UNIVERSITAT DE BARCELONA

Local adaptation by birds to human-altered habitats: the great tit and the house sparrow as model species

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Local adaptation by birds to human-altered habitats: The great tit and the house sparrow as model species

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List of papers

This thesis is based on the following papers:

1-**Personality and urbanization: behavioural traits and *DRD4* SNP830 polymorphisms in great tits in Barcelona city**, Riyahi, S., Björklund, M., Mateos-Gonzalez, F., & Senar, J. C., *Journal of Ethology*, 2017, 35(1):101-108.

2- **Combined epigenetic and intraspecific variation of the *DRD4* and *SERT* genes influence novelty seeking behavior in great tit *Parus major***, Riyahi S., Delgado, M. S., Calafell, F., Monk, D.&Senar, J. C., *Epigenetics*, 2015, 10:6,516-525.

3- **No association between the melanocortin-1 receptor (*MC1R*) and black belly stripe size variation in the great tit *Parus major*,** Riyahi S., Bjorklund M., Odeen A. & Senar J.C., *Bird Study*, 2014, 1-3.

4- **Single origin of human commensalism in the house sparrow,** Sætre, G. P.*, Riyahi, S.*, Aliabadian, M., Hermansen, J. S., Hogner, S., Olsson, U. & Elgvin, T. O., (*equal contribution), *Journal of Evolutionary Biology*, 2012, 25(4), 788-796.

5- Natural epigenetic variation within and among six subspecies of the house sparrow *Passer domesticus*, Riyahi S., Vilatersana R., Schrey A., Ghorbani H., Aliabadian M. & Senar J. C., *Under review in the Journal of Experimental Biology*, 2017.

Other papers

- **Beak and skull shapes of human commensal and non-commensal house sparrows *Passer domesticus***, Riyahi S., Hammer Ø., Arbabi T., Sánchez A., Roselaar C. S., Aliabadian M. & Sætre G. P., *BMC evolutionary biology*, 2013, 13(1), 200.

-**Year-Round Preference for Spiders by Mediterranean great tits *Parus major*,** Pagani-Núñez E., Hernández-Gómez S., Riyahi S. & Senar J.C., Ardeola 61(2), 2014, 257-267.

Report of the director

During her doctoral research, **Sepand Riyahi** has published seven papers in peer reviewed journals within the topic of biological sciences, five of which have been included in this thesis. Her research covered a wide range of evolutionary biology topics, including behavioural science, molecular ecology and epigenetics. All the journals where she published have impact factors, some of high relevance, as for instance the main chapter of her doctoral thesis which was published in the **Journal of Epigenetics,** with an impact factor of 5.29, and the chapter published in the **Journal of Evolutionary Biology,** with an impact factor of 2.74. So far, she has accumulated 32 citations, without including auto citations. She is the first author for all of these five papers. Here you have the list of the papers that form part of the Thesis and her involvement in each of them:

1-**Personality and urbanization: behavioural traits and *DRD4* SNP830 polymorphisms in great tits in Barcelona city**, Riyahi, S., Björklund, M., Mateos-Gonzalez, F., & Senar, J. C., *Journal of Ethology*, 2017, 35(1):101-108.

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Designing the study: JCS and **SR**
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Behavioural experiments: **SR**
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Molecular and Statistical analysis: **SR** and JCS and FC
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3- **No association between the melanocortin-1 receptor (*MC1R*) and black belly stripe size variation in the great tit *Parus major*,** Riyahi S., Bjorklund M., Odeen A. & Senar J.C., *Bird Study*, 2014, 1-3.

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4- **Single origin of human commensalism in the house sparrow,** Sætre, G. P.*, Riyahi, S.*, Aliabadian, M., Hermansen, J. S., Hogner, S., Olsson, U. & Elgvin, T. O., (*equal contribution), *Journal of Evolutionary Biology*, 2012, 25(4), 788-796.

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5- Natural epigenetic variation within and among six subspecies of the house sparrow *Passer domesticus*, Riyahi S., Vilatersana R., Schrey A., Ghorbani H., Aliabadian M.& Senar J. C., Under review in the *Journal of Experimental Biology,* 2017.

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Collecting the samples: **SR**, MA and HG
Molecular lab work: **SR** and RV
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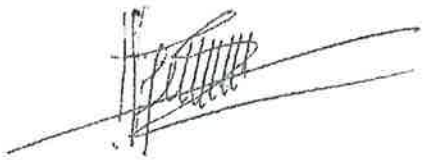

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Abstract

Human-altered environments have expanded rapidly in the past decades and made a huge impact on living organisms. Inhabiting in such a habitat can modify different traits in animals, allowing for a better adaptation to these human-altered environments. In the first part of this thesis I investigated patterns of recent (contemporary) adaptation to urban habitats, focusing on the role of behavioural, genetic and epigenetic variation in great tits. In the second part of the thesis I investigated patterns of adaptation to human-made habitats in a larger time-scale, focusing on the origin and expansion of the house sparrow, which has been for ages a human commensalism. I additionally checked the effect of methylation variation on the rapid expansion and high phenotypic variation of house sparrow in the Palearctic region.

Regarding the great tit I found that urban-dwelling birds are more explorative in novel environments and bolder in front of new objects than forest individuals. I found several epigenetic modifications and genetic polymorphisms possibly related to novelty seeking behaviour in the great tits. Our results suggested that epigenetics adjustment can be different in the urban-dwelling great tits in comparison to the forest birds. Furthermore, we investigated the possible polymorphisms in the Melanocortin-1 gene in the great tit to relate it with the size of the black belly stripe, which had previously been found to differ between urban and forest individuals. However, results showed that there is no polymorphism in this gene in relation to the size of the black belly stripe.

In relation to the house sparrow, we found that the commensalism of this species with humans has a single origin and probably initiated in the Middle East. Then, it expanded rapidly in the Palearctic region with the aid of agriculture and human civilizations expansion. In addition, we discovered that the genome-wide methylation pattern of house sparrows has a general stability within five subspecies in the Middle East but we found high level of variation at the individual level within populations which likely happened randomly rather than due to selection.

Keywords: local adaptation, Epigenetics, DNA methylation, mitochondrial genes, personality traits, morphometry, plumage coloration, melanin coloration, *SERT*, *DRD4*, *MC1R*.

General introduction

Human activity is directly impacting on a large and growing proportion of the world, threatening biodiversity (McKee et al. 2004, Turner et al. 2004, McKinney 2009). Given the high speed at which anthropogenic change takes place, it was previously thought that most species would be unable to adapt to this change. However, recent research has found that species can adapt very quickly to human induced environmental changes, so that contemporary evolution may be the rule rather than the exception (Carroll et al. 2007). As a consequence, these changes represent a potential source of selection and a new avenue to study local adaptation (Carroll et al. 2007, McKinney 2009, Donihue and Lambert 2014, Alberti et al. 2017, Hendry et al. 2017).

1. Local adaptation

Local adaptation is a process whereby individuals of a local population exhibit higher fitness in their local environment compared with individuals from a different population and environment (Kawecki and Ebert 2004) . Divergent local adaptation arising from differences between environments has been recorded in many organisms including vertebrates and plants. Local adaptation results from the interaction between multiple evolutionary forces (e.g. selection, genetic drift, mutation, migration). Whether local adaptation actually evolves, and to what extent, however, depends on the balance among different evolutionary forces. A key prerequisite for the emergence of local adaptation is the existence of a spatially heterogeneous environment generating a heterogeneous selective pressure (Nosil 2012). Local adaptation measures the match between adaptive genetic variation and environmental variation and can be estimated by measuring the fitness of populations in their own habitat and when transplanted to other habitats (Blanquart et al. 2013).

Spatial scale affects local adaptation (Hanski et al. 2010). For example, positive correlations between range size and the number of ecological or geographic races have been observed (Blair 1950) .Evidence suggests that the adaptive divergence that drives ecological speciation is more likely happens at larger scales, and might require some minimum area size. Organisms with weaker gene flow are able to differentiate at finer spatial scales than are those with stronger gene flow, leading to increased speciation rates and higher taxonomic diversity within a given area. Variation

among taxa in the strength of gene flow could be caused by several factors, including differences in dispersal ability, in the degree of habitat specificity (which controls which habitats will act as barriers to dispersal (Thorpe 1945). For example the minimum island size for speciation in birds is around 50 km (Kisel and Barraclough 2010).

In closed populations allele frequencies change predominantly through drift and selection. In large populations selection typically dominates allele frequency changes for alleles subject to natural selection. On the contrary, in small populations genetic drift is usually the dominant force causing allele frequency change, even for alleles subject to natural selection. So, selection is less effective in small than large populations. The ability of drift to overcome selection depends on the selective advantage or disadvantage of the allele (Frankham et al. 2004).

Local adaptation is critical for species persistence in the face of rapid environmental change, but its genetic basis is not well understood. In this thesis one of the main focus is to search for the background of phenotypic alteration in urban habitats. This variation can be due to plasticity processes (Tuomainen and Candolin 2011, Sol et al. 2013) or, more interestingly, to microevolutionary responses related to adaptation to urban habitats (i.e.: local adaptation) (Brown 2012). In this thesis we focus on these microevolutionary responses. One of the easiest ways to identify loci involved in local adaptation is to search for correlation between allele frequencies and important ecological variables or search for extreme allele frequency differences between geographic regions (Coop et al. 2010). This is one of the approaches we have followed. In addition we also searched for the role of epigenetic modification in local adaptation.

2. Urbanization

In recent decades, human activities have converted extensive amounts of natural habitats to urban and agricultural land (United Nations 2014). Changes in food resources, predator communities and exposure to environmental contaminants are among the most important alterations which impact animals in these areas. Also urbanization leads to habitat fragmentation. Most of the cities, act as a physical barrier to dispersal for animals. Thus, we have patches of isolated populations with limited gene flow (Marzluff et al. 2001, Candolin and Wong 2012, Elmqvist et al. 2013, Gil and

Brumm 2014). This fact can create inbreeding pressure and increase genetic divergence between populations (Frankham 2006).

Nevertheless, in spite that these altered habitats impair the development of many species, some other species flourish in urban or agricultural habitats, to the point that several of them highly adapted to these new habitats (Alberti et al. 2017, Hendry et al. 2017). Humans have therefore been claimed to be the "world's greatest evolutionary force" (Palumbi 2001). The birds which can flourish and manage to persist in urban habitat, often at higher population densities than in their native habitats, are called "urban winners" (Sorace 2002, Stracey and Robinson 2012). We have several examples of these winners: the Black bird *Turdus merula* is by no doubt one of the most familiar urban species (Luniak et al. 1990) and the House sparrow *Passer domesticus* is one of the most common birds in the cities and agriculture areas over the world (Summers-Smith 1963).

The reasons of why organisms differ in their tolerance to environmental modification is still quite unknown. Some work has suggested that the birds which can settle in the cities have some behavioural preconditions (Shochat et al. 2006). However, and additionally, it is likely that there are genetic differences underlying this adaptation (Mueller et al. 2013b), though it has not been well-studied. The behavioural response to the modified habitat is due to the change in the physiology of organisms (e.g. Partecke et al. 2006). These physiological alterations can either be due to i) plastic response of organism to environmental stimuli, ii) selection for specific alleles or iii) it can be because of epigenetic effects. Most examples of adaptation to the urban habitat relate to plastic responses as for example the learning new behaviours (Sol et al. 2013) .

However, since there are remarkable ecological differences between urban and wild habitats, most of the species which colonize towns require some phenotypic adjustment to the novel urban environment (Partecke 2013). This kind of species probably undergo some microevolutionary changes and may show some shifts in life history traits. Many studies until now have shown that populations which interact with human-altered habitats may differ in morphology, behaviour, physiology and life history traits (e.g. Quinn et al. 2001; Huey et al. 2000). Examples include modification in song structure (Slabbekoorn and Peet 2003), earlier time of the dawn chorus due

to artificial light (Miller 2006), or some personality differences such as shorter flight initiation distances in urban birds (Møller 2008). Recent work is focusing on investigating the possible genetic basis of these behavioural differences, and several recent studies provide some evidence of local adaptation and rapid evolution in the urban areas. For example Mueller et al. (2013b) found consistent genetic divergence between candidate genes of harm avoidance (*SERT* gene) in multiple urban-rural population pairs of the common blackbird. They concluded that the genetic structure of European blackbird populations is shaped not only by genetic drift associated with founder events during the urbanization process (Evans et al. 2009) but also by adaptive genetic shifts and selection on the genotypes influencing specific behavioural traits, such as harm avoidance.

In this thesis we aim to continue the same road to uncover the effect of urbanization on the phenotypic variation of two species of birds which represent two stages in local adaptation to man-made areas: the great tit *Parus major*, that probably has entered into cities in the last 100 years, and the house sparrow *Passer domesticus*, as an example of an ancient adaptation to human settlings. The work on great tits focuses on the search for the genetic background of intra-specific behavioural and plumage variation in relation to urbanization. Because colonization in the urban areas is mainly a behavioural phenomenon, we specially consider the role of behaviour in shaping adaptive processes of this species to the urban environment. In relation to house sparrows, we focus on phylogeographic patterns in the native range of the species in the Palearctic, to reveal the origin of human commensalism in this species.

3. Objective

The main aim of this thesis was to investigate the mechanisms of local adaptation during the process of urbanization.

In the first chapter of my thesis, the aim was to search for intra-specific variation in the personality traits of urban and forest great tits and to link these differences to environmental and heritable factors. We were interested in understanding how phenotypic traits evolved by the interaction between environmental, genetic and epigenetic factors. As a first study, we tested the relationship between personality and *DRD4* gene using standard tests of boldness and exploratory behaviour in the captive condition (**paper1**). We explored these personality differences comparing a forest

and two urban populations. We also analysed variations in the frequency of one SNP in the exon 3 of dopamine receptor D4 gene (*DRD4* SNP830), previously found to relate to personality (Fidler et al. 2007, Korsten et al. 2010, Mueller et al. 2013a), and we tested the relationship between this gene and personality scores in our population to examine if selection acts differently on urban and forest birds (**paper1**). After that, we performed methylation profiling of two candidate genes for personality traits, namely *DRD4* and *SERT,* to ascertain whether personality traits and behaviour within different habitats have evolved with the aid of epigenetic variation (**paper2**). In fact, we searched whether the *DRD4* and *SERT* promoters were differently methylated in urban and forest habitats and if the novelty seeking behaviour was related to the DNA methylation level in the promoter of these two genes. In addition we wanted to answer if the allelic states at nearby SNPs associated with methylation level of *SERT* and *DRD4* promoters (**paper2**).

In chapter three, we searched for the link between Melanocortin-1 (*MC1R*) polymorphisms and black belly melanin coloration of the great tits. The Melanocortin-1 encodes the receptor protein for melanocyte-stimulating hormone and also controls melanogenesis. We chose great tit from Barcelona city and forest nearby since previous data showed that the size of the black belly stripe was smaller in the urban-dwelling great tits in Barcelona compared to the forest population (Senar et al. 2014) and we wanted to find whether this difference had a genetic basis (**paper 3**).

In chapter four, I investigated the origin of human commensalism in the House sparrow in the Palearctic region (**paper4**). We analysed genetic variation of mitochondrial DNA control region and three nuclear loci to estimate the level of genetic differentiation between subspecies and describe the phylogeographic pattern of this species.

Finally, in the last chapter of my thesis, I investigated the genome-wide methylation pattern of six subspecies of house sparrow in the Palearctic region using methylation sensitive AFLP method to evaluate the importance of epigenetic variation in the rapid expansion of house sparrow (**paper 5**).

4. Model species: the great tit and the house sparrow

The great tit is a small passerine bird from the Paridae family. It's a common species throughout Europe, the Middle East, Central and Northern Asia, and parts of North Africa in any sort of woodland, but can easily live in any habitat (Perrins 1979). It's generally resident and do not migrate and itis a well-adapted species to the urbanized habitats. The great tit has an olive upperparts and yellow underparts and black belly stripe, with a striking glossy black head with white cheeks. The male individuals have a clearly larger black belly stripe compare to the females.

Previous work on the great tit, that is commonly used as a model in urban ecological research, has revealed differences in morphology, physiology, life-history, and various behaviors, including song, exploratory tendency, neophobia, aggression, risk-taking, and problem solving performance (Slabbekoorn and Peet 2003, Björklund et al. 2010, Bueno–Enciso et al. 2015, Riyahi et al. 2015, Demeyrier et al. 2016, Tryjanowski et al. 2016, Preiszner et al. 2017, Riyahi et al. 2017, Sprau et al. 2017, Watson et al. 2017) .

The house sparrow is a small passerine bird from the *Passer* genus and Passeridae family (old world sparrows). It has a natural distribution in Palearctic and Oriental with long history of introduction to America, Africa and Australia (Summers-Smith 1963). The house sparrow is now one of the world's most broadly distributed vertebrate species and has been introduced to a great part of its current range. Males and females are easily distinguished; males have a black bib, a grey crown with chestnut sides, and white cheeks. Females and juveniles have a duskier appearance, and lack the black bib seen in males. Ubiquitous house sparrow is highly urbanized and is among the most prominent birds in the cities and agricultural areas.

The house sparrow is also a model species in urban ecology research and on the study of adaptation to human-altered habitats (Liker et al. 2008, Bókony et al. 2010, Holand et al. 2011, Seress et al. 2011, Bókony et al. 2012, Schrey et al. 2014, Papp et al. 2015).

Chapter 1. Evolutionary Genomics of personality traits in the great tit

1.1 Introduction

1.1.1 Natural selection and personality traits in the great tit

In the ethological literature the term of "personality" or "behavioural syndrome" or "temperament" is used to refer to consistent behavioural differences among individuals in relation to different types of behaviour which appear correlated (Dall et al. 2004, Sih et al. 2004a, Sih et al. 2004b, Dingemanse and Réale 2005, Bell and Sih 2007, Réale et al. 2007). In many animal species, individuals differ in activity, aggressiveness, risk-taking and exploratory behaviour, and these behaviours are often positively correlated with each other. One of the most stable personality characters that have been widely studied is the bold-shy continuum. Boldness usually refers to the tendency to take risks in both familiar and unfamiliar situations while shyness refers to the reluctance to take such risks (Zuckerman and Przewuzman 1979, Zuckerman 1994). In the great tit researchers discovered that individuals could also be assigned either to a "fast" personality or to a "slow" one. The "fast" birds quickly approached novel objects and explored new environments in few minutes and they were also more aggressive compared to the "slow" birds (Verbeek et al. 1994, Verbeek et al. 1996) while "slow" great tits approached novel objects slowly and explored the new environments more carefully. Fast individuals were also bold in front of new situations. These individuals have also been termed as "proactive" within the coping style literature, in contrast to shy and slow individuals which are termed reactive (Coppens et al. 2010).

Interestingly, the personality of the different individuals has a profound effect on the fitness of the individuals (Dingemanse et al. 2004, Dingemanse and Réale 2005a, Smith and Blumstein 2007, Cote et al. 2008) and even more importantly, personality has a heritable basis in the great tit (Dingemanse et al. 2002, Drent et al. 2003, van Oers et al. 2004a, van Oers et al. 2004b). Therefore, these traits are able to pass to the next generation via genetic or epigenetic markers and that's how selection can favour one trait to the other ones. The behavioural traits are the outcomes of adjusting animals to specific situations with the aid of long-term selection pressures. Now most of researchers believe that intra-specific variation in behavioural phenotypes is fundamentally

adaptive and different personalities have different selective advantages under different circumstances (Dall et al. 2004, Biro and Stamps 2008, McNamara et al. 2009, Quinn et al. 2009).

1.1.2 Measuring the avian personality in the captivity

It was in 1994, when for the first time, Verbeek and her colleagues described two experiments for measuring exploration behaviour of great tits in captivity (Verbeek et al. 1994). For measuring the exploration in the novel environment, they made a small room with 5 artificial wooden trees and released the great tits each by each in the room and measured the time it took a bird to visit all of the five trees. The second experiment was reaction to a novel object in a familiar environment. They put a pen light battery on a perch and counted the time of approach to that perch (Verbeek et al. 1994). After that, these two experiments were used as a routine method for estimating novelty seeking behaviour of the birds in the captivity until 2002, when Dingemanse and his colleagues modified the method of measuring exploratory behaviour in the novel environment because in their study, many individuals (48%) did not reach the fifth tree within the 10-min observation period. This time, they counted the total number of flights and hops within the first 2 min after entering to the room as an index of exploratory behaviour ('exploratory score'). Here we used a similar method for measuring the exploratory behaviour (Dingemanse et al. 2002).

1.1.3 Candidate genes for personality traits

Personality is shaped by both genetic and environmental factors. Phenotypic selection will have evolutionary consequences only when the phenotypic variation is heritable (Fisher 1930, Endler 1986). For this reason, researchers working on personality devoted many efforts to measure individual consistency of exploratory behaviour (repeatability) and estimated the heritability with parent–offspring regressions and sibling analyses. Finally, consistency and heritability in the exploratory behaviour was found (Dingemanse et al. 2002, Carere et al. 2005).

After these first analyses, molecular genetics researchers focused in identifying specific genes for quantitative traits. During the last two decades, several studies have been testing the association

between personality traits in human with neurotransmitter-related genes (e.g. Benjamin et al. 1996; Ebstein 2006). But only very recently, researchers extended their studies to other kind of vertebrates including primates and bird (e.g. Momozawa et al. 2005; Bailey et al. 2007; Inoue-Murayama 2009). The first candidate genes identified were components of the monoamine neurotransmitter pathways, such as serotonin and dopamine (e. g. Kluger et al. 2002). The serotonergic system is involved in mood, anxiety, and aggression. Temperamental predisposition and behaviour are likely to be influenced by genetic variations of serotonergic genes – i.e., serotonin-metabolizing enzymes, tryptophan hydroxylase and monoamine oxidase (*MAO*), catechol-Omethyltransferase (*COMT*), 14 kinds of serotonin receptor (5-hydroxytryptamine, or *5HT*) and serotonin transporter (*SERT*).The dopaminergic system is involved in the brain's reward system and addictive behaviour (D'Souza and Craig 2008). Human or animal behaviour is also influenced by dopaminergic genes such as tyrosine hydroxylase (*TH*), dopamine receptors (*DRD*), and dopamine transporter (*DAT*). Noradrenergic and g-aminobutyric acid (*GABA*) ergic genes are also involved in behaviour (D'Souza and Craig 2008). In our study we focused on two candidate genes of personality: Dopamine receptor D4 (*DRD4*) and *SERT* (also known as *SLC6A4*).

Single nucleotide polymorphisms (SNPs) within the third exon of the *DRD4* gene are related to variation in novelty seeking behavior in humans and other mammals (e. g. Schinka et al. 2002; Bailey et al. 2007). In birds, the effect of Dopamine receptor D4 (*DRD4*) and serotonin transporter (*SERT*) genes on the personality trait have been described (Fidler et al. 2007, Korsten et al. 2010, Mueller et al. 2013a). *DRD4* gene is a candidate gene for novelty seeking and exploratory behaviour (Fidler et al. 2007, Korsten et al. 2010) and *SERT* gene is a candidate gene for harm avoidance behaviour and adaptation to novel environments such as urban areas (Mueller et al. 2013b).For this reason, in this study we focused on *DRD4* and *SERT* genes.

1.1.4 *DRD4* SNP830

In the last decade, several studies have reported that polymorphisms in the exon encoding the *DRD4* receptor third intracellular loop are associated with personality variation among humans, monkeys and horses (e.g. Kluger et al. 2002; Ito et al. 2004; Momozawa et al. 2005). Later on,

Fidler and his colleagues (2007) obtained the great tit *DRD4* orthologue cDNA and described the SNP830 in the exon 3 of *DRD4* gene as a candidate gene for novelty-seeking behaviour.

Fidler and his colleagues (2007) hand-reared the great tits in captivity and used selective breeding based on the early exploratory behaviour (EBB) score over four generations. Based on the two standard personality experiments in captivity: (i).Exploration score in a novel environment and (ii).Latency to approach a novel object, they divided the birds into fast and slow explorers. They found an association between EBB score and *DRD4*SNP830 genotypes. After that, Korsten and his colleagues (2010) repeated this study with four wild-caught populations of great tits, but they found a link between *DRD4* SNP830 and exploration score only in one of the populations and in this population allelic stats at the SNP830 explained only 4.5–6.0% of the total variation in behaviour. As a consequence, currently it is said that many other loci, with small effects, can contribute to differences in boldness among individuals (Mueller et al. 2013a).

1.1.5 Epigenetics

Epigenetics refers to collective heritable changes in phenotype due to processes that arise independent of primary DNA sequence. This reversible and heritable phenomena ranges from DNA methylation to histone modifications to prions. Epigenetic processes occur in diverse organisms and control a vast array of biological functions, such as tissue/organ regeneration, X-chromosome inactivation, stem cell differentiation, genomic imprinting, and aging (Tollefsbol 2010).

In most eukaryotes DNA methylation, the most studied of the different epigenetic processes, consists in the transfer of a methyl moiety from S-adenosylmethionine (SAM) to the 5-position of cytosines in certain CpG dinucleotides. This reaction is catalysed by DNA methyltransferase (DNMT) enzymes. CpG dinucleotides are gathered in clusters called CpG islands which are unequally distributed across genome. There are approximately 30,000 CpG island in the human genome and 50-60% of these are found within the promoter regions of genes. CpG islands are primarily unmethylated in normal tissues while the aberrant methylation of CpG islands is clearly related with disease such as cancer (Lai et al. 2005). The most significant aspect of DNA

methylation, which can also influence such processes as X chromosome inactivation and cellular differentiation, is its effects on gene expression. In general, the more methylated a gene regulatory region, the more likely it is that the gene activity will become down-regulated and *vice versa*.

One of the ways to detect gene-environment interaction is investigating epigenetic modifications. Epigenetic marks are controlling the activity of transcription factors with the environmental stimuli. In fact, epigenetic signals can determine the capacity for environmental regulation of the genome. In addition, epigenetic marks are directly altered in early life by environmental events and thus influence the development of individual differences in specific neural functions that underlie cognition and emotion (Zhang and Meaney 2010). Thus, any change in environmental condition can potentially modify the phenotype through epigenetic modification.

As we know, natural selection can be triggered by epigenetic as well as genetic variation. As a result, DNA methylation is a potential source of inter-individual phenotypic variation (Bossdorf et al. 2008). Recent studies comparing plants in different environments came to the conclusion that the genome contains single site methylation polymorphisms in addition to SNPs (Schmitz et al. 2013). These polymorphisms at the epigenetic level allow individuals in the populations to react differently to fluctuating environments (Duncan et al. 2014). Considering that, epigenetic modifications can occur much faster than genetic divergence (Rando and Verstrepen 2007), methylation might have a role in shaping personality traits in newly formed environments. We believe that this can be a fructiferous future avenue of new research and hypothesized that part of the variation in personality or morphology between urbanized and non-urbanizes populations in our two study species could have an epigenetic basis.

1.1.6 Aims

Our first aim was to test for a relationship between SNP830 *DRD4* gene and personality scores (i.e. latency to approach a novel object and exploration scores in a novel environment), comparing an urban great tit population (Barcelona city) and a forest population (Collserola National Park) (**paper 1**).

In the next step (**paper2**) we searched for DNA methylation variation inside *DRD4* and *SERT* genes (two candidate genes of personality traits) in urban and forest great tits. Here our aim was to found the possible link between novelty seeking behaviour and level of DNA methylation in the *SERT* and *DRD4* promoters. In addition, we were interested to find whether inter-individual methylation variation can be due to habitat differences. Furthermore, we wanted to check if allelic states at nearby SNPs can be associated with methylation level of *DRD4* and *SERT*. Finally, we searched if the methylation level at nearby CpG site of SNP830 *DRD4* could affect exploratory behaviour. We selected this SNP because it was a plausible candidate gene of novelty seeking behaviour (Fidler et al. 2007, Korsten et al. 2010).

1.2 Materials and methods

A total of 130 wild great tits were captured between October and March in 2012 and 2013 and were transferred into captivity for the behavioural experiments. Forest birds (N= 74) were captured at the Can Cata study area located in Collserola National Park, 3 km from Barcelona city and urban birds (N=56) were captured at two parks in Barcelona city: Ciutadella Park (21 birds) and Sarria-Setmenat park, in the suburban area (35 birds). We determined the age and sex of the birds at capture according to Svensson (1992). For measuring the novelty seeking and exploratory behaviours we used the standard methods described in the introduction of this chapter. We made two behavioural experiments in captivity: i). latency to approach a novel object and ii).exploration scores in a novel environment. Blood samples were collected from each individual great tits for DNA analyses. DNA was extracted using Ecogen Master Pure DNA Purification kit (MCD85201). Birds were released into the wild after two days.

1.2.1 Behavioural experiments

Two standard tests were performed. Novel object test and novel environment test. These two tests are currently the standard method to quantify novelty seeking behaviour under standardized captive condition (Verbeek et al. 1994, Dingemanse et al. 2002, Drent et al. 2003). Previous work has found that these two measurements are repeatable and consistent within individuals (Dingemanse et al. 2002). Each bird was individually tested in the whole sequence of trials and

used only once. On the morning of the second day after capture (8–12 a.m.), we performed a standard novel object test inside the individual enclosure (1 × 1 × 1.5 m) using a pen light battery as a novel object. The pen was put on a feeder containing 6-7 waxworms (Drent et al 2003, Verbeek et al 1994). We measured the latency of the focal bird to approach the feeder (in seconds) within a period of 10 minutes. Birds were then allowed to continue with their activities for one hour in the individual enclosure.

We then performed a standard novel environment test (Verbeek et al 1994). Birds were introduced individually into an individual enclosure (50 × 40 × 40 cm) within the experimental room. After 30 minutes, the individual enclosures were opened with a remote control string to allow the bird access to the observation room. The size of the room was 3 × 2 × 2 m and contained 5 artificial trees, as described in Verbeek et al. (1994). We observed the birds through a one-way glass and recorded their movements on video camera. The number of flights and hops within the first 2 min after entering the room was used as the exploration score (Dingemanse et al 2002). The exploration score was standardized by date using the residuals from the regression between exploration score (dependent variable) and number of days from 1st September (independent variable). Data was standardized within each year. This standardization allowed to account for any possible seasonal temporal trend in exploration score (Dingemanse et al 2002, Quinn et al 2009).

1.2.2 Gene sequencing

The genotyping for SNP830 of *DRD4* in all the samples was performed following the protocol described by Fidler et al. (2007). Additional polymorphisms within the *DRD4* and *SERT* loci were genotyped by standard PCR amplification and direct sequencing of the resulting amplicons. The sequence electropherograms were interrogated using Sequencherv4.6 (Gene Codes Corporation, MI, AnnArbor, USA) to distinguish heterozygous and homozygous samples. The sequence of *SERT* gene and SNPs has been deposited in GenBank: accession number KP869099.

1.2.3 Epigenetic methods

We applied two well-known methods for estimating the DNA methylation status across the genes including Bisulfite conversion and Pyrosequencing.

Bisulfite conversion is a method that deaminates unmethylated cytosine to produce uracil in DNA and is considered the "gold standard" for downstream applications to assess DNA methylation status. Methylated cytosines are protected from the conversion to uracil, allowing the use of direct sequencing to determine the locations of unmethylated cytosines and 5-methylcytosines at single-nucleotide resolution (Zymo Research 2017)

Analysis of DNA methylation patterns by pyrosequencing combines a simple reaction protocol with reproducible and accurate measures of the degree of methylation at several CpGs in close proximity with high quantitative resolution. After bisulfite treatment and PCR, the degree of each methylation at each CpG position in a sequence is determined from the ratio of T and C (Tost and Gut 2007).

1.2.4 Statistical methods

The promoter sequence of the *SERT* gene was not available for the great tit. Thus, to find the great tit *SERT* orthologue, we performed sequence homology analysis with BLAST2 to compare the genomic sequence of blackbirds (*Turdus merula*) with additional bird species to identify the conserved region to design the primer for *SERT* promoter. We performed sequencing for this part of the *SERT* gene. This 850 bp amplicon consist of 770bp within the proximal promoter and part of *SERT* exon1. We utilized the CpG island finder tool (http://dbcat.cgm.ntu.edu.tw) to screen for CpG islands. We check the relation between exploration score and DNA methylation of *SERT* and *DRD4* using General Linear Model (GLM) in STATISTICA 8 software (StatSoft 2013). Test of the link between SNPs and exploration behaviour within the *SERT* and *DRD4* promoter and also SNP830 *DRD4* was done similarly.

Regarding the latency to approach the novel object, we performed COX proportional hazards regression model because our data included censored observations (birds that did not approach the novel object).

1.3 Results and Discussion

1.3.1 DRD4 SNP830 polymorphisms

Urban and forest populations did not significantly deviate from the Hardy-Weinberg equilibrium (Urban: χ^2 = 0.01, p=0.90, N=173; Forest χ^2 = 1.35, p=0.24, N=218). However, we found differences in genotype frequencies of *DRD4* SNP830, with a lower proportion of the T-allele in urban birds than in forest habitat birds (forest: 0.53, urban: 0.42; χ^2 = 9.42, df =1, p=0.002).

The exploration score was not related to *DRD4* SNP830 polymorphisms, standardizing for habitat effects. This result was consistent both for an additive and a T-dominant model (paper 1). However, the additive model showed an interaction between genotype and habitat (paper 1). The analysis of the variation in exploration score by habitat showed that while exploration was not related to genotype in the forest birds, the relationship was close to significance in the urban habitat, so that heterozygotes showed a higher exploration score than homozygotes (figure1-1).

Latency to approach a novel object was not related to *DRD4* SNP830 polymorphisms, standardizing for habitat effects (Cox Additive model: χ^2= 1.31, p=0.52; Cox T-dominant model: z = −0.61, p=0.54, N=126).

So, in our populations there was no association between *DRD4* SNP830 polymorphism and exploratory behaviour. In general, the SNP830 genotype explained 4.5-6.0% of the total variation in exploratory behaviour. So, the effect of this SNP in the exploration behaviour is very low and also this effect changes between environments.

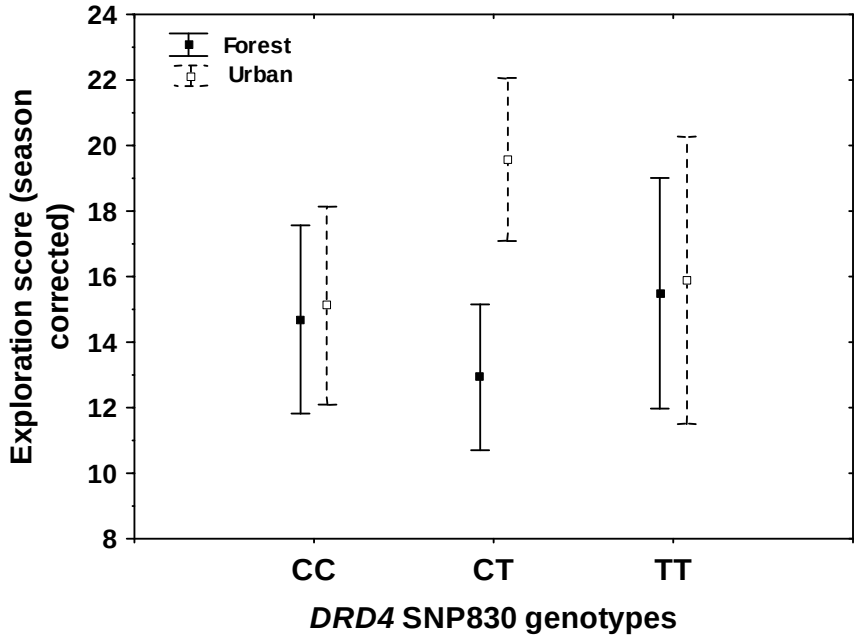

Figure 1-1. Exploration scores (corrected by seasonal trend) of wild great tits in forest and urban habitats in relation to the *DRD4* SNP830 genotype. Black circles and black lines represent forest great tits and white squares and dashed lines represent urban great tits

Our results therefore show that the relationship between *DRD4* SNP830 polymorphism and personality is not sufficiently clear to allow for generalizations, which is in line with more recent studies (Fidler et al. 2007, Korsten et al. 2010, Tschirren and Bensch 2010, Mueller et al. 2013a). Future research considering additional candidate genetic and epigenetic variants within serotonergic and other dopaminergic genes may show a closer association with personality (Mueller et al. 2013a, Riyahi et al. 2015, Verhulst et al. 2016).

1.3.2 Epigenetic effects

Regarding the *DRD4*, we found only 1 % difference in the methylation level between urban and forest populations (figure 1-2). Differential *DRD4* methylation level couldn't explain inter-individual variation of personality.

However regarding the *SERT* gene, our results showed that within the *SERT* promoter there are only two CpG sites. The first CpG site was 83% (SE= 0.26%) methylated while methylation level

was ~2% higher in urban great tits than forest ones. The second CpG site is an allele-specific methylation site (ASM).That means a genomic variant (SNP290) were located at the site and can abolish CpG site (G nucleotide is replaced by an A variant) hence any change in this SNP polymorphisms would change the methylation level at this site. Great tits with homozygous A allele were ~8.1% (SE=0.55%) methylated, GA heterozygous individuals were ~31.3% methylated (SE= 1.03%) and homozygous G birds were ~49.0% methylated (SE=0.85%) (figure 1-3). Our results showed a marginally significant association between exploratory behaviour in the urban-dwelling great tits and methylation level at the CpG site. In addition, we found that urban-dwelling birds in average were ~3.9% hypermethylated than forest birds at this site.
Finally, our results showed that urban birds were more methylated at both *DRD4* and *SERT* genes than forest ones. These variations in a small scale highlight the role of urbanization as one of main environmental stimuli in the new era.

Furthermore, sequencing the *DRD4* and *SERT* promoter allowed us to identify genetic polymorphisms (SNPs) within the promoter of these two genes and enabled us to find a relationship between methylation level and SNPs and also to check the association between SNPs and personality traits. We identified 7 SNPs in the *DRD4* promoter and 11 SNPs in the *SERT* promoter. From these SNPs, only *SERT* SNP234 showed association with latency to approach the novel object: TT great tits approached faster to a novel object than TA ones. TT birds were more frequent within the urban habitat than TA birds. The shift in the allele frequency at this SNP could be an evidence of adaptation of this species to the urban area.

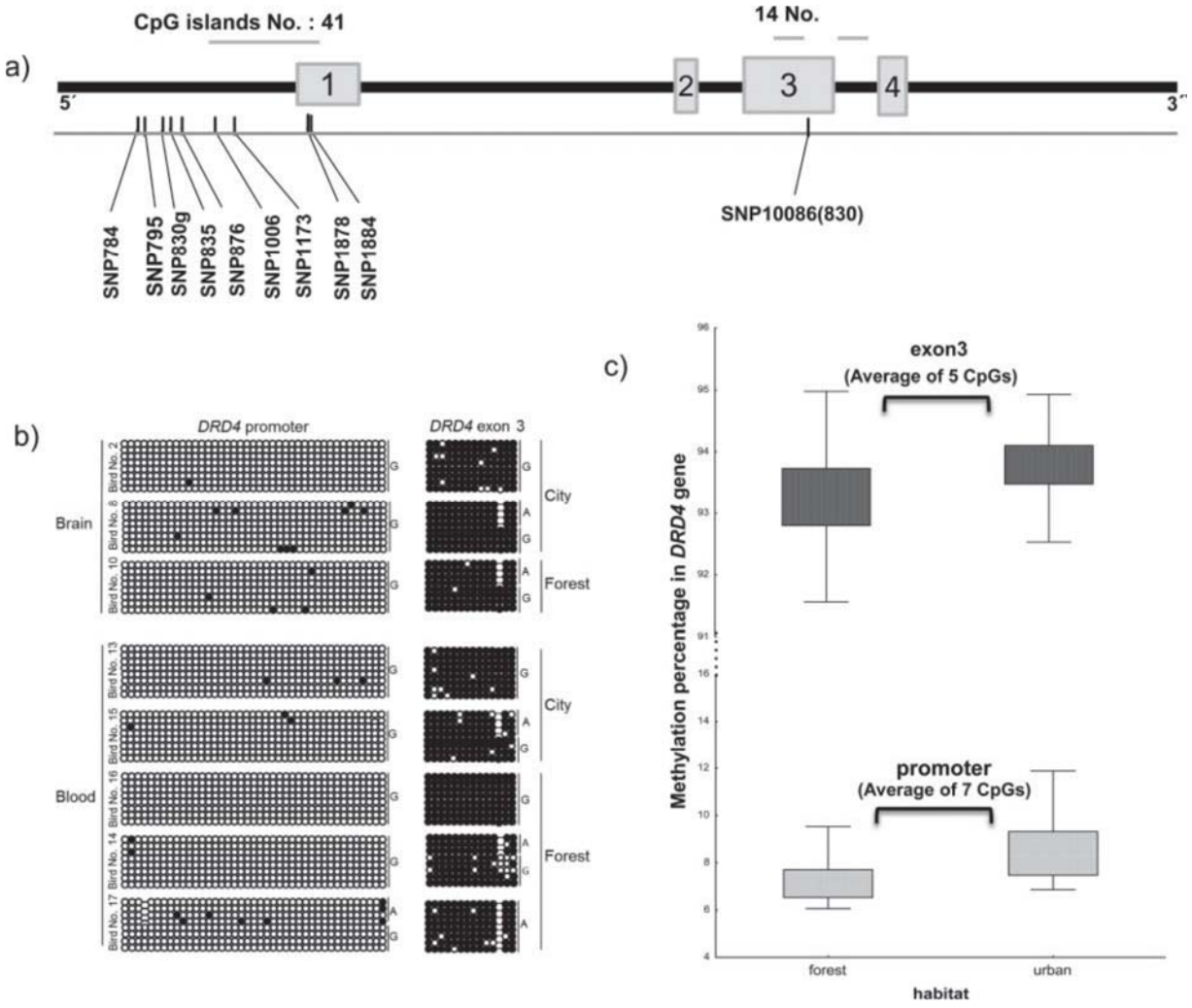

Figure 1-2. Structure and methylation profile of the *DRD4* gene. (a) Schematic representation of the *DRD4* gene. Horizontal bars show the number and location of CpG islands and grey boxes represent exons. The location of SNPs is shown by black bars. (b) Methylation status at *DRD4* locus in brain and blood-derived DNA samples. The left panel shows the methylation profile of the promoter and the right panel shows the results for the CpG island within exon 3. Each circle represents a single CpG dinucleotide on a DNA strand. (●): methylated cytosine; (○): unmethylated cytosine. (c) Methylation percentage of *DRD4* promoter and exon 3 regions comparing urban and forest populations. Differences are significant (see text).

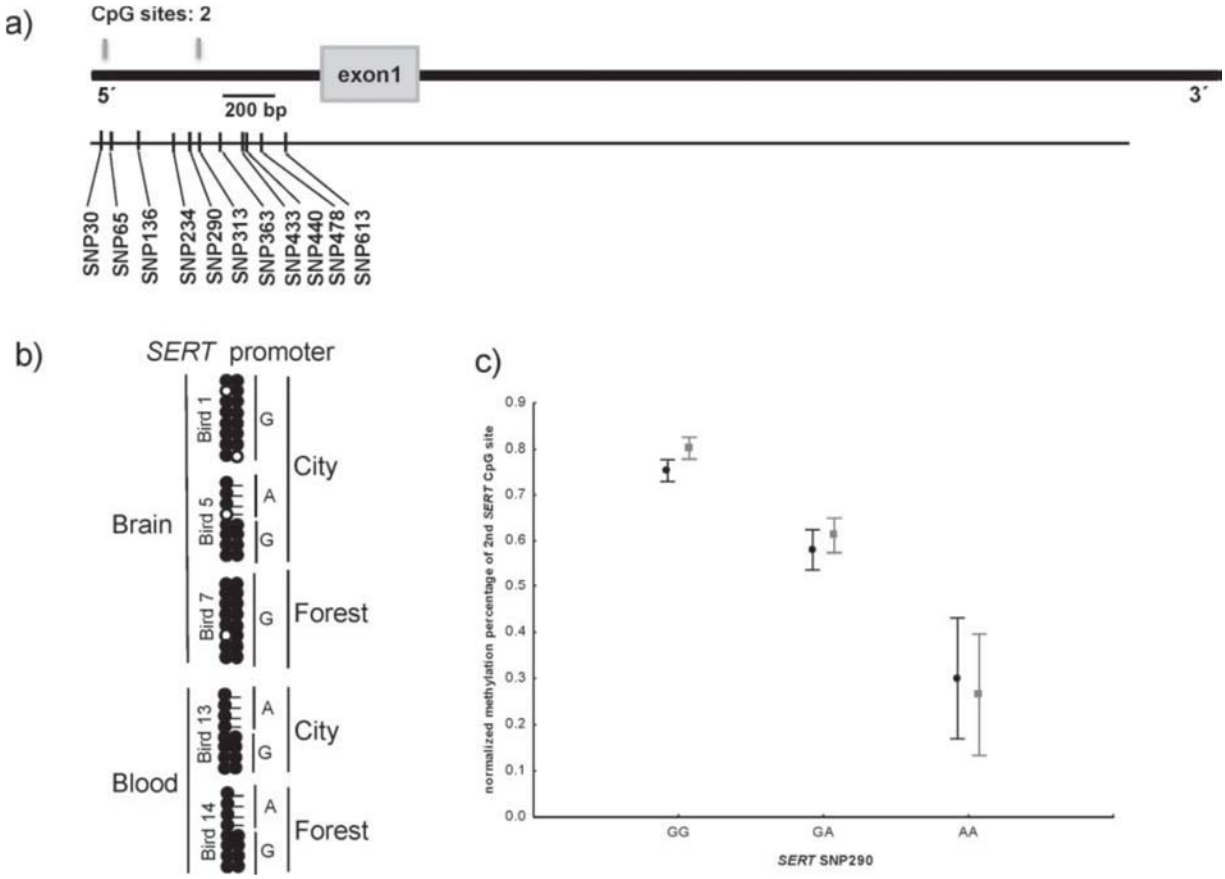

Figure 1-3. Structure and methylation profile of the *SERT* promoter. (a) The Schematic representation of *SERT* gene. Grey boxes showing the location of CpG sites and the grey box represents the exon. The location of SNPs is shown by black bars. (b) The methylation status at 2 CpG sites in the *SERT* promoter in brain and blood-derived samples. (c) Variation in methylation percentage of second CpG site in the *SERT* promoter, according to habitat and SNP290 genotype. The black data points represent forest birds and the grey urban birds. Methylation percentage was both affected by genotype and habitat, but the lack of significant interaction suggests that the difference between habitats was similar for the different genotypes (see text for tests).

Chapter 2. An attempt to identify the genetic basis of the size variation in the black belly melanin stripe of the great tit

2.1 Plumage coloration

Plumage coloration of the feathers is resulting of two factors: 1) Chemical coloration, 2) structural coloration and 3) combination of both types. Chemical coloration is caused by pigments or biochromes. Each pigment absorbs specific wave lengths within the visible spectrum and reflects the remaining light waves to the eye of the observer as colour. Two main chemical colours are carotenoid and melanin pigments. Red, orange and yellow colours in most cases produce by carotenoids and black, grey and brown are mostly produce by melanin pigments. Melanins are relatively insoluble pigments appearing as granules. Melanins are synthesized from amino acids in special pigment cells which called melanocytes. The melanistic colours depend on the amount of pigment deposition and the size of the pigment granules. For example black coloration is due to large amount of pigment which absorbs all light waves (Pettingill 2013). Two different types of melanin coloration are eumelanin and pheomelanin. Brown, grey and black colorations are due to the deposition of eumelanin pigments, while reddish-brown colorations are produced by pheomelanin. Differences in melanin-based coloration are caused by switches of eumelanin to pheomelanin production or by changes in feather keratin structure, melanoblast migration and differentiation, as well as melanosome structure (Roulin and Ducrest 2013).

Plumage coloration is relevant to our thesis because previous work has found that plumage coloration is often under natural selection. The great tit, our model species, has a black belly melanin stripe. This stripe varies in size between sexes and also between individuals of the same sex (Gosler 1993). Males with larger stripes display higher survival (Senar et al. 2014) and higher breeding success (Norris 1990a, b) . The size of this black stripe is in part genetically determined (Quesada and Senar 2009), which means that this character can potentially be under selection. In fact, recently it has been found that the pattern and direction of this selection can vary in relation to urbanization, so that survival prospects in forest great tits increased the larger their breast stripe size (i.e., directional positive selection), but the reverse was found for urban birds, with individuals displaying smaller stripe sizes showing higher survival (i.e., directional negative selection) (Senar

et al. 2014). Identifying the genetic background on the variation on the size of the great tit breast stripe and relating it to allelic frequencies in urban and forest habitats was therefore a main aim of this work.

2.2 Candidate gene for black belly melanin stripe in the great tit

Our candidate gene for variation in the size of the great tit stripe size was the melanocortin receptor 1 (*MC1R*) gene. Mutations in this gene have frequently been found to cause a shift in the degree of melanism in vertebrates including birds and is a great example of direct relation between genotype and phenotype (Ducrest et al. 2008, Hubbard et al. 2010).There are a lot of studies available on this topic which relate inter and intra-specific variation of melanin-base coloration to the polymorphisms inside *MC1R* gene (e.g. Theron et al. 2001; Pointer and Mundy 2008; Baião and Parker 2012). Also several other genes (for example *TYR*, *TYRP1*, *DCT*) have been found which can modify eumelanin and pheomelanin coloration (Chang et al. 2006, Minvielle et al. 2010, Lehtonen et al. 2012).

In order to find a relationship between great tit *MC1R* genetic polymorphisms and the size of the black belly stripe, we compared the urban population in Barcelona city with another one in a forest nearby (**paper 3**).

2.3 Material and Methods

We sequenced the *MC1R* gene for 46 individuals with a different size in the black belly stripe from Barcelona city and Collserola forest area.

2.4 Results and Discussion

Our results showed that for *MC1R* gene there was no genetic polymorphisms neither intra nor inter-populations. All of the great tit individuals displayed the same sequence for this gene. This means that we were unable to ascertain the genetic basis of variation in the size of the black breast stripe between urban and forest populations.

Chapter 3. The geographic origin of commensalism in the house sparrow

3.1 Evolutionary history of the house sparrow

The house sparrow is highly urbanized and one of the most successful birds in urban and agricultural habitats. The house sparrow has a natural distribution in the Palearctic and Oriental with a long history of introductions into America, Africa and Australia. Its large natural distribution was probably facilitated by commensalism with human agriculture that expanded from the Middle East probably in the Tigris-Euphrates valley of Mesopotamia about 10,000 years ago (Johnston and Klitz 1977). This valley probably is one of the first places where man evolved from a nomadic hunter and gatherer to a sedentary agriculturalist (Summers-Smith 1988). Vaurie (1949, 1956) suggested that house sparrow can be divided into two main lineages based on some morphological differences and due to periodic glacial advances and recessions long before the advent of agriculture: *domesticus* group and *indicus* group. Another possibility is that the *domesticus* and *indicus* groups may have begun the commensalism with humans independently in two regions (Vaurie and Koelz 1949). Vaurie (1956) defined 11 subspecies in the house sparrow, with five subspecies belonging to *domesticus* group and the six others belonging to the *indicus* group. All of these subspecies are sedentary, except *P. d. bactrianus* and *P. d. parkini* which are migratory. *P. d. bactrianus* subspecies breeds from southern Kazakhstan (from the northern end of the Caspian Sea east to Karaganda) southward through Uzbekistan, Kyrgyzstan, Tajikistan, and Turkmenistan into central Afghanistan (Summers-Smith 1988, 1995). It spends the winter on the plains of northern Pakistan and north western India. This species shows different habitat preferences compare to the other subspecies of house sparrow and breeds mostly in natural and semi-natural grassland habitat and it's not closely associated with humans (Gavrilov and Korelov 1968, Yakobi 1979, Summers-Smith 1988).

The aim of this study was to answer whether the origin of human commensalism in the house sparrow in the Palearctic region arose a single time or multiple times independently (**paper 4**).

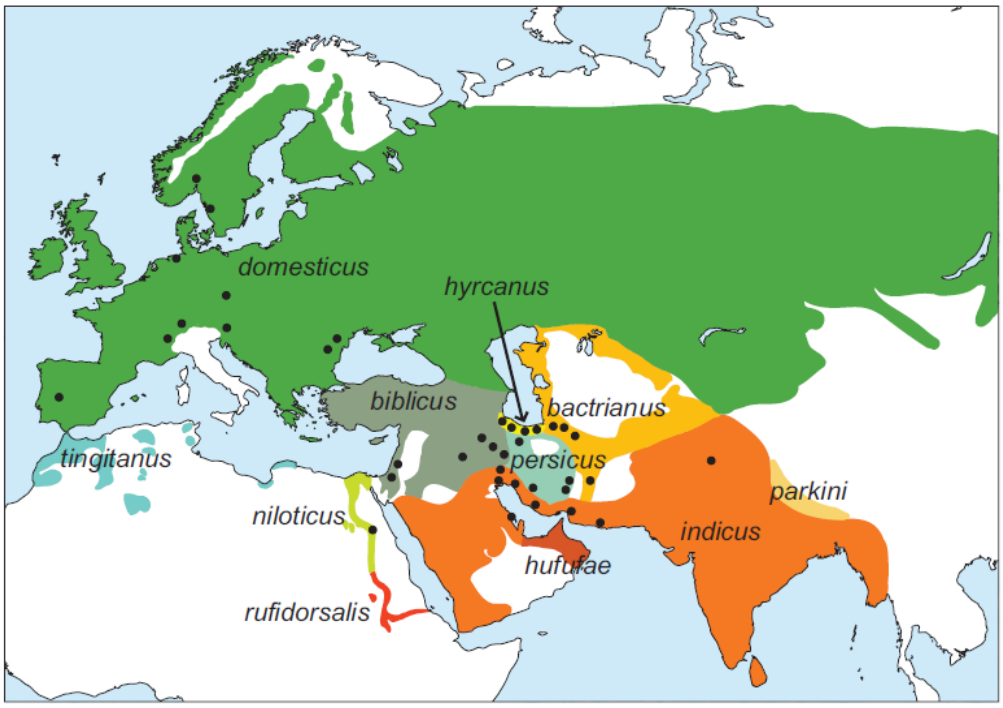

Figure3-1. Sampling locations (black dots) and geographical distribution of 11 subspecies of the house sparrow *Passer domesticus*. Subspecies in the domesticus group are in green/blue tones and subspecies in the indicus group are in red/orange/yellow tones. After Vaurie (1949, 1956), Summers-Smith (1988) and Cramp & Perrins (1993). Note that delimitation to subspecies and hence distribution varies somewhat between different authors. Moreover, zones of intergradation between subspecies are not indicated, and therefore, the map should be considered approximate.

3.2 Materials and Methods

We collected 181 samples of House sparrows from 37 locations in 17 countries, including seven of the 11 subspecies: *domesticus*, *biblicus*, *persicus*, *niloticus*, *indicus*, *bactrianus* and *hyrcanus* (Figure 3-1). After DNA extraction from blood or feather samples, we amplified a 667 bp fragment of the control region (mitochondrial DNA) using H1248 and L437 primers (Tarr 1995) and three nuclear loci including: BRM (Z-linked gene), CKB (Autosomal) and LAMA (Autosomal). The sequences were aligned using Sequencher ver4.8 (Gene Codes Corp., Ann Arbor, MI, USA). Nucleotide variation and population differentiation and Tajima's D test were calculated using DNAsp 5.0 (Librado and Rozas 2009). Genetic structure was analysed using *F*-statistics and

analyses of molecular variance (AMOVA) using Arlequin 3.1 (Excoffier et al. 2006). In addition, haplotype network for the control region was constructed using the minimum-spanning network method (MINSPNET in Arlequin 3.1). Finally, we calculated the mismatch-distribution using Arlequin 3.1 (Excoffier et al. 2006). For more details please check **paper 4**.

3.3 Results and Discussion

The descriptive statistics on genetic variation among different subspecies of house sparrow is displayed in table 3-1. Genetic variation was higher in the *indicus* group than in the *domesticus*group. Tajima's D was significantly negative in five subspecies (*P. d. domesticus, biblicus, indicus, bactrianus and hyrcanus*). The negative Tajima's D means that the population size may be increasing due to recent population expansion or we may have evidence for purifying selection at this locus. The minimum-spanning network showed a star-shaped topology with one very common haplotype surrounded by many rare and closely derived haplotypes (figure 3-2). The negative Tajima's D and the star shaped minimum-spanning network and also our result of mistmatch-distribution model, all confirmed the occurrence of recent population expansion in this species. So, we found no evidence for any split between eastern and western subspecies (*domesticus* and *indicus* group).

In this study, we found out that the human commensalism of the house sparrow in the Palearctic region has a single origin. So, commensalism with humans have evolved from a single origin in the Middle East and expanded rapidly with the expansion of agriculture and human civilizations. So, morphological differences between the subspecies represent very recent differentiation after the advent of agriculture.

Table 3-1. Descriptive statistics on genetic variation among house sparrow (*Passer domesticus*) subspecies.

Locus	Taxon	Length (bp)	*N*†	Hd‡	π††	θ_W‡‡	S§	Π¶	Tajima's *D*∫
CR (mtDNA)	*P. d. domesticus*	667	60	0.629	0.00133	0.00456	14	0.887	−2.075*
	P. d. biblicus	667	25	0.540	0.00094	0.00240	6	0.627	−1.811*
	P. d. persicus	667	13	0.500	0.00081	0.00097	2	0.538	−0.462
	P. d. niloticus	667	5	0.900	0.00211	0.00217	3	1.400	−0.175
	P. d. hyrcanus	667	11	0.618	0.00219	0.00413	8	1.455	−1.934*
	P. d. bactrianus	667	15	0.657	0.00140	0.00325	7	0.933	−2.040*
	P. d. indicus	667	28	0.638	0.00180	0.00587	15	1.198	−2.354**
	Domesticus group	667	103	0.619	0.00125	0.00676	23	0.829	−2.388**
	Indicus group	667	54	0.628	0.00177	0.00911	27	1.177	−2.611***
	Total	667	157	0.622	0.00143	0.01188	43	0.951	−2.628***
BRM (Z-linked)	*P. d. domesticus*	269	51	0.700	0.00671	0.00930	11	1.790	−0.778
	P. d. biblicus	269	31	0.695	0.00808	0.00572	6	2.151	1.223
	P. d. persicus	269	16	0.525	0.00989	0.00567	5	2.625	2.446*
	P. d. niloticus	269	5	0.400	0.00149	0.00179	1	0.400	−0.817
	P. d. hyrcanus	269	9	0.944	0.01194	0.01269	9	3.139	−0.684
	P. d. bactrianus	269	27	0.772	0.01070	0.01189	12	2.877	−0.253
	P. d. indicus	269	32	0.685	0.00794	0.00744	7	2.121	0.203
	Domesticus group	269	103	0.779	0.00790	0.00737	10	2.101	0.240
	Indicus group	269	68	0.760	0.00904	0.01547	19	2.415	−1.285
	Total	269	171	0.782	0.00858	0.01587	23	2.284	−1.274
CKB (Autosomal)	*P. d. domesticus*	372	90	0.751	0.00413	0.00648	12	1.537	−0.945
	P. d. biblicus	372	26	0.818	0.00544	0.01009	14	2.025	−1.542
	P. d. persicus	372	16	0.850	0.00435	0.00574	7	1.608	−0.837
	P. d. niloticus	372	10	0.933	0.00578	0.00869	9	2.133	−1.442
	P. d. hyrcanus	372	6	0.933	0.00705	0.00715	6	2.600	−0.060
	P. d. bactrianus	372	24	0.721	0.00327	0.00510	7	1.210	−1.108
	P. d. indicus	372	36	0.830	0.00486	0.00592	9	1.806	−0.501
	Domesticus-group	372	142	0.783	0.00449	0.01209	24	1.669	−1.754(*)
	Indicus group	372	66	0.806	0.00472	0.00867	15	1.754	−1.301
	Total	372	208	0.791	0.00461	0.01327	28	1.706	−1.789*
LAMA (Autosomal)	*P. d. domesticus*	362	44	0.729	0.00450	0.00583	9	1.609	−0.638
	P. d. biblicus	362	24	0.909	0.00707	0.00908	12	2.529	−0.731
	P. d. persicus	362	6	0.889	0.00658	0.00594	6	2.356	0.458
	P. d. niloticus	362	4	0.833	0.00325	0.00304	2	1.167	0.592
	P. d. hyrcanus	362	8	0.929	0.00699	0.00758	7	2.500	−0.352
	P. d. bactrianus	362	18	0.941	0.00955	0.01240	13	3.405	−0.831
	P. d. indicus	362	38	0.849	0.00707	0.01152	17	2.528	−1.228
	Domesticus group	362	82	0.822	0.00570	0.01033	18	2.038	−1.277
	Indicus group	362	64	0.882	0.00770	0.01460	24	2.751	−1.439
	Total	362	146	0.850	0.00661	0.01516	29	2.359	−1.593(*)

(*) $0.1 < P < 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
†No. of individuals (CR) or no. of haploid sequences (the other loci).
‡Haplotype diversity (Nei, 1987).
††Nucleotide diversity.
‡‡The population mutation parameter theta, estimated from no. of segregating sites.
§No. of segregating sites.
¶Average number of nucleotide differences between sequence pairs (Nei, 1987).
∫Tajima (1989).

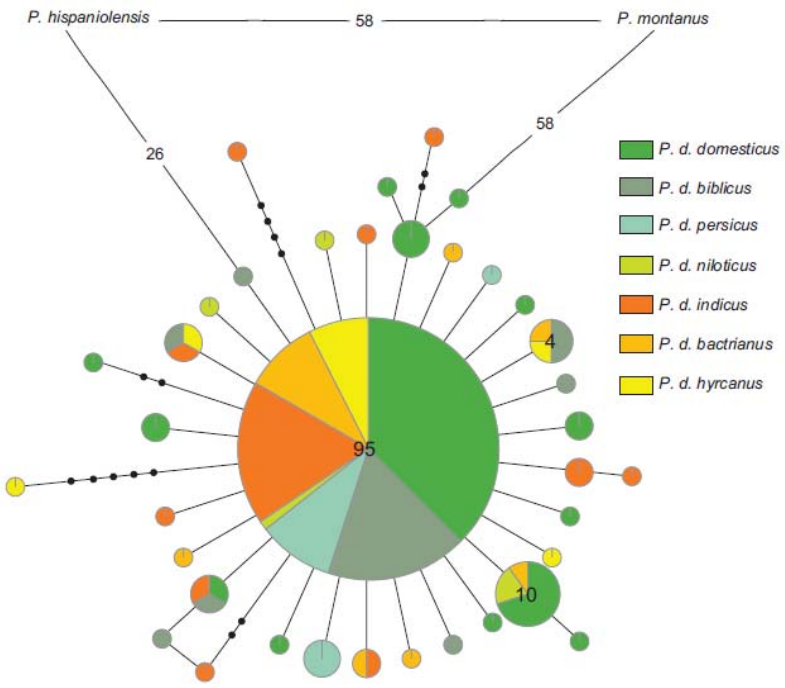

Figure 3-2. Minimum-spanning network of the mitochondrial control region haplotypes found among 157 house sparrows (*Passer domesticus*) from Europe, Asia and North Africa. One Spanish sparrow (*P. hispaniolensis*) and one tree sparrow (*P. montanus*) are included as outgroups. Each circle represents one haplotype and the size of the circle indicates the number of individuals sharing it (also indicated with a number in the three most common haplotypes: 4, 10 and 95 individuals). Colours refer to the seven subspecies included as indicated to the right. Subspecies belonging to the domesticus group are in green tones, and those belonging to the indicus group are in orange/yellow tones. The number of mutational steps between haplotypes is indicated with a line (1 step), dots (2–5 steps) or numbers (more than 5 steps).

Chapter 4. Natural epigenetic variation within and among six subspecies of house sparrow *Passer domesticus*

4.1 Epigenetic variation and local adaptation

Epigenetic processes have an important role in regulating and shaping gene expression. DNA methylation is one of the best-studied epigenetic mechanisms and it is a crucial process in natural selection and evolution because it allows organisms to adapt rapidly to environmental fluctuations by modifying phenotypic traits, either via phenotypic plasticity or developmental flexibility (Schlichting and Wund 2014).

Range expansions, either natural or anthropogenic, are an ideal ecological process to study the role of DNA methylation in local adaptation. In fact, epigenetics could explain the success of some of these expansions, in spite that very often, genetic variability seems to be low and not directly involved in the process (Schrey et al. 2014). The house sparrow *Passer domesticus* is a remarkable bird species regarding range expansion and adaptation. The species originated probably in the Middle East (e.g. Johnston and Klitz 1977; Summers-Smith 1988). Iran is a key place of transition for this species (Vaurie 1956), and several subspecies evolved in this area, probably due to complex topographic features of the Iranian plateau (Misonne 1959). In fact, several zones of intergradations have been described for the different subspecies of the house sparrow in this area (Vaurie and Koelz 1949, Vaurie 1956), so that the geographic distribution map of the species is just approximate (figure 4-1). The species extended naturally into Eurasia, with the aid of human commensalism (Summers-Smith 1988, Anderson 2006, Saetre et al. 2012). Later, it was additionally introduced by humans into America, North Africa and also Australia, being now one of the most broadly distributed vertebrate species (Anderson 2006). Eleven subspecies have been recognized, mainly on the basis of plumage coloration and body size (Vaurie and Koelz 1949, Vaurie 1956, Summers-Smith 1988). Earlier studies on several mitochondrial and nuclear loci showed that human commensalism in the house sparrow has a single origin and evolved around 10,000 years ago after advent of agriculture in the Middle East (Saetre et al. 2012). Analyses on microsatellite markers showed high differentiation in the native populations of house sparrow while genetic diversity was higher in the populations at lower latitudes (Schrey et al. 2011).

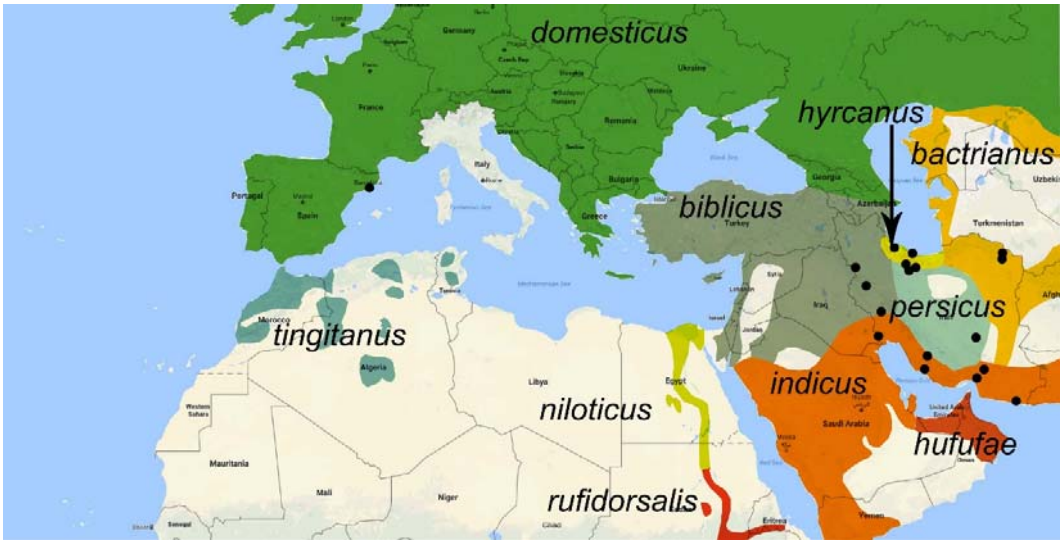

Figure 4-1. Sampling locations (black dots) and geographical distribution of 10 subspecies of house sparrow based on Vaurie (1949, 1956) and Summers-Smith (1988). An additional subspecies (*P. d. parkini*, from the Himalayas) is not shown in this map.

The different native subspecies of the house sparrow show a high level of phenotypic variation (e.g. in plumage coloration, body size and wing length), and the differences have been suggested to be the result from adaptation to the different environmental conditions (Vaurie 1956, Anderson 2006). Outstanding phenotypic differences have also been described between introduced and source house sparrow populations, supporting the adaptive capacity of the species in response to novel or changing environments (see for example Johnston and Selander 1964; Johnston and Klitz 1977; Hamilton and Johnston 1978; Anderson 2006; Johnston and Selander 2008) however, the role of epigenetic changes in shaping these differences is still unknown.

In this study we estimated genome-wide methylation variation within and between five subspecies of house sparrow across Middle East, within the Iranian plateau, to evaluate the role of epigenetic modification in local adaptation and rapid expansion of the species in its native range. Additionally we sampled the subspecies from Spain, which was used as an out-group. We searched for intraspecific methylation variation of house sparrow with the aid of methylation-sensitive AFLP (MSAP) method. This method is a kind of genome-wide fingerprinting method and currently it is

the most common technique in ecological epigenetic studies (Reyna-Lopez et al. 1997). In addition, we searched for correlation between morphometric characters and methylation characters to evaluate the effect of DNA methylation variation on phenotypic traits.

4.2 Material and Methods

We collected 84 muscle tissues from 14 localities in the Palearctic region (figure 4-1). 71 samples from 13 localities in Iran including five of the 11 subspecies: *P. d. bactrianus* (*N* = 10), *biblicus* (*N* = 17), *hyrcanus* (*N* = 4), *indicus* (*N* = 16) and *persicus* (*N* = 24). In addition, 13 house sparrow muscle tissue belonging to *P. d. domesticus* subspecies, deposited at the Natural History Museum of Barcelona (Catalonia, Spain). DNA was extracted from muscle tissues preserved in ethanol using Ecogen MasterPure DNA Purification kit (MCD85201).

The Methylation Sensitive Amplified Polymorphism (MSAP) is a modified method of AFLP uses the *Eco*RI enzyme (rare cutter) and substituting methylation sensitive isoschizomeric enzymes *Msp*I and *Hpa*II for *Mse*I. The *Msp*I and *Hpa*II enzymes have different sensitivities to cytosine methylation. Both enzymes cut the same restriction sequences (5´-CCGG-3´) but *Hpa*II is sensitive to methylation of the internal cytosine at both stands whereas *Msp*I is sensitive to methylation of the external cytosine in any strand (Reyna-Lopez et al. 1997). MSAP detects methylation state of a particular locus in a banding pattern (Salmon et al. 2008).

DNA methylation could differ among tissue due to different gene expression pattern in each tissue. Therefore, we used the same tissue (muscle tissue) and with same DNA extraction kit for all of our individuals. We attempted to optimize MSAP protocol from Liebl et al. (2013). For checking the protocol please see **paper 5**.

Fragments electrophoresis was performed at the Parque Cientifico de Madrid (Spain) using ABI 3730 capillary sequencer (Applied Biosystem). Individual PCR products were analysed along with GeneScan 500-LIZ size standards (Applied Biosystems). PeakScanner v.2.0 software (Applied Biosystems) was used to read the MSAP electropherograms, to detect peaks and calculate their intensity and size. Light peak smoothing was applied to the electropherograms and we changed

minimum peak half width to 1 and minimum peak threshold to 100 for each dye (6-FAM and VIC). Binning and scoring were performed in RawGeno v.2.0 (R CRAN library; Arrigo et al. 2009) with this setting: minimum bin width of 1 bp , maximum bin width of 2 bp, scoring range of 50-500 bp and filtering threshold of the minimal fluorescence was set to 90 relative fluorescent units (rfu). We used the option of review binning in the RawGeno to manually check all the position of the peaks as well as peaks width. After saving the new binning format the binary matrix was generated with RawGeno. The 10% of samples were replicated to check the repeatability of AFLP data generating and scoring. After generating the binary matrix for each primer for all the individuals, with put the binary matrix of repeated individuals together to compare the scoring similarity for all of the peaks. The peak with different scoring pattern for the same individuals, were checked visually by GeneMarker v.1.85 (SoftGenetics) for all of the individuals.

4.2.1 Data analyses

The MSAP matrix was pooled from three primer combinations to generate a single matrix. This matrix was analyzed using Msap v.1.1.0 software package for the programming environment (Pérez-Figueroa 2013). This software analyses the MSAP binary matrix based on four types of methylation pattern according to the presence or absence of one or both fragments of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I. This software also estimates the overall methylation variation using the Shannon diversity index and the frequency (expressed as a percentage) of the four types of methylation pattern in subspecies. Also, we conducted principal coordinate analyses (PCoA) to explore epigenetic variation between subspecies of house sparrow.

In addition, we performed AMOVA (analyses of molecular variance, Excoffier et al. 1992) using Msap software. Using a binary matrix with the methylated loci (1 for methylated and 0 for not methylated), we carried out locus-by-locus AMOVA using GenAlex v.6.502 software (Peakall and Smouse 2006) only with the five Middle East subspecies data considering subspecies, commensal *versus* non-commensal and sex as grouping variables. We estimated statistical significance following 9999 permutations for all analyses. After identifying loci that had significant ϕ_{ST}, we also used a Bayesian likelihood method implemented in Bayescan v.2.1 (Foll and Gaggiotti 2008) to identify the outlier loci as those potentially under selection.

To evaluate the relation between phenotypic variation and epigenetic variation, we measured six morphometric characters for 61 individuals of the Middle East samples, including beak length from nostril, beak length from skull, wing length, tarsus length, body length and tail length. Normality of all of these morphometric characters was evaluated. Then we performed discriminant and canonical discriminant function analyses considering subspecies as grouping variable to evaluate the level of morphological divergence among subspecies. Then we performed Pearson correlation between all, for morphometric characters and methylation percentage each by each. To standardize the morphometric variable such as bill length based on the body size, we extracted the residuals of log-log regression of bill length from skull *versus* tarsus length and we performed the Pearson correlation between these residuals and methylation percentage. All of the morphological analyses were performed using Statistica v.8.0 (StatSoft 2013). To read in more detail about the data analysis please check paper 5.

4.3 Results and discussion

We detected a total of 101 loci using the 3 selective primer combinations; 64 were methylation-susceptible loci (MSL) and 37 were non-methylated loci (NML). The frequency of polymorphic MSL was 100% and Shannon's diversity index was 0.56 ± 0.13 (mean ± SD). In all subspecies, the highest percentage of loci screened were unmethylated (range 39.8 to 46.6%), while the frequency of the two distinguishable methylation states totally ranged from 26.4% to 28.9% (Table 4-1). Principal coordinate analyses (PCoA) detect only a slightly differentiation between *P. d. domesticus* and the other subspecies (Fig. 2 in paper 5).Using the 6 subspecies studied, *Passer d. domesticus* showed the highest level of differentiation, as it was differentiated from all other subspecies (Table 4-2). *P. d. biblicus* also showed significant differentiation from *bactrianus* and *persicus* (Table 4-2). Analyses of molecular variance (AMOVA) present a differentiation among subspecies (ϕ_{ST} = 0.07, $P < 0.0001$; Table 4-3) and also revealed that a greater portion of the epigenetic differentiation was attributed to differences within subspecies rather than among subspecies (Table 4-3). Considering only the Middle East subspecies, we detect also significant differentiation among subspecies (ϕ_{ST} = 0.022, $P < 0.040$) and commensal *versus* non-commensal (ϕ_{ST} = 0.019, $P < 0.045$) (Table 4-3). And from the methylated matrix using the locus-by-locus AMOVA analyses, we detect seven loci (subspecies as grouping criteria), three loci (in relation

with commensalism as grouping criteria), and three loci (sex as grouping criteria) with significant differentiation respectively (Table 4-3). However, no locus with significant ϕ_{ST} had positive Bayes factors. To read more in detail regarding the results please check paper 5.

Table 4-1. Frequency of methylation states at the target sequence for each subspecies of house sparrow (*P. domesticus*).

Target state (band pattern)	***bactrianus***	***biblicus***	***hyrcanus***	***indicus***	***persicus***	***domesticus***
Unmethylated (HPA+/MSP+)	46.56	39.80	46.48	41.11	41.41	43.75
Hemimethylated(HPA+/MSP−)	18.44	20.31	20.31	18.65	20.18	14.54
Internal C methylation (HPA −/ MSP+)	7.97	7.45	6.64	9.77	8.79	12.86
Full methylation (HPA − /MSP−)	27.03	32.45	26.56	30.47	29.62	28.85

In conclusion this study found variation in DNA methylation was largely independent of subspecies designation. Notably, DNA methylation of the European subspecies was differentiated from all other subspecies, and the non-commensal and migratory subspecies had the second highest level of differentiation. At some loci, DNA methylation was differentiated based on subspecies and presence of commensal relationship with humans, Sex of individuals also generated differences in DNA methylation. Further, significant correlation was found between morphological traits and percentage of DNA methylation, and between geographical distance and percentage of DNA methylation.

Table 4-2. Pairwise AMOVAs between the pairs of subspecies of *Passer domesticus*. *P* values are in parenthesis. Bold ϕ_{ST} values have significant *P* values ($P< 0.05$).

	bactrianus	***biblicus***	***hyrcanus***	***indicus***	***persicus***	***domesticus***
bactrianus						
biblicus	**0.106 (0.0002)**					
hyrcanus	0.002 (0.46)	0.040 (0.17)				
indicus	0.037 (0.05)	0.008 (0.28)	0.006 (0.40)			
persicus	0.003 (0.39)	**0.055 (0.0009)**	0.013 (0.33)	0.023 (0.06)		
domesticus	**0.161 (<0.0001)**	**0.156 (<0.0001)**	**0.163 (0.0007)**	**0.129 (<0.0001)**	**0.115 (<0.0001)**	

Table 4-3. Analyses of molecular variance (AMOVA) based on the MSAP data. Bold ϕ_{ST} values have significant *P* values ($P< 0.05$).

Grouping variables	Sources of variation	df	Sum of squares	MSD	Variation	Φ_{ST} (P value)	N 1oci with significant Φ_{ST}
Including all subspecies (6 subspecies)							
Subspecies	Among subspecies	5	106.9	21.38	0.80	**0.070** (< 0.0001)	----
	Within subspecies	78	831.4	10.66	10.66		
Including Middle East subspecies (5 subspecies)							
Subspecies	Among subspecies	4	59.60	14.90	0.26	**0.022** (< 0.040)	7 (29, 32, 37, 52, 68, 71, 75)
	Within subspecies	66	752.32	11.40	11.40		
Commensal *vs* non-commensal	Among groups	1	15.35	15.35	0.22	**0.019** (< 0.045)	3 (29, 58, 71)
	Within groups	69	796,57	11.54	11.54		
Sex	Among groups	1	12.96	12.96	0.05	0.004 (= 0.231)	3 (39, 75, 100)
	Within groups	62	709.62	11.45	11.45		

5. General discussion

5.1 Isolation by adaptation and urbanization

Population genetic differentiation and structure is typically related to several habitat features such as geographic distances among populations (e.g. Barlow et al. 2011) presence of physical barriers and habitat fragmentation (Cushman 2006, Sork and Smouse 2006, Porlier et al. 2009).

However, in recent years, evidence is growing on the fact that adaptive divergence can also appear as a result of ecological differences between populations, even between populations located at very short distances (Nosil 2012). This has been termed "isolation by adaptation"(Nosil et al. 2008). This is for instance the case of several highly mobile passerine species such as Crossbills *Loxia curvirostra*, Citril finches *Serinus citrinella* and Blue tits *Cyanistes caeruleus* in which adaptive divergence can occur due to habitat and ecology differences rather than isolation by geographic distance and physical barriers. In fact, local adaptation can reduce gene flow among populations located in different habitats (e.g. Senar et al. 2006; Edelaar et al. 2012; Porlier et al. 2012). Thus, local adaptation may lead to phenotype and genotype differentiation in response to different environmental stimuli.

One of the more severe habitat fragmentation appears because of urbanization. Urbanization affects on population dynamics, colonization probabilities and reproduction output even on highly mobile species (Delaney 2013, Gil and Brumm 2014). In big cities, strong and significant genetic structure has even been reported for some species (Partecke 2013). This is also the case of the great tit, one of our model species, in which low gene flow between the city and the nearby forests, in addition to inbreeding pressure, has produced this genetic differentiation (Björklund et al. 2010). The next step, however, is to know the mechanisms by which the differentiation takes place. To link genetic to behavioural and morphological variation, so that we can understand why the individuals differentiate and whether this change is adaptive. This was the main aim of this thesis.

5.1.1 The role of personality in adaptation to urban habitats

The most general and easiest form of adaptation to the urban habitat is by changes in the behaviour of the individual (Sol et al. 2013). Most of these changes have been attributed to phenotypic

plasticity, that is, changes in the individuals in response to environmental alteration without genetic changes. This happens when the same genotype may produce alternative phenotypes in relation to environmental cues to enhance fitness and facilitate the persistence of populations in novel environments (e.g. Baldwin et al. 1902 ; Schlichting and Pigliucci 1998).

However, there are some behaviours which are consistent between individuals and that show correlated patterns. These behaviours define the "personality" of the individual. In recent years it has been found that these behaviours have a profound effect of the prospects of success and fitness of the different individuals (Wilson et al. 1994, Dall et al. 2004, Dingemanse and Réale 2005a). Personality has also a heritable component (Dingemanse et al. 2002, Drent et al. 2003, van Oers et al. 2004b, Quinn et al. 2016), which means that this "trait" and be under micro-evolutionary selection. This is why we focused on the study of personality in urban great tits as way to understand adaptive differentiation of urban birds.

In chapter one we showed that urban and woodland populations of great tits in the Barcelona area differ in novelty seeking behaviour. On average, urban-dwelling birds were bolder, showing shorter latencies to approach a novel object than forest great tits and exploring novel environments faster than forest birds. We are not sure if all of the urban great tits from different cities show the same behaviour but these kind of transformations in the personality traits have been reported before in several species such as dark-eyed juncos *Junco hyemalis* and song sparrows *Melospiza melodia* (Scales et al. 2011, Atwell et al. 2012). A high exploration rate, boldness and risk taking, commonly termed proactivity (Coppens et al. 2010), can be advantageous in the urban setting (Wilson et al. 1994). Proactive individuals, for instance, more rapidly discover and use new food resources than reactive birds (van Overveld and Matthysen 2010), which could help urban dwellers to take profit of the new feeding opportunities that a city can provide (e.g. Fisher and Hinde 1949). Our finding thus add to the general view that urban birds are considered to adapt to human disturbance as a process of local adaptation (Partecke et al. 2006, Sol et al. 2013). Work is now needed to show that the difference between urban and forest great tits in proactive behaviour is caused by divergent selection. Analyses relating survival (and breeding success) to different personality styles, according to degree of urbanization, would allow to test for that (see Senar et al. 2014).

5.1.2 Urbanization changes the rules and brakes the personality syndrome

Personality is defined as a suite of fixed and correlated behaviours. In fact, the correlation between all the different behaviours is a main assumption to be able to speak of a personality syndrome (Sih et al. 2004b, Groothuis and Carere 2005). At first, we expected to record a pattern of correlated behaviours in our two populations of great tits, the forest and the urban one. However, data showed that the correlation between exploratory behaviours was only found in the forest population. The urban population, in spite of showing a behavioural frequency different from the forest one, with a higher prominence of exploratory behaviours and boldness, did not show a personality syndrome with a high correlation between the different exploratory behaviours.

Nevertheless, a more detailed examination of the topic suggests that the lack of a syndrome could in fact be adaptive. The existence of behavioural syndromes sometimes can limit behavioural plasticity and this creates some behaviours which seems non-adaptive and non-optimal. In addition, limited plasticity could be a problem in rapidly changing habitats such as big cities. Theoretical work on the topic has in fact found that if there is a maladaptive behavioural correlation, then selection, with the aid of genetic modifiers, can decouple the correlation, facilitating local adaptation (Sih et al. 2004a, Karlsson Green et al. 2016). Recent empirical work on other species, supports this view (Evans et al. 2010, Scales et al. 2011, Bókony et al. 2012).

5.1.3 In search of a genetic link in differences in personality between urban and forest birds

A good way to show that differences in personality between urban and forest great tits is the result of microevolutionary responses is to find out a genetic link to the differences. Previous work had suggested that SNP830 *DRD4* gene in great tits was linked to exploratory behaviour (Fidler et al. 2007, Korsten et al. 2010). So, we searched for a relationship between SNP830 *DRD4* gene and personality scores in our two populations (paper 1). However, we did not found any significant relationship between the polymorphism in this gene and novelty seeking scores. Now we know that the relationship between genes and behaviour appears far more complex than previously described (Tschirren and Bensch 2010) and our work is consistent with recent findings that the

relationship between SNP830 *DRD4* and novelty seeking score holds in some populations but not in others (Korsten et al. 2010). Apparently, the link between *DRD4* gene and personality is context-dependent, probably mediated by certain characteristics of the habitat at a local scale (Mueller et al. 2013a)

After analysing the *DRD4* gene, we sequenced the *SERT* promoter for our samples, to search for any genetic polymorphisms which possibly can have a role in novelty seeking behaviour. We identified 11 SNPs in the promoter. Finally, we found a significant correlation between one SNP in the *SERT* promoter (SNP234) with novelty seeking behaviour.

5.2 Can epigenetic modifications regulate personality traits?

Epigenetic modification of gene expression, rather than genetic factors *per se*, can be responsible for the rapid adaptive response of birds to novel environments (Jaenisch and Bird 2003). Epigenetic marks can be triggered by environmental effects and can lead to permanent changes in gene expression, which can affect the phenotype of an organism. Thus, epigenetic modifications have been suggested as key in plasticity allowing individuals to adapt themselves with the environmental challenges without changes in their genetic structure. Trans-generational transmission of epigenetic information may allow that this adaptive information regarding environment passes to the next generations (e.g. Anway et al. 2005; Stouder and Paoloni-Giacobino 2010).

In this thesis we found that methylation was ~1-4% higher in urban than in forest birds, for all loci and tissues analysed. Moreover, we also found in urban great tits, a positive relationship between methylation level of the *SERT* second CpG site and exploration score. Both of these results suggest that epigenetic modifications (here DNA methylation level) is influenced by environmental conditions. Many studies have now confirmed that environmental exposures during early development can influence gene regulation through epigenetic mechanisms and this will affect the adult phenotypes (Frésard et al. 2013). Our work also confirms the role of environmental cues and epigenetics in shaping personality traits (Yauk et al. 2008, Wong et al. 2010). All of this makes me to suggest that epigenetic adjustments probably have an important role in allowing urban birds

to better adapt to novel environments in just a few generations, with this flexibility settling in the populations by trans-generational inheritance of these epigenetic marks.

5.3 Is *MC1R* gene polymorphisms responsible for regulating the size of black belly coloration in great tits?

The size of melanin-based black belly stripe in the great tits changes with age (Adamik and Vaňáková 2011) and it is also sex-dependent (Slagsvold 1993). The male has more glossy coloration probably because sex-limited expression (i.e. expressed to a larger extent in one sex compared to the other sex) or sex-linked inherited genes (Roulin and Ducrest 2013). The black belly stripe is in part a genetically determined trait (Norris 1993, Quesada and Senar 2009) and may also have an environmental component (Fitze and Richner 2002). Recent work showed that the size of the stripe was related to urbanization through a process of divergent selection, with urban great tits displaying smaller stripes (Senar et al. 2014) .Recent work also showed that the size of the stripe in great tits was also related to personality, with birds displaying smaller stripes being bolder (Nicolaus et al. 2016). We found therefore highly relevant to search for the genetics basis of variation in great tit black stripe and to relate it to urbanization.

MC1R gene or Melanocortin-1 is one of the main genes that regulate melanin coloration (Roulin and Ducrest 2013). Other genes such as *TYR* and *TYRP1* also regulate the eumelangenesis mechanism. Our aim was therefore to relate *MC1R* gene polymorphism to urbanization (paper 3). However, we found that there was no genetic polymorphisms in the *MC1R* gene which could affect the size of black belly coloration in the great tits. So, we suggested that several other factors are affecting the size of this stripe in the great tit, such as level of hormones and also epigenetic marks. For the next studies, we suggest to investigate other mechanisms of gene expression alterations such as mRNA expression and/or epigenetic alteration in the *MC1R* gene promoter (Fontanesi et al. 2010, Emaresi et al. 2013, Scriba et al. 2013).

5.4 Does the house sparrow have a single origin in the Palearctic?

The house sparrow represents a classic example of an ancient adaptation of birds to human-made habitat modifications. However, it is not yet clear how this process evolved. Vaurie (1949, 1956) suggested that house sparrows split into two main lineages due to periodic isolation in association with a glacial advance long before the advent of agriculture. However, Johnston and Klitz (1977) suggested that commensalism of house sparrow with humans probably arose with the advent of agriculture in early human civilization and from a single origin. Here (paper 4) we used sequence analysis of one mitochondrial marker (control region) and three nuclear makers to find the phylogeographic pattern of house sparrows within Palearctic region and to clarify which hypothesis better explains the current phylogeographic situation of the house sparrow.

The minimum-spanning network of the control region marker showed a star-shaped topology with one common haplotype surrounded by many rare haplotypes. In addition, the Tajima's D was negative for five out of seven subspecies for this genetic marker. In addition we found considerable allele sharing at all the four genetic markers. All of these results suggested that house sparrow subspecies differentiated recently and from a single source. The mismatch-distribution model estimated the expansion of house sparrow occurred approximately 3000-7500 years ago. Following the rise of human agriculture and permanent settlement around 10'000 years ago, human populations expanded throughout the Palearctic and Oriental regions (see e.g. Atkinson et al. 2008). Human population expansion and increase food availability facilitated the rapid expansion of house sparrow.

5.5 Can genome-wide methylation level of house sparrow subspecies explain patterns of adaptation to man-made habitats?

House sparrow is one of the most successfully urban-adapted species which have been flourished in the urban environments probably due to its fitness to consume cultivated cereals (Riyahi et al. 2013) and abandonment of migratory behaviour despite the other closely related species. Since epigenetic modifications enable species to adapt with new environmental conditions rapidly, investigating epigenetic marks in this kind of species can be very interesting. In addition, evidences showed that epigenetic markers have an important role for adaptation and expansion of

introduced species (Liebl et al. 2013, Schrey et al. 2014, Schrey et al. 2016). In this study we found that despite the high phenotypic variation between these subspecies, the general DNA methylation profile has a very similar pattern and variation in DNA methylation did not match subspecies designations. The European subspecies (*P. d. domesticus*), in contrast, was differentiated from all other subspecies in DNA methylation. However, when we checked our results locus by locus, from 101 loci in our dataset we detected seven loci which showed differentiation based on subspecies and also three loci which showed alteration in the methylation level in relation to human commensalism.

The difference we detected between the European and the Middle East populations indicates that at a broad-scale significant differences are present. This is further supported by the significant differences detected among commensal and non-commensal subspecies.

Our results may be surprising, initially. However, Trucchi et al (2016) recently showed that while some loci have variable DNA methylation, perhaps due to environmental stimuli, a large proportion of the methylome is typically stable. Our results support this finding and showed that genome-wide methylation patterns in the house sparrow remained highly stable over a broad range.

6. Concluding remarks

Anthropogenic changes are causing huge alterations in ecosystems and many animals undergo modifications to better survive in this "new world". In this thesis we have documented several of these modifications, showing that they have both a genetic and an epigenetic basis and therefore, may be potentially selected from a micro-evolutionary point of view. Here we focused mostly on behavioural traits because colonization in urban areas is mainly a behavioural phenomenon.

Selective pressures favour different personalities in the wild and there is no ideal phenotype. We showed that some personality traits can be different in the urban habitats compared to the wild habitats and animals with these behaviours may have a higher fitness in these novel environments. We also found out that epigenetic modification (including DNA methylation) may help organisms to adapt rapidly in fluctuating environments such as big cities. We realized that epigenetic processes are crucial in natural selection and can influence morphological and phenotypical traits.

We also showed the great impact of human civilizations on the expansion and evolution of the ubiquitous house sparrow. In addition, we revealed that although there is a general stability in the genome-wide methylation pattern of the house sparrow among different subspecies, there are some epiloci which differentially evolved in different populations. We suggest that these epiloci may have a role in phenotypic alterations which exist in different habitats.

Overall, for future genotype–phenotype association studies, we recommend to researchers to consider epigenetics modifications as part of their research before they come to any conclusion.

Now new generation technologies such as epiRAD (Schield et al. 2016) are more suitable tools to investigate the link between DNA methylation states and phenotypic traits. Also the great tit whole genome assembly (Laine et al. 2016) has been released recently which will help scientists to more deeply integrate ecology, evolution and behaviour together.

7. Resumen en Castellano

7.1 Introduccion

La adaptación local es un proceso por el cual los individuos exhiben mayor éxito reproductivo en su ambiente local que individuos de poblaciones y ambientes diferentes (Kawecki and Ebert 2004). La adaptación local es crítica para la persistencia de las especies debido ante rápidos cambios ambientales. Aunque este concepto ha sido tradicionalmente considerado como una forma de mejorar el éxito reproductivo de acuerdo con condiciones naturales particulares, las enormes transformaciones producidas por los humanos en la naturales pueden también promover (o hacer necesaria) la adaptación local.

Los humanos han sido proclamados la mayor fuerza evolutiva del planeta debido a los grandes cambios que introducimos en los ecosistemas naturales (Palumbi 2001). Entre esos cambios, los ambientes modificados por los humanos se han expandido rápidamente en décadas pasados, produciendo un impacto tremendo en los seres vivos. Estos ambientes modificados por los humanos pueden ser considerados, por lo tanto, nuevos ecosistemas (Pataki 2015), que muestran marcadas diferencias con los hábitats naturales (Aronson et al. 2014). Las especies que colonizan estos ambientes pueden requerir ajustes fenotípicos a estas nuevas condiciones (Partecke 2013). Los colonizadores de ambientes urbanos desarrollan probablemente cambios micro-evolutivos que podrían implicar cambios en sus rasgos (o características) vitales. Aunque investigaciones sobre estas cuestiones son todavía escasas, ha habido reportes de modificaciones en la estructura del canto (Slabbekoorn and Peet 2003), un avance en el coro del amanecer debido a la luz artificial (Miller 2006), y diferencias en "personalidad" como una distancia de iniciación del vuelo más corta en aves urbanas (Møller 2008) o personalidades más atrevidas en lagartijas urbanas (Lapiedra et al. 2017).

Los cambios en los rasgos vitales en hábitats modificados por los humanos pueden ser causados por: 1- respuestas plásticas de los organismos a estímulos ambientales, 2- selección por alelos específicos o, 3- efectos epigenéticos (Partecke 2013). Recientemente, ha habido intentos para entender la base genética de las adaptaciones locales, proporcionando evidencias de adaptación local y rápidas respuestas evolutivas a la urbanización (e. g. van Dongen et al. 2015; Watson et al. 2017). Por el contrario, los intentos para identificar polimorfismos genéticos asociados de manera

consistente con rasgos de la personalidad ha tenido escaso éxito hasta ahora (van Oers and Mueller 2010, Balestri et al. 2014).

Además de polimorfismos genéticos, las modificaciones epigenéticas parecen un buen candidato para jugar un papel clave en explicar la variación en personalidad (Ledón-Rettig 2013). Marcas epigenéticas, incluyendo la metilación del ADN o la modificación de histonas, pueden ser activadas por efectos ambientales, y llevar a cambios permanentes en la expresión de genes, afectando el fenotipo de un organismo. Debido a este hecho, la epigenética es un proceso crucial para la selección natural y la evolución porque permite al organismo adaptarse rápidamente a fluctuaciones ambientales a través de la modificación de rasgos fenotípicos, ya sea a través de la plasticidad fenotípica o de la flexibilidad durante el desarrollo (Schlichting and Wund 2014).

La relación entre la variación en la metilación de AND y el comportamiento ha sido investigado recientemente en animales humanos y no humanos (e. g. Duclot and Kabbaj 2013; Kumsta et al. 2013). Sin embargo, se sabe muy poco sobre la capacidad de la epigenética para explicar la variación en rasgos comportamentales en animales salvajes. Por ejemplo, un reciente estudio de Verhulst et al (2016) mostró que linajes de carbonero común (*Parus major*), artificialmente seleccionados para divergir en su comportamiento exploratorio durante cuatro generaciones, difirieron en sus niveles de metilación de ADN en el gen receptor de dopamina D4 (*DRD4*). Por lo tanto, es necesario investigar la relación entre la metilación de ADN y las adaptaciones comportamentales y morfológicas a los ambientes modificados por los humanos.

7.2 Objetivo

En la primera parte de esta tesis he investigado los patrones de adaptación contemporánea a hábitats urbanos, centrándome en el papel de la variación comportamental, genética y epigenética en el carbonero común. En la segunda parte de esta tesis he investigado los patrones de adaptación a ambientes modificados por los humanos en una escala temporal más grande, centrándome en el origen y la expansión del gorrión común *Passer domesticus*, que ha sido comensal de la especie humana durante milenios. Adicionalmente, comprobé el efecto de la variación en metilación en la rápida expansión y elevada variabilidad fenotípica del gorrión común en el Paleártico.

7.3 Resultados Y discusión

En el primer capítulo de mi tesis, investigamos la variación intraespecífica en rasgos de personalidad (comportamiento de exploración y "búsqueda en nuevas condiciones "[novelty seeking]) de carboneros comunes *Parus major* para ligar estas diferencias a factores ambientales y heredados. Además, testamos la relación entre personalidad y el gen *DRD4* usando pruebas estándar del comportamiento de "atrevimiento" [boldness] y de exploración en aves cautivas. Exploramos esas diferencias de personalidad comparando una población forestal y dos poblaciones urbanas. También analizamos la variación en la frecuencia de un SNP en el exón 3 del gen receptor de dopamina D4 (*DRD4* SNP830), que ha sido relacionado previamente con la personalidad (Fidler et al. 2007, Korsten et al. 2010, Mueller et al. 2013a). Investigamos las relaciones entre este gen y los resultados de las pruebas de personalidad en nuestra población para determinar si hay selección en rasgos de personalidad en aves urbanas y forestales. Encontramos que las aves urbanas fueron más exploradoras en nuevos ambientes y más atrevidas enfrente de un objeto nuevo que las aves forestales. En esta población no había correlación entre los polimorfismos de *DRD4* SNP830 y los valores de comportamiento. Los valores de exploración se correlacionaron con el nivel de atrevimiento en aves forestales pero no urbanas. Nuestros resultados sugieren que nuevas presiones selectivas en ambientes urbanos favorecen el desacoplamiento de rasgos del comportamiento que generalmente forman un síndrome comportamental en la naturaleza (artículo 1).

En el segundo capítulo, realizamos los perfiles de metilación de dos genes candidatos para rasgos de personalidad, *DRD4* and *SERT*, en el carbonero común *Parus major*, para determinar si los rasgos de personalidad y el comportamiento ha evolucionado con la ayuda de la variación genética en distintos hábitats. De hecho, buscamos si los promotores *DRD4* and *SERT* metilaron de manera diferente en carboneros forestales y urbanos y si los comportamientos de "búsqueda en nuevas condiciones" y de exploración estaban relacionados con el nivel de metilación del ADN en el promotor de estos dos genes. Adicionalmente, intentamos responder si los estados alélicos en el cercano SNPs se asociaron con los niveles de metilación en los promotores *SERT* and *DRD4*. Encontramos varias modificaciones epigenéticas y polimorfismos genéticos posiblemente relacionados con el comportamiento de "búsqueda en nuevas condiciones" en carboneros comunes. Además, la metilación fue del 1-4% más elevada en aves urbanas comparadas con aves

forestales, para todos los tejidos y loci analizados, lo que sugiere que esta modificación epigenética está influenciada por las condiciones ambientales. Nuestros resultados sugieren que el ajuste epigenético puede ser diferente en carboneros urbanos y forestales (artículo 2).

En el tercer capítulo, exploramos las relaciones entre polimorfismos de Melanocortin-1 (*MC1R*) y la coloración pectoral negra basada en melaninas de los carboneros comunes. Melanocortin-1 (*MC1R*) codifica la proteína receptora para la hormona estimuladora de melanocitos y controla la melanogénesis. Elegimos carboneros comunes de la ciudad de Barcelona y un bosque cercano porque nuestros daos muestras que el tamaño de la franja pectoral negra en más pequeña en carboneros urbanos que en carboneros forestales. Nuestro objetivo era encontrar el efecto genético subyacente a este patrón. Nuestros resultados mostraron que no hay polimorfismo en este gen en relación con el tamaño de la franja pectoral negra. Por lo tanto, sugerimos que diversos factores afectan el tamaño de esta franja en el carbonero común, tales como el nivel de hormonas o marcas epigenéticas (artículo 3).

En el cuarto capítulo, investigamos el origen del comensalismo hacia los humanos en el gorrión común en la región Paleártica. Analizamos la variación genética de la región control del ADN mitocondrial y de tres loci nucleares para estimar el nivel de diferenciación genética entre subespecies y para describir el patrón filogeográfico de esta especie. Encontramos que el comensalismo hacia los humanos tiene un solo origen en esta especie. Este proceso se inició probablemente en Oriente Medio y se expandió rápidamente en el Paleártico con la ayuda de la agricultura y la expansión de la civilización humana (artículo 4).

En el último capítulo de mi tesis, investigamos los patrones de metilación a lo largo de todo el genoma de seis subespecies de gorrión común en la región Paleártica usando el método de la metilación sensitiva AFLP (MASP) para evaluar la importancia de la variación epigenética en la rápida expansión del gorrión común. Descubrimos que el patrón de metilación a lo largo de todo el genoma de los gorriones comunes es estable en cinco subespecies del Oriente Medio. Detectamos una correlación entre el nivel de metilación y algunos rasgos morfológicos como la longitud del pico estandarizada. Por lo tanto, sugerimos que parte de la elevada variación

morfológica en poblaciones nativas del gorrión común está influenciada por regiones metiladas diferencialmente en loci específicos a lo largo del genoma. También detectamos que siete loci metilados diferencialmente divergieron entre subespecies y, además, tres loci difirieron entre subespecies comensales y no comensales. Por lo tanto, la técnica MSAP detectó diferencias de mayor escala entre subespecies europeas y migratorias, no comensales, pero no detecto diferencias a menor escala entre otras subespecies del Oriente Medio (artículo 5).

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PAPER 1

Personality and urbanization: behavioural traits and *DRD4* SNP830 polymorphisms in great tits in Barcelona city

Resumen en castellano

La mayoría de ejemplos de adaptación al entorno urbano están relacionados con procesos de plasticidad, más que con la selección natural. Sin embargo, la personalidad, definida como diferencias consistentes a nivel individual en el comportamiento relacionado con la exploración, la precaución, y la neofobia, es un buen rasgo candidato para estudiar la selección natural en relación al entorno urbano, debido a su variación heredable. El objetivo de este estudio fue analizar variaciones en la personalidad mediante la comparación de poblaciones urbanas y forestales del carbonero común *Parus major*, usando tests estándares para medir el comportamiento exploratorio y la audacia. Estudiamos la personalidad en 130 carboneros silvestres capturados en la ciudad de Barcelona y en los bosques cercanos, y encontramos que los pájaros urbanos eran más exploradores, y mostraban más audacia al ser enfrentados con objetos nuevos, que los pájaros forestales. Las frecuencias genotípicas del polimorfismo SNP830 del gen *DRD4*, una región asociada con variaciones en la personalidad, difirieron significativamente entre las poblaciones urbanas y forestales. Sin embargo, las medidas del comportamiento no correlacionaron con este polimorfismo en nuestra población. Las medidas de la exploración correlacionaron con la audacia en los pájaros forestales pero no en los urbanos. Nuestros resultados sugieren que las nuevas presiones de selección del entorno urbano favorecen el desacoplamiento de rasgos conductuales que con frecuencia forman síndromes conductuales en la naturaleza.

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ARTICLE

Personality and urbanization: behavioural traits and *DRD4* SNP830 polymorphisms in great tits in Barcelona city

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Abstract Most examples of adaptation to the urban environment relate to plasticity processes rather than to natural selection. Personality, however, defined as consistent individual differences in behaviour related to exploration, caution, and neophobia, is a good behavioural candidate character to study natural selection in relation to the urban habitat due to its heritable variation. The aim of this paper was to analyse variation in personality by comparing urban and forest great tits *Parus major* using standard tests of exploratory behaviour and boldness. We studied personality in 130 wild great tits captured in Barcelona city and nearby forests and found that urban birds were more explorative and bolder towards a novel object than forest birds. Genotype frequencies of the *DRD4* SNP830 polymorphism, a gene region often associated with personality variation, varied significantly between forest and urban birds. Behavioural scores, however, were not correlated with this polymorphism in our population. Exploration scores correlated to boldness for forest birds but not for urban birds. Our findings suggest that the novel selection pressures of the urban environment favour the decoupling of behavioural traits that commonly form behavioural syndromes in the wild.

Keywords *Parus major* · Urban · Boldness · Exploratory behaviour · Candidate gene

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Introduction

The world is currently undergoing an unprecedented urbanization process (Gaston 2010; Marzluff et al. 2001). Over 50 % of the world's population now resides in urban areas and numbers are rapidly increasing (United Nations 2014). Such rural–urban shifts in population have traditionally been considered to have detrimental effects on wildlife, preventing their colonization. Nevertheless, there is increasing evidence that some species adapt to the urban environment, leading to such trait divergence that urban and natural populations differ in demography, morphology, communication, physiology and genetic structure (Evans 2010; Møller 2012; Shochat et al. 2006). The changes may be so dramatic that the urban habitat is now considered a new selection pressure, and urban adaptation is studied as a natural experiment in contemporary evolution (Evans et al. 2009, 2010b; Garroway and Sheldon 2013; Hahs and Evans 2015; Shochat et al. 2006).

Most examples of adaptation to the urban environment refer to changes in behaviour (Tuomainen and Candolin 2012). Such changes mainly occur via phenotypic

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plasticity, or via microevolutionary responses to natural selection (Brown 2012). Although most behavioural adjustments reported to date relate to plasticity processes (Sol et al. 2013; Tuomainen and Candolin 2012), only microevolutionary responses can prompt divergent selection and hence fix the adaptations in the urban populations (Ryan and Partan 2014).

Personality is a good behavioural candidate to study microevolutionary responses related to adaptation to an urban habitat. Defined as consistent individual differences in behaviour related to exploration, caution, and neophobia (Reale et al. 2007), personality has been found to have marked heritable variation (Dingemanse et al. 2002; Drent et al. 2003; Van Oers et al. 2004). Several studies have analysed differences in personality between urban and forest conspecifics. It has been observed that bolder and less neophobic individuals may be better competitors in urban habitats (Ryan and Partan 2014), but some findings have been contradictory. Studies on blackbirds *Turdus merula* have found urban birds to be neophobic (Miranda et al. 2013) while studies on house sparrows *Passer domesticus* have found the reverse (Bókony et al. 2012). Work on song *Melospiza melodia* and house sparrows has found that urban birds have higher levels of territorial aggression than forest birds (Evans et al. 2010a; Scales et al. 2011) while the reverse has been found in house finches *Haemorhous mexicanus* (Hasegawa et al. 2014). Further research is therefore needed to improve our understanding of this topic.

The great tit *Parus major* is an ideal model species, and a large body of work on personality in this species has already been amassed (e.g. Carere et al. 2005; Dingemanse et al. 2002; Verbeek et al. 1994). Nevertheless, the behavioural differences between urban and non-urban individuals in this species are insufficiently understood. For instance, it has been found that the exploratory behaviour of great tits in new environments and their boldness towards novel objects are typically correlated, along with other behaviours, forming a behavioural syndrome (Marchetti et al. 2000; Verbeek et al. 1994). However, although these suites of correlated behaviours might be favoured by selection in some contexts (Bell 2005; Bell and Sih 2007), they could quickly become maladaptive in a new, fast-changing environment, such as an urban habitat, if certain traits do not decouple (Sih et al. 2003). This decoupling process has recently been demonstrated at the genetic level in a freshwater isopod (Karlsson Green et al. 2016) and in small passerine birds in relation to behavioural traits such as territorial aggression, flight-initiation distance, and reaction to a dummy predator (Bókony et al. 2012; Evans et al. 2010a; Scales et al. 2011). To what point this decoupling appears in urban great tit populations in relation to exploratory behaviour and boldness is unknown.

Another question arises in relation to the genetic basis of personality. Polymorphisms in the SNP830 region of the *DRD4* gene have been related to a novelty-seeking personality in some populations of this species (Fidler et al. 2007; Timm et al. 2015), but we do not know if these polymorphisms translate accordingly to birds living in an urban habitat.

The aim of this paper was to analyse variation in personality, comparing wild-caught urban and forest great tits using standard tests of exploratory behaviour and boldness. We first predicted that urban and non-urban great tits would differ in their exploratory behaviour in a new environment and in boldness towards a novel object. Second, we predicted that great tits in the urban environment would show a lack of correlation between these two behaviours. Finally, we predicted that if the *DRD4* gene is also related to personality differences in our populations, urban and non-urban birds would show different allele frequencies of *DRD4* genotypes. We tested these predictions by comparing great tits in Barcelona city with great tits in nearby forests. These populations are particularly relevant for testing these predictions because urban great tits in Barcelona are genetically different from their forest counterparts, and because gene flow between forest and urban populations is limited despite the short distance between the two (Björklund et al. 2010).

Materials and methods

General field methods

Behavioural analyses were based on 130 great tits captured in the wild between October 2012 and March 2013 and October 2013 and March 2014. All birds were individually marked with numbered metal rings and numbered polyvinylchloride rings. Since great tits in our study area are highly sedentary, the sample of birds we captured is likely representative of the population present in the area during the breeding season (Pagani-Núñez and Senar 2014). Birds were trapped using baited funnel traps (Senar et al. 1997). Forest birds ($n = 74$) were captured at the Can Cata study area located in Collserola National Park, 3 km from Barcelona city. This area consists of a mixed forest, dominated by pure oak (*Quercus ilex* and *Quercus cerrioides*) stands at the bottom of the valleys and Aleppo pine (*Pinus halepensis*) forests in the hills. Urban birds were captured at two parks in Barcelona city, Ciutadella Park (21 birds) and Desert de Sarria-Setmenat Park (35 birds). Ciutadella Park is situated in the city centre and is one of the largest (30 ha) and oldest parks in Barcelona. Despite its size, this park is surrounded by buildings on all sides. It is an open-plan park with a high number of visitors each

day, so it does not resemble a natural forest. Throughout the text, we refer to this park as the 'city centre area'. Desert de Sarria-Setmenat Park is located on the northwest outskirts of Barcelona city (a suburban area). It is partly a natural forest, but there are several buildings within the grounds, as well as some orchards, and many visitors. In the text, we refer to this park as the 'suburban area'. Detailed descriptions of these two parks can be found in Björklund et al. (2010).

We determined the age and sex of the birds at capture according to Svensson (1992) and Jenni and Winkler (1994). We distinguished two age classes: yearlings (Euring codes 3 and 5, after their partial moult and before their first complete post-breeding moult), and adults (Euring codes 4 and >5, after their first complete post-breeding moult). Sample size for age × sex combinatory categories were: yearling males = 30, yearling females = 34, adult males = 38, adult females = 28.

Housing

Birds were individually housed under natural daylight in outdoor enclosures of 1 × 1 × 1.5 m at the Natural History Museum of Barcelona in Ciutadella Park. Each enclosure was connected to an observation room with one-way glass. The great tits had access to water ad libitum and were fed peanuts using a wire feeder hanging from the roof of the cage. They were regularly supplemented with mealworms *Tenebrio molitor* and waxworms *Galleria mellonella* provided on a white feeder located on the floor of the cage. This diet provides essential nutrients for the species (Finke 2002) and has been successfully used in the past for similar studies on the great tit (Cole and Quinn 2012). Each enclosure contained three perches and a nest box for sleeping.

Behavioural experiments

On the first day after capture, the birds were fed waxworms from the white feeder inside the individual enclosure. On the morning of the second day (8–12 a.m.), we performed a standard novel object test (Verbeek et al. 1994) using a pen light as a novel object. The pen was placed on the white feeder that contained six or seven waxworms (Drent et al. 2003; Verbeek et al. 1994). We measured the latency of the focal bird to approach the feeder (in seconds) within a period of 10 min. The test thus measures the boldness or neophobia of the bird in front of a novel (possibly dangerous) object. Birds were then left to continue with their activities for 1 h in the individual enclosure.

We next performed a standard novel environment test (Verbeek et al. 1994). Birds were introduced into another individual enclosure (50 × 40 × 40 cm) within the experimental room. After 30 min, the individual enclosures were opened, by means of a remote control string, to allow the birds access to the observation room. The size of the room was 3 × 2 × 2 m and it contained five artificial trees, as described in Verbeek et al. (1994). We observed the birds through one-way glass and recorded their movements on video camera. The number of flights and hops within the first 2 min after entering the room was used as the exploration score (Dingemanse et al. 2002). The test thus measures the tendency and predisposition of the bird to explore a novel environment. The exploration score was standardized by date using the residuals from the regression between exploration score (dependent variable) and number of days from 1 September (independent variable). Data were standardized within each year. This standardization allowed us to account for any possible seasonal variation in the exploration score (Dingemanse et al. 2002; Quinn et al. 2009). As this standardization is used in similar studies (e.g. (Dingemanse et al. 2002; Quinn et al. 2009), our results allow for comparisons with previous papers.

Each bird was individually tested in the whole sequence of trials and used only once.

Molecular methods

We collected blood samples for DNA extraction from 126 of the 130 great tits (we were unable to extract DNA in four birds). Samples were stored in pure ethanol. DNA was extracted using an Ecogen Master Pure DNA Purification Kit (MCD85201). We used the primers and the polymerase chain reaction (PCR) protocol described in Fidler et al. (2007) to detect the *DRD4* SNP830 polymorphisms. DNA extractions and PCRs were done at the molecular laboratory at the Institut Botànic de Barcelona. The samples were run on a MegaBACE at Uppsala University. In addition to the birds that we brought into captivity for behavioural experiments ($n = 126$), we took advantage of the blood we had collected in previous periods (2003–2011) from urban and forest great tits, so that we finally genotyped 391 individuals for SNP830 *DRD4* (218 individuals from the forest and 173 individuals from the city). The birds sampled in previous years were collected from the same localities, using a similar protocol to that used in the behavioural analyses.

Statistical analyses

Birds were used only once in each of the personality experiments to avoid pseudo-replication. We repeated the exploration score assay for 15 individuals and repeatability was on average 40 %, comparable with previous studies in great tits (Dingemanse et al. 2002).

The relationship between exploration score and habitat was analysed using ANOVA models, with the exploration score as the dependent variable, and age, sex and habitat (forest/suburban/city centre) as categorical independent variables. In relation to the novel object test (i.e. boldness), the response included censored observations (birds that did not approach the novel object). As applying standard statistical methods to censored data, or not taking them into account, can lead to biased estimates, we applied a stratified Cox proportional hazards regression model (Budaev 1997). Latency for approaching the novel object was the dependent variable, analysed using Cox analysis according to age, sex and habitat (forest, suburban or city centre) as independent variables.

To analyse the correlation between boldness and exploration, we again used a stratified Cox proportional hazards regression model, with latency for approaching a novel object (i.e. boldness) as the dependent variable, and exploration score, sex and age as independent variables. Sex and age were converted to dummy 0/1 variables to allow a regression approach. Since in this part of the analysis we needed to analyse the correlation within each habitat separately, and since sample size for the forest area was 73 birds, while for the city centre and suburban area it was only 21 and 36 birds, respectively, lack of correlation between boldness and exploration could be due to a small sample size rather than to a genuine lack of correlation. For this reason we decided in this and the following analyses to pool city centre and suburban birds into a single urban class ($n = 57$), thereby increasing the power of our tests.

To investigate the association between the *DRD4* SNP830 genotype and the exploration score, we applied a generalized linear mixed model in the R package glmmADMB (Skaug et al. 2013). Two genetic models were used, including the additive genetic effect of the SNP830 alleles (additive genetic model) and the dominance effect of the T allele (dominant-T genetic model), following Fidler et al. (2007). We used a Cox proportional hazards analysis to assess the association between boldness and *DRD4* SNP830. All the analyses considered exploration score and latency to approach the novel object as dependent variables, and habitat and genotypes as categorical factors. We used the χ^2-test to see whether genotype frequencies in each population followed the Hardy-Weinberg equilibrium and to test whether the genotype frequencies differed between habitats.

Statistical analyses were performed using STATISTICA 8 (StatSoft 2013) and R software (R Development Core Team 2011).

Ethical note

The test procedure involved a period of 48 h in which birds were removed from their natural environment, following a procedure similar to that of Dingemanse et al. (2002). All the birds were released on the second day at the site of capture, where a supply of peanuts was provided to allow birds to feed before dusk. As in the study of Dingemanse et al. (2002), we found no adverse effects regarding the test procedure. Changes in body weight between capture and release ($x \pm$ SE change; males, 0.95 ± 0.03 g; females, 0.93 ± 0.08 g) were well within the great tits' natural range of diurnal mass fluctuations (Balen 1967; Macleod et al. 2005), and within the range found in similar experiments in captivity (Cole et al. 2011; Dingemanse et al. 2002). Previous work has shown that male great tits do not usually lose their territory when removed for less than 48 h (Dingemanse et al. 2002; Krebs 1982). Behaviour during captivity was closely monitored to detect any deterioration in health, but birds invariably began feeding shortly after they were transferred into their housing cages and no health problems were detected. We extracted 30–50 µl of blood from each bird by puncturing the ulnar vein. This quantity and procedure are similar to those used in other studies on great tits and have previously been shown not to harm the birds (Lubjuhn 1998). Permission for handling, transport, blood taking and short-term housing of free-ranging great tits was granted by the Department of Environment and Housing of the Generalitat de Catalunya (2012-SF/518, 2013-SF/677 and 2014-SF/090).

Results

Behavioural responses in an urbanization gradient

We found that great tits from urban localities (both the city centre and suburban area) were more explorative than forest birds ($F_{2,118} = 4.59$, $p = 0.01$, $n = 130$) (Fig. 1). The exploration score between birds from the city centre and the suburban area did not differ ($F_{1,55} = 0.12$, $p = 0.73$, $n = 57$). The exploration score was independent of the sex and age of the birds (sex, $F_{1,118} = 1.55$, $p = 0.22$; age, $F_{1,118} = 0.39$, $p = 0.53$; interaction, $F_{1,118} = 0.92$, $p = 0.92$, $n = 130$).

Great tits differed in time to approach the novel object according to locality (Cox analysis $\chi^2 = 6.13$; $p = 0.047$) (Fig. 2). City centre urban birds approached the novel object faster than forest birds, and suburban birds showed intermediate values between city centre and forest birds (Fig. 2). Although it did not reach significance, there was a

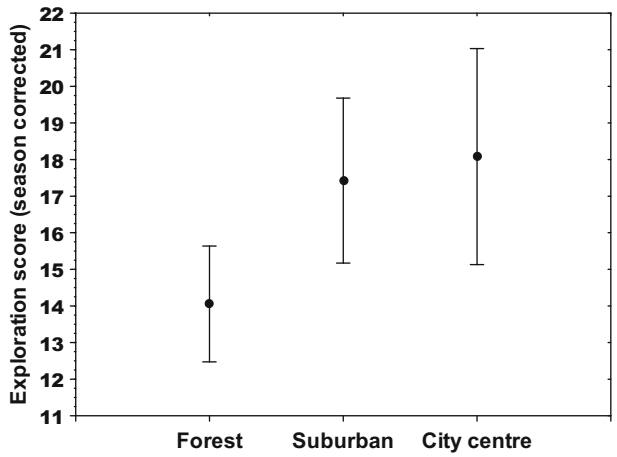

Fig. 1 Exploration score of wild great tits (corrected by seasonal trend), measured as the number of movements during a period of 2 min in a standard room with five artificial trees, according to a gradient of urbanization from the city centre to the suburban area, and then to nearby forests

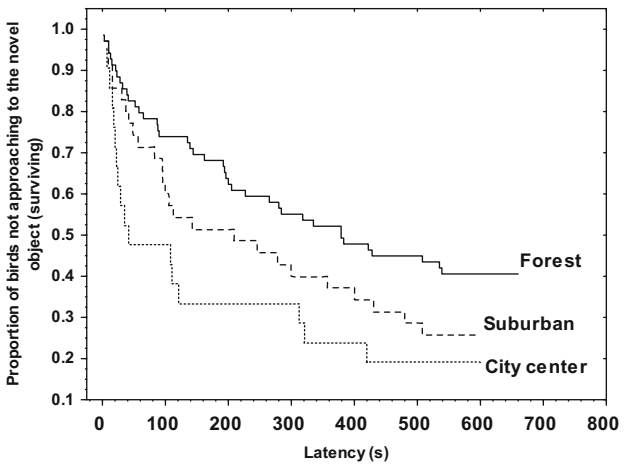

Fig. 2 Survivorship plot function based on Cox analysis for latency to approach a novel object (a penlight battery on the feeder) by wild great tits, according to an urbanization gradient. The figure shows the proportion of great tits that did not approach the object (*surviving*) in the 10-min time interval. City centre birds approached the novel object faster than suburban and forest birds

tendency for males to approach the novel object faster than females (sex, $Z = 1.94$; $p = 0.05$). No significant age effect was detected in latency to approach the novel object (age, $Z = 1.36$; $p = 0.17$).

Correlation between behavioural responses

As explained in the Materials and methods, here and in the following analyses we pooled birds from the city centre and suburban area into a single urban class to increase the power of our tests. When testing whether the two measures of personality (time to approach the novel object and exploration rate) were correlated, we found a significant interaction between exploratory behaviour and habitat (Cox analysis Wald statistic = 7.66; $p = 0.01$; $n = 130$; dependent variable, time to approach a novel object; independent factors, exploration score, habitat, sex and age). Hence we analysed the relationship between response to a novel object and exploratory behaviour separately for the two habitats (urban vs. forest). Exploratory behaviour correlated with response to a novel object for the forest habitat (Cox analysis Wald statistic = 9.27; $p = 0.002$; $n = 73$), but the correlation was weaker and insignificant for the urban habitat (Cox analysis Wald statistic = 0.42; $p = 0.51$; $n = 57$) (in both cases standardizing for sex and age effects, both of which were non-significant).

DRD4 SNP830 polymorphisms

Urban and forest populations did not significantly deviate from the Hardy-Weinberg equilibrium (urban, $\chi^2 = 0.01$, $p = 0.90$, $n = 173$; forest, $\chi^2 = 1.35$, $p = 0.24$, $n = 218$). However, genotype frequencies differed between the two habitats ($\chi^2 = 9.997$, $df = 2$, $p = 0.006$; Table 1). This was mainly due to a higher proportion of the T allele in the birds in the forest habitat than in birds in the urban habitat (forest, 0.53; urban, 0.42; $\chi^2 = 9.42$, $df = 1$, $p = 0.002$).

Association between genetic polymorphisms and behaviour

The exploration score was not related to *DRD4* SNP830 polymorphisms, standardizing for habitat effects. This result was consistent both for an additive and a T-dominant model (Table 2). However, the additive model showed an interaction between genotype and habitat (Table 2). The analysis of the variation in exploration score by habitat showed that while exploration was not related to genotype in the forest birds, the relationship was close to significance in the urban habitat, thus heterozygotes showed a higher exploration score than homozygotes (Table 2; Fig. 3).

Latency to approach a novel object was not related to *DRD4* SNP830 polymorphisms, standardizing for habitat effects (Cox Additive model, $\chi^2 = 1.31$, $p = 0.52$; Cox T-dominant model, $Z = -0.61$, $p = 0.54$, $n = 126$).

Table 1 Cross tabulation of the number of great tits with the three possible *DRD4* SNP830 genotypes according to habitat [forest vs. urban (urban and suburban birds pooled)]

Genotypes	Forest		Urban	
	Obs.	Exp.	Obs.	Exp.
CC	52	61	58	49
CT	100	103	85	82
TT	66	54	30	42

Observed (*Obs.*) and expected (*Exp.*) frequencies are provided

Table 2 Results from the general linear model analysis of the variation in exploratory behaviour (movements within 2 min in a standard experimental room, standardized by date) in relation to *DRD4* SNP830 genotypes (CC, CT and TT) and habitat (forest vs. city (urban and suburban birds pooled)]

	β	SE *β*	*F*	*df*	*p*
Additive genetic model					
Habitat	0.18	0.09	3.63	1.120	0.06
Genotype	0.07	0.09	0.5	2.120	0.61
Habitat × genotype	0.25	0.10	3.41	2.120	0.04
Forest					
Genotype	0.17	0.13	0.86	2.67	0.43
City					
Genotype	0.31	0.13	3.11	2.53	0.05
Dominant T genetic model					
Habitat	0.2	0.09	4.47	1.122	0.04
Genotype	0.09	0.09	0.97	1.122	0.33
Habitat × genotype	0.17	0.09	3.19	1.122	0.08

Analyses are provided both for an additive genetic model and for a dominance effect of the T allele (*Dominant T genetic model*). Since the interaction between habitat and genotype was significant for the additive model, we provide additional tests of variation in the exploration rate by genotype in the two habitats separately. We found this variation was close to significance in the city birds but not in the forest birds

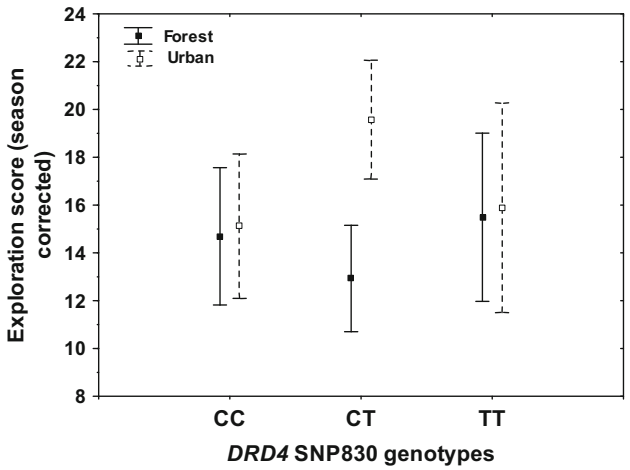

Fig. 3 Exploration scores (corrected by seasonal trend) of wild great tits in forest and urban habitats in relation to the *DRD4* SNP830 genotype. *Black circles* and *black lines* represent forest great tits and *white squares* and *dashed lines* represent urban great tits

Discussion

Previous work on great tits in Barcelona city has shown that urban birds and forest birds are subjected to different selection pressures (Senar et al. 2014), to the point that they have diverged genetically (Björklund et al. 2010). Because personality is a heritable trait subject to natural selection (Dingemanse et al. 2002; Dingemanse and Réale 2005; Drent et al. 2003), one of the differences between urban and forest great tits could depend on personality (Senar et al. 2014). Accordingly, our results show that urban great tits are more explorative and less neophobic than forest birds. Although our results agree with those found in house sparrows *Passer domesticus* (Bókony et al. 2012), they contrast with those of urban blackbirds that have been found to show higher levels of neophobia than forest birds (Miranda et al. 2013). These findings show that, although natural selection can shape the personality of urban birds, the specific manifestation of personality can vary between species, perhaps because the adaptive advantage of different personalities in different environments can vary according to the ecology of the different species.

Combinations of different behavioural traits might evolve together through correlational selection, creating adaptive behavioural syndromes. If the conditions (e.g. environment) change, and selection starts acting negatively on one of the traits, the existing genetic correlation could hamper the other traits from reaching their optimal expression, and the behavioural syndrome would become an evolutionary constraint (Lynch and Walsh 1998). A recent view, however, suggests that natural selection could favour genetic modifiers that would decouple maladaptive syndromes, promoting adaptive phenotypes with the right set of behavioural traits (Karlsson Green et al. 2016; Sih et al. 2004). Decoupled correlations between personality traits could therefore be a sign of an adaptive process in the urban environment. In our experiment, exploratory behaviour correlated with boldness in the forest birds, providing evidence of a behavioural syndrome, but this correlation disappeared for the urban-dwelling great tits. Hence, and similarly to other species, behavioural traits that usually correlate in the forest or rural habitats decoupled in the highly variable urban habitat, supporting the view that birds are showing a microevolutionary process of adaptation to cities (Bókony et al. 2012; Evans et al. 2010a; Scales et al. 2011).

Although urban tits in the city of Barcelona were more explorative and showed a shorter time to approach novel objects than forest birds, the former have been shown to display a higher stress response to capture than forest birds (Torné-Noguera et al. 2014). This suggests that although great tits are highly explorative in the urban habitat, where it may be advantageous, they are also highly reactive when facing potential dangers, perhaps because dangers are more variable, less abundant and less predictable in the city (Adams et al. 2006). Another apparently contradictory result relates to dispersal behaviour. More exploratory birds have been shown to disperse larger distances than less exploratory birds (Dingemanse et al. 2003; Korsten et al. 2013; Quinn et al. 2011; Roulin 2013; Saino et al. 2014).

Hence we would expect a higher dispersal in our urban great tits than in the forest great tits. However, in a previous study, we found just the reverse: urban birds in Barcelona showed lower levels of dispersal than forest birds (Björklund et al. 2010). Altogether, this again supports an adaptive view of behavioural correlations weakening in urban habitats (Bókony et al. 2012; Scales et al. 2011; Sih et al. 2004).

According to our prediction, we found differences between urban and forest birds in genotype frequencies of *DRD4* SNP830. These results, however, were contradictory: urban birds, more exploratory and bolder than forest birds, showed a lower proportion of the T allele than their forest counterparts. Surprisingly, in previous studies (Fidler et al. 2007; Korsten et al. 2010), the T allele was associated instead with higher exploratory behaviour. We found that urban heterozygotes had a higher exploration score than forest ones. This could be related to gene-environment interactions (Mueller et al. 2013), but recent work has shown that methylation at this locus is not related to the patterns found (Riyahi et al. 2015), so that no clear explanation is yet available. In any case, exploration and boldness scores showed no clear correlation with the *DRD4* SNP830 in our populations, so we cannot interpret these differences in genotype frequencies in a behavioural context. Clearly, and as recently stated, the relationship between the *DRD4* SNP830 polymorphism and personality is not sufficiently clear to allow generalizations (Fidler et al. 2007; Korsten et al. 2010; Mueller et al. 2013; Tschirren and Bensch 2010). Future research considering additional candidate genetic and epigenetic variants within serotonergic and other dopaminergic genes may show a closer association with personality (Mueller et al. 2013; Riyahi et al. 2015).

In conclusion, great tits in the city of Barcelona were more explorative and showed a shorter time to approach novel objects than forest birds. Our results suggest that behavioural traits that commonly form a behavioural syndrome in the wild can decouple in urban environments (Bókony et al. 2012; Evans et al. 2010a; Scales et al. 2011). This decoupling may help to enhance adaptation to urban habitats.

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PAPER 2

Combined epigenetic and intraspecific variation of the *DRD4* and *SERT* genes influence novelty seeking behavior in great tit *Parus major*

Resumen en castellano

La metilación del ADN es uno de los mecanismos epigenéticos principales que regulan la expresión génica y es una vía importante para crear variación fenotípica. En el presente estudio, hemos descrito el perfil de metilación de dos genes candidatos asociados a rasgos de personalidad, *DRD4* y *SERT*, en el carbonero común (*Parus major*) para determinar si los rasgos de personalidad y comportamiento entre diferentes hábitats han evolucionado bajo la influencia de la variación epigenética. Hemos realizado PCRs de bisulfito y secuenciación especifica de hebra para determinar el perfil de metilación de los dinucleótidos CpG en los promotores de *DRD4* y *SERT*, así como también en la isla CpG localizada el exón 3 de *DRD4*. Además, mediante pirosecuenciación, cuantificamos el nivel total de metilación en cada sitio CpG. Nuestros resultados indicaron que la metilación era 1-4% mayor en la población de aves urbana que en la procedente del bosque para todos los *loci* y tejidos analizados, sugiriendo que éstas modificaciones epigenéticas están influenciadas por condiciones ambientales. El cribado de la secuencia de ADN genómico del promotor *SERT* reveló que se trata de una región pobre en sitios CpGs. La metilación en dinucleótidos CpGs individuales localizada a 288 pb desde el inicio de la transcripción se relacionó con el comportamiento de exploración en aves urbanas. Además, los genotipos del polimorfismo SNP234 localizado en el promotor mínimo de *SERT* correlacionaron significativamente con el comportamiento de búsqueda de novedad bajo cautividad, siendo el alelo asociado a este comportamiento más frecuente en la población de aves urbana. En conclusión, parece ser que tanto la variabilidad genética como la metilación del gen *SERT* tienen un papel importante en la formación de rasgos de personalidad del carbonero común, mientras que la variación genética y de metilación del gen *DRD4* no se encuentran muy implicadas en los rasgos de personalidad ni comportamiento.

RESEARCH PAPER

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Combined epigenetic and intraspecific variation of the *DRD4* and *SERT* genes influence novelty seeking behavior in great tit *Parus major*

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Keywords: *DRD4*, methylation, novelty seeking, personality traits, *SERT*, urbanization

DNA methylation is one of the main epigenetic mechanisms that can regulate gene expression and is an important means for creating phenotypic variation. In the present study, we performed methylation profiling of 2 candidate genes for personality traits, namely *DRD4* and *SERT*, in the great tit *Parus major* to ascertain whether personality traits and behavior within different habitats have evolved with the aid of epigenetic variation. We applied bisulphite PCR and strand-specific sequencing to determine the methylation profile of the CpG dinucleotides in the *DRD4* and *SERT* promoters and also in the CpG island overlapping *DRD4* exon 3. Furthermore, we performed pyrosequencing to quantify the total methylation levels at each CpG location. Our results indicated that methylation was ~1–4% higher in urban than in forest birds, for all loci and tissues analyzed, suggesting that this epigenetic modification is influenced by environmental conditions. Screening of genomic DNA sequence revealed that the *SERT* promoter is CpG poor region. The methylation at a single CpG dinucleotide located 288 bp from the transcription start site was related to exploration score in urban birds. In addition, the genotypes of the *SERT* polymorphism SNP234 located within the minimal promoter were significantly correlated with novelty seeking behavior in captivity, with the allele increasing this behavior being more frequent in urban birds. As a conclusion, it seems that both genetic and methylation variability of the *SERT* gene have an important role in shaping personality traits in great tits, whereas genetic and methylation variation at the *DRD4* gene is not strongly involved in behavior and personality traits.

Introduction

The behavioral system of vertebrates is thought to be controlled via the dopaminergic and serotonergic neurogenic systems.[1] Dopamine receptor D4 (*DRD4*) and serotonin transporter (*SERT*) are 2 prime candidate genes for personality traits. Single nucleotide polymorphisms (SNPs) within the third exon of the *DRD4* gene are related to variation in novelty seeking behavior in humans and other mammals.[2-9] In birds, the great tit (*Parus major*) is a classic model species to study avian personality, and an association between *DRD4* SNP830 and exploratory behavior has also been described.[10]

However, the relationship between genes and behavior appears far more complex than previously described, as the relationship holds in some populations but not in others.[11] This made Mueller et al. [12] suggest that the link between *DRD4* gene and personality is context-dependent, probably mediated by certain characteristics of habitat at a local scale. In a recent study, we found that urban and forest great tits differed greatly in novelty seeking behavior, but we were unable to link this variation in behavior to *DRD4* SNP830 (Riyahi & Senar personal observation). Heterogeneous association of *DRD4* gene with exploratory behavior in different environments suggests a possible gene-gene or gene-environment interaction.[11]

In addition to *DRD4*, the *SERT* (also known as *SLC6A4*) gene is recognized as a candidate gene for anxiety, harm avoidance, and other behavioral syndromes in humans. A recent study on 12 paired urban and rural populations of the blackbird *Turdus merula* found a highly significant divergence at an exonic microsatellite of *SERT* gene between habitats.[13] They concluded that the *SERT* gene can be considered as one of the candidate loci for local adaptation to novel conditions and urbanization. Hence, the possible gene-environment interaction has been suggested in both *DRD4* and *SERT* genes. One of the ways to detect gene-environment interaction is investigating epigenetic modifications. DNA methylation is one of several epigenetic modifications that can directly affect gene expression. Any change in environmental condition can potentially modify the phenotype through epigenetic

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modification.[14-16] In many cases, epigenetic marks are not inherited in a Mendelian fashion but different lines of evidence suggest that genetics can influence the epigenetic marks.[17,18] Currently, several studies have shown the effect of the genotype on the epigenotype. Particularly, SNPs can influence DNA methylation, a phenomenon referred to as allele-specific methylation (ASM) or methylation quantitative trait loci (meQTL).[19]

Associations of DNA methylation in the *SERT* and *DRD4* genes and personality have been described before in humans. In humans, prenatal exposure to maternal depression affects methylation patterns of the *SERT* promoter.[20,21] Moreover, the methylation level of the *SERT* promoter was associated with abuse during childhood.[22] In addition, the DNA methylation level of both *DRD4* and *SERT* genes were negatively associated with attention deficit hyperactivity disorder symptom score.[23] Furthermore, in genetically identical twins, methylation differences of *SERT* and *DRD4* genes have been described, which are mostly attributable to environmental factors.[24] As a result, methylation is a strong candidate mechanism to explain differences in personality between habitats. The urban habitat, due to rapid changes in diet as well as environmental pollution, is undoubtedly impacting not only the human epigenome, but also the evolution of many other species. Since urban habitats have independently been found to affect levels of DNA methylation through pollution effects[25] and also personality traits,[13] we hypothesize that differences in personality between urban and forest *Parus major* populations could be related to variation in methylation.

Since the promoter region of a gene primarily influences transcript expression, in this study we determine the methylation patterns of the *DRD4* and *SERT* gene promoters in the great tit from 2 different environments, city and forest. In addition, we investigate the potential function of SNP830 of the *DRD4* gene, which has previously been found to relate to exploratory behavior,[10,11] by looking at the DNA methylation profile of the flanking interval. The great tit inhabiting Barcelona city is an ideal model species to test for that, since the urban population is genetically isolated from the surrounding forest populations[26] and urban birds display higher levels of exploration and novelty seeking behavior compared to the nearby forest (Riyahi & Senar personal observations).[27]

This study aims to answer the following specific questions: (1) Are *DRD4* and *SERT* promoters differently methylated in urban and forest great tits? (2) Is novelty seeking behavior linked to the level of DNA methylation in the promoter of these 2 genes? (3) Is methylation of *SERT* and *DRD4* promoters associated with allelic states at nearby SNPs? In particular, does the synonymous substitution SNP830 in the *DRD4* gene, associated with diversity in the exploratory behavior in the great tit,[10,11] exert its effect by influencing methylation at nearby CpG sites?

Methods

DNA sample set

We obtained blood samples from 96 different birds that were captured from October to March in 2012 and 2013 for personality experiments in captivity. The sample was composed by 46 forest birds from Can Catà field station, located in Collserola National Park, close to Barcelona city (3 km). It is a mixed forest consisting mainly of pure oak (*Quercus ilex* and *Quercus cerrioides*) located at the bottom of the valleys and Aleppo pine (*Pinus halepensis*) forests in the upper hills. A further 50 city birds were captured at 2 different urban parks in Barcelona city (Ciutadella Park and Setmenat-Sarria Park). The blood samples were stored in pure ethanol at 4°C. Finally, we collected brain biopsies from 13 great tits (from city and forest) that died during fieldwork or in captivity. DNA was extracted using Ecogen MasterPure DNA Purification kit (MCD85201).

Gene sequencing

The genotyping for SNP830 of *DRD4* in all the samples was performed following the protocol described by Fidler et al.[10] Additional polymorphisms within the *DRD4* and *SERT* loci were genotyped by standard PCR amplification and direct sequencing of the resulting amplicons. Approximately 200 ng genomic DNA was amplified in 25 μl reactions containing 1x NH4 reaction buffer, 25 ng of each oligonucleotide primer, 0.2 mM of each dNTP, 1.5 mM MgCl2 and 2 units DNA Taq DNA Polymerase (Bioline). PCR was performed for 35 cycles (see **Table 5** for primer sequences). The resulting amplicons were purified using ethanol precipitation and subsequently sequenced in both directions using the BigDye Terminator reaction kits on an ABI 3730 DNA analyzer (PE Bio-systems). The sequence electropherograms were interrogated using Sequencherv4.6 (Gene Codes Corporation, MI, Ann Arbor, USA) to distinguish heterozygous and homozygous samples. The sequence of *SERT* gene and SNPs has been deposited in GenBank: accession number KP869099.

Bisulphite treatment and strand-specific methylation analysis

Approximately 1 μg DNA was subjected to sodium bisulphite treatment and purified using the EZ GOLD methylation kit (ZYMO, Orange, CA, USA) and was used for bisulphite PCR analysis. Bisulphite PCR primers for each region (see **Table 5** for primer sequences) were used with Hotstar Taq polymerase (Qiagen, Crawley, UK) at 40 cycles and the resulting PCR product cloned into pGEM-T easy vector (Promega, Madrid, Spain).

Quantitative methylation pyrosequencing

Pyrosequencing was used as an accurate method of quantifying total methylation at CpG dinucleotides within a PCR amplicon. Standard bisulphite PCR was used to amplify across the region of interest with the exception that the reverse primers were biotinylated. The entire biotinylated PCR product (diluted to 40 μl) was mixed with 38 μl of binding buffer and 2 μl (10 mg/ml) streptavidin-coated polystyrene beads. Bead-amplicon complexes were captured on a vacuum prep tool (Qiagen, Crawley, UK) and the PCR products denatured using 0.2 M NaOH. The denatured DNA was resuspended in 40 pmol of sequencing primer dissolved in 12 μl water and primers annealing was achieved by heating the sample to 80°C for 2 min before cooling to room temperature. For

sequencing, internal forward primers were designed to the complementary strand (see **Table 5**). The pyrosequencing reaction was carried out on a PyroMark Q96 instrument (Qiagen, Crawley, UK). The interrogated peak heights of C/T variants at CpG dinucleotides were determined using the pyrosequencing commercial software.

Phenotyping

Two different standard tests were performed. Each bird was tested alone in the whole sequence of tests and used only once. On the morning of the second day after capture, we performed a standard novel object tests inside the individual cages (1 × 1 × 1.5 m) using a pen light battery put on the feeder where we had previously introduced a few mealworms.[28,29] We measured the latency to approach the feeder (in seconds) within a period of 10 min. Birds were then allowed to continue with their activities for one hour.

We then performed a standard novel environment test.[29] Birds were first introduced into an individual cage (100 × 40 × 40 cm) within the experimental room. After 30 min, the individual cages were opened with a remote control string to allow the birds to fly into the observation room. The size of the room was 3 × 2 × 2 m and contained 5 artificial trees, as in Verbeek et al.[29] We observed the birds from a one-way screen and we recorded movement of birds with a video camera. Number of flights and hops within the first 2 min after entering the room was used as exploration score.[30] Exploration score was standardized by date (days from September 1) to account for within-season temporal trends.[30,31]

Statistical methods

Comparisons between genomic DNA of different species were performed using BLASTN (http://www.ncbi.nlm.nih.gov). Screening for CpG islands (>200 bp; obs/exp 0.6; GC content >50%) utilized the CpG island finder bioinformatics tool (http://dbcat.cgm.ntu.edu.tw). To normalize the data, the average value of DNA methylation percentage were arcsin transformed. The relation between exploration score and DNA methylation of *SERT* and *DRD4* was tested using General Linear Model (GLM) in STATISTICA 8 software (StatSoft 2013). In relation to the novel object test, the response included censored observations (birds that did not approach the novel object). Applying standard statistical methods to censored data, or not taking them into account, can lead to biased estimates. Hence we applied a stratified Cox proportional hazards regression model, a specialized nonparametric regression survival analysis which deals with this problem,[32] to check the latency to approach to the novel object according to DNA methylation. We included the methylation levels of *SERT* and *DRD4* as independent variables and habitat (forest vs. city) as a grouping variable.

The association of each SNP with average CpG DNA methylation percentage of *DRD4* promoter was tested using GLM in STATISTICA 8 software. In addition, we used the same method to reveal SNP associations within the *SERT* promoter and DNA methylation.

Haplotypes were reconstructed with the PHASE program.[33] Population genetic statistics and neutrality tests such as Tajima's D were calculated using DNAsp5.10[34] and Arlequin3.11[35] software packages. Linkage disequilibrium was determined using Haploview.[36] Chi-square test was performed to check whether genotype frequencies of each SNP in each population followed the Hardy-Weinberg equilibrium and to test whether the genotypes frequencies differed between habitats. We used a Cox proportional hazards analysis to assess the association between neophobia (novel object test) and *DRD4* and *SERT* different SNPs one by one, and we estimated the association between exploration score in a novel environment and *DRD4* and *SERT* SNPs with General Linear model (GLM). Cox and GLM analysis were done using STATISTICA 8 (StatSoft 2013) and R software (R Development Core Team 2011).

Results

Extensive genotyping of the *DRD4* promoter interval in great tit

We designed 2 PCR assays which resulted in amplicons covering ~1.5 kb of the *DRD4* exon 1 and proximal promoter interval. Subsequent sequencing of the PCR products revealed 9 SNPs, 7 located in the promoter region (detailed in **Table 1**).

Table 1. Allele names and minor allele frequency (MAF) of each SNP for the *DRD4* gene in each population. Population sample size: 46 forest great tits and 50 urban great tits.

DRD4 locus	Major/minor allele(s)	location	MAF (forest)	MAF (urban)	HW *P*-value (forest)	HW *P*-value (urban)
1. SNP784	G/A	promoter	0	0.057	1	<0.001
2. SNP795	G/A	promoter	0.026	0.029	1	1
3. SNP830g	G/T	promoter	0	0.057	1	<0.001
4. SNP835	T/G	promoter	0.079	0.057	<0.001	<0.001
5.SNP876	T/G	promoter	0	0.057	1	<0.001
6. SNP1006	G/A	promoter	0.132	0.129	1	1
7. SNP1173	C/G	promoter	0.368	0.414	1	0.256
8. SNP1878	C/T	exon1	0.042	0.014	1	1
9. SNP1884	C/T	exon1	0	0.014	1	1
10.SNP830(10086)	C/T	exon3	0.180	0.150	0.97	0.98

Four of the SNP variants (SNP784, SNP830, SNP835, and SNP876) deviated from Hardy-Weinberg equilibrium in our urban population. Allele frequencies showed significant differences between the urban and the forest population only for SNP830 (10086), which is located in exon 3 ($\chi^2_2 = 9.99$, $P < 0.01$). Contrary to other northern Europe populations,[11,12] the In/del ID15 was not observed in our populations. Two SNPs were observed in exon 1 of the *DRD4* gene. One of these coding polymorphisms, SNP1884, resulted in an amino acid substitution, a change from alanine to valine in amino acid position 11. A valine at this position is conserved among passerine birds, while it is not found in other avian orders,[37] which makes the functional significance of this amino acid change uncertain. However, the functional prediction returned by SIFT (http://sift.jcvi.org) was "likely benign."

Characterization of the *SERT* promoter in great tit

The promoter sequence of the *SERT* gene has not previously been reported for the great tit. As a prerequisite for identifying possible genetic variants at this interval, we performed sequence homology comparisons between additional bird species for which genomic sequence was available to enable us to identify the orthologous region in great tits. We performed BLAST2 sequence analysis comparing the genomic sequence of common blackbird (*Turdus merula*) for which the *SERT* promoter interval was already available (Genbank accession number KC584781.1). We subsequently designed PCR primers to conserved regions identified in multiple species [Collared flycatcher (*Ficedula albicollis*), Atlantic canary (*Serinus canaria*), American crow (*Corvus brachyrhynchos*), Rock dove (*Columba livia*)] and performed PCR on DNA derived from great tits. The resulting ~850 bp

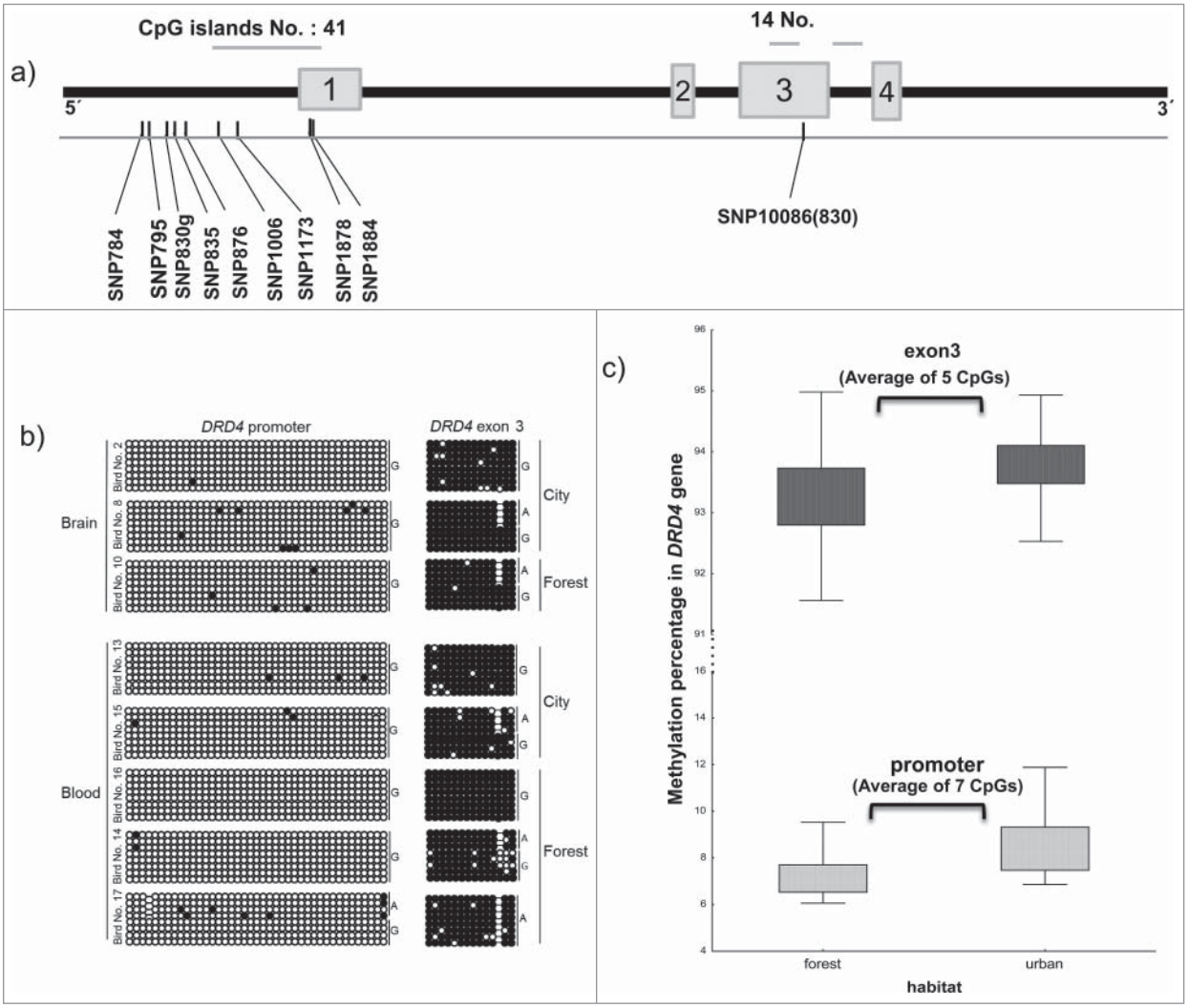

Figure 1. Structure and methylation profile of the *DRD4* gene. (**a**) Schematic representation of the *DRD4* gene. Horizontal bars show the number and location of CpG islands and gray boxes represent exons. The location of SNPs is shown by black bars. (**b**) Methylation status at *DRD4* locus in brain and blood-derived DNA samples. The left panel shows the methylation profile of the promoter and the right panel shows the results for the CpG island within exon 3. Each circle represents a single CpG dinucleotide on a DNA strand. (●): methylated cytosine; (○): unmethylated cytosine. (**c**) Methylation percentage of *DRD4* promoter and exon 3 regions comparing urban and forest populations. Differences are significant (see text).

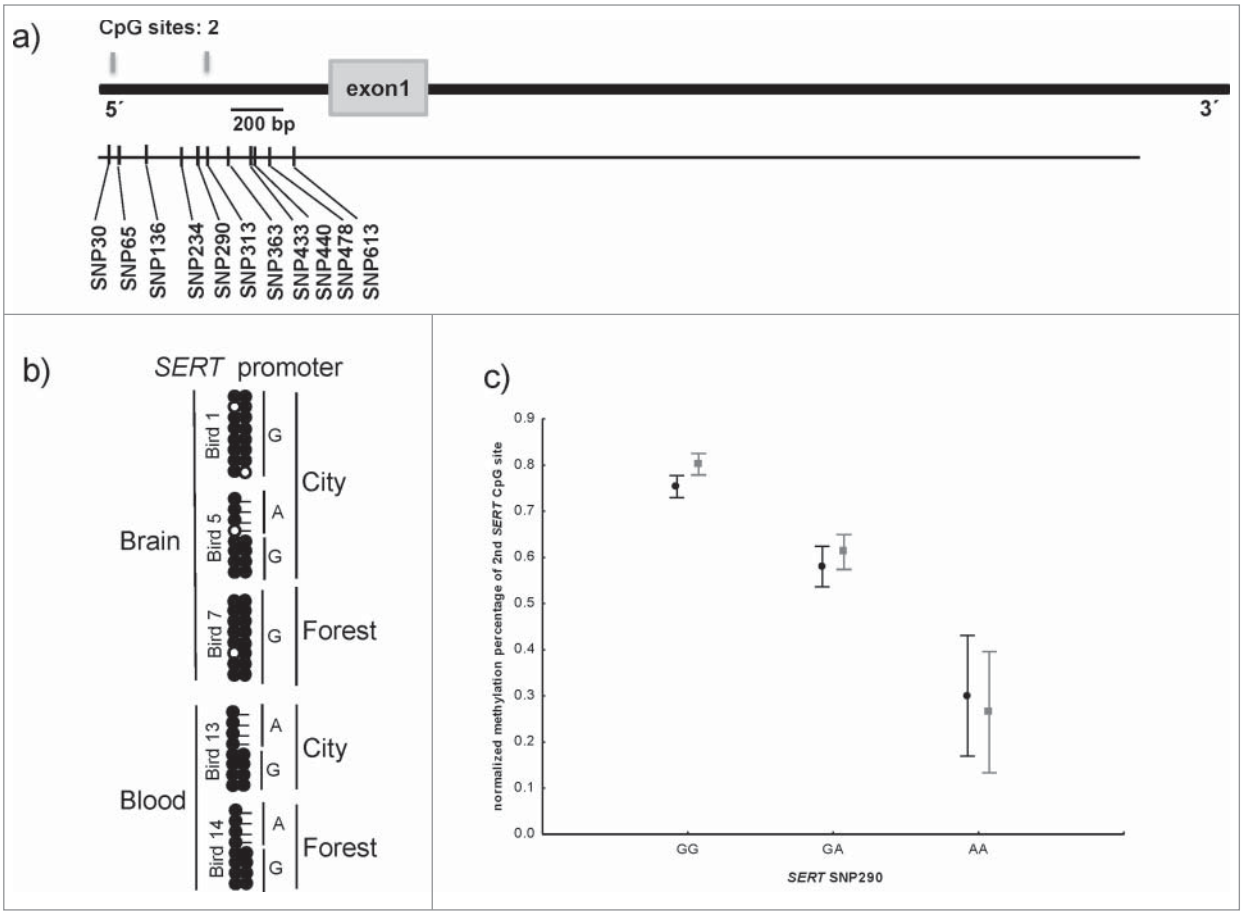

Figure 2. Structure and methylation profile of the *SERT* promoter. (**a**) The Schematic representation of *SERT* gene. Gray boxes showing the location of CpG sites and the gray box represents the exon. The location of SNPs is shown by black bars. (**b**) The methylation status at 2 CpG sites in the *SERT* promoter in brain and blood-derived samples. (**c**) Variation in methylation percentage of second CpG site in the *SERT* promoter, according to habitat and SNP290 genotype. The black data points represent forest birds and the gray urban birds. Methylation percentage was both affected by genotype and habitat, but the lack of significant interaction suggests that the difference between habitats was similar for the different genotypes (see text for tests).

PCR product was sequenced, revealing that the amplicon comprised *SERT* exon 1 and a 770 bp interval within the proximal promoter (**Fig. 2a**), which contained 11 SNP variants in our populations (**Table 2**). All of the SNPs were in the Hardy-Weinberg equilibrium except SNP65, in both populations. Allele frequencies were significantly different between urban and forest populations in SNP136 and SNP234 ($\chi^2_2 = 6.45$, $P = 0.039$; $\chi^2_1 = 9.96$, $P < 0.001$, respectively), so that AA genotype for

Table 2. Allele names and minor allele frequency (MAF) of each SNP for the *SERT* gene in each population. Population sample size: 46 forest great tits and 50 urban great tits.

SERT locus	Major/minor allele(s)	location	MAF (forest)	MAF (urban)	HW *P*-value (forest)	HW *P*-value (urban)
1. SNP30	t/g	promoter	0.088	0.022	1	1
2. SNP65	c/t	promoter	0.188	0.267	0.002	0.011
3. SNP136	a/g	promoter	0.238	0.111	1	0.144
4. SNP234	t/a	promoter	0.212	0.067	0.229	1
5. SNP290	g/a	promoter	0.138	0.156	1	1
6. SNP313	g/a	promoter	0.075	0.078	1	0.445
7. SNP363	a/g	promoter	0.05	0	0.150	1
8. SNP433	c/t	promoter	0	0.011	1	1
9. SNP440	c/a	promoter	0.262	0.211	0.476	0.573
10. SNP478	c/t	promoter	0.275	0.300	0.755	1
11. SNP613	g/t	promoter	0.295	0.350	1	0.972

SNP136 and TT genotype for SNP234 were more frequent in the urban population while GA (SNP136) and TA (SNP234) genotypes were more abundant in forest birds (**Table 2**).

Identification of CpG islands in great tit

Approximately 60% of gene promoters are associated with DNA sequences rich in CpG dinucleotides, termed CpG islands. These genomic features are usually unmethylated and permissive to transcription. Tissue-specific difference in expression can be explained by different methylation profiles with highly methylated regions being associated with robust gene silencing. To determine the methylation profiles of the *DRD4* and *SERT* promoters, we first performed a bioinformatics screen for CpG islands. This revealed that the *DRD4* locus contains 3 CpG islands. The largest encompasses the first exon and 5′UTR (1094 bp; GC 66%), whereas 2 smaller CpG island are intergenic overlapping exon 3 (350 bp; GC 52%) and within intron 3 (262 bp; GC 86%), respectively. Interestingly the same analysis on *SERT* sequence failed to identify any CpG islands, the interval being CpG sparse, containing only 2 CpG dinucleotides.

Methylation profiling of the *DRD4* promoter and exon 3 CpG islands

To determine the methylation profile of the 2 largest CpG islands in the *DRD4* locus in brain and blood-derived DNA, we performed bisulphite PCR and sequencing. To ensure that we would identify any genotype-dependent methylation effects, we performed the analysis on birds with different genotype combinations. This revealed that the *DRD4* promoter interval was robustly unmethylated in both blood and brain, whereas the CpG island overlapping exon 3 was fully methylated. The overall methylation profiles in samples with different genotypes were similar; however, 2 of the SNPs abolish CpG dinucleotides (SNP1878 and SNP1884) that would indirectly affect methylation at these sites. We observed no association between the synonymous SNP830 genotype and the methylation surrounding exon 3, suggesting that variation in methylation due to genotype does not explain the function for this SNP in relation to personality.

To allow for accurate quantification of methylation in an extended cohort of birds from different environments, we optimized the bisulphite PCRs for pyrosequencing. This allowed for 7 of the 41 CpGs included in the *DRD4* promoter PCR amplicons to be measured, while 5 of the 14 CpGs within the exon 3 CpG island PCR product would be quantified. The average DNA methylation level across all CpGs was 8% (SE = 0.16%) for *DRD4* promoter and 93% (SE = 0.08%) for the *DRD4* exon 3. Furthermore this analysis revealed, despite similar methylation profiles at these 2 genomic locations, that urban-dwelling great tits exhibited ~1% higher methylation levels than forest-dwelling birds (*DRD4* promoter: urban = 8.61±0.24, forest = 7.38 ± 0.18, F = 16.48, df = 1,89, $P < 0.005$; *DRD4* exon3: urban = 93.82 ± 0.09, forest = 93.19 ± 0.13, F = 14, df = 1,93, $P < 0.005$).

Allele-specific methylation at the *SERT* promoter in great tit

Bisulphite PCR encompassing the 2 CpG dinucleotides within the *SERT* gene promoter revealed that both sites were fully methylated in brain and blood-derived DNA samples consistent with reports for CpG-poor promoters.[38] One of the CpG dinucleotides, located 288 bp from the transcriptional start site (TSS), is abolished by a genomic variant (SNP290) in which the G nucleotide is replaced by an A variant, therefore behaving as a site of ASM. Bisulphite pyrosequencing enabled us to quantify the methylation of the 2 CpG dinucleotides in our extended cohort. Consistent with the allele-specific bisulphite PCR results, average methylation at the first CpG site within the *SERT* promoter was 83% (SE = 0.26%), with methylation level being ~2% higher in urban-dwelling than in forest great tits (F = 8.9, df = 1,94, $P < 0.005$). However, the methylation levels of the second CpG site clearly stratified into three groups dictated by SNP290 genotype: birds homozygous for the A allele were ~8.1% (SE = 0.55%) methylated, GA heterozygous individuals were ~31.3% methylated (SE = 1.03%) and homozygous G birds were ~49.0% methylated (SE = 0.85%) (**Fig. 2c**). Methylation percentage at *SERT* second CpG site was not only significantly affected by genotype but also by habitat, so that city birds showed ~3.9% hypermethylation than forest birds (urban: 46.89 ± 1.40%; forest: 43.01 ±1.49 %) (habitat: F = 5.69, df = 1,78, $P < 0.05$; SNP290 genotype: F = 116.74, df = 1,78, $P < 0.005$; habitat × genotype: F = 0.26, df = 1,78, $P = 0.60$) (we only considered here GA and GG genotypes because the AA genotype had a too small frequency in our populations to provide enough sample size). A similar genotype-associated methylation profile was also observed for SNP440 and SNP478, located 150–188 bp from SNP290, presumably because the variants are in linkage disequilibrium and the methylation profile is dictated by SNP290.

Table 3. Result of GLM on the variation in exploration score (season corrected) in relation to methylation. Results for the methylation percentage of (**A**) *DRD4* promoter, (**B**) methylation percentage of *DRD4* exon 3, (**C**) methylation percentage of *SERT* first CpG site and (**D**) methylation percentage of second *SERT* CpG site and habitat (forest vs. urban).

		F	Df	*P*
A)				
	Habitat	0.04	1,58	0.85
	methylation of *DRD4* promoter	0.3	1,58	0.58
	Habitat x methylation of *DRD4* promoter	0.11	1,58	0.74
B)				
	Habitat	2.77	1,62	0.10
	methylation of *DRD4* exon3	3.08	1,62	0.08
	Habitat x methylation of *DRD4* exon3	2.82	1,62	0.10
C)				
	Habitat	0.36	1,63	0.55
	methylation of *SERT* first CpG site	0.12	1,63	0.73
	Habitat x methylation of *SERT* first CpG site	0.42	1,63	0.52
D)				
	Habitat	2.84	1,59	0.10
	methylation of *SERT* second CpG	0.49	1,59	0.49
	Habitat x methylation of *SERT* second CpG site	3.9	1,59	<0.05

Trait association

DNA methylation in the *DRD4* promoter or in *DRD4* exon 3 was not significantly associated with personality scores, measured as time to approach to a novel object and exploration score in a novel environment (**Table 3a and b**). However, we found that percentage of methylation in *SERT* second CpG site was related to exploration score. Nevertheless, the relationship was complex, with exploration score showing a significant interaction between habitat and methylation percentage (**Table 3d**). Although no relationship was found between exploration score and methylation for forest birds (**Fig. 3b**), the relationship was marginally significant for urban birds (**Table 4**, **Fig. 3a**). No relationship was found for *SERT* first CpG site (**Table 3c**).

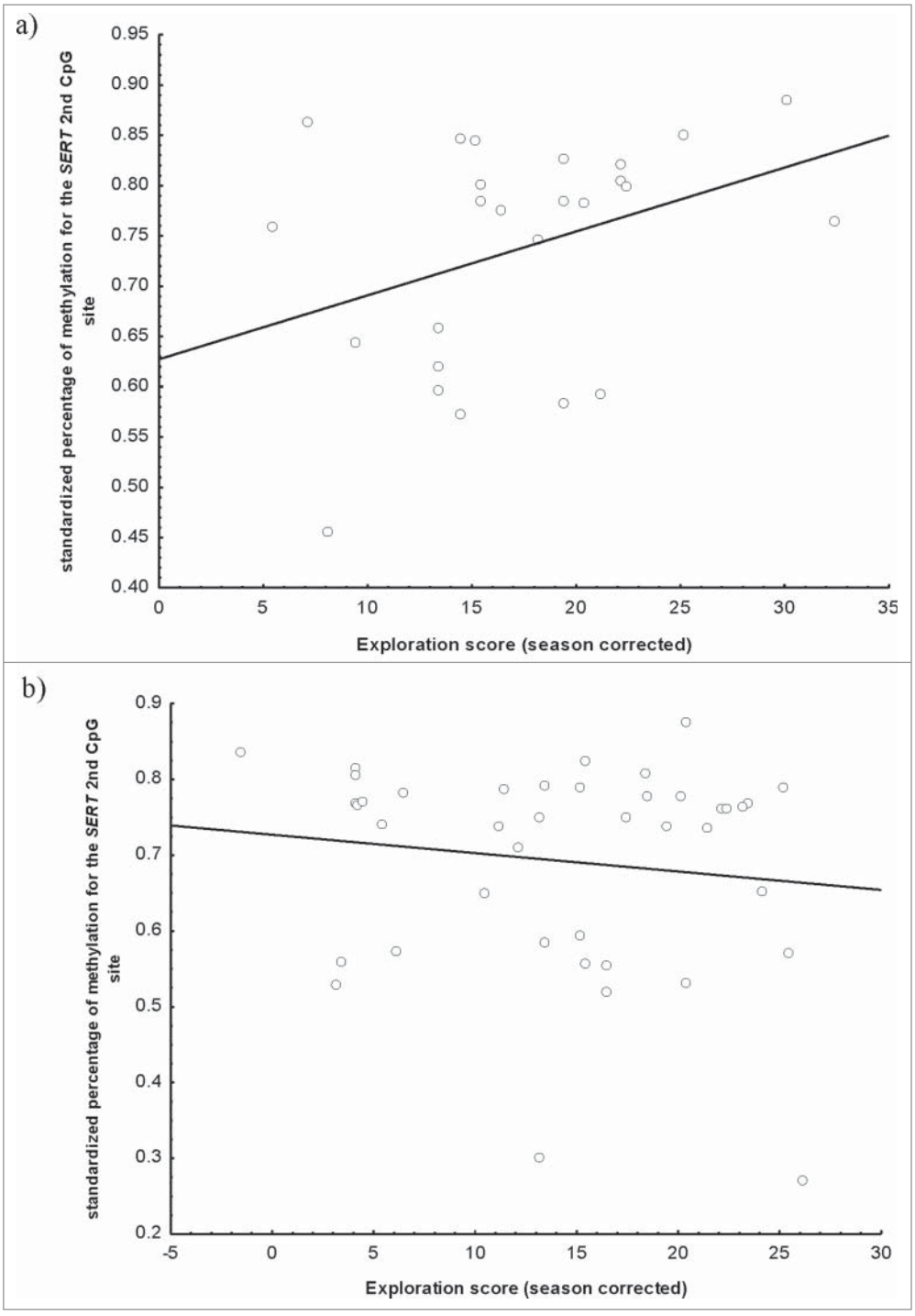

Figure 3. Exploration score of wild great tits, measured as number of movements during 2 min in a standard room with five artificial trees, in relation to methylation level of *SERT* second CpG site. We provide independent figures for urban (**a**) and forest (**b**) great tits (urban: r = 0.37, $P = 0.06$; forest: r = −0.13, $P = 0.40$).

No SNPs in the *DRD4* gene or in the *SERT* promoter were significantly associated with the behavioral traits tested (all $P > 0.42$), with one remarkable exception: *SERT* SNP234. This SNP showed highly significant association with the novel object test, with birds with TT genotype approaching faster to the novel object (Cox analysis $Z = 2.03$; $P < 0.05$; **Fig. 4**). The explained variance in response to novel object by SNP234 was 7%. Accordingly, and as shown before, the TT genotype was also significantly more frequent in the urban population while TA heterozygotes were more abundant in forest birds. The Tajima's D neutrality tests were not significant for *SERT* SNP234 in either population.

Discussion

Recent studies in human and laboratory animals have found a relationship between methylation changes and intraspecific variation in personality traits. For instance, rats treated with endocrine-disrupting chemicals showed changes in mate choice behavior due to methylation variation, even after 3 generations.[39] Here we found, for the first time in a free-living bird species, that variations in methylation levels, via a region of allele-specific methylation (ASM) within the *SERT* promoter, may be related to personality traits and may modify exploratory behavior in the urban-dwelling great tits. In

Table 4. Result of GLM on the variation in exploration score (season corrected) in relation to methylation percentage of *SERT* second CpG site. The results are provided for the 2 sampled populations: forest and urban.

Population	F	Df	*P*
Forest	1.03	1,38	0.32
Urban	3.57	1,23	0.07

addition, our results showed that methylation variation could be relevant in urbanized areas.

Methylation, habitat, and novelty seeking behavior

DRD4 and *SERT* have both previously been suggested as 2 prime candidate genes for personality traits and, therefore, have been the focus of our research. Regarding the *DRD4* gene, the only epigenetic difference we observed was associated with habitat and not tissue-specificity or underlying genotype. The ~1% difference in methylation between urban and forest-dwelling birds did not explain personality variations between habitats.

In *SERT* promoter, we found a similar ~2% hypermethylation in urban compared to forest birds at CpG dinucleotide located nearest transcriptional start site (TSS). However, intraspecific variation in methylation was far more extreme at the second CpG site, with levels dictated by the genotype of a single SNP irrespective of the habitat. The higher level of methylation within *DRD4* and *SERT* loci in urban birds might be due to some environmental conditions, for instance pollution. In support of this, air pollution has been previously shown to be related to whole genome hypermethylation in the sperm DNA of mice.[25] It remains to be determined whether additional loci show this habitat-associated gain in methylation, and if this occurs genome-wide in our great tit population.

Interestingly, we found that the relationship between methylation levels of the *SERT* second CpG site and exploration score interacted significantly with habitat, with more explorative urban birds tending to have higher levels of methylation at this site; however, no relationship was found for forest birds. Although there is no available study on wild birds, studies in human have shown how early environmental conditions affect *SERT* methylation differences between individuals and, hence, account for some inter-individual phenotypic variation.[40,41] This could explain habitat differences we have found in this study. Furthermore, the importance of early stress in shaping higher methylation in serotonin transporter gene and, consequently, health composite scores has been reported recently in monkeys.[42] Therefore, we suggest that early environmental conditions in the city may adjust methylation at this particular CpG site in the *SERT* gene, and this may affect adult behavior.

Differential methylation in the promoter of the *SERT* gene may lead to alteration in different gene expression levels (mRNA and protein). Nevertheless, the mechanism by which allele-specific methylation can affect *SERT* gene expression and, consequently, personality is still unclear. Future studies should provide further insights into the underlying mechanisms by which genetic and epigenetic modification in the *SERT* gene interact to shape personality traits.

Table 5. List of primers. The primers used for bisulphite conversion and pyrosequencing are marked in bold font

DRD4 genotype promoter 1F	GGGAAGGACAGTGCTTGGATCTG
DRD4 genotype promoter 1R	AGTGCTCTAGATAAGTTGGCAAATGCA
DRD4 genotype promoter exon 1F	CTCGGAGTAGACGTAGAGGGGCAGGAC
DRD4 genotype promoter exon 1R	AGGCTCCTCCCGCGGCTCGGCGGGCA
DRD4 promoter Bis F	**AGTAGTTGGTGGTGGTTTTGA**
DRD4 pyro seq F	**TGAGGAGGATGAGGAGGATGT**
DRD4 promoter Bis R	**CTAACATTACTTAAAAAACAACCC**
DRD4 exon 3 Bis F	**TGGGATAAGTTGGTATATTTTTTAT**
DRD4 pyro seq F	**TTTTAGTTATTTTTGATAGTTATT**
DRD4 exon 3 Bis R	**CAAAAAACTATATCACCCCCCAACC**
SERT gentype F	CATCTTCTCCTTTGCTACAGCC
SERT genotype R	ACAGAGCCTCAGAAGTTAGTTGA
SERT promoter Bis F	**TTAGGGGTTTTGTTTTATTGTTTGTG**
SERT pyro seq 1F	**GAGATTTGTTTTGGTTAT**
SERT pyro seq 2F	**GGTTATTAATTATTATAGTA**
SERT promoter Bis R	**CAAATTACCTACTCCATAATTC**

SERT SNPs and novelty seeking behavior

Previous studies had tried to link novelty seeking behavior and genetic variability. However, although earlier works found an association between *DRD4* SNP830 polymorphism and novelty seeking in great tits,[10] this association was only found in one population.[11,12,43] More recently, the *SERT* gene has been suggested as one of the candidate genes for local adaptation to novel conditions and urbanization.[13] Since we found that one of the main differences between urban and forest great tits probably relied on personality,[27,44] we thought that it could be valuable to look for a direct relationship between *SERT* polymorphisms, in addition to *DRD4*, and personality. Out of 21 SNPs (10 SNPs in *DRD4* and 11 SNPs in *SERT*), we found a highly significant association

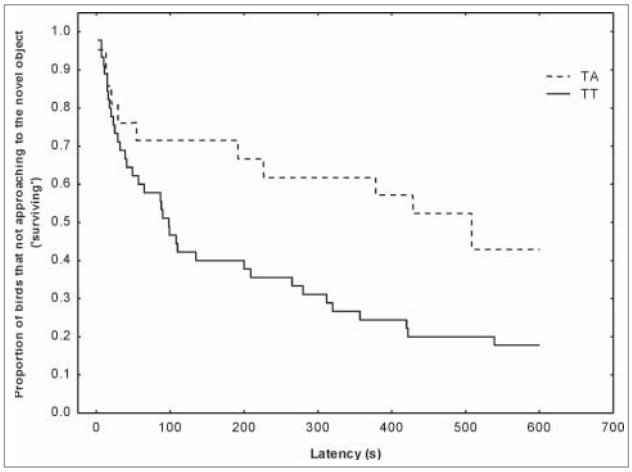

Figure 4. Survivorship plot function for latency to approach a novel object (a penlight battery on the feeder) by wild great tits according to *SERT* SNP234 genotypes. Black line represents TT birds and dash line represents TA birds. The figure shows the proportion of great tits that do not approach the object ('surviving') up to the respective time interval.

between *SERT* SNP234 and novelty seeking behavior: TT birds approached faster to a novel object than TA birds. Accordingly, TT birds were more frequent within the urban habitat. To explore whether this polymorphism is indeed adaptive, we performed Tajima's D neutrality tests, which were not significant in either population. In this case, adaptation would mean an increase in a pre-existing polymorphism, and natural selection based on standing variation is notoriously difficult to detect by neutrality testing.[45] The actual mechanism by which SNP324 affects personality remains to be known. Non-synonymous substitutions and distinctive codon bias have been reported in the same region of *DRD4* in Passeriform species, which may have contributed to the degree of behavioral diversity and potential for adapting to the novel environments such as big cities.[37]

Final considerations

As we know, natural selection can be triggered by epigenetic as well as genetic variation. As a result, DNA methylation is a potential source of inter-individual phenotypic variation.[46] Recent studies comparing plants in different environments came to the conclusion that the genome contains single site methylation polymorphisms in addition to SNPs.[47] These polymorphisms at the epigenetic level allow individuals in the populations to react differently to fluctuating environments.[48] Considering that, epigenetic modifications can occur much faster than genetic divergence,[49] methylation might have a role in shaping personality traits in newly formed environments. We believe that this can be a fructiferous future avenue of new research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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PAPER 3

No association between the melanocortin-1 receptor (*MC1R*) and black belly stripe size variation in the great Tit *Parus major*

Resumen en castellano

El carbonero común *Parus major* exhibe una banda negra de melanina en el pecho (corbata negra o raya negra del vientre), de alta variabilidad y cuyo tamaño está correlacionado con el éxito reproductor, la supervivencia, y la dominancia en los machos. Investigamos las asociaciones entre polimorfismos en el receptor melanocortina-1 (*MC1R*), que tiene una función importante en la coloración de la melanina, y el tamaño de la banda negra, pero no detectamos ningún polimorfismo en este gen. Por lo tanto, es posible que la variación en el tamaño de la banda de melanina esté regulada mediante variación genética en otros genes, o mediante modificación de la expresión génica en la vía de la melanocortina y melanogénesis.

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SHORT REPORT

No association between the melanocortin-1 receptor (*MC1R*) and black belly stripe size variation in the Great Tit *Parus major*

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Capsule The Great Tit *Parus major* displays a black melanin breast patch stripe (black tie or black belly stripe) which shows great variation and its size correlates with male breeding success, survival and dominance. We investigated for associations between the melanocortin-1 receptor (*MC1R*) polymorphism, which has an important function in melanin colouration, and the size of the black belly stripe but were unable to detect any polymorphism in this gene. Variation in the size of the melanin-based black belly stripe may therefore be regulated through genetic variation at other genes or via modification of the gene expression inside the melanocortin system and melanogenesis.

In recent years, several studies have investigated the genetics of melanin colouration in vertebrates. The melanocortin-1 receptor (*MC1R*) gene has been found to have an important function in melanin colouration (Roulin & Ducrest 2013). It can trigger eumelanin biosynthesis through activation of the intracellular cAMP signal pathway of melanocytes. Variation in the *MC1R* gene usually leads to a change in the production of both eumelanin and pheomelanin (Robbins *et al.* 1993).

The relatively large number of studies about genetics of colouration, up to now, have found an association between *MC1R* variants and plumage changes in different morphs in birds, mammals and reptiles (Ducrest *et al.* 2008, Hubbard *et al.* 2010). Several intraspecific studies in birds revealed that the amino acid substitutions within the *MC1R* gene are responsible for the different morphs in Lesser Snow Geese *Anser c. caerulescens*, Arctic Skuas *Stercorarius parasiticus* (Mundy *et al.* 2004), Bananaquits *Coereba flaveola* (Theron *et al.* 2001) and Red-footed Boobies *Sula sula* (Baião *et al* 2007). However, there is little information on how natural selection can genetically determine the size of melanin patches.

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The Great Tit *Parus major* displays a black melanin breast stripe (black tie or black belly stripe) which shows great variation between sexes and between individuals within the same sex (Gosler 1993). Males with bigger black belly stripes display higher survival (Senar *et al.* 2014) and higher breeding success (Norris 1990a, 1990b). The black belly stripe is in part a genetically determined trait (Norris 1993, Quesada & Senar 2009) and may also have an environmental component (Fitze & Richner 2002) which suggests that the variation continuum in size of the black belly stripe should have a genetic background. The Great Tit may therefore be an ideal species to test whether the variation found in the size of the black belly stripe is explained by *MC1R* polymorphism.

We used male Great Tit blood samples collected in Barcelona city and in Collserola national park close to Barcelona city. We measured the size of the black belly stripe from digital photographs of the breast, using an image tool program (Figuerola & Senar 2000). We selected 20 birds which had a large stripe (mean area = 474 mm^2, se = 17.8), 16 with a medium one (mean area = 344 mm^2, se = 7.6) and 10 with a small one (mean area = 272 mm^2, se = 6.0), compared to the average value of the population (average value = 366 mm^2, se = 62.34, total sample size = 220).

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We extracted the DNA from each individual and amplified the 817 bp of the coding region of *MC1R* gene including all sites known to have a function in colouration using these set of primers: MSHR72 (5'-ATGCCAGTGAGGGCAACCA-3') and MSHR9 (5'-CTGGCTCCGGAAGGCATAGAT-3') (Mundy *et al.* 2004). Polymerase chain reactions (PCRs) were performed in a 25-µl total reaction containing: 0.1 µl polymerase (Thermoprime Plus DNA polymerase 5U/µl,), 2.5 µl 10 × reaction buffer, 1.5 µl 25 mM magnesium chloride, 0.05 µl of each dNTP (25 mM), 1 µl of 10 µM each primer and 25–100 ng DNA. PCRs were performed with the following cycling parameters:

94°C for 2 minutes, 35 x: (94°C for 30 seconds, 63°C for 45 seconds, 72°C for 90 seconds), 72°C for 5 minutes. PCR products were directly sequenced by cycle sequencing using Big Dye v.2 (PE Biosystems) under standard conditions, and run on an ABI 377 sequencer. Sequences were edited in sequencher.

Belly stripe size of Great Tits ($n = 46$) was not associated with the melanocortin-1 receptor (*MC1R*) gene polymorphism, because all the sequences were equal. This suggests that variation in stripe size in Great Tits is not due to genetic variation of the *MC1R* gene. Future studies should focus on additional melanogenesis genes, including *MITF*, *ASIP*, *TYR* and *TYRP1*, as earlier found in other species (Chang *et al.* 2006, Minvielle *et al.* 2010, Lehtonen *et al.* 2011). Furthermore, other mechanisms of gene expression alterations such as mRNA expression and/or epigenetic alteration in the *MC1R* gene promoter should be considered (Fontanesi *et al.* 2010, Emaresi *et al.* 2013, Scriba *et al.* 2013). Finally, we suggest that the architecture of phenotypic variation for this kind of small-scale variation such as black belly colouration may have a regulatory background rather than genetic polymorphism in the coding regions.

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PAPER 4

Single origin of human commensalism in the house sparrow

Resumen en castellano

La distribución actual y ubicua del gorrión común (*Passer domesticus*) es una consecuencia de su relación comensal con los humanos. Se ha especulado que, mucho antes del advenimiento de la agricultura, un avance de los glaciares resultó en dos distribuciones disyuntas del gorrión común ancestral – una en el Oriente Medio y otra en el subcontinente indio. La diferenciación durante este periodo dio lugar a dos principales grupos de subespecies: el domesticus, y el indicus. Según esta hipótesis, el comensalismo con los humanos habría evolucionado independientemente en ambas regiones, por lo menos dos veces. Una hipótesis alternativa es que las diferencias morfológicas entre las subespecies representan una diferenciación muy reciente que tuvo lugar después de expandir desde un único origen. Para poner a prueba estas dos hipótesis, hemos analizado la variación genética en la región control del ADN mitocondrial y en tres loci nucleares de varias poblaciones de gorrión común en Europa, Asia, y el norte de África. No encontramos diferencias entre los grupos indicus y domesticus, lo cual apoya la hipótesis de un único origen. Una de las subespecies del grupo indicus, *P. d. bactrianus*, difiere ecológicamente de otros gorriones comunes al ser migratorio y al reproducirse preferentemente en hábitats naturales. Sugerimos que *bactrianus* representa una población relicta de la ancestral, la cual no era comensal. Cuando la sociedad desarrolló la agricultura en el Oriente Medio hace unos 10000 años, una población de gorrión común local, similar a la *bactrianus*, se adaptó al nuevo entorno y se convirtió eventualmente en un comensal sedentario del humano. Con la expansión de la agricultura y de la civilización humana, el gorrión común experimentó una correlacionada y masiva expansión en distribución geográfica y en números. El patrón de variación genética analizado aquí es consistente con este escenario.

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Single origin of human commensalism in the house sparrow

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Abstract

The current, virtually worldwide distribution of the house sparrow (*Passer domesticus*) is a result of its commensal relationship with humans. It has been suggested that long before the advent of agriculture, an early glacial advance resulted in two disjunct ranges of ancestral house sparrows – one in the Middle East and another on the Indian subcontinent. Differentiation during this period of isolation resulted in two major groups of subspecies: the domesticus group and the indicus group. According to this hypothesis, commensalism with humans would have evolved independently in the two regions and at least twice. An alternative hypothesis is that morphological differences between the subspecies represent very recent differentiation, following expansions from a single source. To test between these hypotheses, we analysed genetic variation at the mitochondrial DNA control region and at three nuclear loci from several house sparrow populations in Europe, Asia and North Africa. No differentiation between the indicus and domesticus groups was found, supporting the single origin hypothesis. One of the subspecies in the indicus group, *P. d. bactrianus*, differs ecologically from other house sparrows in being migratory and in preferentially breeding in natural habitat. We suggest that *bactrianus* represents a relict population of the ancestral, noncommensal house sparrow. When agricultural societies developed in the Middle East about 10 000 years ago, a local house sparrow population of the *bactrianus* type adapted to the novel environment and eventually became a sedentary, human commensal. As agriculture and human civilizations expanded, house sparrows experienced a correlated and massive expansion in range and numbers. The pattern of genetic variation analysed here is consistent with this scenario.

Introduction

Humans have a large impact on the distribution and abundance of a range of organisms worldwide. Although human activities have a negative impact on many organisms, some species have taken advantage of our artificial habitats, have evolved adaptations to cope with them and thrive as commensals or parasites in our immediate vicinity (Tchernov, 1991; Kauserud *et al.*, 2007). The house sparrow (*Passer domesticus*) provides a familiar example of such a relationship. Over most of its current breeding range, this bird occurs solely in man-made habitat, including farmland and cities, where it feeds off our crops and spillings and nests in buildings and other human constructions. In these regions, house sparrows will go locally extinct if humans abandon an area; they have become obligate commensals (Summers-Smith, 1963; Anderson, 2006).

Human commensalism explains the wide distribution of the house sparrow. Throughout most of the Palearctic and Oriental regions, the house sparrow is found

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wherever humans live and has more recently also been introduced to the Americas, South Africa, Australia and New Zealand where it has expanded greatly (Summers-Smith, 1988; Anderson, 2006). The house sparrow's commensal relationship with humans probably arose with the advent of agriculture in early human civilizations (Johnston & Klitz, 1977; Hermansen *et al.*, 2011). However, it has been unclear whether commensalism evolved only once or independently in different regions (Anderson, 2006).

Vaurie (1949, 1956) suggested that the house sparrow split into two main lineages, following periodic isolation in association with an early glacial advance long before the advent of agriculture: the domesticus group of the Palearctic region and the indicus group of the Oriental region (see Fig. 1 for a distribution map). This hypothesis is based on the presence of some morphological differences between the two groups. The subspecies in the indicus group are on average smaller, have smaller beaks, are more richly coloured on the upper side, and have lighter cheeks and bellies than those in the domesticus group (Summers-Smith, 1988). In addition, results from studies of a newly formed contact zone between nominate *P. d. domesticus* and the subspecies *P. d. bactrianus* of the indicus group in southern Kazakhstan have been interpreted as further support for the hypothesis of a deep split as little or no interbreeding between individual *P. d. domesticus* and *P. d. bactrianus* was observed (Gavrilov & Korelov, 1968; Yakobi, 1979). The *bactrianus* subspecies is ecologically different from all other house sparrow subspecies. It is migratory, wintering on the Indian subcontinent, whereas other house sparrows are sedentary (Summers-Smith, 1988). Furthermore, *bactrianus* is not closely associated with humans. They preferentially breed in natural or semi-natural grassland habitat, e.g. along riverbeds and lakes, and are replaced by tree sparrows *P. montanus* or other house sparrow subspecies in cities and villages (Gavrilov & Korelov, 1968; Yakobi, 1979; Summers-Smith, 1988; G-P Sætre, S Riyahi, M Aliabadian, JS Hermansen & SA Sæther, personal observations).

The other subspecies in the indicus group are ecologically similar to those in the domesticus group, however. Broad zones of intergradation between the two groups are found elsewhere, such as in southeastern Iran between *P. d. persicus* (*domesticus* group) and *P. d. indicus* (Summers-Smith, 1988).

A split between the domesticus and indicus groups that predates the advent of agriculture as suggested by Vaurie (1949) would imply that commensalism with humans arose independently in the two regions (Anderson, 2006). We will refer to this hypothesis as the independent origin hypothesis. An alternative hypothesis is that the morphological differences between the subspecies represent very recent differentiation, following expansions from a single source (the single origin hypothesis, Johnston &

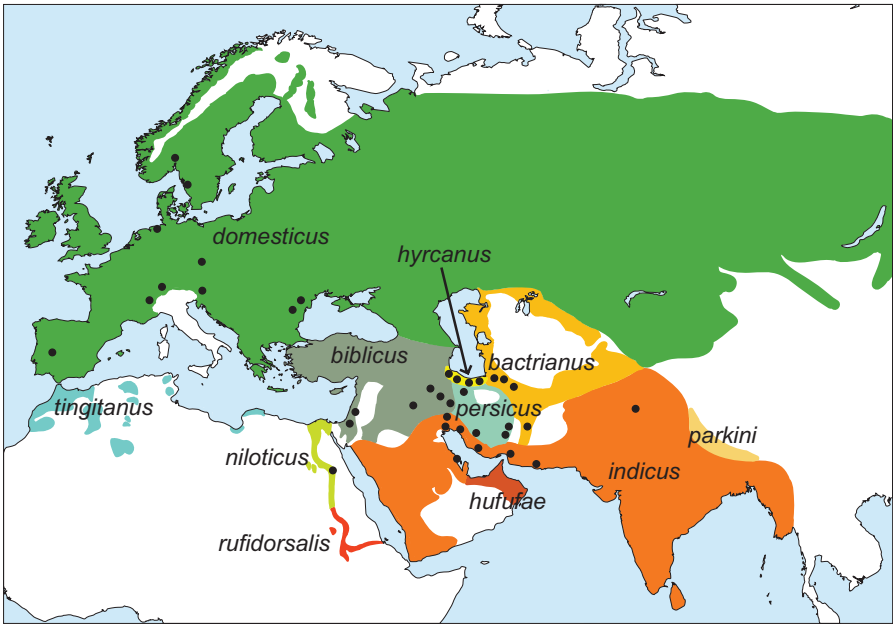

Fig. 1 Sampling locations (black dots) and geographical distribution of 11 subspecies of the house sparrow *Passer domesticus*. Subspecies in the domesticus group are in green/blue tones and subspecies in the indicus group are in red/orange/yellow tones. After Vaurie (1949, 1956), Summers-Smith (1988) and Cramp & Perrins (1993). Note that delimitation to subspecies and hence distribution varies somewhat between different authors. Moreover, zones of intergradation between subspecies are not indicated, and therefore, the map should be considered approximate.

Klitz, 1977). Here, we test between the single and independent origin hypotheses by means of sequence analysis of the rapidly evolving control region of the mitochondrial genome (mtDNA) as well as intron sequences of three nuclear genes.

From the single origin hypothesis, we predict little genetic differentiation between the subspecies and the two main groups of subspecies. House sparrow populations would be expected to exhibit extensive allele sharing across the entire distribution. Finally, we would expect to find genetic evidence consistent with one population expansion associated with the spread and expansion of human societies the last approximately 10 000 years.

Alternatively, from the independent origin hypothesis, we predict significant genetic differentiation between the subspecies, and particularly between the eastern indicus and western domesticus groups of subspecies. We may also expect more complex demographic signals due for instance to effects of secondary contact and introgression between differentiated genomes.

Materials and methods

A total of 181 samples of house sparrows were obtained from 37 locations in 17 countries (Fig. 1; Table S1). The samples include birds from seven of the 11 subspecies recognized by Vaurie (1949, 1956) and Summers-Smith (1988): *domesticus*, *biblicus*, *persicus* and *niloticus* of the domesticus group, and *indicus*, *bactrianus* and *hyrcanus* of the indicus group (Fig. 1). In addition one Spanish sparrow (*P. hispaniolensis*) and one tree sparrow (*P. montanus*) were included for outgroup rooting (Table S1).

We sampled either 20–50 μL of blood or 2–3 feathers (secondaries and tail feathers) from each individual bird. Feathers were stored in small paper bags at ambient temperature. Blood was taken by puncturing a brachial vein and transferred to a 1 mL tube with a standard buffer for storage. Birds were released immediately following blood or feather sampling.

DNA was extracted using the NucleoSpin Kit (Macherey-Nagel GmbH & Co, Düren, Germany) or the isolation robot GeneMole (Mole Genetics AS, Lysaker, Norway), following the manufacturers instructions. We amplified a 667 bp fragment of the mitochondrial genome (domains II and III of the control region (CR)) using the primers H1248 5′-CAT CTT CAG TGT CAT GCT-3′ and L437 5′-CTC ACG AGA ACC GAG CTA CT- 3′ (Tarr, 1995) as well as intron sequences from the Z-linked gene SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2 (BRM) (F: 5′-AGC ACC TTT GAA CAG TGG TT-3′and R: 5′-TAC TTT ATG GAG ACG ACG GA-3′) and the ausomal genes Creatin kinase, brain (CKB) (F: 5′- GGT TGA TAA TCC TGG TAA AAT GCA-3′and R: 5′-GAT GGC CTG GAG TGG TAA TAA AGT-3′) and Lamin A (LAMA) (F: 5′-CCA AGA AGC AGC TGC AGG ATG AGA TGC-3′and R: 5′-CTG CCG CCC GTT GTC GAT CTC CAC CA-3). The amplification reactions were performed in 15 μL reaction volumes, consisting of 7.1 μL mqH_2O, 1.5 μL of each primer (5 μM), 2.25 μL dNTPs solution (2 μM), 1.5 μL 10× buffer, 0.15 μL Ext Polymerase (Finnzymes Oy, Espoo, Finland) and 1 μL of genomic DNA (≈20 ng μL^{-1}). The nuclear loci were chosen from a larger set of markers analysed in house sparrows and Spanish sparrows (Elgvin *et al.*, 2011). We specifically chose markers that did not show any evidence of having been subjected to selection for the present study.

The PCR protocol consisted of an initial denaturation step of 94 °C for 90 s, followed by 30 cycles of 30 s at 94 °C (denaturation), 30 s at 58–60 °C (annealing) and 100 s at 72 °C (extension). The PCR products were sequenced using an ABI 3730 automated sequencer (Applied Biosystems). The sequences have been deposited in GenBank: accession numbers JQ280945-JQ281101, JQ305166-JQ305690 and JQ327855-JQ327856. Sample sizes vary between the markers due to differences in phase (mitochondrial, autosomal and sex-linked), but also because of depletion of DNA due to small aliquots provided by colleagues or poor yield of DNA isolated from feather pens or due to difficulties in base calling because of heterozygosity at multiple insertion/deletion (indel) sites.

The sequences were aligned using SEQUENCHER ver. 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). For nuclear sequences with more than one heterozygous site, haplotypes were assigned statistically using PHASE 2.1.1 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003), using 10 000 iterations, a thinning interval set to 1 and burnin set to 1000). We used default values for all other parameters.

Descriptive statistics of nucleotide variation and population differentiation as well as Tajima's *D* tests (Tajima, 1989) were calculated for the seven subspecies separately, for the domesticus and indicus groups and for all the house sparrow samples combined, using DNASP5.0 (Librado & Rozas, 2009).

Genetic structure was analysed using *F*-statistics and analyses of molecular variance (AMOVA) using ARLEQUIN 3.1 (Excoffier *et al.*, 2006), where we assigned the individuals to their respective populations (sampling location) as well as their affinity to the proposed two main groups of subspecies, the domesticus and indicus groups. Separate analyses were conducted for each of the four loci due to differences in phase (haploid, sex-linked and autosomal) and sample size.

Haplotype network for the mitochondrial CR-fragment was constructed using the minimum-spanning network method (MINSPNET in ARLEQUIN 3.1). To test for a population expansion and its timing, we used a mismatch-distribution approach on the mtDNA dataset. We calculated the mismatch distributions as implemented in ARLEQUIN 3.1 (Excoffier *et al.*, 2006). The mismatch-distribution model assumes that an ancestral

stationary population at equilibrium experiences a sudden expansion in population size t generations ago, after which the population reaches a new stationary phase. Based on the mean and variance of all the pairwise haplotype differences, the general nonlinear least-square approach is used to estimate three demographic parameters: $\tau = 2\mu t$, $\theta_0 = 2\mu N_0$ and $\theta_1 = 2\mu N_1$, where μ is the mutation rate for the haplotype, θ is population mutation parameters and N is the population sizes before and after the expansion. Approximate confidence intervals for the three parameters are obtained by a parametric bootstrap approach (Excoffier *et al.*, 2006). To get an approximate estimate of the time since divergence in years based on τ, we assumed a standard molecular clock of 2% sequence divergence per million years and a generation time of 1 year (see Voje *et al.*, 2009 for further details).

Results

Number of polymorphic sites (S), average number of nucleotide differences (Π), nucleotide diversity (π), and the population mutation parameter theta estimated from S (θ_W), as well as Tajima's D tests are summarized in Table 1. Genetic variation was somewhat higher in the indicus group than in the domesticus group. For the mitochondrial control region (CR), estimates of Tajima's D were significantly negative in five subspecies (*domesticus*, *biblicus*, *indicus*, *bactrianus* and *hyrcanus*), although not significantly different from zero in *persicus* or *niloticus* (Table 1). Tajima's D were consequently significantly negative for both the domesticus and indicus groups as well as across all the house sparrow samples for this marker. Tajima's D were overall negative also at the nuclear markers but rarely significantly so (Table 1). A negative Tajima's D results from an excess of rare polymorphisms relative to neutral expectation, typically caused by a recent population expansion (a genome-wide signal) or a selective sweep (a local signal).

At the mitochondrial CR, we identified a total of 43 variable sites and 37 different haplotypes among 157 sequences. A minimum-spanning network showed a classical star-shaped topology (Fig. 2). The most common haplotype was shared by 95 of the 157 individuals, including individuals from all the seven subspecies investigated across the domesticus and indicus groups. The remaining haplotypes can be derived from this sequence through 1–5 nucleotide substitutions. Birds from the domesticus and indicus groups also shared several of these less common haplotypes.

Our data from CR was consistent with a sudden expansion mismatch-distribution model (sum of squared deviation (SSD) = 0.0031, P (simulated SSD ≥ observed SSD) = 0.166; Harpending's raggedness index (rag) = 0.083, P (simulated rag ≥ obs rag = 0.069)). The estimated time since divergence [90% CI] as calculated from τ, was t = 4 343 [3 016, 7 350] years ago.

There were no fixed nucleotide differences between any of the subspecies or between the domesticus and indicus group, and we found considerable allele sharing at all the four markers (Table S2). Analyses of molecular variance showed that ≈ 90–96% of the genetic variance could be explained by differences between individuals within populations at the four different markers, ≈ 3–8% could be explained by differences between populations within the domesticus and indicus groups, and only ≈ 0–4% could be explained by differences between the two groups (Table 2). Global F_{ST} (fixation index between the populations) was significantly different from zero at three of four markers. F_{SC} (fixation index among populations within the domesticus and indicus groups) was significantly positive at the CR- and the BRM locus but not at the other two markers, whereas F_{CT} (fixation index between the domesticus and indicus group) was only significantly different from zero at BRM (Table 3).

Discussion

Our results are consistent with a single origin of human commensalism in the house sparrow. All house sparrow subspecies cluster together as one large genetic group and we found no evidence for any split between eastern and western subspecies. Our results are further consistent with a recent population expansion of the house sparrow. Our minimum-spanning network of the mitochondrial CR-marker has the classical expansion signal of a star-shaped topology with one very common haplotype surrounded by many rare and closely derived haplotypes. Furthermore, estimates of Tajima's D at the CR-marker were significantly negative in five of the seven subspecies (*domesticus*, *biblicus*, *indicus*, *bactrianus* and *hyrcanus*), again consistent with a population expansion. Finally, our CR-data were consistent with a sudden population expansion according to a mismatch-distribution model. Estimates from the latter model suggest that the expansion occurred approximately 3000–7500 years ago. Hence, we consider it likely that the expansion signal is a correlated response to the increased amount of food made available to sparrows, following the rise of human agriculture and permanent settlements from about 10 000 years ago, the subsequent spread of civilization throughout the Palearctic and Oriental regions and the corresponding expansion of human population sizes in these regions (see e.g. Atkinson *et al.*, 2008).

Although we can positively rule out the hypothesis that there was an ancient split between eastern and western house sparrow subspecies long before the rise of agriculture, one could argue that commensalism might have developed independently from different source populations if these events occurred very recently. We consider the single origin hypothesis much more likely, however. Our markers exhibited considerable genetic variation, sufficient to detect population structuring. Accordingly, the hypothesized independent source populations would

Table 1 Descriptive statistics on genetic variation among house sparrow (*Passer domesticus*) subspecies.

Locus	Taxon	Length (bp)	*N*†	Hd‡	π††	θ_W‡‡	S§	Π¶	Tajima's *D*∫
CR (mtDNA)	*P. d. domesticus*	667	60	0.629	0.00133	0.00456	14	0.887	−2.075*
	P. d. biblicus	667	25	0.540	0.00094	0.00240	6	0.627	−1.811*
	P. d. persicus	667	13	0.500	0.00081	0.00097	2	0.538	−0.462
	P. d. niloticus	667	5	0.900	0.00211	0.00217	3	1.400	−0.175
	P. d. hyrcanus	667	11	0.618	0.00219	0.00413	8	1.455	−1.934*
	P. d. bactrianus	667	15	0.657	0.00140	0.00325	7	0.933	−2.040*
	P. d. indicus	667	28	0.638	0.00180	0.00587	15	1.198	−2.354**
	Domesticus group	667	103	0.619	0.00125	0.00676	23	0.829	−2.388**
	Indicus group	667	54	0.628	0.00177	0.00911	27	1.177	−2.611***
	Total	667	157	0.622	0.00143	0.01188	43	0.951	−2.628***
BRM (Z-linked)	*P. d. domesticus*	269	51	0.700	0.00671	0.00930	11	1.790	−0.778
	P. d. biblicus	269	31	0.695	0.00808	0.00572	6	2.151	1.223
	P. d. persicus	269	16	0.525	0.00989	0.00567	5	2.625	2.446*
	P. d. niloticus	269	5	0.400	0.00149	0.00179	1	0.400	−0.817
	P. d. hyrcanus	269	9	0.944	0.01194	0.01269	9	3.139	−0.684
	P. d. bactrianus	269	27	0.772	0.01070	0.01189	12	2.877	−0.253
	P. d. indicus	269	32	0.685	0.00794	0.00744	7	2.121	0.203
	Domesticus group	269	103	0.779	0.00790	0.00737	10	2.101	0.240
	Indicus group	269	68	0.760	0.00904	0.01547	19	2.415	−1.285
	Total	269	171	0.782	0.00858	0.01587	23	2.284	−1.274
CKB (Autosomal)	*P. d. domesticus*	372	90	0.751	0.00413	0.00648	12	1.537	−0.945
	P. d. biblicus	372	26	0.818	0.00544	0.01009	14	2.025	−1.542
	P. d. persicus	372	16	0.850	0.00435	0.00574	7	1.608	−0.837
	P. d. niloticus	372	10	0.933	0.00578	0.00869	9	2.133	−1.442
	P. d. hyrcanus	372	6	0.933	0.00705	0.00715	6	2.600	−0.060
	P. d. bactrianus	372	24	0.721	0.00327	0.00510	7	1.210	−1.108
	P. d. indicus	372	36	0.830	0.00486	0.00592	9	1.806	−0.501
	Domesticus-group	372	142	0.783	0.00449	0.01209	24	1.669	−1.754(*)
	Indicus group	372	66	0.806	0.00472	0.00867	15	1.754	−1.301
	Total	372	208	0.791	0.00461	0.01327	28	1.706	−1.789*
LAMA (Autosomal)	*P. d. domesticus*	362	44	0.729	0.00450	0.00583	9	1.609	−0.638
	P. d. biblicus	362	24	0.909	0.00707	0.00908	12	2.529	−0.731
	P. d. persicus	362	6	0.889	0.00658	0.00594	6	2.356	0.458
	P. d. niloticus	362	4	0.833	0.00325	0.00304	2	1.167	0.592
	P. d. hyrcanus	362	8	0.929	0.00699	0.00758	7	2.500	−0.352
	P. d. bactrianus	362	18	0.941	0.00955	0.01240	13	3.405	−0.831
	P. d. indicus	362	38	0.849	0.00707	0.01152	17	2.528	−1.228
	Domesticus group	362	82	0.822	0.00570	0.01033	18	2.038	−1.277
	Indicus group	362	64	0.882	0.00770	0.01460	24	2.751	−1.439
	Total	362	146	0.850	0.00661	0.01516	29	2.359	−1.593(*)

(*) $0.1 < P < 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

†No. of individuals (CR) or no. of haploid sequences (the other loci).

‡Haplotype diversity (Nei, 1987).

††Nucleotide diversity.

‡‡The population mutation parameter theta, estimated from no. of segregating sites.

§No. of segregating sites.

¶Average number of nucleotide differences between sequence pairs (Nei, 1987).

∫Tajima (1989).

have had to be basically identical genetically, or in other words, they would belong to the same population according to normal population genetic convention.

We did not find an equally clear expansion signal at the nuclear markers compared to the mitochondrial one, however. Although Tajima's *D* were overall negative also at the latter markers, they were rarely significantly different from zero. The allele frequency spectrum of mitochondrial genetic markers is, however, likely to be more sensitive to changes in population size than a nuclear one. This is in part because the effective population size of a mitochondrial gene is only 1/4th that of a nuclear,

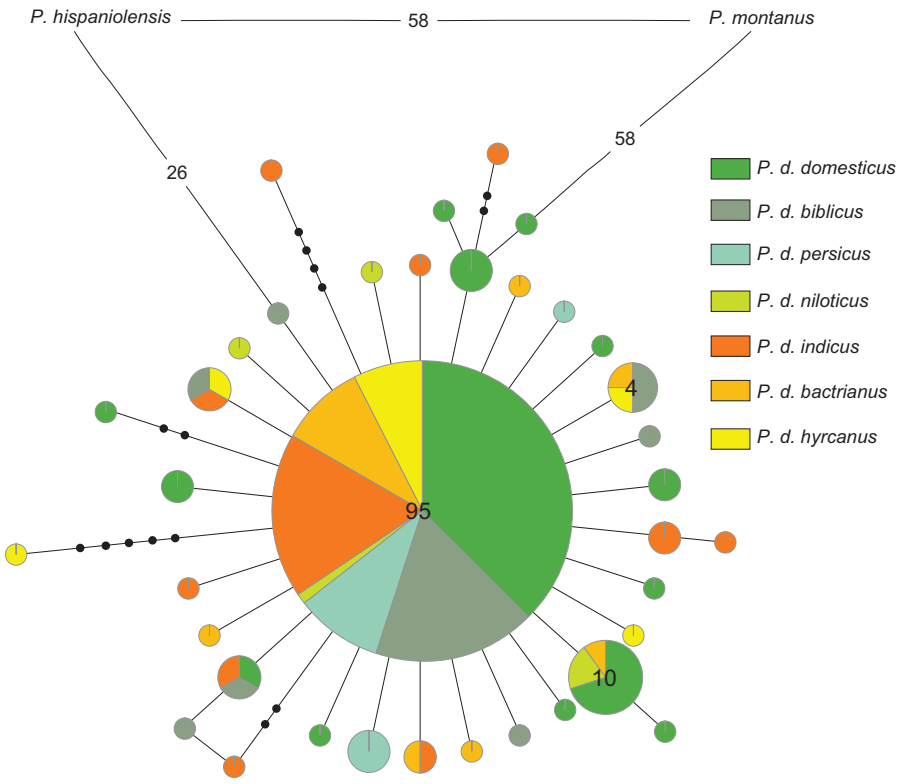

Fig. 2 Minimum-spanning network of the mitochondrial control region haplotypes found among 157 house sparrows (*Passer domesticus*) from Europe, Asia and North Africa. One Spanish sparrow (*P. hispaniolensis*) and one tree sparrow (*P. montanus*) are included as outgroups. Each circle represents one haplotype and the size of the circle indicates the number of individuals sharing it (also indicated with a number in the three most common haplotypes: 4, 10 and 95 individuals). Colours refer to the seven subspecies included as indicated to the right. Subspecies belonging to the domesticus group are in green tones, and those belonging to the indicus group are in orange/yellow tones. The number of mutational steps between haplotypes is indicated with a line (1 step), dots (2–5 steps) or numbers (more than 5 steps).

autosomal gene, due to the haploid nature and maternal inheritance of the mitochondrial genome. Hence, allelic variation is much more likely to get lost by genetic drift during periods of low population size at a mitochondrial gene compared to a nuclear one. Additionally, mutation rates are overall much higher at the mitochondrial compared to the nuclear genome. Hence, following a population expansion, mutant alleles will appear more rapidly at a mitochondrial gene compared to a nuclear one, hence yielding a larger shift in the allele frequency spectrum towards rare alleles (e.g. Fay & Wu, 1999).

The Middle East has been pinpointed as a likely place of origin for the house sparrow's commensal relationship with humans (e.g. Johnston & Klitz, 1977; Summers-Smith, 1988). The earliest fossil evidence of house sparrows stems from Bethlehem, Israel, and is estimated to be 400 000 years old (Tchernov, 1962). Moreover, all early house sparrow fossils (older than 10 000 years) are from the Middle East (Tchernov, 1962; Ericson *et al.*, 1997), placing the species ancestral range close to, or in the region where human agricultural societies first developed, some 10 000 years ago (Johnston & Klitz, 1977). Early *Passer* sparrow fossils have been found also in Maghreb and on the Iberian Peninsula, but these are most likely ancestral Spanish sparrows *P. hispaniolensis*, according to morphological comparisons with contemporary birds (Ericson *et al.*, 1997). Interestingly, in Italy, hybridization between presumably once resident Spanish sparrows and house sparrows that came with the advance of agriculture some 8000 years ago has led to the formation of a novel, stable hybrid lineage, the Italian sparrow *P. italiae* that now replaces the house sparrow on the Italian peninsula and some Mediterranean islands (Elgvin *et al.*, 2011; Hermansen *et al.*, 2011).

An organism that expands from an ancestral source will typically lose genetic variation away from the centre of origin (e.g. Avise, 2000; Hewitt, 2000). According to our analyses, the genetic diversity among house sparrows is higher in the Middle East than elsewhere, but not dramatically so. Interestingly, a similar pattern was found in a study comparing native and recently introduced populations of house sparrows (Schrey *et al.*, 2011). For a future study, we would recommend a dense and balanced sampling scheme to identify possible diversity

Table 2 Analyses of molecular variance (AMOVA) comparing house sparrow populations (*Passer domesticus*) belonging to two proposed groups of subspecies, the domesticus group and the indicus group.

Locus	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
CR (mtDNA)	Among groups	1	0.774	0.00220 Va	0.46
	Among populations within groups	35	20.832	0.03679 Vb	7.65
	Within populations	119	52.599	0.44201 Vc	91.89
	Total	155	74.205	0.44201	100
BRM (Z-linked)	Among groups	1	6.176	0.05436 Va	4.46
	Among populations within groups	31	45.749	0.07753 Vb	6.36
	Within populations	138	150.029	1.08717 Vc	89.18
	Total	170	201.918	1.08717	100
CKB (Autosomal)	Among groups	1	1.0819	0.00883 Va	1.03
	Among populations within groups	27	26.217	0.02034 Vb	2.37
	Within populations	179	148.488	0.82954 Vc	96.60
	Total	207	176.524	0.85871	100
LAMA (Autosomal)	Among groups	1	1.862	0.00398 Va	0.33
	Among populations within groups	24	34.751	0.05871 Vb	4.93
	Within populations	120	135.442	1.12868 Vc	94.74
	Total	145	172.055	1. 19137	100

Table 3 *F*-statistics among house sparrow populations (*Passer domesticus*) belonging to two proposed groups of subspecies, the domesticus group and the indicus group.

Locus	F_{ST} – among populations	F_{CT} – among groups	F_{SC} – among populations within groups
CR (mtDNA)	0.0811*	0.0046	0.0769*
BRM (Z-linked)	0.1082**	0.0446*	0.0666*
CKB (autosomal)	0.0340	0.0103	0.0239
LAMA (autosomal)	0.0526*	0.0033	0.0495

*$P < 0.05$, **$P < 0.01$

hotspots at a more fine-grained scale and hence likely places of origin of human commensalism in the house sparrow.

Our results suggest that the morphological differences between sparrows from different regions represent divergence that has taken place after the initial expansion. We find little or no genetic differentiation between the subspecies at our loci, suggesting that there has not been sufficient time for the build-up of neutral genetic structure, although the populations have diverged phenotypically. Rapid divergence in plumage traits, body size and/or beak size has been observed in other bird taxa, such as the yellow wagtail species complex *Motacilla flava* (Ödeen & Björklund, 2003) and the Darwin's finches *Geospiza* spp. (Grant & Grant, 2002). Johnston and Selander and co-workers described considerable geographical variation in body size and plumage colour among house sparrow populations across North America in a series of papers during the 1960s and 1970s, i.e. only some 110 years after the species was first introduced successfully to the continent in 1853 (e.g. Johnston & Selander, 1964, 1973; Selander & Johnston, 1967). Given the great variation in environmental conditions across the species range in the Old World and the time available for evolutionary change since the initial expansion (~10 000 years), it is not surprising that phenotypic differentiation in Eurasia exceeds that observed in North America.

The difference in ecology between *P. d. bactrianus* and other house sparrows is most intriguing. Unlike other house sparrows, the *bactrianus* is migratory and thrives in natural grassland habitat (e.g. Gavrilov & Korelov, 1968; Yakobi, 1979; Summers-Smith, 1988; G-P Sætre, S Riyahi, M Aliabadian, JS Hermansen & SA Sæther, personal observations). As nominate *domesticus* expanded eastwards in Russia and neighbouring countries during the previous century (Summers-Smith, 1988), it eventually came into contact with local *bactrianus* sparrows in southern Kazakhstan. Gavrilov & Korelov (1968) and Yakobi (1979) studied this contact zone. They observed strong assortative mating between local *domesticus* and *bactrianus* and hence suggested that they belong to different species (named *P. domesticus* and *P. indicus* respectively, due to the proposed affinity of *bactrianus* with the *indicus* group). According to our analysis, *bactrianus* is genetically similar to other house sparrows, suggesting that the ecological separation is of recent origin. The ecological difference itself may actually explain why the two types do not interbreed: *bactrianus* would avoid the breeding sites that *domesticus* prefers and *vice versa* (i.e. away from vs. among human settlements).

We suggest that *bactrianus* is a relict population of the ancestral, noncommensal house sparrow. According to the single origin hypothesis, *bactrianus* would share a recent common ancestor with the radiating commensal sparrows and should therefore cluster genetically with all the other sparrows at genes that are neutral with respect to the ecological transition, consistent with our results. An interesting follow-up study would be to compare commensal and noncommensal sparrows at genes that are likely to associate with the ecological differences. From the single origin hypothesis, we would predict that any genetic adaptation to human commensalism would be shared by all house sparrows, save *bactrianus*, whereas convergent evolution (the same phenotype derived from independent and possibly different genetic changes) would be expected under the independent origin hypothesis.

When sedentary, agricultural human societies first established in the Middle East from about 10 000 years ago, a novel niche developed. This niche included year-round supply of food due to storage of cereals and other food items and feeding of domestic animals, and safe nest sites in cracks and cavities in human buildings. We suggest that some local house sparrow population with the ancestral '*bactrianus* ecology' adapted to this novel niche and eventually became sedentary, obligate human commensals. Correlated events in the history of both humans and sparrows should thus turn out to have dramatic demographic consequences for both species.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sampling scheme.

Table S2 Shared and fixed polymorphisms and genetic differentiation between pairs of proposed subspecies and groups of subspecies of the house sparrow (*Passer domesticus*).

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PAPER 5

Natural epigenetic variation within and among six subspecies of the house sparrow *Passer domesticus*

Resumen en castellano

Las modificaciones epigenéticas pueden responder rápidamente ante cambios ambientales, y dar lugar a variaciones fenotípicas de acuerdo con el estímulo ambiental. Una de las marcas epigenéticas más estudiadas es la metilación del ADN. En este estudio, usamos la técnica MSAP para investigar la variación natural de la metilación dentro y entre subespecies del gorrión común *Passer domesticus*. Nos centramos en cinco subespecies procedentes del Oriente Medio, porque presentan gran variación a nivel de características ecológicas y porque esta región sea el origen probable de la relación comensal entre gorriones y humanos.

Como grupo externo, también analizamos a gorriones comunes procedentes de España. El nivel de variación en la metilación del ADN era similar entre las cinco subespecies del Oriente Medio, a pesar de la gran variación fenotípica y ambiental; sin embargo, la subespecie no comensal se diferenciaba de otras subespecies del Oriente Medio. Además, la subespecie europea estaba diferenciada de todas las demás subespecies analizadas. Nuestros resultados indican que la variación en la metilación del ADN no refleja estrictamente las designaciones taxonómicas. Detectamos una correlación entre el nivel de metilación y algunos rasgos morfológicos, como la longitud estandarizada del pico, y sugerimos que parte de la gran variación morfológica de las poblaciones nativas de gorrión común se ve influenciada por metilación diferencial en loci específicos a lo largo del genoma. También detectamos siete loci con metilación diferencial en función de la subespecie, y tres loci diferenciados en función del estado de comensalismo. Por lo tanto, la técnica MSAP pudo detectar diferencias a gran escala entre las subespecies europea y no comensales, pero no detectó diferencias a pequeña escala entre las demás subespecies del Oriente Medio.

Natural epigenetic variation within and among six subspecies of the house sparrow *Passer domesticus*

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Abstract

Epigenetic modifications can respond rapidly to environmental changes and can shape phenotypic variation in accordance with environmental stimuli. One of the most studied epigenetic marks is DNA methylation. In the present study we used the MSAP technique to investigate the natural methylation variation within and among subspecies of the house sparrow *Passer domesticus*. We focused on five subspecies from the Middle East because they show great variation in many ecological traits and because this region is the probable origin for the house sparrow's commensal relationship with humans.

We additionally analyzed house sparrows from Spain as an outgroup. The level of variation in DNA methylation was similar among the five house sparrow subspecies from Middle East despite high phenotypic and environmental variation; yet the non-commensal subspecies was differentiated from some other Middle Eastern subspecies. Further, the European subspecies, was differentiated from all other subspecies screened. Our results indicate that variation in DNA methylation does not strictly follow subspecies designations. We have detected correlation between methylation level and some morphological traits such as standardized bill length and we suggest that part of the high morphological variation in the native populations of the house sparrow is influenced by differentially methylated regions in specific loci throughout the genome. We also detected seven differentially methylated loci diverged by subspecies and also three loci differentiated by commensal or non-commensal status. Therefore, the MSAP technique was able to detect larger-scale differences among the European and non-commensal subspecies, but did not detect finer-scale differences among the other Middle Eastern subspecies.

KEY WORDS: DNA methylation, environmental adaptation, epigenetic variation, house sparrow, MSAP.

Introduction

Epigenetic processes can affect gene expression in response to developmental and environmental stimuli (Richards 2011). DNA methylation has become one of the most intensively studied epigenetic mechanisms. It is a crucial process in natural selection and evolution because it allows organisms to adapt rapidly to environmental fluctuations by modifying phenotypic traits, either via phenotypic plasticity or developmental flexibility (Schlichting and Wund 2014).

An ideal ecological process to study the role of DNA methylation in local adaptation is range expansions, either natural or anthropogenic. Epigenetics could explain the success of some of these expansions, as genetic variability is often decreased and in many cases selection for genetic variants is unlikely to be directly involved in short timescale expansions (Schrey et al. 2014). The house sparrow *Passer domesticus* is a remarkable bird species regarding range expansion and adaptation. The species probably originated in the Middle East (e.g. Johnston and Klitz 1977; Summers-Smith 1988). Iran is a key place of transition for this species (Vaurie 1956), and several subspecies evolved in this area, probably due to complex topographic features of the Iranian plateau (Misonne 1959). As several zones of intergradation have been described for the subspecies of the house sparrow in this area (Vaurie 1949; Vaurie and Koelz 1956), the geographic distribution map of the species is approximate (Fig. 1). The species extended naturally into Eurasia with the aid of human commensalism (Summers-Smith 1988; Anderson 2006; Sætre et al. 2012). It was later introduced by humans into America, North Africa and Australia, now being one of the most broadly distributed vertebrate species (Anderson 2006). Eleven subspecies have been recognized, mainly on the basis of plumage coloration and body size (Vaurie and Koelz 1949; Vaurie 1956; Summers-Smith 1988). Earlier studies on several mitochondrial and nuclear loci showed that human commensalism in the house sparrow has a single origin and evolved around 10,000 years ago after the advent of agriculture in the Middle East (Sætre et al. 2012). Analyses on microsatellite markers have shown high differentiation in the native populations of house sparrow while genetic diversity appears to be higher in populations at lower latitudes (Schrey et al. 2011).

The different native subspecies of the house sparrow show a high level of phenotypic variation (e.g. in plumage coloration, body size and wing length), considered to possibly be the result of adaptation to different environmental conditions (Vaurie 1956; Anderson 2006). Outstanding intraspecific methylation variation of house sparrow with the aid of methylation-sensitive AFLP (Pérez-Figueroa 2013) method. This approach is a kind of genome-wide fingerprinting method and it is currently the most common technique in ecological epigenetic studies (Reyna-Lopez et al. 1997). We also searched for a correlation between morphometric characters and methylation characters to evaluate the effect of DNA methylation variation on phenotypic traits. Finally, we were interested to assess whether there is any correlation between DNA methylation and geographical or habitat characteristics.

Materials and Methods

We collected muscle tissue from 84 samples of house sparrow from 18 localities in the Palearctic region (Fig. 1). Of these 84, 71 samples were from 17 localities in Iran, and encompassed five of the 11

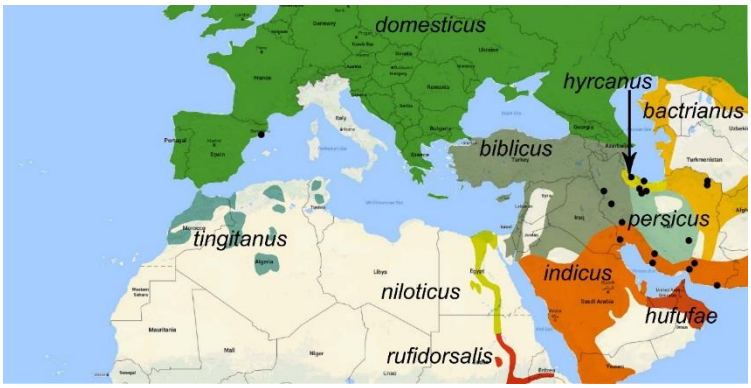

Figure 1. Sampling locations (black dots) and geographical distribution of 10 subspecies of house sparrow based on Vaurie (1949, 1956) and Summers-Smith (1988). An additional subspecies (*P. d. parkini*, from the Himalayas) is not shown in this map.

recognized subspecies: *P. d. bactrianus* (*N* = 10), *biblicus* (*N* = 17), *hyrcanus* (*N* = 4), *indicus* (*N* = 16) and *persicus* (*N* = 24). All of the Iranian samples were deposited at the Ferdowsi University of Mashhad (Mashhad, Iran). In addition, muscle tissue from 13 house sparrows belonging to *P. d. domesticus* subspecies was deposited at the Natural History Museum of Barcelona (Catalonia, Spain). DNA was extracted from muscle tissue preserved in ethanol using the MasterPure DNA Purification Kit (Epicenter). DNA quality and quantity were verified using a NanoDrop 1000 v.3.7.1 spectrophotometer (Thermo Fisher Scientific). DNA methylation differs among tissues due to different gene expression patterns in each tissue. Therefore, we used the same tissue (muscle tissue) and the same DNA extraction kit for all our individuals.

Methylation Sensitive Amplified Polymorphism (MSAP) (Pérez-Figueroa 2013) is a modified version method of the amplified fragment length polymorphism (AFLP) that uses the *Eco*RI enzyme (rare cutter) and substitutes methylation sensitive isoschizomeric enzymes *Msp*I and *Hpa*II for *Mse*I. The *Msp*I and *Hpa*II enzymes have different sensitivities to cytosine methylation. Both enzymes cut the same restriction sequences (CCGG) but *Hpa*II is sensitive to methylation of the internal cytosine at both strands whereas *Msp*I is sensitive to methylation of the external cytosine at any one strand (Reyna-Lopez et al. 1997). MSAP detects the methylation state of a particular locus in a banding pattern (Salmon et al. 2008).

Table 1. List of preselective and selective primers

Primer name	Sequence
Preselective primer F: EcoRI-0	5'-GACTGCGTACCAATTC-3'
Preselective primer R: *Hpa*-0	5'-ATCATGAGTCCTGCTCGG-3'
Selective primers	**Sequence**
Primer 1 F: Hpa-TCAT (VIC)	5'-CATGAGTCCTGCTCGGTCAT-3'
Primer 1 R: EcoRI-CGCTG	5'-GACTGCGTACCAATTCCGCTG-3'
Primer 2 F: Hpa-TCCA (6-FAM)	5'-CATGAGTCCTGCTCGGTCCA-3'
Primer 2 R: *Eco*RI-CGCTG	5'-GACTGCGTACCAATTCCGCTG-3'
Primer 3 F: Hpa-TCAC (6-FAM)	5'-CATGAGTCCTGCTCGGTCAC-3'
Primer 3 R: *Eco*RI-CGCT	5'-GACTGCGTACCAATTCCGCT-3'

We optimized the MSAP protocol from Liebl et al. (2013). For each individual, 250 ng of DNA were digested with 10 U of *Eco*RI (Roche Diagnostics) and *Msp*I (New England Biolabs) and independently with *Eco*RI and *Hpa*II (New England Biolabs) in a final volume of 20 µl. We incubated the restriction digests at 37 °C for 6 hours. We then ligated double-stranded *Eco*RI and *Msp*I/*Hpa*II adaptors to the digested fragments in a final volume of 40 µl containing 5 µmol of the *Eco*RI adaptor, 50 µmol of the *Msp*I/*Hpa*II

adaptor, 0.5 µl BSA (1 mg/ml), 4 µl 10X T4 buffer (Roche Diagnostics) and 1 U of T4 DNA ligase (Roche Diagnostics). We incubated the ligation reaction at 16 ℃ for 16 hours. We performed preselective PCR using 10 µl of a 1:5 dilution of the ligation reaction as template in a PCR at 30 µl final volume containing 1.25 mM of $MgCl_2$, 167 µM of each dNTP, 0.67 µM of *Eco*RI-0 and *Hpa*-0 primers (Table 1) , 2.5 µl 10X PCR buffer II (Fisher, 1973 1969 /id) and 0.5 U of AmpliTaq polymerase (Applied Biosystems). We used a preselective PCR thermal profile of 72 ℃ for 2 min, 30 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s, 72 ℃ for 2 min, and a final step of 72 ℃ for 10 min. We screened a total of three selective primer combinations (Table 1). We performed selective PCR using 5 µl of a 1:9 dilution of the preselective PCR as template in a PCR at 25 µl final volume containing 1.5 mM $MgCl_2$, 300 µM of each dNTP, 0.1 µM of EcoRI primers and 0.15 µM of Hpa primers and 2.5 µl 10× Gold Buffer (Applied Biosystems) and 1 U of AmpliTaq Gold polymerase (Fisher, 1973 1969 /id). We used a selective PCR thermal profile of 95 ℃ for 10 min, 13 cycles of 94 ℃ for 30 s, 56-65 ℃ for 30 s (decrease the temperature with 0.7 ℃ each cycle), 72 ℃ for 2 min, then 40 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s and 72 ℃ for 2 min and a final step of 70 ℃ for 5 min. Fragment electrophoresis was performed at the Parque Cientifico de Madrid (Spain) using ABI 3730 capillary sequencer (Fisher, 1973 1969 /id). Individual PCR products were analyzed along with GeneScan 500-LIZ size standards (Applied Biosystems). PeakScanner v.2.0 software (Applied Biosystems) was used to read the MSAP electropherograms and to detect peaks and calculate their intensity and size. Light peak smoothing was applied to the electropherograms and we changed minimum peak half width to 1 and minimum peak threshold to 100 for each dye (6-FAM and VIC). Binning and scoring were performed in RawGeno v.2.0 (R CRAN library; Arrigo et al. 2009). We replicated 10% of samples to check the repeatability of AFLP data generating and scoring. The peaks with a different scoring pattern for the same individuals were checked visually by GeneMarker v.1.85 (SoftGenetics) and eliminated the peaks that inconsistently amplified.

Data analyses

The MSAP matrix was pooled from three primer combinations to generate a single matrix. This matrix was analyzed using Msap v.1.1.0 software package for the programming environment R (Pérez-Figueroa 2013). This software analyses the MSAP binary matrix based on four types of methylation pattern according to the presence or absence of one or both fragments of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I: (1/1) presence of both fragments means unmethylated, (1/0) presence of fragment only in *Eco*RI/*Hpa*II means hemimethylated(CHG methylation), (0/1) presence of fragment only in *Eco*RI/*Msp*I means internal cytosine methylation (CG methylation), and (0/0) absence of both fragments is uninformative because the fragment can be absent or hypermethylated. This software also estimates the overall methylation variation using the Shannon diversity index and the frequency (expressed as a percentage) of the four types of methylation pattern in subspecies. Also, we conducted principal coordinate analyses (PCoA) to explore epigenetic variation between subspecies of house sparrow.

In addition, we performed AMOVA (analyses of molecular variance, Excoffier et al. 1992) using Msap software. Using a binary matrix with the methylated loci (1 for methylated and 0 for not methylated), we

carried out locus-by-locus AMOVA using GenAlex v.6.502 software (Peakall and Smouse 2012) only with the five Middle East subspecies data considering subspecies, commensal *versus* non-commensal and sex as grouping variables. We estimated statistical significance following 9999 permutations for all analyses. After identifying loci that had significant ϕ_{ST}, we also used a Bayesian likelihood method implemented in Bayescan v.2.1 (Foll 2008) to identify the outlier loci as those potentially under selection.

To evaluate the relation between phenotypic variation and epigenetic variation, we measured six morphometric characters for 61 individuals of the Middle East samples, including beak length from nostril, beak length from skull, wing length, tarsus length, body length and tail length. Normality of all of these morphometric characters was evaluated. Then we performed discriminant and canonical discriminant function analyses considering subspecies as grouping variable to evaluate the level of morphological divergence among subspecies. Then we performed Pearson correlation between all, for morphometric characters and methylation percentage each by each. To standardize the morphometric variable such as bill length based on the body size, we extracted the residuals of log-log regression of bill length from skull *versus* tarsus length and we performed the Pearson correlation between these residuals and methylation percentage. All of the morphological analyses were performed using Statistica v.8.0 (StatSoft 2013).

We built a distance matrix based on Euclidean distance using the six morphometric characters. We also estimated the epigenetic distance for all individuals based on MSAP markers generated from MSAPcalc.r (Schulz et al. 2013) with the function Extract-MSAP-epigenotypes and parameters Epicode=″Mix1″, delete.monomorphic.loci=TRUE and MinPoly=2. Finally, we used Mantel's test to assess correlations between epigenetic and morphologic distances using GenAlex software.

To explore sex-based variation in DNA methylation, we made linear regression analysis between percentage of methylation and sex using using Statistica software v.8.0 (StatSoft 2013). The percentage of methylation was also used to test for correlation with several environmental factors. We selected a set of 21 environmental layers that may potentially influence the distribution of methylation values. This set was composed of the following variables: 19 climatic variables representing annual trends (e.g., mean annual temperature, annual precipitation), seasonality (e.g., annual range in temperature and precipitation), and extreme or limiting factors (e.g. temperature of the coldest and warmest month, and precipitation of the wet and dry quarters) downloaded from the WorldClim website (http://www.worldclim.org), the elevation also extracted from WorldClim, and the index of anthropogenic impact called global human footprint (WCS/CIESIN 2005). For each occurrence point sampled of Middle East *P. domesticus* subspecies the values for each variable were extract with ArcGIS v.10.2.2 software (Environmental System Research Institute; http://www.esri.com). In order to reduce sampling bias and spatial autocorrelation, we only retain 16 occurrence points that were geographically separated from each other and not sampled in the same locality with equal coordinates. Finally, a Pearson correlation analysis was performed with SPSS v.17.0 (SPSS Inc.) between each pairwise of genetic and environmental data.

Mantel tests were also performed to determine the relationship between the DNA methylation differentiation (ϕ_{ST} estimated through MSAP software) and the geographic distance per population pairs between Middle East populations, using 1000 permutations with the Isolation by Distance Web (IBDWS v.3.23; Jensen et al. 2005).

Results

We detected a total of 101 loci using the 3 selective primer combinations; 64 were methylation-susceptible loci (MSL) and 37 were non-methylated loci (NML). The frequency of polymorphic MSL was 100% and Shannon's diversity index was 0.56 ± 0.13 (mean ± SD). In all subspecies, the highest percentage of loci screened were unmethylated (range 39.8 to 46.6%), while the frequency of the two distinguishable methylation states totally ranged from 26.4% to 28.9% (Table 2). Principal coordinate analyses (PCoA) detect only a slightly differentiation between *P. d. domesticus* and the other subspecies (Fig. 2).Using the 6 subspecies studied, *Passer d. domesticus* showed the highest level of differentiation, as it was differentiated from all other subspecies (Table 3). *P. d. biblicus* also showed significant differentiation from *bactrianus* and *persicus* (Table 3). Analyses of molecular variance (AMOVA) present a differentiation among subspecies (ϕ_{ST} = 0.07, P < 0.0001; Table 4) and also revealed that a greater portion of the epigenetic differentiation was attributed to differences within subspecies rather than among subspecies (Table 4). Considering only the Middle East subspecies, we detect also significant differentiation among subspecies (ϕ_{ST} = 0.022, P < 0.040) and commensal *versus* non-commensal (ϕ_{ST} = 0.019, P < 0.045) (Table 4). And from the methylated matrix using the locus-by-locus AMOVA analyses, we detect seven loci (subspecies as grouping criteria),

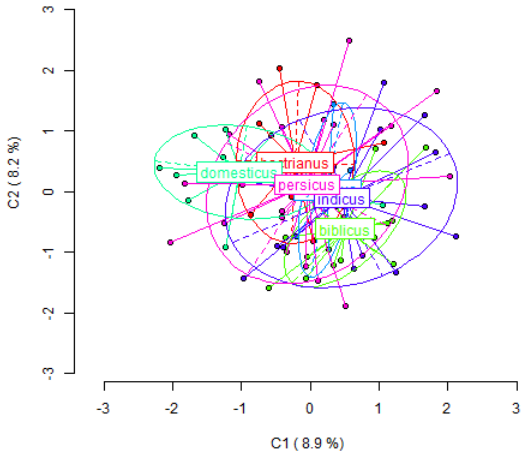

Figure 2. Results of principal coordinate analysis (PCoA) for the epigenetic variation (MSL) among six subspecies of house sparrow. The percentage of variance shown is that explained by the first two coordinates (C1 and C2) (8.9% and 8.2%). The label for each group shows the centroid for each group. Ellipses represent the dispersion associated with each value.

three loci (in relation with commensalism as grouping criteria), and three loci (sex as grouping criteria) with significant differentiation respectively (Table 4). However, no locus with significant ϕ_{ST} had positive Bayes factors.

Regarding the morphological results, all of the morphological variables were normal. Discriminant function analysis on the six morphological loci detected morphological divergence between five Middle East subspecies based on bill length from skull, wing length and tarsus length (Wilks' Lambda: 0.20, $F_{24,158}$ = 3.95, P < 0.001, N = 55). Generally, *biblicus* and *indicus* have the bigger body size compared to the other Middle East subspecies. Figure 4 shows a scatterplot resulting from canonical discriminant function analysis for the first and second components. The first and second statistically significant function (P < 0.05) explained 61.5 % and 32.8 % of the total variance. More information about

morphological variation of these subspecies is available in Riyahi et al. (2013).

Relating the sum of methylation percentage to different morphologic characters showed an almost significant correlation between bill length from skull (standardized based on tarsus length) and percentage of hemimethylation + internal C methylation (r = 0.22, *P* = 0.078, Fig. 4). Finally, Mantel test revealed significant correlation between epigenetic and morphometric distance (r = 0.1127, *P* = 0.030).

Some of the differences that we detected within subspecies can be due to variation of methylation

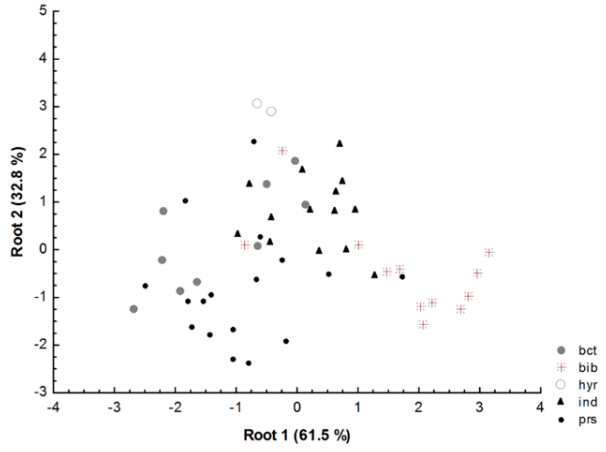

Figure 3. Scatter plot of Canonical Discriminant Function analysis shows divergence among Middle East subspecies base on morphologic characters.

percentage between different sexes. The female showed higher methylation level especially when we considered hemimethylation level ($F_{1,61}$ = 4.777, *P* = 0.033).

Regarding correlations between methylation and environmental layers, we did not find any significant relation between both datasets (all *P* values > 0.05). Thus, the influence of the environment in methylation content could be discarded with the data obtained.

Mantel test showed a significant positive correlation between the pairwise epigenetic differentiation (ϕ_{ST}) and the geographic distance within the Middle East region (r = 0.198, *P* = 0.05).

Discussion

Living in human-modified habitats forces urban species to exhibit physiological, behavioral and morphological plasticities in response to anthropogenic changes (e.g. Shochat et al. 2006; Kempenaers et al. 2010). The house sparrow is one of the most successfully urban-adapted species. Its flourishing in urban environments is probably due to its ability to consume cultivated cereals, and its abandonment of migratory behavior (Riyahi et al. 2013), but the high success of such urban-adapted species suggests they may have additional abilities to adapt rapidly to new environmental conditions (e.g. Sih et al. 2004; Møller 2009). These abilities may be related to epigenetic changes, which play an important role in adaptation and expansion of introduced species (Liebl et al. 2013; Schrey et al. 2014, 2016) and also in the domestication process (Nätt et al. 2012). For instance, the comparison of the expression and methylation arrays of domestic chickens and red junglefowls confirmed the role of domestication processes in shaping the methylation profile of the former (Nätt et al. 2012). The authors of this study analyzed methylation and expression variation within selective sweep regions. From 145 promoter regions, they found that average methylation levels were 79% hypermethylated in the junglefowls and 21% hypomethylated in the domestic chicken. Their study is an example of how artificial selection in animals

affects the biological profiles of species and how this can generate a species with favorable traits. The case of the house sparrow and its commensalism with humans is similar to domestication in some aspects. Both flourish in human habitats, one with the aid of artificial selection and the other by natural selection and obligatory commensalism. In fact, adaptation to urban life has recently been described as a process of domestication (Møller 2012).

The present study is one of the first to investigate natural epigenetic variation among different avian subspecies (Schrey et al. 2012; Liebl et al. 2013). We estimated genome-wide methylation level in six subspecies of the house sparrow, five from Iran (*P. d. bactrianus*, *biblicus*, *hyrcanus*, *indicus*, *persicus*) and one from Catalonia, Spain (*P. d. domesticus*). All these subspecies, *w*ith the exception of *P. d. bactrianus*, are human commensals that inhabit cities and suburbs. These subspecies inhabit a broad range of environmental conditions: high arid desert in the center of the country (*P. d. persicus*), humid and dense Hyrcanian forest on the Caspian coast (*P. d. hyrcanus*) and semi-humid Oak forest in the western Iran (*P. d. biblicus*), tropical coast of the Persian Gulf and Gulf of Oman (*P. d. indicus*), and sporadic woodlands and semi-arid steppes in eastern Iran (Firouz 2000). Despite the high environmental diversity, the methylation pattern was highly similar among all the Iranian house sparrows. Our results indicate that variation in DNA methylation does not match subspecies designations. We identified a stable pattern in DNA methylation among all commensal Iranian subspecies of house sparrow.

Interestingly, the Iranian subspecies *P. d. bactrianus* is non-commensal, breeds in natural or semi-natural grassland habitats (e.g. along riverbeds and lakes), is migratory, and is replaced by tree sparrows *P. montanus* or other house sparrow subspecies in cities and villages (Gavrilov and Korelov 1968; Yakobi 1979; Summers-Smith 1988; Sætre et al. 2012). We detected the second highest level of epigenetic differentiation in comparisons with this subspecies. This suggests that some portion of the observed variation in DNA methylation could be attributed to differences in autecology of this subspecies.

The European subspecies (*P. d. domesticus*), in contrast, was differentiated from all other subspecies in DNA methylation. The European house sparrow highlights the lack of differentiation among the majority of Iranian subspecies. Because we detected significant differentiation among this subspecies and all others, we support the ability of MSAP to detect differences at this magnitude, with this sample size. Thus, it is not the case that we failed to detect significant differences among other subspecies primarily based on a lack of power. The difference we detected between the European and the Middle East populations indicates that at a broad-scale significant differences are present. This is further supported by the significant differences detected among commensal and non-commensal subspecies.

Our results may be surprising, initially. However, Trucchi et al (2016) recently showed that while some loci have variable DNA methylation, perhaps due to environmental stimuli, a large proportion of the methylome is typically stable. Our results support this finding and showed that genome-wide methylation patterns in the house sparrow remained highly stable over a broad range. From 101 loci in our dataset, we detected seven loci which show differentiation based on subspecies and also three loci which show alteration in the methylation level in relation to human

commensalism. Taken together, our results suggest that the methylome has a high level of variation at the inter-individual level in the native populations of house sparrow.

Our results provide some evidence for correlation between DNA methylation and phenotypic traits. We discovered that the standardized bill length for the Middle East samples is related positively to the DNA methylation percentage. Our data suggests that bill length is plastic and probably adaptive trait which can be regulated directly by environmental stimuli via environmentally-induced DNA methylation.

We acknowledge that failing to detect significant differences in DNA methylation among house sparrow subspecies across diverse habitats does not indicate that no such differences exist. Techniques that screen a much larger portion of the genome and those that provide more detailed information as to what is methylated, such as reduced representation bisulphite sequencing, or epiRAD (Baerwald et al. 2015), are likely to detect additional variation in DNA methylation. Further, these techniques may be more appropriate to investigate the link between DNA methylation states and phenotypic traits (Nätt et al. 2012; Baerwald et al. 2015; Riyahi et al. 2015; Trucchi et al. 2016).

In conclusion this study found variation in DNA methylation was largely independent of subspecies designation. Notably, DNA methylation of the European subspecies was differentiated from all other subspecies, and the non-commensal and migratory subspecies had the second highest level of differentiation. At some loci, DNA methylation was Differentiated based on subspecies and presence of commensal relationship with humans, Sex of individuals also generated differences in DNA methylation. Further, significant correlation was found between morphological traits and percentage of DNA methylation, and between geographical distance and percentage of DNA methylation. However, there were no clear effects of habitat detected in these data. Together, these results support the potential for epigenetic variation to be independent form genetic variation. The overall pattern of DNA methylation was not detected to follow differences in habitat. Yet, several other biological phenomena, including morphology, sex, and commensalism, were associated with variation in DNA methylation.

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Competing interests

No competing interests declared.

Author contributions

S. R. designed the lab experiments, analyzed the data and wrote the manuscript. J.C.S. supervised the study and revised the manuscript. R.V. designed the lab experiments, analyzed the data and revised the manuscript. A.S. revised the manuscript. M. A. and H. G. N. provided the samples.

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Table 2. Frequency of methylation states at the target sequence for each subspecies of house sparrow (*P. domesticus*).

Target state (band pattern)	***bactrianus***	***biblicus***	***hyrcanus***	***indicus***	***persicus***	***domesticus***
Unmethylated (HPA+/MSP+)	46.56	39.80	46.48	41.11	41.41	43.75
Hemimethylated (HPA+/MSP−)	18.44	20.31	20.31	18.65	20.18	14.54
Internal C methylation (HPA −/ MSP+)	7.97	7.45	6.64	9.77	8.79	12.86
Full methylation or mutation (HPA −/MSP−)	27.03	32.45	26.56	30.47	29.62	28.85

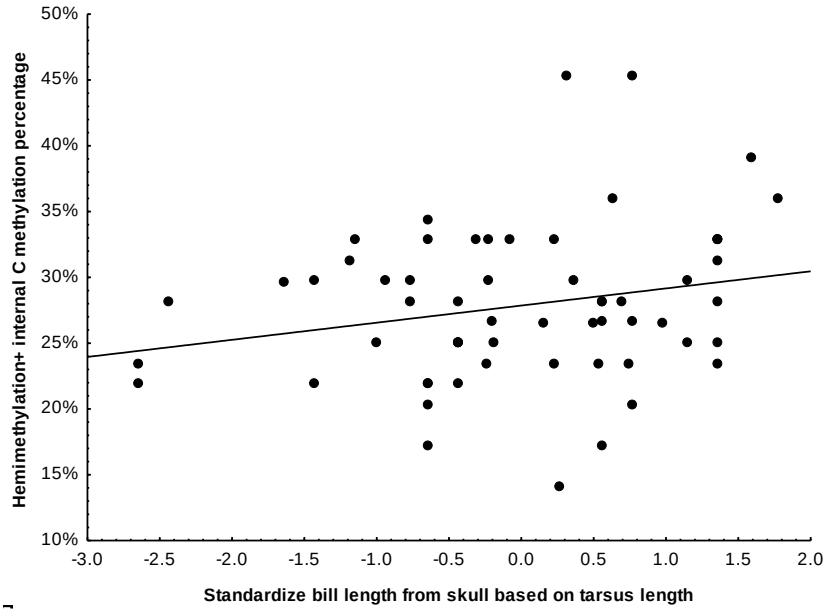

Figure 4. Relation between residuals of bill length from skull versus tarsus length regression and hemimethylation+ internal C methylation percentage.

Table 3. Pairwise AMOVAs between the pairs of subspecies of *Passer domesticus*. *P* values are in parenthesis. Bold ϕ_{ST} values have significant *P* values ($P< 0.05$).

	bactrianus	***biblicus***	***hyrcanus***	***indicus***	***persicus***	***domesticus***
bactrianus						
biblicus	**0.106 (0.0002)**					
hyrcanus	0.002 (0.46)	0.040 (0.17)				
indicus	0.037 (0.05)	0.008 (0.28)	0.006 (0.40)			
persicus	0.003 (0.39)	**0.055 (0.0009)**	0.013 (0.33)	0.023 (0.06)		
domesticus	**0.161 (<0.0001)**	**0.156 (<0.0001)**	**0.163 (0.0007)**	**0.129 (<0.0001)**	**0.115 (<0.0001)**	

Table 4. Analyses of molecular variance (AMOVA) based on the MSAP data. Bold ϕ_{ST} values have significant *P* values ($P< 0.05$).

Grouping variables	Sources of variation	df	Sum of squares	MSD	Variation	Φ_{ST} (P value)	N 1oci with significant Φ_{ST}
Including all subspecies (6 subspecies)							
Subspecies	Among subspecies	5	106.9	21.38	0.80	**0.070** (< 0.0001)	----
	Within subspecies	78	831.4	10.66	10.66		
Including Middle East subspecies (5 subspecies)							
Subspecies	Among subspecies	4	59.60	14.90	0.26	**0.022** (< 0.040)	7 (29, 32, 37, 52, 68, 71, 75)
	Within subspecies	66	752.32	11.40	11.40		
Commensal *vs* non-commensal	Among groups	1	15.35	15.35	0.22	**0.019** (< 0.045)	3 (29, 58, 71)
	Within groups	69	796,57	11.54	11.54		
Sex	Among groups	1	12.96	12.96	0.05	0.004 (= 0.231)	3 (39, 75, 100)
	Within groups	62	709.62	11.45	11.45		

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