Avian evolutionary genomics:
studies of *Ficedula* flycatchers

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AVIAN EVOLUTIONARY GENOMICS: STUDIES OF *FICEDULA* FLYCATCHERS

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The thesis is based on the following articles, which will be referred to in the text by the Roman numerals:

I. Buggiotti L, Hellström MA, and Primmer CR. 2006. Characterization of the first growth hormone gene sequence for a passerine bird--the pied flycatcher (*Ficedula hypoleuca*). *DNA Sequence* 17:401-6.

II. Buggiotti L and Primmer CR. 2006. Molecular evolution of the avian growth hormone gene and comparison with its mammalian counterpart. *Journal of Evolutionary Biology* 19:844-54.

III. Buggiotti L, Bures S, and Primmer CR. Characterisation of Z-linked genes in *Ficedula* flycatchers and their possible role in post-zygotic isolation. *Manuscript*.

IV. Buggiotti L, Saetre G-P, Bures S, and Primmer CR. The genetic basis of colouration and reinforcement in *Ficedula* flycatchers: a splicing variant in the sex-linked *TYRP1* is a strong candidate for contributing to pre-zygotic isolation in a flycatcher hybrid zone. *Manuscript*.

V. Buggiotti L, Corthals GL, Saloniemi I, Primmer CR, and Leder EH. Proteomic analyses reveal novel protein expression patterns in natural *Ficedula* flycatcher hybrids. *Manuscript*.

VI. Buggiotti L, Corthals GL, Kovonen P, Bures S, Primmer CR, and Leder EH. Identification of differentially expressed proteins in *Ficedula* flycatchers. *Manuscript*.

ABSTRACT

In this thesis, different genetic tools are used to investigate both natural variation and speciation in the *Ficedula* flycatcher system: pied (*Ficedula hypoleuca*) and collared (*F. albicollis*) flycatchers. The molecular evolution of a gene involved in postnatal body growth, *GH*, has shown high degree of conservation at the mature protein between birds and mammals, whereas the variation observed in its signal peptide seems to be adaptive in pied flycatcher (I & II). Speciation is the process by which reproductive barriers to gene flow evolve between populations, and understanding the mechanisms involved in pre- and post-zygotic isolation have been investigated in *Ficedula* flycatchers. The Z chromosome has been suggested to be the hotspot for genes involved in speciation, thus sequencing of 13 Z-linked coding genes from the two species in allopatry and sympatry was conducted (III). Surprisingly, the majority of Z-linked genes seemed to be highly conserved, suggesting instead a potential involvement of regulatory regions. Previous studies have shown that genes involved in hybrid fitness, female preferences and male plumage colouration are sex-linked. Hence, three pigmentation genes have been investigated: *MC1R*, *AGRP*, and *TYRP1*. Of these three genes, *TYRP1* was identified as a strong candidate to be associated with black-brown plumage variation in sympatric populations, and hence is a strong candidate for a gene contributing to pre-zygotic isolation (IV). In sympatric areas, where pied and collared flycatchers have overlapping breeding areas, hybridization sometimes occurs leading to the production of unfit hybrids. By using a proteomic approach a novel expression pattern in hybrids was revealed compared to the parental species (V) and differentially expressed proteins subsequently identified by sequence similarity (VI). In conclusion, the Z chromosome appears to play an important role in flycatcher speciation, but probably not at the coding level. In addition the novel expression patterns might give new insights into the maladaptive hybrids.

CONTENTS

INTRODUCTION

Evolutionary genomics

Evolutionary genomics represents a growing field that has undergone tremendous progress during the last decade. The key word in evolutionary genomics is "*integration*", in fact only the integration of the fields of molecular functional genetics, ecology and evolution will provide a comprehensive understanding of organismal biology. In particular, molecular functional genetics has the potential to reveal the genes and genetic variation responsible for phenotypic output. Ecology gives insights concerning interactions among individuals and species and their environments, as well as how species survive and reproduce. And evolution provides details of how forces such as selection, genetic drift, and evolutionary history have shaped the pattern of variation at either or both the molecular and phenotypic levels. Although gaps still exist between these fields the growing understanding of genes, their diversity and regulation, and how they work together in networks of interacting elements, has allowed the emergence of a new era for biology. In fact, the genome is coming to be seen as a complex net of information enabling the formation of a distinct phenotype, and the challenge is then to be able to use this net of information as a link between genomic and phenotypic diversity within species.

During the last decade, whole-genome sequences of various organisms (a total of 665 genomes published to date, where only 72 are Eukaryotes; www.genomesonline.org), written in the nucleotide code, have enabled organism comparisons and opened the door to a wider understanding of some of the genetic differences underlying the molecular evolution patterns of the organisms observed today. We are now living in the post-genomic era where all the information available for a variety of organisms is waiting to be explored. The hidden key for this exploration is the integration of the already established wealth of knowledge in the above mentioned fields. In fact, the nucleotide code itself, retaining all the genetic building block information, might not be easy to interpret, hence a cooperative effort will provide a greater understanding of the biological history of a given organism.

Evolutionary genomics studies in model vs non model organisms

Our biological knowledge is mainly based on model organisms such as the fruit fly (*Drosophila melanogaster*), yeast *(Saccharomyces cerevisiae)*, roundworm (*Caenorhabditis elegans*), mustard plant (*Arabidopsis thaliana*), zebrafish (*Danio rerio*), chicken (*Gallus gallus*), mouse (*Mus musculus*), and human (*Homo sapiens*). Although these have helped unravel some of the intricate mysteries of cell communication, genetics, and embryonic development, a wealth of evolutionary and ecological questions remain unaddressed, as does understanding the forces that create and maintain phenotypic variation. In fact, understanding how organisms respond to different environments and interact among themselves at the genomic level is still a big challenge for biology today (Crawford 2001, Rockman and Kruglyak 2006).

In the post-genomic era, transfer of the knowledge gained from model organisms to non-model organisms is providing insight into the ecology and evolution of different lineages, with the potential of revealing biological mechanisms as yet unknown. Although living creatures look and behave in many different ways, all of their genomes consist of DNA and/or RNA, and genomic data have revealed that genes are remarkably conserved throughout different species. However, it is not all about genes, as their regulation seems to be the main factor that gives rise to the astonishing diversity of creatures. Thus, genomic sequence information and new technological and bioinformatics platforms now enable comprehensive surveys of neutral and adaptive variation in model *vs* non-model organisms. Many interesting behavioural, physiological or ecological traits and responses are poorly expressed or absent in the genetic model organisms, but the genomic information now available can be used as exploratory tools in non-model organisms (Ungerer et al. 2007). In addition, functional genomics, in terms of expression profiles (gene/protein expression) represents an example of a synergistic way to infer adaptive variation in non-model organisms in their natural settings. Only by increasing the application of the newly developed techniques to non-model organisms will it be possible to shed light on environmental modifications of gene expression (Feder and Mitchell-Olds 2003, Thomas and Klaper 2004).

The question of whether there is a need for non-model organisms to become model organisms is controversial and in my opinion unnecessary. In fact, we expect a multidisciplinary approach to provide the answer instead. In particular, the combination of organismal analysis with molecular genetics and genomics, laboratory experiments (model organisms) along with studies in natural settings (non-model organisms) will enable the understanding of organismal biology.

Molecular evolution in birds: an overview

In molecular evolutionary genetics understanding how various evolutionary forces interact to determine the amount and type of genetic variation in natural populations is of great interest. Thus, the sequencing of the chicken (*Gallus gallus*) genome opened the door for a new bird-era allowing comparative studies between avian and non-avian genomes and among natural bird populations. In fact, the chicken genome represents the first and most important source of bird genomic data available so far (ICGSC 2004) and constitutes a very useful tool for exploring molecular evolution in a wide range of avian species.

Although the chicken genome represents the most important source of data for the inference of molecular evolution at DNA, RNA, or protein levels within avian species, sequence data from other birds are also important. Genomic sequences and expressed sequence tags (ESTs) are now available from various species, leading to an exponential increase in molecular evolution studies in birds (Cogburn et al. 2003, Agate et al. 2004, Edwards and Dillon 2004, Hillier et al. 2004, Mello 2004, Sundstrom et al. 2004, Berlin and Ellegren 2006). In addition, a survey to study the genetic basis of phenotypic traits (ICPMC 2004) revealed 2.8 million single nucleotide polymorphisms (SNPs), highlighting the high level of genetic diversity in chicken. This finding is certainly of great interest as

the SNPs can be used as genetic markers in population genetic studies, providing insights for genetic variability across genomes in natural bird populations. Another feature of the chicken genomic information is that it enhances the possibility of expression analysis studies (Abzhanov et al. 2006, Kaiser 2006), which integrated with avian molecular evolution studies has the potential to provide answers to fundamental questions such as the identification of molecular changes and forces responsible for various phenotypes in birds. In addition, with the up-coming zebra finch (*Taeniopygia guttata*) whole genome sequence the possibility to understand the intimacy of molecular evolution in birds will become a reality.

Positive selection

The molecular evolution field emerged during the 1960s with the aim being to understand the structure and function of nucleic acids and proteins. The recent advances in genomics have led to an increase in the number of studies in this field with the focus being to understand the extent of adaptive molecular evolution versus neutral drift, and the forces responsible for genotype-phenotype relationships. The neutral theory of molecular evolution (Kimura 1983) suggests that the majority of molecular differences that are fixed over evolutionary time are selectively neutral, leading to the generalization that coding regions are under purifying selection due to their functional constraints.

The evolutionary forces determining the amount and type of genetic variation can be divided into two main classes: purifying selection (eliminates deleterious mutations) and positive selection (favours advantageous mutations). The role of positive selection has been debated for many years without a clear resolution. In fact, different methods have been used to identify the rate of adaptive evolution in coding sequence at the molecular level (Suzuki and Gojobori 1999, Kreitman 2000, Bamshad and Wooding 2003, Suzuki 2004, Massingham and Goldman 2005, Pond and Frost 2005, Zhang et al. 2005), with the non-synonymous to synonymous substitution ratio (d_N/d_S or Ka/Ks) being the most frequently used. Accordingly, when the ratio is > 1 non-synonymous substitutions occur more often than synonymous substitutions and they are driven to fixation by positive selection, whereas when the ratio is < 1 purifying selection is acting to remove deleterious mutations. This test can be considered to be conservative as the majority of non-synonymous substitutions are expected to be deleterious, hence the general trend is that d_N tends to be lower than d_S unless there is adaptive evolution. However, this comparison might underestimate the extent of purifying selection on coding sequences. By using this approach many genes have been suggested to have evolved under the forces of positive selection (Wittbrodt et al. 1989, Ting et al. 1998, Fossella et al. 2000, Barbash et al. 2003, Presgraves et al. 2003, Swanson et al. 2003). In birds, where the chromosomes are classified into macrochromosomes (1-5) and microchromosome (6-38) according to their different lengths, it has been observed that the d_N/d_S ratio is higher for genes on macrochromosomes than on microchromosomes (Axelsson et al. 2005), suggesting that fast evolving genes are not randomly distributed in the genome.

However, natural selection favouring phenotypic adaptations might follow

different routes other than only repeated amino acid (aa) replacement. In fact, it might well be that many changes at the phenotypic level are caused by different expression patterns (Carroll 2005), hence regulatory sequences might be of central importance for the different selection modes. Recently, Hughes (2007) discussed the misguided quest for positive selection, suggesting that one possible explanation for persisting in using the d_N/d_S model for detecting positive selection, even when it is unlikely to be applicable, is a case of "l'*effect réverbére*: for the proverbial drunk who searches for his lost keys under the streetlamp, not because that is where he lost them but because the light is better there". Thus, only with advances in statistical methods, combined with the generation of new genomics resources, might we be able to shed light along the entire street, and hence find the right key.

Comparative approach: Aves vs Mammalia

Whole genome sequencing provides detailed sequence information for comparative studies of genome evolution beyond the level of individual genes. The chicken has a relatively small genome of about 1200 million base pairs (Mbp), about 40% of the size of the human genome. One distinct feature of avian genomes is that they contain chromosomes of different lengths, classified as macrochromosomes and microchromosomes, whereas mammalian chromosomes are more equal in size. A comparison of about 7000 orthologous genes shared between human and chicken revealed a mean d_N/d_S of 0.06 (ICPMC 2004), whereas in comparisons of mouse-rat and human-chimpanzee genes mean d_N/d_S values were 0.13 and 0.2,

respectively (CSAC 2005). In addition, the mean was higher for genes on macrochromosomes than on microchromosomes suggesting that the fast evolving genes are not randomly distributed across the genome.

Another peculiar future of the avian genome is represented by the different sex-chromosome system, ZW, compared to mammals, XY. Birds differ from mammals in that the female is the heterogametic sex (ZW) while males are homogametic (ZZ). It has been shown that the avian sex chromosomes evolved from different pairs of autosomes than the mammalian sex chromosomes (Fridolfsson et al. 1998), and comparison of the chicken Z chromosome to the mammalian genome found it to be orthologous to human chromosome 9 (Nanda et al. 1999, Nanda et al. 2000). According to the faster-male theory, where mutations are usually occurring faster in males due to physical factors such as spermatogenesis, and considering that males are the homogametic sex in birds, selection of favourable semi-recessive mutations on the Z chromosome would likely to be increased in females (Charlesworth et al. 1987). Interestingly, it seems that the level of genetic diversity is reduced on the Z chromosome (Sundstrom et al. 2004, Borge et al. 2005), likely explained by the lower recombination rate compared to the autosomes. An alternative explanation may be that as the sex chromosomes are enriched with genes crucial for reproductive isolation (Price and Bouvier 2002) among closely related species (see below), a selective sweep might reduce the level of polymorphism, hence the Z chromosome is of special interest for dissecting the molecular evolution of potential "speciation genes".

Speciation and evolution of the isolation barriers

In the course of evolution the event of the formation of a new species, and thus increasing biodiversity, is called speciation. After almost 150 years from publication of *On the origin of the species* (Darwin 1859), the understanding of speciation still remains one of the major challenges faced by evolutionary biology. The exciting research by Dobzhansky (1937) brought to the modern age the reproductive isolation species concept that was later incorporated into Mayr's biological species concept, stating that "species are groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1963).

Despite the huge variety of examples of speciation in nature and the advances in genetic and molecular techniques, surprisingly little is known about the mechanisms involved in speciation. By dissecting the genetics of speciation, studies on *Drosophila* have given insight into "speciation genes" potentially involved in reproductive isolation (Barbash et al. 2003, Gavrilets 2003, Presgraves et al. 2003, Sun et al. 2004, Ortiz-Barrientos and Noor 2005). However, one of the lessons we've learned from those studies is that only by understanding the mode of speciation can we get a clearer picture of the mechanisms and causes behind this phenomenon, and that this model system is limited for investigating the basis of speciation in natural settings.

Not surprisingly, a large number of factors, such as geographical isolation, sexual selection, and natural selection, contribute to the speciation process. From a genetic perspective reproductive barriers to gene exchange may be considered as the main players in the field, however, due to both selection on genes and gene flow, it's very difficult to evaluate and disentangle their effects on maintaining different genotypes. In fact, according to the allopatric speciation model (Coyne 1992, Gavrilets 2003, Coyne and Orr 2004) the process of speciation occurs when subpopulations of a single ancestral population become geographically isolated and embark on different evolutionary trajectories. During separate long-term evolution in geographic isolation, reproductive isolation evolves as a by-product of divergence in phenotypic and genotypic aspects of an organism. When secondary contact occurs between the diverging species, and thus their ranges overlap (sympatry), the production of unfit hybrids may reinforce reproductive isolation.

This reproductive isolation can take broadly two forms: pre-zygotic barriers, which prevent the formation of hybrid zygotes, and post-zygotic barriers, which act on hybrid fitness as well as hybrid sterility or inviability. In both cases the barriers ensure that the species remain genetically different, as they prevent gene flow in sympatry (where the species co-exist), allowing independent evolutionary fates. In addition, pre- and post-zygotic isolating mechanisms arise as by-product of genetic divergence in allopatry, and their evolution can be accelerated by divergent selection. However, hybridization and introgression occur sometimes, and thus natural hybrid zones are the ideal arenas to investigate the mechanisms behind reproductive isolation, providing valuable genetic information on the natural history of speciation and its causes (Edwards et al. 2005, Arnold and Meyer 2006, Noor and Feder 2006).

Pre-zygotic isolation

Pre-zygotic isolation can arise when populations are separated in space or time. Under natural selection allopatric species might evolve differences leading to reduced hybrid fitness after secondary contact that subsequently favour reinforcement (Dobzhansky 1937, Butlin 1987), which is the process by which mating discrimination is increased by natural selection acting against the production of unfit hybrids. Species can be recognized by their morphological characteristics and if two geographically isolated lineages diverge in male traits and female preferences, they are likely to be sexually isolated when their ranges subsequently overlap (Turelli et al. 2001). The hypothesis that pre-zygotic isolating mechanisms can be selectively strengthened, or reinforced, along the edges of a hybrid zone can be traced to Dobzhansky's (1940) writings on speciation. This process typically results in reproductive character displacement, a pattern of "greater divergence of an isolating trait in areas of sympatry between closely related taxa than in areas of allopatry" (Howard 1993). However, it should be highlighted that other processes, such as selection for specialization to different ecological conditions, might affect the pattern of character displacement (Rundle and Schluter 1998).

Reinforcement is an important component of speciation theory as it's the only speciation mechanism which involves natural selection directly for reproductive isolation (Kirkpatrick and Ravigne 2002, Servedio and Noor 2003, Butlin 2006, Lemmon and Kirkpatrick 2006). Although over the past decades theoretical and empirical studies (Howard 1993, Liou and Price 1994, Coyne and Orr 1997, Rundle and Schluter 1998, Noor 1999, Turelli et al. 2001, Butlin 2002, Pfennig 2003, Ritchie and Noor 2004, Servedio 2007) have provided strong evidence supporting the reinforcement hypothesis, it still remains one of the most intensely debated topics in speciation theory (Dobzhansky 1937, Servedio and Noor 2003, Ortiz-Barrientos et al. 2004, Servedio 2004).

Surprisingly little information exists concerning post-copulatory pre-zygotic barriers (between insemination and fertilization stages), with the major focus being invertebrates such as *Drosophila* (Coyne and Orr 1997, Noor 1999, Coyne and Orr 2004). It has been suggested that only a few genes are involved in post-copulatory pre-zygotic barriers, and potential candidates are the reproductive proteins involved in gametic interactions as it's been shown that they evolve rapidly (Vacquier 1998, Swanson et al. 2001). However, the source of this selection is not well known, but potentially due to sexual selection and inbreeding avoidance (Grant and Grant 1997, Servedio 2001, Woodruff and Thompson 2002, Lorch and Servedio 2005, Rundle and Nosil 2005, Price 2006, Birkhead and Brillard 2007).

Post-zygotic isolation

Pot-zygotic isolation is an important aspect of the process of speciation, where barriers such as hybrid sterility and inviability inhibit gene-flow between species through hybrids as a result of genetic incompatibilities between genomes that are expressed when they are brought together. The evolution of hybrid fitness problems likely reflects the gradual accumulation of deleterious epistatic interactions between species (Dobzhansky 1937, Muller 1940, 1942, Noor 1999). Such hybrid incompatibilities accumulate

as a side effect of normal adaptive or neutral divergence (Dobzhansky 1937, Muller 1940, 1942, Orr and Turelli 1996, Orr 1997). Although this classical Dobzhansky-Muller model highlights the role of epistasis in speciation, and that the evolution of reproductive isolation needs to be opposed by natural selection, it's still unclear what forces drive isolation. One possibility is that alleles causing hybrid problems have little or no effect on fitness in their parental species and randomly drift to fixation (Coyne 1992, Orr and Turelli 2001). An alternative possibility is that the causative genes for speciation are driven to fixation by various forms of selection, and there is evidence that sexual selection may be one of those (Wu and Johnson 1996, Presgraves and Orr 1998). This topic has inspired biologists for centuries and in 1922 Haldane formulated an observation known as Haldane's rule: the fact that when one hybrid sex is sterile or inviable, it is usually the heterogametic (XY or ZW) sex (Haldane 1922).

Haldane's rule is now known to hold across a wide range of organisms (Coyne 1992, Orr and Turelli 1996, True et al. 1996, Coyne and Orr 1997, Laurie 1997) and it's been shown to be almost an obligate phase of speciation in Drosophila (Coyne and Orr 1997). The ubiquity of Haldane's rule suggests that the types of genetic changes underlying it, and thus underlying post-zygotic isolation, may be similar in most or all organisms (Coyne 1992, Laurie 1997, Orr 1997, Turelli 1998, Orr and Presgraves 2000, Price and Bouvier 2002, Turelli and Moyle 2007). It's now agreed by many that Haldane's rule is a composite phenomenon reflecting the confluence of several evolutionary and genetic factors. In fact it combines dominance theory, which hypothesizes that most hybrid incompatibilities act as partial recessives in hybrids (Muller 1940, 1942, Turelli and Orr 1995, 2000), and faster-male evolution, hypothesizing that genes expressed only in males evolve faster than genes also expressed in females (due to more intense sexual selection in males) and that spermatogenesis may be more sensitive to the genetic perturbation experienced by hybrids (Hollocher and Wu 1996, True et al. 1996). However, the latter theory cannot explain the rule in taxa with heterogametic females as the female hybrids should be the sterile sex (Orr and Turelli 1996).

Importance of sex-linked genes in speciation

Sex chromosomes seem to be enriched with genes controlling traits associated with post-zygotic isolation, like hybrid sterility, as well as with pre-zygotic isolation, like preferences and secondary sexual traits important for mate recognition (Civetta and Singh 1998, Reinhold 1998, Hurst and Randerson 1999, Saifi and Chandra 1999, Ritchie 2000, Noor et al. 2001, Wang et al. 2001, Saetre et al. 2003, Tao and Hartl 2003, Borge et al. 2005), suggesting them to be the hotspot for genes associated with speciation. Hence understanding the evolution of sex-linked genes may be crucial for understanding the development of reproductive barriers. Coevolution patterns between pre- and post-zygotic barriers to gene flow have been hypothesized, suggesting that this might be enhanced by sex-linkage of genes affecting mate recognition and hybrid viability (Servedio and Saetre 2003). According to this model the genes involved in pre-zygotic isolation are linked to those controlling post-zygotic isolation by a positive feedback loop leading to increased accumulation of these genes on

the sex chromosomes. However, sex chromosomes have arisen independently in many taxonomic groups, thus the number of genes involved in reproductive isolation are likely to be different between the two main systems – XY and ZW.

Theoretical models have predicted that if the majority of mutations are recessive, selection will be more efficient on the sex chromosomes than on the autosomes: the fast-X effect (Charlesworth et al. 1987). On the other hand, Orr and Betancourt (2001) found that evolution from standing genetic variation always proceeds more slowly at sex-linked than at autosomal genes. Accordingly, there are several studies that have reached different conclusions using the same approach as in *Drosophila* (Betancourt et al. 2002, Counterman et al. 2004, Thornton et al. 2006) and humans (Bustamante et al. 2005, Lu and Wu 2005), which leaves the question of whether there really is a faster or slower sex-effect still open.

Research aims

The young emerging field of avian evolutionary genomics has mainly inspired and been the challenge of this thesis. The sequencing of the chicken genome, soon after I started this work, provided new tools to play with at the genomic level. Using comparative approaches, the challenges of investigating the natural variation observed in nature at the genetic level have become a reality for *Ficedula* flycatchers. In this thesis I also addressed one of the major issues in evolutionary biology, the causes of speciation. This has proven to be difficult to answer since speciation is usually slow and therefore unobservable in real time, and it's not possible to draw broad conclusions about the causes and the importance of any form of reproductive isolation without using a comparative approach. The research aims of this thesis can be summarized as follows:

1. To investigate the molecular evolution of a widely studied gene involved in postnatal body growth: the *growth hormone* (*GH*) gene, and its comparison between Aves and Mammalia.

2. To understand what causes incomplete reproductive isolation barriers in *Ficedula* flycatchers and which genes are involved. Thus investigations of the evolution and genetic architecture of the traits involved in pre-zygotic isolation as well as candidate genes potentially involved in post-zygotic isolation have been conducted in the natural laboratory of the *Ficedula* flycatchers system.

3. To disentangle the causes and forces leading to the production of unfit hybrids, comparisons of protein expression patterns between hybrids and parental species were conducted in the *Ficedula* flycatcher natural hybrid zone in the Czech Republic.

MATERIAL & METHODS

"Non-model" system: *Ficedula flycatchers*

The bird species complex characterized by pied (*Ficedula hypoleuca*) and collared flycatchers (*F. albicollis*) represents the non-model system of this thesis. Flycatchers are small migratory passerine birds, belonging to the family *Muscicapidae*, breeding in Europe during spring and summer, and spending the rest of the year in tropical habitats in Africa. The plumage trait in pied flycatcher males is variable, ranging from black to brown, while collared flycatcher male plumage spans from black to gray with a large white forehead patch and white neck collar (Fig. 1). Despite the distinct plumage variation in males, the females of the two species are highly similar.

Pied and collared flycatchers have overlapping breeding areas in Central and Eastern Europe and on two islands in the Baltic Sea (Gotland & Öland; Fig. 1). For this work, sympatric pied and collared flycatcher populations have been sampled in the Czech Republic, whereas the allopatric pied flycatchers were collected in both Norway and Finland, and allopatric collared flycatchers were sampled in Italy (Fig. 1).

In the overlapping breeding areas hybridization sometimes occurs (2-7%) resulting in the production of unfit offspring, where females are sterile and males have reduced fitness (Alatalo et al. 1990, Saetre et al. 1997, Veen et al. 2001). This follows Haldane's rule (Haldane 1922) which states that the hybrids of the heterogametic sex (ZW, female) are often sterile or inviable (Alatalo et al. 1990, Saetre 1999, Veen et al. 2001). The two species are thought to have come into secondary contact after the last glaciation period (Saetre et al. 2001). The hybrids produced are intermediate in morphology between the two parental species, characterized by intermediate plumage traits and mixed songs (Saetre et al. 2003, Haavie et al. 2004).
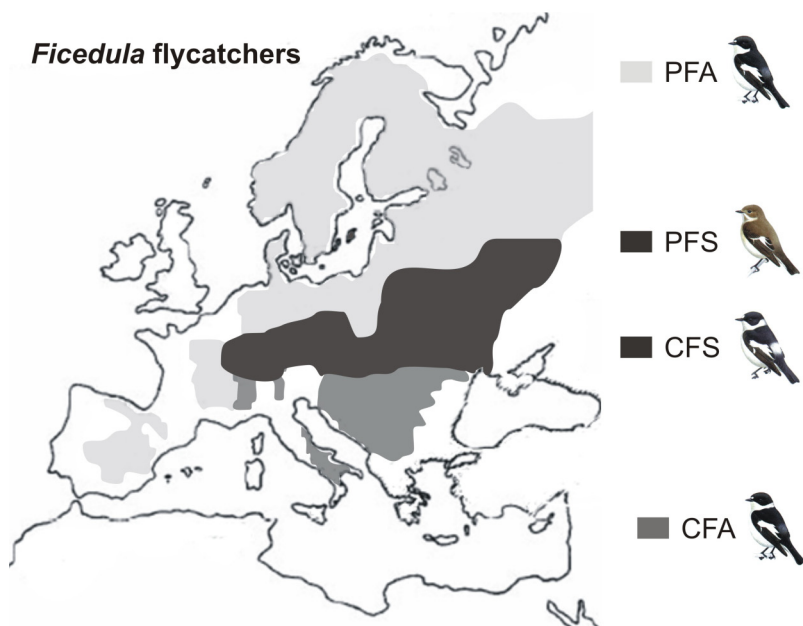

Ficedula flycatchers

PFA

PFS

CFS

CFA

Fig. 1.
The European geographical distributions of the pied and collared flycatcher. Abbreviations are as follows: PFA, allopatric pied flycatcher; PFS, sympatric pied flycatcher; CFA, allopatric collared flycatcher; CFS, sympatric collared flycatcher.

The European *Ficedula* flycatchers represent one of the most convincing examples of reinforcement described to date (Ungerer and Rieseberg 2005, Butlin 2006). Also in accordance with reinforcement theory is the observation of strong character displacement of plumage and song traits (Haavie et al. 2004) in sympatric populations (Saetre et al. 1997). Recent studies have shown that genes involved in hybrid fitness, male plumage colour and female preferences are linked to the Z chromosome (Saetre et al. 2003, Saether et al. 2007). Additional studies highlight that in regions where these two sister species have overlapping breeding areas there is extensive gene flow at autosomal genes, whereas introgression at the Z-chromosome is almost absent (Saetre et al. 2003, Borge et al. 2005). Taken together these findings suggest the sex chromosomes to be the hotspots for genes involved in maintaining the species reproductive barriers. Although the evolutionary fate of genetic incompatibilities is unpredictable when species exchange genes in the wild, the physical linkage of traits involved in pre- and post-zygotic isolation restricts the recombination between these traits, which has been one of the major theoretical obstacles against the theory of reinforcement (Felsenstein 1981). Thus, reinforcement, character displacement of male plumage colouration, and sex-linked genes in *Ficedula* flycatchers, had set the scene for this thesis.

DNA-RNA-Protein: bridging the gaps

The genetic information is carried in DNA language that must be decoded, with the creation of RNA templates, to the executive level, in the form of protein. In this thesis I tried to integrate different molecular approaches spanning from DNA through RNA to protein levels, to obtain a wider understanding of the molecular evolution, genetic variation, and expression pattern in *Ficedula* flycatchers (Fig. 2).
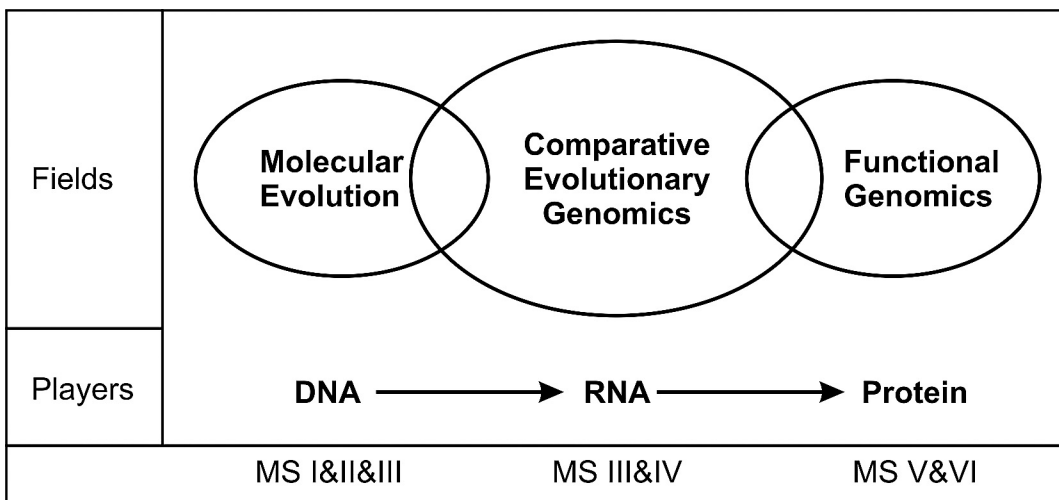

Fig. 2. DNA-RNA-Protein: bridging the gaps.

The avian molecular evolution of a well characterized gene in mammalian species, the *GH* gene, was first characterized in pied flycatcher and investigated at the DNA level, providing detailed information about the genetic variation within avian and between avian and mammalian species. The characterization of Z-linked candidate coding genes potentially involved in the speciation process was carried out using mRNA, with the main aim being to investigate the variation at the coding level. However, when I started this PhD surprisingly little genetic information was available for avian species and only after the release of the chicken genome was the identification of sex-linked genes in *Ficedula* flycatchers possible. In addition, as proteins retain the "executive power", an exploration of the different expression patterns between hybrids and parental species in flycatchers was conducted.

RESULTS & DISCUSSION

General summary of the overall studies

When I first started this work there was very little avian genomic data available, mostly limited to domesticated bird species (such as chicken and turkey) due to their economical importance. This thesis begins with the characterization (**I**) and subsequent molecular evolution analysis (**II**) of the growth hormone (*GH*) gene in passerine birds, where the forces under which this gene has evolved have been investigated and also compared with mammalian species. Previous studies on *Ficedula* flycatchers (Saetre et al. 2003, Borge et al. 2005), and the availability of the chicken genome, inspired the following chapter of this thesis which focussed on the molecular evolution of candidate coding sex-linked genes (**III**) in order to gain new insights into post-zygotic isolation mechanisms. As regards to the genetics of speciation in *Ficedula* flycatchers new insights into pre-zygotic isolation are given by the exploration of three genes involved in the pigmentation pathway (**IV**). In the final chapters of the thesis the comparisons of protein expression patterns between the hybrids and parental species revealed a unique hybrid expression pattern (**V**) as well as allowed the identification of differentially expressed proteins (**VI**).

Characterization and molecular evolution of the avian growth hormone gene

The ecological and evolutionary wealth of information available on passerine birds (Lundberg and Alatalo 1992) is uneven if compared with genomic information. Thus, I begun with the characterization in pied flycatcher (*Ficedula hypoleuca*) of the *GH* gene, suggested to be involved mainly in postnatal body growth (Etherton and Bauman 1998) and in a variety of secondary functions such as reproduction, aging and egg production (Aramburo et al. 2000, Ip et al. 2001, Zhao et al. 2004). Not surprisingly, due to its importance, the molecular evolution of the mammalian *GH* gene has been extensively studied in a wide range of vertebrate species (Wallis 1996, Lioupis et al. 1997, Forsyth and Wallis 2002) with the evolutionary rate being generally slow, but characterized by several bursts of rapid change in mammals (Wallis 1996, Wallis and Wallis 2001, Wallis et al. 2001). However, the pattern of evolution of the avian *GH* gene may differ from that observed in mammals as may its function.
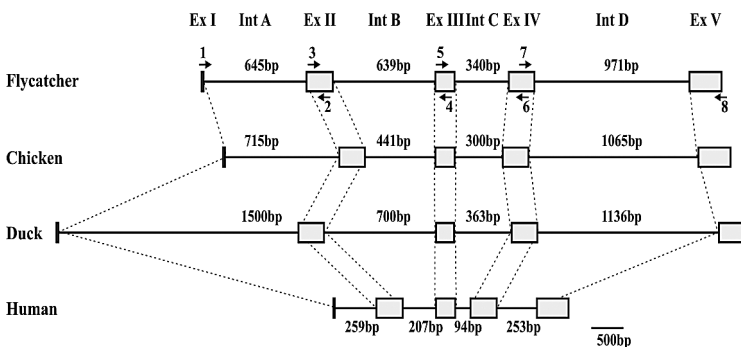

Fig. 1.
Schematic comparison of the *GH* gene exon-intron structures among pied flycatcher, chicken, duck and human (Chapter **I**).

In fact, while *GH* has been demonstrated to have an important role in postnatal body development in mammals, its role in growth rate regulation in birds is possibly reduced, as it has been shown that exogenous *GH* exhibits no effect on growth during the early post-hatch growing period (Zhao et al. 2004). The characterization of the *GH* gene sequence in pied flycatcher revealed that the overall organization of the gene was very similar to that of other available avian genomic sequences (Fig. 1), characterized by five exons and four introns, with the intron lengths being very different than those of the other two bird species, chicken and duck (**I**). This gene led to the formation of a protein characterized by a signal peptide in its N-terminal end which has been shown to be crucial for the correct cleavage of the protein. It has been suggested that this signal peptide may be involved in post-translational modification leading to the observed structural diversity of avian *GH* (Williams et al. 2000, Martinez-Coria et al. 2002). This led us to hypothesize that the increased variation in signal peptide sequences may instead be adaptive.
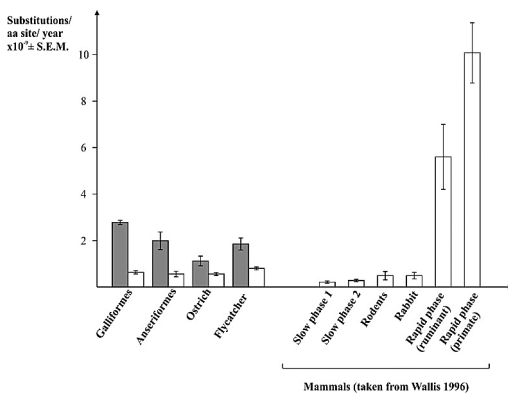
The investigation of the molecular evolution of the avian and mammalian *GH* genes was performed (**II**) using a maximum-likelihood codon based method, to infer potential positively selected sites (Yang 1997, Suzuki and Gojobori 1999). A total of six avian *GH* coding sequences were included, representing the minimum number allowed for the analysis (Wong et al. 2004). Overall, the molecular evolutionary rate of the *GH* gene in birds included in the study has been considerably more constant than in mammals. Wallis (1996) estimated that the evolutionary rate of mammalian *GH* varied up to 25-50 fold, while in the avian *GH* genes studied here the maximum rate of variation between lineages was only 1.4 fold (Fig. 2). Results of the codon-site method indicated that the majority of the amino acid (aa) sites in the mature avian *GH* protein have been subjected to purifying selection, with the ω value of almost 99% of aa sites being estimated to be ≤ 0.15 (Fig. 3A). Therefore, it's clear that the rapid bursts of *GH* evolution observed in several mammalian lineages (Wallis 1994, Lioupis et al. 1997, Wallis 2001, Wallis and Wallis 2001, Wallis et al. 2001) and some fish lineages (Wallis 1996) are not evident in the current data-set of mature avian *GH* proteins. Interestingly, the site-specific maximum likelihood analyses conducted with the mammalian *GH* data-set failed to detect any positively selected aa sites in the mature protein despite the bursts of rapid evolution observed in ruminants and primates, suggested to be due to positive selection (Wallis 1994). One likely explanation might be that in some regions of the mammalian data-set there is saturation of sequence substitutions.
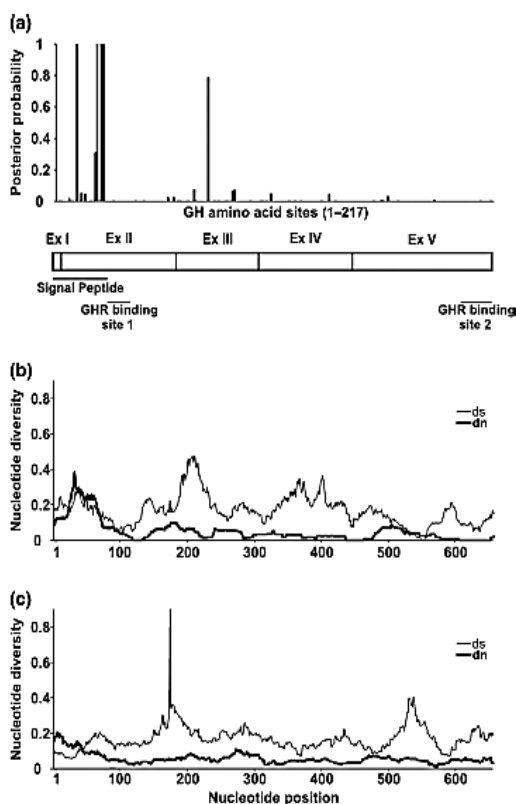

Fig. 2. Rate of molecular evolution of avian and mammalian *GH* genes. Grey bars represent the rate of molecular evolution of the *GH* signal peptide; white bars represent the rate of molecular evolution of the mature *GH* protein (Chapter **II**).

Fig. 3. Identification of aa sites under positive selection in the *GH* gene. **A)** Posterior probabilities of site classes along the avian *GH* gene under the discrete model (M3). Sliding window analysis of the average number of synonymous substitutions per synonymous site (d_S) and non-synonymous substitutions per non-synonymous site (d_N) for **B)** avian and **C)** mammalian *GH* gene sequence data-sets (Chapter **II**).

A major finding in this study was that four of the 27 avian signal peptide codons were estimated to have been affected by positive selection (Fig. 3A), with two of these sites being in positions important for the cleavage of the protein to occur correctly (von Heijne 1988, Jain et al. 1994). Taking together the high level of non-synonymous variation observed at these sites in birds, and the fact that these sites were identified as being positively selected by the codon-site model, one potential implication is that signal peptide

sequence variation in birds is, in fact, adaptive. The great interest surrounding the field of positive selection and its detection from coding sequences is of great interest in evolutionary biology. However, the analytical method that's best suited to this task is still disputed (Suzuki and Nei 2002, Yang and Swanson 2002, Suzuki and Nei 2004), with the main argument being over the use of likelihood and parsimony methods (Wong et al. 2004, Zhang 2004). Therefore, the answer to the question "positive selection or relaxed negative selection?" still awaits further development of statistical methods.

Molecular evolution of Z-linked genes in *Ficedula* flycatchers

The speciation process, viewed as the formation of reproductive barriers between populations to prevent gene flow, represents one important area of research in evolutionary biology ever since Darwin introduced the concept (Darwin 1859). Its understanding still lacks full comprehension, mostly due to the lack of detection of the forces involved with the tools currently available. However, the increased availability of genomic information has increased the potential for comparative studies across a wide range of different taxa, leading to a better understanding of the mode of evolution. In particular, the availability of the chicken genome together with recent findings related to speciation in *Ficedula* flycatchers (Saetre et al. 2003, Borge et al. 2005) inspired this study. The latter studies have shown that in regions where pied and collared flycatchers have overlapping breeding areas there is extensive gene flow at autosomal genes, whereas introgression at the Z-chromosome is almost absent (Saetre et al. 2003, Borge et al. 2005), with

a likely explanation being that the Z-chromosome is the hotspot for genes involved in maintaining species barriers, further suggesting that inter-species incompatibilities in sex-linked genes are involved in completing the final stages of speciation in these two species. This has led to the hypothesis that Z-linked genes might retain the potential to be involved in maintaining the reproductive barriers in *Ficedula* flycatchers, and are therefore evolving faster in sympatric areas compared to allopatric settings (**III**). Thus, several sex-linked candidate genes were investigated among *Ficedula* flycatcher populations (**III**).

Overall, 13 Z-linked coding genes (14289bp, which represents ~70% of the homologous chicken genes on the Z-chromosome) have been identified in pied and collared flycatchers in the different settings (allopatry and sympatry). A pairwise maximum likelihood method (**II**) was used to infer sites potentially under positive selection among pied and collared flycatchers, both in allopatry and sympatry, and the genes resulted to be highly conserved, with six of the 13 Z-linked coding genes without non-synonymous substitutions (Fig. 4).

Human (*Homo sapiens*) and chimpanzee (*Pan troglodytes*) were then chosen to be the mammalian counterparts for further comparisons of the level of molecular evolution of the sex chromosomes between Mammalia and Aves. However, since the synteny of the Z and X chromosomes is not conserved (Nanda et al. 1999, Nanda et al. 2002), with the majority of Z-linked genes found on human chromosomes 5 and 9, the X-linked data set from (Lu and Wu 2005) was used for the comparison. The average d_N/d_S for the X-linked genes were almost five times higher than for Z-linked genes (Fig. 5).

Fig. 4. Schematic representation of the 13 Z-linked genes and their locations on the Z chromosome, based on the *Gallus gallus* genome. Symbols indicate the position of the observed non-synonymous substitutions.

* CFS
• CFA
∧ PFA
PFS

Although the majority of the coding genes examined here seem to be highly conserved among the different *Ficedula* flycatcher comparisons, *neurotrophic-tyrosine-kinase, receptor-2* (*NTRK2*) and *very low density lipoprotein receptor* (*VLDLR*) revealed a d_N/d_S of 1.07 (between allopatric and sympatric collared flycatchers) and 0.99 (between allopatric pied and collared flycatchers), respectively, suggesting their potential of being under positive selection. It has to be mentioned that the method used to investigate the molecular evolution in these Z-linked genes is mainly concerned with the mode of evolution at the protein level. Accordingly, it assumes that ratios between non-synonymous and synonymous substitutions bigger than 1 can be interpreted as positive selection, and thus doesn't consider potential interactions between genes as well as the importance of the regulatory elements. Only recently has there been some attention given to the potential selective forces driving synonymous substitutions (Hoffman and Birney 2007, Resch et al. 2007), assuming that the substitution rate at intronic sequences is the neutral rate. Hence, synonymous substitutions might indeed be involved in the stability of the mRNA, influencing folding and splicing, and consequently at the expression level (**IV**).

Nevertheless the non-synonymous and synonymous substitutions found in this study warrant further investigation of additional individuals to determine whether they may be potentially important for maintaining post-zygotic reproductive barriers in this interesting system.
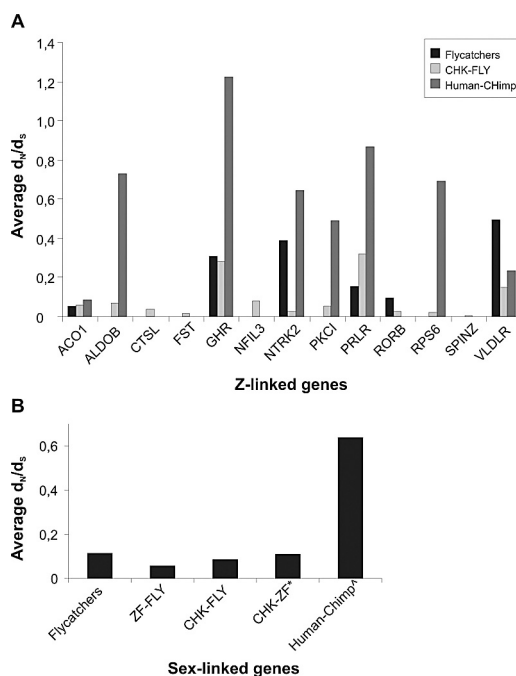
* Chicken-zebra finch (CHK-ZF) data taken from (Mank et al 2007)
^ Human-chimp data taken from (Lu & Wu 2005)

Fig. 5. A) d_N/d_S averages for Z-linked genes in birds and mammals. **B)** d_N/d_S averages for sex-linked genes (Chapter **III**).

The genetic basis of colouration and reinforcement in Ficedula flycatchers

In chapter **III** the aim was to investigate sex-linked genes potentially involved in post-zygotic isolation, while in this chapter (**IV**) the main aim was to investigate the genetic basis of reinforcement – the increase in pre-zygotic reproductive isolation between two populations due to natural selection that results from a reduction in the fitness of hybrids (Dobzhansky 1937, Servedio and Noor 2003, Ortiz-Barrientos et al. 2004, Servedio 2004), which is one of the big challenges in evolutionary biology (Servedio 2007). Hence genes potentially involved in plumage colour variation observed in sympatric *Ficedula* flycatchers, one of the most convincing examples of reinforcement described to date (Ungerer and Rieseberg 2005, Butlin 2006), have been investigated. One of the remarkable cases of character displacement in this system is in plumage colouration. In addition, it has been demonstrated that pied flycatcher females in sympatry prefer dull brown males as mates and that the resulting character displacement helps species recognition (Saetre et al. 1997). Accordingly, three genes considered as candidates for controlling black-brown plumage colouration, and hence character displacement, in *Ficedula* flycatchers was investigated: *melanocortin receptor 1* (*MC1R*), *agouti related protein* (*AGRP*), and *tyrosinase related protein 1* (*TYRP1*). Although research into the genetics of colouration have a long history in mammalian species (Sturm et al. 2001, Bennett and Lamoreux 2003, Rosenblum et al. 2004, Hoekstra 2006), associations between variations in gene sequences and plumage colouration have been reported in avian species only recently (Theron et al.

2001, Mundy et al. 2003, Mundy et al. 2004, Mundy 2005, Nadeau et al. 2007a & b), with the *MC1R* gene being one of the most intensely studied in birds.

Unlike a number of other avian species (Mundy 2005), no clear 1 to 1 association between *MC1R* sequence and male plumage colour variation in pied and collared flycatchers was found. Analysis of 22 avian species using a maximum likelihood codon-based method (**II & III**) to infer potentially positively selected sites was conducted. However, it revealed no indications of positive selection, and the overall d_N/d_S ratio was well below 1, ranging between 0.04 and 0.11, suggesting that *MC1R* has generally evolved under strong purifying selection. This result was in agreement with a recent study on the evolution of avian pigmentation, with a major focus on galliform species (Nadeau et al. 2007b), where the authors found no category of sites under positive selection in the *MC1R* gene when using site-specific analysis. However, in the same study, a lineage specific approach revealed a significant relationship between dichromotism and changes at the *MC1R* gene in galliform species, although the d_N/d_S values ranged between 0 to 0.1. Moreover, no assocation between plumage colour and *AGRP1* variation was observed.

The sex-linked *TYRP1* was instead identified as a strong candidate associated with black-brown plumage variation in sympatric pied flycatchers, and hence is a strong candidate for a gene contributing to pre-zygotic isolation. In fact, two splice variants of the sex-linked *TYRP1* gene segregating in the sympatric pied flycatcher population and a strong association between individual *TYRP1* transcript lengths and plumage colouration in pied flycatchers were found (Fig 6 and 7).
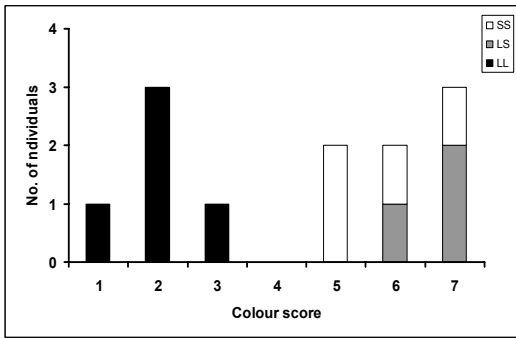
Fig. 6. Association between male plumage colouration and *TYRP1* transcript lengths in pied flycatchers (Chapter **III**).

One transcript is 536 aa in length and is similar in structure to the polypeptide characterized in other birds, while the second transcript, most likely the result of a splicing error, results in much shorter (420 aa) transcript lacking the C-terminal end found to be essential for trafficking to the melanosome (Vijayasaradhi et al. 1995, Sarangarajan and Boissy 2001).
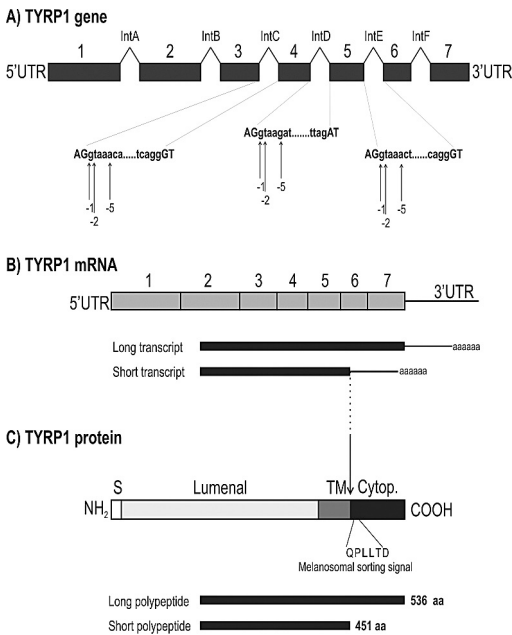
This provides a likely functional mechanism which is consistent with the evolution of character displacement i.e. selection for brown male plumage in pied flycatchers in the Czech Republic sympatric population. Hence, *TYRP1* can be considered as a strong candidate to be one of the first speciation genes identified in any vertebrate species.

Fig. 7. Schematic representation of the *TYRP1* gene in *Ficedula* flycatchers. A) *TYRP1* genomic region; intron-exon boundaries are shown with bars and lines, respectively, as well as the nucleotide positions important for the splicing (position -1, -3, -5). B) *TYRP1* coding region; the four Ficedula flycatcher forms and the transcript length, respectively, are illustrated. C) Potential translated *TYRP1* protein; the domains are shown with different grey colours and in the cytoplasmatic domain the sequence essential for melanosomal recognition is illustrated (Chapter **IV**).

Proteomic analyses in natural Ficedula flycatcher hybrids

In the final chapters of this thesis the potential of proteomics has been used to investigate the evolution of gene function, providing a unique perspective into ecological functional genomics in natural populations of *Ficedula* flycatchers that allowed firstly investigation of the variation of protein expression between and within *Ficedula* flycatchers and their hybrids (**V**), and secondly, by combining two-dimensional electrophoresis (2-DE) (Klose 1975, O'Farrell 1975) with mass spectrometry techniques, identification of the differentially expressed protein (**VI**). This combination might allow qualitative and quantitative measurements of protein expression that can be subsequently linked to the corresponding gene and used to understand its function (Ideker et al. 2001). As mentioned above (**III & IV**), speciation can be viewed as the build-up of pre- and post-zygotic reproductive barriers between populations where natural selection should favour traits that reinforce reproductive species barriers (Dobzhansky 1937). Hence, examining the implications of heterospecific pairing in natural populations might provide valuable insights into the mechanisms behind the evolution of reproductive isolation.

An ANOVA analysis of 116 protein spots was conducted and 61 protein spots were detected to be significantly differentially expressed among the following comparisons: hybrids and the sympatric pied flycatchers, hybrids and the sympatric collared flycatchers, pied and collared flycatchers in allopatry, pied and collared flycatchers in sympatry, within pied flycatchers, and within collared flycatchers. Comparison of the protein expression profiles of liver tissue between the hybrids and the parental species revealed that the differences are probably not due to environmental factors as both allopatric and sympatric individuals showed the similar patterns of expression for the majority of the protein spots estimated to be significantly different by the ANOVA analysis (Fig. 8). The novel hybrid expression pattern can be explained as the heterologous chromosome complement resulting in interactions between transcription factors of one species and the regulatory regions of the other. Thus, changes at the regulatory regions of genes might play a crucial role in the phenotypic diversity between species (Belting et al. 1998, Carroll 2005, Gompel et al. 2005, Wratten et al. 2006).

A step toward a greater understanding of protein variation in *Ficedula* flycatchers and the production of unfit hybrids is given by the significantly different protein spots identified for the trio comparisons of the sympatric area, PFS-HYB-CFS. The last chapter of this thesis (**VI**), identifying by sequence similarity of differentially expressed proteins, shows that the vast majority of the identified peptides (**VI**) fall into one of two main functional classes: binding and catalysis (Fig. 9). One likely explanation might be that the annotated proteins for these two categories in the public databases may certainly be more accurate in several organisms, chicken in particular, than for smaller functional classes; hence the *Ficedula* flycatcher peptides falling into these categories have had higher chances to be identified.
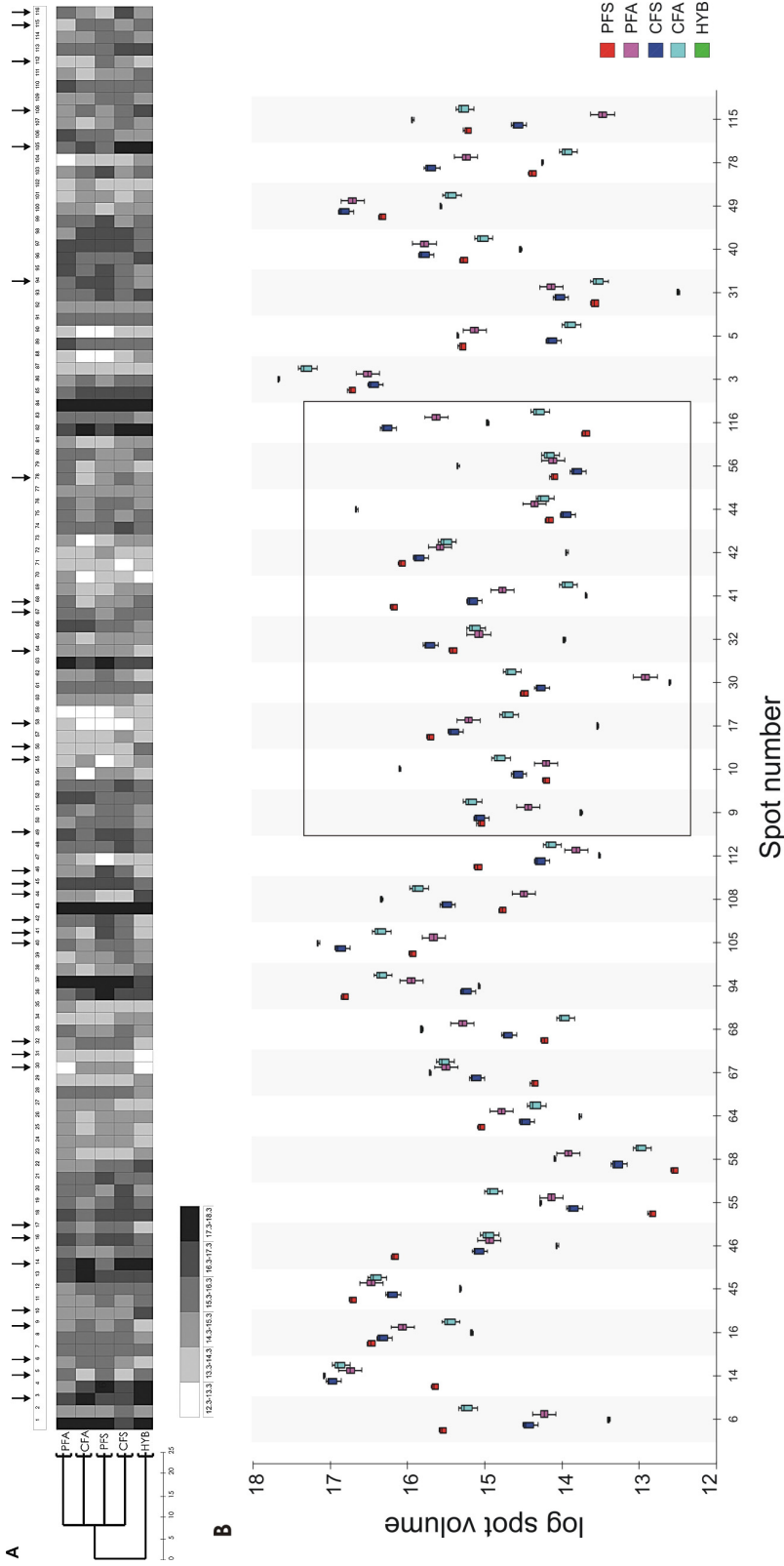
Fig. 8. Results of the protein expression study in Chapter **V**. A) Dendogram constructed using squared Euclidian distances and median method for all 116 spots. Arrows indicates the spots which were significant for the sympatric area. B) Box plot of the significant spot from the sympatric area. Significant spots shared by PFS-HYB and CFS-HYB comparisons are indicated in the box.

Notably, conserved and non-conserved protein spots were included in the identification analysis, although the conserved were only 23% of the total proteins submitted for identification. Consequently, it's hard to draw conclusions about the conserved *vs* non-conserved protein spots comparison as the different functional classes identified as well as the sub-categories are represented in both of the cases (Fig. 9). Unfortunately, but not unexpectedly, more than 55% of the flycatcher protein spots failed to be identified by one of the databases although they showed good mass spectra characteristics.

The most likely explanation for this result is that these proteins are sufficiently diverged from chicken and zebra finch that they aren't recognized through homology similarity searches based on mass spectra. Only manual sequencing or the completion of a whole-genome sequence from a more closely related species, e.g. zebra finch, will allow these proteins to be identified and would enable a better understanding of the *Ficedula* flycatchers liver proteome and its potential involvement in the production of unfit hybrids.

Differentially expressed proteins
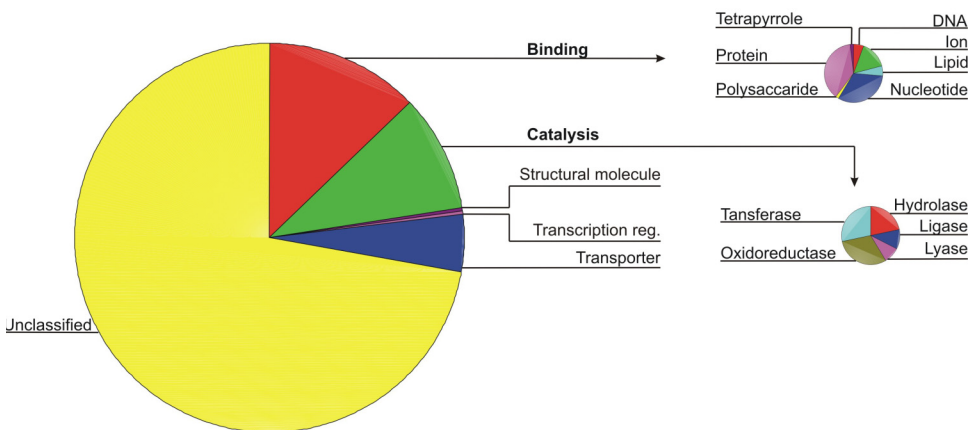
Conserved proteins
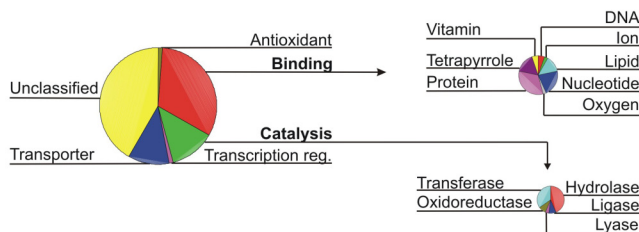
Fig. 9. Differentially expressed and conserved proteins in the *Ficedula* flycatcher liver proteome (Chapter **VI**).

FUTURE PROSPECTS

The results I obtained during this thesis will contribute to enrich the knowledge of avian evolutionary genomics. As I mentioned earlier, the key word in this thesis is integration, and I strongly believe that only by combining different areas of biology and approaches will it be possible to reach a complete understanding of the forces, genes, and molecules involved in speciation. As evolutionary biologists we are interested in patterns or rules that might characterize the genes underlying speciation, and the *Ficedula* flycatcher system provided the ideal system to investigate those.

When I started this work I was expecting to answer many questions which I tried to address using different molecular and genomics tools, travelling from the molecular evolution of single genes, to comparative genomics of several coding sequences, to the discovery of novel expression patterns in natural populations. However, many more questions have been raised. I hope that this work has added a little stone to the big castle and can be considered as both a continuation and starting point for future studies. In particular, in *Ficedula* flycatchers, the first proteomic approach has given new insights about differentially expressed proteins between hybrids and parental species which might then be deeply investigated at the genomic level. In addition, functional studies using the *TYRP1* gene will enable a better understanding of its role in the pigmentation pathways in flycatchers as well as its importance in pre-zygotic isolation. And the Z-linked genes identified can be investigated in a broader scale to be able to link genotypes with phenotypes, also in other passerine species.

Finally, I think that the chapter on the genetics of speciation of the *Ficedula* flycatchers system has just begun and many more exciting chapters will follow as soon as the first passerine genome (zebra finch – *Taenopigia guttata*) becomes available.

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