crows. *HPGDS* had the strongest signal of underexpression in grey among all genes, and was along with *NDP* moreover also detected in a genome scan for high divergence regions between carrion and hooded crows (see Paper IV).

Concluding Remarks and Future Prospects

In this thesis, a series of studies on speciation in carrion and hooded crows were performed, which hopefully have the potential to establish *Corvus* crows as an exciting model system for speciation genetic research. We have first investigated the genetic basis of plumage divergence between carrion and hooded crows using a candidate gene approach. Nucleotide divergence was confirmed to be low and there was no evidence for any of the sequenced genes to be associated with colour differences. We subsequently followed up on this work in papers IV and V with genome-scale methods.

For methodological evaluation of our bioinformatic pipelines, we next performed a simulation study to assess how variation in transcriptome complexity and bioinformatic workflow affects the accuracy of gene expression profiling with RNA-seq. We generally found reassuring robustness in the inference of gene expression levels and differential expression, underlining the usefulness of the RNA-seq approach, and we made a number of specific recommendations. Future work should in particular focus on improving annotation routines for *de novo* assemblies, and on improving inference of transcript isoforms from sequence data.

The third project compared the corticosterone stress response of carrion and hooded crows. In accordance with the hypothesis that the degree of melanisation and physiological traits are correlated due to pleiotropy in the melanocortin system, we found a higher stress response in hooded crows. In pituitary, expression of *POMC,* the gene that produces melanocortins, was potentially higher in carrrion crow. Future work should focus on assessing whether other behavioural and physiological traits that are under melanocortin control differ consistently between carrion and hooded crows. In addition, potential differential expression of *POMC* should be further investigated with larger sample sizes, as well as the expression levels of other genes in the melanocortin system such as those mediating the posttranslational modifications that produce melanocortins. Eventually, protein abundance data on the melanocortins will be needed to assess the putative pleiotropic role of this gene.

Next, we investigated genomic divergence by first assembling a hooded crow reference genome followed by whole-genome resequencing of samples from four European populations. Northern European carrion crows were more similar to hooded crows than to Spanish carrion crows, pointing towards rampant introgression far beyond the hybrid zone. Nevertheless, we

detected several narrow genomic regions with high between-taxon diver-divergence, and these were potentially associated with phenotypic traits. These exciting findings are worth following up on with several lines of future work. First, sequencing of crows from potential hooded crow refugial areas (Italy, the Balkans, and the Middle East) can further confirm the presence of extensive gene flow in northern Europe. Second, genes shown to harbour exceptionally high divergence, and others with a potential link to colouration, should be investigated in more detail. Third, crows from around an independent hybrid zone in Siberia as well as from potential eastern carrion crow refugial areas can be sequenced to compare the genomic landscape of divergence with that in Europe. Fourth, the current data, ideally combined with more extensive geographical sampling, should be used to perform demographic modeling to quantify levels of gene flow and divergence times between carrion and hooded crows.

Finally, we compared whole-transcriptome gene expression profiles between and within carrion and hooded crows, focusing on skin with developing feathers. We used a design that allowed differentiating between taxon-specific, region-specific (body patterning) and colour-specific effects. Widespread underexpression of genes in the melanogenesis pathway was associated with grey colour, and we pinpointed several candidates that may contribute to colour differences between carrion and hooded crows. Several of these (*NDP*, *HPGDS*, and *POMC*) were moreover also corroborated by results from papers III and IV. Future work should follow up on these candidates and it will be important to accurately differentiate transcript isoforms and study their expression levels separately. Further, performing similar experiments on other sister taxa in the genus *Corvus* with divergent colouration will enable comparisons of the genetic basis of colour divergence across taxon pairs. More generally, studying speciation genetics also in other *Corvus* species will provide a unique comparative framework, and the work presented in this thesis facilitates investigations in those species.

Sammanfattning på Svenska

Under processen då arter bildas divergerar tidigare fritt korsande populationer och reproduktionsbarriärer byggs upp mellan dem. Nyckelfrågor i forskningen runt artbildningsprocessen är till exempel: vilka karaktärer och underliggande genomiska element är det som divergerar, vilka är de evolutionära krafter som driver och underhåller divergensen och vilken roll spelar genflöde mellan populationerna i artbildningen? Så kallade "artbildningsgener" – gener som bidrar till att reproduktionsbarriärer bildas och upprätthålls – har identifierats i ett antal modellarter men då främst för postzygotisk inkompatibilitet medan den genetiska bakgrunden till prezygotisk inkompatibilitet är i stort sett outforskad.

I denna avhandling har jag undersökt artbildningsgenetiken hos två (under)arter av kråka, *Corvus* (*corone) corone* (gråkråka) och *C. (c.) cornix* (svartkråka). Dessa två taxa skiljer sig markant i färgen på fjäderdräkten; svartkråkor är helt svarta medan gråkråkor har en i huvudsak grå kropp med svart huva, svarta vingar och svart stjärt. De två (under)arterna möts i en smal, väldefinierad och stabil hybridzon som går genom Europa. Tidigare genetiska undersökningar har visat mycket låga nivåer av genetisk skillnad mellan dessa två taxa. De viktigaste komponenterna som bidrar till isolering verkar vara partnerval och andra former av socialt urval som baseras på fjäderdräktens färg, pigmenteringen kan alltså potentiellt vara en karaktär som driver artbildningen.

Jag började med att undersöka den genetiska bakgrunden till färgskillnaden mellan gråkråka och svartkråka genom att sekvensera ett antal så kallade kandidatgener – gener som vi vet på något vis påverkar pigmenteringen i andra arter. Kroppens pigmentering som är grå hos gråkråka och svart hos svartkråka skiljer sig på grund av skillnader i mängden eumelanin. Genetiken bakom produktionen av melaninbaserad pigmentering i djur är relativt välkänd tack vare ett sekels forskning på bland annat möss. Jag använde information från dessa studier för att välja en uppsättning potentiella kandidatgener som skulle kunna påverka skillnaden i pigmentering mellan gråkråka och svartkråka. Nivåerna av genetisk divergens bekräftades vara mycket låga mellan gråkråka och svartkråka och jag fann inte några belägg för att någon av de undersökta generna påverkar färgskillnaden mellan dessa taxa. Detta kan innebära att färgskillnaderna beror på mutationer i andra gener än de jag undersökte, eller att de mutationer som påverkar pigmenteringen låg utanför de regioner som jag

undersökte och att låg kopplingsojämvikt gjorde att vi inte kunde upptäcka dem – det senare är särskilt troligt för stora gener och/eller gener med avskilt belägna regulatoriska mutationer.

I nästa steg utförde jag en simuleringsstudie för att undersöka prestandan hos transkriptom som genererats med ny sekvenseringsteknik (så kallad Next-Generation Sequencing, NGS) för tidigare genetiskt okaraktäriserade arter. Vi frågade oss hur variation i exempelvis transkriptomets komplexitet och det bioinformatiska arbetsflödet påverkade riktigheten av antalet sekvenser för en specifik region och skillnaden i genuttryck mellan testgrupper. Vi fann att metoderna är relativt robusta när det gäller att uppskatta genuttryck och våra slutsatser var ett antal rekommendationer för hur man kan gå tillväga för att utnyttja NGS för att karaktärisera transkriptom. Ett exempel är att det var bättre att utnyttja eventuellt tillgängliga genomiska resurser från besläktade arter än att försöka sätta ihop ett transkriptom helt från början, detta gäller även över relativt stora avstånd mellan studieart och tillgänglig resursart.

Flera gener i melanokortinsystemet har multipla funktioner och en hypotes är att skillnader i melanokortinsystemets gener kan ge skillnader i såväl pigmentering som stressrespons. För att undersöka det testade jag i min tredje studie om det fanns skillnader i kortikosteronnivåer mellan kråkor och svartkråkor som utsattes för stress och fann en signifikant högre kortikosteronnivåer i gråkråka. Dessutom undersökte jag genuttryck i framhjärnan och hypofysen och fann att uttryck av genen *POMC* i hypofysen eventuellt kan förklara skillnader i såväl pigmentering som stressrespons.

I min fjärde studie undersökte jag genetisk divergens mellan gråkråka och svartkråka över hela arvsmassan. Med hjälp av NGS sekvenserade vi hela arvsmassan för en enskild gråkråka och använde den som referens för att kartlägga variation i totalt 60 individer representerande både gråkråka och svartkråka insamlade på vardera två olika lokaler i Europa. Vi fann att nordeuropeiska svartkråkor var mer lika gråkråkor än spanska svartkråkor. Detta tyder på att genflöde och genetisk introgression sker långt utanför den nuvarande hybridzonen och att det leder till den generella genetiska likheten mellan vanlig gråkråka och svartkråka som observerats i tidigare undersökningar. Vi upptäckte också flera korta regioner i arvsmassan där den genetiska divergensen är hög mellan gråkråka och svartkråka, det gällde även mellan populationer i Nordeuropa. Dessa regioner verkar vara resistenta mot genflöde och kan associeras med de fenotypiska särdrag som karaktäriserar dessa två taxa.

Slutligen jämförde jag genuttrycksprofiler mellan kråkor och svartkråkor med speciellt fokus på de gener som uttrycks i fjäderfolliklar i skinnet. Jag använde mig av en 2x2 design för att differentiera mellan taxonspecifika (gråkråka jämfört med svartkråka), kroppsregionspecifika (skinnprover från bålen jämfört med skinnprover från huvudet) och färgspecifika effekter. Skillnader i genuttryck mellan taxa var generellt mycket låg i alla undersökta

vävnader: framhjärnan, levern, könsorganen och skinnet. När jag jämförde genuttryck i skinnet mellan bålen och huvudet fann jag bara en handfull gener med skillnad i uttryck mellan taxa medan hundratals gener hade skilda uttryck mellan skinnregionerna, eventuellt som en konsekvens av skillnader i pigmentering. Vi fann att ett generellt lägre uttryck av gener i melaninkaskaden var kopplat till grå färg och vi fann flera kandidatgener (till exempel *MITF*, *HPGDS* och *POMC*), som skulle kunna orsaka skillnader i uttrycksmönster och därigenom bidra till färgskillnader mellan kråkor och svartkråkor.

Sammanfattningsvis har min forskning bidragit till att etablera gråkråka och svartkråka som ett intressant system för att studera genetiken vid ett tidigt stadium av artbildningsprocessen där det fortfarande sker genflöde mellan taxa. Min avhandling har belyst fysiologiska, och speciellt genetiska, skillnader mellan gråkråka och svartkråka och etablerat kopplingar till kandidatgener för pigmentering och stressrespons. Framtida forskning bör nu fokusera på att göra mer omfattande geografiska insamlingar av prover, identifiera vilka mutationer i kandidatgenerna som eventuellt ligger bakom skillnader i pigmentering och stressrespons och göra parallella jämförelser i andra kända system där helsvarta och partiellt svarta kråkarter samexisterar.

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