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El efecto de genes candidato sobre la expresión de la actividad migratoria en currucas capirotadas (*Sylvia atracapilla*) de la Península Ibéricas

The effect of candidate genes on the expression of migratory behaviour in Eurasian blackcaps (*Sylvia atracapilla*) from the Iberian Peninsula

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Table of contents

Summary 6

Resumen 8

General section

Introduction 12
Aims 15
Methods 17
Conclusions 20

Section I Associations between genes and migratory behavior in Iberian blackcaps

- Chapter 1: *Association between variation in candidate genes and migratory behaviour in Iberian blackcap populations* 27
- Supplementary material 43

- Chapter 2: *Candidate genes do not predict variation in zugunruhe expressed under different environmental conditions in Iberian blackcaps* 71
- Supplementary material 85

- Chapter 3: *Association between molecular variation and migration strategies in a partially migratory bird population in the wild* 93
- Supplementary material 103

Section II Testing the hypothesis of indirect genetic effects on migration through correlations with personality traits

- Chapter 4: *The association between genetic markers, dominance and personality-related traits in a migratory bird species* 111
- Supplementary material 127

Section III A detailed look at the candidate gene G3PD in blackcaps

- Chapter 5: *Glycerol-3-phosphate dehydrogenase (G3PD) sequence polymorphisms cannot explain allozymic variation associated with migratory behaviour in the blackcap (Sylvia atricapilla)* 133
- Supplementary material 143

References 153

Acknowledgements / Agradeciemientos / Dankwoord / Anerkennungen 165

"Without data you're just another person with an opinion"
William Edwards Deming

Summary

Migration is central to the life-history of many animals of all taxa. Heritable genetic differences have been identified as major causes of differences in the expression of specific migratory behaviour, yet our knowledge of its molecular bases remains limited. In this thesis, I try to explore in more detail the extent of genes affecting migratory behaviour.
Studies on a wide range of species and taxa have found several genes that influence various aspects of migratory behaviour. Here, I tested for associations between candidate genes and migratory behaviour under various conditions in order to determine whether genetic effects are consistent throughout populations, migratory traits and environmental conditions. For doing this, I used a bird species that previously has been used as a model for migration studies: the European blackcap (*Sylvia atricapilla*, Linneaus 1758). On the Iberian Peninsula, this species has populations with all possible migratory strategies, resident, partially migratory, completely migratory. Having such a wide range of behaviours within one species on a small geographical scale makes it a very interesting model to study the effects of genes on migratory behaviour, since it eliminates cross-species comparative noise and allows us to minimize a number of other factors potentially confounded in large-scale studies like, for instance, geographic variation due to gene flow and colonization history.

I investigated possible associations between genes and migratory behaviour using a set of candidate genes, three of which have been related to circadian behaviour (CLOCK, ADCYAP1 and NPAS2) and one related to harm avoidance behaviour (SERT). I tested for associations between these genes and migration in three different sets of samples, covering different levels (populational and individual) and different components of migratory behaviour:
(a) Samples that were collected in the field in 21 Iberian populations. Migration status of each population was determined using wing measurements and data on presence and absence in winter.
(b) Birds of 3 populations displaying marked differences in migratory tendency in the field. Individuals were kept in cages, where migratory restlessness was measured in a controlled environment.
(c) Birds from a partially migratory population were monitored in the wild. The migration strategy of each individual bird was determined using ringing and observational data on presence and absence in the area.

The results show that several genetic markers were associated with the migration strategy of wild Iberian blackcap populations. The marker that explained the most variation in migration strategy was the candidate gene ADCYAP1. CLOCK also differed significantly among populations differing in migration. Combining data on ADCYAP1 and CLOCK proved to be a good predictor of a populations' migration strategy.
The investigation of migratory activity of individual birds kept in captivity did not support these findings. I found associations between various measurements of migratory restlessness and polymorphisms at different loci, including a number of presumably neutral markers, but not at ADCYAP1 and CLOCK. However, none of these associations were consistent across populations and measurements of activity. In the study on blackcaps from a partially migratory population in the wild, there was no evidence for an association between individual migration strategy and ADCYAP1. In this analysis the association with CLOCK was weak and did not reach significance.

One explanation for these apparently inconsistent results, which seem to depend on environmental circumstances, could be that Iberian blackcaps are close to the threshold of migration and that they are, therefore, influenced by environmental conditions to a higher degree than elsewhere in the blackcap range.

I also looked into the possibility that genes affect migratory behaviour indirectly. That is, that genes affecting other behavioural traits linked to migration, like personality traits, could have an effect on the propensity to migrate. For this, I tested the effects of the previously mentioned set of candidate genes on six different personality axes (2 exploration-memory axes, 1 dominance, 3 moderate stress response). The results suggest that it is unlikely that migratory behaviour is better explained by variation in personality or dominance, or genes underlying either of those, then by genes acting on migratory behaviour "directly", at least for the genes studied here.

In blackcaps, allozymic variants of a possible candidate gene, G3PD, were previously found to be linked to migratory behaviour. It was hypothesized that genetic variation on this locus could be shaped by selection for migration. Here, I studied sequence differences at this gene with the aim of identifying the molecular bases underlying variation at this enzyme, and for designing a molecular marker that could help to study this polymorphism on a large scale. I studied two G3PD genes, one on chromosome 7 and one on linkage group 22. However, I could not demonstrate a link between protein variation and sequence variation at these genes. Assuming that there are more exonic regions than the ones I've been able to sequence, it seems likely that the mutation underlying the G3PD-S allozyme could be located on one of these exons. Other sequencing techniques should be applied to clarify this.

Overall, the findings of this thesis, compared to previous studies, indicate that whether or not a gene has an effect on the expression of migratory behaviour depends on several factors, like the exact behavioural trait studied, the environmental conditions in which the behaviour is expressed, the species and the geographical range considered. For this reason, the same gene can have a large effect on variation in the expression of migratory behaviour in one study but have no effects in another study. This makes it very difficult to advance in understanding the genetic control of migration. Future studies should try to standardize the behavioural traits and the environmental conditions in which the traits are measured as much as possible. Moreover, they should aim at repeating studies in the same species considering potential geographic and temporal variation. This will allow obtaining a better understanding of the genetic control of migration.

Resumen

La migración es un aspecto fundamental en la vida de muchos animales de todo tipo de taxones. Se ha establecido que las diferencias genéticas son la mayor causa en las variaciones en la expresión del comportamiento migratorio, pero nuestro conocimiento sobre las bases moleculares es aun limitado. En esta tesis, trato de explorar en mayor detalle el alcance de los genes que afectan al comportamiento migratorio.
Diversos estudios realizados en un amplio rango de especies y taxones han permitido encontrar diversos genes que afectan a distintos aspectos del comportamiento migratorio. En este trabajo, compruebo la asociación entre varios genes candidatos y el comportamiento migratorio bajo diversas condiciones, con el objetivo de determinar si los efectos genéticos son consistentes entre poblaciones, entre rasgos migratorios y entre condiciones ambientales. Para ello, he utilizado una especie de ave que previamente había sido empleada como modelo para estudios migratorios: la curruca capirotada (*Sylvia atricapilla,* Linneaus 1758). En la Península Ibérica, esta especie tiene poblaciones con todas las posibles estrategias migratorias: pueden ser residentes, migradores parciales o migradores completos. El poseer este rango de comportamientos en la misma especie, en una escala geográfica pequeña, lo convierte en un modelo muy interesante para estudiar los efectos de los genes en el comportamiento migratorio; de esta manera se elimina el ruido de estudios comparativos entre especies, además de permitir minimizar otros posibles factores de confusión típicos de los estudios a gran escala, como, por ejemplo, la variación geográfica debido al flujo de genes o patrones de diferenciación que son debidos a la historia de colonización.

He investigado la posible asociación entre genes y el comportamiento migratorio utilizando un grupo de genes candidatos, tres de los cuales están relacionados con los ritmos circadianos (CLOCK, ADCYAP1 y NPAS2) y uno relacionado con el comportamiento de evitación del daño (SERT). He comprobado la asociación entre estos genes y la migración en tres grupos diferentes de muestras, asi cubriendo distintos niveles (poblacionales e individuales) y diferentes componentes del comportamiento migratorio:
(a) Muestras recogidas en el campo en 21 poblaciones ibéricas. El carácter migratorio de cada población fue establecido usando medidas del ala y datos de presencia o ausencia en invierno.
(b) Capturando individuos de 3 poblaciones con diferencias marcadas en su comportamiento migratorio. Los individuos se mantuvieron en jaulas, donde se midió su actividad migradora (*zugunruhe*) en un ambiente controlado.
(c) Individuos de una población parcialmente migradora fueron estudiados en el campo. La estrategia migratoria de cada individuo fue determinada mediante anillamiento y datos observacionales sobre su presencia o ausencia en el área.

Los resultados muestran que varios marcadores genéticos están asociados con la estrategia migratoria de las poblaciones de currucas capirotadas ibéricas estudiadas en su entorno natural. El marcador que explica la mayor variación en la estrategia migratoria fue el gen candidato ADCYAP1, aunque CLOCK también difiere significativamente entre poblaciones con distinto comportamiento migratorio. La información combinada sobre ADCYAP1 y CLOCK ha demostrado ser un buen predictor de la estrategias migratoria de una población.
La investigación de la actividad migratoria en individuos mantenidos en cautividad no ha apoyado estos hallazgos. He encontrado asociaciones entre varias medidas de la actividad migradora y polomorfismos en diferentes loci, incluyendo varios marcadores presumiblemente neutrales, pero no con ADCYAP1 y CLOCK. Sin embargo, ninguna de estas asociaciones fue consistente entre poblaciones y medidas de la actividad migratoria. En el estudio realizado en el campo en poblaciones parcialmente migradoras, no

hay evidencia de una asociación entre la estrategia de migración individual y ADCYAP1. En este análisis la asociación con CLOCK era débil y no llegó al nivel de significción.

Una explicación para estos resultados aparentemente inconsistentes, podría ser la dependencia de la expresión de la actividad migradora de las circunstancias ambientales. Parece que las currucas capirotadas ibéricas están cerca del umbral de migración y están por ello influenciadas en mayor grado por las condiciones ambientales que currucas capirotadas de otras zonas geográficas.

También se estudió la posibilidad de que los genes afecten al comportamiento migratorio indirectamente. Esto se podría dar si genes que afecten a otros rasgos del comportamiento, como distintas características de la personalidad, tuvieran un efecto sobre la probabilidad de expresar actividad migradora. Para comprobar esta hipótesis, he analizado el efecto de los grupos de genes antes comentados en seis ejes diferentes de la personalidad (2 ejes de la exploración-memoria, 1 de la dominancia y 3 de la respuesta a estrés moderado). Los resultados sugieren que es poco probable que el comportamiento migratorio esté mejor explicado por la variación en típos de personalidad o dominancia, o por los genes subyacentes a estos rasgos, que por los genes que actúan sobre el comportamiento migratorio“directamente”, al menos en el caso de los genes aquí estudiados.

En el pasado se encontró en la curruca capirotada que las aloenzimas de un gen candidato, G3PD, estaban relacionadas con el comportamiento migratorio. Se planteó la hipótesis de que la variación genética en este locus está determinada por la selección sobre la conducta migratoria. He analizado las diferencias en la secuencia de este gen con el propósito de identificar las bases moleculares de la variación de esta enzima, para poder diseñar un marcador molecular que pueda ayudar a estudiar su polimorfismo a gran escala. He estudiado dos genes G3PD, uno en el cromosoma 7 y otro en el grupo de genes ligados 22. No he podido demostrar una relación entre la variación proteica y la variación en la secuencia de estos genes. Asumiendo que existen más regiones exónicas que las que he sido capaz de secuenciar, parece que las mutaciones subyacentes a la aloenzima G3PD-S puede estar localizada en uno de estos exones. Para aclarar este aspecto, lo más indicado sería utilizar otras técnicas de secuenciación.

En general, los resultados de esta tesis, comparados con los de estudios previos, indican que hay diversos factores que intervienen para que un gen tenga un efecto en la expresión del comportamiento migratorio: la especie, el rango geográfico de estudio, las condiciones ambientales y la variable comportamental estudiada. Por esto, el mismo gen puede tener un gran efecto en el comportamiento migratorio en un estudio, pero no tenerlo en otro. Esto hace muy difícil el avance en la comprensión del control genético de la migración. En futuros estudios, debería intentarse estandarizar lo máximo posible los rasgos comportamentales a estudiar y las condiciones ambientales en las cuales las características son medidas. Más aun, se debería intentar repetir los estudios en la misma especie considerando potenciales variaciones geográficas y temporales. Esto permitirá obtener un mejor conocimiento del control genético de la migración.

Introduction

Migration qualifies as one of the most conspicuous and widespread phenomena in nature. It is found throughout many taxa and has evolved into many forms; various distances, through different media and using multiple modes of locomotion and transport (e.g. Harker 1958, Dingle 1996, Alerstam *et al.* 2003, Wilcove & Wikelski 2008, Hansson & Åkesson 2014). Migration is central to the life-history adaptations of many animals. The behaviour, and its control, will consequently influence other fields of research within biology, like response to climate change, speciation and conservation biology (Liedvogel *et al.* 2011).

One of the central questions in migration research is "Why do animals migrate?". Various reasons to migrate have been proposed. Probably the most obvious reasons are environmental factors like food availability that drive animals to migrate (e.g. Boyle & Conway 2007, Wysujack *et al*. 2009). Population dynamics can also play a role when making the decision whether to migrate or not. Density or dominance status, for instance, could cause an animal to have better survival chances elsewhere (e.g. Berthold 2001). When food is scarce the food sources tend to get occupied by more dominant individuals. Subordinates tend to migrate in order to avoid competition that could be more costly than migration itself (Ketterson and Nolan Jr, 1979, Marra, 2000).

But, while the ecological causes of variation in migratory behaviour have been under investigation for many years, studies on the genetic bases of migration have been scarce. In the last 30 years, however, a number of studies investigating the evolutionary genetics of migration, based on phenotypic differences among species, populations and individuals, have been conducted (see, for instance, Berthold & Pulido 1994; Pulido 2007, 2011; Hecht *et al.* 2015). One of the first pieces of evidence was provided by common-garden and cross-breeding experiments among groups of European blackcaps (*Sylvia atricapilla*) that differed in migratory direction and activity, which indicated a strong genetic basis of among-population difference in migratory behaviour, at least in small passerines (Berthold & Querner 1981*,* Berthold *et al.* 1990*,* Berthold 1991, reviewed in Pulido & Berthold 2003). Moreover, high genetic correlations among incidence, amount, intensity and timing of migratory activity in blackcaps suggested that these components of migratory behaviour are influenced by common genetic mechanisms (Pulido *et al.* 1996; Pulido & Berthold 2003*,* 2010).
Whether or not this genetic potential is expressed could be dependent of environmental factors. A study on blackcaps showed that whether or not a bird migrates not only depends on its genetic breeding value determining the amount of migratory activity it will produce and the position of a threshold that determines whether the activity will be expressed, but also on environmental effects (Pulido & Berthold 2010, Pulido 2011). There is suggestive evidence that genes controlling the position of this threshold and the amount of activity are very tightly linked (Pulido *et al.* 1996), and that the position of the threshold and the activity displayed are modified by environmental variation. The effects of the environmental variation on the migration threshold are largest in individuals and populations close to the migration threshold (Pulido 2011).

Recently, a few studies have started to investigate the molecular genetic bases of variation in migratory behaviour (e.g. Mueller *et al.* 2011, Hecht 2013, and reviewed by Liedvogel *et al.* 2011).
A general pattern found in birds, fish and insects is that intraspecific differences in migratory traits either

do not, or only weakly, correlate with overall genetic differentiation at neutral markers (Buerkle 1999, Bench *et al.* 1999, 2002, Pérez-Tris *et al.* 2004, O'Malley and Banks 2007, 2008). This could mean that phenotypic variation in migratory traits results from selection on relatively few genomic regions or loci. These could however alter the expression of many genes downstream as the result of a cascade reaction (Liedvogel *et al.* 2011; see also Bensch *et al.* 2002; 2009).

One approach to investigate the genetic background of traits is a so-called "bottom-up approach", where the first step is to investigate variation in the genes. Then, the genetic variation is used to explain the variation found in phenotypes. This method became on the rise when molecular techniques and the field of genomics developed rapidly facilitating such approaches (Boake *et al.* 2002). One bottom-up approach is the candidate gene approach, in which candidates for trait loci are selected on the basis of knowledge on their effects on similar phenotypes in model species like humans, fowl or mice. This method avoids the time consuming process of a genome wide search and requires little prior sequence information; making it an attractive method to use when working on a non-model species (Fitzpatrick *et al.* 2005).

In a first study using a candidate-gene approach for identifying "migration genes" in blackcaps, polymorphisms in six previously identified candidate genes for behavioural traits, which could be linked to migration were investigated. Allelic variation at one of these candidate loci, the ADCYAP1 gene, significantly correlated with variation in the amount of migratory restlessness - i.e., a measure of migratory activity displayed by migratory songbirds in captivity (see, Berthold 2001; Pulido 2011) - within populations and with migratoriness (i.e, the proportion of migrants and the distance moved) across populations (Mueller *et al.* 2011). This study stimulated a number of other studies using candidate genes. However, the results of these studies regularly have been fairly inconsistent with previous research. For instance, another study on blackcaps did not confirm the results on ADCYAP1 (Mettler *et al.* 2015), which may be due to studying other migratory traits (spring arrival) than those studied by Mueller *et al.* (2011). Such inconsistencies make generalizing results about gene effects problematic. Further research is needed to better understand the conditions under which migratory behaviour is expressed.

Aims

The general aim of this thesis is to investigate possible genetic influences on the expression of migratory behaviour and on the control of the threshold of migration. In order to do so I applied several approaches:

Section 1

Firstly I aimed to test for associations between candidate loci and migration in the blackcap, a species that shows a wide array of different migratory strategies on a small geographical scale. I looked for associations between migratory behaviour and genes using three approaches. (1) I tested for associations in a large sample set of wild birds for which we did not know the individual behaviour but only knew the general migration strategy of each population. (2) I tested for associations in birds of various populations with different migratory strategies held in captivity. With this approach, I could measure every detail of each individual's migratory behaviour. (3) I tested for associations between genes and behaviour in a wild, partially migratory, population where we knew the behaviour of each individual under natural conditions, due to intensive monitoring in the field.
By combining these three approaches I aimed at unravelling and studying possible condition-dependency of the associations between genes, migratory behaviour and the migration threshold. Since the analyses of candidate genes have yielded rather inconsistent results across different studies, I hoped gaining a better understanding of genetic effects by investigating the same genes in different sample sets, considering different behavioural measurements and under different environmental circumstances. Another aim of this approach was to get more insight into the question to what extent results based on migratory restlessness data are valid for migratory behaviour expressed in the wild, i.e. whether captivity alters the expression of migratory behaviour.

Section 2

Secondly I investigated to what extent genes could influence migratory behaviour indirectly due to effects of these genes on other behavioural traits. Since previously found associations between genetic markers and migratory behaviour have been weak and the candidate genes with the strongest and most consistent association are not specific "migration genes", it could be possible that these genes primarily affect "general" behavioural traits, reflected in animal personalities, and that the effects of these genes on migratory behaviour is indirect, due to the link between personality traits and migration. I also looked at genes not known for affecting migration, but known for affecting personalities, to see whether they might indirectly influence migratory behaviour. I aimed to test the effects of these "migration" and "personality" genes on personality traits and compare them to their effect on migratory traits.

Section 3

Thirdly, I studied sequence differences underlying the allozymic variants of a possible candidate gene that were previously found to be linked to migratory behaviour in the study species. It was hypothesized that genetic variation on this locus could be shaped by selection for migration. By combining the knowledge on variation in proteins and variation in genetic sequence, the mechanism underlying selection might become clearer. Another aim of this study was to develop a marker to test for the polymorphism using smaller, non-invasive samples allowing us to screen large numbers of birds with different migration strategies to study the association between this gene and migratory behaviour on a large scale.

Methods

Iberian blackcaps

Throughout this study (but in chapter 5), I only used blackcaps from a small part of its distribution, i.e. from the Iberian Peninsula. Iberian blackcaps show all possible migratory strategies on a relatively small spatial scale (see, for instance, Pérez-Tris & Tellería 2002). This allows us to minimize a number of potential factors confounding large-scale studies like, for instance, geographic variation due to gene flow and colonization history, and differentiation due to selection on other traits. Also, Iberia has been a refugium to the blackcap during the Pleistocene. For this reason Iberian blackcaps have an older and more complex history of among-population divergence than northern populations (Perez-Tris *et al.* 2004). In addition, high levels of genetic variation found in this area could result in different associations with genes due to its history. Therefore, it's interesting to test whether associations found in studies covering the entire blackcap distribution are consistently found throughout the species or whether it depends on the sample used.

Sampling and data collection

Our main source for DNA was blood. A blood sample (ca 50 µl) was taken from each individual by either puncturing the brachial vein, or by extraction from the jugular vein with a syringe. Blood was stored in alcohol in a freezer (at -20^{o}C) until lab-work commenced. DNA extractions were made using a *Blood and Tissue Kit* (Macherry & Nagel).

In the field, we determined the migration strategy of birds by using either of two methods: (1) In 21 Iberian locations, birds were captured in their presumptive breeding area before migration started (i.e. in July – mid August, for details, see Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015). As birds were caught only once in the field, it was impossible to determine their migratory behaviour based solely on capture data (see de la Hera *et al.* 2014, Morganti *et al.* 2015). Migration status of each population was therefore determined using wing measurements (according to Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015), and data on presence and absence in winter (SEO/BirdLife, 2012; de la Hera *et al.* 2014). (2). In one particular population in Cocentaina (Valencia), we demonstrated that it was partially migratory by performing a long term, high intensity capture-recapture effort. We combined the resulting presence-absence data with morphometric measurements of the wing to determine the migration status of each individual bird (see Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015, 2017).

In captivity, we measured individual migratory behaviour in greater detail. Fledglings from three populations, of different migratory strategies, were caught at the end of the breeding season and brought to a facility at the Casa de Campo in Madrid. The birds were kept indoors, in individual cages. Activity inside these cages was measured as the amount of perch contacts recorded through micro switches placed under two movable perches that were connected to the Microscript recording system (Berthold *et al.* 1972). This nocturnal activity, also called Zugunruhe or migratory restlessness, is a measure of migratory activity displayed by migratory songbirds in captivity (see, Berthold 2001; Pulido 2011) and is correlated to migratory activity in the wild (see, for instance, Berthold 1973; Berthold 1988; Eikenaar *et al.* 2014). Activity was monitored from September through May. We also kept some birds captive in outdoor aviaries where birds were exposed to another, more natural environment. Activity in aviaries was monitored using infrared surveillance cameras.

From the data collected from the captive birds we obtained information on the timing of migration (the onset and the end), the intensity and amount of activity and the type of activity (for more details, see Bulaic 2015).

Candidate genes

In this study I investigated a particular set of candidate genes. Most of these genes have previously been studied by Mueller *et al.* (2011). Each of this genes had its own reason as for why it was interesting for us to look at it in more detail. ADCYAP1, CLOCK and NPAS2 have been related to circadian behaviour. SERT and DRD4 have been found to be associated to personality-related behaviour. These could possibly affect the personalities of the birds, thereby indirectly influencing migratory behaviour. G3PD is involved in the synthesis of lipids and in the gluconeogenesis of fats, which could be important in the preparation for migration or the use of fat reserves during migration.

ADCYAP1

A recent study in blackcaps found that allelic variation at the ADCYAP1, adenylate cyclase activating polypeptide, gene significantly correlated with variation in the amount of migratory restlessness within populations and with migratoriness (i.e., the proportion of migrants and the distance moved) across populations (Mueller *et al.* 2011). Another study on blackcaps did not confirm these results (Mettler *et al.* 2015), which may be due to studying other migratory traits (spring arrival) than those studied by Mueller *et al.* (2011). A study in two North American junco species trying to replicate this study partly confirmed the association between ADCYAP1 and migratory behaviour (Peterson *et al.* 2013).

CLOCK

In fish, O'Malley *et al.* (2010) found evidence for an effect of the CLOCK gene on seasonal adaptation for migration and an influence on geographical variation in reproductive timing in several migratory species of salmon. In juncos, migration distance was found to be associated with CLOCK-gene variation. The results of this study indicated that individuals with long CLOCK alleles than birds with shorter alleles, but only within two sub-specific groups and not across the whole genus (Peterson *et al.* 2013). Other studies that found CLOCK to be associated with migratory behaviour focussed on traits related to the timing of migration or breeding. In barn swallows (*Hirundo rustica*), individuals with longer CLOCK alleles migrated later (Saino *et al.* 2015).

NPAS2

The neuronal PAS domain protein 2 (NPAS2) likely functions as a molecular clock operative in the mammalian forebrain. Its amino acid sequence is highly related to CLOCK (Reick *et al.* 2001). NPAS2 deficient mice for example show a changed locomotor activity pattern as well as an altered adaptability to a rapid shift in external light schedules (jet lag) and daytime feeding paradigms (Dudley *at al.* 2003).

SERT

Results on the serotonin transporter gene (SERT) are inconsistent when slightly different traits are measured (e.g. harm avoidance, neurotisms; Munafo *et al.* 2009). Though, in general, SERT has been shown to be associated with anxiety-related traits (Eley & Plomin 1997, Gordon & Hen 2004).

DRD4

In humans polymorphisms of the DRD4 gene have accounted for 3% of the novelty seeking behaviour (Munafo *et al.* 2008). Studies on great tits, *Parus major*, showed that the allele frequency of an exonic SNP in the DRD4 gene was associated with exploratory behaviour (Fidler *et al.* 2007) though this polymorphism could be linked to the functional variant in some, but not all populations, or the association might depend on the environment, since it was not found across populations (Korsten *et al.* 2010).

G3PD

Allozyme variation at the G3PD, Glycerol-3-phosphate dehydrogenase , locus in blackcaps was found to be strongly associated with non-migratory behaviour. The slow variant of this enzyme was only found in populations with non-migratory individuals (Pulido 1994). The hypothesis that this enzyme plays a central role in the migration metabolism of birds was supported by the fact that G3PD is involved in the synthesis of lipids and gluconeogenesis from fats in chicken (Harding *et al.* 1975). Since fats are the main fuel of migratory birds (Berthold 1996), G3PD could play a central role either in the building up of fat reserves before migration or in the generation of glucose during migration.

Apart from the candidate genes I also analysed a set of 8 anonymous, neutral microsatellite loci (Syl1, Syl2, Syl4, Syl5, Syl6, Syl9, Ppi2 and Pca8), to evaluate the null hypothesis of no association between allelic variation and migratory behaviour. A sexing marker, P2P8 (Griffiths *et al.* 1998), was only used to sex the birds. The selection of these neutral markers was based on Segelbacher *et al.* (2008) and Steinmeyer *et al.* (2009).

Laboratory analysis

For analysing variation at the candidate genes ADCYAP1, CLOCK, NPAS2 and SERT exonic micosatellites were used (following Mueller *et al.* 2011). A microsatellite in the DRD4 candidate gene is not known, therefore I tried to sequence blackcap specific SNPs at exon 3 of the DRD4 gene identified in another blackcap study (Mueller *et al.* 2011). For G3PD there is no marker, nor an alternative method to screen large sample sets. Therefore I attempted to design a genetic marker identifying different allozymes. The idea was to sequence the G3PD gene and search for polymorphisms that associate with the variation found in the allozymes. Afterwards, I would design a marker for this polymorphism.

In the microsatellite analysis, minimum, maximum and mean allele lengths, as well as major allele scores (the number of copies of the most common allele an individual possesses; see Mueller *et al.* 2013) were used to test for associations between behaviour and genes. In order to find out whether partial migrants are more similar to residents or migrants they were treated in various ways: separate, grouped with the residents, and grouped with the migrants.

I tested for independance of molecular and genetic variance by looking for allelic differences between groups of birds with differing migration strategy or activity. I wanted to see whether there are linear relationships between activity measurements and allele lengths. To do so, I used various statistical approaches, including contingency tests, mulitple regression analyses and AMOVAs.

Conclusions

Overall, the findings of this thesis, compared to previous studies, suggest that whether or not a gene has an effect on the expression of migratory behaviour and/or on its potential to express migratory behaviour, depends on several factors, like the exact behavioural trait studied, the population, and geographical range considered, etc. The same gene can appear to be irrelevant in one study, but influential in the next. This makes it particularly difficult to advance in understanding the genetic control of migration. Future studies should try to standardize the behavioural traits measured as much as possible. Moreover, they should aim at repeating studies in the same species considering potential geographical and temporal variation. This will allow controlling for these effects and unravelling variation in the genetic control of migration.

A summary of the conclusions of this thesis;

Section I

I: Several genetic markers were associated with the migration strategy of wild Iberian blackcap populations. The marker that explained the most variation in migration behaviour was the candidate gene ADCYAP1 (Chapter 1). This finding concurs with the results of a previous, Europe-wide, study on blackcaps. Individual behaviour in a partially migratory population was not associated with ADCYAP1 allele length (Chapter 3). The fact that northern blackcaps that winter at the study site did not differ from the residents at the ADCYAP1 locus, may indicate that the wintering birds are likely to breed in areas close to this wintering area.

II: Another candidate gene that showed discriminative powers in wild populations was CLOCK (Chapter 1). Though, when looking at individual behaviour in a partially migratory population, the association was only near significance (Chapter 3). Combining data on ADCYAP1 and CLOCK proved to be a good predictor of a populations' migration strategy (Chapter 1).

III: Associations between genes and individual migration behaviour in captivity, Zugunruhe –migratory restlessness-, were not consistent (Chapter 2). The fact that this is different from the associations found in wild populations (Chapter 1) is probably due to the fact that Iberian blackcaps are close to the threshold of migration and they are, therefore, expected to be influenced by environmental conditions to a higher degree than elsewhere in the blackcap distribution. This idea is supported by the results of the study on the wild partially migratory population (Chapter 3) since genetic differences between birds of different migration strategies could not be detected, making it more plausible that their behaviour is more dependent of environmental factors.

IV: Candidate marker SERT was found to be correlated with the type of behaviour displayed in outdoor aviaries (Chapter 2). SERT, has previously been found to be a candidate gene controlling harm avoidance behaviour and was shown to be highly significant with habitat type. Adding my results to those findings it appears that SERT might be more influential on behavioural matters, but not so much on the decision making and regulatory process of whether to migrate or not.

V: It appears that this study provides more evidence for the idea that Zugunruhe -migratory restlessness- might be a good measure for actual migration behaviour in the wild for long distance migrants, but not for short distance migrants (Chapter 2), which are close to the migration threshold.

VI: It is also possible that due to the Iberian peninsula having been the refugium for blackcaps in the ice age, that Iberian blackcaps have a much larger genetic variation making it possible that the set of genes controlling migration behaviour is different from the set of genes in blackcaps elsewhere in Europe. This might include genes I did not test for. In the case that migration in this population is controlled by different genes it would explain the discrepancy with the results from previous studies (Chapters 1, 2, 3).

VII: This study shows the difficulties of detecting differences between residents and migrants in a partially migratory population. To come to more clear conclusions and predictions on the control of migration I propose doing a similar study in a partially migratory population with a high proportion of migrants (Chapter 3).

Section II

VIII; NPAS2 proved not to be a very informative candidate marker in my thesis due to its low variability in alleles and high homozygosity (Chapters 1, 2, 3, and 4).

IX: Our analyses show that it is unlikely that migratory behaviour is better explained by variation in personality or dominance, or by genes underlying either of those, then by genes acting on migratory behaviour "directly", at least for the genes studied here (Chapter 4).

X: There is no evidence that there is one behavioural syndrome with which all behaviours are correlated. Instead of one clear behavioural axis, there appear to be three major axes in this study; (1) dominance, (2) moderate stress and (3) memory + exploration (Chapter 4).

Section III

XI: Single-site polymorphisms, involving changes of electric charges, at the two G3PD genes under investigation could not explain allozyme variation at this locus. However it is likely that the mutation underlying the G3PD-S allozyme is located on the exons that I could not sequence. Other sequencing techniques should be applied to clarify this (Chapter 5).

Section 1

Associations between genes and migratory behavior in Iberian blackcaps

Chapter 1

Association between variation in candidate genes and migratory behaviour in Iberian blackcap populations

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(unpublished manuscript)

Abstract

Heritable components have been identified as major causes of differences in the expression of specific migratory behaviour yet our knowledge of its molecular bases remains limited. A first study on blackcaps (Sylvia atricapilla) demonstrated an association both between among-population and within-population variation in migratory behaviour/nocturnal restlessness and mean allele length on the candidate gene locus ADCYAP1. Studies on other species could not fully replicate this result. Here, we aim at investigating the association between candidate loci and migratory behaviour in the blackcap on a smaller geographic scale to assess the generality of this association We genotyped 564 birds from 21 Iberian populations using 13 microsatellite loci, 4 of which were candidate gene loci (ADCYAP1, CLOCK, NPAS2 and SERT). Our results confirm previous findings that on average longer alleles at the ADCYAP1 locus are found in the more migratory populations. In addition to the results from the study in blackcaps at a continental scale, we found an association between allelic variation at the CLOCK gene with variation in migratory behaviour on the Iberian Peninsula. Apart from these two candidate genes, Syl9, a supposedly neutral marker, showed an association with migration. We discuss possible explanations for this finding. By combining allele data on ADCYAP1 and CLOCK it was possible to predict with high accuracy whether an Iberian blackcap population was sedentary or migratory. The results of our study confirm the importance of ADCYAP1 for the expression of migratory behaviour in the blackcap but also suggest that the strength of associations of migratory behaviour with candidate or other genes may depend on the scale or geographic region under investigation.

Introduction

Migration is central to the life-histories of many animals (Newton 2008, Dingle 2014). But, while the environmental causes of variation in migratory behaviour have been under investigation for many years, studies on the genetic bases of migration have been scarce. In the last 30 years, however, a number of studies investigating the evolutionary genetics of migration, based on phenotypic differences among species, populations and individuals, have been conducted (see, for instance, Berthold & Pulido 1994; Pulido 2007, 2011; Hecht et al. 2015). One of the first pieces of evidence was provided by cross-breeding experiments among groups of European blackcaps (*Sylvia atricapilla*) that differed in migratory direction and activity, which indicated a strong genetic basis of among-population difference in migratory behaviour, at least in small passerines (Berthold & Querner. 1981*,* Berthold *et al.* 1990*,* Berthold 1991, reviewed in Pulido & Berthold 2003). Moreover, high genetic correlations among incidence, amount, intensity and timing of migratory activity in blackcaps suggested that these components of migratory behaviour are influenced by common genetic mechanisms (Pulido *et al.* 1996; Pulido & Berthold 2003*, 2010*). Based on these and other results, it has been predicted that selection can change migratory behaviour and that among-population differences in migratory behaviour can evolve within few generations, especially in species that show a continuous scale of migration strategies (Berthold & Pulido 1994*;* Pulido and Berthold 1999, 2003, 2010; Pulido *et al.* 2001; Pulido 2007). A number of studies confirmed these predictions by demonstrating rapid evolutionary change in migratory behaviour (Berthold *et al.* 1992, Outlaw and Voelker 2006, Rolhausen *et al.* 2009, Pulido and Berthold 2010). One of the best examples for rapid evolutionary change in migration has been the strong reduction of migratory activity that recently has been observed in central European blackcap populations, presumably in response to climate change (Pulido & Berthold 2010). New wintering areas were established in only a few decades, involving evolutionary changes in migration distance and direction (Berthold *et al.* 1992, Bearhop *et al.* 2005, Rolhausen *et al.* 2009)

Recently, a few studies have started to investigate the molecular genetic bases of variation in migratory behaviour (e.g. Müller *et al.* 2011, Hecht 2013, and reviewed by Liedvogel *et al.* 2011). A general pattern found in birds, fish and insects is that intraspecific differences in migratory traits either do not, or only weakly, correlate with overall genetic differentiation at neutral markers (Buerkle 1999, Bench *et al.* 1999, 2002, Pérez-Tris *et al.* 2004, O'Malley and Banks 2007, 2008). This could mean that phenotypic variation in migratory traits results from selection on relatively few genomic regions or loci. These could however alter the expression of many genes downstream as the result of a cascade reaction (Liedvogel 2011). To identify the genetic regions involved in the expression of migratory behaviour several methods have been used: Analyses of mtDNA or microsatellites (Helbig 1996), amplified fragment length polymorphism, AFLP (Albert *et al.* 2006, Bensch *et al.* 2009) and candidate gene analyses (Mueller *et al.* 2011, O'Malley and Banks 2008, O'Malley *et al.* 2010). In fish, O'Malley *et al.* (2010) found evidence for an effect of the CLOCK gene on seasonal adaptation for migration and an influence on geographical variation in reproductive timing in several migratory species of salmon. In birds, a recent study in blackcaps investigated polymorphisms in six previously identified candidate genes for behavioural traits, which could be linked to migration. Allelic variation at one of these candidate loci, the ADCYAP1 gene, significantly correlated with variation in the amount of migratory restlessness - i.e., a measure of migratory activity displayed by migratory songbirds in captivity (see, Berthold 2001; Pulido 2011) - within populations and with migratoriness (i.e, the proportion of migrants and the distance moved) across

populations (Mueller *et al.* 2011). Another study on blackcaps did not confirm these results (Mettler *et al.* 2015), which may be due to studying other migratory traits (spring arrival) than those studied by Mueller *et al.* (2011). A study in two North American junco species trying to replicate this study partly confirmed the association between ADCYAP1 and migratory behaviour (Peterson et al. 2013).

In the present study, we aim at further testing the association between genetic variation at the ADCYAP1 locus and among-population variation in migratoriness , but on a smaller geographical scale than in the original study (Mueller *et al.* (2011). Iberian blackcap populations, populations evolved into a pleistocene refugium, and therefore having an older and more complex history of among-population divergence than northern populations, could have different associations with genes due to its history. Therefore it's interesting to test whether the association found is consistently found throughout the species or whether it depends on the sample used.
Apart from ADCYAP1 we aim to explore the relationships between other relevant candidate genes, even though they haven't shown consistent results across various studies, and migration to get an idea how general these relationships might be, and which genes are most often associated with migration.
Over its entire range, the blackcap shows a wide range of migration strategies (resident, partially migratory and completely migratory populations exist), which has made it the preferred study species for investigating the genetics and evolution of migratory behaviour (Berthold 2003, Pulido 2007). Studying migration in a species like this eliminates cross-species comparative noise. Since Iberian blackcaps show all the possible migratory strategies on a relatively small spatial scale, we can minimize a number of potential factors confounding large-scale studies like, for instance, geographic variation due to gene flow and colonization history and differentiation due to selection on other traits.

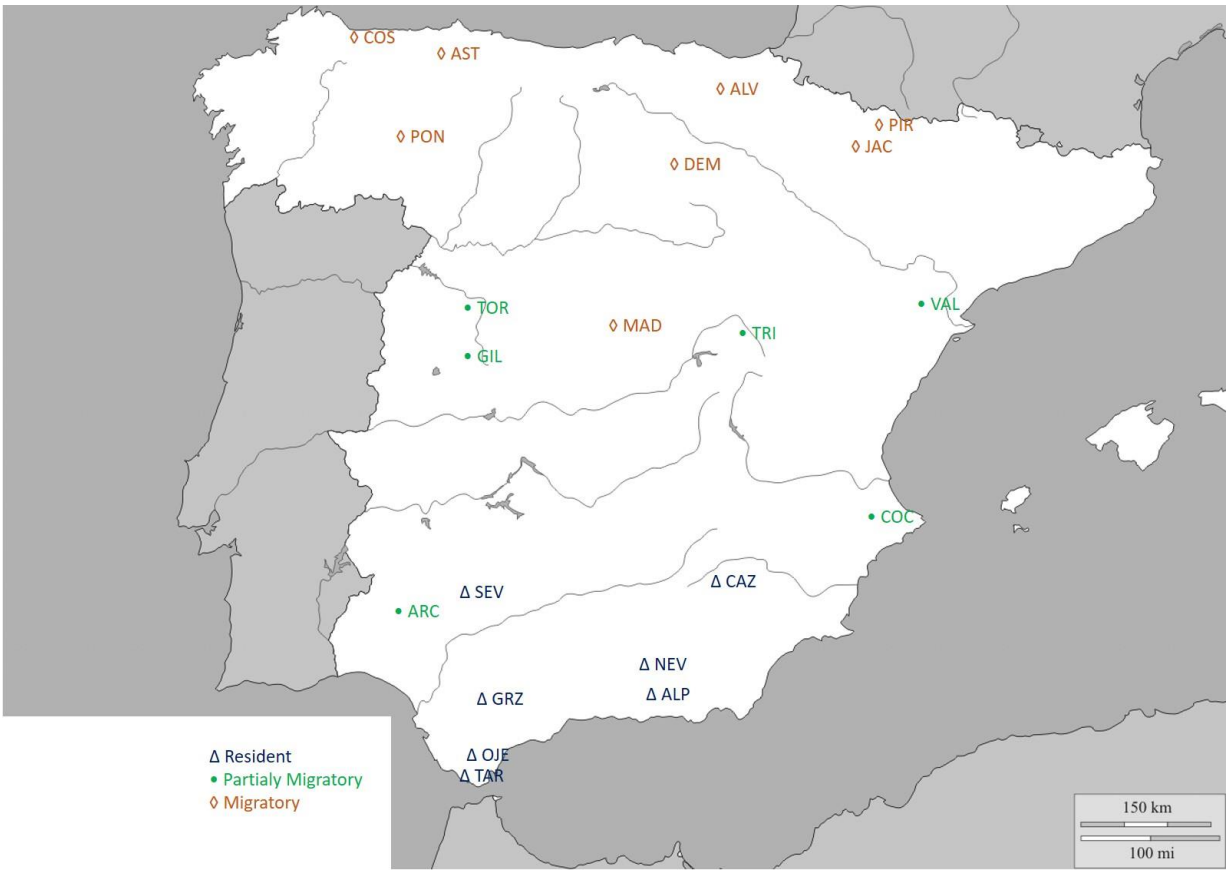
Material & Methods

Sampling

Blood samples from 564 individuals of 21 Iberian populations were taken over a period of six years (2008-2013), see figure 1 and table 1. All birds were captured in the breeding area before migration started (i.e. in July – mid August, for details, see Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015). The blackcap populations sampled exhibit all possible migration patterns, like residency, partial migration and complete migration, on the Iberian peninsula (Pulido 2007*,* Pérez-Tris *et al*. 2004). Hence; birds from populations with all possible migration strategies were represented in our dataset (See table 1 with the map for more detailed information). As birds were caught only once in the field, it was impossible to determine their migratory behaviour based solely on capture data (see de la Hera *et al.* 2014, Morganti *et al.* 2015). Migration status of each population was therefore determined using wing measurements (according to Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015), and data on presence and absence in winter (SEO/BirdLife, 2012).

Table 1. Overview of the populations studied, and their characteristics. Migration status: 0= sedentary, 1= partially migratory, 2= completely migratory. Map IDs in the table correspond to locations on the map below.

Map ID	Population	N	Sampling Year	Lattitude	Longitude	Altitude	Migration Status
TAR	Tarifa	22	2010-2013	36.05583	-5.62944	2	0
ALP	Alpujarras	11	2008	36.94125	-3.36744	1115	0
GRZ	Sierra de Grazalema	30	2008	36.75944	-5.48981	608	0
CAZ	Sierra de Cazorla	19	2011	38.01804	-2.86436	699	0
NEV	Sierra Nevada	14	2008	37.13295	-3.44642	1532	0
SEV	Sierra Norte de Sevilla	38	2011	37.94260	-5.73409	516	0
OJE	Sierra Ojén	12	2008	36.15944	-5.58056	186	0
COC	Cocentaina	90	2010-2013	38.72418	-0.43461	436	1
ARC	Aracena	25	2008	37.87863	-6.65345	636	1
GIL	Gilbuena	32	2011	40.41306	-5.61000	1042	1
TOR	Santa Marta de Tormes	30	2009	40.95250	-5.62944	778	1
TRI	Trillo	21	2011	40.72151	-2.61549	785	1
VAL	Valderobles	19	2009	40.85000	0.15000	575	1
MAD	Madrid	35	2010-2013	40.94806	-3.80111	1121	2
AST	Asturias	22	2009	43.38972	-6.00611	61	2
ALV	Alava	30	2008	42.89183	-2.51861	562	2
JAC	Jaca	14	2009	42.55000	-0.58333	829	2
PIR	Pirineos	25	2011	42.71842	-0.29978	1096	2
PON	Ponferada	36	2008	42.54667	-6.53333	563	2
COS	San Cosme de Barreiros	19	2011	43.54529	-7.23566	28	2
DEM	Sierra de la Demanda	20	2008	42.29024	-3.24542	1304	2



Genotyping

We took a blood sample (ca 50 µl) from each individual by either puncturing the brachial vein, or by extraction from the jugular vein with a syringe. Blood was stored in alcohol in a freezer (at -20ºC) until lab-work commenced. DNA extractions were made using a *Blood and Tissue Kit* (Macherry & Nagel). Following Mueller *et al.* (2011), we investigated four candidate genes, three of which have been related to circadian behaviour (CLOCK, ADCYAP1 and NPAS2) and one related to harm avoidance behaviour (SERT). For the analysis of variation at each of these genes we used exonic microsatellites (Mueller *et al.* 2011). For evaluating the null expectation we further analysed 8 anonymous, neutral microsatellite loci (Syl1, Syl2, Syl4, Syl5, Syl6, Syl9, Ppi2 and Pca8). The sexing marker P2P8 (Griffiths *et al.* 1998) was only used to sex the birds. Selection of the markers was partially based on Segelbacher *et al.* (2008) and Steinmeyer *et al.* (2009) (for more information see Supplementary table S1). All samples (N=564) were genotyped for these 13 microsatellites using an ABI 3100 sequencer (Applied Biosystems) and analysed in Genemapper® (Version 4.1, Applied Biosystems).

In order to compare and pool our data with the data obtained by Mueller et al. (2011), for which a different machine was used, we re-ran several of our samples side by side with samples used by Mueller *et al.* (2011) on the same machine to make a conversion table. (see Supplementary table S2, for allele frequencies of both studies including a conversion table, and Supplementary table S3 for allele frequencies for each population).

Data Analyses

Most statistical analyses were conducted using SPSS version 20 (IBM Corporation). Analysis of molecular variance (AMOVA) and basic population-genetic analyses were done in Arlequin 3.11 (Excoffier et al. 2005).

Kolmogorov-Smirnoff tests showed that the frequency distributions of allele lengths of the microsatellite data did not deviate from normality (Supplementary table S4 shows allele frequency tables per locus per population). Tests for Hardy Weinberg Equilibrium were performed using GenAlEx 6.502 (Peakall *et al.* 2012). Linkage disequilibrium was tested using Genepop V4 (Raymond and Rousset 1995). (Supplementary table S5 Shows summaries of HWE tests, Supplementary table S6 shows Linkage tables for each population). One of the neutral markers; Syl4, was not in HWE in most populations. This locus was therefore excluded from subsequent analyses.

For assessing the association between allele frequency differences and differences in migratory behaviour between populations we used generalised linear models (GLMs) with migration status as the response variable. We had several approaches to group our data. This was done to limit the possible effect of populations erroneously being classified a different migration status and to attempt to be able to distinguish between real genetic associations and effects by e.g. latitude. In the first approach, we considered “migration status” as a categorical variable with 3 groups (sedentary, partially migratory and migratory). In the three other approaches, we used a binary classification of migration status of the populations. In the second approach, “Migration Status 0/2”, we only compared sedentary populations with the completely migratory ones (partial migrants were excluded). In the third, “Migration Status 1”, we grouped the partially migratory populations with the sedentary ones and compared this group with the migratory populations. In the fourth and last, “Migration Status 2”, we grouped partial migrants with

migrants and compared this group with sedentary populations. As a measure for allele frequency, we used mean population allele length and the major allele value. The latter is the number of copies of the most common allele a birds has (Mueller *et al.* 2013). At some loci, we found more than one most common allele. For these loci we repeated the major allele analyses for all alleles that had the highest frequency.
In the analyses, in which we studied the effects on mean allele length, a univariate GLM was used considering migration status as the fixed factor and longitude and/or latitude as covariates. In the analyses where we studied the effects on the major allele value, a Monte Carlo approach was used entering migration status as the fixed factor and the major allele value as the dependent variable in the model.

We further conducted one-way Anovas for determining heterogeneity in the distribution of allele frequencies among groups of populations with different migratory behaviour (i.e. migrants, partial migrants, and residents). The Anovas were performed using minimum, maximum and mean allele lengths, and major allele value as dependent variables. In addition, we conducted a t-test for identifying differences in mean allele length between populations of different migration status. In this analysis, we considered only the binary variables, "Migration Status 0/2", "Migration Status 1" and "Migration Status 2", which we had previously defined (see above) as grouping factors.

We conducted Analyses of Molecular Variance (AMOVAS) to analyse population structure (Excoffier *et al.* 1992). By comparing differentiation at three different levels, among groups/among populations within groups/among individuals within populations, we can identify the importance of the candidate loci for migration or adaptation to the environment. We grouped populations according to six different grouping criteria (Altitude, Latitude, Migration status, Migration status 0/2, Migration status 1, Migration status 2). The distribution of molecular variance for each of these grouping criteria was calculated using AMOVAs for three sets of microsatellite markers: All markers together, only the candidate markers and only the neutral markers. This was done to see whether genetic structure in candidate markers is better explained by these grouping criteria than the neutral markers, as we would expect molecular variance to increase if genes are under selection, as expected for candidate genes. The AMOVAs were run in Arlequin.

Results

Association between genetic variation and migration status

The results of the univariate GLMs show that, of all the loci, mean allele length of ADCYAP1 together with the longitude of the population best explains the variation found in migratory behaviour among populations in all three groupings of migratory status ("Migration status 0/2": Longitude $p = 0.003$ and Migration $p = 0.001$. "Migration status 1": Longitude $p < 0.001$ and Migration $p = 0.005$. "Migration status 2": Longitude $p = 0.004$ and Migration $p = 0.006$.). Also allelic variation at the candidate gene CLOCK and at the neutral marker Syl9 explain some of the variation found in migratory behaviour (E.g. Syl9 at "Migration status 2, Longitude $p = 0.002$ and Migration $p < 0.001$" (see table 2 and Supplementary table S7 for detailed results).

Table 2; Results of GLMs on mean allele length and migration status 2 (= sedentary against partially migratory + migratory populations pooled.) Numbers show the probability of the mean allele length of each locus to explain variation in migration taking longitude, latitude or both in consideration (Results of GLMs on other groupings of populations are given in supplementary table S7).

	Migration Status 2						
					Longitude+Latitude		
Allele	Longitude	Migration	Latitude	Migration	Longitude+l	Latitude	Migration
ADCYAP1	**0.004**	**0.006**	0.595	**0.036**	**0.002**	0.185	0.420
CLOCK	0.872	**0.001**	0.475	**0.004**	0.980	0.486	**0.007**
NPAS2	0.177	**0.000**	0.192	0.089	0.294	0.323	**0.049**
SERT	0.965	0.389	0.627	0.794	0.861	0.607	0.859
PC8	**0.022**	**0.016**	0.665	0.117	**0.013**	0.291	**0.017**
Ppi2	0.276	0.082	0.074	0.769	0.515	0.120	0.976
Syl 1	0.540	0.369	0.446	0.964	0.667	0.533	0.905
Syl 2	0.080	**0.007**	0.775	**0.013**	0.083	0.862	0.098
Syl 5	**0.047**	**0.008**	0.063	0.516	0.120	0.165	0.495
Syl 6	0.415	0.507	0.252	0.250	0.250	0.160	0.135
SYL 9	**0.002**	**0.000**	0.802	**0.001**	**0.001**	0.558	**0.039**

The contingency analyses yielded a number of significant associations between migration status and major allele effects . However, the association of migration with major allele effects at ADCYAP1, NPAS2 and Syl1 were consistent whichever way we grouped migratory status. When looking at "Migration Status 2" ADCYAP1 $p < 0{,}001$; CLOCK $p = 0.001$; NPAS2 $p < 0.001$ and Syl1 has two major alleles, but is significant either way. Either $p = 0.002$ or $p = 0.04$. This suggest that these loci best explain variation in migratory behaviour (For more detailed results see Supplementary table S8)

One way Anovas showed that at several candidate gene loci there were differences in allele length between populations of different migration status. Apart from differences between migratory and sedentary populations at candidate-gene loci, allele lengths also differed significantly at several neutral loci: particularly, Syl9 which was significant for all different allele measurements. For candidate genes we found differences between populations with different migratory status in ADCYAP1 for all except for the minimum allele length, in CLOCK and NPAS2 for all except the maximum allele length (see Supplementary table S9)
The comparison of mean allele lengths between migratory and non-migratory populations gave similar results with most of the candidate markers showing a significant difference in allele length between the migration groups. NPAS2 and Syl5 yielded significant results for all three methods of grouping populations. ADCYAP1, CLOCK, and Syl9 were significant for two of the groupings (for all results see Supplementary table S10).

The Amovas used to calculate molecular variance showed that "migration status" was the criterion for grouping populations that explained the highest proportion of the variance in allele frequencies. We calculated a ratio of among-population to among-groups variances for evaluating the importance of groupings for the genetic differentiation (all markers: 0.55; candidate markers: 6.08; neutral markers; 0.27. Differences became even clearer when we grouped population using "Migration Status 2": all markers: 1.04; candidate markers: 8.39; neutral markers: 0.59), grouping by differences in latitude (all markers: 0.53; candidate markers: 1.91; neutral markers; 0.37) gave a very similar result. This shows that the latitude and migration status of a population are correlated, but that "migration status" had a higher

effect on population differentiation at candidate loci than latitude. Altitude did hardly explain any variance among populations (ratio of all markers: 0; candidate markers: -0.26; neutral markers; 0.07). In all analyses, the proportion of molecular variance explained by migration status was always highest in ADCYAP1. Among-population differentiation was highest and most significant at this locus. NPAS2 also showed high scores, however this is a locus with very few alleles which makes it unsuitable to draw any conclusions using variation at this locus. Apart from the candidate loci a neutral locus, Syl9, showed a strong differentiation if populations are grouped by migration status (For more detailed results see table 3 and Supplementary table S11).

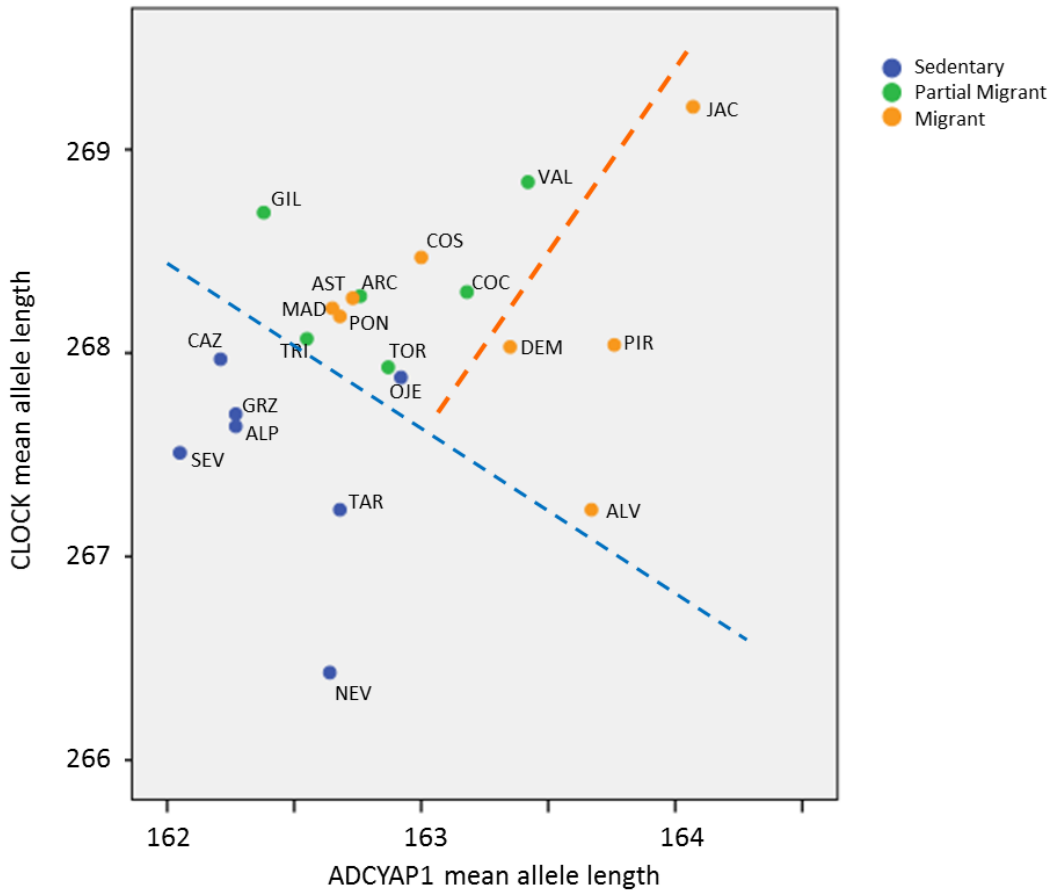
Table 3; Ratio of among-group to among-population variance component (AMOVA) considering different sets of genes and assuming different groupings of populations. (Migration Status = 3 migration statuses separate, Migration Status 1 = sedentary + partial migrants / migrants, Migration Status 2 = sedentary / partial migrants + migrants, Migration Status 0/2 = sedentary / migrants (thus excluding the partial migrants). Note; For more detailed results of the AMOVAs, see supplementary table S11.

	all	candidate	neutral
Altitude	0.000	-0.265	0.071
Latitude	0.532	1.907	0.371
Migration Status	0.546	6.083	0.275
Migration Status 1	0.067	0.490	-0.007
Migration Status 2	1.043	8.393	0.592
Migration Status 0/2	0.960	6.550	0.507

Identification of migratory and sedentary populations using genetic markers (alleles at candidate loci)

A plot of the mean allele length of the two, seemingly, most important candidate genes; ADCYAP1 and CLOCK showed a clear distinction between sedentary populations and the rest (Figure 1). This confirms the result of our previous analyses (Tables 2,3 and Supplementary tables S10,S11) that partially migratory populations appear to group with migratory populations rather than with sedentary ones. This figure suggests that we can predict the migration strategy (completely and partially migratory versus sedentary) of a population if we know the mean allele lengths of a population at these two loci.
Using logistic regression models we determined what percentage of populations would be assigned to the correct migratory status based on microsatellite data. Supplementary table S12 shows an overview of the results for all the loci and several combinations. Candidate loci can predict high amounts of populations correctly. Several combinations of candidate loci allow to make a 100% correct prediction. All neutral loci combined also make predictions that are 100% correct even though most neutral loci perform poorly on their own. This could be due to a latitudinal cline. However the result in neutral loci rests mainly on Syl9, a neutral locus that is strongly associated with migration status (see above). When we test the neutral loci excluding Syl9, the correct predictions sink down to 74.5% and 60.3% depending on which migration status grouping is being used (Migration status 2 and Migration status 0/2 respectively). Syl9 combined with a candidate locus also gives 100% correct predictions (Supplementary table S12 and Supplementary figure S13).

Figure 1; Separation of Iberian blackcap populations differing in migratory behaviour by mean allele length at the ADCYAP1 and CLOCK gene. Mean allele length of these two loci predict migration status with 95.2% correctness.



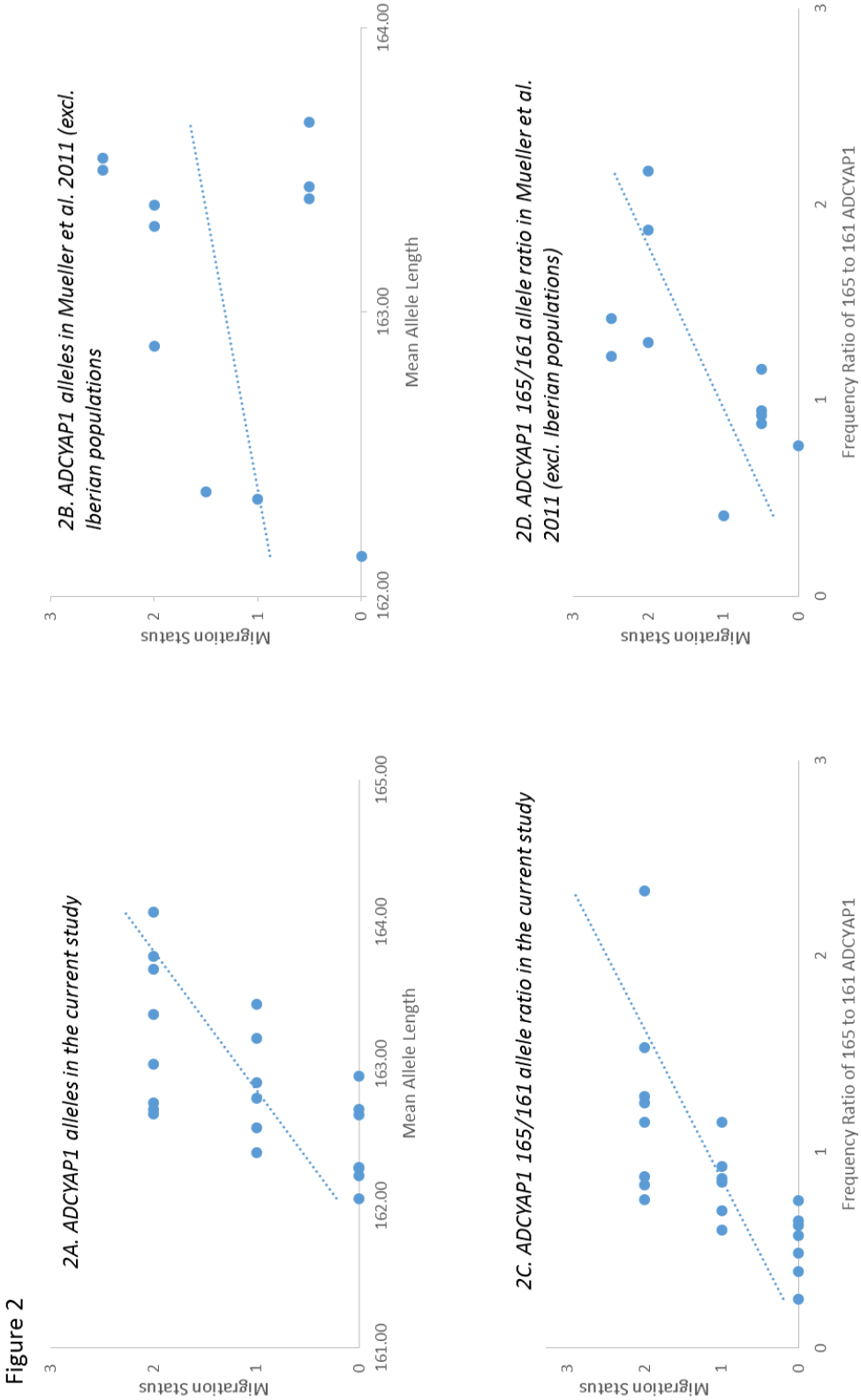
Analysis of combined dataset

When analysing the combined ADCYAP1 dataset *(i.e this study and the study by Mueller et al. 2011)*, we see that there are two clear most common (major) alleles for this locus within the complete European dataset. The dataset from the Iberian populations on its own showed the same pattern (Supplementary table S2).

When mean allele length of the Iberian populations is plotted against the migration status it clearly shows that the more migratory the population is, the longer the average ACDYAP1 alleles are (See figure 2A. See supplementary table S3 for ADCYAP1 allele frequencies per population). This is in accordance with the findings of Mueller *et al.* (2011) (Figure 2B). Also the cline of most common allele ratios is similar when comparing our findings to Mueller *et al.* (2011) (Figures 2C & 2D) Generally no changes in the allelic distributions were found when comparing blackcaps from the three areas (Madrid, Catalonia, Gibraltar/Tarifa) that were sampled in two studies 20 years apart. However, we found a significant difference in the distribution of alleles between the Gibraltar (samples in 1992) and the Tarifa samples, which geographically, are located 17 kilometres apart (Pearson exact test: p=0.001) (The results of other comparisons are Madrid-Madrid p=0.184, and Catalonia-Valderobles p=0.598).

Figure 2.

Figure 2



Discussion

Summary of results and comparison with Mueller et al. 2011 and other studies

Our study shows that in Iberian blackcaps, in most of the investigated candidate genes allele frequencies differ between populations with different migration statuses. Analyses of molecular variance showed that migration status and not geographic features (e.g. latitude or altitude), explains the highest proportion of molecular among-population variance (about 1.5%) at candidate loci, suggesting that genetic differentiation among populations is associated with selection processes favouring different migration strategies. Among candidate genes, the strongest differentiation was found in ADCYAP1, for which the more migratory populations had the longer alleles. This confirms the previously found association between ADCYAP1 and migratoriness in European blackcaps (Mueller *et al.* 2011), but at a smaller geographic scale. The fact that the strength of the association found in our study is stronger than in the study by Mueller *et al*. (2011) could be due to our study area being smaller and more homogenous. A study on two junco species *(J. hyemalis* and *J. phaeonotus)*, which used a similar approach, yielded different results (Peterson *et al.* 2013). In that study, an association between ADCYAP1 and migration activity was found, but it was inconsistent among species and populations.
A recent study on ADCYAP1 in wild blackcaps showed a possible effect of ADCYAP1 on spring arrival (Mettler *et al.* 2015). Birds with longer alleles arrived earlier. This effect, however, was found only in females with pointed wings. In a study on Wilson's warblers ADCYAP1 was associated with the breeding latitude of long-distant migrant males, while it was not associated with the timing of migration (Bazzi *et al.* 2016). These studies are, however, not comparable to our study or to the studies by Mueller *et al*. (2011) and by Peterson *et al*. (2013) since it studied the association between these candidate genes and arrival time, which is not likely to be controlled by the same set of genes than the amount of migratory activity or the propensity to migrate (see, Pulido & Berthold 2004).

Other markers found to be associated with migratory behaviour in Iberian blackcaps

Apart from ADCYAP1, CLOCK showed to have some discriminative power to separate populations of different migration strategies. We found shorter CLOCK alleles predominantly in sedentary populations. Latitudinal clines in CLOCK allele frequencies occur, also in non-migratory species (e.g. Blue tits, *Cyanistes caeruleus*, Johnsen *et al*. 2006). However in our study the variation found in CLOCK seems more likely to be due to migration, since our AMOVAs show that migration status explains the variation better than latitude. Even though the CLOCK-gene was assumed to be an important candidate locus in various migration studies, Mueller *et al.* (2011) did not find migration-linked variation for CLOCK in the blackcap. In juncos, however, migration distance was found to be associated with CLOCK. The longer the CLOCK alleles the further the bird migrated, but only within two sub-specific groups, not across the whole genus (Peterson *et al.* 2013). Other studies that found CLOCK to be associated with migratory behaviour focussed on traits related to the timing of migration or breeding. In barn swallows (*Hirundo rustica*), individuals with longer CLOCK alleles migrated later (Saino *et al.* 2015).

The third candidate gene that showed a positive correlation with Migration Status in our study was NPAS2. It is however difficult to draw any inferences based on our results since NPAS2 only exhibited 2 alleles in our sample, with more than 90% homozygosity. Thus, it had very little discriminative power.

Surprisingly, we also found Syl9, a supposedly neutral marker, to be a good predictor of migration status.

The association found could be due to chance. With a 0.05 significance level we expect to find 0.65 significant associations by chance alone when testing 13 markers. If the association was not due to chance it might be possible that Syl9 is part of a migration gene, or maybe it's linked to one. The pattern and degree of differentiation at this locus and the association with migration status of the populations, suggests that this locus is not neutral. At first glance, the results of the AMOVAs support this idea. Looking at the results of the AMOVAs, all neutral markers, but Syl9, are weakly linked to migration. In several analyses Syl1 showed a slightly stronger link which, together with the result of the Monte-Carlo test for this locus, could be due to geographical variation. The fact that Syl9, according to the AMOVAs, is in some cases more strongly linked to migration than any other marker, including the candidate genes, makes us believe that this locus is not neutral but associated to migration in Iberian blackcaps. However, in the AMOVAs, among group variation was not consistently greater than among population variation. In fact it was usually the opposite. This means that variation among populations was greater than variation among migration statuses. This indicates that Syl9 is most likely to be correlated with something else, not with migration

How could this association be used to identify migratory and non-migratory blackcap populations

Our results show that it is possible to quite accurately predict whether an Iberian blackcap population is sedentary or a migratory if we know mean allele lengths for ADCYAP1 and CLOCK of a given population. Logistic regression analyses showed that any combination of allele distributions at the candidate genes ADCYAP1, CLOCK and NPAS2 or a combination of one of these genes with Syl9 can accurately predict the migration status of a population. The figure for ADCYAP1 and CLOCK also shows that the distinction between partially migratory and completely migratory is impossible to make based on these data – a result which is obtained using any other combination of discriminative loci. This suggests that, genetically, partially migratory populations are more similar to migrants than to sedentary birds. This could be explained by the possibility that migratory blackcaps adapted to warming at their breeding areas by becoming less migratory (Pulido & Berthold 2010). Hence, it is likely that Iberian partially migratory populations originate from completely migratory populations. Their genetic background thus being more similar to the genetics of migratory populations then to sedentary populations. A previous study on Iberian blackcaps found the same pattern: Wing morphology of birds from a partially migratory population was more similar to migratory than to sedentary blackcaps (Morganti *et al.* 2015).

Shortcomings of this study – What should be done in the future?

Classifying the migration status of birds using presence and absence in winter and on ringing and recapture histories is very difficult and may in some cases, lead to erroneous results. This is particularly the case in populations where there is an influx in winter of birds that breed elsewhere, as is the case in many Iberian regions (see, de la Hera *et al.* 2014 & Morganti *et al.* 2015). Therefore, the classification of populations into sedentary, partially migratory or migratory may not always have been a correct assignation. This could explain the genetic similarity of partially migratory and migratory populations in our results. However, capture-recapture studies in partially migratory populations tend to overestimate the proportion of migrants in the population (see, Morganti *et al.* 2015). Also, our method of grouping the populations in various ways in our analysis should have limited the effect of possible misclassifications For these reasons we believe that the genetic similarity between partial migrants and migrants is not an artefact, but rather a consequence of similar selection or by common ancestry. To exclude the possible error in classifying migration status and to assess the effects of these genes on

migratory behaviour it would be necessary to know the migratory behaviour of each individual, this could either be done using tracking-techniques on birds in the wild (see, for instance, Fudickar *et al.* 2013, who did, however, not genotype birds) or measuring migratory activity of birds in captivity (see, studies by Mueller *et al.* 2011 and Peterson *et al.* 2013). Unfortunately using isotope analysis for determining migration status in iberian blackcaps has proved futile, probably because Iberian blackcaps are most likely short-distance migrants (Morganti *et al.* 2015). In European blackbirds (*Turdus merula*) short distance migrants could not be distinguished from residents using stable isotopes (Fudickar *et al.* 2013)

Our study only shows the current allele frequencies and lengths (samples taken from 2008-2013) of the Iberian populations. It would be very interesting if the spatial pattern of variation we see now is also shown through time when looking at a particular population where the migration strategy has changed recently over the years (see, for instance, Pulido and Berthold 2010). At this moment we could only compare our data with the three Iberian populations used in Mueller *et al.* 2011. (samples taken from 1989-1996) We did not expect to find considerable differences since winter temperatures on the Iberian peninsula have not changed in the past 30 years. The only difference we found was between the two samplings of the southernmost population. In the more recent sampling, alleles were not shorter, as expected for climate change, than they were in the past, just different alleles were more abundant. However, the comparison was not made with samples from the exact same population, which could influence this finding. Since birds might be genetically predisposed to be of a certain habitat and the Gibraltar site is more shrub land like than usual it is possible that these birds have a different pattern of movement. Thereby also altering their re-capture chances. Forrest birds seem to be recaptured more often than shrub land birds (personal observation JP-T). The Gibraltar population has only a very narrow, densely urban land bridge connecting it to the mainland blackcap stronghold. It is possible that gene flow is therefore absent making this population more isolated than previously thought. This would explain morphological differences observed (unpublished personal observation by FP.) A new set of Gibraltar samples could give answers.

A potential problem of our study is the fact that all sedentary populations are in the south of the study area. This makes it hard to distinguish between effects caused by migration and effects caused by the latitude of the population. These do give very similar results, which could be due to colonisation, temperature or selection. Only candidate genes (as expected) were associated with differentiation, not the neutral genes. If the pattern of differentiation observed was due to processes of colonization this should have affected all loci. Temperature seems an unlikely initiator since altitude did not appear to be correlated in our results. Latitude does explain a fairly high amount of variation between populations and groups in just the candidate genes according to our AMOVAs (table 3 and supplementary table S11). However, migration appears to explain a bigger amount, which makes it more plausible that migration is the main selection factor instead of latitude.

How does this result compare to results in other systems?

The fact that our results on, for example, CLOCK do not match with the results found in the study by Mueller *et al.* (2011), even though the same species and behavioural trait were studied using the same genetic markers, is not uncommon. Such inconsistencies among results of candidate gene studies occur quite often. For example, the candidate gene DRD4 was shown to be associated with personality in

captive great tits, *Parus major* (Fidler *et al.* 2007), whereas in wild populations of great tits only one to two out of four populations showed a clear association (Korsten *et al.* 2010).

Making conclusions about the role of genetic control on migratory behaviour is difficult since the studies in this field of research have looked at numerous traits of migratory behaviour (migration propensity, timing, orientation) in a range of taxa. This makes it difficult to generalise the results that have been found (Liedvogel & Lundberg 2014).
It appears that the genetic control of migratory behaviour rests on many different genes each of which have relatively small effects. Hecht *et al.* (2013), for instance, suggested a complex multi-genic system of several loci with small effects distributed throughout the genome shaping migration behavior in rainbow trout (*Oncorhynchus mykiss*). Thus, geographic variation in migratory behaviour could be caused by different genes which may vary among geographic regions, which have different, independent evolutionary histories.

On the other hand, the fact that we confirmed the previously found association between ADCYAP1 and migratory behaviour in blackcaps, at another geographic scale, indicates that this gene is important in the regulation of migratory activity in this species. Even though variation in this gene only explains a small proportion of variation in migratory behaviour, the results of our study strongly support the idea that ADCYAP1 is a gene involved in the control of migratoriness, potentially regulating the factor underlying the amount of migratory activity (see, Pulido 2011).

Contrary to Mueller *et al.* (2011) we found an associations between CLOCK and Syl9 with migratoriness in blackcaps. This suggests that whether or not a gene has an effect on the expression of migratory behaviour and its potential depends on several factors, like the exact behavioural trait studied, the species, and geographical range considered, etc. The same gene can appear to be irrelevant in one study, but influential in the next. This makes it even more difficult to advance in understanding the genetic control of migration. Future studies should try to standardize the behavioural traits measured as much as possible. Moreover, they should aim at repeating studies in the same species considering potential geographical and temporal variation. This will allow delivering new insights into the genetic control of migration.

Supplementary material

Supplementary table S1. The molecular markers used in this study

Gene/Locus	Locus type	No. of observed alleles	Reference
CLOCK	Trinucleotide microsatellite	8	Steinmeyer et al. 2009
ADCYAP1	Dinucleotide microsatellite	13	Steinmeyer et al. 2009
NPAS2	Trinucleotide microsatellite	2	Steinmeyer et al. 2009
SERT_Ex1	Trinucleotide microsatellite	3	C. Hermannstaedter, pers. comm.
Syl1	Tetranucleotide microsatellite	14	Segelbacher et al. 2008
Syl2	Tetranucleotide microsatellite	15	Segelbacher et al. 2008
Syl4	Tetranucleotide microsatellite	20	Segelbacher et al. 2008
Syl5	Dinucleotide microsatellite	24	Segelbacher et al. 2008
Syl6	Tetranucleotide microsatellite	36	Segelbacher et al. 2008
Syl9	Tetranucleotide microsatellite	18	Segelbacher et al. 2008
Ppi2	Dinucleotide microsatellite	21	Martinez et al. 1999; A. Ramirez, pers. comm
Pca8	Dinucleotide microsatellite	4	Dawson et al. 2000; A. Ramirez, pers. comm

Supplementary table S2. ADCYAP1 allele frequencies from this study and Mueller et al. 2011

Allele		This study		Mueller *et al.,* 2011	
This study	Mueller 2011	Number	%	Number	%
x	147	0	0.00	2	0.18
155	151	10	0.89	1	0.09
x	153	0	0.00	5	0.46
157	154	2	0.18	2	0.18
x	155	0	0.00	6	0.55
154	157	49	4.34	55	5.08
161	159	11	0.98	27	2.50
163	**161**	**464**	**41.13**	**410**	**37.89**
165	163	103	9.13	115	10.63
167	**165**	**377**	**33.42**	**375**	**34.66**
x	166	0	0.00	2	0.18
169	167	93	8.24	50	4.62
171	169	16	1.42	18	1.66
x	170	0	0.00	6	0.55
173	171	3	0.27	8	0.74
		1128	100	1082	100
		N=564		N=541	

Samples from our study were processed on a different machine which caused a slight "shift" in allele numbers. Here are the allele frequencies of both studies with both allele numbers. This table serves as a conversion table between the studies. The figures in Supplement S3 were made with our own allele numbers.

Supplementary figures S3. Allele Frequencies at ADCYAP1 for each population. (When comparing these figures to Mueller et al. 2011, please keep the conversion table in supplementary table S2 in mind).

ADCYAP1 frequencies in the sedentary populations:

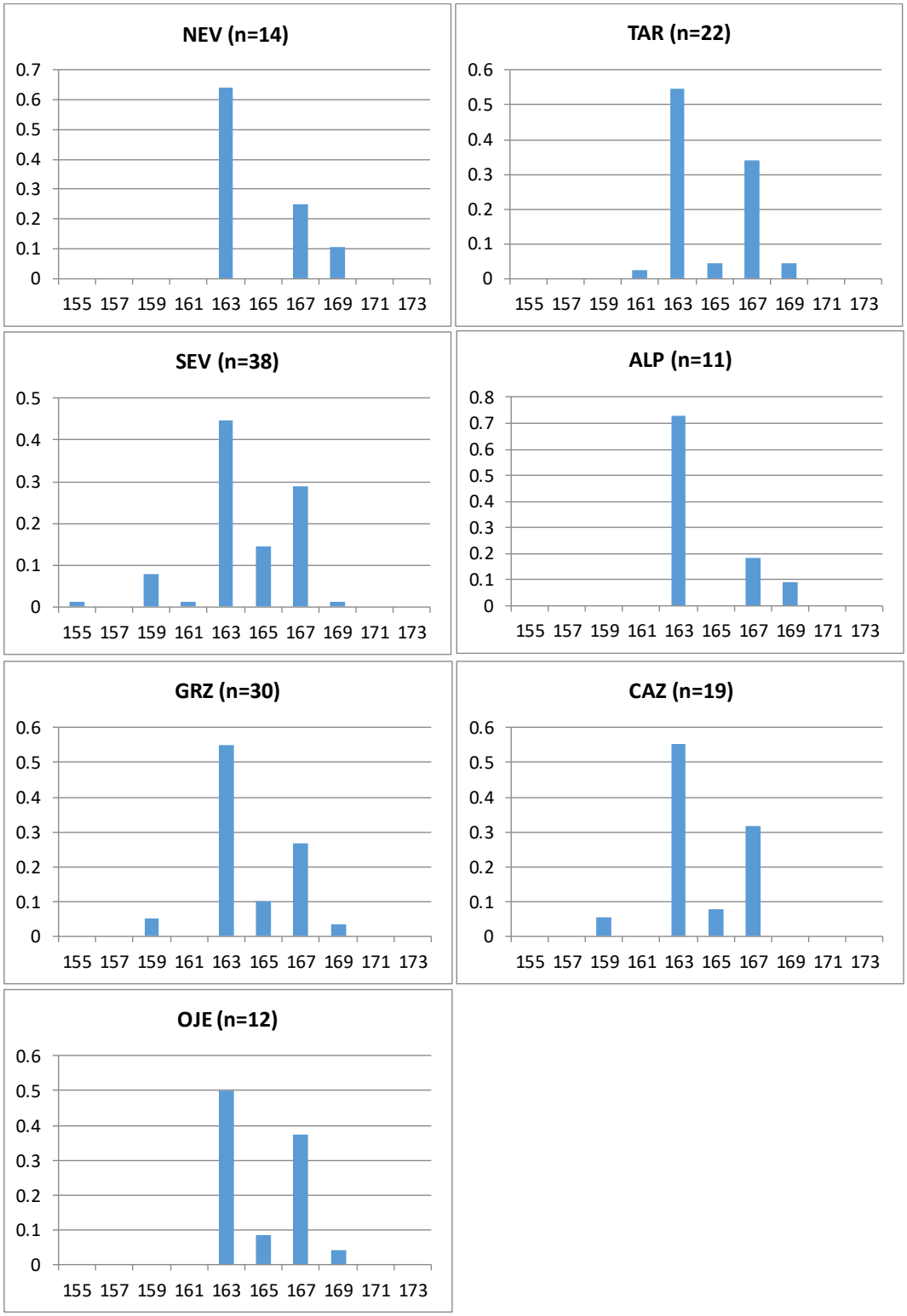


Table S3 (continued)

ADCYAP1 frequencies in the partially migratory populations:

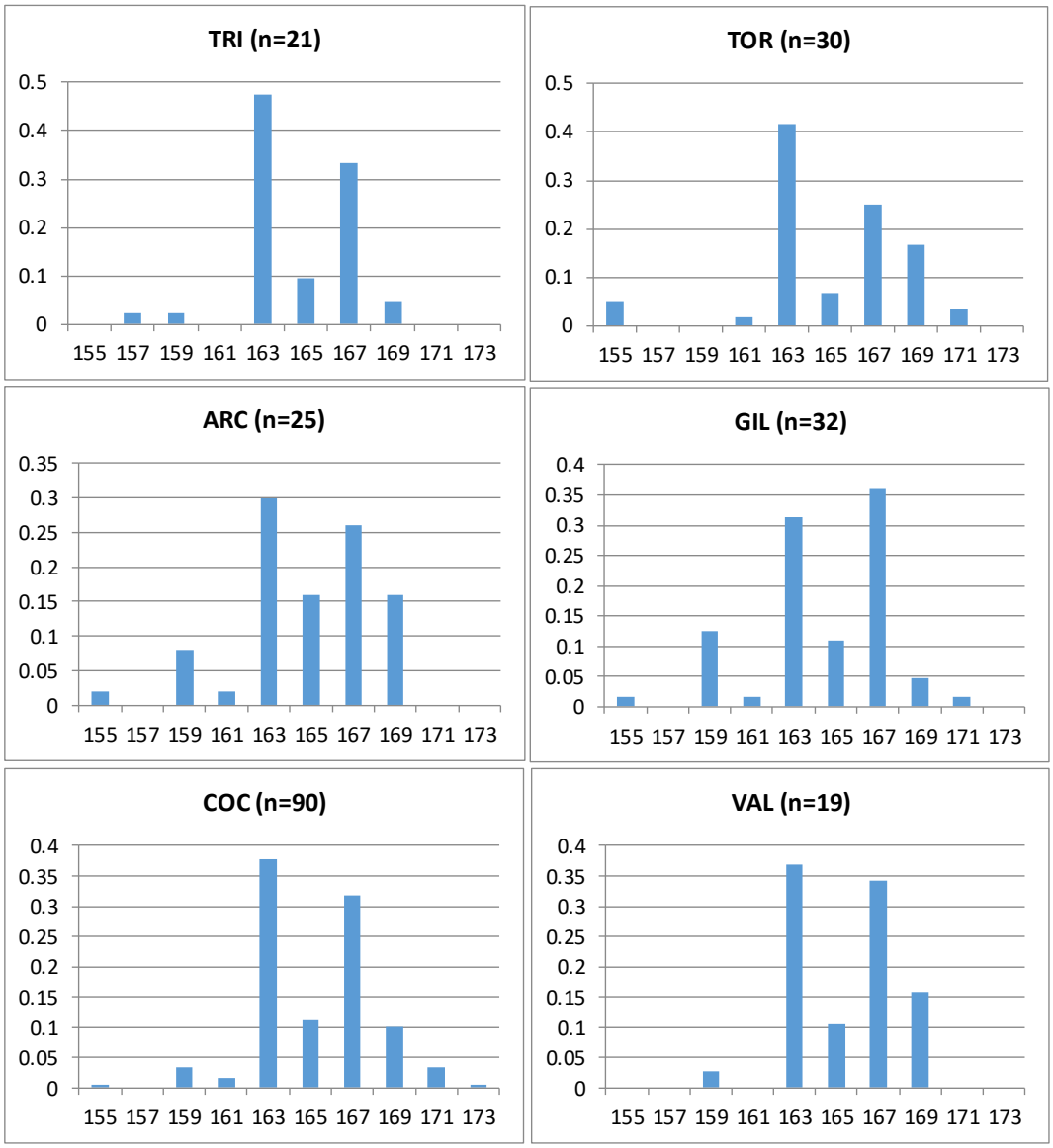
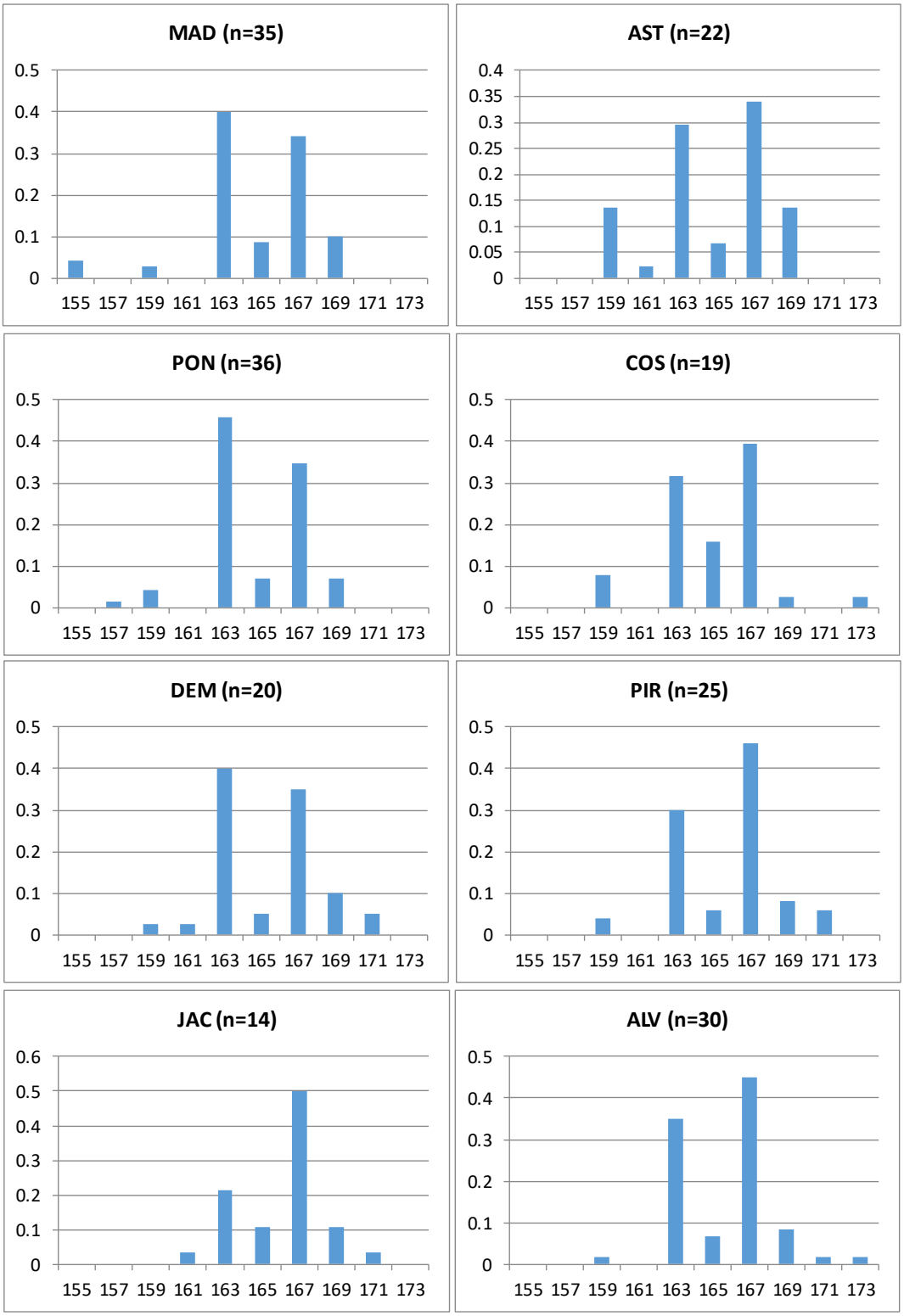


Table S3 (continued)

ADCYAP1 frequencies in the migratory populations:



Supplementary table S4. Allele Frequencies and Sample Size per Population.

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
ADCYAP1	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	155	0.006	0.000	0.043	0.000	0.000	0.000	0.000	0.013	0.000	0.020	0.016	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	157	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000
	159	0.033	0.000	0.029	0.000	0.050	0.053	0.000	0.079	0.000	0.080	0.125	0.000	0.024	0.026	0.136	0.017	0.000	0.040	0.042	0.079	0.025
	161	0.017	0.023	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.020	0.016	0.017	0.000	0.000	0.023	0.000	0.036	0.000	0.000	0.000	0.025
	163	0.378	0.545	0.400	0.727	0.550	0.553	0.643	0.447	0.500	0.300	0.313	0.417	0.476	0.368	0.295	0.350	0.214	0.300	0.458	0.316	0.400
	165	0.111	0.045	0.086	0.000	0.100	0.079	0.000	0.145	0.083	0.160	0.109	0.067	0.095	0.105	0.068	0.067	0.107	0.060	0.069	0.158	0.050
	167	0.317	0.341	0.343	0.182	0.267	0.316	0.250	0.289	0.375	0.260	0.359	0.250	0.333	0.342	0.341	0.450	0.500	0.460	0.347	0.395	0.350
	169	0.100	0.045	0.100	0.091	0.033	0.000	0.107	0.013	0.042	0.160	0.047	0.167	0.048	0.158	0.136	0.083	0.107	0.080	0.069	0.026	0.100
	171	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.033	0.000	0.000	0.000	0.017	0.036	0.060	0.000	0.000	0.050
	173	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.026	0.000
CLOCK	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	256	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	258	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.020	0.016	0.033	0.000	0.000	0.023	0.033	0.000	0.000	0.014	0.026	0.000
	260	0.000	0.068	0.014	0.000	0.000	0.000	0.143	0.013	0.000	0.020	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	263	0.128	0.205	0.171	0.273	0.300	0.184	0.179	0.289	0.250	0.140	0.141	0.183	0.119	0.132	0.091	0.267	0.071	0.160	0.125	0.132	0.175
	266	0.017	0.045	0.000	0.000	0.017	0.000	0.071	0.000	0.000	0.000	0.000	0.017	0.024	0.026	0.068	0.017	0.000	0.020	0.000	0.026	0.025
	269	0.800	0.636	0.743	0.682	0.583	0.789	0.607	0.592	0.667	0.720	0.688	0.683	0.738	0.711	0.727	0.633	0.786	0.800	0.833	0.684	0.775
	272	0.011	0.023	0.029	0.000	0.017	0.026	0.000	0.013	0.042	0.060	0.078	0.017	0.048	0.026	0.091	0.017	0.071	0.020	0.028	0.053	0.000
	275	0.033	0.023	0.029	0.045	0.083	0.000	0.000	0.079	0.042	0.020	0.078	0.067	0.024	0.105	0.000	0.033	0.071	0.000	0.000	0.079	0.025
	280	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NPAS2	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	163	0.039	0.045	0.014	0.273	0.067	0.105	0.000	0.026	0.000	0.000	0.031	0.033	0.000	0.000	0.023	0.000	0.000	0.000	0.014	0.000	0.025
	169	0.961	0.955	0.986	0.727	0.933	0.895	1.000	0.974	1.000	1.000	0.969	0.967	1.000	1.000	0.977	1.000	1.000	1.000	0.986	1.000	0.975
SERT_Ex1	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	314	0.000	0.023	0.014	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	320	1.000	0.955	0.971	1.000	1.000	1.000	1.000	1.000	0.958	1.000	0.984	1.000	1.000	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000
	323	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000
	326	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table S4 (continued)

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
PC8	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	171	0.617	0.568	0.657	0.545	0.550	0.553	0.500	0.724	0.750	0.760	0.734	0.683	0.524	0.632	0.773	0.583	0.714	0.540	0.647	0.632	0.525
	175	0.094	0.000	0.129	0.000	0.017	0.000	0.000	0.013	0.000	0.040	0.063	0.033	0.000	0.079	0.023	0.133	0.036	0.080	0.074	0.026	0.125
	177	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000
	179	0.289	0.432	0.214	0.455	0.433	0.447	0.500	0.263	0.250	0.200	0.203	0.283	0.476	0.289	0.205	0.283	0.250	0.380	0.265	0.342	0.325
	193	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025
Ppi2	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	243	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	251	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.033	0.024	0.000	0.023	0.050	0.000	0.000	0.028	0.079	0.025
	254	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	258	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	259	0.006	0.000	0.043	0.000	0.000	0.000	0.000	0.013	0.000	0.040	0.000	0.017	0.000	0.026	0.114	0.017	0.000	0.000	0.028	0.053	0.050
	261	0.100	0.159	0.214	0.273	0.100	0.105	0.286	0.158	0.208	0.120	0.188	0.150	0.238	0.184	0.205	0.117	0.286	0.260	0.139	0.237	0.050
	263	0.228	0.114	0.114	0.136	0.150	0.184	0.214	0.105	0.333	0.140	0.250	0.150	0.214	0.079	0.068	0.133	0.107	0.020	0.153	0.132	0.125
	266	0.028	0.000	0.000	0.000	0.017	0.000	0.000	0.026	0.042	0.040	0.000	0.033	0.095	0.000	0.114	0.083	0.000	0.100	0.028	0.026	0.025
	268	0.389	0.386	0.400	0.364	0.383	0.316	0.321	0.526	0.167	0.320	0.297	0.367	0.238	0.263	0.273	0.283	0.357	0.420	0.375	0.237	0.425
	270	0.022	0.023	0.000	0.000	0.017	0.000	0.000	0.013	0.000	0.020	0.016	0.017	0.024	0.026	0.000	0.033	0.036	0.000	0.042	0.053	0.050
	273	0.006	0.000	0.014	0.045	0.017	0.053	0.071	0.000	0.000	0.040	0.031	0.033	0.048	0.000	0.000	0.067	0.071	0.000	0.014	0.026	0.025
	275	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	277	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	279	0.011	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	281	0.006	0.000	0.029	0.000	0.050	0.026	0.000	0.013	0.083	0.000	0.000	0.000	0.024	0.053	0.023	0.017	0.000	0.040	0.000	0.000	0.000
	283	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	285	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.026	0.000	0.017	0.036	0.020	0.000	0.000	0.000
	287	0.156	0.273	0.143	0.136	0.250	0.289	0.071	0.132	0.125	0.220	0.172	0.183	0.048	0.158	0.182	0.117	0.107	0.120	0.181	0.158	0.225
	289	0.011	0.023	0.014	0.045	0.000	0.026	0.036	0.013	0.000	0.020	0.031	0.000	0.000	0.079	0.000	0.033	0.000	0.020	0.014	0.000	0.000
	291	0.000	0.023	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.020	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	293	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
Syl1	Genes	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	115	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000
	116	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000
	122	0.056	0.114	0.029	0.000	0.033	0.000	0.107	0.039	0.000	0.060	0.047	0.000	0.071	0.000	0.068	0.017	0.036	0.040	0.069	0.000	0.025
	126	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	130	0.017	0.045	0.000	0.000	0.017	0.000	0.071	0.066	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.020	0.014	0.000	0.000
	134	0.033	0.068	0.014	0.000	0.000	0.053	0.071	0.000	0.083	0.040	0.016	0.033	0.024	0.026	0.045	0.033	0.071	0.080	0.097	0.053	0.000
	138	0.106	0.045	0.100	0.045	0.150	0.105	0.036	0.079	0.167	0.060	0.031	0.117	0.167	0.132	0.295	0.067	0.071	0.160	0.167	0.105	0.125
	143	0.078	0.182	0.143	0.227	0.183	0.263	0.286	0.092	0.250	0.220	0.203	0.250	0.190	0.184	0.068	0.167	0.071	0.180	0.139	0.237	0.150
	147	0.294	0.091	0.229	0.136	0.150	0.053	0.179	0.224	0.125	0.120	0.234	0.183	0.190	0.368	0.182	0.400	0.179	0.180	0.208	0.158	0.275
	152	0.194	0.295	0.114	0.136	0.233	0.158	0.107	0.237	0.125	0.200	0.188	0.133	0.119	0.184	0.045	0.100	0.321	0.160	0.111	0.237	0.150
	156	0.133	0.045	0.300	0.136	0.033	0.158	0.036	0.197	0.167	0.240	0.203	0.117	0.167	0.053	0.159	0.117	0.143	0.120	0.097	0.053	0.200
	160	0.022	0.045	0.029	0.000	0.017	0.026	0.036	0.013	0.042	0.020	0.000	0.050	0.048	0.053	0.023	0.033	0.036	0.040	0.000	0.053	0.050
	164	0.028	0.045	0.000	0.045	0.083	0.079	0.000	0.039	0.000	0.020	0.000	0.067	0.024	0.000	0.091	0.033	0.000	0.000	0.028	0.053	0.025
	168	0.017	0.023	0.014	0.182	0.100	0.026	0.000	0.000	0.000	0.020	0.047	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.028	0.053	0.000
	172	0.011	0.000	0.000	0.091	0.000	0.079	0.036	0.013	0.000	0.000	0.016	0.017	0.000	0.000	0.000	0.017	0.036	0.000	0.014	0.000	0.000
	176	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.017	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000
	179	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000
Syl2	Genes	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	129	0.011	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.023	0.050	0.000	0.000	0.000	0.026	0.000
	133	0.017	0.091	0.014	0.045	0.033	0.079	0.000	0.013	0.167	0.020	0.000	0.017	0.024	0.000	0.000	0.017	0.000	0.000	0.014	0.000	0.000
	138	0.017	0.045	0.114	0.000	0.117	0.105	0.071	0.158	0.042	0.040	0.047	0.067	0.071	0.053	0.045	0.083	0.143	0.040	0.028	0.105	0.000
	140	0.006	0.023	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.020	0.000	0.000	0.024	0.000	0.023	0.000	0.000	0.020	0.014	0.026	0.000
	143	0.089	0.205	0.129	0.182	0.067	0.211	0.143	0.092	0.125	0.200	0.188	0.150	0.071	0.263	0.205	0.167	0.143	0.080	0.167	0.184	0.175
	147	0.261	0.273	0.243	0.273	0.350	0.289	0.214	0.105	0.333	0.220	0.219	0.200	0.238	0.158	0.227	0.167	0.321	0.080	0.264	0.079	0.300
	151	0.228	0.114	0.129	0.136	0.167	0.079	0.286	0.224	0.083	0.220	0.156	0.167	0.095	0.105	0.091	0.167	0.107	0.300	0.167	0.263	0.175
	155	0.133	0.068	0.114	0.000	0.050	0.053	0.071	0.145	0.042	0.100	0.063	0.050	0.119	0.105	0.091	0.100	0.143	0.080	0.111	0.079	0.075
	156	0.022	0.023	0.014	0.227	0.050	0.105	0.036	0.092	0.042	0.120	0.063	0.100	0.048	0.158	0.045	0.100	0.000	0.060	0.069	0.053	0.025
	157	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	159	0.106	0.000	0.114	0.000	0.017	0.000	0.036	0.039	0.000	0.020	0.125	0.100	0.167	0.053	0.091	0.033	0.071	0.080	0.069	0.079	0.100

Table S4 (continued)

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
(Syl2)	**160**	0.006	0.000	0.043	0.045	0.033	0.000	0.000	0.000	0.042	0.000	0.000	0.017	0.000	0.000	0.023	0.033	0.000	0.000	0.000	0.053	0.000
	163	0.044	0.114	0.071	0.091	0.083	0.053	0.107	0.079	0.125	0.020	0.109	0.117	0.048	0.079	0.068	0.017	0.036	0.180	0.056	0.026	0.125
	167	0.039	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.023	0.067	0.000	0.040	0.014	0.000	0.000
	171	0.022	0.023	0.014	0.000	0.000	0.026	0.036	0.013	0.000	0.000	0.016	0.000	0.024	0.000	0.023	0.000	0.036	0.040	0.028	0.000	0.025
	176	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.020	0.016	0.000	0.000	0.026	0.023	0.000	0.000	0.000	0.000	0.026	0.000
Syl4	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	161	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000
	165	0.050	0.023	0.000	0.000	0.050	0.053	0.000	0.000	0.000	0.060	0.016	0.033	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	169	0.011	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.053	0.000	0.000	0.071	0.000	0.000	0.026	0.000
	173	0.006	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.017	0.000	0.000	0.045	0.000	0.000	0.020	0.000	0.053	0.075
	178	0.111	0.227	0.114	0.045	0.183	0.079	0.000	0.053	0.083	0.220	0.078	0.033	0.048	0.132	0.023	0.167	0.107	0.140	0.125	0.079	0.025
	182	0.183	0.432	0.286	0.318	0.200	0.526	0.536	0.263	0.375	0.220	0.313	0.317	0.286	0.316	0.409	0.217	0.393	0.260	0.264	0.342	0.400
	186	0.167	0.136	0.114	0.227	0.133	0.079	0.071	0.092	0.083	0.240	0.141	0.133	0.119	0.158	0.159	0.150	0.036	0.200	0.194	0.158	0.100
	190	0.133	0.114	0.243	0.091	0.200	0.132	0.036	0.263	0.292	0.120	0.203	0.167	0.262	0.079	0.205	0.217	0.179	0.100	0.236	0.158	0.175
	194	0.183	0.045	0.071	0.091	0.133	0.026	0.071	0.197	0.083	0.080	0.094	0.167	0.048	0.053	0.159	0.117	0.000	0.140	0.111	0.132	0.100
	197	0.044	0.023	0.000	0.227	0.100	0.079	0.286	0.118	0.083	0.000	0.063	0.067	0.143	0.132	0.000	0.050	0.107	0.040	0.042	0.000	0.075
	202	0.050	0.000	0.057	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.017	0.024	0.053	0.000	0.017	0.071	0.020	0.000	0.000	0.025
	206	0.017	0.000	0.057	0.000	0.000	0.026	0.000	0.000	0.000	0.040	0.063	0.000	0.024	0.026	0.000	0.050	0.000	0.040	0.014	0.053	0.000
	210	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.025
	214	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.040	0.000	0.000	0.000
	218	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	226	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Syl5	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	143	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	151	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	156	0.034	0.068	0.029	0.091	0.000	0.000	0.036	0.053	0.000	0.063	0.000	0.017	0.000	0.000	0.000	0.017	0.071	0.000	0.042	0.026	0.050
	160	0.135	0.114	0.214	0.091	0.100	0.132	0.214	0.066	0.208	0.104	0.109	0.100	0.048	0.184	0.159	0.117	0.071	0.160	0.083	0.105	0.025
	162	0.006	0.023	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.021	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.025
	164	0.062	0.023	0.100	0.182	0.083	0.105	0.071	0.145	0.042	0.042	0.078	0.050	0.024	0.053	0.114	0.133	0.107	0.080	0.056	0.026	0.075
	166	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
(Syl5)	**168**	0.129	0.000	0.014	0.091	0.117	0.079	0.107	0.039	0.042	0.083	0.063	0.067	0.143	0.105	0.114	0.100	0.143	0.060	0.083	0.026	0.025
	170	0.022	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.078	0.033	0.000	0.000	0.023	0.000	0.000	0.020	0.000	0.026	0.025
	172	0.045	0.091	0.071	0.045	0.050	0.079	0.071	0.145	0.167	0.021	0.063	0.067	0.095	0.026	0.091	0.100	0.000	0.080	0.056	0.105	0.250
	174	0.017	0.023	0.029	0.136	0.033	0.079	0.000	0.013	0.000	0.021	0.031	0.000	0.000	0.053	0.068	0.017	0.000	0.040	0.014	0.000	0.000
	176	0.157	0.136	0.071	0.045	0.100	0.053	0.036	0.132	0.125	0.104	0.047	0.117	0.190	0.158	0.023	0.100	0.071	0.100	0.111	0.079	0.025
	178	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.017	0.000	0.000	0.014	0.000	0.000
	180	0.056	0.114	0.057	0.182	0.217	0.158	0.107	0.039	0.167	0.188	0.078	0.133	0.071	0.184	0.091	0.050	0.107	0.120	0.097	0.000	0.100
	182	0.022	0.114	0.043	0.045	0.067	0.053	0.036	0.039	0.000	0.021	0.094	0.017	0.024	0.026	0.023	0.000	0.000	0.020	0.028	0.000	0.025
	184	0.051	0.114	0.129	0.000	0.067	0.105	0.107	0.224	0.208	0.229	0.125	0.067	0.071	0.053	0.114	0.133	0.179	0.040	0.153	0.211	0.125
	186	0.011	0.045	0.000	0.000	0.050	0.026	0.036	0.000	0.000	0.063	0.000	0.033	0.000	0.000	0.045	0.067	0.000	0.000	0.000	0.000	0.000
	188	0.056	0.023	0.129	0.045	0.067	0.053	0.143	0.026	0.000	0.000	0.094	0.100	0.048	0.026	0.023	0.033	0.036	0.040	0.097	0.105	0.000
	190	0.051	0.091	0.043	0.000	0.017	0.079	0.000	0.039	0.000	0.021	0.031	0.033	0.119	0.026	0.000	0.033	0.071	0.020	0.056	0.026	0.050
	192	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.071	0.000	0.028	0.000	0.025
	194	0.028	0.000	0.029	0.045	0.000	0.000	0.000	0.013	0.000	0.000	0.047	0.067	0.048	0.053	0.023	0.050	0.000	0.040	0.028	0.105	0.025
	196	0.006	0.023	0.000	0.000	0.017	0.000	0.000	0.013	0.000	0.021	0.000	0.017	0.000	0.000	0.000	0.017	0.071	0.000	0.000	0.000	0.025
	198	0.017	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.033	0.095	0.000	0.023	0.000	0.000	0.060	0.014	0.053	0.050
	200	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.023	0.017	0.000	0.020	0.000	0.026	0.000
	202	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.023	0.000	0.000	0.080	0.000	0.026	0.050
	204	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025
	206	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	208	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000
	211	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.026	0.000
Syl6	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	156	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.000
	160	0.006	0.000	0.029	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000
	172	0.017	0.068	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.120	0.000	0.000	0.000	0.026	0.000	0.017	0.036	0.040	0.000	0.000	0.000
	176	0.011	0.045	0.000	0.000	0.017	0.026	0.000	0.053	0.000	0.040	0.031	0.000	0.024	0.053	0.023	0.000	0.036	0.000	0.042	0.000	0.000
	180	0.022	0.045	0.029	0.000	0.017	0.026	0.000	0.053	0.042	0.040	0.078	0.033	0.000	0.053	0.045	0.033	0.071	0.020	0.056	0.000	0.050
	184	0.078	0.023	0.114	0.091	0.067	0.079	0.000	0.132	0.000	0.160	0.141	0.133	0.190	0.132	0.136	0.100	0.107	0.120	0.153	0.237	0.100
	185	0.033	0.045	0.014	0.045	0.000	0.105	0.000	0.026	0.000	0.000	0.031	0.050	0.000	0.026	0.000	0.000	0.036	0.060	0.014	0.000	0.000

Table S4 (continued)

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
(Syl6)	**188**	0.139	0.136	0.100	0.000	0.033	0.158	0.071	0.079	0.292	0.060	0.078	0.083	0.167	0.053	0.023	0.100	0.036	0.040	0.028	0.079	0.025
	189	0.050	0.023	0.014	0.000	0.083	0.000	0.000	0.000	0.042	0.020	0.016	0.033	0.024	0.079	0.045	0.083	0.000	0.100	0.069	0.000	0.025
	192	0.122	0.205	0.171	0.045	0.183	0.105	0.036	0.092	0.083	0.060	0.047	0.150	0.071	0.026	0.091	0.067	0.250	0.100	0.069	0.132	0.175
	193	0.072	0.000	0.014	0.045	0.000	0.026	0.143	0.000	0.000	0.020	0.000	0.000	0.071	0.026	0.023	0.033	0.036	0.000	0.083	0.000	0.125
	196	0.072	0.091	0.100	0.136	0.117	0.000	0.393	0.039	0.083	0.080	0.109	0.067	0.119	0.079	0.068	0.017	0.071	0.140	0.042	0.105	0.175
	197	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.033	0.024	0.026	0.023	0.000	0.036	0.000	0.028	0.000	0.050
	200	0.056	0.091	0.043	0.091	0.067	0.079	0.036	0.224	0.167	0.040	0.109	0.083	0.119	0.079	0.091	0.117	0.000	0.120	0.139	0.158	0.025
	201	0.011	0.000	0.000	0.045	0.067	0.000	0.036	0.013	0.083	0.080	0.031	0.017	0.000	0.026	0.000	0.050	0.071	0.020	0.028	0.053	0.000
	204	0.111	0.045	0.114	0.045	0.100	0.132	0.179	0.092	0.042	0.000	0.156	0.117	0.000	0.000	0.159	0.100	0.036	0.100	0.069	0.132	0.100
	205	0.000	0.000	0.014	0.000	0.017	0.079	0.000	0.000	0.000	0.060	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025
	208	0.050	0.023	0.057	0.000	0.083	0.053	0.036	0.026	0.042	0.060	0.031	0.017	0.024	0.053	0.000	0.067	0.071	0.020	0.042	0.079	0.000
	209	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000
	212	0.033	0.068	0.057	0.227	0.083	0.000	0.071	0.039	0.083	0.060	0.078	0.083	0.071	0.000	0.114	0.117	0.036	0.020	0.028	0.000	0.025
	216	0.006	0.000	0.014	0.045	0.033	0.079	0.000	0.013	0.000	0.000	0.031	0.033	0.024	0.132	0.023	0.000	0.000	0.020	0.000	0.000	0.000
	220	0.017	0.068	0.014	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.016	0.033	0.000	0.053	0.023	0.000	0.000	0.040	0.028	0.000	0.000
	224	0.000	0.000	0.014	0.045	0.000	0.000	0.000	0.013	0.000	0.080	0.000	0.000	0.000	0.000	0.000	0.033	0.036	0.000	0.000	0.000	0.000
	228	0.011	0.000	0.014	0.000	0.033	0.026	0.000	0.013	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.020	0.014	0.000	0.000
	232	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.017	0.000	0.000	0.023	0.017	0.000	0.000	0.000	0.000	0.000
	236	0.000	0.023	0.014	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.026	0.023	0.000	0.000	0.000	0.014	0.000	0.000
	240	0.006	0.000	0.029	0.045	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.024	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.050
	244	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.024	0.000	0.023	0.017	0.000	0.000	0.028	0.000	0.025
	248	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.017	0.024	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	252	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.025
	256	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.017	0.036	0.000	0.000	0.000	0.000
	268	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Syl9	**Genes**	180	44	70	22	60	38	28	76	24	50	64	60	42	38	44	60	28	50	72	38	40
	139	0.039	0.000	0.000	0.000	0.000	0.026	0.000	0.039	0.000	0.000	0.016	0.017	0.071	0.079	0.000	0.017	0.036	0.000	0.028	0.000	0.000
	143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000
	147	0.456	0.795	0.643	0.636	0.783	0.737	0.893	0.592	0.875	0.780	0.625	0.483	0.548	0.526	0.614	0.467	0.500	0.440	0.597	0.658	0.500
	151	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.020	0.014	0.000	0.000

Locus	Allele/n	COC	TAR	MAD	ALP	GRZ	CAZ	NEV	SEV	OJE	ARC	GIL	TOR	TRI	VAL	AST	ALV	JAC	PIR	PON	COS	DEM
(Syl9)	**152**	0.111	0.068	0.086	0.091	0.033	0.000	0.071	0.092	0.000	0.040	0.125	0.250	0.048	0.105	0.023	0.117	0.071	0.100	0.139	0.132	0.100
	157	0.033	0.023	0.029	0.136	0.000	0.026	0.000	0.158	0.000	0.000	0.031	0.033	0.095	0.026	0.023	0.067	0.071	0.140	0.042	0.026	0.050
	161	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	165	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000
	168	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.023	0.017	0.000	0.000	0.000	0.000	0.000
	170	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	172	0.006	0.023	0.043	0.000	0.017	0.053	0.000	0.013	0.000	0.040	0.047	0.000	0.000	0.053	0.000	0.017	0.036	0.060	0.014	0.000	0.125
	176	0.028	0.000	0.000	0.045	0.050	0.053	0.000	0.026	0.042	0.000	0.016	0.000	0.024	0.000	0.000	0.017	0.071	0.000	0.000	0.026	0.000
	179	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000
	181	0.061	0.000	0.043	0.045	0.000	0.026	0.000	0.013	0.042	0.020	0.047	0.050	0.048	0.000	0.000	0.050	0.143	0.040	0.042	0.000	0.025
	184	0.139	0.000	0.029	0.045	0.083	0.026	0.036	0.039	0.000	0.100	0.000	0.083	0.119	0.132	0.205	0.083	0.036	0.100	0.069	0.079	0.100
	187	0.050	0.091	0.086	0.000	0.033	0.000	0.000	0.000	0.042	0.000	0.016	0.017	0.048	0.053	0.068	0.050	0.000	0.060	0.056	0.053	0.075
	192	0.033	0.000	0.029	0.000	0.000	0.026	0.000	0.013	0.000	0.000	0.016	0.033	0.000	0.026	0.045	0.050	0.000	0.020	0.000	0.000	0.025
	196	0.006	0.000	0.014	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000
	200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.016	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

All Loci except the sexing locus

Genes = N x2

*Supplementary table S5. Overview of the test for Hardy-Weinberg Equilibrium. When a locus was monomorphic (mm) in a population, no test could be conducted. Levels of significance: ns = P>0.05; * = P<0.05; ** = P<0.01; *** = P<0.001.*

Pop/Locus	CLOCK	ADCYAP1	NPAS2	SERT	Syl1	Syl2	Syl4	Syl5	Syl6	Syl9	Ppi2	Pca8
COC	ns	ns	ns	mm	ns	ns	***	***	ns	ns	***	***
TAR	ns	ns	ns	ns	ns	*	**	ns	ns	ns	ns	ns
MAD	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
ALP	ns	ns	ns	mm	ns	ns	*	ns	ns	ns	ns	ns
GRZ	ns	*	ns	mm	ns	**	***	ns	ns	ns	ns	ns
CAZ	ns	ns	ns	mm	ns	ns	*	ns	ns	ns	ns	*
NEV	ns	ns	mm	mm	ns	ns	ns	ns	ns	ns	ns	**
SEV	ns	ns	ns	mm	ns	ns	*	ns	ns	ns	ns	ns
OJE	ns	ns	mm	ns	ns	ns	**	ns	ns	ns	ns	ns
ARC	**	ns	mm	mm	ns	**	***	ns	ns	ns	ns	ns
GIL	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	*
TOR	ns	ns	ns	mm	ns	ns	**	ns	ns	**	ns	ns
TRI	ns	ns	mm	mm	ns	ns	ns	ns	ns	ns	ns	ns
VAL	ns	ns	mm	mm	ns	ns	**	ns	ns	*	ns	*
AST	ns	ns	ns	mm	ns	*	**	ns	ns	ns	ns	ns
ALV	ns	ns	mm	ns	ns	ns	ns	ns	ns	ns	ns	**
JAC	ns	ns	mm	mm	ns	ns	ns	ns	ns	ns	ns	ns
PIR	ns	ns	mm	mm	ns	ns	***	ns	ns	ns	ns	ns
PON	ns	ns	ns	mm	ns	ns	*	*	*	***	ns	ns
COS	ns	ns	mm	mm	ns	ns	***	ns	ns	ns	ns	ns
DEM	ns	ns	ns	mm	ns	ns	ns	ns	ns	ns	ns	ns

Supplementary table S6. Deviations from linkage equilibrium. Numbers give error probabilities as determined by Fisher's method Genepop V4 (Raymond and Rousset 1995).

All birds	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.9441	0.0527	0.8803	0.8993	0.4273	0.7763	0.9917	0.9696	1	0.3212
CLOCK	x	x	0.8243	0.7245	0.1684	0.9629	0.9112	0.4415	0.9998	0.9548	0.9045
NPAS	x	x	x	1	0.1637	0.7797	0.9821	0.9332	1	0.9957	0.4345
SERT	x	x	x	x	0.3386	0.731	0.5934	0.8973	0.9803	0.9982	0.868
PC8	x	x	x	x	x	0.5158	0.5147	0.685	1	0.6832	0.4947
Ppi2	x	x	x	x	x	x	0.9954	0.9508	0.8654	1	0.3023
Syl1	x	x	x	x	x	x	x	0.9862	0.9992	0.9991	0.5361
Syl2	x	x	x	x	x	x	x	x	0.9678	0.9964	0.9846
Syl5	x	x	x	x	x	x	x	x	x	1	0.9963
Syl6	x	x	x	x	x	x	x	x	x	x	0.9996
Syl9	x	x	x	x	x	x	x	x	x	x	x

Per population

ALP	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.8845	0.0019	x	0.9199	0.836	no info	1	no info	no info	0.4983
CLOCK	x	x	1	x	0.7265	0.8028	no info	0.4897	no info	no info	0.9801
NPAS	x	x	x	x	0.4152	0.541	no info	1	no info	no info	0.8448
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.3615	no info	0.5864	no info	no info	1
Ppi2	x	x	x	x	x	x	no info	1	no info	no info	1
Syl1	x	x	x	x	x	x	x	x	no info	no info	no info
Syl2	x	x	x	x	x	x	x	x	no info	no info	1
Syl5	x	x	x	x	x	x	x	x	x	no info	no info
Syl6	x	x	x	x	x	x	x	x	x	x	no info
Syl9	x	x	x	x	x	x	x	x	x	x	x

ALV	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.7894	x	1	0.5511	0.5686	0.8113	0.108	0.4205	0.2326	0.5044
CLOCK	x	x	x	0.1982	0.0569	0.6798	0.1238	0.6064	1	0.5128	0.5287
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	0.0678	0.6748	0.4618	0.8023	0.7295	0.8741	0.6368
PC8	x	x	x	x	x	0.5259	0.4202	0.7612	0.6825	0.4267	0.489
Ppi2	x	x	x	x	x	x	1	0.2814	1	1	0.0703
Syl1	x	x	x	x	x	x	x	1	0.1371	1	0.5606
Syl2	x	x	x	x	x	x	x	x	1	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	0.2357
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

ARC	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.7106	x	x	0.5034	0.9337	0.6431	0.5579	0.3674	1	0.2482
CLOCK	x	x	x	x	0.3854	0.5463	0.8829	0.9606	0.1188	0.9351	0.0741
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.6102	0.9934	0.2555	0.8826	0.7163	0.9607
Ppi2	x	x	x	x	x	x	0.2047	1	1	1	0.9211
Syl1	x	x	x	x	x	x	x	0.3504	1	0.2478	0.1849
Syl2	x	x	x	x	x	x	x	x	1	1	0.6927
Syl5	x	x	x	x	x	x	x	x	x	1	0.4971
Syl6	x	x	x	x	x	x	x	x	x	x	0.5562
Syl9	x	x	x	x	x	x	x	x	x	x	x

Table S6 (continued)

AST	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.7762	0.3153	x	0.6947	1	0.3961	0.5272	1	1	0.0165
CLOCK	x	x	1	x	0.4687	0.3339	0.2026	0.1185	1	0.5945	0.8495
NPAS	x	x	x	x	1	0.8184	1	1	1	0.8111	1
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.6317	0.359	0.7042	0.8494	0.7025	0.6919
Ppi2	x	x	x	x	x	x	1	0.3653	0.1274	1	0.6776
Syl1	x	x	x	x	x	x	x	1	1	1	0.4958
Syl2	x	x	x	x	x	x	x	x	0.0728	1	0.8414
Syl5	x	x	x	x	x	x	x	x	x	1	0.0312
Syl6	x	x	x	x	x	x	x	x	x	x	0.3597
Syl9	x	x	x	x	x	x	x	x	x	x	x

CAZ	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.6922	0.5877	x	0.1565	0.7153	0.0373	0.5755	1	no info	0.1214
CLOCK	x	x	0.1742	x	0.8181	0.3957	0.2585	1	1	no info	0.4465
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	0.0587	0.8373	0.5532	0.9939	0.8923	no info	1
PC8	x	x	x	x	x	0.8411	0.09	1	1	no info	1
Ppi2	x	x	x	x	x	x	1	1	1	no info	0.7128
Syl1	x	x	x	x	x	x	x	1	1	no info	0.0316
Syl2	x	x	x	x	x	x	x	x	1	no info	0.8935
Syl5	x	x	x	x	x	x	x	x	x	no info	0.4906
Syl6	x	x	x	x	x	x	x	x	x	x	x
Syl9	x	x	x	x	x	x	x	x	x	x	x

COC	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.4839	0.038	x	0.6557	0.478	0.8951	0.9376	0.7878	0.6434	0.3346
CLOCK	x	x	0.3062	x	0.112	0.6353	0.2653	0.9288	0.6202	0.9525	0.5652
NPAS	x	x	x	x	0.0617	0.5252	0.922	0.8293	0.6054	0.6177	0.9137
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.009	0.124	0.1382	0.598	0.2541	0.9105
Ppi2	x	x	x	x	x	x	0.1845	0.5624	0.3342	0.3908	0.5082
Syl1	x	x	x	x	x	x	x	0.5244	0.8086	0.0782	0.5374
Syl2	x	x	x	x	x	x	x	x	0.6395	0.4987	0.0977
Syl5	x	x	x	x	x	x	x	x	x	1	0.7703
Syl6	x	x	x	x	x	x	x	x	x	x	0.5816
Syl9	x	x	x	x	x	x	x	x	x	x	x

COS	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.8691	x	x	0.6012	0.4256	1	1	0.3204	0.3662	0.6116
CLOCK	x	x	x	x	0.9184	0.3094	1	0.6084	0.5541	0.6623	0.6799
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.9123	0.8134	0.0315	1	1	0.4576
Ppi2	x	x	x	x	x	x	1	1	1	1	1
Syl1	x	x	x	x	x	x	x	1	0.0633	1	0.5719
Syl2	x	x	x	x	x	x	x	x	1	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	0.4684
Syl6	x	x	x	x	x	x	x	x	x	x	0.5817
Syl9	x	x	x	x	x	x	x	x	x	x	x

Table S6 (continued)

DEM	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.118	0.6967	x	0.5957	0.4079	0.6899	0.4885	0.2533	1	0.9501
CLOCK	x	x	0.1534	x	0.341	0.767	0.9168	0.2955	1	1	0.2087
NPAS	x	x	x	x	0.4003	0.353	0.6963	0.4981	0.8017	1	0.5434
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.1214	0.4774	0.6571	0.3787	0.3633	0.7456
Ppi2	x	x	x	x	x	x	0.4307	0.0292	1	1	0.0932
Syl1	x	x	x	x	x	x	x	0.18	1	1	0.5715
Syl2	x	x	x	x	x	x	x	x	1	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

GIL	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.7501	0.2101	0.2781	0.0974	0.1281	0.3033	0.4495	1	1	0.1532
CLOCK	x	x	0.8506	0.5275	0.0073	0.7092	0.2121	0.0779	1	0.0635	0.1768
NPAS	x	x	x	1	0.3694	0.202	0.1963	0.6482	0.7637	0.773	0.5255
SERT	x	x	x	x	0.3748	0.2195	0.4347	1	0.8751	0.6623	0.2555
PC8	x	x	x	x	x	0.4308	0.1209	0.8867	0.7095	0.8646	0.1173
Ppi2	x	x	x	x	x	x	0.339	1	1	1	0.2897
Syl1	x	x	x	x	x	x	x	0.0109	1	0.016	0.1804
Syl2	x	x	x	x	x	x	x	x	0.0418	0.1027	0.5805
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

GRZ	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.9699	0.1422	x	0.9831	0.3523	1	0.5265	0.9412	0.662	0.6609
CLOCK	x	x	0.6649	x	0.1108	0.9809	0.4098	0.0111	0.5035	1	0.5913
NPAS	x	x	x	x	0.1341	0.3791	0.4292	0.1066	1	0.3016	0.044
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.2588	0.5996	0.303	0.9123	0.7656	0.4518
Ppi2	x	x	x	x	x	x	0.6909	0.512	0.2914	1	0.5834
Syl1	x	x	x	x	x	x	x	0.3943	1	1	0.5527
Syl2	x	x	x	x	x	x	x	x	1	0.2549	0.2536
Syl5	x	x	x	x	x	x	x	x	x	1	0.9824
Syl6	x	x	x	x	x	x	x	x	x	x	0.48
Syl9	x	x	x	x	x	x	x	x	x	x	x

JAC	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.5054	x	x	0.5581	0.4739	1	0.5128	0.1657	1	0.7517
CLOCK	x	x	x	x	0.145	0.6003	1	0.4117	1	1	0.4498
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.645	0.3567	0.8656	0.3492	1	0.0637
Ppi2	x	x	x	x	x	x	1	0.0512	0.1158	1	0.5946
Syl1	x	x	x	x	x	x	x	1	1	1	1
Syl2	x	x	x	x	x	x	x	x	0.0494	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

Table S6 (continued)

MAD	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.0962	0.7513	0.4214	0.974	0.0351	0.2706	0.3935	0.668	1	0.6525
CLOCK	x	x	1	0.6914	0.4077	0.0352	0.7456	0.6647	0.8954	0.6154	0.2425
NPAS	x	x	x	1	0.0868	0.7989	1	0.8888	0.6562	0.7087	1
SERT	x	x	x	x	1	0.4214	0.2401	0.818	0.4198	0.932	0.6591
PC8	x	x	x	x	x	0.1437	0.4626	0.1716	0.4667	0.8439	0.6128
Ppi2	x	x	x	x	x	x	0.0804	0.5328	1	1	0.2682
Syl1	x	x	x	x	x	x	x	0.599	1	1	0.4365
Syl2	x	x	x	x	x	x	x	x	1	0.1352	0.2586
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

NEV	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.8209	x	x	0.357	0.0135	0.4094	0.7849	no info	0.3138	0.0971
CLOCK	x	x	x	x	0.375	0.7693	0.3242	0.6331	no info	0.1706	0.5426
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.7797	0.5574	0.0637	no info	0.1905	0.0886
Ppi2	x	x	x	x	x	x	1	1	no info	1	0.4421
Syl1	x	x	x	x	x	x	x	0.0956	no info	1	0.351
Syl2	x	x	x	x	x	x	x	x	no info	0.2393	0.1098
Syl5	x	x	x	x	x	x	x	x	x	no info	no info
Syl6	x	x	x	x	x	x	x	x	x	x	0.656
Syl9	x	x	x	x	x	x	x	x	x	x	x

OJE	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.6312	x	1	0.8847	0.6055	0.1025	0.3481	0.3434	0.3494	0.2344
CLOCK	x	x	x	1	0.3284	0.5213	1	0.09	1	1	1
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	1	1	0.6715	0.5818	0.8316	0.8333	1
PC8	x	x	x	x	x	0.183	0.7247	0.9387	1	1	0.7407
Ppi2	x	x	x	x	x	x	1	1	1	1	0.2692
Syl1	x	x	x	x	x	x	x	1	0.0294	1	0.8283
Syl2	x	x	x	x	x	x	x	x	1	1	0.6687
Syl5	x	x	x	x	x	x	x	x	x	1	0.5487
Syl6	x	x	x	x	x	x	x	x	x	x	0.5412
Syl9	x	x	x	x	x	x	x	x	x	x	x

PIR	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.3152	x	x	0.4391	0.0952	0.4816	0.6502	1	1	0.5676
CLOCK	x	x	x	x	0.5139	0.8561	0.8761	0.9672	0.4759	0.9293	0.9993
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.5591	0.4678	0.6885	1	0.0942	0.0208
Ppi2	x	x	x	x	x	x	0.5043	0.6144	0.0901	0.467	1
Syl1	x	x	x	x	x	x	x	1	1	1	1
Syl2	x	x	x	x	x	x	x	x	1	0.1326	1
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	0.2879
Syl9	x	x	x	x	x	x	x	x	x	x	x

Table S6 (continued)

PON	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.4928	0.6431	x	0.9762	0.3903	0.3588	0.3668	1	0.8199	0.3418
CLOCK	x	x	0.302	x	0.1727	0.7208	0.5152	0.8426	0.9975	0.076	0.2983
NPAS	x	x	x	x	0.264	1	0.8319	0.8834	0.7911	0.665	0.2185
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.2405	0.8618	0.9886	0.6818	0.6948	0.6126
Ppi2	x	x	x	x	x	x	1	1	1	0.4819	0.9095
Syl1	x	x	x	x	x	x	x	1	1	1	0.0506
Syl2	x	x	x	x	x	x	x	x	0.0483	1	0.7245
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	0.8544
Syl9	x	x	x	x	x	x	x	x	x	x	x

SEV	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.2173	0.3277	x	0.5737	0.3054	0.286	0.7716	0.4951	0.8874	0.4929
CLOCK	x	x	0.6883	x	0.5188	0.0973	0.2706	0.3205	0.9272	0.0987	0.394
NPAS	x	x	x	x	0.3251	0.4037	1	0.2608	0.8629	0.6188	0.4893
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.2993	0.5529	0.7425	0.6021	0.0055	0.0482
Ppi2	x	x	x	x	x	x	0.9161	0.6328	0.0412	0.8384	0.1473
Syl1	x	x	x	x	x	x	x	1	1	1	0.3516
Syl2	x	x	x	x	x	x	x	x	1	1	0.423
Syl5	x	x	x	x	x	x	x	x	x	1	0.4829
Syl6	x	x	x	x	x	x	x	x	x	x	0.3333
Syl9	x	x	x	x	x	x	x	x	x	x	x

TAR	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.9536	0.6105	0.6047	0.0728	0.295	0.6879	0.6314	0.5035	0.7813	0.1323
CLOCK	x	x	0.2209	0.416	0.4973	0.5258	0.2702	0.1812	0.5146	1	0.7436
NPAS	x	x	x	1	1	0.7074	0.7209	0.8024	0.9463	0.9762	0.0783
SERT	x	x	x	x	0.147	0.4758	0.4585	0.2413	0.9505	0.9734	0.6582
PC8	x	x	x	x	x	0.8835	0.5539	0.9963	1	1	0.9534
Ppi2	x	x	x	x	x	x	0.3822	0.5889	0.23	1	0.1765
Syl1	x	x	x	x	x	x	x	1	1	1	0.1899
Syl2	x	x	x	x	x	x	x	x	1	1	0.3321
Syl5	x	x	x	x	x	x	x	x	x	1	0.7801
Syl6	x	x	x	x	x	x	x	x	x	x	0.9549
Syl9	x	x	x	x	x	x	x	x	x	x	x

TOR	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.7626	0.4714	x	0.7963	0.9312	0.8277	0.6685	1	1	0.8201
CLOCK	x	x	0.7254	x	0.7828	0.9298	0.5251	0.4462	0.8053	0.8604	0.8984
NPAS	x	x	x	x	1	0.0863	0.3172	0.1673	0.9125	0.7809	0.0536
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.7484	0.1965	0.1952	0.8801	0.118	0.6951
Ppi2	x	x	x	x	x	x	0.5928	0.5083	1	1	0.0116
Syl1	x	x	x	x	x	x	x	0.3555	1	1	0.2733
Syl2	x	x	x	x	x	x	x	x	1	1	0.5427
Syl5	x	x	x	x	x	x	x	x	x	1	0.474
Syl6	x	x	x	x	x	x	x	x	x	x	0.5836
Syl9	x	x	x	x	x	x	x	x	x	x	x

Table S6 (continued)

TRI	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.1515	x	x	0.3648	1	0.6164	1	1	1	0.9922
CLOCK	x	x	x	x	0.7372	0.8264	0.8836	0.8775	0.2816	0.5075	0.7273
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.9352	0.9372	0.1578	1	0.5739	0.1911
Ppi2	x	x	x	x	x	x	0.2595	0.2874	1	1	1
Syl1	x	x	x	x	x	x	x	1	1	1	1
Syl2	x	x	x	x	x	x	x	x	1	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

VAL	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.6734	x	x	0.296	1	0.1636	1	0.1169	1	0.3141
CLOCK	x	x	x	x	0.7381	1	0.9984	1	0.6647	1	0.664
NPAS	x	x	x	x	x	x	x	x	x	x	x
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	1	0.0319	0.7813	0.6779	1	0.7377
Ppi2	x	x	x	x	x	x	1	1	1	1	0.3722
Syl1	x	x	x	x	x	x	x	1	1	1	0.8191
Syl2	x	x	x	x	x	x	x	x	1	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

Supplementary table S7. Results of GLMs on mean allele length and migration status 0/2 (= sedentary populations against migratory populations, excluding partially migratory populations) and migration status 1 (= sedentary + partially migratory populations pooled against migratory populations.) Numbers show the probability of the mean allele length of each locus to explain variation in migration mean allele length explain variation in migration taking longitude, latitude or both in consideration.

	Migration Status 0/2							Migration Status 1						
					Longitude+Latitude							Longitude+Latitude		
Allele	Longitude	Migration	Latitude	Migration	Longitude+	Latitude	Migration	Longitude	Migration	Latitude	Migration	Longitude+	Latitude	Migration
ADCYAP1	**0.003**	**0.001**	0.991	0.253	**0.003**	0.805	0.484	**0.000**	**0.005**	0.093	0.912	**0.000**	0.256	0.492
CLOCK	0.664	**0.007**	0.991	0.446	0.662	0.961	0.414	0.247	0.438	**0.011**	0.099	0.424	**0.017**	0.132
NPAS2	0.362	**0.000**	0.994	0.294	0.364	0.993	0.241	0.979	**0.004**	**0.019**	0.840	0.708	**0.018**	0.798
SERT	0.744	0.645	0.241	0.332	0.668	0.228	0.306	0.759	0.851	0.140	0.271	0.927	0.148	0.284
PC8	0.097	0.076	0.750	0.925	0.102	0.859	0.733	0.099	0.731	0.228	0.469	0.065	0.143	0.319
Ppi2	0.370	0.039	0.499	0.912	0.400	0.548	0.987	0.600	0.089	0.123	0.809	0.448	0.102	0.722
Syl 1	0.138	0.126	0.535	0.349	0.123	0.452	0.247	0.770	0.159	0.880	0.339	0.785	0.913	0.367
Syl 2	0.284	**0.028**	0.698	0.737	0.268	0.630	0.867	**0.008**	0.344	**0.006**	0.074	**0.023**	**0.016**	0.155
Syl 5	0.162	**0.009**	0.416	0.904	0.182	0.486	0.936	0.233	0.092	**0.014**	0.334	0.116	**0.008**	0.231
Syl 6	0.310	0.851	0.567	0.573	0.333	0.626	0.679	0.485	0.480	0.869	0.602	0.466	0.788	0.531
SYL 9	0.136	**0.000**	0.440	0.585	0.118	0.365	0.758	**0.000**	**0.012**	**0.008**	0.380	**0.000**	**0.039**	0.812

Supplementary table S8. Kendall's Tau correlation between major allele scores (= the number of copies of the most common allele) and migration status using different groupings. Significances were determined by Monte Carlo simulations. If loci had more than one major allele, all major alleles were tested.

	Migration Status 0/2		Migration Status 1		Migration Status 2	
Allele	Tau-b Valor	MonteCarlo sig	Tau-b Valor	MonteCarlo sig	Tau-b Valor	MonteCarlo sig
ADCYAP1-163	-0.24	**0**	-0.109	**0.008**	-0.206	**0**
ADCYAP1-167	-0.135	**0.007**	0.118	**0.004**	0.067	0.096
CLOCK-269	0.147	**0.005**	0.06	0.137	0.13	**0.001**
NPAS-169	0.209	**0**	0.122	**0.004**	0.163	**0**
SERT-320	0.021	1	-0.017	0.699	0.043	0.38
PC8-171	0.02	0.701	-0.014	0.728	0.035	0.392
Ppi2-268	-0.046	0.374	-0.006	0.888	-0.05	0.211
Syl 1-147	0.143	**0.007**	0.044	0.289	0.132	**0.002**
Syl 1-152	-0.137	**0.008**	-0.097	**0.021**	-0.086	**0.04**
Syl 2-147	-0.036	0.489	-0.037	0.375	-0.015	0.712
Syl 5-160	0.023	0.683	0.015	0.726	0.016	0.725
Syl 5-176	-0.051	0.348	-0.085	0.042	0.011	0.816
Syl 5-184	0.009	0.88	0.067	0.115	-0.042	0.313
Syl 6-184	0.137	**0.009**	0.065	0.113	0.108	**0.01**
Syl 6-188	-0.115	**0.042**	-0.111	**0.009**	-0.041	0.355
Syl 6-192	0.006	0.946	0.045	0.287	-0.028	0.534
Syl 9-147	-0.231	**0**	-0.073	0.069	-0.22	**0**

Supplementary table S9. One way ANOVAs testing for the effect of migration status on allele measurements of the studied genes. Several loci had more than one major allele. For these we tested all major alleles (see supplementary table S8 for details of these allele numbers).

	Min	Mean	Max	Major 1	Major 2	Major 3
ADCYAP1	0.082	**0.000**	**0.000**	**0.000**	**0.014**	
CLOCK	**0.001**	**0.001**	0.116	**0.002**		
NPAS2	**0.000**	**0.000**	x	**0.000**		
SERT	0.184	0.525	0.902	0.582		
PC8	0.479	0.200	0.183	0.719		
Ppi2	**0.009**	0.161	0.721	0.582		
Syl1	0.388	0.327	0.150	**0.006**	0.055	
Syl2	**0.001**	**0.002**	0.059	0.478		
Syl5	0.653	0.063	**0.009**	0.929	**0.031**	**0.024**
Syl6	0.303	0.634	0.634	**0.025**	**0.029**	0.461
Syl9	**0.020**	**0.000**	**0.000**	**0.000**		

Supplementary table S10. T-test on mean allele lengths of the candidate markers to test for difference in allele length between groups of populations with different migration status. (MigrSta0/2 = Comparison of Sedentary against Migratory. MigrSta1 = Comparison of Sedentary + Partially migratory against Migratory. MigrSta2 = Sedentary against Partially migratory + Migratory).

	MigrSta0/2	MigrSta1	MigrSta2
ADCYAP1	**0.012**	0.401	**0.015**
CLOCK	**0.004**	0.078	**0.002**
NPAS2	**0.000**	**0.000**	**0.000**
SERT	0.159	0.969	0.035
PC8	0.362	0.355	0.381
Ppi2	0.112	0.128	0.230
Syl1	0.199	0.543	0.143
Syl2	0.418	0.189	0.880
Syl5	**0.007**	**0.036**	**0.019**
Syl6	0.276	0.349	0.364
Syl9	**0.000**	0.113	**0.000**

Supplementary table S11. Results of the analyses of molecular variance (AMOVA). Given are the percentages of molecular variation explained by grouping of populations by migration status, altitude and latitude.

Altitude	All			Candidate			Neutral		
Locus	A	B	C	A	B	C	A	B	C
All	0	1.3	98.7	-0.4	1.51	98.9	0.08863	1.25443	98.65694
ADCYAP1	-0.40644	1.34919	99.05725	-0.40644	1.34919	99.05725			
CLOCK	-0.33999	1.29587	99.04412	-0.33999	0.00292	99.04412			
NPAS2	-1.1368	5.46549	95.67131	-1.1368	0.00155	95.67131			
SERT	0.82466	-0.02455	99.19989	0.82466	0	99.19989			
PC8	-0.33857	1.66769	98.67088				-0.33857	1.66769	98.67088
Ppi2	0.11645	0.75775	99.1258				0.11645	0.75775	99.1258
Syl1	0.08542	1.35039	98.56419				0.08542	1.35039	98.56419
Syl2	-0.04485	0.889	99.15585				-0.04485	0.889	99.15585
Syl5	0.1933	0.75978	99.04692				0.1933	0.75978	99.04692
Syl6	0.05796	1.21669	98.72535				0.05796	1.21669	98.72535
Syl9	0.47606	2.71787	96.80606				0.47606	2.71787	96.80606
Sum of Squares	11.333	101.285	3675.204	0.706	19.607	668.943	10.626	81.677	3006.261
Variance Components	-0.00002	0.04377	3.32252	-0.00246	0.00921	0.60428	0.00244	0.03456	2.71823
V.a. P value	0.44575	0	0	0.97947	0	0	0.18182	0	0

Latitude	All			Candidate			Neutral		
Locus	A	B	C	A	B	C	A	B	C
All	0.502	0.94421	98.55379	1.03	0.54	98.43	0.38377	1.03385	98.58238
ADCYAP1	1.1331	0.31219	98.55471	1.1331	0.31219	98.55471			
CLOCK	0.97957	0.40603	98.61441	0.97957	0.40603	98.61441			
NPAS2	0.4836	4.44306	95.07334	0.4836	4.44306	95.07334			
SERT	-0.18097	0.57747	99.6035	-0.18097	0.57747	99.6035			
PC8	0.08969	1.40755	98.50276				0.08969	1.40755	98.50276
Ppi2	0.07622	0.77102	99.15276				0.07622	0.77102	99.15276
Syl1	0.37517	1.13397	98.49086				0.37517	1.13397	98.49086
Syl2	0.1051	0.78881	99.10609				0.1051	0.78881	99.10609
Syl5	0.27232	0.67894	99.04873				0.27232	0.67894	99.04873
Syl6	-0.09535	1.31775	98.7776				-0.09535	1.31775	98.776
Syl9	2.27862	1.37117	96.3502				2.27862	1.37117	96.3502
Sum of Squares	23.471	89.147	3675.204	6.37	13.944	668.943	17.101	75.203	3006.261
Variance Components	0.01692	0.03183	3.32252	0.00634	0.00333	0.60428	0.01058	0.02851	2.71823
V.a. P value	0	0	0	0	0	0	0.00098	0	0

A = Among groups

B = Among populations within groups

C = Within populations

migrStatus	All			Candidate			Neutral		
Locus	A	B	C	A	B	C	A	B	C
All	0.51201	0.93782	98.55017	1.46	0.24	98.3	0.30062	1.09317	98.60622
ADCYAP1	1.62456	-0.03342	98.40885	1.62456	-0.03342	98.40885			
CLOCK	1.27662	0.19756	98.52582	1.27662	0.19756	98.52582			
NPAS2	1.2128	3.9206	94.8666	1.2128	3.9206	94.8666			
SERT	-0.0679	0.49755	99.57034	-0.0679	0.49755	99.57034			
PC8	0.52809	1.09739	98.37452				0.52809	1.09739	98.37452
Ppi2	0.11124	0.74642	99.14234				0.11124	0.74642	99.14234
Syl1	0.1969	1.2606	98.54251				0.1969	1.2606	98.54251
Syl2	0.05306	0.82568	99.12126				0.05306	0.82568	99.12126
Syl5	0.15334	0.76326	99.0834				0.15334	0.76326	99.0834
Syl6	-0.09421	1.31681	98.7774				-0.09421	1.31681	98.7774
Syl9	1.64725	1.8239	96.52885				1.64725	1.8239	96.52885
Sum of Squares	23.765	88.853	3675.204	8.074	12.24	668.943	15.691	76.613	3006.261
Variance Components	0.01726	0.03162	3.32252	0.00898	0.00148	0.60428	0.00829	0.03013	2.71823
V.a. P value	0	0	0	0	0.17302	0	0.00489	0	0

migrStatus1	All			Candidate			Neutral		
Locus	A	B	C	A	B	C	A	B	C
All	0.08397	1.25795	98.65808	0.5	1.02	98.48	-0.00857	1.31001	98.69856
ADCYAP1	0.7405	0.74555	98.51394	0.7405	0.74555	98.51394			
CLOCK	0.09342	1.05289	98.85369	0.09342	1.05289	98.85369			
NPAS2	0.88046	4.33578	94.78375	0.88046	4.33578	94.78375			
SERT	-0.18673	0.54207	99.64466	-0.18673	0.54207	99.64466			
PC8	0.09582	1.42335	98.78084				0.09582	1.42335	98.48084
Ppi2	0.20321	0.72402	99.07277				0.20321	0.72402	99.07277
Syl1	0.04776	1.37601	98.57623				0.04776	1.37601	98.57623
Syl2	-0.17196	0.94854	99.22342				-0.17196	0.94854	99.22342
Syl5	0.03506	0.85406	99.11088				0.03506	0.85406	99.11088
Syl6	-0.06313	1.2817	98.78143				-0.06313	1.2817	98.78143
Syl9	-0.19568	3.09615	97.09953				-0.19568	3.09615	97.09953
Sum of Squares	7.538	105.08	3675.204	2.605	17.709	668.943	4.933	87.37	3006.261
Variance Components	0.00283	0.04236	3.32252	0.00306	0.00629	0.60428	-0.00024	0.03608	2.71823
V.a. P value	0.15543	0	0	0.02737	0.00196	0	0.48778	0	0

A = Among groups

B = Among populations within groups

C = Within populations

* migrStatus = Comparison of the three different migration strategies. MigrStatus1 = Comparison of Sedentary + Partially migratory against Migratory.

Table S11 (continued)

migrStatus2	All			Candidate			Neutral		
Locus	A	B	C	A	B	C	A	B	C
All	0.94425	0.90538	98.15037	2.35	0.28	97.34	0.62009	1.04661	98.3333
ADCYAP1	2.45132	0.09321	97.45547	2.45132	0.09321	97.45547			
CLOCK	2.32519	0.13106	97.54375	2.32519	0.13106	97.54375			
NPAS2	2.59363	3.65403	93.75234	2.59363	3.65403	93.75234			
SERT	0.0638	0.4234	99.51281	0.0638	0.00003	99.51281			
PC8	1.23861	0.9514	97.80999				1.23861	0.9514	97.80999
Ppi2	-0.03311	0.83865	99.19446				-0.03311	0.83865	99.19446
Syl1	0.58727	1.15425	98.25848				0.58727	1.15425	98.25848
Syl2	0.34772	0.7188	98.93347				0.34772	0.7188	98.93347
Syl5	0.07933	0.8385	99.08217				0.007933	0.8385	99.08217
Syl6	-0.00046	1.25041	98.75005				-0.00046	1.25041	98.75005
Syl9	3.05823	1.68898	95.25279				3.05823	1.68898	95.25279
Sum of Squares	18.925	93.692	3675.204	7.119	13.195	668.943	11.806	80.498	3006.261
Variance Components	0.03196	0.03065	3.32252	0.01482	0.00172	0.60428	0.01714	0.02893	2.71823
V.a. P value	0	0	0	0	0.15249	0	0	0	0

migrStatus0/2	All			Candidate			Neutral		
Locus	A	B	C	A	B	C	A	B	C
All	0.9456	0.9852	98.0692	2.62	0.4	96.98	0.56711	1.11767	98.31522
ADCYAP1	3.02625	-0.00205	96.9758	3.02625	-0.00205	96.9758			
CLOCK	2.05717	0.30776	97.63507	2.05717	0.30776	97.63507			
NPAS2	3.17701	5.53478	91.28821	3.17701	0.00173	91.28821			
SERT	-0.1532	0.29245	99.86075	-0.153202	0.00003	99.86075			
PC8	1.38309	0.88823	97.72868				1.38309	0.88823	97.72868
Ppi2	0.12008	0.87323	99.0067				0.12008	0.87323	99.0067
Syl1	0.68064	1.38308	97.93628				0.68064	1.38308	97.93628
Syl2	0.17717	0.90576	98.91708				0.17717	0.90576	98.91708
Syl5	0.005	0.76741	99.22759				0.005	0.76741	99.22759
Syl6	-0.02638	1.5381	98.48828				-0.02638	1.5381	98.48828
Syl9	2.68604	1.45025	95.86371				2.68604	1.45025	95.86371
Sum of Squares	15.825	62.489	2251.226	6.247	9.277	410.453	9.578	53.213	1840.773
Variance Components	0.0318	0.03313	3.29755	0.01624	0.00247	0.60096	0.01555	0.03066	2.69659
V.a. P value	0	0	0	0	0.12805	0	0	0	0

A = Among groups

B = Among populations within groups

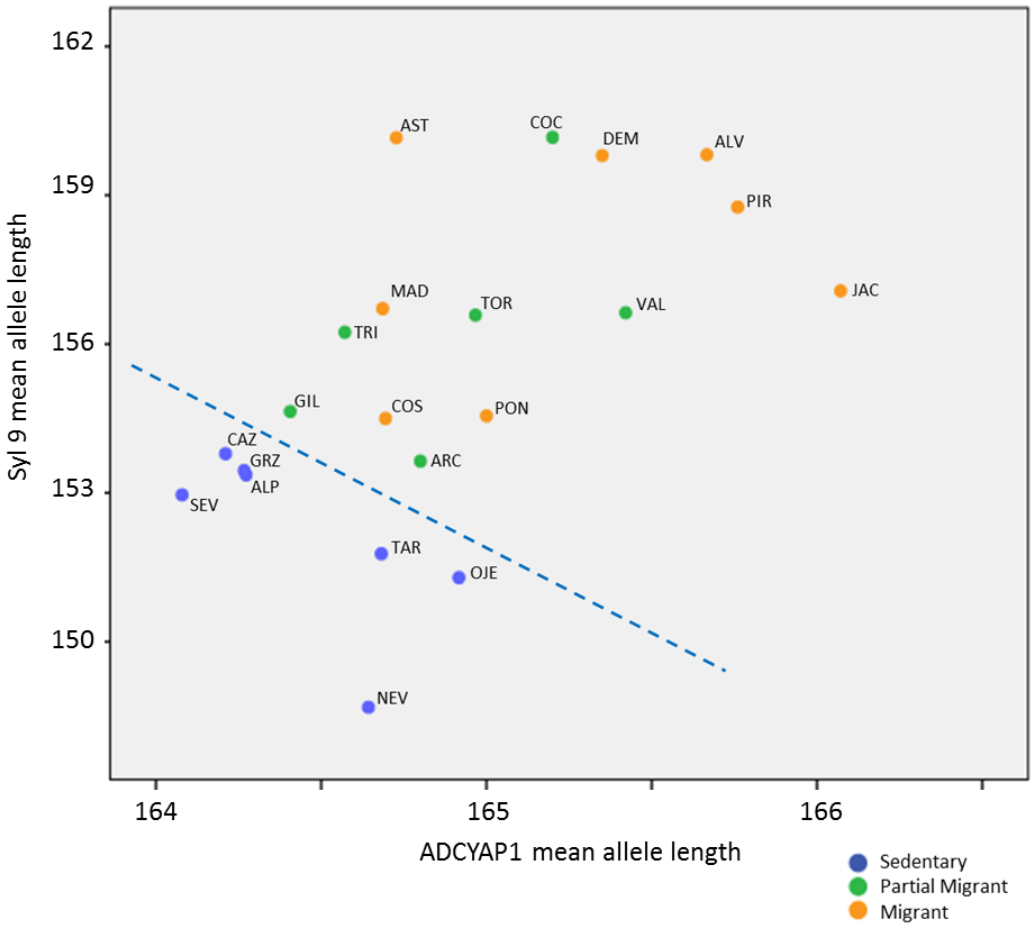
C = Within populations

* migrStatus2 = Comparison of Sedentary against Partialy migratory + Migratory. MigrStatus0/2 = Comparison of Sedentary against Migratory.

Supplementary table S12. Results of logistic regression analyses. The table gives the percentages of populations assigned to the correct migratory status using mean allele length at different loci or combinations of loci as the explanatory variables in the model (Migration Status 2 = Comparison of Sedentary populations against Partially migratory + Migratory populations. Migration Status 0/2 = Comparison of Sedentary against Migratory. (excluding partially migratory populations).

	Migration Status 2				Migration Status 0/2			
Mean allele length of	X^2	Sig	LogLikeli	%	X^2	Sig	LogLikeli	%
All markers	26.734	0.005	0	100	20.728	0.036	0	100
Candidate makers	26.734	0	0	100	20.728	0	0	100
Neutral Markers	26.734	0	0	100	20.728	0.004	0	100
ADCYAP1	10.422	0.001	16.312	81	10.409	0.001	10.318	80
CLOCK	11.095	0.001	15.638	85.7	7.095	0.008	13.633	80
NPAS2	6.798	0.009	19.936	85.7	6.03	0.014	14.698	86.7
SERT	2.421	0.12	24.312	71.4	1.411	0.235	19.317	53.3
PPi2	0.806	0.369	25.928	71.4	1.396	0.237	19.332	60
PC8	3.283	0.07	23.451	76.2	2.216	0.137	18.511	73.3
Syl 1	0.156	0.693	26.577	66.7	0.187	0.665	20.541	60
Syl 2	7.798	0.005	18.936	76.2	4.76	0.029	15.968	66.7
Syl 5	8.916	0.003	17.817	76.2	7.665	0.006	13.063	66.7
Syl6	0.587	0.444	26.147	71.4	0.223	0.637	20.505	46.7
Syl 9	22.404	0	4.33	90.5	20.728	0	0	100
ADCYAP1, CLOCK	25.501	0	5.233	95.2	20.728	0	0	100
ADCYAP1, NPAS2	11.263	0.004	15.471	81	10.539	0.005	10.189	80
CLOCK, NPAS2	16.755	0	9.978	90.5	12.367	0.002	8.361	86.7
ADCYAP1, CLOCK, NPAS2	21.518	0	5.216	95.2	20.728	0	0	100
ADCYAP1, Syl 9	26.734	0	0	100	20.728	0	0	100
CLOCK, Syl 9	26.734	0	0	100	20.728	0	0	100
ADCYAP1, CLOCK, Syl 9	26.734	0	0	100	20.728	0	0	100
ADCYAP1, CLOCK, NPAS2, Syl 9	26.734	0	0	100	20.728	0	0	100

Supplementary Figure S13. Separation of Iberian blackcaps differing in migratory behavior by mean allele length at the ADCYAP1 and Syl9 gene.



Chapter 2

Candidate genes do not predict variation in zugunruhe expressed under different environmental conditions in Iberian blackcaps

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(unpublished manuscript)

Abstract

Iberian blackcaps live close to the threshold of migration. The genetics of migration in this group has so far been limited to field studies. Though it is not clear to what extent migratory activity in captivity (migratory restlessness or Zugunruhe) reflects migratory behaviour in the wild, studying it does make it possible to obtain data impossible to gather in the field. For this reason we monitored Zugunruhe of three Iberian Blackcap populations which, in the wild, exhibit marked differences in migratory behaviour. We tested for correlations between Zugunruhe and a set of genetic markers, as was previously done in natural populations of blackcaps. The three populations in this study have different migratory strategies (sedentary, partially migratory, completely migratory). They were kept under laboratory and under captive outdoor conditions. Their activity was closely monitored and they were screened for a set of 13 microsatellite markers, 4 of which are candidate gene markers.

In neither captive situation was the migratory restlessness correlated to any genetic marker.

A difference in onset of migration was found between populations. However, this difference was not correlated to any of the genetic markers we studied.

Candidate marker SERT was associated with the type of behaviour birds displayed in the outdoor aviaries.

It appears that among population differences in migratory behaviour in Iberian blackcaps, as they were found in the wild, are not expressed under our laboratory conditions. We believe this is the result of co-gradient variation. The right genetics and the right environmental conditions are needed to express the proper behaviour. As a result of excluding environmental influences in our study, behavioural differences between the populations were lacking. Results from laboratory studies on genetic predispositions could therefore not be very informative.

Introduction

Captivity and Zugunruhe

Keeping migratory songbirds in captivity, and measuring the individual migratory activity (migratory restlessness or Zugunruhe) has been the bases for studies on the physiology, genetics and evolution of migration (reviewed by Berthold 2001, Pulido & Berthold 2003). This technique allows obtaining information on individual migratory behaviour (e.g. onset, intensity, duration or migration), which is difficult to obtain accurately in the wild. Studies in captivity can also provide information on the genetic bases of migratory behaviour. For example crossbreeding experiments showed that migration direction is an inherited trait (Berthold & Helbig 1992).
The urge of the birds to migrate manifests itself in seasonal bouts of nocturnal activity called migratory restlessness or "Zugunruhe" (see, Berthold *et al.* 2000). Migratory restlessness can easily be monitored and scored and has therefore been widely used as a measure for migratory activity for captive birds (see, for instance, Bertold 1984, Helm and Gwinner 2006).

However, it has been suggested that migratory restlessness in the laboratory may not reflect actual migratory behaviour in the wild (Helm 2006, Rappole 2013,). This may partly be due to the fact that the threshold of migration may be sensitive to environmental effects, particularly in short-distance migrants. The current model of the control of the expression of migratory activity, the environmental threshold model, predicts that in populations close to the migration threshold the expression of migratory behaviour is primarily determined by variation in environmental conditions (Pulido 2011).
It has previously been shown that blackcap (*Sylvia atricapilla)* populations of the Iberian Peninsula are close to the migration threshold. A study on these birds showed that their migratory restlessness was unrelated to the behaviour of their wild conspecifics. Birds from populations with different migration strategies (completely migratory, partially migratory and sedentary) exhibited similar activity patterns in captivity (Bulaic, 2015).

Molecular genetics of differences in migratory behaviour

To date, the molecular genetic basis of migration is largely unknown (Liedvogel *et al.* 2011). In blackcaps, a study investigating birds from 14 different European populations showed that there was an association between migratory behaviour and allelic variation at a candidate gene, ADCYAP1. It showed both, the association at the continental level, when migratory status of each population was considered and an association of variation at this gene and migratory restlessness at the individual level within two different populations (Mueller *et al.* 2011). The results of this study suggested that ADCYAP1 might act at multiple levels, via a phase-shift of the endogenous oscillator or via a modulation of the downstream processes of the molecular clock, modifying the shift between migratory and non-migratory states. Another study on Juncos found the same association between ADCYAP1 and Zugunruhe, as well as an association of zugunruhe and CLOCK, another candidate marker linked to circadian rhythms. However, results differed when birds where studied in the field or under natural conditions (Peterson *et al.* 2013). The causes of this discrepancy between results on the association between these candidate genes and migratory behaviour expressed in the wild and in captivity is unknown and requires a better understanding of the expression of migratory behaviour.

A recent study on wild Iberian blackcap populations showed that there was an association between

migratory behaviour and variation at the ADCYAP1 locus, confirming the previous result on the association of migratory behaviour and ADCYAP at a continental scale (Chapter 1). It is, however, unknown, if this association persists in birds expressing migratory activity in captivity, i.e. migratory restlessness.

This study

So far the study of the genetics of migration of Iberian blackcaps, which are close to the migration threshold (in which migratory behaviour is likely to be strongly determined by environmental conditions), has been limited to wild populations, looking at the birds at population level. Here, we want to investigate whether the association with ADCYAP1 found by Mueller *et al.* (2011) can also be found in captivity at an individual level. We will use Iberian populations of blackcaps with different migration strategies: one completely migratory, one completely sedentary and one partially migratory. By doing so we can exclude several confounding factors e.g. inter species differences, effects of a large geographical scale.

Moreover, as we know of the importance of environmental conditions for the expression of migratory behaviour in the population of this study, we wanted to test for the association of variation at candidate gene loci with migratory behaviour displayed in captive blackcaps held under more natural conditions So, far, studies on migratory activity in natural or semi-natural environments, like outdoor aviaries, are lacking. Therefore, apart from keeping most of the birds in a regular laboratory environment similar to that of previous captive studies, we kept birds under outdoor conditions. This approach will make it possible to elucidate whether the association of migratory activity and candidate genes persists under different environmental conditions, and, if discrepancies with results obtained in the wild are found, in which case we could study the causes of these discrepancies. This may give us new insights into the control of the expression of migratory behaviour, particularly of migratory restlessness.

Material & Methods

Capture of birds

For 3 consecutive years (2010-2012) fledgling, 2-3 months old, blackcaps, *Sylvia atricapilla*, were caught at their breeding sites before autumn migration (July – mid August) and brought to a facility in a restricted area in the natural park Casa de Campo, Madrid, Spain (40º25'N, 3º45'W) (N=104.). After spring migration, birds were returned to their respective capture sites to be released. The birds originated from three populations on the Iberian Peninsula, each with a different migratory strategy. (1) Madrid, a completely migratory population (N=35), (2) Cocentaina, a partially migratory population (N=47), (3) Tarifa, a sedentary population (N=22). (For a more detailed overview of the sampled birds and populations, see Table 1 with its map). Determining migration status of these populations was based on presence-absence (wintering/breeding) data (SEO/BirdLife, 2012) and wing measurements (according to Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015).

Table 1. Overview of the birds used in this study. Including the map with the location of the completely migratory population of Madrid, the partially migratory population of Cocentaina and the sedentary population of Tarifa.

Indoor Cages

		Madrid	Cocentaina	Tarifa	Total
2010-2011	Male	4	12	4	20
	Female	6	3	4	13
	Total	10	15	8	33
2011-2012	Male	7	14	0	21
	Female	5	6	0	11
	Total	12	20	0	32
2012-2013	Male	3	5	5	13
	Female	2	3	2	7
	Total	5	8	7	20
All Years	Male	14	31	9	54
	Female	13	12	6	31
	Total	27	43	15	85

Outdoor Aviaries

		Madrid	Cocentaina	Tarifa	Total
2012-2013	Male	4	2	2	8
	Female	4	2	5	11
	Total	8	4	7	19

Keeping of birds

Most birds were kept indoors, in cage cages of (45x23x38cm). The holding facility had windows, but two lamps were added to provide the same light intensity as an outdoor situation would. (2 x fluorescent Megaman WL 130 Compact 2000 HPF of 30W, an intensity of 1620 lumen and a colour temperature of 6500K). These lights were switched on and off by an automated system that followed the natural light cycle outside. Sunrise and sunset were imitated by a dusk period. Minimum temperatures were about 4°C warmer than outside and maximum temperatures about 4°C cooler, resulting in the range of 5-36°C. Activity inside the cages was measured as the amount of perch contacts recorded through micro switches placed under two movable perches that were connected to the Microscript recording system (Berthold *et al.* 1972). Activity was monitored from September through May.

In 2012 (until the spring of 2013), 19 birds were kept in outdoor aviaries (more info in table 1) (3x2x2.3m). In this environment, temperatures ranged from 1-40ºC. Nocturnal activity was quantified through video revision. Each aviary was equipped with a surveillance camera on infrared mode. Videos were analysed using a method that entailed not having to watch all video footage in real time. Videos were analysed for alternate nights for the 3 month period (September-November). Activity was quantified by watching the first 30 minutes of each hour for 2 minutes for every 10 minute interval (e.g. 0:00-0:02, 0:10-0:12, 0:20-0:22, 0:30-0:32, 1:00-1:02, etc.). Observed behaviour was assigned to one of three categories. 1) Hopping on the perch. 2) Wing whirring (Vibrating, buzzing of the wings possibly producing sound). 3) Flying. The dominant behaviour was calculated through the frequency of each type. The total amount of activity was the number of active 2 minute intervals.

The birds were fed daily. They received a diet consisting of mealworms, fruit (depending on whichever was in season; apple, persimmon fruit, pomegranate, fig, pear, cherry) and industrially produced birdseed for insectivorous birds with added vitamins (Raff, Patée con insetti), water was provided ad libitum. Individual intake for each type of food was monitored to keep track of health, but also to notice dietary changes in preparation for migration. Monitoring food intake for the outdoor birds proved more difficult since these birds were catching insects to their heart's desire.

DNA sampling

Blood (ca 50 µl) was taken from all birds by either puncturing the brachial vein, or by extracting it from the jugular vein with a syringe. Blood was stored in alcohol in a freezer (at -20ºC) until lab work commenced. DNA extractions were made using a *Blood and Tissue Kit* (Macherry & Nagel).

Genotyping

Following Mueller *et al.* 2011 (and chapter 1) we investigated four candidate genes, three of which have been related to circadian behaviour (CLOCK, ADCYAP1 and NPAS2) and one related to harm-avoidance behaviour (SERT). For each an exonic microsatellite was used (Mueller *et al.* 2011 & chapter 1). For comparative association analyses we used 8 anonymous, neutral microsatellite loci (Syl1, Syl2, Syl4, Syl5, Syl6, Syl9, Ppi2 and Pca8) and the sexing marker P2P8 from Griffiths et al. (1998). This made a set of 13 microsatellite markers. (Selection of the markers was partially based on Segelbacher *et al.* 2008 and Steinmeyer *et al.* 2009) (For more information see Supplementary table S1).
All samples (N=101) were genotyped for these 13 microsatellites using an ABI 3100 sequencer (Applied Biosystems) for the microsatellite fragment analyses. (For details on PCR conditions: see Mueller *et al*. 2011). The result files were analysed in Genemapper

Data Analyses

Most analyses were conducted using SPSS (IBM Corp. version 20). Kolmogorov-Smirnoff tests showed that allele lengths of the microsatellite data were not normally distributed. (Supplementary table S2 shows allele frequency tables per locus per population). For this reason we exclusively used non-parametric tests. Tests for Hardy Weinberg Equilibrium were performed using GenAlEx 6.502 (Peakall *et al.* 2012). Linkage disequilibrium was tested using Genepop V4 (Raymond and Rousset 1995). (Supplementary table S3 shows summaries of HWE tests, Supplementary table S4 shows a Linkage table). One of the neutral markers; Syl4, was not in HWE and therefore excluded from further analysis of this dataset (see, Chapter 1). No linkage between loci was observed.
From the Microscript records and the data extracted from the videos we could obtain information on the

timing of migration (the onset and the end), the intensity and amount of activity and the type of activity. The latter was only scored for the birds what were kept in outdoor aviaries, since they were monitored by cameras. In table 2 we listed the variables we extracted from these data. From these variables we calculated different principle components representing different independent axes of variation in migratory behaviour: For the birds kept indoors, we extracted one principle component for the onset of migration, and two for the amount of activity (one for all activity and one for the period of high intensity activity). For the birds kept outdoors we extracted one principle component for the type of behaviour. (For details of the PCAs, see table 3). Since activity indoors and outdoors was measured using different methods the resulting variables differed. Hence, we used similar variables for quantifying migratory activity in birds kept in the aviaries, but did not conduct principle component analyses.
Yearly variation in activity was not found (Bulaic, 2015), therefore, we did not consider year effects. Birds from the same population, but different years, were grouped together.

Non-parametric, Kolmogorov-smirnov, tests show that the response variables, the activity measurements, are normally distributed; our allele variables were not. Thus for following analysis non-parametric tests were used.

For the caged, indoor, birds we used non parametric Spearman rank tests, and linear regressions to see whether there are any genetic markers that were associated with the activity of the birds. Linear regressions are parametric tests, however it has been shown that deviation from normality of the dependent variable is not critical, thus we do not have to use a non-parametric alternative. The activity measurements were entered into the model as the dependent variables. Mean, maximum and minimum allele length were used as independent variables, as well as a major allele score.

For the aviary, outdoor, birds we started with one-way anovas to look for population differences. After that linear regressions were done, as for the caged birds. And finally some one-way anovas were used to look for any links between the type of behaviour and migration activity.

Table 2. Variables characterizing different components of migratory activity extracted from activity data.

Variable name	in- / outdoor	Activity incl.	Min. # of 30min interv.	Period considered
5-day onset of MA	both	All	5	at least 5 consecutive days at the start of the phase with continuous activity
5-day onset of high MA	both	High	5	at least 5 consecutive days at the start of the phase with continuous activity
3 intervals onset of activity	both	All	3	first part of the night (22:00-02:00h) at the start of the phase with cont. activity
4 intervals onset of activity	both	High	4	first part of the night (22:00-02:00h) at the start of the phase with cont. activity
Continuity indep. 3 intervals onset	both	All	3	at the start of the phase independently of the continuity of activity
Total hopping activity	outdoor	x	x	Weigthed average over hopping activity as the primary and secondary activity
Total wing whirring activity	outdoor	x	x	Weigthed average over whing whirring activity as the primary and secondary act.
Total flying activity	outdoor	x	x	Weigthed average over flying activity as the primary and secondary activity

Variable name	Description
MA= Migration Activity	the sum of 30min periods of night activity during the migration season (=period between the onset and end of migr.)
High MA	MA, of the phase with continuous activity
MA maximum days of activity	MA corrected for the number of days with missing data
MA intensity	MA, excluding days without activity, divided by the number of nights with activity
MA maximum days of higher activity	MA corrected for the number of days with missing data, for the days of continuous activity
MA intensity of higher activity	MA, excluding days without activity, divided by the number of nights with activity, for the days of continuous activity
MA maximum days of activity (filled gaps)	MA with extrapolated data for days with missing data
MA maximum days of higher activity (filled gaps)	MA with extrapolated data for days with missing data, for the days of continuous activity
Onset MA5HA OMA1 (Aviary birds)	Onset in aviary birds
Corr AMA OMA 1 (Aviary birds)	Activity in aviary birds

Table 3. Summary of the results of the principle component analysis. In the subsequent analyses the 4 extracted principle components (PCs) were used. (PC Activity 1 is the PC considering all night activity. PC Activity 2 is the PC representing periods of high activity. See table 2 for more details).

	Indoor / Caged			Outdoor / Aviaries
	PC Onset	PC Activity 1	PC Activity 2	PC Behaviour Type
% of variance	73.1	72.46	20.25	71.96
Eigenvalue	3.657	4.348	1.215	2.159
Factor loadings				
5-day onset of MA	0.78			
5-day onset of high MA	0.875			
3 intervals onset of activity	0.948			
4 intervals onset of activity	0.838			
Continuity independent 3 intervals onset	0.826			
MA maximum days of activity		0.901	-0.41	
MA intensity		0.839	-0.445	
MA maximum days of higher activity		0.877	0.442	
MA intensity of higher activity		0.706	0.574	
MA maximum days of activity (filled gaps)		0.894	-0.426	
MA maximum days of higher activity (filled gaps)		0.874	0.378	
Total hopping activity				0.918
Total wing whirring activity				0.573
Total flying activity				-0.994

Results

Our regression analyses showed several significant models, though a pattern is lacking. The onset of migration is significantly correlated to the mean allele length of Syl2 ($p= 0.021$) and to the major allele score of Syl9 ($p=0.004$). Migration activity is correlated to the major allele score of Syl1 ($p<0.005$) . (an overview of these results; Table 4A for mean allele length and Table 4B for major allele scores). Spearman's rank correlations concur with these results (see table 5)

Aviary birds

One-way anovas with two activity measurements as dependent variables showed no significant population effects (Onset of MA 5 HA, $F = 0.591$ and $p=0.568$. Corr AMA OMA1, $F = 1.365$ and $p=0.292$.).Therefore we lumped all birds together in the following analyses.

The regression analyses shows, in comparison to the caged birds, several more markers with significant correlations to activity. For instance, the candidate markers CLOCK and SERT appear. CLOCK is significant when looking at major allele scores (Activity $p=0.037$). SERT is significant for the principle component for the behaviour types in all possible models, mean allele length and major allele score. $p=0.028$ each time. Several neutral markers complete the set of results. (For more detailed results see table 4C for mean allele lengths and 4D for major allele scores).

Overall, the majority of the significant results are with neutral markers. However, chapter 1 showed that Syl9, also a neutral marker, might not be as neutral as previously thought.

Table 4. Summary of the results of the multiple regression analyses. The best model for different dependent variable (= activity measurements) was obtained by either adding significant variables (forward) or eliminating non-significant variables (backward). Each table gives the variables retained in the final model and their significance, as well as the significance of the model. Models were run considering mean allele lengths at each locus (tables 4A and 4C), or major allele scores (= the number of most common alleles present: tables 4B and 4D) as independent variables.

4A. Caged birds (Mean allele lengths)

Activity measurement	F/B	P model	Marker	P	Marker	P
PC onset	Backward	**0.016**	Syl2	**0.05**	Syl9	0.087
PC onset	Forward	**0.021**	Syl2	**0.021**		
PC activity	Backward	no value	all	1		
PC activity	Forward	x	x	x		
PC high activity	Backward	0.074	Syl5	0.074		
PC high activity	Forward	x	x	x		

4B. Caged birds (Major allele scores)

Activity measurement	F/B	P model	Marker	P	Marker	P
PC onset	Forward	**0.004**	Syl9	**0.004**		
PC onset	Backward	**0.004**	Syl9	**0.004**		
PC activity	Forward	x	x	x		
PC activity	Backward	0.108	Syl2	0.108		
PC high activity	Forward	**0.000**	Syl1	**0.000**		
PC high activity	Backward	**0.000**	Syl1	**0.000**	Syl2	0.066

4C. Aviary birds (Mean allele lengths)

Activity measurement	F/B	P model	Marker	P	Marker	P	Marker	P
Onset MA5HA OMA1	Forward	x						
Onset MA5HA OMA1	Backward	0.110	Syl5	0.11				
Corr AMA OMA 1	Forward	x						
Corr AMA OMA 1	Backward	**0.002**	Sert	**0.016**	Syl6	**0.014**	Syl5	**0**
PC behaviour types	Forward	**0.028**	Sert	**0.028**				
PC behaviour types	Backward	**0.028**	Sert	**0.028**				

4D. Aviary birds (Major allele scores)

Activity measurement	F/B	P model	Marker	P	Marker	P	Marker	P	Marker	P	Marker	P
Onset MA5HA OMA1	Forward	x										
Onset MA5HA OMA1	Backward	0.133	Syl1	0.133								
Corr AMA OMA 1	Forward	x										
Corr AMA OMA 1	Backward	**0.001**	CLOCK	**0.037**	Sert	**0.001**	PC8	**0.05**	Syl1	**0.004**	Syl9	0	Ppi2	0.058
PC behaviour types	Forward	**0.028**	Sert	**0.028**										
PC behaviour types	Backward	**0.028**	Sert	**0.028**										

Table 5. Spearman's rank correlation between alleles at different microsatellite loci and measurements of migratory activity.

	PC_Onset_without2	PC_corrAMA_1	PC_corrAMA_2	ADCYAP1 max	ADCYAP1 min	ADCYAP1 mean	CLOCK max	CLOCK min	CLOCK mean	NPAS2 max	NPAS2 min	NPAS2 mean	SERT max	SERT min	SERT mean
PC_Onset_without2	x	**.044**	.647	.141	.697	.392	.679	.406	.551	.	.941	.941	.691	.367	.724
PC_corrAMA_1	x	x	.969	.452	.191	.236	.307	.496	.201	.	.154	.154	.978	.332	.485
PC_corrAMA_2	x	x	x	.252	.403	.180	.979	.616	.633	.	.427	.427	.562	.233	.212

	PC8 max	PC8 min	PC8 mean	Syl1 max	Syl1 min	Syl1 mean	Syl6 max	Syl6 min	Syl6 mean	Syl9 max	Syl9 min	Syl9 mean	Ppi2 max	Ppi2 min	Ppi2 mean
PC_Onset_without2	.473	.344	.818	.061	.080	.760	.613	.792	.724	**.040**	.318	**.031**	.559	.308	.516
PC_corrAMA_1	.877	.626	.935	.023	.280	.056	.513	.283	.412	.720	.453	.639	.800	.861	.774
PC_corrAMA_2	.345	.742	.439	.194	.717	.920	.809	.613	.772	.452	.761	.494	.688	.458	.732

	Syl2 max	Syl2 min	Syl2 mean	Syl5 max	Syl5 min	Syl5 mean
PC_Onset_without2	**.043**	**.029**	**.014**	.261	.488	.325
PC_corrAMA_1	.580	.513	.404	.506	.926	.715
PC_corrAMA_2	.288	.388	.895	.257	.116	.171

Type of behaviour

From the data on which type of behaviour was the primary and secondary behaviour of a bird and for how long, we could calculate a "weighted behaviour type value" for each of the three behaviours (Hopping, wing whirring, flying). One-way anovas were performed to check for inter-populational behavioural differences. None of the three were significant (Hopping p= 0.098. Wing whirring p= 0.600. Flying p= 0.124). A principle component derived from these three variables still did not show differences between populations (One way anova, n=16 df; 2 F=2.216 p=0.149). Anovas on just the primary behaviour data, show significance for hopping; p= 0.022. (Wing whirring p= 0.775. Flying p= 0.280). This significance seems to depend completely on the difference between birds of the migratory and partially migratory populations.

Discussion

Summary of results and comparison to Chapter 1 & Mueller et al. 2011 (the" blackcap-collection")

We showed here that at individual level there is no association between the migration data and the investigated microsatellite loci for the birds kept in captivity. This result was consistent under both conditions, i.e. in birds kept in indoor cages and individuals kept in outdoor aviaries. Although, our analysis found several significant correlations between the genetic markers and migratory activity, a consistent pattern was lacking. Moreover, most significant results were found in neutral markers, suggesting that they may have been fortuitous. This does not corroborate the results of Mueller *et al.* (2011) who found an association between migratory restlessness in captive birds and the candidate locus ADCYAP1 at an individual level. Neither do our results concur with the results found in chapter 1 and Mueller *et al.* (2011) across wild Iberian populations, plus an association with another candidate gene (CLOCK) and a neutral gene (Syl9). One possible explanation for these results is that the differences found in Chapter 1 were fortuitous and are not linked to migration at all, but rather to another selection factor that we did not study or maybe due to phylogeography. But we consider this an unlikely explanation since results concur with other results obtained in blackcaps on a wider scale. We consider a more likely explanation for the discrepancy between the results of the studies the fact that the Iberian population is near the threshold of blackcap migration. Since birds from these populations, are on the verge of switching from being migratory to becoming sedentary their behaviour is extremely flexible and their decision to migrate is currently probably influenced more by the environment than is the case elsewhere in the blackcaps' European distribution. The fact that blackcaps from the three populations kept under identical environmental conditions, the conditions of the migratory population of Madrid, showed similar migration activity supports this idea (Bulaic, 2015).

How does this result compare to results in other species?

In the passerine genus Junco, which shows considerable geographical variation in migratoriness, ADCYAP1 showed a positive correlation with migratory behaviour only in captive birds, not in their wild populations (Peterson *et al.* 2013). This is the opposite of what we found in Iberian blackcaps, where the association between migratory behaviour and genetic markers was only significant in data from wild populations. However, as chapter 1 proposed, the perceived influence that genetic markers have on migratory behaviour seems to depend greatly on the species and experimental design of the study. This makes it very difficult to generalise and/or compare the findings of any study on the genetics of

migratory behaviour.

Results of the analysis of Behaviour types / SERT

Our analysis of the type of behaviour displayed in the outdoor aviaries showed a correlation between the type of behaviour and the candidate marker SERT. This interaction was found in all models, with mean allele lengths and with major allele scores. This consistency suggests a possible genuine association. In previous studies SERT, a candidate for harm avoidance behaviour, was shown to be highly significant with habitat type (Mueller *et al.* 2013). It appears that SERT might be more influential on behavioural matters, not so much on the decision making and regulatory process of whether to migrate or not.

Shortcomings of this study – What should be done in the future?

At first sight it might seem strange that birds from a presumed sedentary population showed migratory restlessness in captivity. However this is not an uncommon finding. For example, even individuals of an equatorial, non-migratory, songbird species exhibited migratory restlessness in captivity (Helm & Gwinner 2006). Sedentary blackcaps are also known to exhibit migratory restlessness. Birds from the Canary Islands for instance, showed migratory restlessness in captivity despite being from a population that supposedly is completely resident in the wild (Berthold & Querner, 1981). The fact that a sedentary population shows migratory restlessness in captivity is a major drawback for such activity studies and makes it much harder to relate the findings to the situation in the wild.

Changing the captive conditions to match a sedentary habitat would be the ultimate test to see whether the environment is more important than genetics in Iberian populations. We propose keeping birds of the same set of populations as our current study, but at the latitude/in the region where sedentary populations occur. One would expect all birds not to show migratory restlessness.

Whether or not the blackcap is a species where Zugunruhe is a good measure for actual migratory behaviour in the wild might depend on the migration distance of the individual and/or population. Long-distance migrants like e.g. willow warblers (Phylloscopus trochilus) show less variable and more distinct cycles of Zugunruhe than related species, like chiffchaffs (*Phylloscopus collybita*), that travel shorter distances (Gwinner 1972)

In this study we only used one population for each of the possible migratory strategies. This is obviously limited and thus more prone to possible “unfortunate sampling effects”. Perhaps the differences between the populations were completely unrelated to migratory behaviour. Testing a more extensive set of populations could clarify this doubt, however we were unable to execute this for now, due to logistic reasons.

Conclusion

Based on the results from this study we would conclude, contrary to chapter 1, that the migratory strategy of an individual blackcap cannot be determined based on alleles found at certain microsatellite loci, at least not based on the set of markers we used. Whether or not this discrepancy can be accredited to the sole difference of using wild populations opposed to keeping birds under laboratory conditions remains to be seen. The apparent difference between the results of our previous study and our current study could be due to several reasons. Perhaps the fact that Spain is near the threshold of migration in this species makes the animals more sensitive to environmental changes, whereby standardised

laboratory conditions induced similar behaviour in the birds of this study. It appears that sedentary Iberian populations have both, genes and environmental conditions, favouring non-migratory behaviour. At the same time migratory populations have genes and live under conditions fit for active migratory behaviour. This indicated that migration in blackcaps on the Iberian peninsula might be subject to co-gradient variation. More research into the details of how genes and environment together result in behaviour and the possible condition dependence of the expression of this behaviour is needed.

Supplementary material

Supplementary table S1. The molecular markers used in this study.

Gene/Locus	Locus type	No. of observed alleles	Reference
CLOCK	Trinucleotide microsatellite	8	Steinmeyer et al. 2009
ADCYAP1	Dinucleotide microsatellite	13	Steinmeyer et al. 2009
NPAS2	Trinucleotide microsatellite	2	Steinmeyer et al. 2009
SERT_Ex1	Trinucleotide microsatellite	3	C. Hermannstaedter, pers. comm.
Syl1	Tetranucleotide microsatellite	14	Segelbacher et al. 2008
Syl2	Tetranucleotide microsatellite	15	Segelbacher et al. 2008
Syl4	Tetranucleotide microsatellite	20	Segelbacher et al. 2008
Syl5	Dinucleotide microsatellite	24	Segelbacher et al. 2008
Syl6	Tetranucleotide microsatellite	36	Segelbacher et al. 2008
Syl9	Tetranucleotide microsatellite	18	Segelbacher et al. 2008
Ppi2	Dinucleotide microsatellite	21	Martinez et al. 1999; A. Ramirez, pers. comm
Pca8	Dinucleotide microsatellite	4	Dawson et al. 2000; A. Ramirez, pers. comm

Supplementary table S2. Allele frequencies per locus and population.

Candidate Markers				
Locus	**Allele/n**	**COC**	**MAD**	**TAR**
ADC	**Genes**	90	74	44
	155	0.011	0.041	0.000
	159	0.033	0.027	0.000
	161	0.033	0.000	0.023
	163	0.389	0.405	0.545
	165	0.111	0.081	0.045
	167	0.300	0.338	0.341
	169	0.089	0.108	0.045
	171	0.033	0.000	0.000
ClkpolyQ	**Genes**	90	74	44
	256	0.011	0.000	0.000
	258	0.011	0.000	0.000
	260	0.000	0.014	0.068
	263	0.133	0.176	0.205
	266	0.033	0.000	0.045
	269	0.767	0.743	0.636
	272	0.000	0.027	0.023
	275	0.044	0.027	0.023
	280	0.000	0.014	0.000
NPAS2	**Genes**	90	74	44
	163	0.044	0.014	0.045
	169	0.956	0.986	0.955
SERT_Ex1	**Genes**	90	74	44
	314	0.000	0.014	0.023
	320	1.000	0.973	0.955
	323	0.000	0.014	0.000
	326	0.000	0.000	0.023
Neutral Markers				
PC8	**Genes**	90	74	44
	171	0.678	0.662	0.568
	175	0.089	0.135	0.000
	179	0.233	0.203	0.432
Ppi2	**Genes**	90	74	44
	251	0.000	0.014	0.000
	259	0.000	0.041	0.000
	261	0.078	0.216	0.159
	263	0.256	0.135	0.114
	266	0.044	0.000	0.000
	268	0.378	0.378	0.386
	270	0.022	0.000	0.023
	273	0.011	0.014	0.000
	279	0.000	0.014	0.000
	281	0.011	0.027	0.000
	287	0.167	0.149	0.273
	289	0.011	0.014	0.023
	291	0.000	0.000	0.023
	293	0.022	0.000	0.000

Table S2 (continued)

Locus	Allele/n	COC	MAD	TAR
Syl1	**Genes**	90	74	44
	122	0.033	0.027	0.114
	126	0.000	0.027	0.000
	130	0.033	0.000	0.045
	134	0.022	0.014	0.068
	138	0.067	0.108	0.045
	143	0.067	0.149	0.182
	147	0.322	0.243	0.091
	152	0.244	0.108	0.295
	156	0.111	0.284	0.045
	160	0.011	0.027	0.045
	164	0.033	0.000	0.045
	168	0.011	0.014	0.023
	172	0.022	0.000	0.000
	176	0.011	0.000	0.000
	179	0.011	0.000	0.000
Syl2	**Genes**	90	74	44
	129	0.022	0.000	0.000
	133	0.022	0.014	0.091
	138	0.033	0.108	0.045
	140	0.000	0.000	0.023
	143	0.067	0.135	0.205
	147	0.211	0.257	0.273
	151	0.189	0.122	0.114
	155	0.189	0.108	0.068
	156	0.022	0.014	0.023
	159	0.100	0.122	0.000
	160	0.000	0.041	0.000
	163	0.056	0.068	0.114
	167	0.067	0.000	0.023
	171	0.022	0.014	0.023
Syl4	**Genes**	90	74	44
	161	0.011	0.000	0.000
	165	0.067	0.000	0.023
	169	0.022	0.027	0.000
	173	0.011	0.027	0.000
	178	0.089	0.122	0.227
	182	0.189	0.297	0.432
	186	0.211	0.108	0.136
	190	0.089	0.243	0.114
	194	0.133	0.068	0.045
	197	0.056	0.000	0.023
	202	0.033	0.054	0.000
	206	0.033	0.054	0.000
	210	0.022	0.000	0.000
	218	0.033	0.000	0.000

Table S2 (continued)

Locus	Allele/n	COC	MAD	TAR
Syl5	**Genes**	90	74	44
	143	0.011	0.000	0.000
	151	0.011	0.000	0.000
	156	0.045	0.027	0.068
	160	0.045	0.230	0.114
	162	0.000	0.014	0.023
	164	0.068	0.095	0.023
	168	0.182	0.014	0.000
	170	0.011	0.014	0.000
	172	0.034	0.068	0.091
	174	0.000	0.027	0.023
	176	0.193	0.081	0.136
	178	0.023	0.000	0.000
	180	0.045	0.054	0.114
	182	0.023	0.041	0.114
	184	0.057	0.122	0.114
	186	0.000	0.000	0.045
	188	0.068	0.122	0.023
	190	0.068	0.041	0.091
	192	0.045	0.000	0.000
	194	0.023	0.027	0.000
	196	0.011	0.000	0.023
	198	0.011	0.027	0.000
	204	0.023	0.000	0.000
Syl6	**Genes**	90	74	44
	160	0.000	0.027	0.000
	172	0.011	0.000	0.068
	176	0.022	0.000	0.045
	180	0.022	0.027	0.045
	184	0.089	0.108	0.023
	185	0.044	0.014	0.045
	188	0.156	0.095	0.136
	189	0.078	0.014	0.023
	192	0.100	0.189	0.205
	193	0.067	0.014	0.000
	196	0.056	0.108	0.091
	197	0.056	0.000	0.000
	200	0.044	0.041	0.091
	201	0.011	0.000	0.000
	204	0.122	0.108	0.045
	205	0.000	0.014	0.000
	208	0.022	0.068	0.023
	212	0.033	0.054	0.068
	216	0.011	0.014	0.000
	220	0.011	0.014	0.068
	224	0.000	0.014	0.000
	228	0.011	0.014	0.000
	232	0.033	0.000	0.000
	236	0.000	0.014	0.023
	240	0.000	0.027	0.000
	248	0.000	0.014	0.000
	252	0.000	0.014	0.000

Table S2 (continued)

Locus	Allele/n	COC	MAD	TAR
Syl9	**Genes**	90	74	44
	139	0.033	0.000	0.000
	147	0.444	0.662	0.795
	152	0.089	0.081	0.068
	157	0.067	0.027	0.023
	161	0.022	0.000	0.000
	165	0.033	0.000	0.000
	172	0.000	0.041	0.023
	176	0.033	0.000	0.000
	181	0.022	0.041	0.000
	184	0.133	0.027	0.000
	187	0.056	0.081	0.091
	192	0.056	0.027	0.000
	196	0.011	0.014	0.000

S*upplementary table S3. Overview of the test for Hardy-Weinberg Equilibrium. Levels of significance: ns = P>0.05; * = P<0.05; ** = P<0.01; *** = P<0.001. The Cocentaina population (COC) was monomorphic (mm) at the SERT locus.*

pop/locus	**CLOCK**	**ADCYAP1**	**NPAS2**	**SERT**	**Syl1**	**Syl2**	**Syl4**	**Syl5**	**Syl6**	**Syl9**	**Ppi2**	**Pca8**
COC	ns	ns	ns	mm	ns	ns	***	ns	ns	ns	ns	ns
TAR	ns	ns	ns	ns	ns	*	**	ns	ns	ns	ns	ns
MAD	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns

Supplementary table S4. Deviations from linkage equilibrium. Numbers give error probabilities as determined by Fisher's method Genepop V4 (Raymond and Rousset 1995).

All birds	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.1838	0.6659	0.6883	0.2193	0.0523	0.5687	0.9156	0.9008	0.9805	0.3686
CLOCK	x	x	0.3497	0.655	0.1445	0.297	0.5817	0.4382	0.6037	0.9962	0.6557
NPAS	x	x	x	1	0.337	0.9511	0.9951	0.9975	0.7741	0.9068	0.4286
SERT	x	x	x	x	0.4253	0.523	0.5937	0.4374	0.7149	0.9935	0.7741
PC8	x	x	x	x	x	0.0256	0.1227	0.7192	0.7804	0.8516	0.9264
Ppi2	x	x	x	x	x	x	0.429	0.2597	0.5463	0.98	0.4269
Syl1	x	x	x	x	x	x	x	0.9962	1	0.0466	0.2687
Syl2	x	x	x	x	x	x	x	x	1	0.9189	0.4077
Syl5	x	x	x	x	x	x	x	x	x	1	0.9953
Syl6	x	x	x	x	x	x	x	x	x	x	0.5619
Syl9	x	x	x	x	x	x	x	x	x	x	x

Per population

COC	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.2812	0.2896	x	0.2408	0.2765	0.3767	0.9876	1	0.736	0.7975
CLOCK	x	x	0.1587	x	0.0462	0.8794	0.4538	0.5139	0.2384	0.9517	0.6655
NPAS	x	x	x	x	0.4003	0.783	0.9771	0.9611	0.3264	0.5168	0.6745
SERT	x	x	x	x	x	x	x	x	x	x	x
PC8	x	x	x	x	x	0.0055	0.0225	0.389	0.3826	0.3034	0.663
Ppi2	x	x	x	x	x	x	0.8652	0.0599	0.3383	0.5671	0.5802
Syl1	x	x	x	x	x	x	x	1	1	0.0017	0.792
Syl2	x	x	x	x	x	x	x	x	1	1	1
Syl5	x	x	x	x	x	x	x	x	x	1	1
Syl6	x	x	x	x	x	x	x	x	x	x	0.0936
Syl9	x	x	x	x	x	x	x	x	x	x	x

MAD	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.054	0.7314	0.5364	0.9271	0.022	0.291	0.5392	0.7015	1	0.385
CLOCK	x	x	1	0.6728	0.3535	0.0649	0.8431	0.6877	0.738	0.7344	0.2552
NPAS	x	x	x	1	0.0826	0.8129	1	1	0.6242	0.6786	1
SERT	x	x	x	x	1	0.4048	0.5666	0.6899	0.3803	0.8915	0.6252
PC8	x	x	x	x	x	0.1485	0.5226	0.4069	0.5671	0.899	0.6023
Ppi2	x	x	x	x	x	x	0.1945	0.5393	1	1	0.5618
Syl1	x	x	x	x	x	x	x	0.7443	1	1	0.1893
Syl2	x	x	x	x	x	x	x	x	1	0.22	0.12
Syl5	x	x	x	x	x	x	x	x	x	1	0.9394
Syl6	x	x	x	x	x	x	x	x	x	x	1
Syl9	x	x	x	x	x	x	x	x	x	x	x

TAR	ADCYAP1	CLOCK	NPAS	SERT	PC8	Ppi2	Syl1	Syl2	Syl5	Syl6	Syl9
ADCYAP1	x	0.9536	0.6105	0.6141	0.071	0.3062	0.6725	0.6036	0.4807	0.7904	0.1309
CLOCK	x	x	0.2209	0.427	0.5138	0.5185	0.2549	0.1589	0.5579	1	0.7338
NPAS	x	x	x	1	1	0.6991	0.7333	0.8033	0.9508	0.9758	0.0751
SERT	x	x	x	x	0.1461	0.4577	0.4663	0.2277	0.9476	0.971	0.6643
PC8	x	x	x	x	x	0.8885	0.5593	0.9968	1	1	0.9533
Ppi2	x	x	x	x	x	x	0.2991	0.5415	0.2809	1	0.1801
Syl1	x	x	x	x	x	x	x	1	1	1	0.1894
Syl2	x	x	x	x	x	x	x	x	1	1	0.3207
Syl5	x	x	x	x	x	x	x	x	x	1	0.7817
Syl6	x	x	x	x	x	x	x	x	x	x	0.9453
Syl9	x	x	x	x	x	x	x	x	x	x	x

Chapter 3

Association between molecular variation and migration strategies in a partially migratory bird population in the wild

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(unpublished manuscript)

Abstract

Migratory behaviour of wild Iberian blackcap (Sylvia atricapilla) populations has been shown to be associated with genetic variation at several candidate genes. A study on captive birds showed no such association between the markers and individual Zugunruhe. In this study we investigate this association in a wild population in more detail in an attempt to clarify this presumed inconsistency and to see whether we can genetically distinguish the resident birds from the migratory birds. We investigated a set of 13 previously used microsatellite loci, 4 of which are candidate gene loci, in birds from a partially migratory population in eastern Spain, for which migratory behaviour in the wild had been previously determined. Contrary to the study at the population level, we found no association between the candidate genetic markers and migratory behaviour among individuals of this partially migratory population, only an indication of a possible association for the CLOCK gene. This implies that with the markers used we cannot predict the migration status of an individual bird by determining their genotype. A neutral marker, Syl9, showed some discriminative power in this study, though in the opposite direction of previous research, which suggests it is not associated with migratory behaviour, but a sampling artefact. Our results suggest that migratory behaviour on the Iberian peninsula is highly flexible and, due to being close to the threshold of migration it is likely to be more strongly influenced by environmental variation than elsewhere in the blackcaps' distribution range where genetic predisposition has shown to be more important.

Introduction

Migratory behaviour in the blackcap, *Sylvia atricapilla* is well studied. Over the years, this species has increasingly gained importance as a model for studying the control and genetics of avian migratory behaviour. Blackcaps have, for example, shown that migration direction has a genetic component to it (Berthold & Helbig 1992) and that there might be a threshold for migration indicating at a mechanism combining genetics and environmental factors (Pulido 2011). In the last decade, studies on migration in general, not just blackcaps, have increasingly aimed at understanding the molecular genetics underlying differences in behaviour (e.g. reviewed in Liedvogel *et al.* 2011). More recently it was shown that across the Iberian peninsula migratory behaviour of wild populations is associated with several candidate loci (ADCYAP1, CLOCK) and one neutral marker (Syl9) (Chapter 1). This association could not be found when studying migratory restlessness of Iberian blackcaps from different populations held in captivity (Chapter 2). It has been suggested that, even though the populations from which these birds originated show different migration strategies in the wild, they were exhibiting similar activity in captivity due to strong environmental sensitivity, following the environmental threshold model of migration (Pulido 2011). The Iberian Peninsula appears to be the location where populations are on the verge of switching from migratory to sedentary (hence the amount of partial migrants in Iberia), this can cause the birds in this area to be very sensitive to environmental conditions. Thus, keeping birds from one region under the environmental conditions of another region will have an effect on their activity. The association between behaviour and variation at candidate genes found in wild Iberian populations was found earlier in captive European populations in a study by Mueller *et al.* (2011). These were, however populations of blackcaps far from the migration threshold, thus supposedly not prone to have an environmental-dependent expression of migratory behaviour as found in the Iberian populations.

In a previous study (Chapter 1) a migration strategy was assigned to each population, yet variation among individual migratory activity within populations was ignored. In the study of captive blackcaps (Chapter 2), data on individual activity was obtained, yet all birds were kept under the same environmental conditions, making it impossible to study environmental effects on migration and their interaction with genetic effects. In this study, we tested for the association between migratory behaviour and the previously identified genetic markers at the individual level and under natural conditions, the conditions supposedly inducing the among population differences in the expression of migratory behaviour. In this study, we excluded confounding factors from previous studies like the geographic variation and lack of individual activity data (chapter 1) and the use of an a single artificial environment, where the activity displayed by birds does not correlate with actual migratory behaviour in the wild (chapter 2). We predicted that if we found an association between migratory behaviour and genetic markers using this approach, then Zugunruhe is probably a poor measure of migratory behaviour.

Material & Methods

We studied a partially migratory population (Morganti *et al*. 2015) in the east of Spain, along the Serpis river valley in the municipality of Cocentaina (38^{o} 44’ N – 0^{o} 44’ W, Alicante, Spain). The area extends for

about 2,5km along the riverbank covering 77 hectares. Over the course of three years (2010-2013) this population has been subjected to an intensive capture-ringing-recapture effort. We combined presence-absence data with morphometric measurements of the wing to determine the migration status of each individual bird (see Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015, 2017) 70 birds were classified as being either; (a) locally breeding migrants (N=15), (b) locally breeding and all year residents (N=28), (c), non-locally breeding migrants, (wintering in the study area but breeding elsewhere). (N=27), for details , see Morganti *et al.* (2015). (For more details on the breakdown of the samples see table 1).

Table 1. The number and groups of birds used in this study.

	Resident	Migrant	Wintering	Total
Male	20	10	16	46
Female	8	5	11	24
Total	28	15	27	70

DNA sampling

Blood (ca 50 µl) was taken from all birds, captive and wild, by either puncturing their brachial vein, or by extracting it from the jugular vein with a syringe. Blood was stored in alcohol in a freezer (at -20ºC) until labwork commenced. DNA extractions were made using a *Blood and Tissue Kit* (Macherry & Nagel).

Genotyping

Following Mueller *et al.* 2011 and chapter 1 we investigated four candidate genes, three of which have been related to circadian behaviour (CLOCK, ADCYAP1 and NPAS2) and one related to harm avoidance behaviour (SERT). For each an, exonic, microsatellite was used (Mueller *et al.* 2011 & chapter 1). For comparative association analyses we used 8 anonymous, neutral microsatellite loci (Syl1, Syl2, Syl4, Syl5, Syl6, Syl9, Ppi2 and Pca8) and the sexing marker P2P8 from Griffiths *et al.* (1998). This made a set of 13 microsatellite markers. (Selection of the markers was partially based on Segelbacher *et al.* 2008 and Steinmeyer *et al.* 2009) (for more information see Supplementary table S1)
All samples (N=174) were genotyped for these 13 microsatellites using an ABI 3100 sequencer (Applied Biosystems) for the microsatellite fragment analyses. The result files were analysed in Genemapper.

Data Analyses

Most analyses were conducted using SPSS (IBM Corp. version 20). Tests for Hardy Weinberg Equilibrium were performed using GenAlEx 6.502 (Peakall *et al.* 2012) (Supplementary table S2 Shows summaries of HWE tests). Linkage disequilibrium was tested in previous chapters. Linkage between loci was not found. Since we are using a subset her of the previous dataset we do not need to rerun the test.
In order to test for differences in minimum, maximum and mean allele length of all markers between the three groups of birds binary logistic regressions were performed in SPSS on various combinations of the groups of birds. (We compared all possible combinations of two of the three groups. And we compared all possible combinations of two groups merged together with the third group. This gave five binary comparisons). To simplify models by eliminating non-significant effects both the standard “enter” and “forward” methods were used. When all three groups were used in the analysis at the same time, we used a multinomial logistic regression (the dependent variable was the migration status. The min, max,

mean allele lengths were entered in the models as covariates).

Results

Three out of ten regressions were significant, all forward models. The only comparisons not resulting in any significant model, with either of the two methods, were between the resident and the migratory group, and between the resident and migrants + wintering birds together. (For detailed results see table 2). Syl9 is a reoccurring significant marker in the three significant models. In the comparison of residents with migrants CLOCK is also present in one final model, though not significant. According to the results 69.8% of the birds (82.1% of the residents, and 46.7% of the migrants) would be assigned to the correct group when using this model. We plotted Syl9 mean against CLOCK min in figure 1. Since Syl9 was the only marker that was significant we include allele frequency distributions of the three groups in figure 2. Migrants, and residents to a lesser extent, have a second peak around the alleles of 181-184-187, this peak is absent for the wintering birds from northern Europe causing the wintering group to have shorter Syl9 alleles.

Figure 1. Mean allele length of Syl9 plotted against minimum allele length of CLOCK. Resident individuals (= group 1) are marked in blue. Migratory individuals (= group 2) are marked in green.

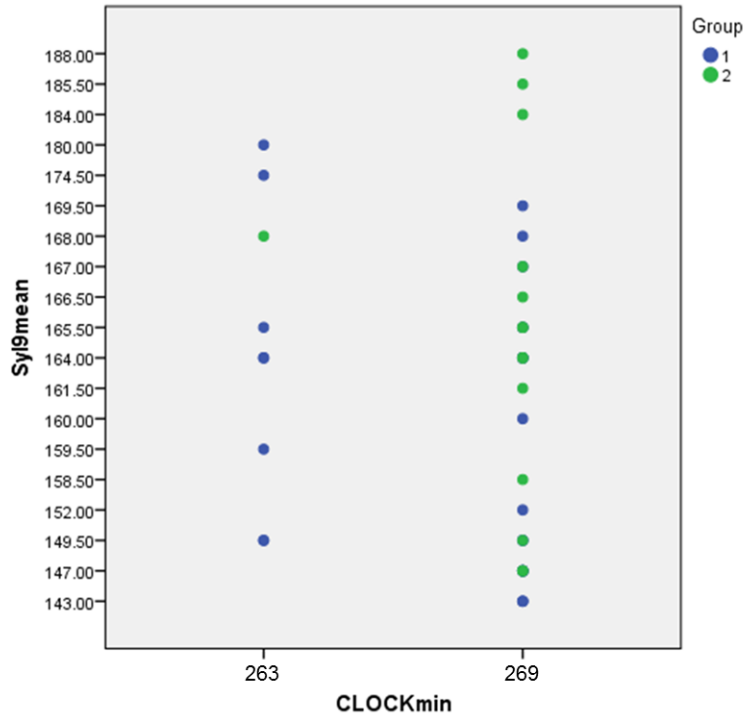


Table 2. Summary of the results of the logistic regression analyses for predicting migrations status using allele lengths at microsatellite loci as predictors. The table gives the best fit of the full model including all variables (Enter) compared to the best model obtained by adding significant variables (forward approach). For each result the variables retained in the model and their significance, as well as the significance of the model are given.

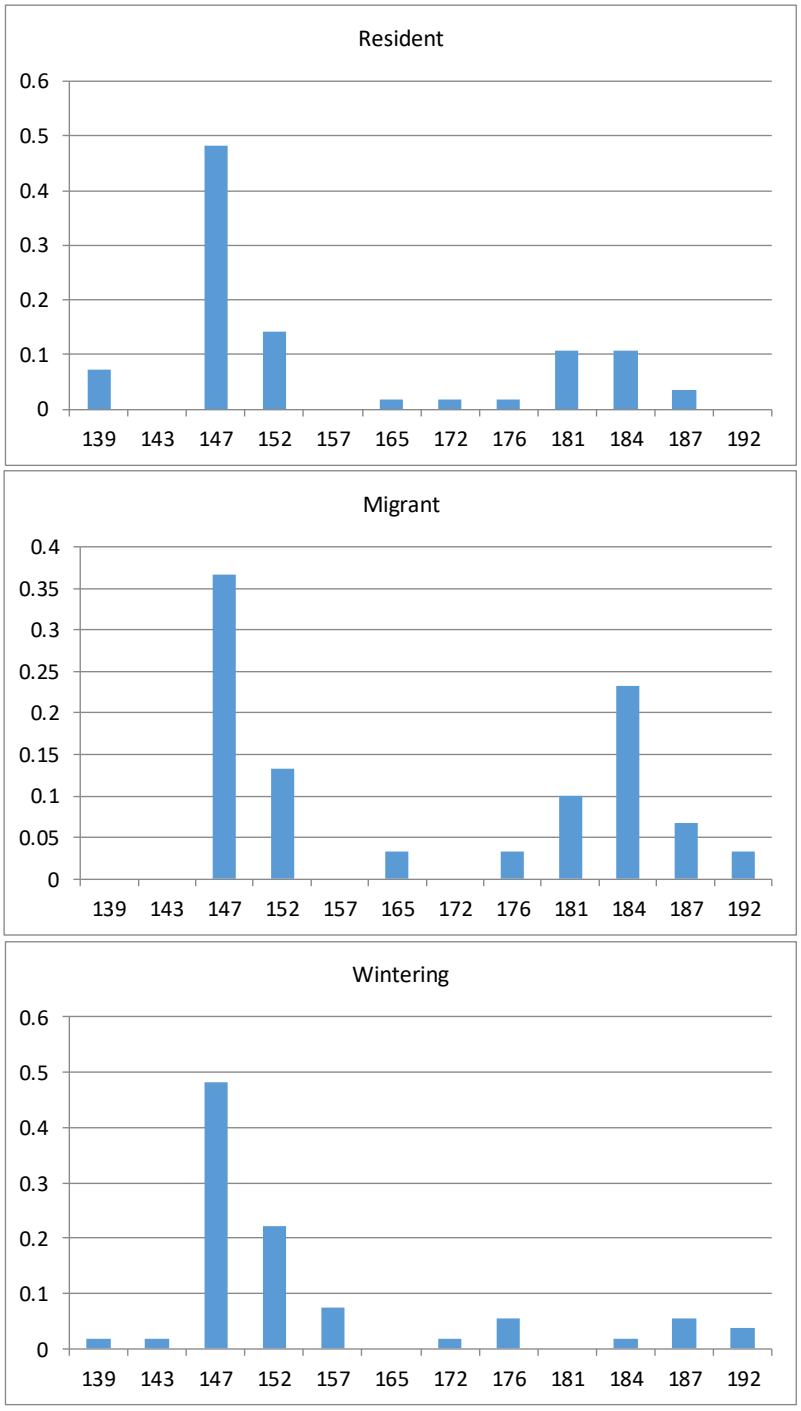
Binary logistic regressions

Comparing	Method	Chi^2	df	sig	-2 log lik	Cox & Snell R	Nagelk erke R	Final step #	Markers left	B	S.E.	Wald	df	sig	Exp(B)	Model log likelihood	Change in -2 log	df	sig of the change
Resident-Migratory	Enter	55.618	21	0	0	0.726	1.000	1	None										
	Forward	9.622	2	**0.008**	45.996	0.201	0.276	2	CLOCK Min	-0.373	0.199	3.528	1	0.06	0.689	-25.496	4.997	1	**0.025**
									Syl9 Mean	-0.078	0.035	5.079	1	**0.024**	0.925	-26.174	6.353	1	**0.012**
Resident-Wintering	Enter	15.607	22	0.835	60.621	0.247	0.329	1	None										
	Forward							0	None	-0.036	0.27	0.018	1	0.893	0.964				
Migratory-Wintering	Enter	54.748	22	0	0	0.728	1.000	1	None										
	Forward	7.152	1	**0.007**	47.595	0.157	0.215	1	Syl9 Mean	-0.078	0.032	5.84	1	**0.016**	0.925	-27.374	7.152	1	**0.007**
(Resident+Migratory)-Wintering	Enter	22.685	22	0.42	70.666	0.277	0.376	1	*Many, but none significant.*										
	Forward	3.925	1	**0.048**	89.426	0.055	0.74	1	Syl9 Max	-0.029	0.015	3.764	1	0.052	0.971	-46.675	3.925	1	**0.048**
Resident-(Migratory+Wintering)	Enter	17.522	22	0.734	76.7	0.221	0.299	1	None										
	Forward							0	None	0.405	0.244	2.762	1	0.097	1.5				

Multinominal logistic regression to compare the 3 groups at once

Comparing	Chi^2	df	Sig	-2 log likelihoo	Final step	Marker sign
Resident-Migratory-Wintering	58.965	44	0.065	90.004	2	None

Figure 2. Allele frequency distributions at Syl9 in 3 groups of blackcaps differing in migratory behaviour or origin.



Discussion

The genetic markers that were found to be associated with migratory strategy of wild Iberian blackcap populations (chapter 1) do not seem to be associated with individual migratory behaviour in blackcaps of a partially migratory population.

Main result + Syl9

The fact that the comparison of resident birds with migratory birds was never significant while others, even migratory birds compared to wintering birds, were is surprising. It's the opposite of what we expected. Resident birds were expected to show the biggest difference to the wintering birds.
Syl9 shows the clearest differences between the groups. Wintering birds have shorter alleles. This is in contrast with the results of Chapter 1, where sedentary birds had the shortest alleles and birds that migrate had longer alleles. This inconsistency indicates that the association found between migratory behaviour and Syl9 is most likely by chance.

CLOCK

The near significant results we found in CLOCK do concur with chapter 1 where migrating populations had longer alleles. In the present study, resident birds had the shortest average mean allele length as was found in the among population comparison (see, chapter 1). Migrant birds had the longest alleles; wintering birds slightly shorter than the migrants, but still longer than residents (for a more detailed view of the allele distributions per group, see Supplementary table S3A). In this study we did not have data on distance or timing of migration, which have been proposed to be the main migratory characters under the influence of CLOCK (eg. Peterson *et al.* 2013 and Saino *et al.* 2015).This means that CLOCK could potentially influence migratory behaviour in multiple ways. However the importance of CLOCK in shaping migratory behaviour is debateable since it has also been shown to exhibit a latitudinal cline, similar to the cline in our study, in a near resident species (Johnsen *et al.* 2007).

ADCYAP1 and comparison with chapter 1

In the distribution of ADCYAP1 we see that the longest alleles are lacking in the wintering birds coming from the north, whereas the migrants lack the shortest alleles (see Supplementary table S3B). This does not seem very informative though, since this also occurred in several populations in chapter 1 without it being associated to a certain migration strategy. It is rather surprising that these birds did not differ at the ADCYAP1 locus, which was significantly associated with migratory behaviour and a latitudinal cline was observed for those in Iberian blackcaps. This may indicate that migrants and residents that winter at our study site do not differ strongly in migratoriness, they may breed in areas close to this wintering area (see also conclusions in Morganti *et al.* 2015).

Comparison with other studies

Our results appear to confirm what van we suggested in chapter 2. Migratory behaviour in Iberian blackcap populations appears to be an extremely flexible trait that depends more on environmental cues than on the genetic predisposition. Genetic differences in this population, even at genes that have previously been found to be involved in the expression of migration, seem not to be the main

determinant of whether a bird migrates or not. This is likely to be because Iberian blackcaps are close to the threshold of migration, which is expected to be associated with strong environmental effects on migration. This would explain why in this study we did not find genetic differences between the three groups.
However, migrants and residents in this population do not differ neither in morphology (Morganti *et al.* 2015), which concurs with results in a blackbird (*Turdus merula*) study by Fudickar & Partecke (2012), nor in habitat size or composition (Morganti *et al.* 2017), suggesting that other environmental factors which have not been studied (e.g. dominance, experience) may determine differences in migratory behaviour in these populations. A study on blackbirds found that whether or not to migrate was sex-biased, with females being more prone to do so (Fudickar *et al.* 2013). In the blackcap population that we studied we found no such pattern.

Shortcomings of this study – What should be done in the future?
We do not know the origin of the birds wintering in our study area, but it is possible that they did not come from very far. If they are southern French birds, or even southern German birds, then they are not expected to show large differences from the Iberian birds at the loci, which might cause such genetic differences to go undetected (see, Mueller *et al.* 2011).
Likewise, the locally breeding migrants might not migrate far (as proposed by Morganti *et al.* 2015). If birds migrated downhill or to the next village they would be classified as full migrants in our study even though they would migrate very short distances. Such short distance migration could be initiated by different causes then long distance migration (e.g. food supplies, dominance/hierarchy). According to the threshold model of migration, such short-distance migrants with a high proportion of residents (> 65%; see Morganti *et al.* 2015) are expected to be phenotypically and genetically very similar to residents (see, Pulido *et al.* 1996, Pulido 2011).
Distinguishing between residents and migrants proved to be quite difficult in our population (69.8% correct assignment, with more than half of the migrants being wrongly labelled as residents). Our population has a high proportion of residency (65%) and is supposedly close to the threshold. According to the threshold model the migrating birds are expected to only migrate short distances. This does seem to concur with our findings.

General conclusion about migration in Iberian blackcaps
Previously candidate genes showed an association with migratory behaviour of wild populations on the Iberian Peninsula (Chapter 1), though birds hailing from several of these populations exhibit similar levels of Zugunruhe in captivity (Chapter 2). This could be due to the fact that they were all kept under the same environmental conditions and since the Iberian blackcaps are near the threshold of migration environmental influences could be more important in shaping the behaviour in these birds then their genetic predisposition. On the other hand it is possible that a different set of genes is responsible for migratory behaviour opposed to for migratoriness/Zugunruhe.
This current study indicates that there are only slight genetic differences between birds of different migration strategy, not nearly as strong and clear as the associations found in Chapter 1. This suggests support to the idea that a population near the threshold of migration is more susceptible to environmental influences than genetic differences, which are likely to be small in partially migratory

populations with a high proportion of residents, causing them to go undetected. However it is also possible that due to Spain having been the refugium for blackcaps in the ice age (Perez-Tris *et al.* 2004), that Spanish blackcaps have a much larger genetic variation making it possible that the set of genes controlling migratory behaviour is different from the set of genes in blackcaps elsewhere in Europe. This might include genes we did not test for. In the case that migration in this population is controlled by different genes it would explain the discrepancy with the results from previous studies.

This study shows the difficulties of detecting differences between residents and migrants in a partially migratory population. To come to more clear conclusions and predictions on the control of migration we propose doing a similar study in a partially migratory population with a high proportion of migrants.

Supplementary material

Supplementary table S1. The molecular markers used in this study

Gene/Locus	Locus type	No. of observed alleles	Reference
CLOCK	Trinucleotide microsatellite	8	Steinmeyer et al. 2009
ADCYAP1	Dinucleotide microsatellite	13	Steinmeyer et al. 2009
NPAS2	Trinucleotide microsatellite	2	Steinmeyer et al. 2009
SERT_Ex1	Trinucleotide microsatellite	3	C. Hermannstaedter, pers. comm.
Syl1	Tetranucleotide microsatellite	14	Segelbacher et al. 2008
Syl2	Tetranucleotide microsatellite	15	Segelbacher et al. 2008
Syl4	Tetranucleotide microsatellite	20	Segelbacher et al. 2008
Syl5	Dinucleotide microsatellite	24	Segelbacher et al. 2008
Syl6	Tetranucleotide microsatellite	36	Segelbacher et al. 2008
Syl9	Tetranucleotide microsatellite	18	Segelbacher et al. 2008
Ppi2	Dinucleotide microsatellite	21	Martinez et al. 1999; A. Ramirez, pers. comm
Pca8	Dinucleotide microsatellite	4	Dawson et al. 2000; A. Ramirez, pers. comm

*Supplementary table S2. Overview of the test for Hardy-Weinberg Equilibrium at different loci and populations. When loci were monomorphic (mm) the test could not be conducted. Levels of significance: ns = P>0.05; * = P<0.05; ** = P<0.01; *** = P<0.001.*

pop/locus	**CLOCK**	**ADCYAP1**	**NPAS2**	**SERT**	**Syl1**	**Syl2**	**Syl4**	**Syl5**	**Syl6**	**Syl9**	**Ppi2**	**Pca8**
MIGRANT	ns	ns	ns	mm	ns	ns	ns	ns	ns	ns	ns	**
RESIDENT	ns	ns	ns	mm	ns	ns	ns	ns	ns	ns	***	**
WINTERING	ns	ns	mm	ns	ns	ns	**	ns	ns	ns	ns	ns

ns = not significant
mm = monomorphic

Supplementary table S3A. Allele frequency distributions at CLOCK in 3 groups of blackcaps differing in migratory behaviour or origin. The numbers in the table represent percentages of the total 140 alleles tested.

Allele	Resident	Migrant	Wintering
263	16.07	3.33	11.11
266	0.00	0.00	3.70
269	80.36	90.00	75.93
272	1.79	3.33	5.56
275	1.79	3.33	0.00
278	0.00	0.00	3.70

Supplementary table S3B. Allele frequency distributions at ADCYAP1 in 3 groups of blackcaps differing in migratory behaviour or origin. The numbers in the table represent percentages of the total 140 alleles tested.

Allele	Resident	Migrant	Wintering
159	5.36	0.00	3.70
161	0.00	0.00	1.85
163	35.71	36.67	29.63
165	8.93	16.67	9.26
167	30.36	40.00	42.59
169	14.29	3.33	12.96
171	3.57	3.33	0.00
173	1.79	0.00	0.00

Section 2

Testing the hypothesis of indirect genetic effects on migration through correlations with personality traits

Chapter 4

The association between genetic markers, dominance and personality-related traits in a migratory bird species

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(unpublished manuscript)

Abstract

Over recent years the influence of genetics on behaviour has been a growing field of research. Differences in behaviours like moderate stress response, dominance or exploration, could in its turn, influence the migratory behaviour of an animal. We looked at personality and dominance differences between individuals of a migratory passerine species, the blackcap (Sylvia atricapilla), which previously has been shown to have genetic differences between populations of different migratory status. If differences in personality traits were the immediate causes of variation in migration we would expect gene-effects mediated through personality traits to have stronger effects on personality and dominance than on migration. The aim of this study was to test this hypothesis by determining the effect of genes affecting migration on personality traits. Over the course of three years, 85 birds of three populations with different migration strategies in the wild were kept in captivity and were subject to several behavioural experiments involving measurements of dominance, memory, exploration and stress response behaviour. We tested for the association of variation in these behavioural traits with genetic variation using a set of 13 microsatellite markers, which included 4 Candidate markers (ADCYAP1, CLOCK, NPAS2 and SERT) and 9 neutral markers. Our analyses revealed that only the candidate maker NPAS2 could be linked to dominance. However due to low variation at this locus and the fact that NPAS2 in past research has not been linked to personalities, but to circadian clocks, suggests that this result may be spurious and needs further investigation. Candidate markers SERT and CLOCK and neutral marker Syl6 could be linked to stress response behaviour during the autumn migration period. SERT, being a marker that has previously been shown to be associated to anxiety related behaviour in other species, seemed promising, though our results are not consistent across our analysis for this marker. CLOCK and Syl6 showed stronger associations, though not enough to use them as predictors for behaviour. We have shown that a behavioural syndrome in Iberian blackcaps seems absent and that migration has stronger associations directly with certain candidate markers then with markers possibly influencing migration through personality traits.

Introduction

The genetic basis of migratory behaviour

Investigating what drives migratory movements in animals, and what part their genetics play in this, could aid in conservation and understanding how animals adapt to changing environments (e.g. Frankham *et al.* 2002, Lande & Shannon 1996). Genes that have been shown to be associated with various facets of behaviour, migration or other, generally only explain low amounts of variation within these traits (e.g. van Oers *et al.* 2004, Mueller *et al.* 2011, Chapter 1) or the genes found to be involved show inconsistent results (e.g. Korsten *et al.* 2010, Mueller *et al.* 2011). A possible explanation for the fact that direct associations between genes and migratory behaviour are often weak or inconsistent could be that these genes influence migratory behaviour indirectly. Possibly these genes are not directly controlling migratory traits but traits that are correlated with migratory behaviour, i.e. as part of a behavioural syndrome (see, Dingle 2006, Dingle & Drake 2007). It has been proposed that individuals display behaviour consistently across different situation (e.g. exploration, aggression, risk taking) resulting in correlations between the functionally different behaviour types. This lack of independency of distinct behaviours is called a behavioural syndrome (Sih *et al.* 2004). Though several studies oppose this idea, showing that the correlations between the different behaviours were generally weak (Garamszegi *et al.* 2012, 2013). Such a behavioural syndrome could have a genetic origin (van Oers *et al.* 2005). Animal personalities are the best known, and widespread, behavioural syndrome. Animal personalities, consistent differences in behaviour (Gosling, 2001, Réale *et al.* 2007), are characterised by being heritable, having a moderate genetic component (Dingemanse *et al.* 2002, van Oers *et al.* 2004, van Oers and Mueller, 2010).

Genetics of personalities

To pinpoint specific regions of the genome involved in personality differences QTL and, or Candidate gene approaches have been used. Two candidate genes have been found to be associated to personality and behaviour: the DRD4 gene and the serotonin transporter gene (SERT) (Savitz & Ramesar, 2004). In humans e.g. polymorphisms of the DRD4 gene have accounted for 3% of the novelty seeking behaviour (Munafo *et al.* 2008). Studies on great tits, *Parus major*, showed that the allele frequency of an exonic SNP in the DRD4 gene was associated with exploratory behaviour (Fidler *et al.* 2007) though this polymorphism could be linked to the functional variant in some, but not all populations, or the association might depend on the environment, since it was not found across populations (Korsten *et al.* 2010).

Results on SERT are inconsistent when slightly different traits are measured (harm avoidance, neurotisms) (Munafo *et al.* 2009) though in general SERT has been shown to be associated with anxiety-related traits (Eley & Plomin 1997, Gordon & Hen 2004).

Animal personalities and their link to migratory behaviour

Various studies show how personality traits could influence migratory behaviour. In freshwater fish for example it is know that boldness influences migratory propensity, with bold animals being more likely to migrate (Chapman *et al.* 2011). In birds, there are indications based on comparative studies that migratory and sedentary species differ in exploratory and neophobic behaviour (e.g. Mettke-Hofmann *et al.* 2005). Also, in blackbirds, *Turdus merula* (Lundberg 1985) and dark eyed juncos, *Junco hyemalis* (Rogers *et al.* 1989), it is the subordinate individuals that are the fraction of the population that migrates

or migrates larger distances than dominant individuals. This follows the idea of the dominance hypothesis of migration which proposes that when food is scarce the food sources get occupied by more dominant individuals. Subordinates tend to migrate in order to avoid competition that could be more costly than migration itself (Ketterson and Nolan Jr, 1979, Marra, 2000).

Personality traits and their potential link to migratory behaviour in blackcaps

In captive Iberian blackcaps, *Sylvia atricapilla*, stress tolerance was negatively correlated with the onset of migration, which differed slightly, but significantly, between populations (Bulaic, 2015). On top of that, birds from sedentary populations coped better with moderate stress situations showing shorter latencies. These birds were also the least flexible; it took them longer to find new sources of food and get used to a new situation (neophobia). (Bulaic, 2015). This concurs with an interspecific experiment where migratory garden warblers (*Sylvia borin*) were quicker in discovering food in a novel environment than sedentary Sardinian warbles (*S. melanocephala*) (Mettke-Hofmann and Gwinner, 2004). This follows the idea that birds in constant environments don't need to have very flexible behavioural responses, since the stimuli do not change (Niemela *et al.* 2013).

From these captive blackcaps, birds with higher dominance scores seemed more likely to be migratory individuals (Bulaic, 2015), which is contrary to the "dominance hypothesis". In a partially migratory, wild, population it was found that during winter the smaller sized residents were dominant over the larger overwintering migratory birds (Morganti *et al.* 2017).

The fact that captive blackcaps with higher dominance scores seemed more affected by moderate stress situations, but less by neophobia (Bulaic, 2015) implies that dominant individuals tend to be fast explorers (Verbeek *et al.* 1996, Boogert *et al.* 2006, David *et al.* 2011) A similar result has been found in great tits, *Parus major* (Dingemanse and de Goede, 2004). A characteristic for dominant and fast exploring birds is rigidness and lower flexibility in adjusting their behaviour in a changed situation, with a tendency to old habits. This would explain the low performance of dominant blackcaps in the moderate stress experiment from Bulaic, 2015 (Benus *et al.* 1991, Verbeek *et al.* 1999).

Aim of this study

Since previously found associations between genetic markers and migratory behaviour were week (see chapter 1), and the candidate genes with the strongest and most consistent association were not specific "migration" genes, it would be possible that these genes primarily affect "general" behavioural traits, reflected in animal personalities, and that the effects of these genes on migratory behaviour is indirect, due to the link between these personality traits and migration. Apart from those genes we also look at genes not known for affecting migration, but known for affecting personalities, to see whether they might indirectly affect migratory behaviour. Here, we aim to test the effect of all these genes on personality traits and compare them to the effect on migratory traits.

Material & Methods

Capture of birds

For 3 consecutive years (2010-2012) fledgling, 2-3 months old, blackcaps, *Sylvia atricapilla*, were caught

at their breeding sites before autumn migration (July - mid August) and brought to a facility in a restricted area in the natural park Casa de Campo, Madrid, Spain (40°25'N, 3°45'W) (N=85). After spring migration, birds were returned to their respective capture sites to be released. The birds originated from three populations on the Iberian Peninsula, each with a different migratory strategy. (1) Madrid (Pinilla del Valle 40°55'N, 3°49'W), a completely migratory population (N=29), (2) Cocentaina (Alicante 38°44'N, 0°26'W), a partially migratory population (N=40), (3) Tarifa (Los Barrios 36°11'N, 5°36'W), a sedentary population (N=16). (For a more detailed overview of the numbers of sampled birds and populations, see Table 1). Determining migration status of these populations was based on Tellería *et al.* 2001, presence-absence (wintering/breeding) data (SEO/BirdLife, 2012) and wing measurements (according to Pérez-Rodríguez *et al.* 2013 and Morganti *et al.* 2015).

Table 1. Summary of the birds used in this study. Given are the number of males and females (M, F) sampled in the 3 populations in different years. Included is a map with the location of the completely migratory population of Madrid, the partially migratory population of Cocentaina and the sedentary population of Tarifa.

Year	Madrid	Cocentaina	Tarifa	Total	
2010	4 , 6	10 , 4	5 , 4	19 , 14	33
2011	7 , 5	15 , 5	0 , 0	22 , 10	32
2012	5 , 2	4 , 2	5 , 2	14 , 6	20
Total	16 , 13	29 , 11	10 , 6	55 , 30	
	29	40	16		85

Keeping of birds

Most birds were kept in indoor cages of (45x23x38cm) (visual and auditory contact between neighbours was present at all times) with a plastic box feeder hanging at the side of the cage. The holding facility had windows, but two lamps were added to provide the same light intensity as an outdoor situation would. (2 x fluorescent Megaman WL 130 Compact 2000 HPF of 30W, an intensity of 1620 lumen and a colour temperature of 6500K). These lights were switched on and off by an automated system that followed the natural light cycle outside. "On" was 10 minutes after dawn, "off" was 10 minutes before dusk, thus leaving 10 minutes of gradual increase and decrease in light between dark and full light intensity. Birds were kept in a restricted facility without cooling, nor heating. Thus they were exposed to a natural temperature regime. Due to the cover from the elements, minimum temperatures were about 4°C

warmer than outside and maximum temperatures about 4°C cooler, resulting in the range of 5-36°C.

For one particular experiment, where we determined dominance, birds had to be kept in pairs for which the indoor cages were too small. Therefore, birds were kept in outdoor aviaries (3x2x2.3m) with 6 perches, a tree and undergrowth. Temperatures ranged from 1-40°C. In these aviaries neighbours could hear each other, but visual contact was blocked.

The birds were fed daily. They received a diet consisting of mealworms, fruit (depending on whichever was in season; apple, persimmon fruit, pomegranate, fig, pear, cherry) and industrially produced birdseed for insectivorous birds with added vitamins (Raff, Patée con insetti), water was provided ad libitum. Individual intake for each type of food was monitored to keep track of health, but also to notice dietary changes in preparation for migration. Monitoring food intake for birds kept outdoors proved more difficult, since these birds were catching insects to their heart's desire.

Behavioural experiments

Four different types of behavioural tests were performed by one of us (MB); (1) dominance, (2) moderate stress, (3) exploration, (4) memory tests. (Table 2 shows the numbers of birds that were used in each experiment).

(1) Dominance experiments were conducted by keeping the birds in pairs in the outside aviaries during spring, i.e. from the beginning of April until the end of May, when spring migration was coming to an end. This period was chosen as more individuals were available. (During autumn, the birds had to be in the indoor cages to accurately monitor their migratory behaviour). Because this spring testing period was close to the breeding season, only pairs of the same sex were put together as to avoid courtship and mating behaviour influencing our results. In the final year birds were also tested during autumn since we started measuring migration activity outside.

Experiments were filmed with hand cameras (Panasonic SDR-H85 and Sony DRC-SX65E) placed inconspicuously outside the aviary.

For the determination of dominance, two individuals were introduced simultaneously to a new, unfamiliar, aviary as to avoid residency effect. Observation of interactions between the birds were registered the first time the birds were introduced and two more times in the two subsequent days. Their staged dyadic encounters were scored for 10 minutes, making it possible to assign a within pair dominance rank. The final dominance rank of each individual was determined by analysing typical dominance interactions such as active and passive displacements where the subordinate bird would repeatedly leave the perching site to make way for the dominant bird (Pravosudov *et al.* 2003, Fox *et al.* 2009). In such a case the approaching bird would be assigned a score of 1, where the leaving bird would get a score of 0.

During the process of establishing the dominance relationship little to no aggression was observed towards the subordinate bird. In total 43 pairs were tested spread over 3 years. To test for possible among-population differences the birds were paired up as follows; Madrid-Tarifa (N=16), Madrid-Cocentaina (N=19), Cocentaina-Tarifa (N=8).

The following 3 experiments were conducted during autumn and spring migration in the indoor cages (From the first week of October until the first week of November and from the last week of February

until the end of March. Ergo; before the dominance tests were conducted). For each of these experiments birds were filmed for of a period of 20 minutes.
(2) The moderate stress experiment was a modification of the feeding protocol. A period of 20-30 minutes of food deprivation was applied before the original feeder, with replenished supply of food, was placed back in its usual place. Three variables were measured. (A) The latency (i.e. the time needed) to approach the feeder for the first time. (B) The total time spent in the feeder before feeding. (C) The number of visits to the feeder before feeding.

(3) In the exploration test, we tested for differences in the way individuals adjust their behaviour to an altered environment. Therefore we placed, after 20-30 minutes of food deprivation, a new type of feeder (a clay dish with an 2cm upright edge) filled with food on the floor of the cage, while the original feeder was put back in its usual place, but without food. In this experiment, the same variables as for the moderate stress experiment were scored, but now for both feeders, the original one and the new one: i.e. the latency to approach the feeder, time spend on/in the feeder before feeding, and the number of visits to the feeder before feeding).

(4) The memory test was similar to the exploration test. The only difference is that now the new feeder was covered by a white piece of paper. The experiment was repeated three times per bird. This elicits the expectation that the birds would grow accustomed to the new location of food, even though it was hidden. The variables measured were the same as in the exploration test.

Table 2. Summary of the birds used for each experiment.

		Madrid	Cocentaina	Tarifa	Total
Dominance	2010	7	10	9	26
	2011	12	18	0	30
	2012	3	2	1	6
	Total	22	30	10	62
Explore / Memory	2010	10	14	9	33
	2011	11	20	0	31
	2012	6	5	7	18
	Total	27	39	16	82
ModStress Autumn	2010	0	0	0	0
	2011	11	19	0	30
	2012	6	6	7	19
	Total	17	25	7	49
ModStress Spring	2010	10	14	9	33
	2011	12	20	0	32
	2012	0	0	0	0
	Total	22	34	9	65

DNA sampling
Blood (ca 50 µl) was taken from all birds by either puncturing the brachial vein, or by extracting it from the jugular vein with a syringe. Blood was stored in alcohol in a freezer (at -20ºC) until lab work commenced. DNA extractions were made using a *Blood and Tissue Kit* (Macherry & Nagel).

Genotyping
Following Mueller *et al.* 2011 (and chapter 1) we investigated four candidate genes, three of which have

been related to circadian behaviour (CLOCK, ADCYAP1 and NPAS2) and one related to harm-avoidance behaviour (SERT). For analysing variation at each of these loci an exonic microsatellite was used (Mueller *et al.* 2011 & chapter 1). For comparative association analyses we used 8 anonymous, neutral microsatellite loci (Syl1, Syl2, Syl4, Syl5, Syl6, Syl9, Ppi2 and Pca8) and the sexing marker P2P8 from Griffiths et al. (1998). This made a set of 13 microsatellite markers. (Selection of the markers was partially based on Segelbacher *et al.* 2008 and Steinmeyer *et al.* 2009) (For more information see Supplementary table S1).
All samples (N=101) were genotyped for these 13 microsatellites using an ABI 3100 sequencer (Applied Biosystems) for the microsatellite fragment analyses. (For details on PCR conditions: see Mueller *et al.* 2011). The result files were analysed in Genemapper.

DRD4

Apart from the microsatellite markers mentioned above we attempted to sequence the polymorphism at the DRD4 locus. Genetic variation at DRD4 has been shown to be associated with variation in personality traits in humans and a number of avian species (e.g. Munafo *et al.* 2008, Fidler *et al.* 2007). Therefore, we aimed at testing for an association of variation in this gene with personality and migratory traits. A microsatellite in the DRD4 candidate gene is not known, therefore we tried to sequence blackcap specific SNPs at exon 3 of the DRD4 gene identified in a previous blackcap study (Mueller *et al.* 2011). For reasons unknown, the laboratory work did not result in usable data. The PCRs failed to produce products fit for sequencing. The laboratory where the work was performed had knowledge and experience with this particular work in blackcaps, since it is the laboratory where the work for the study by Mueller *et al.* 2011 was also conducted., though with our present samples something went amiss. Possibly the different storage method of the blood samples interfered with the subsequent lab analyses, though the extraction method should have cleaned up the sample sufficiently.

Data Analyses

Most analyses were conducted using SPSS (IBM Corp. version 20). The set of birds used in this study is a subset of the birds used in chapters 1 and 2. Tests for Hardy Weinberg Equilibrium were performed using GenAlEx 6.502 (Peakall *et al.* 2012). Linkage disequilibrium was tested using Genepop V4 (Raymond and Rousset 1995). (Details on these tests can be found in chapter 1 and 2).

For each of the behavioural experiments we extracted several variables (see above). Non-parametric, Kolmogorov-Smirnov tests show that the response variables, the behavioural variables, were normally distributed, except for the measurements on moderate stress in spring. Therefore we normalised these spring variables by applying a square root conversion before using them.
To reduce the number of inter-correlated variables, we conducted principle component analyses which allowed us to group different variables and extract several principle components, which represent variation in these variables.
First we tried to make principle components of all variables of all experiments together. There it became clear that the variables of the moderate stress experiment were not correlated to the variables of the exploration or memory tests. As a consequence, we ran a PCA for these variables separately and analysed the results from the moderate-stress experiment separately from the other experiments. Bulaic (2015) found a learning effect in the stress experiment. A result that was confirmed in the present analysis. We, therefore decided to analyse separately the results of the experiments conducted in autumn and spring. Birds tested in spring for the first time, had accustomed to the experimental

conditions (i.e. removal of the feeder, which is the usual routine for provisioning of birds) and to the presence of people, causing shorter latencies and making it impossible to group these data with autumn results. For the birds that were tested multiple times within a season, only the first test was used in this analysis, avoiding interference of a learning effect. Principal component analyses resulted in three principle components for the moderate stress experiment; PC1 Autumn, PC2 Autumn and PC1 Spring (see table 3).
The experiments on exploration and memory showed no learning effects, thus making it possible to group data of birds independent of the season they were collected. In a first attempt of making principle components of these variables it became clear that several variables were highly correlated. Therefore, we deleted variables with low sample sizes which were highly correlated with variables for which had high sample sizes. By deleting these variables we considerably increased samples size without losing information. From the remaining variables we constructed two principle components; PC1 ME and PC2 ME (ME = Memory, Exploration). (for details of all principle components, see table 3).

Table 3. Summary of the results of the principle component analysis (PCA) for the variables of (a) the moderate stress response experiment and (b) the memory and exploratory experiments combined. Separate PCAs were run for the data on moderate stress response experiments conducted in autumn and spring. In subsequent analysis the 5 extracted principle components (PCs) were used. Variables of the moderate stress response experiment conducted in spring were normalised before conducting the PCA.

(a)

		PC1 Autumn	PC2 Autumn	PC1 Spring
	% of variance	59.03	33.746	73.808
	Eigenvalue	1.771	1.012	2.214
	Factor loadings per variable			
Moderate Stress	Latency to feed	0.929	0.183	0.964
	Latency to approach the usual feeder	0.944	-0.042	0.933
	Total time spend around the usual feeder	-0.132	0.988	0.644

(b)

		PC1 ME	PC2 ME
	% of variance	45.061	15.761
	Eigenvalue	3.605	1.261
	Factor loadings per variable		
Memory test	latency to feed from the covered feeder	0.853	0.224
	Number of visits to the usual feeder in the memory-covered feeder- test	0.337	0.797
	Latency to approach the covered feeder	0.835	0.175
	Number of visits to de covered feeder	-0.273	0.578
Exploration test	latency to feed from new feeder	0.854	-0.274
	Number of visits to the usual feeder in the exploration -new feeder- test	0.603	0.163
	Latency to approach the new feeder	0.882	-0.313
	Number of visits to de new feeder	-0.350	0.107

To construct a weighted mean dominance value per bird a pairwise interaction dominance was calculated by dividing the number of wins by the total number of initiated interactions, and weighted by the number of non-initiated interactions, as to correct for a possible bias in dominance scores for individuals with few initiated interactions, with no observed reversals within pairs (Pravosudov *et al.* 2003).

Correlations between all principle components and the dominance variable were tested to see whether these are independent of each other. Table 4 shows they are clear separate measurements.

Linear regressions were performed in order to see whether any genetic marker was associated with the behaviour of the birds. The principle components and the weighted mean dominance were entered into the model as the dependent variables. Mean, maximum and minimum allele length were used as independent variables. Full models and forward and backward methods were applied in SPSS. However, as forward and backward approaches gave very similar results, we only give the results from the forward approach, along with the full models.

Whenever, we found a significant effect in the regression analysis, we performed GLMs to assess the effects of allele frequency differences and their interactions on differences in behaviour. We made several models combining markers that were significant, or near significant, in other regressions.

Table 4. Correlations between the 6 personality variables studied. The tables give Pearson's correlation coefficients, two tailed significances and sample sizes.

		Dominance	PC1 Autumn	PC2 Autumn	PC1 Spring	PC1 ME	PC2 ME
Dominance	Pearson Correlation	1	0.312	-0.120	0.159	-0.013	0.112
	Sig. (2-tailed)		0,073*	n.s	n.s	n.s	n.s
	N	62	34	34	56	61	61
PC1 Autumn	Pearson Correlation		1.000	-0.025	0.119	0.113	0.127
	Sig. (2-tailed)			n.s	n.s	n.s	n.s
	N		49	49	30	46	46
PC2 Autumn	Pearson Correlation			1.000	-0.062	0.208	0.162
	Sig. (2-tailed)				n.s	n.s	n.s
	N			49	30	46	46
PC1 Spring	Pearson Correlation				1.000	0.142	-0.146
	Sig. (2-tailed)					n.s	n.s
	N				65	64	64
PC1 ME	Pearson Correlation					1.000	-0.030
	Sig. (2-tailed)						n.s
	N					82	82
PC2 ME	Pearson Correlation						1.000
	Sig. (2-tailed)						
	N						82

Results

Dominance

Forward regression models showed that dominance seems to be associated to the mean allele length at the NPAS2 locus (p = 0.033) (See table 5). A one way anova with mean allele length as the dependent variable and population as the factor results in p = 0.499. We, thus, can conclude that there were no inter-population differences in mean allele length of NPAS2.

In our first GLM on dominance we entered all variables. This gave a non-significant model with poor AIC and BIC values. In the regressions, mean allele length of NPAS2 was the only significant marker. Therefore, we made GLMs with mean allele length of NPAS2 alone, and it combined with several

markers significant in regressions of other experiments. The GLM on dominance with the mean allele length of NPAS2 is the only significant model (p = 0.029). A one-way anova yielded the same result (dependent variable = dominance, factor = NPAS mean allele length. p = 0.033). Thus, based on these analyses we can conclude that birds with a shorter mean allele length at the NPAS2 locus are more dominant.
GLMs with NPAS2 combined with several other markers what were significant in other experiments did not improve the model (See table 6A).

Table 5. Summary of the results of the multiple regression analyses. Besides a full model with all variables (Enter), the best model for different dependent variables (= personality axes) was obtained by adding significant variables (forward). Each table gives the variables retained in the final model and their significance, as well as the significance of the complete model. Models were run considering mean, minimum and maximum allele lengths as independent variables.

	Final Model					Dependent variables in final model				
	Model	Sum of Squares	df	F	sig	Markers left	B	S.E.	t	sig
Dominance	Full model / Enter	1.536	22	1.056	0.429	*				
	Forward	0.304	1	4.787	*0.03*	Npas2 mean	-0.086	0.039	-2.188	*0.033*
PC1 Autumn	Full model / Enter	32.799	22	2.382	*0.02*	CLOCK Min	-0.127	0.046	-2.783	*0.01*
						Syl4 Min	-0.061	0.021	-2.887	*0.008*
						Syl5 Min	0.045	0.02	2.248	*0.033*
	Forward	13.679	2	8.889	*0*	Syl6 Mean	0.045	0.013	3.537	*0.001*
						CLOCK Mean	-0.157	0.067	-2.358	*0.023*
PC2 Autumn	Full model / Enter	17.2	22	0.682	0.817	*				
	Forward	**				*				
PC1 Spring	Full model / Enter	23.006	22	1.047	0.435	ADCYAP1 Max	0.188	0.089	2.105	*0.041*
	Forward	6.047	1	6.47	*0.01*	Syl1 Min	0.032	0.013	2.544	*0.013*
PC1 ME	Full model / Enter	15.318	22	0.634	0.881	Ppi2 Min	0.05	0.025	2.006	*0.049*
	Forward	**				*				
PC2 ME	Full model / Enter	21.968	22	0.978	0.503	Ppi2 Max	0.031	0.014	2.265	*0.027*
	Forward	4.326	1	4.444	*0.04*	Ppi2 Max	0.023	0.011	2.108	*0.038*

** no significant markers left in the final model*
*** no model fitted due to absence of significant markers*

Moderate stress in autumn
The regression model where all independent variables were entered was significant for the first principle component of the autumn stress experiments; PC1 Autumn (p = 0.018). In this model there were three markers significant; the minimum allele lengths of CLOCK, Syl4 and Syl5.
Forward regressions showed that the mean allele lengths of Syl6 and CLOCK were significant with the same principle component (p = 0.001). Regressions with the second PCA; PC2 Autumn did not produce any significant models (See table 5 for details of the regressions).

A first GLM on PC1 Autumn with all the markers resulted in a significant model (p < 0.001). Within this model several markers were significant, including the mean allele length of candidate marker SERT.
We tested all these significant markers, plus the markers that were significant in any of the regression analyses, in single GLMs. This resulted in only CLOCK and Syl6 being significant, just as in the regression analysis using the “forward” approach did. We made several combinations of markers. Various models

were significant, though the best appeared to be a model with the mean allele lengths of CLOCK and Syl6 ($p < 0.001$) (See table 6B).

Moderate stress in spring

The principle component for moderate stress during the spring season was significant in the forward regression with the minimum allele length of the neutral marker Syl1 (see table 5).
Combining markers to build a GLM model for the spring moderate stress experiment showed that the best model is similar to the result of the regression analysis. Minimum allele length of Syl1 alone ($p = 0.012$) and combined with mean allele length of CLOCK ($p = 0.013$) explain behavioural variation best (See table 6C).

Exploration & Memory

The maximum length of Ppi2, a neutral marker, was significant with the principle component of PC2 ME in the forward regression. (See Table 5).
As with dominance and with moderate stress in spring, the marker significant in the regression analysis also gave the best GLM result. Maximum allele length of the neutral marker Ppi2 $p = 0.035$.
(See table 6D for all the GLMs and their details).

Table 6. Results of the GLMs for the 4 personality axes for which we performed GLMs. For each variable the fit of the best models in ascending order from the model with the lowest to the highest information criteria (AIC and BIC) and the full model are given.

6A. Dominance

Variables included in the model	Δ AIC	Δ BIC
NPAS2 Mean	0	0
NPAS2 Mean, ADCYAP1 Mean, + Interaction	2.87	7.12
NPAS2 Mean, Ppi2 Max, + Interaction	3.58	7.84
NPAS2 Mean, Syl1 Min, + Interaction	3.78	8.03
NPAS2 Mean, CLOCK Mean, + Interaction	3.90	8.15
NPAS2 Mean, Syl6 Mean, + Interaction	3.93	8.19
All; Full model	17.79	62.46

6B. PC1 Autumn

Variables included in the model	Δ AIC	Δ BIC
CLOCK Mean, Syl6 Mean	0	0
CLOCK Mean, Syl6 Mean, + Interaction	1.90	3.79
All; Full model	1.93	39.77
CLOCK Mean, Syl6 Mean, SERT Mean, + Interaction	3.19	6.97
Syl6 Mean	3.59	1.70
CLOCK Mean, CLOCK Min, + Interaction	9.76	11.65
CLOCK Mean	9.79	7.89
CLOCK Min	9.92	8.03
Ppi2 Max	13.54	11.65
Syl1 Min	13.60	11.71
SERT Mean	13.64	11.75
Syl5 Min	13.74	11.85
NPAS2 Mean	13.93	12.04
Syl4 Min	14.01	12.11
SERT Mean, Syl1 Mean, Syl5 Mean, Syl1 Min, Syl4 Min, Syl5 Min, + Interaction	19.23	28.69

6C. PC1 Spring

Variables included in the model	Δ AIC	Δ BIC
Syl1Min, CLOCK Mean	0	1.78
Syl1 Min	0.40	0
Syl1 Min, CLOCK Mean, + Interaction	1.95	5.90
CLOCK Mean	3.60	3.20
CLOCK Min	3.90	3.50
Syl1 Min, ADCYAP1 Max, + Interaction	4.03	7.98
NPAS2 Mean	5.60	5.20
Syl6 Mean	5.73	5.33
ADCYAP1 Max	5.81	5.42
CLOCK Mean, NPAS2 Mean, + Interaction	5.84	9.80
ADCYAP1 Mean	6.19	5.80
SERT Mean	6.24	5.84
ADCYAP1 Min	6.66	6.26
Ppi2 Max	6.75	6.35
All; Full model	20.32	65.59

6D. PC2 ME

Variables included in the model	Δ AIC	Δ BIC
Ppi2 Max	0	0
Ppi2 Mean	0.53	0.53
Ppi2 Max, ADCYAP1 Min, + Interaction	1.72	6.53
Ppi2 Max, Ppi2 Mean, + Interaction	3.07	7.89
Ppi2 Max, CLOCK Mean, + Interaction	3.33	8.14
Syl6 Mean, Ppi2 Max, SERT Mean, + Interaction	3.45	3.45
CLOCK Mean	3.54	3.54
NPAS2 Mean	3.97	3.97
SERT Mean	4.08	4.08
ADCYAP1 Min	4.18	4.18
Syl1 Min	4.19	4.19
Syl2 Min	4.38	4.38
Syl2 Mean	4.39	4.39
All; Full model	20.93	71.47

Discussion

Indirect genetic effects on migration through personality traits

Our results show that it seems unlikely that migratory behaviour is better explained by variation in personality or dominance, or genes underlying either of those, then by genes acting on migratory behaviour "directly", at least for the genes studied here, ergo, those that were previously associated with migratory behaviour (Mueller *et al.* 2011; Chapter 1).

Behavioural syndrome

Bulaic (2015) showed that captive Iberian blackcaps with higher dominance scores tended to be more affected by moderate stress situations, but less by neophobia suggesting a behavioural syndrome. From our results it seems clear that a genetic basis for such a possible behavioural syndrome in Iberian blackcaps is lacking, apart from behaviours displayed in memory and exploration experiments, which were very similar in their set up creating an overlap in variables measured, behavioural traits seem independent. Instead of one clear behavioural axis, several appear in our study; 1 dominance axis, 3 moderate stress axes and 2 memory + exploration axes. This result concurs with the general fining of studies by Garamszegi *et al.* (2012, 2013) that in most studies a clear behavioural syndrome is absent or weak. The lack of integration of behavioural traits, particularly a correlation between general behavioural traits (i.e. dominance or personality), could explain why we did not find no evidence for "indirect genetic effects" on migration.

Association between our genes and personality

In this study, we found an association between the dominance of birds with mean allele length at the NPAS2 locus. The candidate marker NPAS2 (neuronal PAS domain protein 2) has, in past research, been connected to circadian rhythms (Steinmeyer *et al.* 2009) e.g. the timing of breeding in tree swallows, *Tachycineta bicolor* (Bourret and Garant, 2015). In Iberian blackcaps NPAS2 has also been associated with the migration status of wild populations (Chapter 1). Though, like in this study, variation at this locus was very low, with just two alleles present in the sample with a more than 90% homozygosity. This gives the locus a very low discriminative power. Low variation at the NPAS2 locus appears a wide spread feature. In buzzards heterozygosity was also very low. Though juvenile, heterozygous birds significantly dispersed less, staying closer to their natal area, than their homozygous conspecifics (Chakarov *et al.* 2013).

SERT, which has been found to be associated to anxiety response in other studies was significant in only one GLM analysis with our moderate stress experiment in autumn. However, a consistent association between SERT and behaviour is lacking in our results.
A more prominent role in moderate stress behaviour in autumn seems to be for CLOCK and Syl6. Though also for these markers the connection is still not strong enough for the markers to be used as a predictor of behaviour.

Thoughts on DRD4 and shortcomings

It has previously been shown that variation at polymorphisms of the candidate gene DRD4 are not associated directly to differences in migratory behaviour between blackcap populations across Europe, not in migratory distance nor in their Zugunruhe (Mueller *et al.* 2011). However it would be interesting

to see whether DRD4 is associated to personalities or dominance in blackcaps, possibly influencing migratory behaviour indirectly. Thus one shortcoming of this study is the DRD4 polymorphism could not be studied in our sample. Due to time and financial constraints the testing of DRD4 has been temporarily suspended. We do however still aspire to perform this analysis whenever possible, and add it to this study later on to complete the overview.

Another potential influence on the result of this study is the fact that the experiments were done in a captive environment at a location in central Spain. It is has been suggested that it is likely that holding a bird captive at a different location than its origin can influence its migratory activity (Chapter 2). It is possible that a similar change in behaviour occurs in personality traits. The change in latitude, environment, housing, social structure could perhaps have altered the personality of the birds, influencing the outcome of our study. Besides the location of our experiments, maybe the experimental set up was not adequate to test personality traits that related to migration. For future studies it might be interesting to try some aptly designed personality experiments for Iberian blackcaps in the wild. Similar studies have been done in other species (e.g. Dingemanse *et al.* 2002).

Conclusion

It seems that we can safely say that candidate genes like ADCYAP1 and CLOCK seem to be lacking pleotropic effects, only influencing migration in a direct fashion rather than indirectly through behavioural traits. A possible effect of the personality candidate gene DRD4 on migration still remains to be investigated, though it seems unlikely to alter our findings. Besides the lack of a genetic effect on personalities it became clear that Iberian blackcaps lack a general behavioural syndrome.

Supplementary material

Supplementary table S1. The markers used in this study

Gene/Locus	Locus type	No. of observed alleles	Reference
CLOCK	Trinucleotide microsatellite	8	Steinmeyer et al. 2009
ADCYAP1	Dinucleotide microsatellite	13	Steinmeyer et al. 2009
NPAS2	Trinucleotide microsatellite	2	Steinmeyer et al. 2009
SERT_Ex1	Trinucleotide microsatellite	3	C. Hermannstaedter, pers. comm.
Syl1	Tetranucleotide microsatellite	14	Segelbacher et al. 2008
Syl2	Tetranucleotide microsatellite	15	Segelbacher et al. 2008
Syl4	Tetranucleotide microsatellite	20	Segelbacher et al. 2008
Syl5	Dinucleotide microsatellite	24	Segelbacher et al. 2008
Syl6	Tetranucleotide microsatellite	36	Segelbacher et al. 2008
Syl9	Tetranucleotide microsatellite	18	Segelbacher et al. 2008
Ppi2	Dinucleotide microsatellite	21	Martinez et al. 1999; A. Ramirez, pers. comm
Pca8	Dinucleotide microsatellite	4	Dawson et al. 2000; A. Ramirez, pers. comm

Section 3

A detailed look at the candidate gene G3PD in blackcaps

Chapter 5

Glycerol-3-phosphate dehydrogenase (G3PD) sequence polymorphisms cannot explain allozymic variation associated with migratory behaviour in the blackcap (Sylvia atricapilla)

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Abstract

Over recent years, the influence of genes on behaviour has been a growing field of research. Here we studied sequence variation in the G3PD gene in birds. This gene has been hypothesized to be important for modulating the expression of migratory behaviour in birds due to its role in the accumulation or use of fat deposits. In Eurasian blackcaps (Sylvia atricapilla), an allozymic variant of this protein was found in resident populations only, suggesting that there is an association between migratory behaviour and genetic variation at this locus. Here, we aimed at unravelling the genetic basis of this enzyme polymorphism by sequencing the G3PD-gene in blackcaps differing in the number of copies of this allele. We sequenced two versions of the gene, located on different chromosomes, and found a large number of variable sites: 21 for the gene located at the linkage group 22 and 11 on chromosome 7. However, none of these polymorphisms could be linked to the G3PD-allozyme associated with migration. However, compared to the sequences known in other bird species (chicken and zebra finch), the sequence of blackcaps was "missing" 2 exons on either gene. We can't be certain whether these exons are absent in blackcaps or whether our attempts at sequencing them failed for other reasons. Further investigation is needed to clarify this.

Introduction

Investigating what drives migratory movements in animals, and what part their genes play in this, could help understanding how animals adapt to changing environments (e.g. Carlson & Seamons 2008). One approach for studying genes underlying variation in migration is looking at those genes known to encode for proteins which are part of the biochemical pathways involved in the expression of migratory behaviour or the migratory syndrome.

Allozyme studies have been widely used to determine whether selection shapes patterns of genetic variation at loci encoding for proteins of known function (Eanes 1999). For instance, the latitudinal cline of frequencies of the two most common alleles at the alcohol dehydrogenase (ADH) locus in *Drosophila melanogaster* was found to be maintained by selection (e.g. Berry & Kreitman, 1993). In this system, differences in enzyme activity were found to be associated with different allozymes and also with changes in lipid synthesis from ethanol in larvae (Geer *et al.* 1988, Heinstra *et al.* 1987). Latitudinal allozyme clines also were found at the glycerolphosphate dehydrogenase (GPDH) locus in *Drosophila*, with the frequency of the high activity allele increasing when going to the north (Oakeshott *et al.* 1982). In *Drosophila* and other insect species GPDH-variation was found to be linked to variation in flight capacity, which may be explained by its prominent role in the α-glycerophosphate cycle in the adult thoracic flight muscles (see, for instance, Clarke *et al.* 1983, Colgan 1992). This locus could be a potential candidate locus for migratory behaviour in birds. However, the extent to which these results can be generalised to birds is unclear, since insect enzymes at this locus are mitochondrial while it is cytosolic in birds. Therefore, it is likely that the role of this enzyme in insect metabolism is different from its role in bird metabolism.

In birds, the first attempt to link enzyme variation to migratory behaviour was made by one of us (FP). He investigated allozyme variation in the blackcap, *Sylvia atricapilla*, which is a migratory songbird, well used in migration studies. The aim of this project was to identify enzymes under selection and to link them to migratory behaviour. This could help identifying proteins central to the expression of migratory behaviour. In this study, allozyme variation was analysed at 39 loci in 13 populations with varying proportions of migrants. One central result of this study was that a polymorphism at the G3PD locus was strongly associated with non-migratory behaviour, the “slow” allele being found only in resident or partially migratory populations. Moreover, the frequency of this allele increased under artificial selection for sedentariness (Pulido 1994; Pulido *et al*. unpublished). It was hypothesized that this enzyme is under strong selection in migratory populations, where the allozyme would be selected against. The hypothesis that this enzyme plays a central role in the migration metabolism of birds was supported by the fact that G3PD is involved in the synthesis of lipids and gluconeogenesis from fats in birds (Harding *et al*. 1975). Since fats are the main fuel of migratory birds (Berthold 1996), G3PD could play a central role either in the building up of fat reserves before migration or in the generation of glucose during migration. If this holds true, we should expect to find only the most efficient variants of this enzyme (=allozymes) in migratory populations.

The aim of the present study was to sequence the G3PD gene to find sequence differences underlying the allozymic variant that was previously found to be under selection in blackcaps. By combining knowledge on protein variation and variation in genetic sequences the mechanism underlying selection might become clearer. Moreover, if we can pinpoint the site causing different allozymic variants, we

could develop a microsatellite marker to test for the polymorphism using smaller, non-invasive samples. With such a marker we would be able to screen large numbers of birds with different migration strategies and test for an association between the G3PD gene and migration behaviour on a large scale.

Material & Methods

Allozymes

In the 1990's FP performed an allozyme study in the Eurasian blackcap. Sampling was done between 1992 and 1994. Allozyme analyses were performed between 1994 and 1998. He found a strong association of one alloyzmic variant at the G3PD locus with non-migratory behaviour. This "slow" allele was found only in populations with non-migratory individuals. Most common were the 11 and 12 genotypes, of which the "2" allele was another name for the "slow" allele. In addition, various other, rarer genotypes were found: 13, 14, 15, 16, 17, 18, 19, 22. Allozymes on the gel are separated by their electric charge. Therefore, to detect the base substitutions underlying allozyme variation, we must look for polymorphisms in the sequence that code for differences in the amino acid chain that have different loadings (see, Evans 1987).

Selection of samples

Two allozyme types were most common, "1" and "2". For each of these we aimed to have at least 4 full sequences, preferably not all from the same population. Our sample catalogue was the same as used for the study by Mueller *et al.* 2011. We used a sub set of these from Austria, Cape Verde, Catalonia, Gibraltar, Kenya, La Palma, Madrid. The S-allele, "2", which is associated with non-migratory behaviour, was only found in the populations of Madrid, Gibraltar, La Palma and Cape Verde. Samples were collected through the years of 1989-1996 except for the Kenyan birds which were sampled in 2000 (for more details see, Mueller *et al.* 2011). The rarer allozyme types (13, 14, 15,16, 17, 18, 19 and 22) were investigated on the side, but were not considered vital in the first stage of the comparison since they were not associated with migration. These variants were studied to understand the molecular variation underlying allozymic variation at this locus.

After this initial pilot-batch of samples to scan the gene for polymorphisms, we found one polymorphism that matched the pattern in allozyme variation. Thus, for this exon, we increased sample size to 55 individuals.

DNA Extractions

For individuals of the Kenyan population and the samples collected from Cape Verdean population in 2000 we extracted DNA from muscle samples. For individuals of all other populations we used blood samples. Muscle samples were taken from dead birds kept in an ultrafreezer, by puncturing the frozen chest muscle (Fusani & Gwinner 2005), with a biopsy needle. DNA was extracted from this tissue using the DNeasy kit (Qiagen). Blood samples (ca 50 µl) were taken by puncturing the brachial vein. Blood was stored in alcohol in a freezer (at -20ºC) until lab work commenced. DNA extractions were made using a "Blood and Tissue Kit" (Macherry & Nagel).

Selection of the gene

According to the Uniprot website, in humans cytoplasmic G3PD (Enzyme number 1.1.1.8), Glycerol-3-

phosphate dehydrogenase [NAD (+)], is mentioned as GPD1. There is also a GPD1- like protein present in humans.
When searching for G3PD and GPD1 in the UCSC Genome Browser for chickens three versions of the genes appear. They are on chromosomes 2, 7, and on linkage group 22 (LGE22). The GPD1 on chromosome 2 was merely a like homologue. Since the proteins studied in the allozyme study were soluble, we chose for the cytoplasmic versions. Since in the alloyzme study, FP used pectoral muscle tissue for screening G3PD in blackcaps, we used as reference a study on amino-acid sequence of G3PD in chicken muscle (Zucker 1987). The DNA sequence of the blackcap G3PD gene on LGE22 had a higher match with the chicken sequence (98%) than the blackcap G3PD gene on chromosome 7 (85%).

Design of primers

Our initial aim was to sequence the whole gene, i.e. including exons and introns. When we started sequencing the gene on chromosome 7, we therefore designed primers that "overlapped" as to get the complete sequence. This strategy did not prove to be very successful, since sequences broke off in the introns. Therefore, we switched to making intronic primers located as close to the exons as possible. This was the only strategy we used when we started sequencing the gene on LGE22. Primer pairs were designed using the chicken and zebra finch, and in rare instances the turkey, sequences available at the UCSC browser. Primer3 (Koressaar and Remm 2007) aided in selecting primers with matching length and annealing temperatures. Whenever a primer pair did not give the aimed result of a full exon sequence, we would try different strategies to "fill in the gaps"; designing new pairs, trying combinations of new and previously used primers, in rare cases we designed exonic primers. (All primers used and their characteristics are given in Supplementary table S1A for chromosome 7, and Supplementary table S1B for LGE22).

PCR protocol & Sequencing

PCRs were optimised by trial and error starting with a basic mix and a cycling temperature that was the mean of the annealing temperatures of the primers that made a pair (See Supplementary table S2 for an overview of these basic PCR conditions). From there on, temperatures and the number of cycles or the amount of magnesium chloride were adjusted to get the best results. PCR products were put on a gel. The band at the correct base pair length was cut out and extracted with a "QIAquick Gel Extraction Kit" (Qiagen). This product was then washed twice with ethanol, before dissolving the resulting DNA pellet in a small amount of RNAse free water. This product, together with small amounts of the used primers, was sent to Eurofins Genomics Germany, where sequencing took place.

Data Analyses

We examined the sequences and looked for heterozygous sites using Chromas lite 2.0.1 (by Technelysium Pty Ltd). Thereafter, we marked the heterozygous sites and aligned the sequences of the different individuals in the BioEdit sequence alignment editor, version 7.2.5 (Hall 1999), which was used to search for polymorphisms.
We divided all detected polymorphisms into two groups: coding and non-coding polymorphisms, according to whether or not the base change in the DNA sequence altered the amino acid sequence. Since a coding polymorphisms can only match the allozyme variation found if the loading of the protein changes with the amino acid substitution accordingly, we only considered these DNA polymorphisms. We used Fisher Exact Tests to test for possible associations between allozyme variation and DNA polymorphisms causing a change in loading.

Results

The sequence(s) found

For each of the two genes, we successfully sequenced 6 exons. The sequence of these 6 exons on LGE22 had 21 polymorphic sites, 10 of which coded for differences in the amino acid sequence. Of these, 5 nucleotid changes also changed the loading of the protein (see table 1A for the details).
The amino acid sequence of the 6 exons on chromosome 7 had 11 polymorphic sites, 4 of which amino acids. Only 1 of these substitutions changed the loading of the protein (see table 1B for the details).
Table 2 shows the statistical approach, by means of Fisher exact tests, to prove there is no association between polymorphisms that code for different amino acids and change the loading of the protein, and allozyme genotypes, except for the polymorphism on LGE22, exon 2 (p = 0.018).
The amino acid sequences of the gene on LGE22, chromosome 7 and the chicken amino acid sequence found by Zucker are given in Supplementary table S3.

Table 1A. The polymorphisms found in the G3PD exons on linkage group 22.

#	Exon	Base # in the exon	BPs Found	Coding	Possible amino acids	Side chain charge	Site number in table 3
1	2	3	AC / CC	No			
2	2	18	AA / AG / GG	No			
3	2	36	AA / AG / GG	No			
4	2	46	AA / AG / GG	Yes	Glycine (G) / Serine (S)	Neutral / Neutral	29
5	2	61	AC / CC	Yes	Lysine (K) / Glutamine (Q)	Positive / Neutral	34
6	2	150	GG / TT	No			
7	2	158	AA / AG	Yes	Histidine (H) / Agrinine (R)	Pos-neutral / Positive	57
8	2	170	CC / CG	Yes	Proline (P) / Arginine (R)	Neutral / Positive	70
9	3	19	CC / CT	Yes	Phenylalanine (F) / Leucine (L)	Neutral / Neutral	79
10	3	42	CC / CT / TT	No			
11	3	72	CT / TT	No			
12	3	118	CC / GG	Yes	Alanine (A) / Proline (P)	Neutral / Neutral	112
13	3	133	CC / TT	Yes	Phenylalanine (F) / Leucine (L)	Neutral / Neutral	117
14	4	3	CC / TT	No			
15	4	10	AG / GG	Yes	Glutamic acid (E) / Lysine (K)	Negative / Positive	123
16	4	123	AC / CC	Yes	Phenylalanine (F) / Leucine (L)	Neutral / Neutral	160
17	4	138	CC / CT	No			
18	6	108	AA / GG	No			
19	6	165	CC / CT / TT	No			
20	7	16	CC / GG	No			
21	7	23	AG / GG	Yes	Aspartic acid (D) / Glycine (G)	Negative / Neutral	298

Table 1B. The polymorphisms found in the G3PD exons on chromosome 7.

#	Exon	Base # in the exon	BPs Found	Coding	Possible amino acids	Side chain charge	Site # in table 3
1	4	14	AA / GG	Yes	Asparctic acid (D) /Glycine (G)	Negative / Neutral	124
2	4	36	CC / CG	Yes	Isoleucine (I) / Methionine (M)	Neutral / Neutral	131
3	4	51	AA / AG	No			
4	5	27	AC / CC	No			
5	6	6	CC / CT	No			
6	6	48	CC / CT / TT	No			
7	6	66	AA / AG / GG	No			
8	6	116	AG / GG	Yes	Cysteine (C) /Tyrosine (Y)	Neutral / Neutral	242
9	7	84	AG / GG	No			
10	8	43	GG / GT	Yes	Cysteine (C) /Glycine (G) / Valine (V)	Neutral / Neutral / Neutral	331
11	8	44	GG / GT	Yes			

Table 2. Contingency tables showing the relationship between polymorphisms that code for changes in the amino acid chain that alter the loading of the protein and G3PD allozyme genotype. Significance of the association was tested with Fisher's exact test.

(a) LGE 22, Exon 2. Polymorphism number 5 in table 1A. The given table has probability; p = 0.2

Allozyme	# of indiv	AC	CC
11	5	0	5
12	3	0	3
13	2	1	1
All	10	1	9

(b) LGE 22, Exon 2. Polymorphism number 7 in table 1A. The given table has probability; p = 0.2

Allozyme	# of indiv	AA	AG
11	5	5	0
12	3	3	0
13	2	1	1
All	10	9	1

*(c) LGE 22, Exon 2. Polymorphism number 8 in table 1A. The given table has probability; p = 0.018**

Allozyme	# of indiv	CG	GG
11	4	0	4
12	3	3	0
13	1	0	1
All	8	3	5

(d) LGE 22, Exon 4. Polymorphism number 15 in table 1A. The given table has probability; p = 0.7

Allozyme	# of indiv	AG	GG
11	5	1	4
12	2	0	2
13	0	0	0
All	7	1	6

(e) LGE 22, Exon 7. Polymorphism number 21 in table 1A. The given table has probability; p = 0.3

Allozyme	# of indiv	AG	GG
11	4	0	4
12	2	1	1
13	2	0	2
All	8	1	7

(f) Chr 7, Exon 4. Polymorphism number 1 in table 1B. The given table has probability; p = 0.2

Allozyme	# of indiv	AA	GG
11	5	0	5
12	2	0	2
13	2	1	1
All	9	1	8

Exon 2, LGE22

In the initial scan of the sequence of the gene on LGE22 one polymorphism in exon 2 (at the 170th base of exon 2) matched the allozyme variation. This batch of samples consisted of 8 individuals. 4 Birds with allozyme phenotype 11 (3 from Cape Verde, 1 from Kenya), 3 birds with allozyme phenotype 12 (all Cape Verde) and 1 bird with allozyme phenotype 13 (from Kenya). The "12" birds were heterozygous at the site with CG, whereas the "11" and "13" birds were homozygous with GG. This was fully consistent with the hypothesis that this mutation underlies the change in loading in the "2" allele. We thus investigated this site in more detail.

In this further analysis, we sequenced exon 2, which included the SNP associated with the allozyme, for 55 individuals (28 individuals with allozyme phenotype 11, 16 birds with genotype 12, and 11 birds which had one of the rarer phenotypes). In this larger set of samples, also hailing from more populations than the initial set, the analysis of sequence variation was not limited to birds with the "12" allozyme genotype anymore. The result using this enlarged sample indicates that the S allele was not significantly associated with a certain base pair (Fishers exact test; p = 0.082). Also when we expand the table slightly by looking at homozygosity, heterozygosity or absence of the S allele, no significant association is found (see table 3A).

Several birds did not yield reliable sequences (these are not included in the 55 individuals mentioned above). Since was no statistical evidence for an association, we decided not to rerun these. Some of these birds had rare genotypes. Therefore 15, 18 (both limited to the Rybatchy population) and 19 (a single bird in la Palma) are not represented in the overview. (See Supplementary table S4 for the raw data of all 55 birds). We further did not find any evidence for a genetic structure of populations at this locus, SNPs not being confined to particular populations (see table 3B).

Table 3A. Contingency table for the extended dataset showing the relationship between the polymorphism at base pair site 170 of Exon 2 on LGE22 and the G3PD allozyme genotype (i.e. presence/absence of the S=2 allele). Significance of the association was tested with Fisher's extact test (P=0.0119).

	CC	GC	GG
S Homozygote	0	0	2
S Heterozygote	0	4	12
S Absent	0	2	35
All	0	6	49

Table 3B. Contingency table for the extended dataset testing among-population heterogeneity in the distribution of genotypes at position 170 of Exon 2 on LGE22. (Fisher's exact test, P=0.303).

Population	#	CG	GG	Allozyme distribution
Austria	2	1	1	CG; 1 x 14. GG; 1 x 14
Cape Verde	16	3	13	CG; 3 x 12. GG; 8 x 11, 3 x 12, 2 x 22
Catalonia	3	0	3	GG; 2 x 16, 1 x 17
Gibraltar	8	0	8	GG; 5 x 11, 3 x 12
Kenya	8	1	7	CG; 1 x 13. GG; 5 x 11, 2 x 13
La Palma	6	1	5	CG; 1 x 12. GG; 5 x 11
Madrid	12	0	12	GG; 5 x 11, 6 x 12, 1 x 17
All	55	6	49	

Discussion

In the sequences that we obtained of G3PD in the blackcap, we did not find any polymorphism associated with the allozyme variation that was previously established. Therefore, we were unable to develop a marker for G3PD linked to migratory behaviour.

For the failure to find the sequence variation underlying different allozymes at this locus, we could think of two possible explanations:

(1) Limitations of the methods or the design of the study:
In both genes we could only sequence 6 exons, while 8 exons have been described in chickens and zebra finches. Similarly, 8 exons were found in other animal groups (.e.g. *Drosophila*) (See UCSC Genome Browser). In our study, chicken exon 1 and 8 were missing on LGE22 and chicken exon 1 and 3 were missing on chromosome 7. We are not completely sure whether blackcaps actually have less exons or whether we failed to sequence this exons because we did not find the appropriate primers. However, it seems unlikely that blackcaps have a different number of exons, since it has been suggested that the GPDH gene-enzyme system is highly conserved and is evolving slowly (e.g. Bewley *et al.* 1989, Carmon and MacIntyre 2010). If this was the case, it possible that the mutation causing the enzyme polymorphism is on a site locate within one of the missing exons.

(2) Biological reasons due to which we did not find an associated polymorphism:
It is possible that the allozyme polymorphism is not caused by sequence variation. It may, for instance, be generated by secondary protein modifications. However, we have evidence that it is inherited and that it has a Mandelian segregation (Pulido *et al.* unpublished). Also, if this was the case, we would not expect to find differences among populations. A second biological reason could be that the enzyme is synthesized through a process of complex splicing (see, Cook *et al.* 1988). However, given the reliability of results and the normal segregation of G3PD alleles, we would consider this explanation unlikely.

Conclusion
It seems that there is no association between polymorphisms of the G3PD gene and its allozyme variation in blackcaps, at least not for the part of the gene we sequenced. It is likely that the mutation underlying the G3PD-S allozyme is located on one of the four exons that we could not sequence. By sequencing the whole genome or, at least the complete gene, it may be possible to identify the base

substitution causing this change. This may fill in the missing parts of the possibly incomplete sequence that we have at the moment, enabling us to give any definite conclusion about this.

Supplementary material

Supplementary table S1A. The primers used to sequence G3PD on chromosome 7.

Pair	Forward					Reverse					Base pair	PCR
	in/Near Exon		Length	Ann. Temp	Sequence	in/Near Exon		Length	Ann. Temp	Sequence	Length	Temp
1	Intronic	1	21	63	GGGCTGAGAGGYRAMATATGG	Intronic	1	22	63.1	GCAGMAGYCARTGAGAAYTGGA	981	63
2	Intronic	2	27	59.7	RGRGTAGTGGATAAAGCTTTCTGATAA	Exonic	3	19	63.3	TGGCTGTACCAGACGTGGC	2216	61
3	Exonic	3	20	63.9	ACCAGACGTGGCTGAAGCAG	Exonic	4	20	62.3	GTCCTSAKGGCCTGAAGCTC	1368	62
4	Exonic	4	22	61.2	AGTTCTGTGAAACCACCATTGG	Exonic	5	23	61.6	TGATACAGTGGAGMTYTGTGGAG	1753	61
5	Exonic	5	20	59.5	GGAGMTYTGTGGAGCCTTAA	Exonic	6	21	62.2	CCTYATGGARATGGTGGCCTT	1692	61
6	Exonic	6	19	62.2	GARGCCTTTGCTCGCACAG	Exonic	7	26	63	TGGAAAAKGAGATGCTGAATGGACAA	213	63
7	Exonic	7	24	56.8	GCTGAAGTCTACAARATTCTGAAA	Exonic	8	21	57	CAACCATCTACAAGATCTGCT	1440	57
8	Intronic	5	18	63.4	CMCTGGCWTCCAGCAGCA	Intronic	5	26	58.2	GCWGGCAAAGCATTTMAAAATAKYTG	587	61
9	Intronic	6	19	58.9	CTGCTYTGRKKTTTGCCCA	Intronic	6	21	63.2	CAGTATYCCCTCTGAYCC	606	61
10	Intronic	7	19	62.5	GGAAAGCTGTGGGGTTGCT	Intronic	7	24	62.8	AACAACWGCTTTCTACACCTTGGG	474	63
11	Intronic	8	22	60.2	AGMAaGGAAAAGRGGATATGCA	Intronic	8	21	60.5	GGAAAGCTGTGGGGTTGCT	661	61
12	Exonic	7	24	58.1	GCTGAAGTCTACAARATTCTGAAA	Intronic	8	21	60.5	TGAAAACAGTKCCCCTTGTRA	1786	60
13	Intronic	2	19	59.7	CAMGTGGGTCAGAGGAWAA	Intronic	2	20	60.7	AACCCACAGCATCTTCTGGA	1437	60
14	Intronic	3	20	60.2	GCACACAGTTCTCCTGGGTT	Intronic	3	18	59.7	aGCCCTCAGCTGCAYRCT	1327	60
15	Intronic	4	18	60.3	GGCTGTACCAGACGTGGC	Intronic	4	24	59.9	RccCAGTAACATTCTGTTCYTCTC	1929	60
16	Exonic	2	26	63.1	TGTCAAGATGTGGGTATTTGAAGAGA	Exonic	3	19	63.3	TGGCTGTACCAGACGTGGC	1997	63

	Forward				Reverse				
Pair	Near Exon	Length	Ann. Temp	Sequence	Length	Ann. Temp	Sequence	Base pair Length	PCR Temp
1	1	15	56	GYGSCSGGCGCTCAC	20	62	GGGcaCAGCCTTSAAATAGC	115	60
2	2	17	61.4	AGSTCASYGGGARSGCA	17	61.4	GGCAAYTGGTGAGCGCC	691	61
3	3	20	60.3	CCATCTGGTCCTTCATCCAC	18	60.9	CTGGCATCYCACRGGCTG	746	60
4	4	19	59.1	GCTGGCAGGARCTTYYAGM	18	58	SARTGGCAGGATTGGGRTG	527	59
5	5	19	59.9	CCANCRCAGAAACCAGCYC	21	59.5	CTSTGTGGRCCTGCRAATGTT	455	60
6	6	18	62.4	GCTCTGCAGGGAGCWCKG	22	60	CTTCTCCAGCTGCTCAATRGRC	803	62
7	7	18	58.2	AGTGCYRYARCCCAGCTC	23	54.9	ATCTGATASACAGCWGTGAAGAG	416	59
8	8	21	58.5	ATTGAGCAGCTGGAGAAGGAG	20	58.3	CTGGACTCCTYMCAARGCTG	503	60
9	1	20	60.3	AGGTCACTCTCCTCCTTGAG	19	58.8	CTCCAGCACCCACATGTTC	2500	60
10	1	22	59.4	CTCTCCTCCTTGAGGAAGTTAC	19	58.8	CCAGCACCCACATGTTCAC	2500	59
11	8	17	57.6	GGCAGAAGCTGCAGGGT	18	58.2	GAAGATGCTCAGCCCTGC	647	58
12	1	19	70.1	CGATGGCCGAGCCCCTGCG	19	73.1	GSMCGGASCGGTGCCCGGT	336	72
13	2	21	61.3	GCWGCAGCTCATCCMARMACA	21	61.2	GAARGTKTGCATCGTGGGCTC	739	62
14	7	x	x	with the previously used Forward	23	58	TTCTCCACTGCATTCTTGCTCTT	181	59
15	7	x	x	with the previously used Forward	20	56.8	CAGCACTCACTTCTCCACTG	191	59
16	7	24	60.5	ATTGAGCAGCTGGAGAAGGAGATG	22	61.5	CTCAGGGTGGTTCTGGAGACAC	379	62
17	8	22	57.8	AGAGCAAGAATGCAGTGGAGAA	18	60.3	TGGTGTRGGYRGGAGCTC	413	59

Supplementary table S1B. The primers used to sequence G3PD on Linkage group 22.

Supplementary table S2. The basic PCR conditions used in this study.

PCR Mix, in µl	
DNA	1
H_20	12.5
2.5Mm $MgCl_2$	1.6
10xP + NH_4	2
dNTPs	0.4
Primer Forward	1
Primer Reverse	1
Taq	0.5
total	20

PCR programm		
95°C	5'	
94°C	30''	33 cycles
*°C	30''	
72°C	1'	
72°C	15'	
4°C	∞	

** mean annealing temperature of the primers*

Supplementary table S3. Amino acid sequences coding for protein synthesis on Chromosome 7 & LGE 22, including the chicken sequence from Zucker (1987)

	Position Amino Acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Zucker	Chicken	G	G	K	K	V	C	I	V	G	S	G	N	W	G	S	A	J	A	K	I	V	G	S	N	A	A	R
LGE22	Blackcap	~	~	~	~	~	~	~	~	~	~	~	~	~	G	S	A	I	A	K	I	V	G	S	N	A	A	R
Chr7	Blackcap	~	~	~	~	~	~	~	~	~	~	~	~	~	G	S	A	I	A	R	I	I	G	K	N	V	Q	K
	Exons	Exon 1													Exon 2													

	Position Amino Acid	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
Zucker	Chicken	L	T	T	F	E	N	T	V	N	M	W	V	L	E	E	E	V	G	G	R	R	L	T	E	J	J	N
LGE22	Blackcap	L	S/G	S	F	E	S	Q/K	V	N	M	W	V	L	E	E	E	V	G	G	R	R	L	T	D	I	I	N
Chr7	Blackcap	S	N	R	F	D	P	T	V	K	M	W	V	F	E	E	I	I	N	G	R	K	L	S	E	I	I	N
	Exons	Exon 2																										

	Position Amino Acid	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81
Zucker	Chicken	T	E	H	E	N	V	K	Y	L	P	G	H	K	L	P	P	N	V	V	A	E	P	D	L	V	K	A
LGE22	Blackcap	T	E	H/R	E	N	V	K	Y	L	P	G	H	K	L	P	P/R	N	V	V	A	E	P	D	L	L/F	K	A
Chr7	Blackcap	Q	E	H	E	N	V	K	Y	L	P	G	Y	K	I	P	H	N	V	~	~	~	~	~	~	~	~	~
	Exons	Exon 2													Exon 3													

	Position Amino Acid	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
Zucker	Chicken	A	A	G	A	D	I	L	L	F	V	V	P	H	Q	F	I	G	K	V	C	D	E	J	K	A	H	V
LGE22	Blackcap	C	A	G	A	D	I	L	L	F	V	V	P	H	Q	F	I	G	K	V	C	D	Q	L	K	G	H	V
Chr7	Blackcap	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~
	Exons	Exon 3																										

	Position Amino Acid	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135
Zucker	Chicken	K	A	G	A	I	G	M	S	L	I	K	G	V	D	E	G	P	D	G	L	R	L	I	S	D	I	I
LGE22	Blackcap	K	K	E	A/P	V	G	M	S	L/F	I	K	G	V	D	E/K	G	P	D	G	L	R	L	I	S	D	I	I
Chr7	Blackcap	~	~	~	~	~	~	~	~	~	~	~	G	I	D	E	G/D	P	D	G	L	K	L	I/M	S	D	L	I
	Exons	Exon 3								Exon 4																		

	Position Amino Acid	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162
Zucker	Chicken	H	E	K	L	G	I	E	M	S	V	L	M	G	A	N	I	A	S	E	V	A	E	E	K	F	C	E
LGE22	Blackcap	R	E	K	L	G	I	E	M	N	V	L	M	G	A	N	I	A	T	E	V	A	E	E	K	F/L	C	E
Chr7	Blackcap	R	E	Q	L	K	I	E	M	S	V	L	M	G	A	N	I	A	K	E	V	A	D	E	K	F	C	E
	Exons	Exon 4																										

	Position Amino Acid	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189
Zucker	Chicken	T	T	I	G	C	K	N	A	Q	Y	G	Q	I	L	K	E	L	M	Q	T	P	N	F	R	V	T	V
LGE22	Blackcap	T	T	I	G	C	K	N	T	K	H	G	Q	M	L	K	D	L	M	Q	T	P	N	F	R	V	S	V
Chr7	Blackcap	T	T	I	G	C	K	N	K	T	Q	G	E	I	F	K	E	L	M	Q	T	P	N	F	R	I	T	V
	Exons	Exon 4				Exon 5																						

	Position Amino Acid	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216
Zucker	Chicken	V	Q	E	A	D	T	V	E	I	C	G	A	L	K	N	I	V	A	V	G	A	G	F	C	D	G	L
LGE22	Blackcap	V	Q	E	A	D	T	V	E	I	C	G	A	L	K	N	V	V	A	V	G	A	G	F	C	D	G	L
Chr7	Blackcap	V	L	D	S	D	T	V	E	L	C	G	A	L	K	N	I	V	A	V	G	A	G	F	C	D	G	L
	Exons	Exon 5														Exon 6												

	Position Amino Acid	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243
Zucker	Chicken	G	F	G	D	N	T	K	A	A	V	I	R	L	G	L	M	E	M	I	S	F	A	K	I	F	C	K
LGE22	Blackcap	G	Y	G	D	N	T	K	A	A	V	I	R	L	G	L	M	E	M	I	G	F	A	K	L	F	C	K
Chr7	Blackcap	S	F	G	D	N	T	K	A	A	V	I	R	L	G	L	M	E	M	V	A	F	A	K	M	F	C/Y	K
	Exons	Exon 6																										

	Position Amino Acid	244	245	246	247	248	249	250	251	252	253	254	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271
Zucker	Chicken	G	P	V	T	P	S	T	F	L	E	S	G	V	A	D	L	I	T	T	C	Y	G	G	R	N	R	K
LGE22	Blackcap	G	S	V	T	S	S	T	F	L	E	S	G	V	A	D	L	I	T	T	C	Y	G	G	R	N	R	K
Chr7	Blackcap	G	P	V	S	T	A	T	F	L	E	S	G	V	A	D	L	I	T	T	C	Y	G	G	R	N	R	K
	Exons	Exon 6																										

Table S3 (continued)

	Position Amino Acid	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298
Zucker	Chicken	V	A	E	A	F	A	K	T	G	K	S	I	E	Q	L	E	K	E	M	L	Q/N	G	Q	K	L	Q	G
LGE22	Blackcap	V	A	E	A	F	A	K	T	G	K	~	~	~	~	~	~	~	~	~	M	N	G	Q	K	L	Q	D/G
Chr7	Blackcap	V	A	E	A	F	A	R	T	G	K	S	I	E	E	L	E	K	E	M	L	N	G	Q	K	L	Q	G
	Exons	Exon 6										Exon 7																

	Position Amino Acid	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325
Zucker	Chicken	P	Q	T	S	A	E	L	N	H	I	L	K	T	K	N	M	V	D	K	F	P	L	F	T	A	V	Y
LGE22	Blackcap	P	Q	T	S	A	E	L	H	R	I	L	K	S	K	N	A	V	E	~	~	~	~	~	~	~	~	~
Chr7	Blackcap	P	Q	T	S	A	E	V	Y	K	I	L	K	Q	K	N	M	L	Q	R	F	P	L	F	T	A	I	Y
	Exons	Exon 7																		Exon 8								

	Position Amino Acid	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348
Zucker	Chicken	Q	I	C	Y	E	G	K	P	V	S	D	V	I	K	C	L	Q	N	H	P	E	H	M
LGE22	Blackcap	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~
Chr7	Blackcap	K	I	C	Y	E	G/V/C	R	S	I	Q	D	F	I	M	C	L	Q	N	H	P	E	H	M
	Exons	Exon 8																						

Supplementary table S4. Genotypes at base pair site 170 of exon 2 on LGE22 and G3PD allozyme genotypes in birds of the extended dataset.

Bird	Population	Allozyme	Base Pair
1	Austria	14	CG
2	Austria	14	GG
3	Cape Verde	11	GG
4	Cape Verde	11	GG
5	Cape Verde	11	GG
6	Cape Verde	11	GG
7	Cape Verde	11	GG
8	Cape Verde	11	GG
9	Cape Verde	11	GG
10	Cape Verde	11	GG
11	Cape Verde	12	CG
12	Cape Verde	12	CG
13	Cape Verde	12	CG
14	Cape Verde	12	GG
15	Cape Verde	12	GG
16	Cape Verde	12	GG
17	Cape Verde	22	GG
18	Cape Verde	22	GG
19	Catalonia	16	GG
20	Catalonia	16	GG
21	Catalonia	17	GG
22	Gibraltar	11	GG
23	Gibraltar	11	GG
24	Gibraltar	11	GG
25	Gibraltar	11	GG
26	Gibraltar	11	GG
27	Gibraltar	12	GG
28	Gibraltar	12	GG
29	Gibraltar	12	GG
30	Kenya	11	GG
31	Kenya	11	GG
32	Kenya	11	GG
33	Kenya	11	GG
34	Kenya	11	GG
35	Kenya	13	CG
36	Kenya	13	GG
37	Kenya	13	GG
38	La Palma	11	GG
39	La Palma	11	GG
40	La Palma	11	GG
41	La Palma	11	GG
42	La Palma	11	GG
43	La Palma	12	CG
44	Madrid	11	GG
45	Madrid	11	GG
46	Madrid	11	GG
47	Madrid	11	GG
48	Madrid	11	GG
49	Madrid	12	GG
50	Madrid	12	GG
51	Madrid	12	GG
52	Madrid	12	GG
53	Madrid	12	GG
54	Madrid	12	GG
55	Madrid	17	GG

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Jon, faith brought us together, and even though our start seemed a bit awkward, I now consider you one of my best friends. Thanks for all the lovely visits and unusual conversations we’ve had. Let’s think about where to meet next! Jenny thanks for the amazing drawings for my thesis and the painting on my wall!

Que lastima para mi que has ido a Berlin, Fernando. Pero me da igual, ahora hay un razon más de visitor a Berlin más a menudo. Es siempre un placer de estar contigo. Siempre interesante y muy relajado. Tengo muchos recuerdos bonitos de las montañas, el teatro y de Mallorca, …
Diego, gracias por ser tú. Nuestro viaje nocturno a La Pedriza con las cabras luchando por la mañana es una de las cosas más chulas que he hecho.

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