Avian Genome Evolution - Gene Expression, Gene Divergence and Sexual Dimorphism

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Avian Genome Evolution

Gene Expression, Gene Divergence and Sexual Dimorphism

Introduction

The extensive differences between males and females have always fascinated scientists. They include for example the differences in body size in the black widow spider, the elaborate displays and plumage of male birds of paradise and the antlers of moose and red deer (see e. g. Andersson M. 1994). We know today that they arise despite virtually identical DNA sequences. Sex-limited genes only occur in species with genetically determined sex and even in such species they are very few. The extensive amount of sexual dimorphism in the natural world must therefore to a large extent be related to how genes are used and not to which genes the different sexes possess.

Sex-biased gene expression, i.e. when the amount of transcription (or 'usage') of a gene is different between the sexes, is very common. In fact, it seems large parts of the genome of many species is sex-biased at one point or another (Ellegren & Parsch 2007). Large-scale studies of how the sexes differ in their utilization of the genome are still relatively uncommon and have long been limited to the few model species for which the entire genome has been sequenced.

Sex chromosomes contain the genes that determine the sex of an individual. They are common in many taxa and have evolved independently many times (Charlesworth 1996; Rice 1996). The presence of genes which determine sex have had a profound effect on sex chromosomes and selection, as well as other genetic processes, have affected them in a very different way compared to the rest of the genome (see paper 7). Sex chromosomes are highly implicated in the regulation of sexual dimorphism as they harbor many sex biased genes. Moreover, they are also of key importance in processes like adaptive evolution and speciation as they evolve rapidly, are conductive to sexual selection and harbor genes linked to reproduction (Ellegren and Parsch 2007; paper 2, 5 and 6). The study of sex chromosomes is therefore a natural bridge between the topic of sex-biased gene expression and the studies of DNA sequence divergence between species.

Studies of the level of sequence divergence of entire chromosomes and genomes between species can give valuable information concerning how conserved genomes are and which parts of the genome that are highly essential. Moreover, such studies can shed light on how the evolutionary rate of genes is affected by the size and type of chromosome on which they reside and by the way they are regulated (if they have sex-biased gene expression, if they have a high or low general level of gene expression). This type of knowledge about genomes can not only elucidate processes like for example the evolution of genetic sex or sexual selection but can potentially increase our understanding of the mechanisms behind genetic diseases linked to the level of gene expression (like cancer) or the mechanisms behind diseases caused by changes in chromosomal constitution (like Turner's or Down's syndrome).

Research aiming at elucidating how the patterns of gene expression and gene sequence divergence vary on a genome-wide level has been made feasible the last few years, as it is now possible to study thousands of genes in parallel. This thesis

has utilized a large-scale microarray containing over 15 000 genes in the bird genome (Box 3) and has focused on: (i) the extent of sex-biased gene expression in the brain, (ii) the implications of sex-biased gene expression on sex chromosomes, (iii) the rate of sequence divergence of sex chromosomes and autosomes, and (iv) how the sequence divergence of single genes has been affected by their chromosomal location and gene expression patterns.

As the following sections continue to outline the different studies and objectives in more detail the most extensively used scientifical and methodological terms will be explained in glossary boxes.

Section 1. Introductions to the subjects of sex-biased gene expression and sex chromosome evolution

1.1 Sexual dimorphism in gene expression

Sexual dimorphism, i.e., systematic differences between the sexes within a species, occurs in most taxa and is extensive in many. It includes behaviour, like the elaborate songs in some male birds which is not displayed by females, and secondary sexual characters like differences in size or the tail of the peacock (reviewed in; Andersson M. 1994). The DNA sequences in the sexes is virtually identical so sexual dimorphism must to a large extent arise through differences between the sexes in how genes are used, like differences in the gene expression level (Connallon & Knowles 2005; Rinn & Snyder 2005). In line with this there is extensive sexual dimorphism in gene expression in many species (Ellegren & Parsch 2007; Mank et al. 2008; Marinotti et al. 2006; Parisi et al. 2004; Ranz et al. 2003; Reinke et al. 2004; Rinn & Snyder 2005; Yang et al. 2004). Moreover, sex-biased gene expression (Box 1) is linked to reproduction and subject to strong selection (Dauwalder et al. 2002; Drapeau et al. 2003; Good & Nachman 2005; Jagadeeshan & Singh 2005; Kadener et al. 2006; Khaitovich et al. 2005; Proschel et al. 2006; Swanson & Vacquier 2002; Torgerson et al. 2002). The differences between gene expression levels in the sexes are therefore not just implicated in the systematic differences between the sexes in morphology and behavior, but are also linked to sexual selection and how it affects species divergence and speciation. Sexually selected traits are often sexually antagonistic, for example male display traits (like the peacocks tail) which increase the reproductive success of a male but only entail costs for females (Rice 1984; Rice & Chippindale 2001; Seger & Trivers 1986). Sex-biased gene expression is one way of resolving such sexually antagonistic selection because a lower gene expression in the sex which suffers the cost can lead to a lower expression of the harmful trait (Ellegren & Parsch 2007). Hence, there is a link between sexually selected traits and sex-biased gene expression. Moreover, such sexually selected traits, like male song or male plumage in birds, are often involved in species recognition and therefore directly influence the process of speciation (Servedio & Saetre 2003). Genes with sex-biased gene expression are therefore more likely than unbiased genes to play a role in sexual selection and speciation.

1.2 The evolution of sex chromosomes

The occurrence of sex-determining mutations has strongly affected the evolutionary processes on sex chromosomes subjecting them to selection pressures which are highly different from those faced by other chromosomes in the genome. Paper 7 describes the evolution of sex chromosomes in more detail. Here I will focus on briefly explaining the processes which will be much discussed in this thesis (see also Box 2).

The two types of sex determination. The sex determining genes on the sex chromosomes can determine either male or female sex. This gives rise to two types of sex determination, so called XY sex determination, and so called ZW sex determination. Sex chromosomes in both systems are believed to have evolved in similar ways (Fridolfsson et al. 1998; Rice 1984) and both X/Y chromosomes and Z/W chromosomes have evolved independently many times (Charlesworth 1996; Rice 1996).

The two types of sex chromosomes. There are two distinct types of sex chromosomes, those

which harbor the sex determining genes (Y and W chromosomes) and their homologues (X and Z chromosomes). Sex chromosomes are inherited in two homologous copies by offspring just like all other chromosomes (one copy from mom and one from dad; Box 1). In XY species, such as for example mammals and *Drosophila*, individuals who inherit one Y chromosome and one

Box 1: General terms

Autosome:
A regular chromosome in the genome, present in one maternal and one paternal copy containing the same genes.

Gene expression level:
The level of transcription of a gene (i.e. the amount of raw material available for the production of the protein which the gene codes for).

Homologous genes:
In a diploid organism each chromosome is present in one paternal and one maternal copy. Each gene is thereby present in two homologous copies, one on each of the two chromosomes.

Male-biased mutation rate:
Due to a higher number of meiotic cell divisions in the male- than in the female germline, genes will often have a higher mutation rate when it is present in a male. This will have a large effect on sex chromosomes due to the fact that they reside more often in one sex than the other. Male-biased mutation rates are present in many taxa (e. g. mammals and birds) but absent or very weak in others (often species with short generation time like *Drosophila*).

Sexually antagonistic genes:
Genes that are beneficial to one sex but detrimental to the other.

Sexually antagonistic selection:
A gene faces sexually antagonistic selection if there are opposing selection pressures in males and females. This occurs if the sexes have different adaptive optima for the gene.

Sex-biased gene expression:
Uneven levels of transcription (activity) of a gene in the sexes. This can be due to higher activity per gene copy in one sex than in the other, or to transcription from genes that have a higher gene copy number in one sex than the other.

Sequence divergence:
Used in a general manner in this thesis, referring to the number of changes (substitutions or mutations) between two species in a DNA sequence.

Recombination:
The crossing over between homologous chromosomes, leading to exchange of genetic information between the two and to more genetic diversity in the next generation.

mRNA:
mRNAs are copies of genes created (transcribed) in order to serve as 'blue prints' for the production of proteins.

Box 2: Terms related to sex chromosome evolution

Accumulation of sexually antagonistic genes on sex chromosomes:
X or Z is present twice as often in one sex than in the other. This will lead to more selection on dominant or partly dominant mutations in the homogametic sex (XX or ZZ) and such mutations will therefore remain in the population even if they are harmful to the other sex (XY or ZW). Recessive mutations however, will face immediate selection in the heterogametic sex (XY or ZW) and will not be expressed in the homogametic sex until they have reached a frequency where individuals are homozygous. Recessive X- or Z-linked mutations will therefore remain in the population if they are advantageous to XY-males or ZW-females regardless of their effect on the homogametic sex. Hence, both recessive and dominant antagonistic mutations stand a higher chance of being fixed on sex chromosomes.

Dosage compensation:
A mechanism which alters the gene expression of sex-linked genes in the heterogametic sex (XY-males or ZW-females). This compensation is expected to evolve in response to the change in gene copy number caused by the loss of genes on the degrading Y and W chromosomes. Changes in gene copy numbers for entire chromosomes are generally lethal as they disturb the balance in genetic networks. In order to avoid disruption of genetic networks as Y and W chromosomes degrade, XY-males and ZW-females are expected to increased gene expression on the copy of each gene which they retain on the X or Z chromosome. The end result of such compensation in XY-species is a situation where X chromosome-linked gene expression from the single X chromosome in males is equal to that produced from the two X chromosomes in females.

Homogametic sex:
The sex that carries two copies of the X or Z chromosome (XX-females and ZZ-males).

Heterogametic sex:
The sex that is determined by the Y or W chromosome and also carries one copy of the X or Z chromosome (XY-males and ZW-females).

Fast X or fast Z effect: the rapid evolution of sex chromosomes:
Sex chromosomes are expected to evolve fast due to:
Hemizygous exposure: In the heterogametic sex (XY or ZW) the X or Z chromosome is only present in one copy. This means that all alleles regardless of dominance coefficient will be expressed. This 'hemizygous' exposure of recessive alleles will lead to a higher fixation rate of recessive beneficial alleles on sex chromosomes.
The small population size of sex chromosomes: X and Z chromosomes are present in on average 1.5 copies per individual (and Y and W in 0.5 copies) and they thereby have a lower population size than autosomes, which are present in two copies in each individual. This will lead to fixation of more mutations due to drift on sex chromosomes.

Sex chromosomes:
Originally homologous pairs of autosomes, X/Y or Z/W sex chromosomes are today harboring the genes that determine sex. The Y chromosome is male-determining while the W chromosome is female-determining.

Box 2. Continued.
XY sex determination:
The sex determining system in species with X and Y sex chromosomes, where females are the homogametic sex (XX) and males are the heterogametic sex (XY). The Y chromosome is only present in males while the X chromosome is present two thirds of time in females. XY sex determining systems include for example mammals, *Drosophila* and *C. elegans*.

ZW sex determination:
The sex determining system in species with Z and W sex chromosomes, where males are the homogametic sex (ZZ) and females are the heterogametic sex (ZW). The W chromosome is only present in females while the Z chromosome is present two thirds of the time in males. ZW sex determining systems include for example birds, many reptiles and many insect- and fish-species.

X chromosome become males (XY), while those that inherit two X chromosomes become females (XX). In ZW species, such as for example birds, individuals who inherit one W and one Z chromosome become females (ZW), while those that inherit two Z chromosomes become males (ZZ). The fact that Y chromosomes determines males while W chromosomes determine females opens up for studies of whether sex chromosomes evolve differentially in males and females (as Y is present in males 100% of the time, X is in males 33% of the time, Z is in males 66% of the time while W is only present in females)

The degradation of Y and W chromosomes. Y and W chromosomes degrade. The genes on these chromosomes accumulate mutations, loose function and are therefore silenced. This process has proceeded in many species until large parts of Y and W chromosomes have literally disappeared and they are now small, gene poor chromosomes harboring a low number of genes besides the sex determining genes (for details see box 1 in paper 7 and; Bergero & Charlesworth 2008). This is due to the fact that Y and W chromosomes do not experience any recombination. Recombination is the process by which the maternal and the paternal copies of each chromosome in an individual cross-over and exchange genetical material with each other. Recombination spreads detrimental ('harmful') and beneficial ('good') versions of each gene among individuals of the next generation. Selection will remove individuals with harmful genes while beneficial genes will remain in the population. However, since the Y and W chromosomes have largely ceased to recombine with their homologues X and Z, deleterious mutations accumulate on these chromosomes eventually causing their degradation. In contrast, Z and X chromosomes still recombine when they are present in XX or ZZ individuals and hence do not degrade.

The evolution of dosage compensation. Changes in gene expression level of many genes or for entire chromosomes are often lethal since they cause chain reactions in essential genetic networks, where the expression level of one gene directly influences the expression levels of others (Jeong et al. 2001; Lindsley et al. 1972; Lu & Oliver 2001; Pauli et al. 1993; Rosenbusch 2004). Hence, XY-males and ZW-females face a dilemma when Y and W chromosomes degrade. In order to avoid disruption of essential networks they must compensate for the loss of gene expression incurred by the loss of genes on Y and W. They have been selected to achieve such compensation by increasing the gene expression on the copy of each gene which they retain on their single X or Z chromosome. This compensatory process is referred to as *dosage compensation* as it compensates for loss of gene dose as Y- and W-linked genes disappear. In XY-males dosage compensation has led to a level of gene expression from their single X chromosome which is equal to that produced from the two X chromosomes in females (for the controversies regarding such compensation in ZW-females, see paper 3, 7 and results below).

Sex chromosome evolution has thereby led to a situation where Y and W chromosomes are limited to one sex and are small and degraded while Z and W chromosomes have faced selection for dos-

Box 3: Methodological terms

EST sequence:
An Expressed Sequence Tag (EST) is a DNA sequence copy of a mRNA (see Box 1).

Gene Expression Microarray:
DNA (and RNA) sequences form spontaneous bonds with each other when the sequences are complementary. In the cell nucleus DNA is present in two such complementary strains, bond together in a double helix. Microarrays utilize this natural propensity of DNA to bind together. On a gene expression microarray DNA sequences which are complementary to mRNAs are chemically attached to a glass plate in a fashion so that each mRNA is represented by many copies. All mRNA present in a tissue of interest is then extracted producing a sample which contains different amounts of mRNA for different genes (the amount represents the expression level (activity) of the gene). This sample of mRNA is labeled with a fluorescent molecule and then allowed to form spontaneous bonds (i. e. hybridize) with the sequences on the glass plate. The glass plate is then scanned and the fluorescence intensity of each mRNA is calculated, thereby giving a relative measure of the gene expression level.

Comparative Genome Hybridization (CGH):
A term used in this thesis to denote an experiment where we: (i) extracted genomic DNA from a wild bird species, and (ii) allowed this DNA to hybridize with the Lund-zf microarray. ESTs are represented on the array by 11 separate short sequences (so called probes). After hybridization of DNA to the array we calculated how many of the 11 probes which hybridized with signals stronger than background. We then ranked ESTs as having 0–11 of the probes significant. This rank denotes the relative level of divergence between the wild species and the zebra finch as probes with 3 or more substitutions will not yield significant signals. Hence, the rank of 0 to 11 significant probes is determined by the number of substitutions between the zebra finch sequences on the array and the DNA of the wild species. The rank can be used to: (i) identify conserved or rapidly evolving genes, and (ii) avoiding analysis of highly diverged ESTs on the Lund-zf array during gene expression experiments using wild birds. Gene expression analyses on such highly diverged sequences should be avoided as such ESTs will look like they are low expressed when in fact they simply hybridize poorly due to a high number of substitutions.

age compensation in XY-males and ZW-females. Moreover, X and Z chromosomes are have different copy numbers in the sexes and are therefore exposed to different degrees of selection in males and females (Z is in males 66% of the time while X is in females 66% of the time).

The sex-bias in transmission pattern of Z and X chromosomes in turn leads to selection pressures causing these sex chromosomes to: (i) be particularly conductive to accumulation of sexually antagonistic genes (Box 2; Charlesworth et al. 1987; Rice 1984), (ii) harbor a large proportion of all genes with sex-biased gene expression (Ellegren & Parsch 2007), (iii) evolve rapidly (Box 2; Presgraves 2008; Vicoso & Charlesworth 2006), and (iv) be highly implicated in adaptive evolution and speciation (Coyne 1985; Dobzhansky 1974; Presgraves 2008; Templeton 1977).

2. An outline of the avian model system

2.1 The avian genome

The avian genome is about a third of the size of that of the human and has evolved separately from the mammalian genomes for about 310 million years (International Chicken Genome Sequencing Consortium, ICGSC, 2004). It contains approximately one billion base pairs of DNA sequence and between 20 000 and 23 000

genes (www.ncbi.nlm.nih.gov/mapview/; ICGSC 2004). The gene content (synteny) on avian autosomes and sex chromosomes is highly conserved between Galliformes (the chicken *Gallus gallus*) and Passeriformes (the zebra finch, *Taeniopygia guttata*) with the exception of a fission of the chicken chromosome 1 into two separate chromosomes in the passerine bird lineage and a fusion of two ancestral chromosomes forming the chicken chromosome 4 (Dawson et al. 2007; Derjusheva et al. 2004; Griffin et al. 2007; Backstrom et al. 2006). In fact, the level of synteny in the avian genome is remarkably high compared to that of mammals (Dawson 2007, Stapely 2008).

The chicken has 38 pairs of autosomal chromosomes compared to the 22 in humans and there is extensive variation in chromosome size in the bird genome, spanning almost two orders of magnitude from the large macrochromosomes to the small microchromosomes (ICGSC 2004). This variation in chromosome size is correlated with GC content, recombination rate and gene density (ICGSC 2004).

The field of avian genomics is young but has steadily grown since the release of the chicken genome in 2004 (ICGSC 2004). The second fully sequenced bird genome (that of a songbird, the zebra finch) will be released within weeks of the print of this thesis and with the arrival of this second genome, evolutionary studies of birds will now fully hit the era of genomics. This thesis is clearly a sign of this transformation as it studies gene expression, gene divergence and chromosomal divergence for almost the entire passerine genome (about 15 800 of the 20 000 to 23 000 genes).

Domesticated model species like the chicken and the zebra finch are invaluable resources but can never replace studies which address selection and evolution in the wild. Classical studies on wild birds have investigated microevolutionary patterns based on morphological and behavioral traits, as well as measured fitness parameters (e.g Abzhanov et al. 2006; Baker & Fox 1978; Grant 1999). It will be extremely valuable to combine such studies in the wild birds with the now rapidly accumulating avian genomic resources, to address fundamental questions at the interface of evolution, genetics and ecology.

2.2. Birds as a model for studies of sex-biased gene expression and sex chromosome evolution

Birds are among the taxa with the highest degree of sexual dimorphism, noted already by Darwin who wrote in his Descent of Man: 'Secondary sexual characters are more diversified and conspicuous in birds than in any other class of animals' (Darwin 1874). Moreover, birds have a ZW sex determination system which allows for interesting comparisons which the extensively studied XY systems (like mammals and *Drosophila*; Box 2).

Furthermore, passerine birds are particularly interesting for several reasons. The zebra finch, which will be the second fully sequenced bird, is a passerine and the passerine brain (in particular that of the zebra finch and the canary) has long been a model for studies of the neurological development of the brain and how this relates to learning and behavior (Barnea et al. 2006; Li et al. 2000; Tchernichovski et al. 2001). Moreover, passerine birds include many important model species for studies of evolutionary biology in the wild such as the Darwin's finches (Abzhanov et al. 2006; Abzhanov et al. 2004; Grant 1999), the collared flycatcher (Gustafsson et al. 1995; Qvarnstrom et al. 2006), the great tit (Drent P.J. et al. 2003; Postma & van Noordwijk 2005) and the great reed warbler (Bensch et al. 1998; Bensch et al. 2007; Hasselquist et al. 1996). Hence, passerine birds can potentially shed light on many aspects of evolution for which the domesticated chicken is not suitable and with the upcoming availability of a passerine genome, avian genes and chromosomes can now be studied under more natural circumstances. This thesis is a first step in that direction as it studies gene divergence and gene expression for thousands of genes not only in the zebra finch but also in wild passerine species.

3. Our approach

This work has used the passerine genome in order to study the pattern of sex-biased gene expression, the evolution of the avian Z chromosome and the sequence divergence level of passerine genes in relation to their chromosomal location and their gene expression. We have done the following:

(i) Designed a microarray which contains large parts of the zebra finch genome (the Lund-zfa array; 15 800 genes; paper 1).
(ii) Optimized and verified the usefulness of this microarray for the zebra finch and for studies of wild passerine bird species (paper 1).
(iii) Studied the extent of sexually dimorphic gene expression in the brain of the zebra finch and a wild warbler, the common whitethroat (paper 2).
(iv) Related this sex-bias in gene expression to the process of dosage compensation (paper 3).
(v) Described a neo-sex chromosome in wild passerine birds; one of the autosomes in the zebra finch has become a sex chromosome in warblers (paper 4).
(vi) Investigated how chromosomal location and gene expression level affects the degree of sequence evolution of genes in passerine birds (paper 5).
(vii) Studied how the sequence divergence of genes on the avian Z chromosome is affected by sex-bias in gene expression and by general gene expression level (paper 6).
(viii) Summarized the implications this work and recent work of others in an opinion paper (paper 7).

We have focused some of our efforts on non model birds and have so far run gene expression studies on common whitethroats, *Sylvia communis*, and willow warblers, *Phylloscopus trochilus* as well as DNA hybridizations in order to study gene divergence for the common whitethroat, the willow warbler, the great reed warbler, *Acrocephalus arundinaces*, the blue tit, *Cyanistes caeruleus*, and the crow, *Corvus corvus*. Data from the common whitethroat, the willow warbler and the crow is included in this thesis.

4. Results and Discussion

4.1. A microarray for large-scale genomic and transcriptional analyses of the zebra finch (*Taeniopygia guttata*) and other passerines (Paper 1)

This first study aimed at evaluating the quality of the Lund-zfa microarray, both as a platform for zebra finch studies and for studies of other passerines. We did this by evaluating which probes and ESTs (each EST is represented by 11 probes on Affymetrix arrays, see section 6. 4) that hybridized significantly when genomic DNA was hybridized on the microarray (a Comparative Genome Hybridization, CGH, approach; Box 3; section 6.6). We demonstrated that zebra finch DNA hybridized well to a vast majority of all ESTs on the Lund-zfa array and moreover, that DNA of a wild species of warbler, the common whitethroat, also gave strong hybridization signals (it hybridized significantly to 96% of all ESTs).

We concluded that the array was of high technical quality, and that it was highly likely to yield good and reliable gene expression data for studies of wild passerine bird species in general. The study also set the framework for filtering procedures to detect all probes which do not yield significant signals when DNA samples of a wild passerine is hybridized to the array (see CGH in Box 3). Such non-significant probes can be removed from downstream analyses of gene expression, thereby avoiding that analyses on sequences with a high number of substitutions confuse the interpretation of results (applied in paper 2–4). Moreover, these 'non-hybridizing' probes can also be used to identify ESTs with a high degree of sequence divergence between the zebra finch array and the bird species hybridized to the array (applied in paper 5–6). The possibility of using such filtering procedures and the high hybridization efficiency of the common whitethroat DNA opens up for the possibility to use the microarray technique in areas such as avian behavioral ecology and comparative genomic studies.

4.2. The sex-biased brain: sexual dimorphism in gene expression in two species of songbirds (Paper 2)

Sex-biased gene expression in the brain has been linked to behavioral differences between males and females and is therefore of particular interest to behavioral ecologists (Dauwalder et al. 2002; Drapeau et al. 2003; Irizarry et al. 2003; Kadener et al. 2006).

In our second study we examined the extent of sexually dimorphic gene expression in the brain of two species of songbirds, the zebra finch and the

common whitethroat. Sex-bias in gene expression was extensive in the passerine brain, comprising several hundreds of genes in both species studied and most of these genes were sex-biased in both species. The majority of genes with sex-biased gene expression had higher expression in males than in females, and there was a large excess of Z-linkage among the sex-biased genes identified.

Previous studies have shown that the avian Z chromosome has a very high degree of male-biased gene expression (Box 2; Ellegren et al. 2007; Itoh et al. 2007). Our study confirms this based on a much higher number of genes in the zebra finch (which was previously studied by Itoh et al 2007 for 40 Z-linked genes) as well as for a new species, the common whitethroat. Due to the large degree of male-biased gene expression on the avian Z chromosome (paper 2; Ellegren et al. 2007; Itoh et al. 2007) it has been suggested that female birds have inefficient or even absent dosage compensation (Box 2; Ellegren et al. 2007; Itoh et al. 2007). Our results demonstrate that if the male-bias on avian Z chromosomes is due to a low degree of dosage compensation in ZW-females then the capacity to compensate is likely to vary between species. We base this conclusion on the fact that we found extensive Z-linkage and male-bias not only among genes which were sex-biased in both species in our study, but also among the genes which were sex-biased only in one of the species (Figure 1).

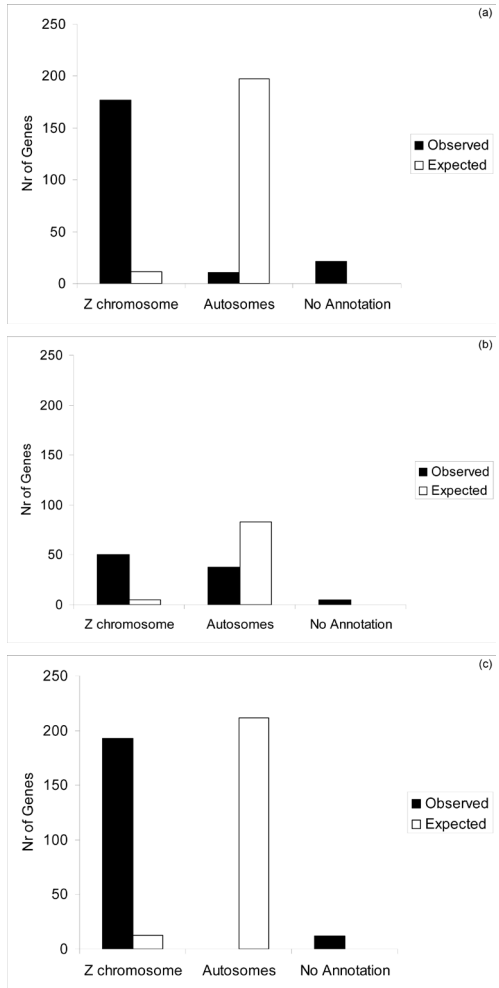


Figure 1. Chromosome annotation of (a): the 212 genes that were sex-biased only the zebra finch, (b): the 93 genes that were sex-biased only in the common whitethroat and (c): the 205 genes that were sex-biased in both species.

4.3. Male-biased gene expression and dosage compensation on the avian Z-chromosome (Paper 3)

As mentioned, the presence of hundreds of male-biased genes on Z has previously been described as a general lack of dosage compensation in birds (Box 2; Ellegren et al. 2007; Itoh et al. 2007). In this paper we studied the degree of male-biased gene expression on the Z chromosome in more detail, using the same two species of songbirds as in paper two (the zebra finch and the common whitethroat). We concluded that: (i) even if females have consistently lower gene expression than males for many Z-linked genes their ratios of Z-linked to autosomal gene expression (Z:A ratios) indicate that they do achieve dosage compensation, and (ii) male-bias on the Z chromosome increases with increasing gene expression level (Figure 2).

Dosage compensation is considered essential for species with differentiated sex chromosomes because changes in gene expression level for many genes will disrupt the balance in genetic networks (Lindsley et al. 1972; Lu & Oliver 2001; Lynch 2007; Pauli et al. 1993; Rosenbusch 2004). Hence, when ZW-females lose gene expression from W-linked genes (due to the degradation of

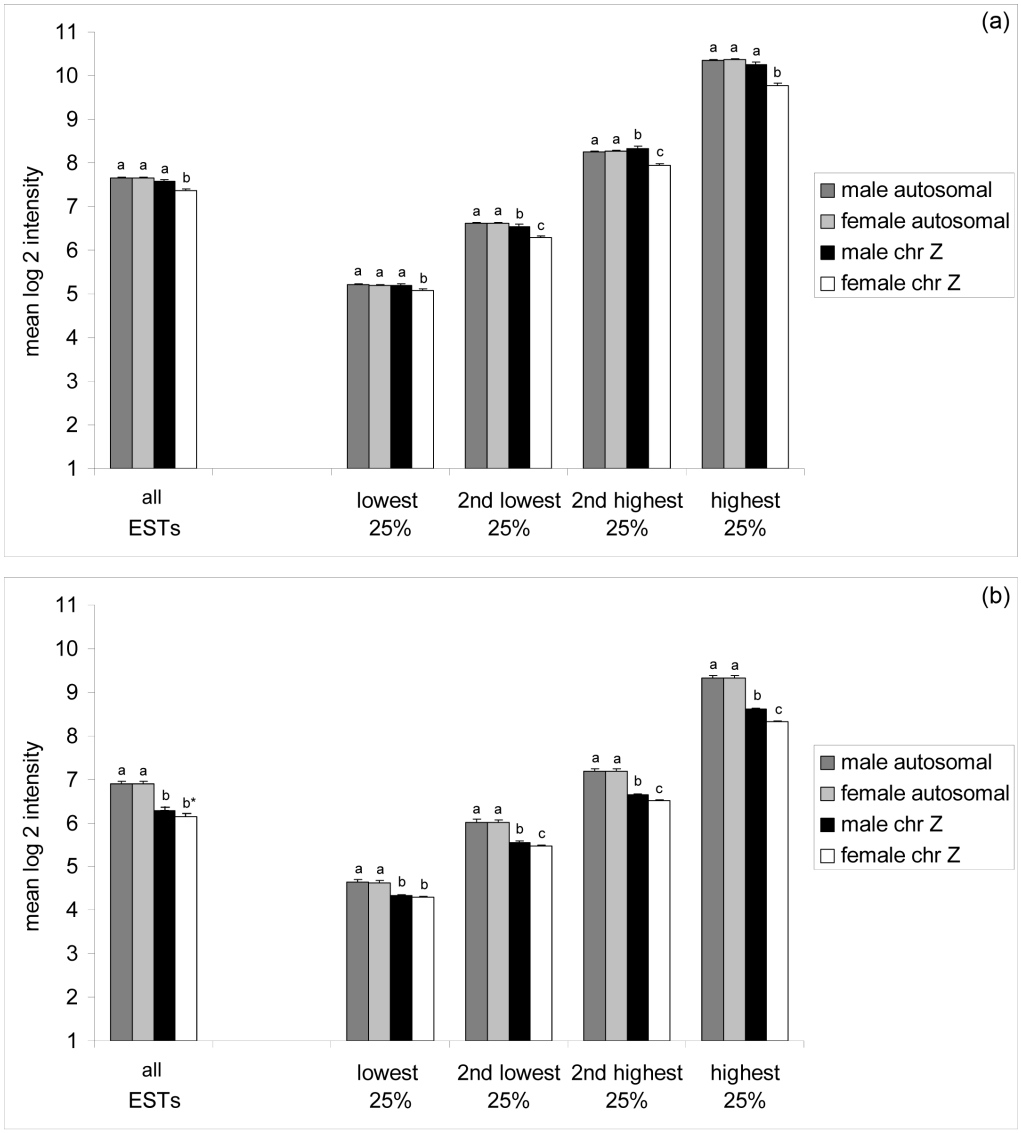


Figure 2. Mean (+SE) gene expression intensity of autosomal and Z-linked ESTs in (a) the zebra finch and (b) the common whitethroat. Data for all ESTs and when divided into quartiles by mean gene expression intensity from lowest 25% to highest 25% are given. Significant differences between categories are indicated (a, b and c).

this chromosome; Box 2) they should face strong selection to compensate for that loss by increasing their expression on the Z-linked homologues. Our results indicate that avian females *have* faced such selection and in essence succeeded in balancing their genetic networks. Z:A ratios of gene expression vary between 0.89 to 1.01 for both sexes in both species studied, and this is well within ratios of X-linked to autosomal gene expression in mammals (where males are considered to have effective dosage compensation; Itoh et al. 2007). In fact, for the zebra finch the variation in expres-

sion between the Z chromosome and the autosomes in the brain was maximum 5% in either sex. The mouse (*Mus musculus*) has a 30% higher X-linked than autosomal gene expression in the brain while human X-linked expression in the hypothalamus is 3–5% higher than autosomal (see Itoh et al. 2007). Hence, we argue that the difference between Z chromosomes and X chromosomes is not centred around to what extent sex-linked and autosomal expression is balanced but rather around the fact that ZW-females achieve such balance without matching their gene expression level exactly to that of the males. Moreover, it is highly interesting that there is a close link between gene expression level and the extent of male-biased gene expression. A link between expression level and the degree of male-bias has previously been found in another ZW-species, the silkworm (*Bombyx mori*; Zha et al. 2009) and indications of this pattern has also been found in the chicken (Melamed & Arnold 2007). These findings suggest that there might be systematic differences between male-biased Z-linked and unbiased Z-linked genes.

4.4 First evidence of a neo-sex chromosome in birds (Paper 4)

The analyses of sex-biased gene expression we undertook in paper 2 highlighted an unexpected and interesting difference between the zebra finch and the common whitethroat. When sex-biased genes on autosomes were studied in more detail there were 25 significantly sex-biased genes on one of the presumed autosomes (chromosome 4a) in the common whitethroat but none of these genes were sex-biased in the zebra finch. These 25 genes where all located within the first 10 Mb of chromosome 4a (chromosome position based on the zebra finch genome). When DNA from another warbler species, the great reed warbler, *Acrocephalus arundinaceus*, were screened for 18 microsatellites and introns present on Tgu4 and Tgu4a, the two markers which were located within the first 10 Mb on Tgu4a were sex-linked (Figure 3). We concluded that Tgu4a has become a neo-sex chromosome in warblers. Sex chromosome evolution has been a topic of much research and debate for decades, and neo-sex chromosomes provide unique opportunities for detailed tests of many of the hypothesis proposed in this field of research.

Even if neo-sex chromosomes have been extensively studied in for example the fruit fly (*Drosophila* spp.) they are very uncommon among mammals and birds which have highly stable genome structures (van Doorn & Kirkpatrick 2007). Tgu4a is not sex linked in the chicken, the zebra finch, the pied flycatcher (*Ficedula albicollis*) or the blue tit (*Cyanistes caeruleus*), but in both warbler species (*Sylvidae*) studied, and we can therefore conclude that it is between 24 and 47 million years old. This is young compared to the ancestral Z and W chromosomes in birds which are potentially as old as 150 million years (Lawson-Handley et al. 2004; Matsubara et al. 2006; Nam & Ellegren 2008). A neo-sex chromosome in birds will give scientists unique possibilities to study how Z and W chromosomes evolve and compare that to studies of neo-X and neo-Y chromosomes in for example *Drosophila*. Studies of how sex chromosomes evolve when they are present in male contra female environments will thereby be possible (remember that Y is present 100% of the time in males, Z is 66% in males, X is 33% in males while W is 0% in males). Tgu4a will therefore provide a unique opportunity to study which role sexual selection plays in the evolution of sex chromosomes.

4.5. Sex-linkage, Size and Gene Expression Signals: the Rate of Evolution on Avian Chromosomes (Paper 5)

In this study we used the Lund-zf array in order to study genetic divergence on a genome-wide level in passerine birds. We utilized the fact that the Lund-zf microarray design represents each EST with 11 short (25 bp long) probes. This has allowed us to assess the degree of divergence in 11 separate parts of each EST, and thereby in over 140 000 markers in the passerine genome (Box 3; section 6.6 below). We hybridized genomic DNA of the willow warbler (*Phylloscopus trochilus*) and the crow (*Corvus corvus*) on the array according to our CGH approach (Box 3; section 6.6). Each probe was ranked according to whether or not it hybridized with significantly higher intensities than background signal. Each

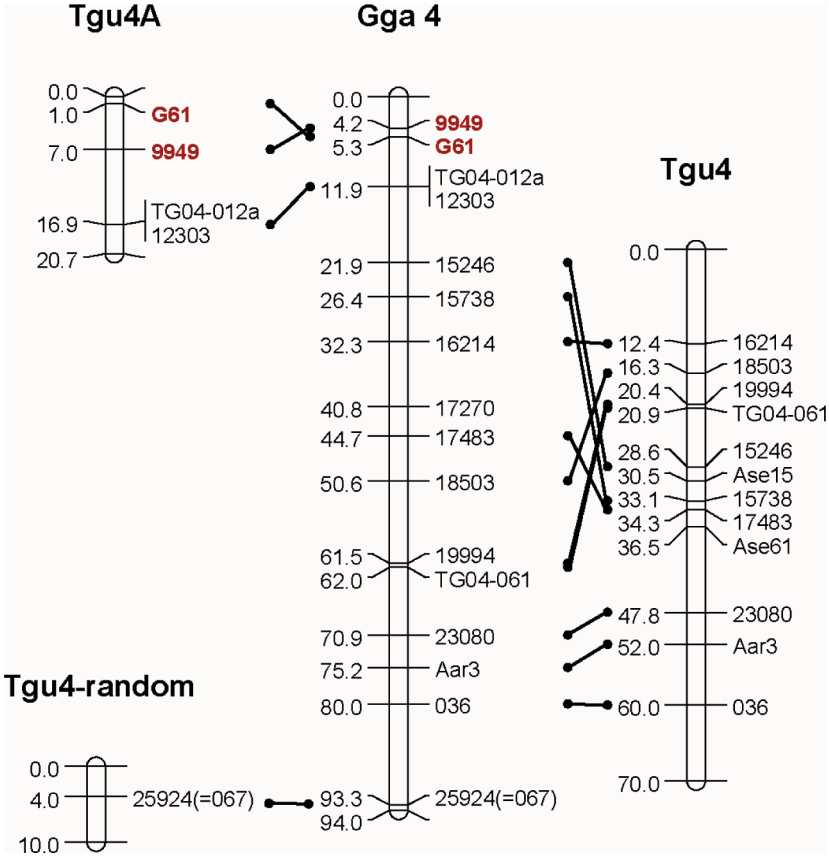


Figure 3. Loci evaluated for sex-linkage in the great reed warbler and their position on the zebra finch (*Tgu4*, *Tgu4a* and *Tgu4_random*) and chicken genome assembly (*Gga4*), respectively. Two loci highlighted in red, *G61* and *09949*, show sex-linkage in the great reed warbler.

EST was then ranked as having 0 to 11 of all its probes hybridizing significantly. We were thereby able to rank 12220 autosomal and 687 Z-linked ESTs according to their level of divergence from the zebra finch genome. We found the following: (i) the avian Z chromosome evolved much faster than autosomal chromosomes (Figure 4), (ii) the level of divergence was positively correlated with chromosome size (Figure 5), and (iii) genes of medium expression levels showed the highest level of conservation (Figure 6).

The rapidly evolving Z chromosome. Z-linked and X-linked genes are expected to evolve faster than autosomal genes, referred to as the 'fast X' or 'fast Z effect' (Box 2; Presgraves 2008; Vicoso & Charlesworth 2006). This is due to hemizygous exposure, where recessive alleles are always expressed in XY-males and ZW-females and therefore have a higher probability of fixation if they are beneficial (Charlesworth et al. 1987). Moreover, Z and X chromosomes are only present in on average 1.5 copies in each individual while autosomes are present in two copies and this lower populations size of sex chromosomes should lead to fixation of more mutations due to drift (Charlesworth et al. 1987; Ellegren 2008). A fast evolution of Z chromosomes should further be reinforced by male-bias in mutation rate (Box 1), a phenomenon which is present in several taxa including birds (Axelsson et al. 2004; Ellegren & Fridolfsson 1997; Li et al. 2002; Makova & Li 2002). Z chromosomes will evolve fast in the

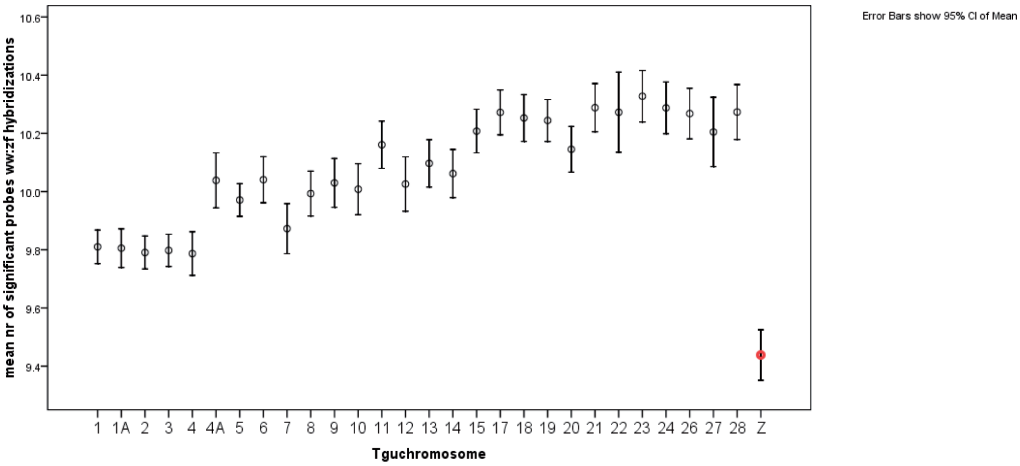


Figure 4. Mean number of significantly hybridizing probes when willow warbler DNA was hybridized to zebra finch sequences on the Lund-zf array (see also Figure 1b in paper 5). A low number of significant probes indicates a high degree of sequence divergence as probes with 3 or more substitutions between the species will not yield significant signals.

presence of a male mutation bias since they are present twice as often in males as in females (Fitzpatrick & Hall 2004).

Previous studies of 28 Z-linked introns between the chicken and the turkey and 172 Z-linked genes between the chicken and the zebra finch have demonstrated a fast Z effect in birds (Axelsson et al. 2004; Mank et al. 2007) and together with our study covering 687 Z-linked sequences it is becoming increasingly clear that the fast Z effect is a major factor on the avian Z and it is likely to have substantially influenced the evolution of this chromosome. The fast Z effect is thereby a potential key player when it comes to

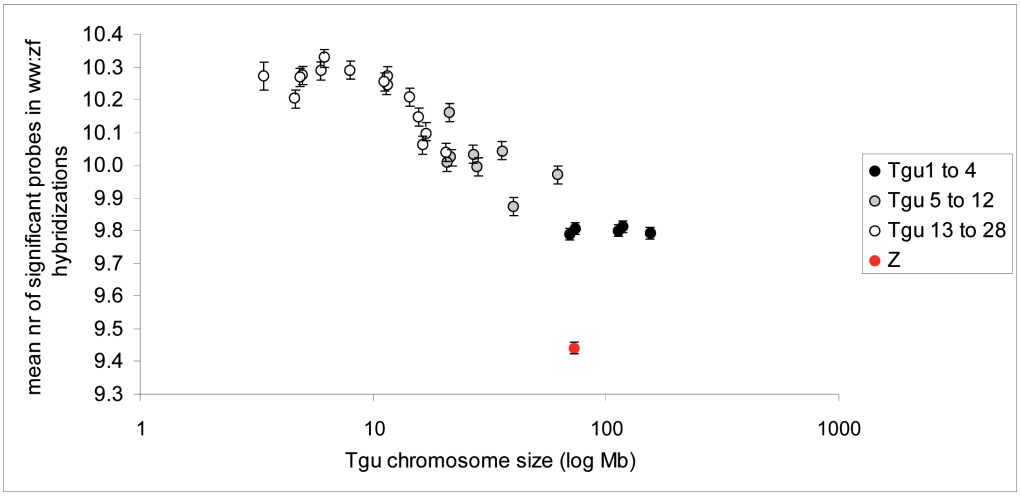


Figure 5. Mean number of significantly hybridizing probes and how it relates to chromosome size when willow warbler DNA was hybridized to the zebra finch sequences on the Lund-zf array (see also Figure 3b in paper 5). A low number of significant probes indicates a high degree of sequence divergence as probes with 3 or more substitutions between the species will not yield significant signals.

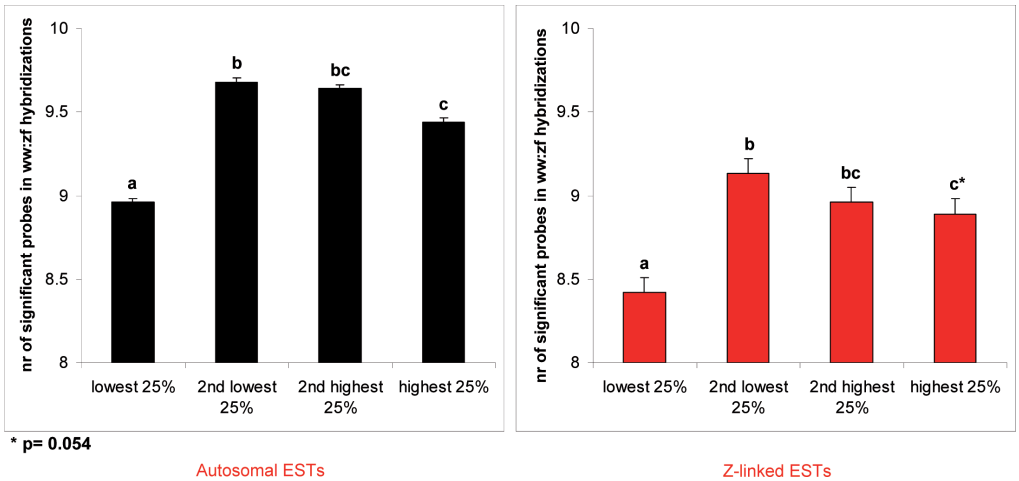


Figure 6. Mean number of significantly hybridizing probes divided into quartiles by gene expression intensity from lowest 25% to highest 25%. A low number of significant probes indicates a high degree of sequence divergence as probes with 3 or more substitutions between the species will not yield significant signals. This figure represents the number of significantly hybridizing probes when willow warbler DNA is hybridized to the zebra finch sequences on the Lund-zf array (see also Figure 6b in paper 5).

explaining why the avian Z chromosome seems to have played such a large and substantial part in avian adaptive evolution and speciation (Ellegren 2008; Qvarnstrom & Bailey 2009; Saether et al. 2007).

The link between divergence rate and chromosome size. Chromosome size in the avian genome spans almost two orders of magnitude (ICGSC 2004). Recombination rate, GC content, gene density and substitution rate are negatively correlated with size of avian chromosomes so that microchromosomes have the highest recombination and substitution rates (Axelsson et al. 2005; ICGSC 2004). However, even if avian microchromosomes have higher general substitution rates than macrochromosomes they have lower ratios of non-synonymous to synonymous substitutions (dN/dS; Axelsson et al. 2005; ICGSC 2004), indicating either a higher degree of purifying selection on small chromosomes or less efficient selection on macrochromosomes. The differences in dN/dS are likely to be associated with the differences in recombination rates on avian chromosomes. The efficiency of selection covaries with recombination rate (Hill & Robertson 1996; Nordborg et al. 1996) and, hence, it is possible that deleterious mutations on the highly recombining microchromosomes are more easily purged (ICGSC 2004). Moreover, background selection and selective sweeps are more common in regions of lower recombination (Ellegren 2007). These processes lead to an increase in the number of fixed weakly deleterious alleles at loci that are linked to selected alleles (Charlesworth et al. 2003; Smith & Haigh 1974), and thereby to a potentially higher dN on the avian macrochromosomes which have a lower recombination rate.

Our results (which measured synonymous and non-synonomous substitutions simultaneously), show that if dS is higher in coding regions on passerine microchromosomes than on macrochromosomes then this effect is weak compared to the effects caused by purifying selection on microcromosomes and/or the fixation of deleterious alleles on macrochromosomes (Figure 5).

The effect of gene expression level on divergence rates. We found that the degree of conservation is highest for ESTs with medium expression levels on both the Z chromosome and the autosomes (Figure 6). These results differs somewhat from previous studies which have shown that sequence and protein evolution decrease with increasing gene expression (Duret & Mouchiroud 2000; Pal et al. 2001; Subramanian & Kumar 2004) . The

sample size of our study is more than double the sample size of any previous study and it is therefore possible that higher statistical power allowed us to detect this interesting pattern. Moreover, the pattern we have found could at least to some extent be bird-specific (previous studies have not been focused on birds). There are two reasons why we believe that our results can be interpreted as showing a higher degree of directional selection at highly expressed genes or a higher degree of stabilizing selection at genes with medium expression levels. Or, in other words, that it is the number of non-synonymous mutations rather than synonymous mutations that is higher in highly expressed than medium expressed genes.

First, a previous study in mammals has measured the rate of synonymous substitutions (dS) separately and found no significant correlation with gene expression level even though dN/dS decreased with increasing gene expression (Duret & Mouchiroud 2000). Secondly, if dS is affected by the level of gene expression the expected pattern would be a lower divergence level of highly expressed genes and not a higher divergence as we observed. This is due to a process known as codon bias, where some codon triplets corresponding to a certain amino acid is more efficiently translated than others, leading to potential selection against certain synonymous substitutions as gene expression increases (Akashi 1994; Sharp et al. 1995). Hence, we find it plausible that the lower degree of divergence in genes with medium expression levels is linked to a lower number of non-synonymous substitutions in such genes.

4.6. Divergence of individual genes on the avian Z chromosome is determined by sex-bias and gene expression level (Paper 6)

In this paper we studied patterns of divergence on the Z chromosome in more detail by comparing unbiased and male-biased Z-linked genes. We did this in two ways: (i) by using the same dataset on divergence levels of ESTs as we used in paper 5, and (ii) by studying the number of synonymous and non-synonymous substitutions in 37 Z-linked orthologous genes between the zebra finch and the chicken. We found that Z-linked ESTs evolved faster than autosomal ESTs regardless of whether or not they were male-biased and that within the Z chromosome, male-biased ESTs evolved faster than unbiased ESTs. Moreover, male-biased Z-linked ESTs and genes evolved at the same rates regardless of gene expression level while for unbiased Z-linked ESTs and genes expression was a predictor of sequence divergence (Figure 7). Hence, in this study we provided further evidence for the fact that the fast Z effect is substantial in birds. We confirmed that it is not driven by the sex-bias on this chromosome but that unbiased Z-linked genes also evolve faster than autosomal genes. Moreover, we demonstrated that male-biased Z-linked genes are clearly affected by different selection pressures than unbiased Z-linked genes.

4.7. On sex chromosome evolution: trade-offs between gene dose and sexual antagonism (Paper 7)

Based on results in this thesis and on other recent studies in birds we propose the following in this opinion paper:

1. In contrast to previous suggestions, the extensive male-bias in gene expression found on Z chromosomes (Ellegren et al. 2007; Itoh et al. 2007; Zha et al. 2009; paper 3) cannot be explained simply by an inability to dosage compensate in ZW-females. Rather it is likely to be caused by properties of male-biased genes which make them unsuitable for high expression in females. This is supported by results demonstrating that: (i) female birds do dosage compensate to a high extent (Itoh et al. 2007; paper 3), (ii) male-biased and unbiased Z-linked genes have different gene expression levels (Melamed & Arnold 2007; Zha et al. 2009; paper 3), (iii) male-biased and unbiased Z-linked genes have different levels of divergence (paper 6), and (iv) male-biased and unbiased Z-linked genes belong to different functional categories (Melamed & Arnold 2007; Zha et al. 2009).
2. Two processes are likely to have interacted and caused the extensive male-bias in gene expression on Z chromosomes. First, Z chromosomes are more conductive to certain types of sexual selection than X chro-

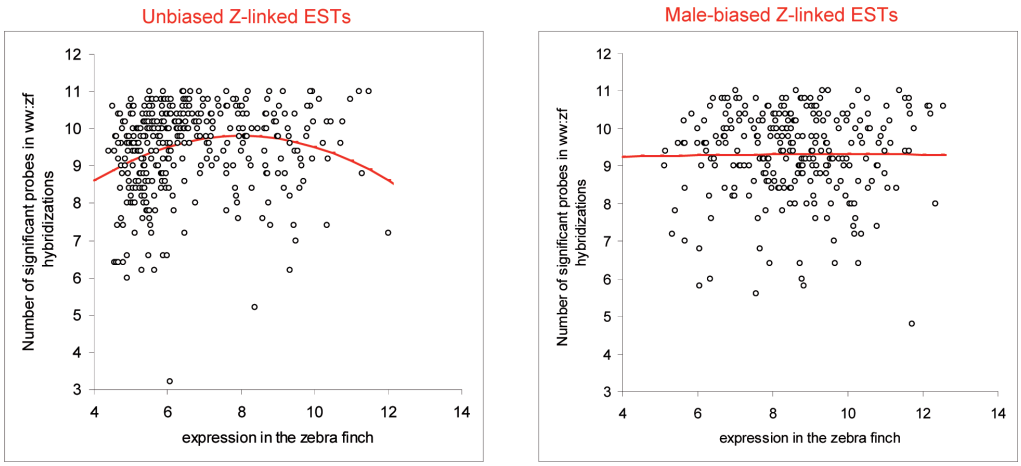


Figure 7. Association between number of significantly hybridizing probes and gene expression in the zebra finch when DNA from the willow warbler is hybridized to the zebra finch sequences on the Lund-zf array (see also Figure 3b in paper 6). Curves represent the association between number of significantly hybridizing probes and gene expression as given by the parameter estimates from a GLM with the number of significant probes as dependent variable. The following independent variables were added to the GLM: mean level of gene expression (both as the original value and as the quadratic term), type (unbiased or male-biased) and the interactions between type and the two expression terms. In these GLMs, both interaction terms between gene expression type were significant (ww:zf: $p < 0.02$; table 1 in paper 3).

mosomes. Such sexually selected alleles are likely to be sexually antagonistic and detrimental to females (Rice 1984; Rice & Chippindale 2001; Seger & Trivers 1986). Secondly, a male-bias in mutation rate, known from many taxa, is likely to have lead to the following scenario: Y chromosomes (100% in males) degraded faster than W chromosomes (0% in males) and selection for dosage compensation is thereby likely to have taken place at an earlier point in time on X than Z chromosomes. X-linked genes (33% in males and thereby relatively slowly diverging) were largely unchanged as XY-males were selected for compensation. However, when ZW-females were selected for dosage compensation Z (66% in males) had evolved rapidly while being particularly conductive to sexual selection in males. Hence, ZW-females likely faced a trade off between retaining gene expression dose and avoiding expression of traits which compromised female function.

We therefore argue that higher conductivity to sexual selection and male-biased mutation rates are proximate mechanisms which can explain why the male-bias on Z chromosomes is not mirrored by an equally extensive female-bias on X chromosomes in mammals, *Drosophila* and *C. elegans*.

5. Implications of this thesis

Apart from the comments and discussion we have included in paper 7 there are some obvious conclusions that can be drawn from the results in this thesis.

First, there are some obvious areas in which continued research could be focused. For example, the extent of male-biased gene expression (dosage compensation) on Z chromosomes needs to be studied in more species, in particular in other taxa than birds.

Moreover, the neo-sex chromosome in birds which we identified in paper 4 (Tgu4a) will open up for studies of: (i) the degree of sequence differentiation between the neo-W and neo-Z homologues, (ii) the degree of degradation of the neo-W chromosome, (iii) the processes by which this degradation occurs, and (iv) the rate of evolu-

tion and the dosage compensation on the neo-Z homologues. Moreover, since Tgu4a, which corresponds to ancestral chromosome 10 in the avian genome, display several interesting characteristics also in species where it is not sex-linked (see paper 4) further studies into its gene content should be highly interesting.

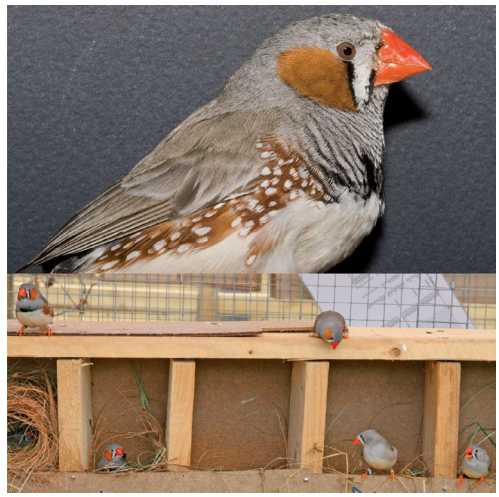
Secondly, existing microarray resources for the zebra finch can be used effectively and successfully for wild non-model passerine birds. Warblers (Sylvidae) are separated from the zebra finch by 24 to 47 million years (Barker et al. 2004; Jonsson & Fjeldsa 2006; Zuccon 2007). We have demonstrated that gene expression studies of warblers on the Lund-zf array are reliable and that such expression studies of wild birds have power enough to yield highly interesting results (paper 2, 3 and 4). Moreover, microarrays with several short probes for each gene can be used to study sequence divergence between species (paper 5 and 6). This method can also be used for filtering of gene expression data of wild birds, making such studies more reliable. Hence, for the next coming decade, until methods like for example 454 sequencing becomes more affordable and manageable, a microarray approach to studying gene expression and gene divergence of wild birds might be a favorable option for many scientists.

Apart from the work included in this thesis we have extensive data on sequence divergence in more species as well as gene expression data on inbreeding in zebra finches and on migrating willow warblers, clearly illustrating the wide scope of the microarray approach in avian genomics.

6.Methods

6.1 RNA samples

The zebra finch (Picture 1): Total RNA from telencephalon of 6 female and 6 male zebra finches were used in paper 2, 3, 5 and 6. Zebra finches were housed at Professor Art Arnolds lab at the University of California, Los Angeles (UCLA). All birds were hatched at the aviary and sacrificed by decapitation as adults (>90 days of age). All birds were healthy at the time of sacrifice. Full telencephalon was removed from the skull intact and flash frozen on dry ice.



Picture 1. The zebra finch, *Taeniopygia guttata*. Pictures are from the population at Lund University (Stensoffa).

The common whitethroat (Picture 2): The common whitethroat gene expression data which has been included in manuscript 2, 3 and 4 corresponds to hybridizations of 22 samples (extracted from full brain of 11 females and 11 males) The common whitethroat (*Sylivia communis*) is a warbler of the family *Sylviidae*, a seasonal breeder and a long distance migratory bird. The species breeds in Europe and winters in Africa south of Sahara. Birds were caught in the wild on two locations, in southern Sweden and central Nigeria, using mist-nets and playback song (Picture 3). All birds caught were adult, in Africa at least six months old and in Sweden at least one year old. The entire brain was transferred into a tube containing RNA later™ RNA stabilization Reagent immediately after sacrifice. See paper 2 for details regarding sample handling.

6.2 Extractions of RNA samples

The zebra finch: Total RNA was extracted using the protocol for TRI Reagent (Applied Biosystems/Ambion). All samples were DNase I treated (with Turbo DNase I; Ambion) and cleaned up using a QIAgen RNeasy spin column.

The common whitethroat: Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit

Picture 2. The common whitethroat, *Sylvia communis*. Picture taken of a wintering bird caught in the ringing effort at A. P. Leventis Ornithological Institute (APLORI) in central Nigeria.

(QIAGEN, cat no: 74804). The samples were extracted following instructions in the protocol supplied with the kit (step 9 to 17). See paper 2 for details regarding extractions.

6.3 DNA samples

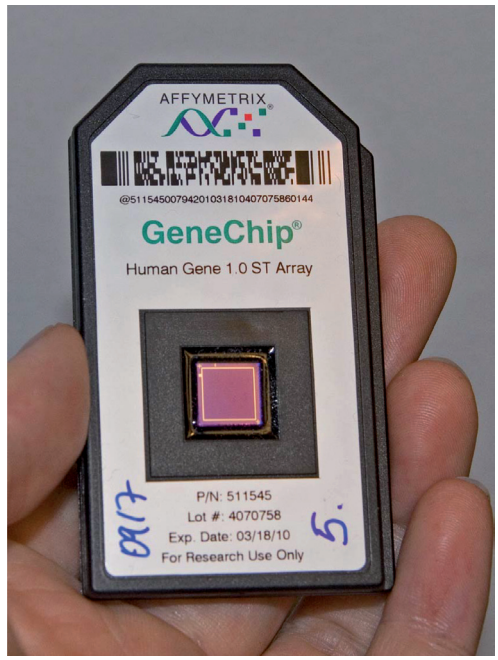
The zebra finch: Data from hybridizations of zebra finch DNA to the Lund-zf array has been used in paper 1 – 6. Zebra finch DNA was extracted from blood collected from an indivudal of the captive population at Lund University (Stensoffa).

Wild passerines: Data from hybridizations of common whitethroat DNA has been used in paper 1 – 4 while data from hybridizations of willow warbler and crow DNA was used in paper 5 and 6. DNA from the common whitethroat, the willow warbler and the crow was extracted from blood collected during mist-netting in Sweden and Denmark. DNA samples were collected in SET buffer and stored at –20 until extraction.

6.4 The Lund-zfa microarray

All studies in this thesis utilized a large-scale microarray for the zebra finch, designed during 2005 and 2006 (the Lund-zfa Affymetrix microarray, Picture 4). The array was designed using over 22000 EST sequences (i. e. sequenced mRNAs) from the ESTIMA database produced by the Songbird Neuro-Genomics Initiative (SoNG initiative; Naurin et al. 2008; Replogle et al. 2008). The array is an Affymetrix costum array (www.affymetrix.com). For the design Affymetrix was provided with EST sequences and did the following, according to their standard procedures and my instructions: (i) BLASTed all sequences against each other and removed all redundancies, (ii) started from the 3 prime end of the sequenc-

Picture 3. Mist-netting to catch wild common whitethroats. Approximately 5 nets were used per field site and playback song was played at each net. Picture taken during fieldwork for this thesis at the Jos Plateau in central Nigeria in 2006. Picture taken by Oskar Brattström and features Sara Naurin (right) and Juliana Dänhardt (left).



Picture 4. An Affymetrix gene expression microarray. This particular array is the current human chip and contains 28 869 genes. The Lund-zf array, featuring over 22 000 EST sequences corresponding to about 15800 genes, looks just like the chip on the picture. All the gene sequences on an Affymetrix chip are fastened chemically in the small square in the middle of the array. See Box 3 for an explanation of what microarrays do and section 6.4 for description of how the Lund-zf array was designed. The array at the picture has been used in an experiment at the Swegene Centre for Integrative Biology at Lund University (SCIBLU) where all the experiments in this thesis have been hybridized.

es and designed 11 probes (25 base pairs long) for each EST sequence, (iii) designed probes to have comparable GC content, and (iv) created a design of the array where the 11 probes representing an EST was distributed across the array surface in a fashion so that their hybridization is independent of the other probes representing the same EST. The resulting microarray is a standard gene expression chip, designed to hybridize with cDNA (mRNA) according to Affymetrix's standard protocols for gene expression studies and can thereby be used by any of their service providers using standard equipment. However, it does not include Mis-Match probes, a feature normally included by Affymetrix and which is often used in background corrections, and normalization and background correction procedures therefore differs somewhat from those used on an commercial Affymetrix chip (see paper 1 and 2).

6.5 Gene expression studies on the Lund-zf array

Samples containing total RNA were delivered to an Affymetrix's service provider, the Swegene Center for Integrative Biology at Lund University (SCIBLU genomics, http://www.lth.se/sciblu), where they were hybridized according to standard Affymetrix protocols for RNA. Before hybridization samples were quality checked at SCIBLU using a Nanodrop spectrophotometer and an Agilent 2100 Bioanalyzer. All samples were of high quality with high and comparable

RNA Integrity Numbers (RIN; Schroeder et al. 2006). See paper 2 for details regarding the procedures at SCIBLU.

6.6 Gene divergence (Comparative Genome Hybridization, CGH) studies on the Lund-zf array

DNA extraction and Array Hybridizations. DNA was extracted from blood samples using standard phenol-chlorophorm-isoamylacohol extraction (Sambrook et al. 1989). DNA was labeled and fragmented according to step 7 and 8 of an Affymetrix standard protocol, the GeneChip Mapping 10K 2.0 Assay Manual (www.affymetrix.com). The biotin labeled DNA samples were hybridized on the Lund-zf array at the Swegene Center for Integrative Biology at Lund University (SCIBLU), according to standard Affymetrix protocols for RNA. See paper 1 for details.

Calculation of the background cutoff. In order to rank each probe on the Lund-zf array according to whether it hybridized significantly or not we calculated the mean background signal from all empty features on the array. We used this mean background signal plus two standard deviations, as they conventionally represent the 95% confidence interval, as background cutoff. Probes hybridizing with higher intensities than this cutoff were considered significant. Each EST on the Lund-zf array is represented by 11 probes and all ESTs were ranked as having 0–11 of its probes significant.

A probe is expected to yield significant signals on the Lund-zf array when it has zero, one or more rarely two substitutions compared to the target DNA. If it has three substitutions or more it is expected to be non-significant (see paper 1).

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Svensk sammanfattning

Denna avhandling har fokuserat på tre huvudområden: (i) de skillnader som finns i hur honor och hanar använder sig av generna i genomet, (ii) hur könskromosomer evolverar, samt (iii) hur geners evolutionstakt påverkas av det genuttryck de har och av den kromosom de sitter på. Det tredje området, geners evolutionstakt, är en naturlig förlängning av de övriga studierna eftersom både gener med könsspecifikt uttryck samt gener på könskromosomer evolverar i högre takt i jämförelse med andra gener i genomet.

Könsskillnader i hur gener uttrycks

Könsdimorfism är vanligt hos många arter och omfattar till exempel skillnader i kroppsstorlek, livslängd, beteende eller i morfologiska karaktärer (som horn hos hjortdjur eller fjäderdräkt hos fåglar). Vi vet idag att honor och hanar har i princip identiskt DNA. De finns ytterst få gener som är begränsade enbart till ett kön och de omfattande könsskillnaderna i naturen måste därmed uppstå genom konsekventa skillnader i de båda könens sätt att utnyttja generna. Skillnader mellan könen i hur geners aktivitet regleras har visat sig vara mycket vanligt hos många arter och många gener uppvisar könsspecifikt genuttryck någon gång under livscykeln (könsspecifikt uttryck här innebär inte nödvändigtvis att en gen enbart är uttryckt i ett kön men att den har högre uttryck i det ena könet än det andra). Gener med sådan könsspecifik reglering är ofta knutna till reproduktion, ofta starkt selekterade och de evolverar fort.

Evolution av könskromosomer

Könskromosomer innehåller de gener som avgör en individs kön. Hos arter som till exempel däggdjur och bananfluga bestäms hanligt kön av gener på Y kromosomen och hanar har därmed en Y kromosom och en X kromosom (XY), medan honor har två X kromosomer (XX). Hos fåglar bestäms honligt kön av gener på den så kallade W kromosomen. Honor har därmed en W kromosom och en Z kromosom (ZW) medan hanar har två Z kromosomer (ZZ). Det är vanligt med genetiskt bestämt kön i många organsimgrupper och könskromosomer har uppstått många gånger oberoende av varandra.

Könskromosomer har på grund av att de innehåller könsbestämmande gener påverkats av selektionen på ett unikt vis. Det har lett till att de idag har många intressanta egenskaper. Till exempel så har Y och W kromosomer brutits ned tills att de bara innehåller de könsbestämmande generna och ett fåtal andra gener. Dessutom så har X kromosomer och Z kromosomer utvecklats till att innehålla många gener som är sexuellt antagonistiska, dvs. de är har positiv påverkan på det ena könet men negativ på det andra. X och Z kromosomer innehåller dessutom många gener som har könsspecifikt uttryck (och därmed kodar för könsdimorfism). Det finns också hypoteser som förutsäger att X och Z bör evolvera fort och att de bör ha stor påverkan på artbildningsprocessen.

Det är extra intressant att arbeta med fåglar för att studera könsdimorfism och könskromosomer. Fåglar är en av de grupper som uppvisar störst skillnader mellan könen, ett faktum som uppmärksammades redan av Darwin i slutet av 1800-talet. Det faktum att fåglar har Z och W kromosomer öppnar också för intressanta jämförelser med de mest välstuderade arternas (t.ex. människans) X och Y kromosomer. Sådana jämförelser kan visa hur könskromosomer utvecklas i honor respektive hanar eftersom Y finns enbart i

hanar, X finns 33% av tiden i hanar, Z finns 66% av tiden i hanar medan W enbart finns i honor.

Vi har identifierat hundratals gener med könsspecifikt uttryck i hjärnan hos sångfåglar och konstaterat att de till mycket hög grad är högre uttryckta hos hanar än hos honor. De flesta av dessa gener sitter på Z kromosomen, d.v.s. den könskromosom som finns i två kopior i hanar och bara en i honor. Ett stort antal gener på Z kromosomen med högre uttryck i hanar än i honor har i och med vår studie konstaterats i tre fågelarter och en insekt. Detta mönster finns inte hos arter med X och Y kromosomer (t.ex. människa) och manus 2, 3 samt 7 i avhandlingen diskuterar detta. Vi har också visat att Z-gener som har högre uttryck i hanar generellt är mer höguttryckta (mer 'aktiva') än gener som uttrycks lika i könen. Detta indikerar att det finns systematiska skillnader mellan dessa gruppar av gener.

Vi har även beskrivit en ny könskromosom i fågel, en kromosom som inte finns i kycklingen eller zebrafinken (de två helgenomsekvenserade fågelarterna) men som vi kunnat identifiera i sångare, en fågelgrupp som innehåller några av våra mest välkända trädgårdsfåglar (som t.ex. trädgårdssångare och lövsångare). Den nya könskromosomen har hittills identifierats i två arter sångare, törnsångare och trastsångare. Denna upptäckt är mycket spännande eftersom denna nyupptäckta könskromosom är mellan 24 och 47 millioner år gammal vilket är mycket yngre än de andra könskromosomerna i fågel (som är ungefär 150 millioner år gamla). Det innebär att det kommer vara möjligt att testa många hypoteser kring hur könskromosomer utvecklas på denna nyupptäckta könskromosom.

Geners evolutionstakt i förhållande till hur de används och var i genomet de finns

Kunskap kring de stora övergripande mönster som påverkar geners utveckling är mycket värdefull eftersom det kan ge oss en uppfattning om vilka delar av genomet som är strikt nödvändiga för överlevnad samt vilka delar som har evolverat mycket.

Kunskap om vilka kromosomer och gener som är välbevarade mellan arter, vilka som utvecklas snabbt samt kunskaper kring de egenskaper som kännetecknar välbevarade kontra snabbt evolverande kromosomer är inte bara intressant eftersom det kan ge oss upplysningar om aspekter av evolution som diskuterats av forskare sen Darwins tid men också för att sådan information kan ge oss ledtrådar angående mekanismerna bakom sjukdomar förknippade med att ha för många eller får få kromosomer (t.ex. Downs syndrom).

Information kring hur geners utveckling påverkas av det genuttryck de har kan i sin tur ge oss ledtrådar kring mekanismer bakom sjukdomar som är förknippade med förändringar i geners aktivitet (som t.ex. cancer).

Vi har studerat graden av sekvensevolution mellan arter för över 15000 gener i fågelgenomet.

Vi har undersökt gener med olika uttryck och konstaterat att på autosomala kromosomer (de 'vanliga' kromosomerna) är gener med medelhögt uttryck mest välbevarade medan låguttryckta gener evolverar allra snabbast. Detsamma gäller för de gener på Z kromosomen som har samma uttryck i de båda könen. För de hundratals gener på Z kromosomen som har högre uttryck i hanar däremot, så spelar genuttryck ingen roll, de evolverar i samma takt oavsett vilken uttrycksnivå de har.

Detta är första gången man visat att gener med medelhögt uttryck är de mer välbevarade än höguttryckta gener. Det kan hända att den höga statistiska styrkan i vårt experiment har gjort att vi kunnat upptäcka detta intressanta mönster. Det är också möjligt att mönstret till viss del är specifikt för fåglar då detta är första studien på fågelgenomet.

Det faktum att evolutionstakten hos Z-gener med högre uttryck i hanar inte påverkas av genuttryck tyder på att de utsätts för andra selektionstryck än övriga gener i genomet.

Vi har dessutom undersökt de olika kromosomerna och sett att Z kromosomen evolverar konsekvent snabbare än autosomala kromosomer och att detta gäller för alla gener på Z, oavsett om de har samma uttryck i könen eller ej. Inom Z kromosomen evolverar gener med könsspecifikt uttryck snabbare än övriga gener. För autosomala kromosomer är dessutom sekvensevolutionen direkt länkad till kromosomstorlek så att stora kromosomer evolverar fort medan små kromosomer evolverar långsamt.

Att Z kromosomen i fågel evolverar fort har nu visats av fyra studier (varav två finns i denna avhandling) och vi kan därmed säga att det hos fåglar finns gott om stöd för den hypotes som säger att könskromosomer bör evolvera snabbt. Att geners evolutionstakt påverkas av storleken på kromosomen de sitter på har tidigare visats i kyckling och i och med våra resultat på sångfåglar kan vi konstatera at detta mönster verkar vara generellt i fåglar (kycklingen och sångarna skiljs åt av 100 miljoner års evolution).

Det faktum att de Z-gener som har högre uttryck i hanar än i honor evolverar i annan takt än övriga gener innebär att vi har visat att de generna: (i) har högre uttrycksnivåer än övriga gener på Z , (ii) har en evolutionstakt som inte påverkas av det genuttryck de har, samt (iii) har evolverat snabbare än andra gener i genomet. Det är därmed mycket troligt att de har specifika funktioner som är unika för dem och positiva för hanar (och eventuellt negativa för honor som har lägre uttryck).

Denna avhandling har därmed lagt fram resultat som visar att antalet gener med könsspecifikt uttryck i fågelhjärnan är mycket högt, högre än i däggdjur. Vi har även visat att den könskromosom som kallas Z i fågel innehåller de flesta av generna med könsspecifikt uttryck samt att den evolverar fort. Vi har också identifierat en ny könskromosom i fågel, en upptäckt som öppnar för studier kring hur könskromosomer och könsdimorfism evolverar. Dessutom har vi sett att evolutionstakten hos autosomala gener påverkas starkt av det genuttryck de har och av vilken kromosom de sitter på, samt att evolutionstakten för gener på Z kromosomen är påverkas av om de har könsspecifikt uttryck eller ej. Till sist har vi lagt fram resultat som visar att Z-gener som har högre uttryck i hanar än i honor evolverat på ett unikt vis i fågelgenomet.

Acknowledgements

Knowing that this is what you'll all read, what have I got to say?
Well, first of all. Science is fun. A lot of fun, and don't let anyone tell you otherwise. Luckily, it feels rewarding rather than exhausting to work hard with something you enjoy.
Good science, I believe, is done by people who have managed to keep an open mind. That is not just achieved by questioning what you are told (although that is obviously important) but also by what you choose to expose yourself to. So, my advice is: give it a rest, have a coffee, go shopping, have a beer or two, take time off and travel around the world for a while, get married, have children, make friends. I'm guessing you will be a better scientist for it (or better at whatever it is you do).

Who knows, you might end up driving a Fiat Uno through the Sahara desert (and quickly find out why doing just that is a really bad idea).
You might meet renowned personalities like Harvey the Wonder-Hamster, Boris the Spider and Mr Lariam (avoid the last one), or learn all the reasons why you should not read the booklets left on the side tables at Nigerian hotels.
You might end up taking a tour of a museum somewhere listening to a guide who describes how fossils are created: *'and so the tree fell into a hole and turned into stone',* or you might find yourself reading a report written by a National Park Official who has confused the words gorilla and guerilla: '*the gorillas are threatened but we have also received worrying reports that they are elsewhere being trained in warfare. Perhaps future generations will benefit from their decline'.*
You might end up juggling oranges in front of a village full of strangers when your car has broken down and your driver has taken off (and once again realize that people are always more likely to be generous than dangerous).
Personally I have certainly not always succeeded in keeping an open mind but I do know I have thoroughly enjoyed myself trying.

When I think back on the last few years, people and places, rather than lab work and analyses, keeps filling my head. This book would therefore not have been complete without this chapter.
I will start at the beginning of my work in biology and go from there.

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A microarray for large-scale genomic and transcriptional analyses of the zebra finch (*Taeniopygia guttata*) and other passerines

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TECHNICAL ADVANCES

A microarray for large-scale genomic and transcriptional analyses of the zebra finch (*Taeniopygia guttata*) and other passerines

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Abstract

The microarray technology has revolutionized biological research in the last decade. By monitoring the expression of many genes simultaneously, microarrays can elucidate gene function, as well as scan entire genomes for candidate genes encoding complex traits. However, because of high costs of sequencing and design, microarrays have largely been restricted to a few model species. Cross-species microarray (CSM) analyses, where microarrays are used for other species than the one they were designed for, have had varied success. We have conducted a CSM analysis by hybridizing genomic DNA from the common whitethroat (*Sylvia communis*) on a newly developed Affymetrix array designed for the zebra finch (*Taeniopygia guttata*), the Lund-zf array. The results indicate a very high potential for the zebra finch array to act as a CSM utility in other passerine birds. When hybridizing zebra finch genomic DNA, 98% of the gene representatives had higher signal intensities than the background cut-off, and for the common whitethroat, we found the equivalent proportion to be as high as 96%. This was surprising given the fact that finches and warblers diverged 25–50 million years ago, but may be explained by a relatively low sequence divergence between passerines (89–93%). Passerine birds are widely used in studies of ecology and evolution, and a zebra finch array that can be used for many species may have a large impact on future research directions.

Keywords: Affymetrix, cross-species, gene expression, microarray, *Passeriformes*, *Taeniopygia guttata*

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Microarrays have revolutionized biological research in the last decade (Schena *et al*. 1995; Lockhart *et al*. 1996; Cheung & Spielman 2002; Preuss *et al*. 2004) but the technology is resource demanding, and consequently, there are still many research fields where it has not yet been extensively used, for example behavioural ecology (Whitfield *et al*. 2003; Fitzpatrick *et al*. 2005).

Here, we present a custom-designed Affymetrix (www.affymetrix.com) array for a passerine, the zebra finch (*Taeniopygia guttata;* family Passeridae) [the 'Lund-zf' (Lund zebra finch) array , Table 1] and its potential use for other passerine bird species. The array is based on sequence data for 22 630 expressed sequence tags (EST) that correspond to about 15 840 nonredundant genes expressed in the zebra finch brain (estimated to cover about 65–70% of the expressed genome). Probes were designed and chosen according to Affymetrix standard procedures (see the GENECHIP CUSTOMEXPRESS ARRAY Design Guide). To evaluate the quality of the Lund-zf Affymetrix custom array, we ran a microarray analysis using genomic DNA from zebra finch (*T. guttata;* family Passeridae). We also wanted to evaluate whether the array could work for other passerine birds and therefore performed a similar analysis on a distantly related

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Table 1 Information about the Lund-zf microarray

Affymetrix array name	Lund-zfa530237N (www.biol.lu.se/zooekologi/molecol/medarbetare/filer/array.pdf)
Species	Zebra finch, *Taeniopygia guttata*
Array type	Affymetrix custom design NIMBLEEXPRESS, perfect match probes only
Sequence type	Expessed sequence tags (ESTs) from brain tissue
Number of EST sequences	22 630 ESTs
Number of probes per sequence	11 (except for 148 ESTs that are represented by 8, 9 or 10 probes)
Length of probes	25 bases
Number of features with probes	254 430
Number of empty features used for background calculations	255 561
Number of nonredundant genes	~15 840
Sequence source	ESTIMA: songbird (http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet = songbird) and NCBI GenBank
Number of sequences annotated	~70%
Controls on the array	Affymetrix amplification and hybridization controls, Affymetrix *Gallus gallus* controls, and zebra finch controls (GAPDH, Histone H3, β-actin)

passerine, the common whitethroat (*Sylvia communis*; family Sylviidae). The split between the Passeroidea (sparrows and relatives, including the zebra finch) and Sylvioidea (warblers including the common whitethroat) should have taken place less then 47–51 million years ago when the parvorder Passerida split from more basal passerines (Jonsson & Fjeldsa 2006) but most likely before 24 million years ago when a dated split between starlings and thrushes has been estimated to have taken place (Zuccon 2007). In order to estimate the sequence similarity between avian species, we compared zebra finch ESTs to ESTs of the chicken (*Gallus gallus*) and of the house finch (*Carpodacus mexicanus*), respectively (ESTs available on GenBank). We used BLAST analyses to detect homologous single-copy sequences (*E*-value < 1E-10). We only compared EST alignments of at least 100-bp length, because this length was sufficient to verify that homologous genes were being compared also for the most diverged sequences. The sequence similarity between the zebra finch and the chicken was 89.5% (range: 81–99%; data from 97 alignments spread across the chicken genome with intervals of about 10 Mb), whereas the similarity between the zebra finch and the house finch was 92.8% (range: 88–100%; data from 42 alignments). The sequence similarity between the zebra finch and the common whitethroat should be between these two values, but closer to that between the two finches than to that between the zebra finch and the chicken, considering the fact that the split between the chicken and other passerines has been estimated to about 100 million years ago (Van Tuinen & Hedges 2001; Pereira & Baker 2006). These results lead to the conclusion that all passerine birds are likely to have a sequence similarity of at least around 89%. Hence, if a zebra finch microarray can be used successfully for a warbler like the common whitethroat, it should be possible to use it for most other passerine species yielding comparable, or better, results.

Materials and methods

Extraction and hybridization

DNA was extracted from 20 µL blood from one adult zebra finch male and one adult common whitethroat male with standard phenol–chloroform–isoamylalcohol extraction (Sambrook *et al*. 1989). After diluting samples in 45 µL of EB buffer (QIAGEN P/N 120002), DNA was labelled and fragmented according to Step 7 and Step 8 of an Affymetrix standard protocol, the GeneChip Mapping 10K 2.0 Assay Manual (www.affymetrix.com). Samples were quantified on an Ultraspec 3000 UV/visible spectrophotmeter (Pharmacia Biotech) before fragmentation and labelling (according to the GeneChip Mapping 10K 2.0 Assay Manual, www.affymetrix.com) and they both contained 20 µg of DNA. After fragmentation and labelling, quantification on a Nanodrop spectrophotometer gave values of 15 µg for the zebra finch and 25 µg for the common whitethroat. We assumed that the second quantification was incorrect and that something had occurred during fragmentation and labelling that disturbed measures on the Nanodrop. Affymetrix does not (according to the 10K 2.0 Assay Manual) recommend a second quantification after the labelling step so there was no easy way to confirm if quantification at that step is problematic. We continued, trusting our initial quantification values, by adding a volume containing 15 of the supposed 20 µg to the standard Affymetrix hybridization mix for gene expression arrays. If however, the second quantification on the Nanodrop spectrophotometer was correct, 14.3 µg was hybridized on the zebra finch array and 19 µg on the common

whitethroat array. Even if the samples contained different amounts of DNA, it is unlikely that this would have any effect on the results because there was most likely a large excess of sample DNA on both arrays. The GeneChip Mapping 10K 2.0 Assay Manual recommends 20 µg of DNA for hybridization to the human SNP (Single Nucleotide Polymorphism) chip called the GeneChip Mapping 10K 2.0 array. However, we hybridized our samples according to standard Affymetrix protocols for RNA hybridizations on an Affymetrix expression array. The starting amount required for hybridizations of labelled DNA on such arrays has not, to our knowledge, been tested statistically or on a large scale. Our expression array is not as dense as the GeneChip Mapping 10K 2.0 Array and does not have nearly as many probes (254 430 compared to 420 000). Even though we have been unable to find any data on how the number of probes affects hybridizations on Affymetrix arrays, half the number of probes would require less DNA to saturate hybridizations. Normally, 10 µg of RNA would be hybridized to an expression chip of the size of the Lund-zf array, and a decrease in signal intensity would most likely not be detectable until sample amount is 5 µg or lower [SCIBLU Microarray Resource Centre (MARC), personal communication]. We conclude that if there was difference in the applied amounts of DNA on our arrays, it is highly unlikely that it had any effects on the measured signal intensities, because any excess of sample is washed away during the strict washing procedures in the Affymetrix protocol.

That our two arrays were saturated with sample DNA is also indicated by the fact that if we would have scaled the zebra finch array according to a possible difference in sample amount, some of the signal intensities on the zebra finch array would most likely had become higher than the scanner could have handled. Judging from the very high signal intensities on the zebra finch array, that is, the array with a possibly lower starting amount, it is not likely that intensities could have increased with a factor of 19/14.3 = 1.329 (the amount in the common whitethroat sample divided by the amount in the zebra finch sample should the second quantification be correct). This would mean that scaling would mimic a scenario that could not logically have taken place on the zebra finch array. Moreover, a scaling of the zebra finch data would have been pointless at this stage. Scaling of the signal intensities in the Affymetrix CEL files would have meant scaling of signals from probes and from the empty features used for background calculations (see below) by exactly the same amount. This would have led to no difference in the number of significant probes identified. We therefore used raw, nonscaled, signal intensities for our further analyses.

The samples were hybridized by an Affymetrix's service provider, the Swegene Center for Integrative Biology at Lund University (SCIBLU MARC, http://www.swegene.org/microarray), according to standard Affymetrix protocols for RNA. The biotin labelled DNA samples were used in the regular hybridization mix for GeneChip Arrays and hybridized onto the Lund-zf Affymetix array overnight in the GeneChip Hybridization oven 640 using standard procedures. The arrays were washed and then stained in a GeneChip Fluidics Station 450. Scanning was carried out with the GeneChip Scanner 3000 and image analysis was performed using GENECHIP OPERATING SOFTWARE. Signal intensities for all features on the array were processed in SPOTFIRE DECISIONSITE for Functional Genomics version 9.0. Data and protocols will be available online at ArrayExpress (www.ebi.ac.uk) with accession number E-MEXP-1181.

Background correction

Each gene representative on an Affymetrix array consists of a so-called probe set of 11 separate probes. Each probe is 25-nucleotide (nt) long. The probes are designed based on sequence information in the 3'–terminal region of the corresponding cDNA and normally a probe set covers a region of approximately 600 bp. The 11 probes in a probe set are positioned as separate features on the array. Because of this physical separation, the 11 probes function as technical replicates of the corresponding gene on the array. Background on Affymetrix arrays is normally estimated using so called mismatch probes (identical to the regular perfect-match probes except for a substitution of the nucleotide in the 13th position). The substitution on the 13th position of the mismatch probes means that while perfect-match probes hybridize on 25 out of 25 bp, mismatch probes hybridize on only 24 out of 25 bp. For cross-species work, when a large number of the perfect-match probes on the array will hybridize with only 24 out of the 25 bp because of sequence divergence between the sequenced and hybridized species, results using mismatch probes will be hard to analyse. Because of this, we did not include mismatch probes on the Lund-zf array. Background was instead estimated as the mean signal intensity from 255 561 features on the chip that contain no probes ('empty features', see Fig. 1) plus two standard deviations and this value was used as cut-off. Only probes with higher intensities than this cut-off was considered significant. Two standard deviations were chosen as they conventionally represent the 95% confidence interval. For both the zebra finch and the common whitethroat sample in our study, about 4% of the features used for background calculation had signal intensities higher than our background cut-off.

The Lund-zf array looks like a 'chessboard' with the white squares being the features containing probes and the dark squares being the empty features used for background calculations (Fig. 1). When hybridization on a probe feature is very high, some bleeding of that signal can occur into empty features. Such bleeding will not affect other probe features to a large extent because other probe features are

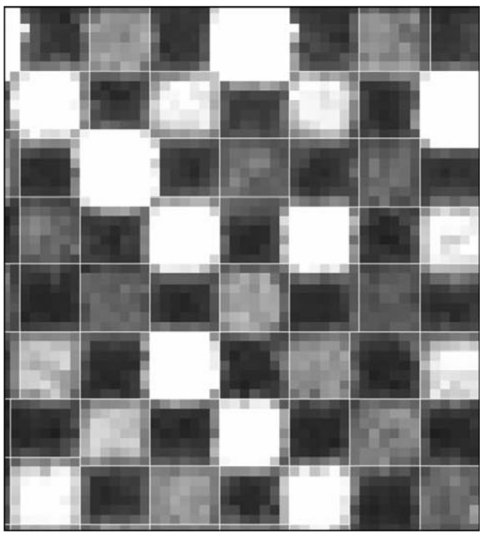

Fig. 1 A scanned image of a Lund-zf array. Every light feature is a region containing thousands of copies of the same probe. Every dark feature is a region where nothing has been spotted, an empty feature used for background calculation on the Lund-zf array.

only present on a diagonal line from the 'bleeding' feature (Fig. 1). Moreover, as can be seen in Fig. 1, within each square in the grid, the image consists of multiple small squares (spots). The spots just adjacent to a grid line in the DAT files will not be used in the calculation of signal intensities in CEL files (GeneChip Expression Analysis Algorithm Tutorial, Affymetrix). This is true for probe features as well as for empty features and will reduce the impact of bleeding. Bleeding might lead to an overestimated background when DNA is hybridized on the array. This is due to the very high degree of hybridization on some probe features that occur during DNA hybridizations. Signal intensities are lower when RNA is hybridized according to normal procedures and hence bleeding will not be a substantial problem in expression studies. Bleeding into empty features was observed for both species in this study but to a larger extent on the zebra finch array where the signal intensities were higher because of species-specific hybridization. This can be seen as an increase in signal intensities in the zebra finch background intensities in Fig. 2.

Results

For the zebra finch, 94.9% of the probes had higher raw signal intensities than the mean of the background plus two standard deviations (241 463 of 254 430 probes in total) in the analysis. The equivalent proportion for the common whitethroat was 92.9% (236 277 of 254 430 probes). Affymetrix has a standard cut-off, used in their GENECHIP OPERATING SOFTWARE when CEL files are transformed into CHP files, of at least eight probes with significant intensities out of the 11 present for each gene. When applying this cut-off, that is, only assigning significance to ESTs for which at least eight out of the 11 probes in the probe set have higher intensities than the background cut-off, a total of 97.6% ESTs were labelled as 'functioning' in the zebra finch. The equivalent proportion in the common whitethroat was 96.0%.

The probe hybridization intensities in the common whitethroat were slightly lower than in the zebra finch (Fig. 2). The lower signal intensities from the common whitethroat were concluded to be due to hybridizations of target DNA having one, or rarely two, substitutions as compared to the zebra finch. Because of the quite high degree of hybridization that normally takes place on Affymetrix mismatch probes, we know that target–probe hybrids with one mismatch are expected to be partially but not fully washed away during the Affymetrix standard washing procedure. Therefore, such probes will yield lower general signal intensity. This is likely to happen because of sequence divergence when another species than zebra finch is hybridized to the array, and explains why the distribution of probe signal intensities from the whitethroat is shifted towards a lower range as compared to the zebra finch (Fig. 2). Targets with two or more mismatches will normally be completely washed away and should not contribute to the distribution of signal intensities in Fig. 2. It should be noted that the data in Fig. 2 represent distributions of probes, not probe sets or ESTs. This has important implications for the interpretation of our results. Probe-set distributions would represent means of 11 probes and such distributions would not be easily comparable to the background distributions, hence we present distributions of probes instead. Distributions of probe sets, as means, would naturally show less difference between species. This means that the Affymetrix approach with several probes per EST or gene increases the likelihood of successful cross-species analyses.

Despite the fact that the probes on the Lund-zf Affymetrix array will hybridize with sequences with one, and sometimes, although probably rarely, two substitutions, the probability of a random hybridization to the bird genome is very low. Bird genome size is highly conserved (Animal Genome Size Database 2007) and we used the binomial distribution to calculate the probability that a random probe, 25-bp long, will hybridize to a genome of 1.2×10^9 bp (the assumed size of the chicken). Under the assumption of an even and random distribution of the four nucleotides, the probability of a perfect match is 1.1×10^{-6}. Even with the allowance of one mismatch per probe, the probability of a probe to hybridize to a random region of the bird genome is only 8.0×10^{-5}. That corresponds to 20 of the 254 430 probes on the chip in this study. If a probe would be allowed to

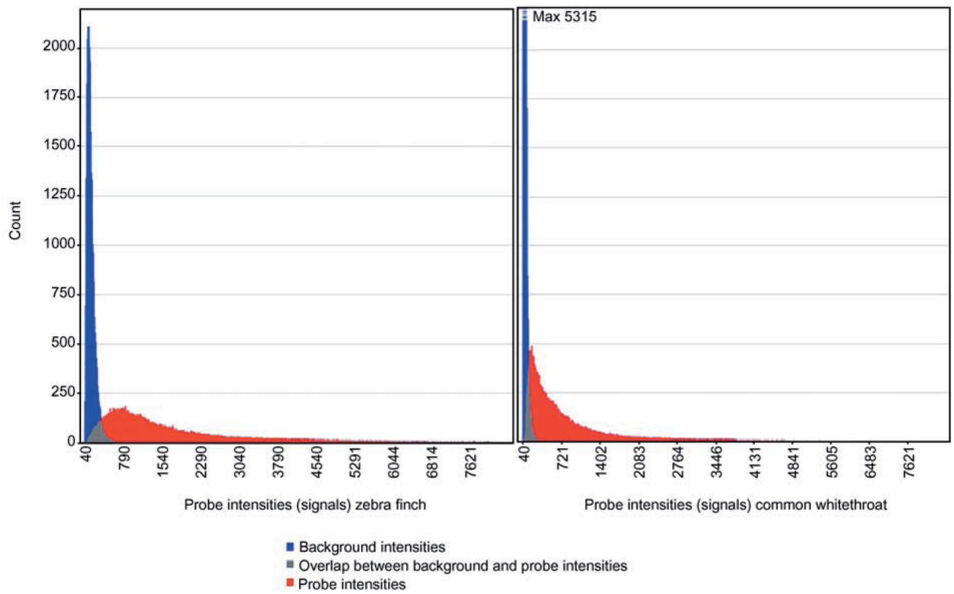

Fig. 2 Histogram showing a count of the values in each column on the *y* axis as a function of raw signal intensity on the *x* axis in zebra finch (left) and common whitethroat (right). For both graphs, the empty bars have been removed and thus *x*-axis scales are not linear. The red distributions represent the hybridizations of all 254 430 probes features, the blue distributions represent the hybridization in the 255 561 empty features on the chip (used for calculating the background threshold), whereas the grey areas indicate overlapping distributions. The graph was produced using SPOTFIRE DECISIONSITE for Functional Genomics version 9.0.

hybridize with a target with two mismatches, then 733 probes on the array would be affected (0.3% of the probes on the array). However, as mentioned above, the vast majority of targets with two mismatches will be washed away during the washing procedure.

Discussion

The high proportion of probes resulting in significant signal intensities when they are hybridized to zebra finch genomic DNA demonstrates that the Lund-zf array is technically of high quality. Moreover, for gene expression analyses, we expect an even higher proportion of probes to provide signals than indicated from our genomic DNA hybridization experiment. This is because the Lund-zf array was designed based on EST (cDNA) sequence information without considering whether a probe is representing a region adjacent to or spanning an intron in the corresponding genomic DNA. When genomic DNA is hybridized, some probes will not yield signals because of the presence of an intron within the corresponding target. On the Lund-zf array, the representation of probes relative the full-length mRNAs is not random, that is, most of the probes are designed based on a 600-bp region upstream the translational stop codon. After examining 40 genes in the chicken (*Gallus gallus*), we found that the number of introns in the 600-bp 3′-terminal region would lead to a maximum number of nonhybridizing probes of 4.5%, considering that the number and position of introns are conserved between chicken and passerines (Primmer *et al*. 2002). It is therefore likely that a large part of the 5.1% of the probes that were not hybridizing in the zebra finch were affected by the presence of introns and to a lesser extent either by poor EST sequence information, probes of low quality or overestimated background due to bleeding.

A remarkable finding was that the probe hybridization success was almost as high for the common whitethroat as for the zebra finch. This was surprising given the fact that finches and warblers diverged 25–59 million years ago, but may be explained by the relatively low sequence divergence between passerines (89–93%). This result strongly suggests that the array can be used for transcriptional analyses of many different passerine species. Moreover, the fact that avian genome size (Animal Genome Size Database 2007), avian chromosome arrangements (Derjusheva *et al*. 2004; Itoh *et al*. 2006) and avian gene order (Backstrom *et al*. 2006; Itoh *et al*. 2006; Dawson *et al*. 2007) are highly conserved

even between distantly related birds suggests that it will be possible to use the physical maps of the chicken and zebra finch genomes to infer locations of specific genes in other bird species. This may turn out to be very useful when interpreting results of cross-species microarray analyses.

The lowered hybridization signal of probes due to substitutions between species will lead to a lower detection rate of low-expressed genes when the array is used for other passerines. This can be seen in Fig. 2 where the common whitethroat probe intensity distribution is tilted towards the lower end of the range. However, Fig. 2 represents distributions of probe signal intensities. As mentioned earlier, the signal of each probe set, or gene representative, is the mean of the signals from the 11 probes representing that probe set. When calculations are based on probe sets, the proportion yielding significant signals will be higher than when based on probes (in the zebra finch 94.9% of the probes are significant leading to significance for 97.6% of the probe sets, the equivalent values for the common whitethroat are 92.9% of the probes and 96.0% of the probe sets). However, even if Affymetrix's approach of multiple probes per gene representative increases the likelihood of success in a cross-species expression study, the mean signal of probe sets will still be lower in the warbler than in the finch, most likely leading to a somewhat lower detection rate among low expressed genes in an expression study. When analysing a cross-species expression study, several approaches are available that will further decrease the difference in performance on the array between the two species. All probe sets that have lost more than three probes, that is below Affymetrix's standard cut-off of at least eight functioning probes, can be removed. It is also possible to filter out all probes that did not hybridize when genomic DNA was hybridized on the array. That means that all probe sets that are above the Affymetrix cut-off of eight functioning probes, but that still have one to three probes with non-significant intensities, can be analysed without a lowered mean intensity because of lack of hybridization on single probes. Moreover, from initial genomic microarray analysis of a passerine species of interest, like the one performed here, single probes can be identified that have lower (but still significant) signal intensities than other probes for the same gene representative (Caceres *et al*. 2003; Hsieh *et al*. 2003). Such probes are likely to have one or more substitutions in their sequence and could potentially be flagged or excluded in the following transcriptional analyses. Using these approaches, expression data can be filtered based on previous DNA hybridization results, leading to more reliable gene-expression results. The possibility of such filtering is one of the strengths of the Affymetrix setup with multiple probes. This, combined with the high degree of cross-species hybridization in the common whitethroat, indicates that studies of other passerine birds using Affymetrix arrays for zebra finch have a very high probability of being successful.

To conclude, the Lund-zf Affymetrix custom array, that successfully work for at least 98% and 96% of the 23 136 gene representatives in zebra finch and common whitethroat, respectively, has a very high potential to act as a successful cross-species microarray (CSM) utility and thus offers a unique opportunity to conduct global transcriptional analyses in passerine birds. This paves the way for applications of the microarray technology in research fields where it has not yet been extensively used, for example avian behavioural ecology and comparative genomics.

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II

The sex-biased brain: sexual dimorphism in gene expression in two species of songbirds

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The sex-biased brain: sexual dimorphism in gene expression in two species of songbirds

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Sexual dimorphism is widespread in many taxa. Since the sexes have virtually identical DNA sequences these differences arise from processes involving gene regulation. In accordance, sexual dimorphism in gene expression is common and extensive. Often it is linked to reproduction and therefore under strong selection. Sex-biased gene expression in the brain has a direct link to sex-dimorphic behaviors, and is particularly likely to be involved in population differentiation and speciation. Studies of gene expression in related species can shed light on in which genes the pattern of sexual dimorphism is conserved and which genes that are likely to be hotspots for speciation. We have studied the extent of sex dimorphism in gene expression in the brain of two species of songbirds, the zebra finch (*Taeniopygia guttata*) and the common whitethroat (*Sylvia communis*), using large-scale microarray technology. Sexual dimorphism in gene expression was extensive in both species, and predominantly sex-linked: most genes identified were male-biased and Z-linked and, to a large extent, the same genes were identified in the two species. This corroborates recent results in the chicken and the zebra finch which suggest that female birds do not fully dosage compensate Z-linked genes. Interestingly, there was a high number of male-biased Z-linked genes not only among genes that were sex-biased in both species but also among genes that were sex-biased only in one species. This suggests that the two species dosage compensate a partly different set of genes on the Z chromosome. It is possible that this pattern reflects differences in the essentiality or the level of sexual antagonism of these genes in the respective species. Such differences might correspond to genes with different rates of evolution related to sex dimorphism in the avian brain.

Introduction

Sexual dimorphism, i.e. systematic differences between the sexes within a species, is a well known phenomenon that occurs in most taxa. Some of the more conspicuous examples of sexual dimorphism are the appearance of miniature parasitic males in anglerfish, the tail of the peacock, and the song of the male nightingale (reviewed in Andersson M. 1994). Sexual dimorphism oc-

curs even though the sexes have virtually identical DNA sequences. Hence, sexual dimorphism must in most cases arise due to mechanisms involving gene regulation and gene expression (Connallon & Knowles 2005; Rinn & Snyder 2005). In line with this, recent research has revealed that a high degree of sex-biased gene expression is a common feature in many different species (Ellegren & Parsch 2007; Mank et al. 2008; Marinotti et al. 2006; Parisi et al. 2004; Ranz et al. 2003; Reinke et al. 2004; Rinn & Snyder 2005; Yang et al. 2004).

Sex-biased gene expression is tightly linked to reproduction (Dauwalder et al. 2002; Kadener et al. 2006; Drapeau et al. 2003) and is therefore subject to strong selection (Good & Nachman 2005; Jagadeeshan & Singh 2005; Khaitovich et al. 2005; Proschel et al. 2006; Swanson & Vacquier 2002; Torgerson et al. 2002). Sex-biased gene expression in the brain is of particular interest since it is linked to behavioural differences between males and females (Irizarry et al. 2003; Dauwalder et al 2002; Drapeau et al. 2003; Kadener et al. 2006). Hence, sexual dimorphism in gene expression is widespread and of general interest since it is likely to be of large importance for sexual selection and speciation (Ellegren & Parsch 2007).

Studies of sex-biased gene expression in the brain will shed light on these processes as well as increase our understanding of the evolutionary basis of sex-specific behaviours.

Genes with sex-biased gene expression are expected to be non-randomly distributed in the genome, with an enrichment of such genes on sex-chromosomes (X and Z chromosomes; Charlesworth et al. 1987; Ellegren & Parsch 2007; Rice 1984). This is due to the sex-bias in transmission pattern of these chromosomes where one sex carries two copies and the other sex only one copy. The uneven dose of X and Z chromosomes between the sexes should lead to an accumulation of sexually antagonistic genes on these chromosomes, i.e. genes that are beneficial to one sex but harmful to the other (Charlesworth et al. 1987; Kaiser & Ellegren 2006; Rice 1984).

Dominant or partly dominant mutations on X or Z chromosomes are exposed to selection twice as often in the homogametic as in the heterogametic sex. Such a mutation is therefore expected to go to fixation if it is beneficial to homogametic individuals (XX or ZZ) even if it is harmful to the opposite sex (Charlesworth et al. 1987; Rice 1984). Sex-linked recessive mutations, on the other hand, will always be exposed to selection in the heterogametic sex (XY or ZW). Thus, a recessive antagonistic mutation will easily reach fixation if it is beneficial to heterogametic individuals (Charlesworth et al. 1987; Rice 1984). Once antagonistic alleles have been fixed, selection for down-regulation of such alleles in the sex that carries the cost is expected to occur (Ellegren & Parsch 2007). This process will induce or increase sex-bias in gene expression and sex chromosomes should thereby be enriched for sex-biased genes.

Microarray technology makes it possible to study gene expression of tens of thousands of genes simultaneously. We have used microarrays to study the extent of sexual dimorphism in gene expression in the brain of two bird species, the zebra finch (*Taeniopygia guttata*) and the common whitethroat (*Sylvia communis*).

Birds are excellent model systems for this type of study due to the combination of extreme sexdimorphism in morphology and behaviour (Andersson M. 1994), conserved genome structure (Dawson et al. 2007; Derjusheva et al. 2004; Itoh et al. 2006), and female heterogamety (females are ZW; males ZZ). Comparisons with extensively studied XY models like mammals and *Drosophila* (where males are heterogametic, XY, while females are XX), thereby makes it possible to distinguish between characteristics attributed to sex and those caused by heterogamety.

At present large scale genomic resources are only available for two bird species, the chicken (*Gallus gallus*) and the zebra finch (www.ncbi.nlm.nih.gov/genome/guide). Despite 80 – 100 million years of independent evolution (Shetty et al. 1999; van Tuinen et al. 2000), the Galliformes and Passeriformes have highly conserved genome structure with few interchromosomal rearrangements (Backstrom et al. 2006; Dawson et al. 2007; Derjusheva et al. 2004; Itoh et al. 2006). The highly conserved genome structure in birds opens up the possibility of using the genome structure of chicken and zebra finches as templates for synteny and gene order for a number of species related to these birds. In the present study we have used an Affymetrix array designed for the

zebra finch (corresponding to about 15800 genes; Naurin et al. 2008) in order to study gene expression in the zebra finch and the common whitethroat. These species of passerine birds are separated by approximately 24–51 million years of evolution (Jonsson & Fjeldsa 2006; Zuccon 2007). The extent to which these species share patterns of sex-biased gene expression in the brain could shed light on the flexibility, or degree of conservation, of the gene expression profile underlying sexually dimorphic behaviours in birds.

Materials and Methods

Zebra Finches

Total RNA from full telencephalon of 6 female and 6 male zebra finches (*Taeniopygia guttata*) were used in this study.

Birds were reared at professor Art Arnolds lab at UCLA. Birds were housed indoors in flight cages holding 30 same-sexed individuals. 350 additional birds of both sexes were within visual and acoustic but not physical contact of the study animals. Cages were kept in an 800 square meter room with a light regime of 12 hours of artificial light (7AM-7PM) followed by 12 hours of dark. All birds were hatched at the aviary and sacrificed by decapitation as adults (>90 days of age) by the same person. All birds were healthy at the time of sacrifice (feeding on their own; feathers were not fluffed; keel was not visible through feathers).

Full Telencephalon was removed from the skull intact and flash frozen on dry ice. Samples were stored at –80 until total RNA was extracted using the protocol for TRI Reagent (Applied Biosystems/Ambion). Whole telencephalon was rapidly lysed (less than 1 minute) using a dounce homogenizer, extracted, precipitated, and re-suspended in depc-treated water. All samples were DNase I treated (after extraction from tissue) with Turbo DNase I (Ambion) 37û × 30 and then cleaned up using a QIAgen RNeasy spin column, eluting with nuclease-free water. Quality of total RNA was determined visually by formaldehyde gel (ribosomal bands showed no evidence of smearing/degradation) and using the Nanodrop ND-1000 spectrophotometer (260/280 ratio >1.9). Extractions and DNase treatment were done randomly to avoid batch effects. Samples were shipped on dry ice to the SCIBLU genomics facility in Lund, Sweden, where hybridizations were performed (see hybridizations below). All samples had good quality total RNA with high and comparable RNA Integrity Numbers (RIN: Schroeder et al. 2006) when tested at SCIBLU.

Common Whitethroats

Total RNA from full brain of 12 female and 12 male common whitethroats (*Sylivia communis*) were used in this study. The common whitethroat is a warbler of the family *Sylviidae*, a seasonal breeder and a long distance migratory bird. The species breeds in Europe (May–July) and winters in Africa south of Sahara (Oct–April). Birds were caught on two locations, in southern Sweden (Skåne: 55°42′16 N, 13°25′52 E) and central Nigeria (Plateau State: 9°2′29 N, 8°58′90 E). Birds from both locations were used in order to increase the sample size as much as possible and in order to avoid producing candidate genes from exclusively breeding or exclusively wintering birds in comparison with the lab-reared zebra finches.

Field methods used for all common whitethroats regardless of location

Birds were caught in the wild, using mist-nets and playback song. Swedish birds were caught in the end of May 2005 (first bird caught on the 19th of May, last on the 30th of May, all birds were caught prior to any egg laying) and Nigerian birds were caught in one session in the end of February and the beginning of March 2005 (first bird caught 25th of February, last on the 3rd of March) and in a second session in the end of January 2006 (first bird caught 21st of January, last bird on the 25th of January).

Birds sacrificed were in good condition (feathers were not ruffled; keel was not visible through the feathers; behavior prior to capture was normal). All birds caught were adult, in Africa at least six months old and in Sweden at least one year old.

All birds were hanging in the mist-net less than 15 minutes, and they were then kept in a dark bag in a quiet location in the shade for 5–10 minutes before being sacrificed. Birds were weighed before

decapitation (handling time 1–2 minutes). The entire brain was transferred into a tube containing RNA later™ RNA stabilization Reagent (Qiagen, cat. no: 76106). Samples were kept in the field for 1 to 8 hours (10–25°C), in 4–8°C for one day to three weeks, in –20°C for three months and then in –80°C until extraction. This is within recommendations for RNAlater stabilizing reagent (see the RNAlater Handbook supplied with the buffer).

Extractions of common whitethroat samples

All Common whitethroat samples were extracted using the RNeasy Lipid Tissue Mini Kit (QIAGEN, cat no: 74804). The brains were removed from the RNAlater buffer and the full brain was homogenized in 1ml QIAzol Lysis Reagent per 100 mg tissue (QIAGEN, supplied with kit) using a TissueLyser (QIAGEN cat no: 85220). The weight of the brains varied between 404 grams and 515 grams with a mean of 463.5 grams. The spin-columns of the kit are not optimized for more than 100 mg tissue so homogenized samples were divided into 4 or 5 separate 1.5 ml tubes containing 1 ml sample before continuing with the protocol. After adding 200 µl of chloroform, the samples were centrifuged at 13 000 rpm in 4°C for 15 minutes. Only 2 of the 4 to 5 tubes were then extracted on spin-columns, the rest were re-frozen. The samples were extracted following the exact instructions in the protocol supplied with the kit (step 9 to 17). QIAGEN also offers a RNase-Free DNase set (cat no: 79254) for use as an integrated step in the protocol. All common whitethroat samples were treated with DNase this way.

The brain of each individual was extracted one at a time as above and quality checked in batches of four. Extractions were randomized to avoid batch effects. Samples were quality checked on a formaldehyde agarose gel (no smearing of ribosomal bands was visible) and 260/280 ratios were checked on an Ultraspec 3000 spectrophotometer (all values were between 2.0 and 2.1).

The array

The Lund-zfa array is an custom Affymetrix array produced for the zebra finch, for detailed description see (Naurin et al. 2008). It contains 22630 ESTs corresponding to about 15800 non-redundant genes. The array contains no Affymetrix Mis-Match (MM) probes.

Hybridizations

High quality total RNA samples representing each individual (24 common whitethroat samples and 12 zebra finch samples) were delivered to an Affymetrix's service provider, the Swegene Center for Integrative Biology at Lund University (SCIBLU genomics, http://www.lth.se/sciblu), where they were hybridized according to standard Affymetrix protocols for RNA. Before hybridization they were once again quality checked at SCIBLU using a Nanodrop spectrophotometer and an Agilent 2100 Bioanalyzer. All samples were of high quality with high and comparable RNA Integrity Numbers (RIN: Schroeder et al. 2006) when quality was checked at the SCIBLU genomics facility in Lund. 5 µg total RNA from each sample was used in the regular protocols for GeneChip Arrays and hybridized onto the Lund-zf Affymetrix array overnight in the GeneChip® Hybridisation oven 6400 using standard procedures. The arrays were washed and then stained in a GeneChip® Fluidics Station 450. These procedures were randomized when possible to avoid batch effects. Scanning was carried out with the GeneChip® Scanner 3000 and image analysis was performed using GeneChip® Operating Software.

Analyses

Since the Lund-zfa array contains no Mis-Match (MM) probes the CEL files carry only information about the PM probes corresponding to the ESTs on the array. CEL files were imported into GeneSpring GX 7.3.1 and RMA normalized. RMA normalization requires no MM probe signals. Signal intensities for all ESTs on the array were then filtered (see below) and Quality control was performed with Expression Console™ 1.0.2467.39138 (Affymetrix) on RMA normalized data. For 34 samples, Affymetrix amplification and hybridization controls showed normal patterns and internal controls showed normal 3'/5' ratios. Correlation plots of biological replicates showed high correlations for both data

sets. Two samples showed a somewhat deviating profile, two common whitethroats, one male and one female. In the case of the male, the sample had degraded somewhat between QC controls at Lund University and at SCIBLU. We found no explanation as to why the female sample was deviating but assumed that something in the sample was interfering with hybridization. The two problematic samples were excluded from all downstream analyses.

Filtering of data

Signal data for all arrays was filtered to remove the ESTs with large standard deviation. This was done to remove any potential noise in the data and all ESTs with a standard deviation larger than 30% of the median value for the signal was removed from the analyses. In this filtration 2055 ESTs were removed from the analysis of zebra finch arrays and 577 ESTs were removed from the analyses of common whitethroat arrays. Furthermore, all common whitethroat arrays were filtered based on the data produced in a Comparative Genome Hybridization, CGH (Naurin et al. 2008). This was done in order to avoid analysing probes with a high degree of sequence divergence and 9827 probes that had non-significant signals in the common whitethroat CGH analysis were removed from downstream analyses. These represent all probes in the CGH analyses that did not hybridize significantly when DNA of the common whitethroat was hybridized to the array but did hybridize significantly when zebra finch DNA was hybridized. The 9827 probes are likely to represent parts of genes on the array that does not function for the common whitethroat due to sequence divergence between the species. The fact that only 9827 out of the over 300 000 probes were lost in the CGH anlyses means that the cross-species hybridisation in this study should not lead to unreliable results for the whitethroat. All ESTs were flagged according to how many probes they had lost after the 9827 probes were removed. This provided us with a number between 11 (= retained all 11 probes) to 0 (= had lost all 11 probes). Only ESTs retaining at least 8 out of the 11 probes were analysed, the rest (554 ESTs) were removed from all downstream analyses of the common whitethroat arrays. The choice of this cut off was motivated by the fact that Affymetrix normally allow analyses on probe sets that retain significant signals on least 8 probes (www.affymetrix.com/index.affx). 4968 ESTs lost one probe in the filtering above but were still analysed, 1662 lost 2 probes and 595 lost 3 probes. After filtering 21081 ESTs remained to be analysed on the zebra finch arrays and 22005 on the common whitethroat arrays.

The common whitethroat hybridization efficiency

The fact that only 554 ESTs were removed in the filtering above confirms that the common whitethroat samples perform well on the chip (Naurin et al. 2008). The efficiency of our filtering is further confirmed when hybridization-performance of whitethroat RNA is studied in detail. Out of the 268 ESTs that were sex-biased only in the zebra finch in this study (see below) no more than 14 had been filtered away.

When these 14 ESTs were excluded from analyzes the remaining 254 ESTs had not lost significantly more probes than the ESTs biased in both species (t-test, p= 0.379). This confirms the efficiency of the filters we used for the whitethroat data.

Moreover, even if mean raw signal of the common whitethroat chips (308) was lower than the mean of zebra finch chips (533) prior to filtering mean hybridization signal in the common whitethroat was not significantly different between the different categories of genes identified in this study (genes that were sex-biased only in the finch, genes sex-biased in both species or genes sex-biased only in the whitethroat). In conclusion, even if the common whitethroat has a handicap on the array due to sequence divergence, this handicap is small and can be effectively controlled via filtering, and hence the common whitethroat data used for further analyses in the present study are reliable and of high quality.

Significance Analysis of Microarrays

Significance Analyses of Microarray (SAM) is a statistical approach to find genes with significant differences in expression in sets of microarray experiments (Tusher et al. 2001). Input data is

gene expression measurements and response variables for each experiment. SAM computes a statistic d_i for each gene *i*, measuring the strength of the relationship between gene expression and the response variable. It uses permutations to assess which genes are significant. Filtered data was imported into Microsoft Office Excel 2003 and analysed using the Significance Analysis of Microarrays 3.02 plugin. Two class (unpaired) tests were run with False Discovery Rates (FDR) set as close to 3% as possible for all analyses. SAM was set to 500 permutations.

All analyses were done on log 2 signals with the response variable being sex, and separately for the two species. Males were entered as control group and females as treatment group. No Fold Change (FC) criterion was specified in SAM. This was due to the expected level of sexual dimorphism in the brain. For many studies of sexual dimorphism in gene expression, no genes with a FC lower than a prior set level has been listed as significant. This level is often quite high (2 fold, eg; Zhang et al. 2004; Zhang & Parsch 2005). However, many of these studies have been conducted on gonads, where one would expect the levels of sex-biased gene expression to be at their highest. In the brain, most gene expression differences between the sexes are not expected to be that high (Mank et al. 2007). We have accepted all genes identified by the permutation test in SAM as significant. In order to facilitate comparisons with other studies of gene expression in birds the Fold Changes given here were calculated separately on unlogged data for the significant genes, as mean male expression over mean female expression (The FC's calculated in SAM not used). The genes in this study have Fold Changes ranging from 0.5 to 1.2 in the zebra finch and 0.3 to 1.7 in the common whitethroat.

Annotations

Estimations of redundancies amongst the ESTs on the array have been made using the annotations produced for each sequence in the ESTIMA: songbird build 2 assembly (Replogle et al. 2008). Non-redundancies were then confirmed with data from a BLAST against the 3.2.4 build of the zebra finch genome, where ESTs listed with the same chicken-TC-id in ESTIMA also had hits against the same chromosome and position in the BLAST. The ESTS with no annotation in the assembly are simply listed as non-redundant genes here.

To generate correct annotation of chromosome and position, BLAST analyses against the 3. 2. 4 build of the zebra finch genome were used (http://www.ncbi.nlm.nih.gov/genome/guide/finch/) and genes with non significant hits (i.e. with E-values > $1*10^{-20}$) in this BLAST has been labeled no annotation. This annotation gave 1075 ESTs with significant Z hits on the array (4.6%), 17954 ESTs with significant autosomal hits (77%), and 4107 ESTs with no significant annotation.

Results

Results for the Zebra Finch

The SAM analyses were performed on all ESTs remaining after filtering (n = 21 081). 509 ESTs (2.2% of all ESTs on the array; Figure 1a) were found to be differentially expressed between the sexes at 3.9% false discovery rate (Table 1, the delta parameter was 1.278 for the zebra finch data), and the expected number of false discoveries in this data should therefore be less than 20 ESTs. After annotations, the significant ESTs were found to correspond to 417 non-redundant genes. 64 out of the 417 non-redundant genes were represented by more than one significantly sex-biased EST. Such genes are represented by at least two separate ESTs on the Lund-zfa array. 15 out of these 64 genes where represented by both female-biased and male-biased ESTs ('ambiguous' genes; Table 2); and 14 out of the 15 ambiguous genes were Z-linked. Female-biased ESTs in such genes had lower mean identity to the Z-sequence in the zebra finch draft (90%) then the male-biased parts (98%), indicating that some female-biased ESTs might in fact be sequences from W homologues. When these ESTs are instead blasted against the chicken genome (http://www.ncbi.nlm.nih.gov/projects/genome/guide/chicken/), three have significant hits on both Z and W.

The majority of the genes identified to be differentially expressed between the sexes were Z-linked (Figure 2a) and both male-biased and

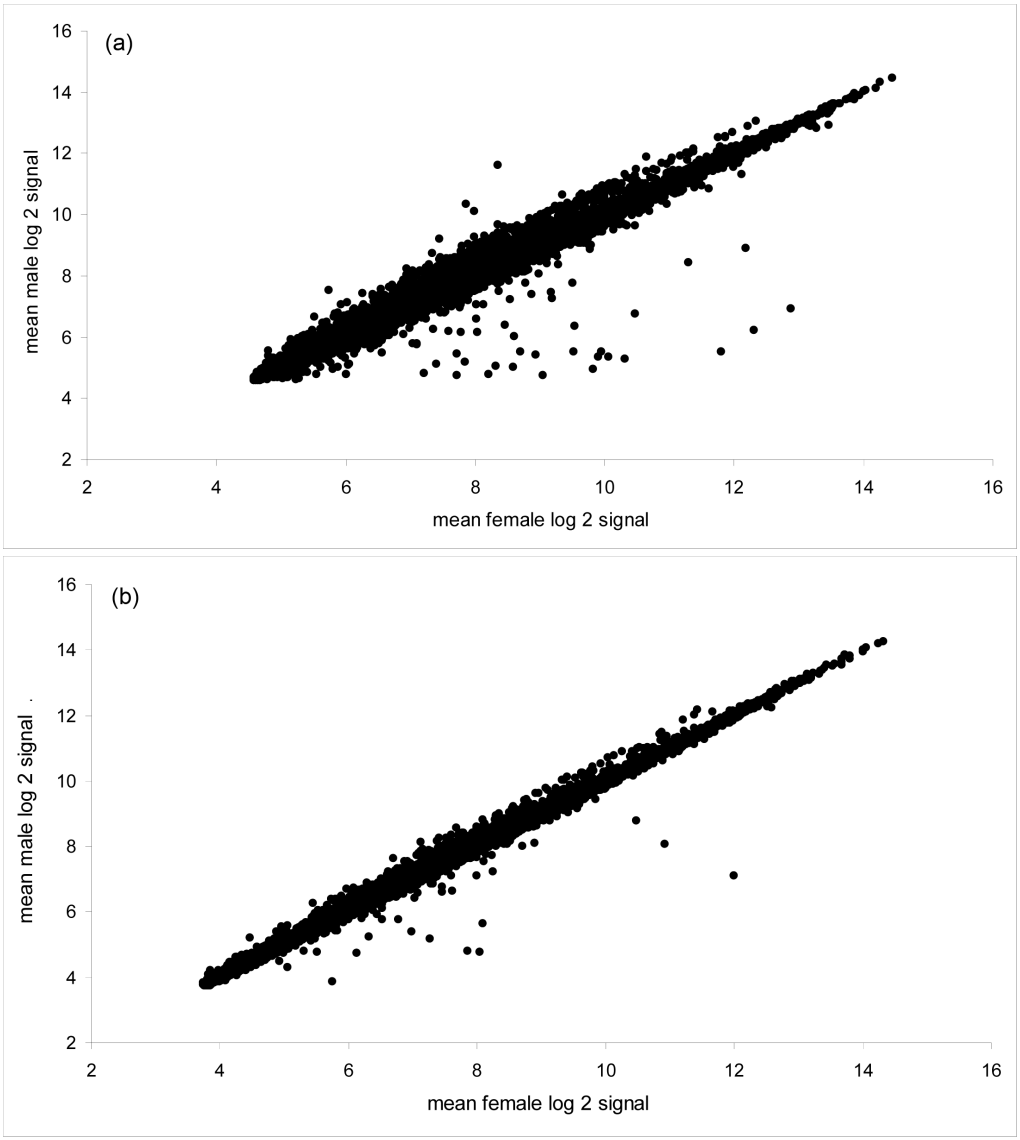

Figure 1. Female gene expression signal versus male signal for all ESTs on the Lund-zf array in (**a**): the zebra finch and (**b**): the common whitethroat.

female-biased genes showed a non random distribution across chromosomes, with an overrepresentation on the Z-chromosome (Table 3).

92% of identified genes were male-biased in expression (Table 1) and male-biased genes had a mean FC (male over female expression ratio) of 1.08 while female-biased genes had a mean FC of 0.77.

Results for the Common Whitethroat

As mentioned above, two common whitethroat samples did not give high quality results on the arrays and the sample sizes in the groups of common whitethroats are therefore: 11 males (6 Nigerian and 5 Swedish) and 11 females (5 Nigerian

Table 1. A total of 509 probe sets corresponding to 417 genes were identified in the zebra finch, and 345 probe sets corresponding to 299 genes in the common whitethroat (see also Table 2).

		nr ESTs	nr non-redundant genes	% of all sex-biased ESTs	% of all sex-biased genes	mean FC non-redundant genes
Zebra Finch						
	male-biased genes	460	383	90	95	1.08
	female-biased genes	49	19	9.6	4.7	0.77
Common Whitethroat						
	male-biased genes	318	271	92	94	1.32
	female-biased genes	27	16	7.8	5.6	0.66

and 6 Swedish). SAM analyses were performed on all ESTs remaining after filtering (n = 22 005) and were first run for all Swedish birds versus all Nigerian birds in order to make sure that genes that are differentially expressed between season/population was not included in the analyses of sex dimorphism. 52 genes were identified as differentially expressed between the two seasons/populations at false discovery rate 3.8, delta 0.61 (results from that comparison will not be included here) but since none of those genes were also identified in the comparison between the sexes no exclusion of data for the analyses of sex-dimorphism was done.

345 ESTs (1.5% of all ESTs on the array; Figure 1b) were found to be differentially expressed between the 11 male and the 11 female samples at false discovery rate 3.5 (the delta parameter was 0.848 for the common whitethroat data) and the expected number of false discoveries in this data should therefore be less then 13 ESTs. After annotations the significant ESTs were found to correspond to 299 non-redundant genes. 36 out of the 299 non-redundant genes were found to be represented by more than one significant sex-biased EST on the Lund-zfa array. 12 out of the significant genes are represented by both female-biased and male-biased ESTs and therefore included in the 'ambiguous' genes group (Table 2).

Also in the common whitethroat, the majority of the genes identified to be differentially expressed between the sexes where Z-linked (Figure 2b) and male-biased genes showed a non-random distribution across chromosomes (Table 3). Female-biased genes however, were not non-randomly distributed in the genome.

Table 2. 15 gene units with ambiguous regulation (i.e., genes represented by both male-biased and female-biased ESTs) were identified in the zebra finch and 12 in the common whitethroat.

		nr ESTs	nr non-redundant genes	% of all sex-biased ESTs	% of all sex-biased genes
Zebra Finch					
	male-biased part	22	15	4.3	3.6
	female-biased part	28	15	5.5	3.6
Common Whitethroat					
	male-biased part	14	12	2.3	4
	female-biased part	11	12	3.2	4

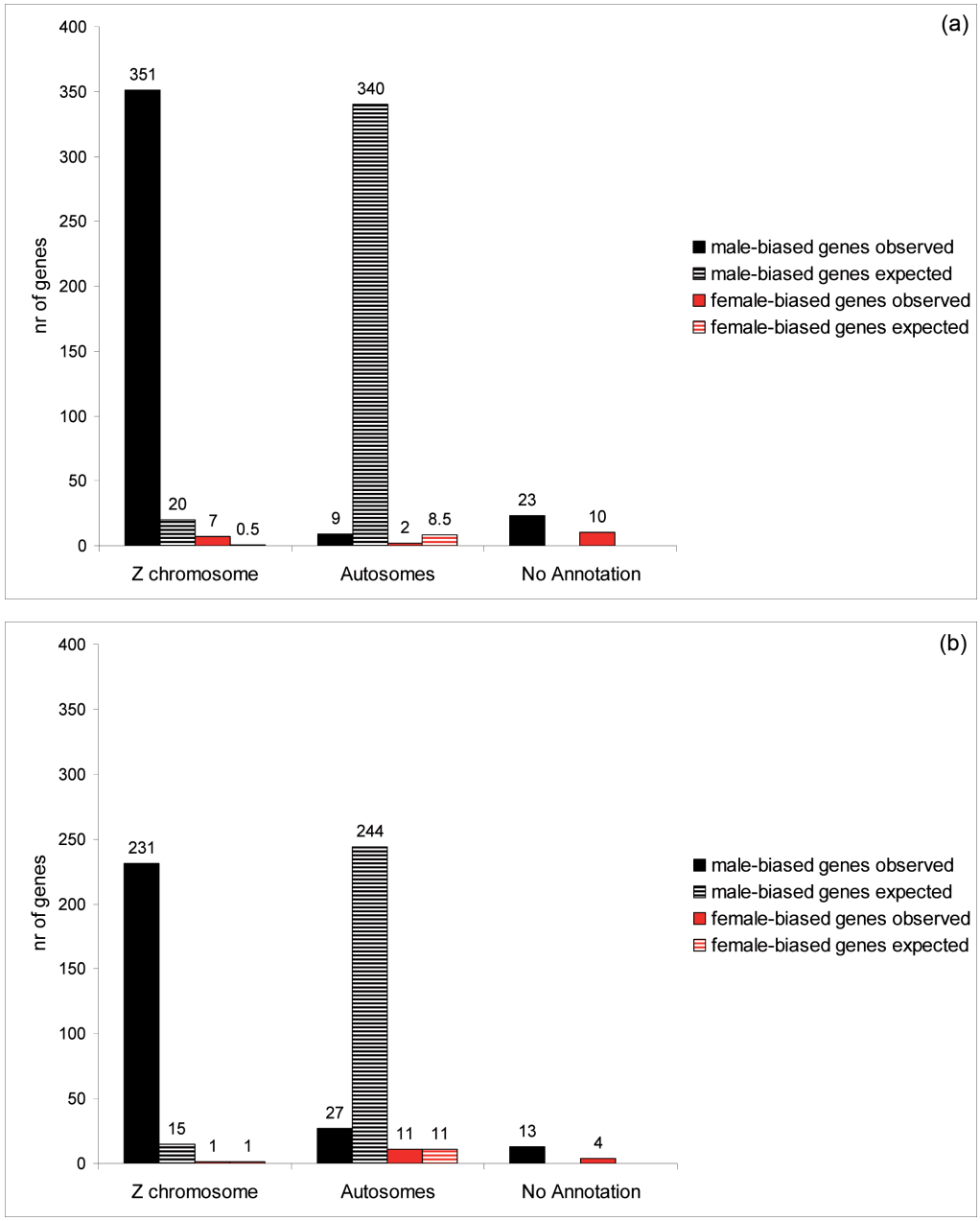

Figure 2. A histogram showing the observed and expected genomic distribution of sex-biased genes. The number of sex-biased genes for each chromosomal category in (**a**): zebra finch and (**b**): common whitethroat. Annotations achieved by BLASTs of the EST sequences on the array against the draft, build 3.2.4, of the zebra finch genome. Significant hits have a hit of e-20 or lower, no anno here indicates that the EST sequence has a significant hit against chrUn or no significant hit against any of the zebra finch chromosomes. Numbers above columns gives nr of genes in each category.

Table 3. Observed and expected genomic distribution of all annotated sex-biased genes that show non-ambiguous regulation. Nr of genes in parentheses.

	zebra finch observed	common whitethroat observed	Expected if distribution was random
female-biased genes			
Z-linked	0.777 (7)	0.056 (1)	0.056
Autosomal	0.222 (2)	0.0944 (11)	0.944
p value Fisher exact test	0.0055	NS	
male-biased genes			
Z-linked	0.975 (351)	0.895 (231)	0.056
Autosomal	0.0025 (9)	0.105 (27)	0.944
p value Fisher exact test	1.30E-165	3.00E-94	

91% of identified genes where male-biased in expression (Table 1) and male-biased genes had a mean FC of 1.32 while female-biased genes had a mean FC of 0.66.

Comparison of sex-biased gene expression in the zebra finch and in the common whitethroat

205 non-redundant genes were identified as differentially expressed between the sexes in both the zebra finch and the common whitethroat (Figure 3). None of the 205 genes or the ESTs corresponding to them showed reversal in sex-bias, meaning that they were biased for the same sex in both species. 12 out of the 205 genes that are shared between the species showed ambiguous regulation; these genes represent the 12 ambiguous genes identified in the common whitethroat data. 212 non-redundant genes were found to be sex-biased only in the zebra finch (Figure 3a) and 93 were sex-biased only in the common whitethroat (Figure 3b). Only 4 of the 205 genes biased in both species where female-biased and 180 of them (88%) were both male-biased and Z-linked (Figure 3c). The proportion of male-biased and Z-linked genes was lower among the genes only biased in the finch (80%, 171 genes) and in the genes only biased in the warbler (45%, 43 genes). Moreover, there were no autosomal genes that were biased in both species (Figure 3c).

Discussion

We found extensive sexual dimorphism in gene expression in the brain of both species of songbirds. In light of the difference between the zebra finch and the common whitethroat, in terms of evolutionary time, sexual behavior, morphology, and sample handling it is remarkable that as many as 49% of the genes identified as sex-biased in the zebra finch was also identified as sex-biased in the common whitethroat (69% of all genes identified in the warbler was also found in the finch). These 205 genes did not only show sex-bias in both species, none of them showed a reversal in sex-bias; i.e., if they were female-biased in the finch they were also female-biased in the warbler, if they were male-biased in the finch, they were male-biased in the warbler.

Differences in gene expression between species and populations can be extensive (Ellegren & Parsch 2007; Mank et al. 2008; Marinotti et al. 2006; Parisi et al. 2004; Ranz et al. 2003; Reinke et al. 2004; Yang et al. 2006). Moreover, sex-biased genes are known to evolve rapidly and to reverse their sex-bias between different species

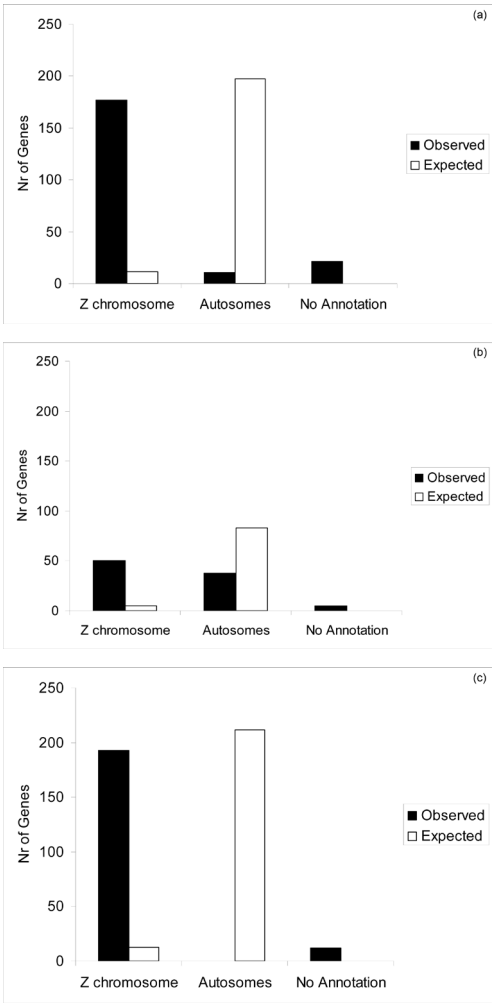

Figure 3. Chromosome annotation of (**a**): the 212 genes that were sex-biased only the zebra finch, (**b**): the 93 genes that were sex-biased only in the common whitethroat and (**c**): the 205 genes that were sex-biased in both species.

(Ellegren & Parsch 2007). It is therefore interesting that the two passerine species studied here show such high similarity in sex-bias of genes in the brain, and that not a single gene with reversed expression pattern was identified.

Given the high similarity in the results for the two study species, the 212 genes that were sex-biased only in the zebra finch are likely to represent hot spots for evolution of gene regulation on the Z chromosome. These genes did not have a lower degree of hybridization in the common whitethroat than genes with significant sex-bias in that species, indicating that they to a large extent represent 'true' differences between the species (as opposed to having been missed in the whitethroat due to sequence dissimilarities).

93 genes were identified as sex-biased only in the common whitethroat. The whitethroat samples contained the entire brain, however, while only the telencephalon was hybridized from the finches. This means that 'unique' common whitethroat genes will belong to two categories: (i) telencephalon species specific genes and (ii) genes implemented in the regulation of sex dimorphism in other parts of the brain.

Male-biased genes dominated the data sets in this study, and this is similar to previous study in chicken (Kaiser & Ellegren 2006). The Z chromosome was enriched with male-biased genes in both the zebra finch and the common whitethroat, and in the zebra finch female-biased genes were also overrepresented on the Z chromosome. This is in line with theory predicting pronounced sexual antagonism, and thereby an enrichment of genes with sex-biased expression, on the Z chromosome (Charlesworth et al. 1987; Rice 1984). However, as previous studies of the zebra finch and the chicken have shown, birds might lack effective dosage compensation (Ellegren et al. 2007; Itoh et al. 2007). This has important implications for our results, both in terms of the number of male-biased genes and in terms of the similarities between the species.

The relevance of inefficient dosage compensation

Absence of dosage compensation will lead to a generally higher expression of Z-linked genes in males compared to females, simply due to the double dose of Z in males. The expression level is expected to be between 1.4 to 2 fold higher in males than in females for Z-linked loci if compensation is lacking (Ellegren et al. 2007; Itoh et al. 2007; Mank 2009). It is therefore possible that the double dose of Z in avian males explains the large number of male-biased Z-linked genes in our data sets. A lack of dosage compensation would make it impossible to separate between

(i) genes that are essential to male-specific morphology and behavior and are thus up-regulated in males, and (ii) genes that have male-biased expression due to Z-linkage and a double dose in males.

Do the two species differ in which genes they dosage compensate?

There was a high degree of male-biased Z-linked genes in all three categories of genes identified here. It was most pronounced among genes biased in both species (88%) but still extensive among genes biased only in one species (80% of genes biased only in the zebra finch and 45% of genes biased only in the common whitethroat were Z-linked and male-biased). This suggests that there might be species-specific differences in which parts of the Z chromosome that are dosage compensated. This is highly interesting as the extent of compensation could be associated with the extent of sexual dimorphism and species differentiation. Finches and warblers separated 24–51 million years ago (Jonsson and Fjeldsa 2006; Zuccon, 2007; Ericson et al. 2006) and may have taken different routes during the evolution of sex-chromosomes and dosage compensation of essential genes. It is possible, therefore, that some genes are compensated to a higher extent in one species than the other and that this pattern represents the essentiality of the genes in the respective species. Hence, we suggest that the differences between the species, whether produced by actual sex-dimorphic regulation or by differences in the ability to compensate, reflect the occurrence of species-specific hot spots for the evolution of sex dimorphism in the avian brain, including hot-spots in the evolution of sex-dimorphic behavior.

How much do females suffer from low Z-linked gene expression?

If a large proportion of the male-biased genes identified in this study are poorly compensated in females rather than specifically up-regulated by males; that leads to questions regarding detrimental effects of lower Z-linked gene expression on females. Aneuploidy, having a lower or higher copy number of a part of the genome, is normally lethal due to effects of gene dose on crucial networks (Jeong et al. 2001; Lindsley et al. 1972; Lu & Oliver 2001; Pauli et al. 1993; Rosenbusch 2004). In line with this, dosage compensation is wide-spread in other taxa (Birchler et al. 2006; Mank 2009; Marin et al. 2000). It is likely therefore that the most essential genes should show a higher degree of dosage compensation in female birds. There is some evidence that this is the case in chicken (Ellegren et al. 2007) but more comprehensive studies are needed. It seems likely that a lowered expression on hundreds of Z-lined loci would have an effect on the female phenotype. If that is the case, then sex dimorphism in birds is perhaps not selected only via regulation of genes with sex-specific tasks but also via selection for different levels of dosage compensation of Z-linked genes in females.

Sexual antagonism on the Z chromosome

There is one other potential explanation for a large amount of sex-bias on the Z chromosome. If a gene is antagonistic, i.e. its expression is favorable for one sex and harmful for the other, then the sex for which it is harmful would be expected to down-regulate the expression of that gene (Charlesworth et al. 1987; Ellegren & Parsch 2007; Rice 1984). This down-regulation will induce or increase sex-bias. Theory predicts that antagonistic genes should aggregate on the Z (or X) chromosomes. This is due to the uneven dose of sex chromosomes between the sexes, which will lead to more selection of dominant mutations in males and of recessive mutations in females (Charlesworth et al. 1987; Rice 1984). How much antagonism contributes to sex-bias in the avian brain is difficult to say at present. However, a recent study identified quite a large number of sexually antagonistic genes on the Z chromosome in chicken (Mank & Ellegren 2009b).

Conclusion

We found a high degree of sexual dimorphic gene expression in the brain of both passerine species studied here. Given that the brain is not expected to be nearly as sexually dimorphic in gene expression as specialized tissues, like testes and ovaries, the identification of several hundred genes with

significantly different expression between the sexes is highly interesting and implies that the avian brain is truly affected by the sex of the brain cells.

These and other recent results on gene expression in birds (Ellegren et al. 2007; Itoh et al. 2007; Mank & Ellegren 2009a; Mank & Ellegren 2009b) leads to questions concerning to what extent the pronounced sexual dimorphism in morphology and behavior in birds can be attributed to the polarization of sexually dependent gene expression in the avian brain.

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III

Male-biased gene expression and dosage compensation on the avian Z chromosome

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Male-biased gene expression and dosage compensation on the avian Z chromosome

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X-linked gene expression in species with XY sex determination is equal between the sexes and balanced with autosomal expression even though males have only one X chromosome while females have two. However, species with Z and W sex chromosomes do not achieve completely balanced gene expression between the sexes on their Z chromosome. We have studied Z-linked gene expression in two songbirds using a large-scale microarray spotted with zebra finch EST sequences. Ratios of Z to autosomal gene expression for all gene-categories were close to one in both sexes and the male-bias in expression on Z-linked genes increased with increasing gene expression level in both species. Low-expressed genes showed a higher degree of dosage compensation than high-expressed genes. Moreover, sequence divergence on the Z chromosome was high suggesting a higher degree of evolution on Z than on autosomes. Our results suggest that (i) females have up-regulated gene expression on Z to balance Z-linked and autosomal expression but have not fully dosage compensated highly expressed genes and (ii) that highly expressed Z-linked genes are hotspots the evolution of sexual dimorphism in passerine birds.

Introduction

The X or Z chromosome is present in one copy in the heterogametic sex (XY or ZW) and in two copies in the homogametic sex (XX or ZZ). For most X-linked genes in XY-males, however, expression is not lower than it is in XX-females (Birchler et al. 2006; Marin et al. 2000). Moreover, mean gene expression on the single X chromosome in males is similar to that on autosomes (Gupta et al. 2006; Nguyen & Disteche 2006). The mechanisms resulting in this balance in expression between the sexes, and between sex-linked and autosomal expression, are referred to as dosage compensation (Lucchesi et al. 2005; Mank 2009; Straub & Becker 2007). It is believed that dosage compensation occurs because changes in gene copy number have detrimental effects on gene expression in crucial genetic networks (Lindsley et al. 1972; Lu & Oliver 2001; Lynch 2007; Pauli et al. 1993; Rosenbusch 2004).

X is homologous to Y, and prior to the early stages of sex chromosome evolution each gene had a homologous copy on both chromosomes.

Over time, the Y chromosome has degraded and it is believed that the loss of gene expression on Y-linked homologues induced selection for dosage compensation in XY-males. Degradation of Y is caused by ceased recombination between X and Y chromosomes (Bergero & Charlesworth 2008), which lead to accumulation of deleterious mutations and loss of functionality at most genes on Y chromosomes (Charlesworth & Charlesworth 2000; Charlesworth et al. 2005). According to present knowledge, Z and W chromosomes are expected to evolve in much the same way as X and Y (Ellegren & Parsch 2007; Rice 1984).

Dosage compensation has evolved to an effective state in XY-systems as diverse as mammals, *Drosophila* spp., and *Caenorhabditis elegans*, indicating that selection for retaining original expression-levels of sex-linked genes has always followed when Y chromosomes degraded. W chromosomes in species with ZW sex-determination cease to recombine and degrade much like Y chromosomes. If dosage compensation is an essential by-product of such degradation, and indeed it has long been considered that this is the case, then ZW-females should compensate their single Z chromosome as W degrades, just as XY-males compensate X. It has recently been shown, however, that the chicken (*Gallus gallus*) and the zebra finch (*Taeniopygia guttata*), where females are ZW and males ZZ, have a large degree of male-biased gene expression on their Z chromosome (Ellegren et al. 2007; Itoh et al. 2007) distinguishing them from XY-species. This suggests that female birds are (i) either inefficient in their dosage compensation or (ii) achieve enough compensation without balancing their Z-linked expression to that of the males. Furthermore, a large degree of male-biased gene expression on the Z chromosome was recently demonstrated in another ZW-species, the silkworm, *Bombyx mori* (Zha et al. 2009). The large degree of male-biased gene expression on Z chromosomes is intriguing considering the fact that X-linked gene expression is highly balanced between males and females in all XY-species studied so far, even though the different X chromosomes are of different origin.

We have studied the extent of dosage compensation in two passerine birds, the zebra finch (*Taeniopygia guttata*) and the common whitethroat (*Sylvia communis*). In a previous study we found significant and extensive male-bias in gene expression on the Z chromosome in the brain of these two songbirds (Naurin et al. 2009). 90% of the 509 ESTs that were significantly sex-biased in the zebra finch and 92% of the 345 ESTs that were sex biased in the common whitethroat were Z-linked and male-biased. Dosage compensation in the zebra finch has previously been adressed by Itoh et al. in an expression study of 40 Z-linked and 84 autosomal ESTs (Itoh et al. 2007). Here we use microarray technology to quantify expression on 1104 Z-linked and 18520 autosomal ESTs in both study species. By shedding light on the extent of dosage compensation on a larger number of Z-linked ESTs in the zebra finch and in a previously unstudied warbler, we add substantial new data concerning the extent of dosage compensation of the avian Z chromosome. Our present results suggest that the pattern of Z-linked gene expression is complex with important differences in the degree of male-bias in genes with high and low gene expression. Furthermore, our comparative approach sheds light on the overall rate of molecular evolution on autosomes and sex chromosomes in ZW-systems.

Materials and Methods

We have analyzed results from hybridizations of brain tissue from 12 adult zebra finches (6 males and 6 females) and 22 adult common whitethroats (11 males and 11 females). For detailed information regarding sample handling, quality control results, hybridization and initial analysis of data, including normalization and filtering, see Naurin et al. (2009). Background correction in these datasets is the same as in (Naurin et al. 2009) in all aspects but one; in the present analyses we have also removed the 2% of ESTs with the lowest signals. This is in line with Affymetrix's standard approach where the 2% lowest probes are removed (which for standard arrays is performed before any background correction according to hybridization on mis-match probes). This was done in order to avoid analyzes of ESTs with signals close to the background level.

The Lund-zfa array

The Lund-zfa array is an Affymetrix custom array that consists of 22360 zebra finch ESTs, representing approximately 15800 genes (Naurin et al. 2008; Replogle et al. 2008). All ESTs have been BLASTed against the 3.2.4 build of the zebra finch genome (Naurin et al. 2009) and hits in that BLAST was considered significant if the E-value was ≤ 10^{-20} or lower. 1104 of the ESTs with signals higher than background cutoff in our data set have significant hits against the Z chromosome and 18520 ESTs have significant autosomal hits. Out of these significantly annotated ESTs 22 were identified as Z-linked and female-biased in a previous study of sexual dimorphism in gene expression (Naurin et al. 2009). These ESTs had lower identities with the Z chromosome than male-biased ESTs (90% for female biased ESTs and 99% for male-biased ESTs) and three of them had significant hits against the W chromosome in chicken (*Gallus gallus*: Naurin et al. 2009). In order to avoid analyzing potential W gene expression we excluded these ESTs from the present analyses (including these ESTs did not qualitatively affect our results).

The Common Whitethroat Hybridization Efficiency

A lower degree of hybridization on microarray probes caused by sequence divergence between whitethroat RNA and the microarray can be efficiently controlled via filtering. See Naurin et al. (2009; 2008) for detailed description of the comparative genome hybridization (CGH) of the common whitethroat and how data generated from that study can be used for filtering.

Analyses of Dosage Compensation

Log 2 signal intensity data for all non-redundant genes with a significant BLAST against either an autosome or the Z chromosome were imported into SPSS Statistics 17.0. For each species, one-way ANOVAs were conducted using signal intensities comparing Z-linked versus autosomal ESTs for females and males respectively, as well as the intensity of Z-linked ESTs in females versus Z-linked intensity in males. Fold Change (FC) was calculated as the mean unlogged male / mean unlogged female signal intensities. Mean FC for Z-linked ESTs versus autosomal ESTs was tested with a one-way ANOVA. Kolmogorov-Smirnov two sample tests were used in order to test whether or not the distribution of Fold Changes was significantly different for Z-linked and autosomal ESTs. All results as listed below remained unchanged when non-parametric tests were performed instead of ANOVAs.

General Linear Models (GLM) were used in order to test whether or not male-biased gene expression increased with increasing signal on Z-linked genes. Residuals for all GLMs were normally distributed.

Results

Analyses of dosage compensation

In the zebra finch, total Z-linked gene expression was significantly lower in females (7.26 ± 0.056) than in males (7.57 ± 0.060; $F_{1, 2208}$=14.2, p < 0.0001; Figure 1a). Moreover, gene expression in females was significantly lower for Z-linked genes (7.26 ± 0.056) than autosomal genes (7.66 ± 015; $F_{1, 12900}$=42.9, p < 0.0001; Figure 1). Male Z-linked expression (7.57 ± 0.060) was not significantly different form autosomal expression (7.65 ± 0.014; $F_{1, 12900}$=1.72, p = 0.192).

Fold Change (FC; male / female signal) was significantly higher for Z (1.31 ± 0.01) than for autosomes (0.997 ± 0.0007; $F_{1, 19623}$=6686, p < 0.0001; Figure 2a), and the distribution of FC-values was significantly different for Z-linked and autosomal ESTs (p < 0.0001; Kolmogorov Smirnov two sample test; Figure 3a).

For the common whitethroat, total Z-linked gene expression tended to be lower in females (6.14 ± 0.048) than in males (6.28 ± 0.05; $F_{1, 2208}$= 3.73, p = 0.053; Figure 1b). As in the zebra finch, female intensity in the common whitethroat was significantly lower for Z-linked (6.14 ± 0.048) than autosomal genes (6.90 ± 0.013; $F_{1, 19623}$=181.4, p < 0.0001; Figure 1b). Male Z-linked intensity in the common whitethroat (6.28 ± 0.05) was also significantly lower than autosomal expression (6.89 ± 0.013; $F_{1, 19623}$=120,

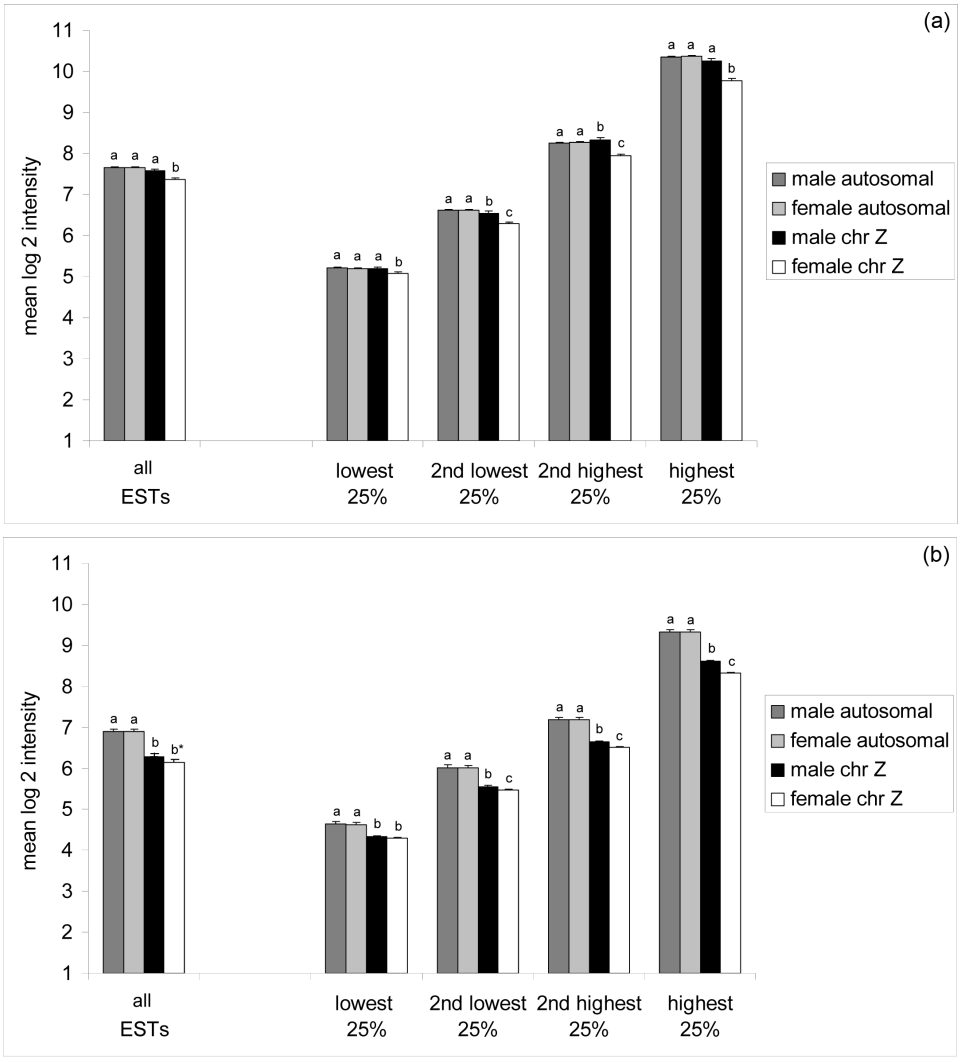

Figure 1. Mean gene expression intensity of autosomal and Z-linked ESTs in (a) the zebra finch and (b) the common whitethroat. Data for all ESTs and when divided into quartiles by mean gene expression intensity from lowest 25% to highest 25% are given. Significant differences between categories are indicated (a, b and c)

$p < 0.0001$; Figure 1b) a pattern that is different from that found in the zebra finch.

Fold Change (FC) was significantly higher for Z (1.12 ± 0.005) than for autosomes (0.998 ± 0.0005; $F_{1, 19623}=2809$, $p < 0.0001$; Figure 2b). The distribution of FC for Z-linked genes was significantly different from the autosomal distribution ($p < 0.0001$; Kolmogorov-Smirnov two sample test; Figure 3b).

Male-biased gene expression and general expression levels

We tested how the male-bias in gene expression on the Z chromosome was related to the general gene expression level. The degree of Z-linked male-bias increased with increasing gene expression levels (Figure 1 and 4); and this pattern was significant in both species. In GLMs with male

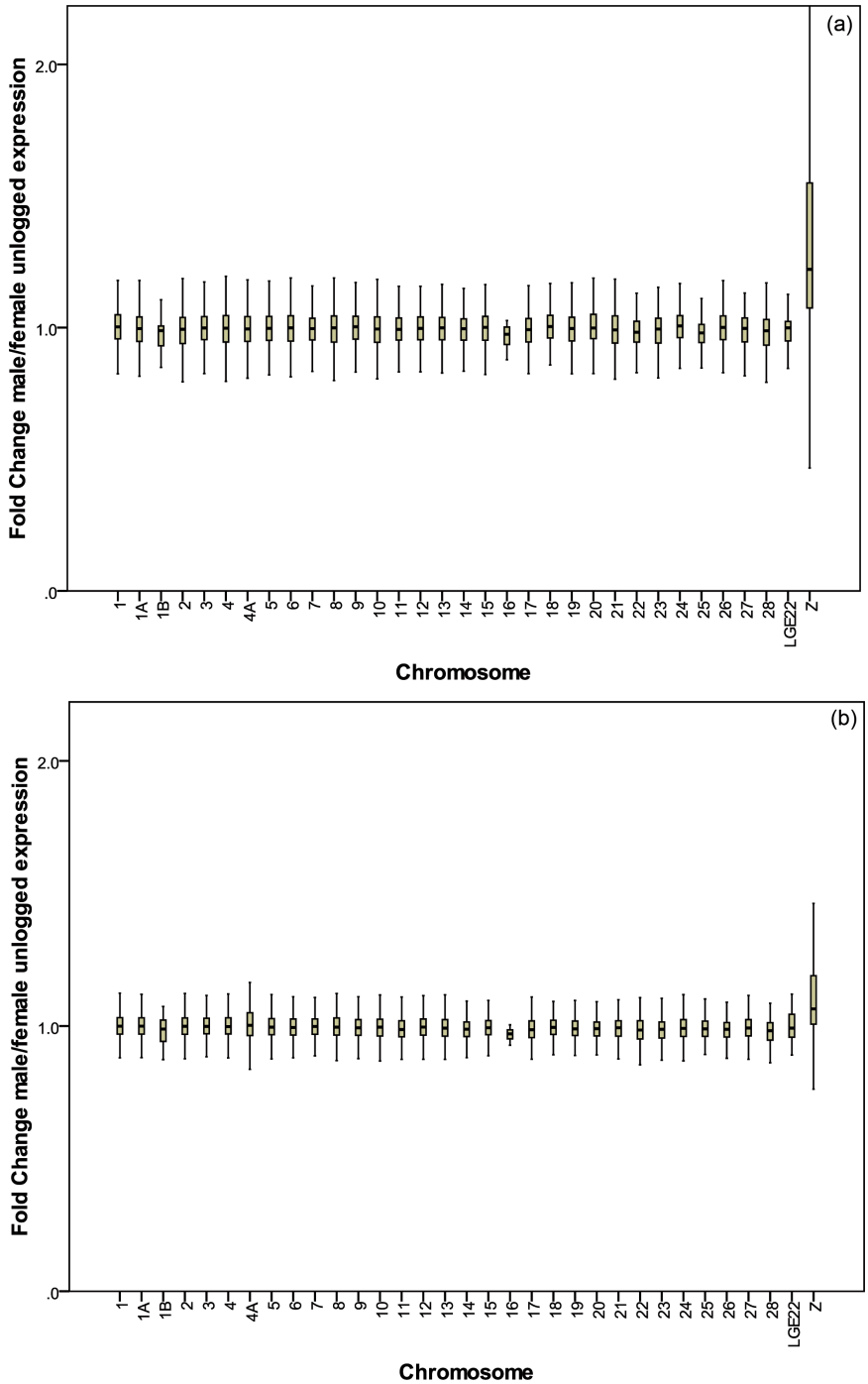

Figure 2. Box plot of Fold Change (FC) for individual chromosomes for the zebra finch (a) and the common whitethroat (b).

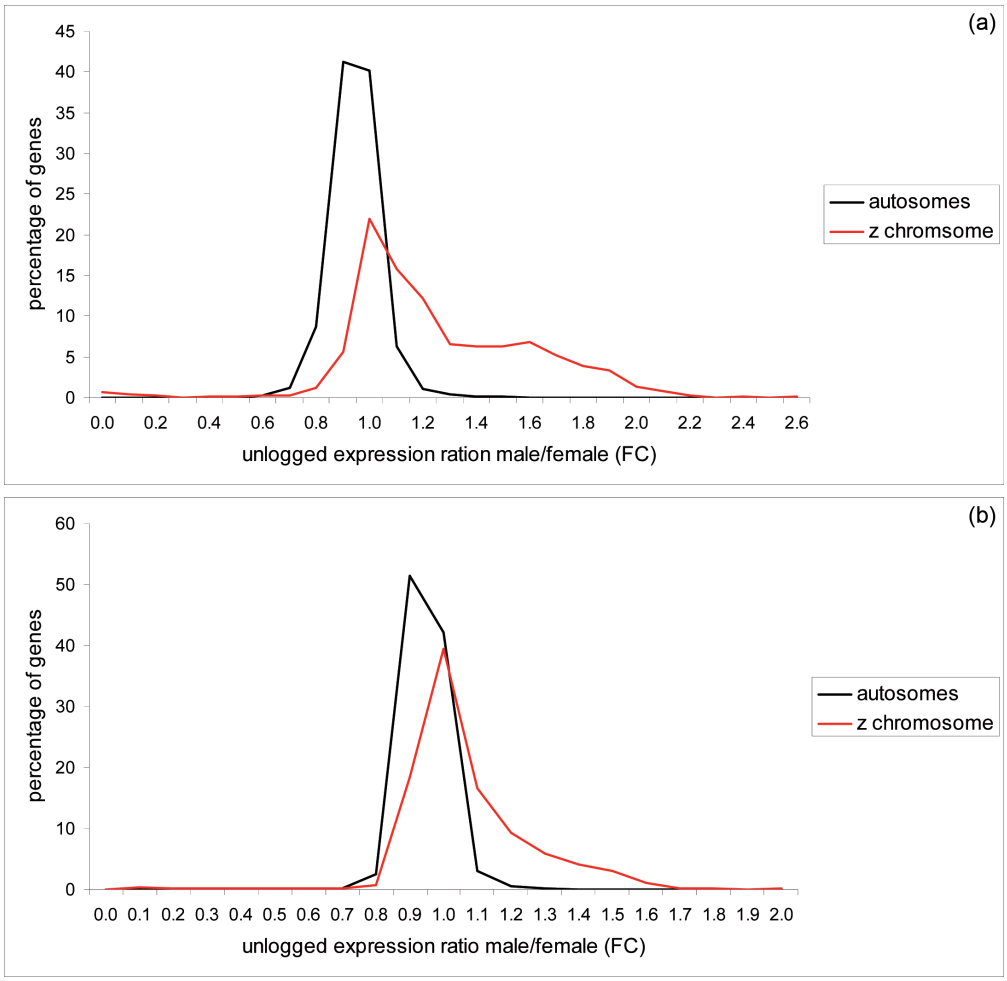

Figure 3. Distributions of FC for (a) the zebra finch and (b) the common whitethroat

intensity as dependent variable, and female intensity, chromosome type (Z or autosomal) and their interaction term as independent variables, the interaction term was highly significant (zebra finch: $p<0.0001$, Table 1; common whitethroat: $p<0.0001$, Table 2). In other words, female Z-linked expression did not increase as rapidly with increasing general gene expression as female autosomal expression (and thereby increased slower than male Z-linked and autosomal expression as well). All results from GLMs remained significant even if the 25% of the dataset with the lowest expression was removed, and were therefore not related to detection rates of the microarray. Linear regressions between male and female expression levels showed that Z-linked expression had steeper slopes (higher b coefficients) than autosomal expression in both species (zebra finch: Z-linked = 1.068 ± 0.005, autosomal = 0.992 ± 0.001; common whitethroat: Z-linked = 1.053 ± 0.004, autosomal = 0.998 ± 0.001 < 0.0001; Figure 4).

Z-linked in relation to autosomal gene expression

In order to evaluate how the pattern of gene expression on the Z chromosome relates to autosomal signal we divided the Z-linked and the

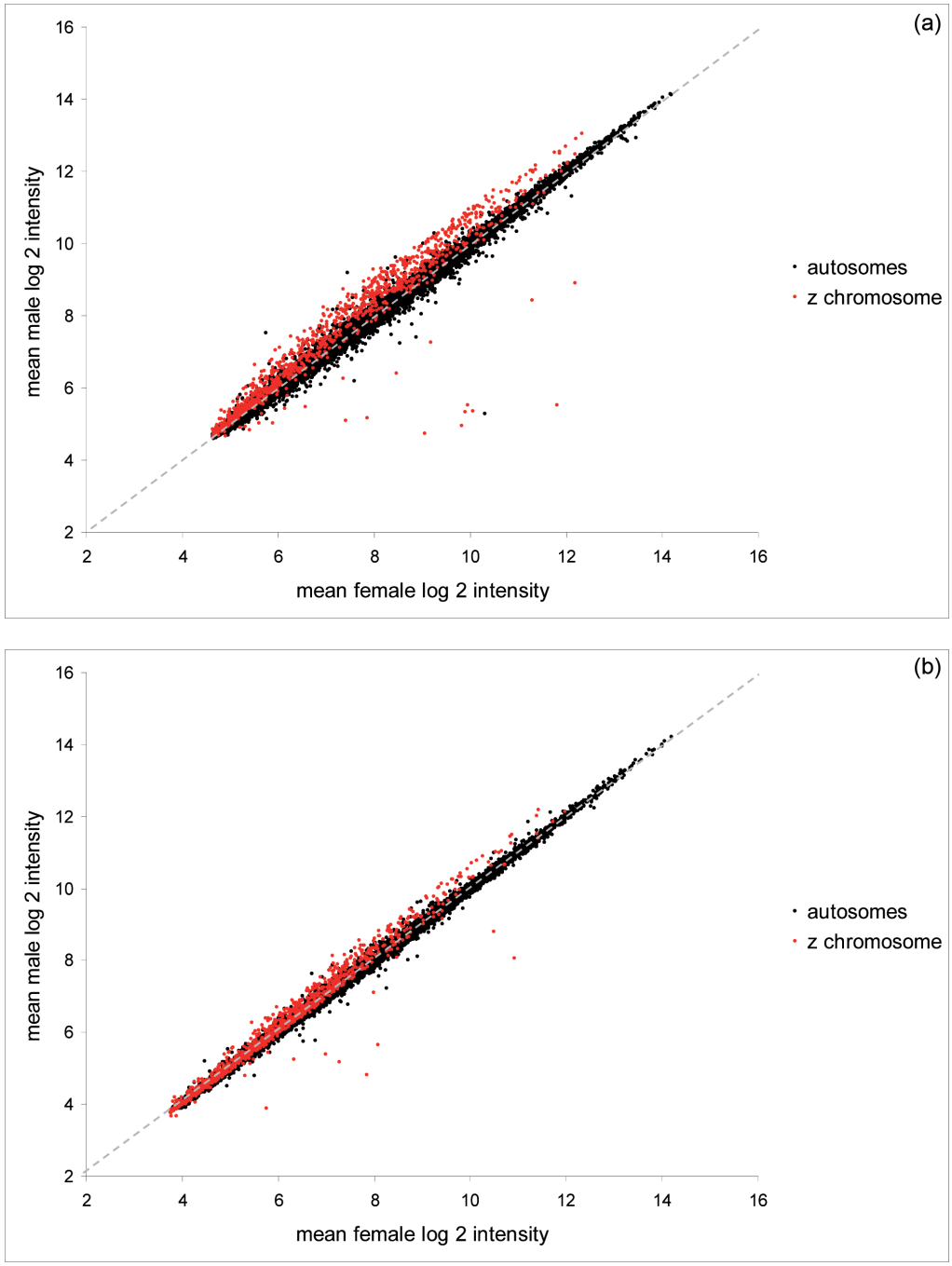

Figure 4. Male signal intensity plotted against female signal intensity for (a) the zebra finch and (b) the common whitethroat.

Table 1. Results from a General Linear Model with male expression intensity as dependent variable, and female expression intensity, chromosome type (Z or autosomal) and their interaction term as independent variables. Data from the zebra finch.

Tests of Between-Subjects Effects

Dependent Variable:mean male log 2 signal

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	46413.570[a]	3	15471.190	626361.172	.000	.994	1879083.517	1.000
Intercept	.277	1	.277	11.195	.001	.001	11.195	.917
meanfemalelog2signal	14568.511	1	14568.511	589815.614	.000	.981	589815.614	1.000
chromosome type	1.821	1	1.821	73.710	.000	.006	73.710	1.000
chromosome type * meanfemalelog2signal	19.949	1	19.949	807.635	.000	.066	807.635	1.000
Error	284.422	11515	.025					
Total	712863.264	11519						
Corrected Total	46697.992	11518						

a. R Squared = .994 (Adjusted R Squared = .994)
b. Computed using alpha = .05

Table 2. Results from a General Linear Model with male expression intensity as dependent variable, and female expression intensity, chromosome type (Z or autosomal) and their interaction term as independent variables. Data from the common whitethroat.

Tests of Between-Subjects Effects

Dependent Variable:mean male log 2 signal

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Corrected Model	39293.390[a]	3	13097.797	1310155.370	.000	.997	3930466.111	1.000
Intercept	1.703	1	1.703	170.371	.000	.015	170.371	1.000
meanfemalelog2signal	10846.411	1	10846.411	1084952.240	.000	.989	1084952.240	1.000
chromosome type	2.406	1	2.406	240.692	.000	.020	240.692	1.000
chromosome type * meanfemalelog2signal	7.975	1	7.975	797.772	.000	.065	797.772	1.000
Error	115.247	11528	.010					
Total	563563.689	11532						
Corrected Total	39408.637	11531						

a. R Squared = .997 (Adjusted R Squared = .997)
b. Computed using alpha = .05

autosomal dataset into four equal parts by sorting all ESTs by mean log 2 signal (calculated for all individuals regardless of sex). Each quartile was then analyzed separately (Figure 1). In the zebra finch female Z-linked signal was significantly lower than male Z-linked and autosomal signal in all four groups (all $p < 0.0001$; Table 3) Male signal intensity differed significantly from mean autosomal signal in the quartile with the second lowest expression, where is was slightly lower than autosomal ($p = 0.013$; Table 3), and in the quartile with the second highest expression where it was slightly higher than mean autosomal signal ($p = 0.008$; Table 3). Overall, the male signal closely followed that of the autosomes while female signal was close to male and autosomal signal only amongst the genes with lowest expression levels and then became progressively lower with higher mean expression in comparison to both male Z-linked and auosomal signals (Figure 1a).

In the common whitethroat, Z-linked signal was significantly lower than autosomal signal in both sexes in all four quartiles ($p < 0.0001$; Table 4; Figure 1b). Female Z-linked signal was significantly lower than male Z-linked signal in the three groups with highest signal intensities ($p < 0.0001$ for the highest and second highest and $p = 0.002$ for the second lowest group) but not significantly different in the lowest quartile ($p = 0.223$: Table 4; Figure 1b).

Analyses of sequence divergence for common whitethroat and zebra finch using array data

Mean number of significantly hybridizing probes for Z-linked genes (9.82 ± 0.040) in our CGH study (Naurin et al. 2008) was significantly lower than mean number of hybridizing probes on autosomes (10.23 ± 0.009; $F_{1, 19615}$, $p < 0.0001$). This result suggests a high rate of sequence evolution on Z. Thus, our common whitethroat data might be affected by the high degree of sequence divergence on the Z chromosome. We therefore re-analyzed the data set for the common whitethroat, this time using only ESTs that had significant hybridization on all of the eleven probes for each EST on the array in a previous Comprative Genome Hybridization (CGH) study (Naurin et al. 2008). This data set included10370 autosomal and 443 Z-linked ESTs. For this data set the results remained unchanged. However, as expected if Z evolves faster than the autosomes, Z-linked expression was affected to the highest degree by the removal of ESTs with some degree of sequence divergence (i. e. only using data for ESTs that hybridized at all 11 probes). Female Z-linked intensity increased by 8.4%, male Z-linked intensity increased by 8.6% while autosomal signal intensities increased by 5% in both sexes.

Ratios of Z-linked to autosomal expression

In order to further clarify how Z-linked expression differs from autosomal we calculated ratios of mean Z-linked over mean autosomal expression (Z:A ratios) for the data set in total and for the four quartiles separately. All Z:A ratios for both sexes in both species fell within the interval of 0.89 and 1.01, hence Z-linked expression varied with a maximum of 11% from autosomal expression. For the zebra finch, the largest difference between Z-linked and autosomal expression was a ratio of 0.95; that is, a 5% difference between Z-linked and autosomal expression only. Individual autosomes in our dataset varied with a maximum of 4.7%. Hence, Z-linked gene expression was very close to mean autosomal expression and in many cases fall within variation of individual autosomes.

Discussion

Our results clearly demonstrate that male-biased gene expression on the avian Z chromosome cannot be entirely explained by a simple effect of the double dose of Z chromosomes in males compared to females. Ratios of Z-linked to autosomal gene expression (Z:A) were close to one for all types of genes in both sexes (zebra finch; 0.94–0.98 in females, 0.99–1.01 in males, common whitethroat; 0.89–0.92 in females, 0.92–0.93 in males). Thus, we conclude that females of these two passerine species have up-regulated their gene expression on Z from a 50% dose, most likely in order to increase the balance between Z-linked and autosomal expression (i.e. dosage compensation). However, there is still extensive and sig-

Table 3a. Results for one-way ANOVAS of female Z-linked and autosomal expression; male Z-linked and autosomal expression; and female Z-linked and male Z-linked expression. Data listed for all four quartiles in the zebra finch.

		female Z vs autosomes	male Z vs autosomes	female Z vs male Z
lowest 25%				
	p value	<0.0001	0.304	<0.0001
	F	24.6	1.06	14.3
	df	1	1	1
2nd lowest 25%				
	p value	<0.0001	0.013	<0.0001
	F	127.9	6.24	33.3
	df	1	1	1
2nd highest 25%				
	p value	<0.0001	0.008	<0.0001
	F	114.2	7.03	56.5
	df	1	1	1
highest 25%				
	p value	<0.0001	0.121	<0.0001
	F	94.4	2.41	40.2
	df	1	1	1

Table 3b. Descriptives of gene expression data in all four quartiles in the zebra finch.

		female Z	male Z	female autosomes	male autosomes
lowest 25%					
	mean signal	5.07	5.18	5.19	5.20
	standard error	0.020	0.022	0.008	0.008
2nd lowest 25%					
	mean signal	7.93	8.33	8.27	8.24
	standard error	0.036	0.041	0.009	0.009
2nd highest 25%					
	mean signal	6.28	6.54	6.62	6.62
	standard error	0.029	0.033	0.009	0.009
highest 25%					
	mean signal	9.76	10.3	10.4	10.3
	standard error	0.054	0.054	0.019	0.019

Table 4a. Results for one-way ANOVAS of female Z-linked and autosomal expression; male Z-linked and autosomal expression; and female Z-linked and male Z-linked expression. Data listed for all four quartiles in the common whitethroat.

		female Z vs autosomes	male Z vs autosomes	female Z vs male Z
lowest 25%				
	p value	<0.0001	<0.0001	0.223
	F	103.2	80.3	1.48
	df	1	1	1
2nd lowest 25%				
	p value	<0.0001	<0.0001	0.002
	F	697.5	495.7	9.91
	df	1	1	1
2nd highest 25%				
	p value	<0.0001	<0.0001	<0.0001
	F	756.5	482.6	16.60
	df	1	1	1
highest 25%				
	p value	<0.0001	<0.0001	<0.0001
	F	179.3	90.3	9.88
	df	1	1	1

Table 4b. Descriptives of gene expression data in all four quartiles in the common whitethroat.

		female Z	male Z	female autosomes	male autosomes
lowest 25%					
	mean signal	4.28	4.32	4.62	4.63
	standard error	0.023	0.022	0.023	0.010
2nd lowest 25%					
	mean signal	5.47	5.56	6.01	6.01
	standard error	0.019	0.020	0.006	0.006
2nd highest 25%					
	mean signal	6.52	6.65	7.19	7.19
	standard error	0.043	0.044	0.013	0.013
highest 25%					
	mean signal	8.32	8.60	9.32	9.32
	standard error	0.063	0.064	0.023	0.023

nificant male-bias in Z-linked gene expression in both species (Naurin et al. 2009) and, interestingly, male-bias increased with increasing gene expression level in this study from a more or less sex-balanced expression for genes with low expression. Together with the indications of increasing male-bias with increased gene expression found by Melamed and Arnold (2007) in the chicken, our results indicate that the pattern of less dosage compensation for highly expressed genes is general in birds.

Thus, in female birds the balance between Z-linked and autosomal expression has not been fully reached for highly expressed genes. Moreover, analyses based on the number of significantly hybridizing probes in our previous CGH study (Naurin et al. 2008), suggest a high degree of divergence between the common whitethroat and the zebra finch on the Z chromosome. Male Z-linked expression in the common whitethroat differs more from autsosomal expression in highly expressed genes (Figure 1) which suggests that divergence is more pronounced for highly expressed genes. The high rate of molecular evolution on Z is highly interesting because of its potential role in the evolution of male-specific traits and thereby in the speciation process. Recent results demonstrate that Z is highly implicated in adaptive evolution, perhaps to a larger extent than X in e. g. mammals (Ellegren 2008; Qvarnstrom & Bailey 2009). Moreover, a study in *Ficedula* flycatchers has demonstrated that genes involved in speciation were predominantely Z-linked and coded for sex-specific traits, like male-plumage characteristics and female species recognition (Saether et al. 2007).

Our results lead to questions regarding: (i) to what extent must the heterogametic sex (XY-males and ZW-females) up-regulate sex-linked gene expression in order to be considered dosage compensated, and (ii) to what degree does sexual conflict and sexual selection affect the patterns of male-bias on Z chromosomes?

When is an organism dosage compensated?

In our view, the most plausible interpretation of the present results is that the zebra finch and the common whitethroat do achieve dosage compensation. All Z:A ratios are close to one and well withing X:A ratios of mammals, considered to be dosage compensated, as listed by Itoh et. al. (2007). For the zebra finch the variation in expression between the Z chromosome and the autosomes in the brain was maximum 5% in either sex. The mouse (*Mus musculus*) has a 30% higher X-linked than autosomal gene expression in the brain while human X-linked expression in the hypothalamus is 3–5% higher than autosomal (Itoh et al. 2007). Hence, it seems likely that birds do not suffer from more imbalance in genetic networks than mammals. Instead, the difference between the avian Z chromosome and the X chromosome in mammals centers around the male-biased expression in highly expressed genes on Z.

Potential explanations for male-biased gene expression on Z

Even if it seems unlikely that the somewhat lower Z-linked than autosomal expression in females should disrupt critical networks, there must be a compelling reason for why females do not balance their Z-linked expression to that of the autosomes for highly expressed genes. This pattern is especially interesting in the light of the fact that they do achieve a high degree of balance for genes with low expression. We suggest three possible explanations for this:

First, the mechanism of dosage compensation could be general and upregulate Z-linked genes along the chromosome in females to a degree equal to the level of low to medium expressed genes (but not to the level of highly expressed genes). However, this explanation seems unlikely as the level of compensation is a highly variable process in birds and varies between tissues and age groups in a way that indicates that selection for up-regulation has taken place on a gene to gene basis (Itoh et al. 2007; Mank & Ellegren 2009a).

Second, selection for higher expression in females might 'spill over' into males, thereby increasing their gene expression as well. If increased expression is detrimental in males then Z-linked genes might face further selection for down-regulation. These opposing selection pressures might explain why females have not fully

balanced all Z-linked genes. It is not immediately clear however, why this should be more common in highly expressed genes. Moreover, if males are more sensitive to increased expression in high-expressed than in low-expressed genes it might be expected that selection in females might push male expression somewhat higher than autosomal for low-expressed genes at least, a pattern not seen in our data.

Third, some property of many of the highly expressed genes makes them less suitable for high expression in females. Females have faced selection for up-regulation of Z-homologues as W degraded. However, Z is not W, the two chromosomes had evolved separately for some time before selection for dosage compensation took place. Male-biased gene expression and the potential for high divergence in highly expressed Z-linked genes give rise to an obvious question: Have these Z-linked genes evolved to increase male-function due to a high degree of sexual selection on males, and when females cannot rely on W any longer, are they forced to accept lower levels of compensation in order to avoid malfunction of these male-adapted genes in females?

Z and X chromosomes are expected to accumulate sexually antagonistic mutations (Charlesworth et al. 1987; Rice 1984). This is due to their uneven dose between the sexes which for the Z chromosome leads to; (i) twice as much selection on dominant mutations in males and (ii) stronger selection on recessive mutations in females. Hence, selection in females cannot remove male beneficial dominant mutations from the population while selection in males cannot remove recessive female beneficial mutations (Charlesworth & Charlesworth 1980; Ellegren & Parsch 2007; Rice 1984). Therefore, an accumulation of sexual antagonism is expected on the X and Z chromosome.

A higher degree of sexual antagonism on Z than on X could potentially explain why there is a large degree of male-bias on Z. Present results do neither prove nor disapprove that there is a higher degree of sexual antagonism on Z than on X chromosomes. However, a recent study in chicken found that female detrimental antagonism was overrepresented on the Z chromosome and the authors therefore concluded that dominant mutations might have been more common than recessive on Z (Mank & Ellegren 2009b). An overrepresentation of female-detrimental antagonism, indication of a high frequency of dominant mutations together with the generally fast evolution of Z lend some support to the hypothesis that sexual antagonism harmful to females might contribute to the high degree of male-biased gene expression on Z, but more studies of dosage compensation and sexual antagonism on both Z and X chromosomes in more species are needed to elucidate these processes.

Results in this study together with results from Melamed and Arnolds (2007) and Zha et al. (2009) hint at a potential role for sexual conflict on Z chromosomes. Present results and results in Melamed and Arnold (2007) suggest that increasing male-bias with increasing expression levels is general in birds. Interestingly the patterns of male-bias is reversed in the the silkworm (*Bombyx mori*; Zha et al. 2009) where male-bias decrease with increasing gene expression level, creating a pattern where male-biased genes are expressed at lower levels than un-biased genes (Zha et al. 2009). Male-biased genes in the silkworm are associated with regulatory activity and motor function, a pattern not seen for un-biased genes. Zha et al. (2009) concluded that low-expressed genes with a higher degree of male-bias were more likely to have evolved for a role in controlling sexual differentiation in the silkworm.

Moreover, Melamed and Arnold (2007) found that there were functional differences between male-biased and unbiased genes on the chicken Z chromosome. Hence, there are three separate reasons why it is difficult to explain the male-bias on Z chromosomes as a simple effect of the fact that females only have one Z chromosome while males have two: (i) females do dosage compensate Z to a high extent; even if highly expressed genes are not fully compensated (present results; Itoh et al. 2007; Zha et al 2009) (ii) male-bias is not evenly distributed among genes with different expression levels; that is females have faced different scenarios for genes with different expression levels on Z when selected for dosage compensation (present results; Melamed and Arnold 2007; Zha et al 2009), and (iii) male-biased and unbiased genes are not only associated with certain expression levels but also have clear functional differences (Melamed and Arnold 2007; Zha

2009). Hence, it is possible that a large degree of selection on some Z-linked genes in males have adapted them to male functions in a way that makes them unsuitable for high expression levels in females.

There is an additional hypothesis regarding the difference between Z and X chromosomes that can be mentioned. Regardless of whether the actual number of sexually antagonistic genes on Z and X is different it is possible that sexual antagonism has been resolved by sex-biased gene expression to a larger extent on Z than on X chromosomes. This difference could be linked to differences in the way dosage compensation has been selected on X and Z, for example to the way the mechanisms for dosage compensation spread across the chromosomes.

Concluding remarks

The high Z:A ratios in this study imply that passerine birds are in fact to a very high degree dosage compensated and suggest that females do not suffer from much imbalance in genetic networks.

Our study further corroborates results showing that the avian Z chromosome evolves fast (Mank 2007; Ellegren 2008) and indicate that highly expressed Z-linked genes might have diverged more than low-expressed genes. Moreover, it is interesting that male-biased genes on Z are highly expressed. Male-biased genes have been shown to evolve fast in several taxa but highly expressed genes normally have a low degree of protein and sequence evolution (Drummond et al. 2005; Duret & Mouchiroud 2000; Pal et al. 2001; Rocha & Danchin 2004; Subramanian & Kumar 2004). Moreover, it has been demonstrated that highly expressed genes have a low degree of expression profile evolution (Liao & Zhang 2006).

Hence, it is surprising to find a large amount of sex-bias in highly expressed genes and that finding, together with the fast evolution of Z, suggests that there has been a high degree of selection on males for Z-linked loci.

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IV

First evidence of a neo-sex chromosome in birds

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Highly differentiated sex chromosomes are common in many species, and the evolutionary process leading to this state include cessation of recombination, degeneration of Y and W chromosomes and the evolution of dosage compensation. It is difficult to study these processes in species where the sex chromosomes are already highly heteromorphic, like for example mammals (where females: XX and males: XY) or birds (where females: ZW and males: ZZ). Studies of sex chromosome evolution have therefore often focused on species where the ancestral chromosomes have fused with autosomal material creating new, much younger, parts of the sex chromosomes (neo-sex chromosomes). Neo-sex chromosomes have been studied extensively in for example *Drosophila*, shedding light on the processes of sex chromosome evolution. We report the first finding of a neo-sex chromosome in birds: Tgu4a, an autosome in the zebra finch genome, is sex-linked in warblers (*Sylviidae*). The first 10 Mb of Tgu4a has highly elevated levels of sex-biased gene expression in the common whitethroat (*Sylvia communis*). 14% of ESTs on Tgu4a shows significant sex-bias compared to only 0.0007% for the regular autosomes. Moreover, both markers present in the first 10 Mb of Tgu4a are sex-linked in the great reed warbler (*Acrocephalus arundinaceus*). Tgu4a shows no evidence of sex-linkage in other passerine species for which linkage maps have been produced, leading to the conclusion that as a neo-sex chromosome it is between 24 and 47 MYA. This finding allows for two types of studies: (i) studies of the evolution of sex chromosomes in birds, a taxa known for its otherwise very stable genome structure and (ii) comparisons of the evolution on these neo-Z and neo-W-chromosomes with the neo-X and neo-Y's in *Drosophila*.

Introduction

The heteromorphic sex chromosomes seen in many species today have evolved from an ancestral pair of homologous autosomes (Charlesworth 1996; Rice 1996). The evolution towards the heteromorphic state most likely began with a mutation on one of the homologues, causing sex to be genetically determined (Bachtrog 2006; Charlesworth 1996; Rice 1996). The sex determined by this new allele can either be males in which case the sex chromosome carrying the mutation is called Y and its homologue X, or females in which case the chromosome with the sex determining allele is called W while the homologue is called Z. XY sex determination is present in taxa like *Drosophila* and mammals, while ZW determination is the general pattern among for example birds.

After the appearance of a novel sex-determining allele, the evolution of sex chromosomes has in many cases lead to two separate heteromorphic and often largely non-recombining sex chromo-

somes. The evolution of sex chromosomes and sex chromosome linked genes appear to involve several patterns and processes, most of which are difficult to study in systems where sex chromosomes have already evolved into highly differentiated chromosomes. The processes involve:

1. **Repression of recombination.** Repression of recombination between sex chromosomes is in many cases an integral part of sex chromosome evolution and in many taxa recombination between X and Y (or Z and W) has largely ceased (Bergero & Charlesworth 2008). In the absence of recombination the X and Z chromosomes recombine when present in XX or ZZ individuals while on the heterogametic chromosome (Y or W) recombination is absent (Bergero & Charlesworth 2008). Repression of recombination between the X and Y (or Z and W) chromosomes is believed to have occurred due to accumulation of sexually antagonistic mutations in linkage with the sex-determining allele (Rice 1987b).
2. **The degeneration of Y and W chromosomes.** Y and W chromosomes have degenerated in many species, leading to the small and gene poor chromosomes seen today. The lack of recombination on Y and W chromosomes is believed to have caused this degradation (Bachtrog 2006; Bergero & Charlesworth 2008). Different hypotheses regarding this process have been put forward (for reviews see: Bachtrog 2006; Charlesworth 1996): (i) a process referred to as Muller's ratchet involving the stochastic loss of Y chromosomes with few deleterious mutations from the population (Charlesworth 1978), (ii) 'background selection', where negative selection on highly deleterious alleles leads to fixation of weakly deleterious alleles (Bachtrog 2006; Charlesworth 1994; Charlesworth 1996), and (iii) 'genetic hitchhiking', i.e., positive selection on beneficial alleles leading to the fixation of weakly deleterious alleles in linkage with the selected gene (Bachtrog 2006; Rice 1987a). It is not known whether negative selection or positive selection plays the largest role in the degeneration of Y and W chromosomes.
3. **The evolution of dosage compensation.** It is believed that the loss of functional homologues on Y chromosomes has led to selection for increased gene expression on X chromosomes (dosage compensation). In many taxa, X-linked gene expression from the single X chromosome in males has been up-regulated and is balanced to X-linked expression in females and expression on autosomes (Charlesworth 1996; Mank 2009). Selection for increased X-linked expression is believed to be caused by the sensitivity of gene networks to changes in gene expression dose (Lindsley et al. 1972; Lu & Oliver 2001; Lynch 2007; Pauli et al. 1993; Rosenbusch 2004). Hence, XY-males and ZW-females can be expected to increase their X- or Z-linked gene expression in order to retain original dose as Y and W degenerates.

Organisms with sex chromosomes in transitory stages of sex chromosome evolution provide excellent models for studies of these patterns and processes. Sex chromosome evolution has been studied in taxa with newly formed sex chromosomes, for example fish, where some species carry XY chromosomes while related species have ZW-determination and where switches between the two is relatively frequent (Ross *et al.* 2009). Recently formed sex chromosomes in plants also provide promising model systems for the study of sex chromosome evolution (Charlesworth 2002; Vyskot & Hobza 2004). Neo-sex chromosomes have also been extensively studied in *Drosophila*. The X chromosome in *D. americana*, and the Y chromosome in *D. miranda*, has fused with an autosome; leading to sex-linkage for genes that were relatively recently autosomal. Such neo-sex chromosomes also provide highly interesting models for the evolution of sex-linked genes.

In birds (ZW) sex chromosomes have been remarkably stable over millions of years, a property they share with sex chromosomes in mammals (van Doorn & Kirkpatrick 2007). This might be due to the presence of a large number of genes necessary for viability or fertility on the sex chromosomes leading to selection for retaining chromosome stability (see Marin & Baker 1998; van Doorn & Kirkpatrick 2007 for other potential reasons for sex chromosome stability). The gene

content (synteny) on autosomes and sex-chromsomes is highly conserved in Galliformes and Passeriformes with the exception of a fission of Gga1 in the passerine bird lineage and a fusion of two ancestral chromosomes forming Gga4 in the chicken lineage (Dawson et al. 2007; Derjusheva et al. 2004; Griffin et al. 2007).

Here we report the presence of a neo-sex chromosome in two species of warblers (*Sylviidae*), the common whitethroat (*Sylvia communis*) and the great reed warbler (*Acrocephalus arundinaceus*) belonging to the order Passeriformes. Passerines also include the zebra finch (*Taeniopygia guttata*), in which the same chromosome, Tgu4a, is autosomal with no indications of sex-linkage. The transition from autosome to sex chromosome has thereby taken place after the split between the warblers and the zebra finch which makes the neo-sex chromosomes between 24–47 million years old (Barker et al. 2004; Jonsson & Fjeldsa 2006; Zuccon 2007). This makes Tgu4a in warblers a young sex chromosome in birds, for which the ancestral Z and W chromosomes are perhaps as old as 150 million years (Lawson-Handley et al. 2004; Matsubara et al. 2006; Nam & Ellegren 2008). Warblers are thereby highly interesting model systems for studies of sex chromosome evolution, allowing for direct comparisons with evolution of neo-Y and neo-X chromosomes in species with male heterogamety. Such comparisons could shed light on how sex chromosomes evolve in predominantly male versus female environments, and thereby potentially explain interesting discrepancies between the XY- and ZW-systems; for instance, why the extent of dosage compensation seems less efficient in the ZW-species than in XY-systems (Ellegren et al. 2007; Itoh et al. 2007; Mank 2009; Melamed & Arnold 2007; Zha et al. 2009).

Interestingly, Tgu4a corresponds to an ancestral microchromosome in the avian genome which has repeatedly fused and split from the ancestral chromosome 4 (Griffin et al. 2007). In chicken these two chromosomes are fused but the arm on Gga4 corresponding to Tgu4a still retains the characteristics typical of avian microchromosomes such as high recombination, high gene density and high G+C-content (ICGSC 2004). No other chromosomes in the avian genome has this pattern of repeated fusions (Griffin et al. 2007) giving rise to obvious questions concerning their gene content. Our finding of sex-linkage of Tgu4a indicates that it might contain genes crucial for reproductive success or genes prone to sexually antagonistic selection.

Methods

Gene expression in the common whitethroat

Samples of the entire brain of 11 female and 11 male common whitethroats were used in the microarray part of this study. The Lund-zfa array is an Affymetrix custom array that consists of 22360 zebra finch ESTs, representing approximately 15800 genes (Naurin et al. 2008; Replogle et al. 2008). All ESTs have been BLASTed against the 3.2.4 build of the zebra finch genome (Naurin *et al.* 2009a), and hits in that BLAST were considered significant if the e-value was $\leq 10^{-20}$. 1104 of the ESTs with significant signals in our data have significant hits against the Z-chromosome and 18520 ESTs have significant autosomal hits. Details regarding microarray hybridization, quality control, filtering of data based on sequence divergence between the common whitethroat and the zebra finch, and analyses of microarray data can be found in Naurin et al. (2009a; 2009b; 2008). Details of identification of significant sex-bias can be found in (Naurin et al. 2009a); where all 25 non-redundant sex-biased genes (17 male biased and 8 female-biased) located on Tgu4a were identified. Out of the 18520 autosomal ESTs, 511 are present on Tgu4a (out of which 197 is located on the first 10 Mb of Tgu4a).

Fold Changes (FC) in this study was calculated as mean male over mean female unlogged signal intensity on the array. One-way ANOVAs were run in order to evaluate whether FC was significantly different between different chromosomal categories (results remained unchanged if non parametric tests were used instead).

Marker analyses in the great reed warbler

A low-density linkage map in the great reed warbler included two microsatellite loci (G61 and Aar3) with orthologues on chicken chromosome

4 (Gga4; Dawson et al. 2007). These two loci are unlinked in the great reed warbler and one of them (G61) is sex-linked. Two other microsatellites (Ase15 and Ase61) are linked to Aar3 in the great reed warbler, and they are not sex-linked (Dawson et al. 2007). In the present study, we examined additional loci with orthologues on Gga4: two microsatellites (TG04-012a and TG04-061; Dawson et al. 2009) and SNPs at 20 introns (06500, 09949, 12303, 15246, 15738, 16214, 17270, 17483, 18503, 19599, 19994, 23080, 23272, 25149, 25171 and 25924 from Backström et al. 2008; and 034, 035, 036, and 037 from M. Stervander and B. Hansson unpubl.; Figure 4). Sex-linkage was determined by genotyping microsatellites and re-sequencing introns in 7 male and 7 female great reed warblers. Both TG04-012a and TG04-061 were polymorphic and easy to score, and 12 of the 20 introns amplified a single PCR-product and gave readable sequences in the great reed warbler. To assign the location of loci that were possible to evaluate for sex-linkage in the great reed warbler, i.e. 6 microsatellites (G61, Aar3, Ase15, Ase61, TG04-012a and TG04-061) and 12 introns (09949, 12303, 15246, 15738, 16214, 17270, 17483, 18503, 19994, 23080, 25924 and 036), in the zebra finch and chicken genome, we ran BLASTN analyses of the GenBank sequence against (i) the zebra finch genome assembly (taeGut3.2.4; http://genome.wustl.edu/tools/blast/) and (ii) the chicken genome assembly (build 2.1; http://www.ensembl.org/Gallus_gallus/blastview). Of these 18 loci, 17 and 16 could be assigned a position in the zebra finch and chicken genome assemblies, respectively.

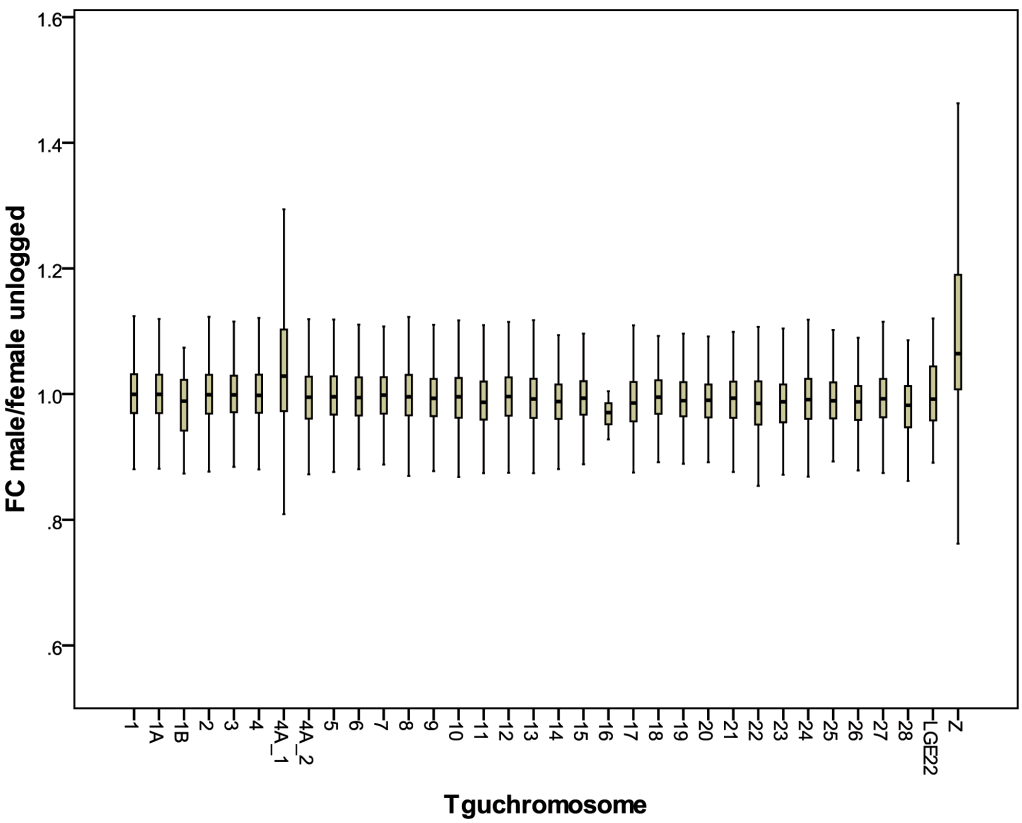

Figure 1. Box plot of Fold Change (FC) on all chromosomes for the common whitethroat. Tgu4a is split in two halves in this figure 4a_1: the first 10 Mb and 4a_2: 10–20.7 Mb. Annotations are taken from the 3.2.4 build of the zebra finch genome.

Results

Gene expression on chromosome 4 and 4a in the common whitethroat

Out of the 40 significantly sex-biased ESTs on the autosomes identified in Naurin et al. (2009a), 27 ESTs (corresponding to 25 non-redundant genes) were situated on Tgu4a in the common whitethroat. Thus, 14% of the 197 ESTs on the first 10 Mb of Tgu4a were sex-biased, whereas the same figures for ESTs on chromosome 4 and e.g. chromosome 1 were 0% and 0.0006%, respectively (mean autosomal = 0.0007%). When the positions of the 25 significantly sex-biased ESTs on the 20.7 Mb large Tgu4a were studied in detail, they all fell within the first 10 Mb. Box plots of Fold Change (FC; male/female expression) for all chromosomes clearly show that the first half of Tgu4a is exceptional in terms of sex-biased expression in the genome of the common whitethroat, surpassed only by the Z chromosome (Figure 1). Moreover, the pattern of elevated sex-bias on the first half of Tgu4a is clearly visible when FC is plotted against chromosomal position on Tgu4a (Figure 2a; compare also with the pattern from Tgu4 and Tgu1 in Figure 2b and 2c). Moreover, scatter plots of gene expression between females and males show that the first half of chromosome 4a has an increased sex-bias (Figure 3a), but does not have the general pattern of high male-bias seen for the Z chromosome (Figure 3b).

A one-way ANOVA showed that FC on the first 10 Mb of Tgu4a (1.03 ± 0.010) was significantly higher than mean FC over all other autosomes (0.99 ± 0.0004; $F_{1,18510} = 89.58$; $p < 0.0001$). The distal part of chromosome 4a (from 10 Mb to the end of the chromosome) did not have significantly higher FC than the other autosomes (results not presented here).

Sex linkage of microsatellite and SNPs on chromosome 4 and 4a in the great reed warbler

Of the 18 loci we evaluated for sex-linkage in the great reed warbler, the microsatellite locus G61 and the SNPs on the intron 09949 showed sex-linked pattern with females being hemizygous and males homo-/heterozygous. Both these loci were located on the first part of the Tgu4a and the distal part of chicken chromosome 4; G61 at positions Tgu4a 1.0 Mb and 09949 at position Tgu4a 7.0 Mb, respectively (Figure 4). The other loci showed no evidence of sex-linkage and were located either on the second part of Tgu4a, on Tgu4 or on Tgu4_random (Figure 4).

Discussion

The first 10 Mb of Tgu4a has an elevated degree of sex-biased gene expression in the common whitethroat. 27 out of 40 significantly sex-biased autosomal ESTs identified in our previous study (Naurin et al. 2009a) map to this part of Tgu4a, and 14% of all ESTs present for this part of Tgu4a on our microarray is thereby significantly sex-biased (compared to the average of 0.0007% on all autosomes). These ESTs represent 25 non-redundant sex-biased genes. Figure 1 and 2 clearly show that the pattern of sex-biased gene expression is remarkably different in the first 10 Mb of Tgu4a, compared to the distal part of the chromosome and other autosomes. Fold Change (male/female signal intensity) was significantly higher on Tgu4a than on the rest of the autosomes, a situation reminiscent of that on the Z chromosome, where male-bias is extensive (Ellegren et al. 2007; Itoh et al. 2007; Naurin et al. 2009b).

Moreover, two of 18 loci located on Tgu4a and Tgu4 (and Tgu4_random) were sex-linked in another warbler species, the great reed warbler, and both these loci were located on the first 10 Mb part of Tgu4a (Figure 4). Thus, the pattern of sex-linkage of loci in the great reed warbler corresponded to the pattern observed in the expression analyses in the common whitethroat.

Hence, our results show that the first 10 Mb of Tgu4a is sex-linked in two species of warblers, the common whitethroat and the great reed warbler.

Tgu4a is not sex linked in the zebra finch (www.ncbi.nlm.nih.gov/genome/guide/finch). Furthermore, there is no evidence of sex-linkage of markers located on Tgu4a in the collared flycatcher *Ficedula albicollis* (Backström et al. 2008) and the blue tit *Cyanistes caeruleus* (Hansson et al. 2009). The blue tit is on the same "Passerida"-branch in the Passerine phylogenetic tree as the

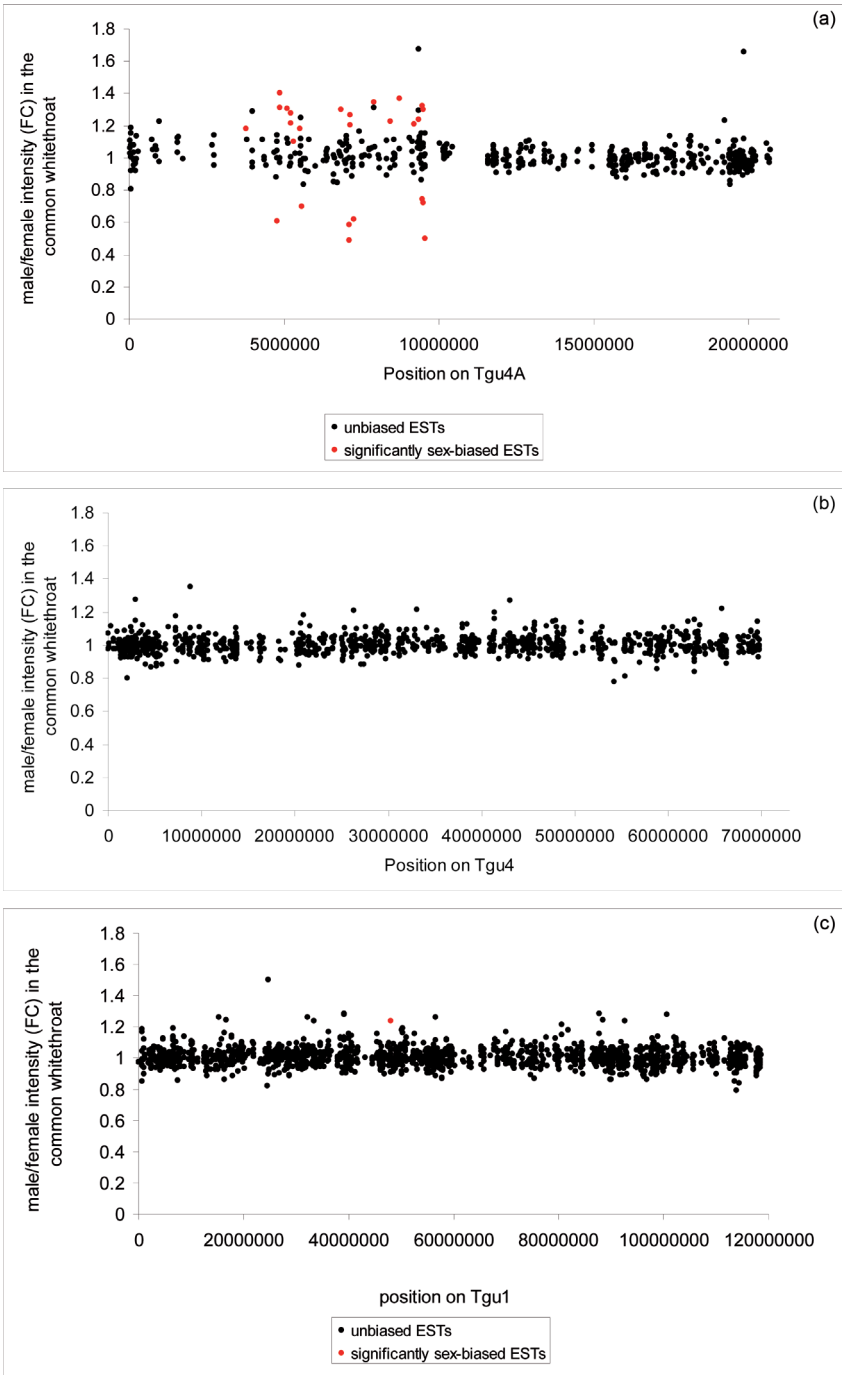

Figure 2. Fold Change (FC) calculated as male/female intensity in the common whitethroat plotted against position on (a) Tgu4a and (b) Tgu4 and (c) Tgu1. Annotations are taken from the 3.2.4 build of the zebra finch genome.

Sylviidae warblers (Baker et al. 2004), whereas the zebra finch and the collared flycatcher is on another branch. The split between the finches (*Fringillidae*; including the zebra finch) and the warblers took place between 47 and 24 MYA (Barker et al. 2004; Jonsson & Fjeldsa 2006; Zuccon 2007). Hence, Tgu4a has existed in its sex-linked form in the *Sylviidae* warblers for a maximum of 47 million years, but is likely to be considerably younger since no evidence of sex-linkage has been found in the blue tit. In comparison to the ancestral avian Z and W, which are potentially as old as 150 million years (Lawson-Handley et al. 2004; Matsubara et al. 2006; Nam & Ellegren 2008), it is therefore a very young sex chromosome.

Several hypothesis regarding changes in the karyotype of the warblers since the split from the finches can be envisioned.

First, regarding the distal part of Tgu4a and the apparent lack of sex-linkage in that region of the chromosome there are two distinct possibilities:

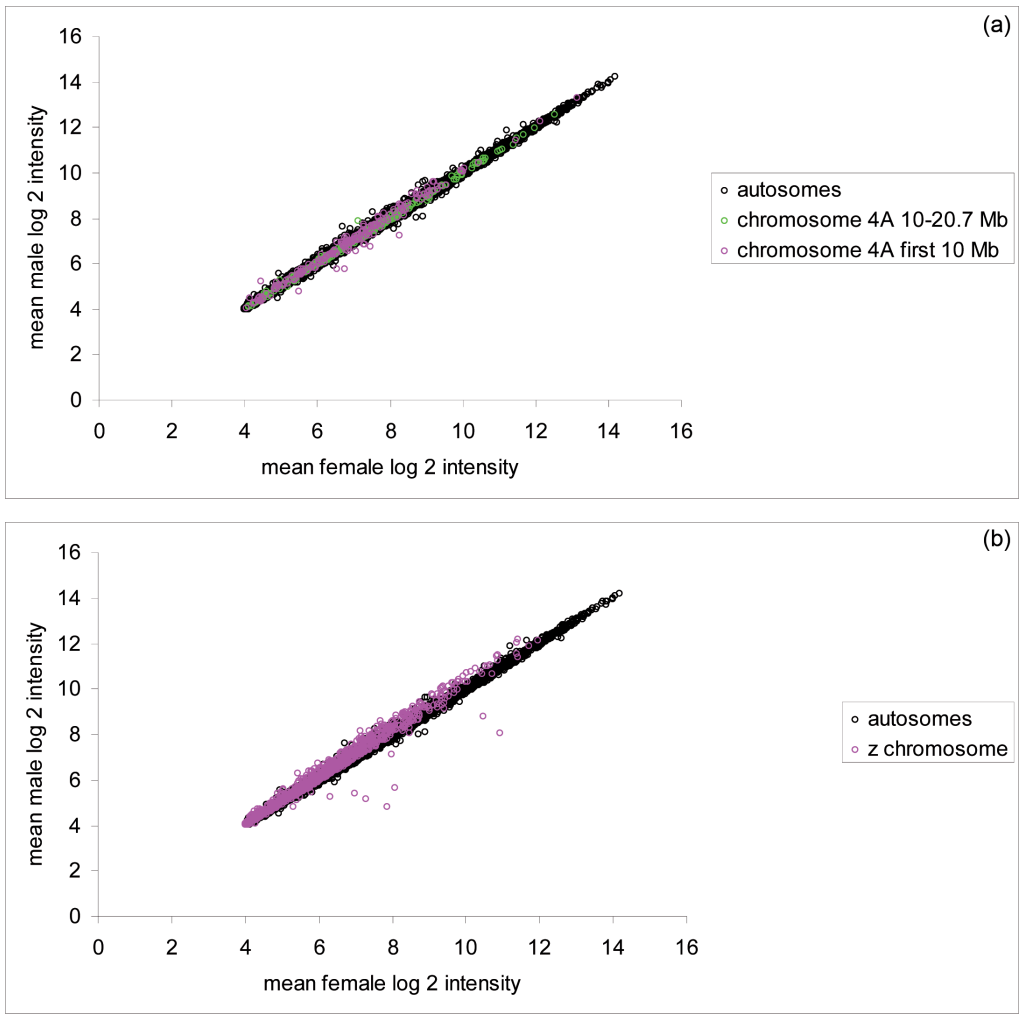

Figure 3. Male log 2 signal intensity plotted against female log 2 signal intensity for (a): chromosome 4a and all autosomes and (b) the Z-chromosome and all autosomes.

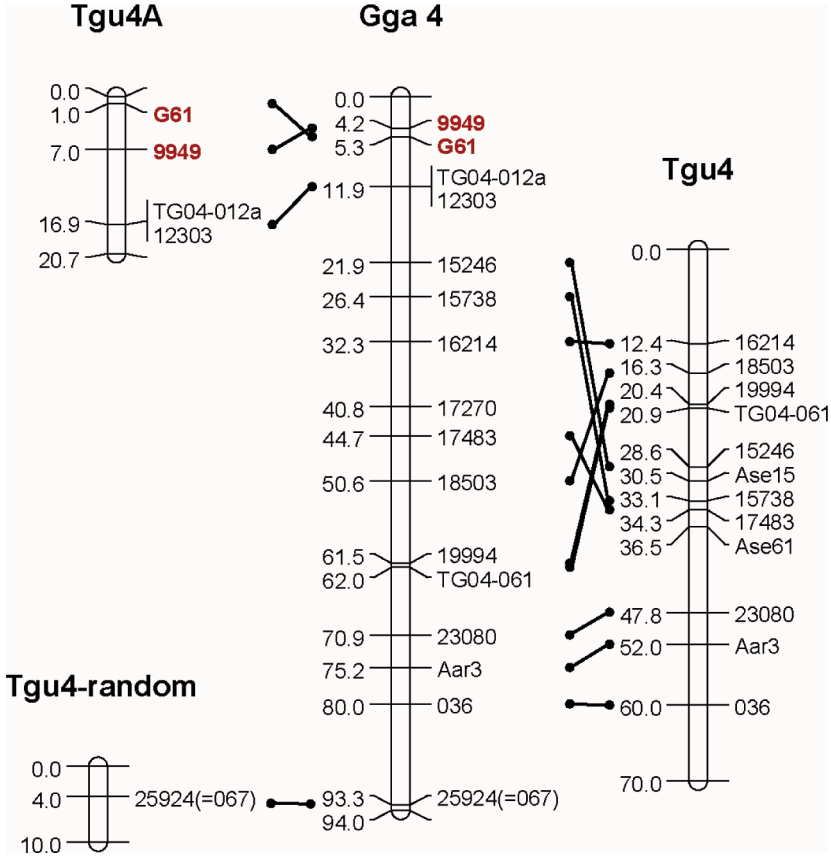

Figure 4. Loci evaluated for sex-linkage in the great reed warbler and their position on the zebra finch (*Tgu4*, *Tgu4a* and *Tgu4_random*) and chicken genome assembly (*Gga4*), respectively. Two loci highlighted in red, *G61* and *09949*, show sex-linkage in the great reed warbler.

1. There might have been another fission of Tgu4a in the warblers, producing two new chromosomes out of which only one has later become sex-linked. This would mean that warblers still have a part of Tgu4a segregating as an autosome.
2. The entire Tgu4a might be sex-linked in warblers. If that is the case then recombination is still present in the distal part of the chromosome, explaining why we have no hemizygous markers in females in that region in the great reed warbler. If the entire Tgu4a is sex-linked then the part of the chromosome covering 10 – 20.7 Mb is now a pseudoautosomal region on a sex chromosome and we would expect that with time recombination would be further repressed in this region, followed by more sex-biased gene expression and differentiation between the neo-Z and neo-W sequences.

Secondly, regarding whether or not Tgu4a is physically linked to the ancestral Z or W chromosomes:

1. **Tgu4a as a separately segregating sex chromosome.** We cannot exclude that Tgu4a is a separate sex chromosome in which case warblers would have multi factorial sex determination ($Z_1 Z_1 Z_2 Z_2$ / $Z_1 Z_2 W_1 W_2$). Theoretically, a new mutation leading to a sex determining allele on an autosome, or a translocation of a sex determining allele from the sex chromosomes to an autosome,

could lead to a situation where both pairs of sex chromosomes remain in the population (van Doorn & Kirkpatrick 2007). It is further possible that the neo-sex chromosomes hijack sex-determination from the ancestral sex chromosomes, thereby leading to a situation where the ancestral W is lost and the ancestral Z becomes an autosome (van Doorn & Kirkpatrick 2007). This is obviously not the case in the warblers, which have a Z chromosome that displays much the same properties as that in the zebra finch (Naurin et al. 2009a; Naurin et al. 2009b). Hence, if Tgu4a is separately segregating it co-exists with the ancestral Z and W.

2. **A fusion between Z and chromosome 4a.** Fusions of autosomes, or parts of autosomes, with sex chromosomes are relatively common (Flores et al. 2008; Steinemann & Steinemann 1998; Veltsos et al. 2008; Wilson et al. 2006). In fact, human sex chromosomes are likely to be the product of at least three such fusions with autosomes (Graves 1995; Graves 1998; Watson et al. 1991). Hence, perhaps a more likely scenario than a separate new sex chromosome is that Tgu4a has actually fused with one of the ancestral sex chromosomes. We have two indications that a fusion between Tgu4a and the Z chromosome might have taken place: (i) in the great reed warbler, there is more statistical support for locus G61 to be linked with the marker Ase50 on one side of the Z chromosome, than with Aar1 on the other side of Z (Hansson et al. 2005), and (ii) a chromosome-aberrant individual great reed warbler female with an AA:ZZW genotype has two alleles at Z-linked markers as well as at the locus G61 (Arlt et al. 2004).
3. **A fusion between W and chromosome 4a.** There are theoretical predictions stating that Y: A fusions should be more common than X:A fusions (Charlesworth & Wall 1999; Charlesworth & Charlesworth 1980). This, together with empirical data showing that X:A fusions have been less common (Luykx 1990; McAllister 2002) means we cannot exclude that Tgu4a originally became sex-linked as a result of a fusion with the W chromosome.

If a fusion between 4a and a sex chromosome has taken place it is possible that 4a is now linked to both sex chromosomes, regardless of with which the original fusion took place. Hypothesis 2 or 3 above would then have been a transient step in the evolution of the sex chromosomes, with the final results being a return to the simple ZZ/ZW sex determination, only with the addition of material from chromosome 4a on both sex chromosomes.

Tgu4a: a special case in avian evolution

Tgu4a corresponds to the ancestral michrochromosome 10 in the avian genome (Griffin et al. 2007). Our results cement the unique role of this chromosome in avian evolution. There are now three separate reasons to believe that this chromosome is of particular interest:

1. As mentioned in the introduction, chromosome 4a corresponds to the p-arm on chromosome 4 in chicken (Gga4) (ICGSC 2004). Gga4 is thereby a result of a fusion between ancestral chromosome 4 and ancestral chromosome 10 (Griffin et al. 2007). The p-arm of Gga4 is very different from the rest of the chromosome (ICGSC 2004). It has a higher recombination rate, higher G+C content, shorter intron lengths and a higher gene density than the rest of Gga4, properties that are typical of michrochromosomes in the avian genome (ICGSC 2004). Hence, it has retained all these characteristics in the chicken even after the fusion with ancestral chromosome 4. It even retains an interstitial telomere adjacent to the centromere (ICGSC 2004).
2. Ancestral chromosome 4, the chromosome with which Tgu4 (ancestral chromosome 10) has fused in the chicken is the most preserved chromosome in the avian genome (Griffin et al. 2007; ICGSC 2004). It is even preserved, without much change, in the human genome (Chowdhary & Raudsepp 2000; Griffin et al. 2007; ICGSC 2004). Hence, there must have been strong selection for retaining this chromosome in its original state. It is therefore peculiar that it is known for repeated fusions (and fis-

sions) with the ancestral chromosome 10 (Griffin et al. 2007). Species where they are fused include the chicken, greylag goose and the African collared dove (Griffin et al. 2007), and several fusions and fissions of these chromosomes must have taken place during avian evolution in order to explain how these species can have fused versions while most of the related species do not (Griffin et al. 2007). So far no plausible explanation for this pattern has been put forward.

3. Our results show a high degree of sex-biased gene expression on Tgu4a, indicating a high degree of sexually antagonistic selection. If the neo-sex chromosomes in warblers are caused by a fusion between Tgu4 and the sex-chromosomes then the presence of sexually antagonistic genes on Tgu4 might have made this fusion beneficial as it creates linkage between these genes and the sex-determining alleles on the sex chromosomes (Charlesworth & Charlesworth 1980).

Hence, Tgu4a (ancestral chromosome 10) is clearly a unique case in avian evolution, it does not only correspond to one of the chromosomes in the only chromosome pair which has experienced multiple fusions and fissions, it has also retained its microchromosomal characteristics even though its linked to a macrochromosome in chicken and it has become a neo-sex chromosome in warblers. Detailed studies of the gene content, gene expression and sequence divergence of this very unique chromosome in more bird species might shed light on what makes it such a special case in the evolution of birds.

Future directions

It should be possible to date the age of chromosome 4a as neo-sex chromosome by successively scanning additional 4a markers for sex-linkage in more species of *Sylviidae* warblers and related taxa. Moreover, segregation and recombination analyses of sex-linked markers can be analyzed with pedigree-based linkage mapping in the great reed warbler to evaluate whether markers at Tgu4a are linked to markers on the Z chromsome (Hansson et al. 2005; Dawson et al. 2007). Sequencing the Z and W homologues of loci located on Tgu4a can reveal the degree of decay on the newly formed W chromosome and the degree of differentiation between homologues on neo-Z and neo-W. RT-PCR with Z and W specific primers can be used to study the extent of dosage compensation of genes with non-functional homologues on W. It would further be highly interesting to study population level variation on 4a-W, and determine if positive selection (in the form of selective sweeps) or negative selection (in the form of purifying selection against deleterious mutations) is more important in causing degradation of this new W chromosome.

Concluding remarks

We have shown that Tgu4a is a potentially highly interesting model for the study of sex chromosome evolution. It does not only allow for tests of the hypotheses listed in the introduction, but also makes comparisons with neo-sex chromosomes in XY sex-determining systems possible. Such studies could shed light on how the evolution of sex chromosomes is affected by sex as opposed to gamety. In other words, Z chromosomes are predominantly selected in males, while X chromosomes are selected two third of the time in females. Y is male-limited while W is female-limited. These differences open up for studies of (i) the degree of relative evolution on neo-X and neo-Z chromosomes, (ii) the degree and mechanisms of degeneration on neo-Y and neo-W chromosomes, and (iii) the degree of dosage compensation on neo-X and neo-Z chromosomes.

Hence, it provides us with an unique opportunity to study which role sexual selection plays in the evolution of sex chromosomes.

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V

Sex-linkage, Size and Gene Expression Signals: the Rate of Evolution on Avian Chromosomes

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Genome-wide studies of divergence can elucidate to what extent the divergence of individual genes is influenced by factors like chromosomal location and gene expression level. They can further shed light on how neutral processes and selection shape the evolution of genomes.

In the present study we explore the patterns of gene divergence in the avian genome by assessing the amount of divergence between passerine bird species for 12 907 ESTs. We evaluated the degree of hybridization between DNA of different passerine bird species for over 140 000 short (25 bp probes) sequences dispersed in these ESTs. The ESTs were categorized according to the degree of hybridization on the probes and ranked from slow to fast diverging. We found that the avian Z chromosome is the fastest evolving chromosome of all, followed by the macrochromosomes and lastly the microchromosomes. In line with theoretical predictions and data from a few previous studies, the fast Z evolution in passerines described in our study suggests that Z-linked genes have played an important role in adaptive evolution and speciation in birds. The increasing degree of sequence divergence with chromosome size further suggests an important role for recombination; with efficient purging of deleterious mutations on the highly recombining microchromosomes and stronger background selection and more selective sweeps on macrochromosomes with their lower degree of recombination. Unexpectedly, our analyses showed that ESTs with medium expression levels were more conserved than low expressed and high expressed ESTs on autosomes as well as on the Z chromosome. Future large-scale divergence and gene expression studies in other taxa are needed to evaluate the generality of this finding.

Introduction

The recent development of new methodology and the rapid accumulation of DNA-sequence data have made it possible to compare rates of evolution between species and taxa on a genome-wide level. Such comparisons enable analyses to pinpoint hotspots for divergence and speciation as well as identify conserved regions potentially harboring essential genes. It also makes it possible to assess the importance of neutral processes as opposed to selection for how evolution has shaped chromosomes and genomes. Several processes stand out as the amount of theory and empirical results now steadily increase.

A high degree of evolution of sex-linked genes

Loci on X chromosomes in species with male heterogamety and on Z chromosomes in species with female heterogamety are expected to evolve

faster than autosomal loci (referred to as the fast X or fast Z effect; Presgraves 2008; Vicoso & Charlesworth 2006). The heterogametic sex (XY males and ZW females) has only one copy of X or Z and is thereby expressing all alleles regardless of their dominance coefficient. Hence, if new mutations on X or Z are beneficial and fully or partially recessive they will immediately face selection in the heterogametic sex which will lead to a higher fixation rate for such mutations (Charlesworth *et al.* 1987). Furthermore, X and Z are only present in on average 1.5 copies in each individual while autosomes are present in 2 copies. This lower population size of sex chromosomes will lead to more fixations of dominant and weakly deleterious mutations due to drift (Charlesworth et al. 1987; Ellegren 2008).

For Z chromosomes the evolutionary rate is also likely to be influenced by a male-bias in mutation rate. This phenomenon is likely to be caused by the higher number of meiotic cell divisions in the male germline and is present in several taxa (Li et al. 2002; Makova & Li 2002) including birds (Axelsson et al. 2004; Ellegren & Fridolfsson 1997). Z chromosomes are expected to evolve fast if there is a male mutation bias since they are present twice as often in males as in females (Fitzpatrick & Hall 2004).

In birds, a fast Z effect has been documented in comparisons of 28 Z-linked introns in the chicken and the turkey (Axelsson et al. 2004) and in comparisons of 172 Z-linked genes between the chicken and the zebra finch (Mank *et al.* 2007).

A low degree of evolution in highly expressed genes

Gene expression level is an important determinant of gene sequence and protein evolution. Studies have shown decreasing levels of protein evolution and non-synonymous substitutions with increasing gene expression in several taxa (Drummond et al. 2005; Duret & Mouchiroud 2000; Pal et al. 2001; Subramanian & Kumar 2004). So far, no straight forward explanation for this pattern has been identified but highly expressed genes are less dispensable (Gu *et al.* 2003) and are more likely to be found in protein-protein interactions (Bloom & Adami 2003). Moreover, the level of gene expression is positively correlated with the level of codon bias (Akashi 1994). If particular codon triplets are more efficiently translated and thereby favored in highly expressed genes then selection against synonymous substitutions might be expected to be higher in highly expressed genes.

Evolutionary rate is linked to chromosome size

Chromosome size is highly variable in the avian genome and spans almost two orders of magnitude (ICGSC 2004). Recombination rate, GC content, gene density and substitution rate are negatively correlated with size of avian chromosomes so that microchromosomes have the highest rates of recombination and substitution (Axelsson et al. 2005; ICGSC 2004). However, even if avian microchromosomes have higher general substitution rates than macrochromosomes they have lower ratios of non-synonymous to synonymous substitutions (dN/dS) (Axelsson et al. 2005; ICGSC 2004), indicating either a higher degree of purifying selection on small chromosomes or less efficient selection on macrochromosomes.

We have investigated these patters in passerine birds by using a microarray for the zebra finch (*Taeniopygia guttata; Fringillidae*) in order to measure the amount of sequence evolution in coding regions of the passerine genome. We have assessed the degree of divergence in 12220 autosomal and 687 Z-linked ESTs by Comparative Genome Hybridization (CGH) analyses. Each of these ESTs is represented by 11 probes (25 bp long) on our microarray (Naurin *et al.* 2008) and we have therefore analyzed 141977 markers in the coding parts of the passerine genome. The ESTs have been ranked as having 0 to 11 probes hybridizing significantly when the DNA of another species was hybridized to the zebra finch array. The probes are expected to yield non-significant signals if there are more than 2 substitutions between the microarray and the DNA hybridized (Naurin et al. 2008). We have analyzed hybridizations of DNA from the willow warbler (*Phylloscopus trochilus; Sylviidae*) and a basal passerine, the crow (*Corvus corvus; Corvidae*). Our results show that: (i) ESTs on the passerine Z-chromosome have a much higher degree of sequence evolution than ESTs on the autosomes, (ii) coding DNA on the passerine microchromosomes evolve at a

slower pace than on macrochromosomes, in fact, chromosome size is highly positively correlated with the degree of general sequence divergence in passerine birds, and (iii) gene expression is a predictor of sequence divergence, however, unexpectedly ESTs of medium expression levels show less sequence evolution than highly expressed ESTs.

Methods

The Lund-zfa array

The Lund-zfa array is an Affymetrix custom array that consists of 22360 zebra finch ESTs, representing approximately 15800 genes (Naurin et al. 2008; Replogle et al. 2008). Each EST is represented by 11 probes (25 bp long) and the probes have been designed to have comparable GC content. Probes are expected to yield significant signals from hybridization with target cDNA (or target genomic DNA) if the match is perfect, includes one substitution or, less frequently, if the target DNA has two substitutions compared to the microarray probe (Naurin et al. 2008). Sequences with three or more substitutions are expected to be washed away during Affymetrix's rigorous washing procedures (Naurin et al. 2008). All ESTs on the array were BLASTed against the 3.2.4 build of the zebra finch genome (Naurin *et al.* 2009) and hits in that BLAST was considered significant if the E-value was ≤ 10^{-20} or lower.

Samples and hybridizations:

For descriptions of the methods for extraction of DNA, labeling of DNA and hybridization of DNA to the array, see Naurin et. al (2008). After hybridizing DNA to the array, a background cutoff was calculated as mean hybridization signal + two standard deviations in all empty features on the array (for details see Naurin et al. 2008). The EST is represented on the microarray by 11 probes each (25 bp long). All probes with signals ≤ the background cutoff were considered non-significant. Each EST was then ranked as having 0 to all of its 11 probes significantly hybridizing (i.e.; having a signal higher than the background cutoff) in the DNA sample hybridized to the microarray. ESTs were thereby ranked on a scale from 0 (very highly diverged) to 11 (very low divergence).

In this study, we used results from five hybridizations of willow warbler DNA and four hybridizations of crow DNA with the Lund-zfa array. Two of the willow warbler samples and two of the crow samples were pooled (willow warbler samples contained 10 individuals and crow samples 12 individuals). For the present study, a mean was taken over all hybridizations for a single species, that is, the analyzed data for the willow warbler is a mean of three single individual hybridizations and two pooled hybridizations (in total 22 individuals, all males); while the crow data consist of a mean over two individual hybridizations and two pooled hybridizations (in total 26 individuals, 16 females and 10 males). We have controlled that the presence of female DNA in the crow samples (females have only on Z chromosome) have no quantitative impact on our results by comparisons of single individual hybridizations of female and male DNA. We concluded that results regarding the degree of hybridization on probes from Z-chromosome are not affected by inclusion of the DNA samples from females and we therefore included results from all crow samples regardless of sex here (results from the comparisons of male and female DNA are not included).

Moreover, in order exclude probes with technical problems and probes with introns in their sequence, we have only analyzed ESTs that had all 11 probes significantly hybridizing when zebra finch DNA is hybridized on the array (for details regarding the zebra finch hybridization see Naurin et al. 2008). We also excluded Tgu-chromosome 1B, 16 and 25 from all downstream analyses due to the low number of ESTs representing these small chromosomes in our data set. After these exclusions, 12220 autosomal ESTs and 687 Z-linked ESTs remained and have been included in further analyzes.

Gene expression data

We have used gene expression data from hybridizations of samples of the telencephalon from 12 zebra finches to the Lund-zfa array, and all information regarding these hybridizations are available in a previous study (Naurin et al. 2009). A

mean signal over all 12 individuals (6 males and 6 females) has been used here and is labeled "expression level in the zebra finch" in our results. Hence, when the term gene expression level is used in this study it refers to gene expression in the brain of adult zebra finches.

Statistical analyses

One way ANOVAs were used to test if the number of significantly hybridizing probes was different between chromosomal categories and between ESTs with different gene expression levels. Pearson correlations were run between the number of significantly hybridizing probes and chromosome size, and between the hybridizations using willow warbler DNA and the hybridizations using crow DNA.

The results of how gene expression level relates to divergence were not quantitatively affected if all redundancies in our EST data set were removed (by excluding ESTs that were within 5000 bp of another EST).

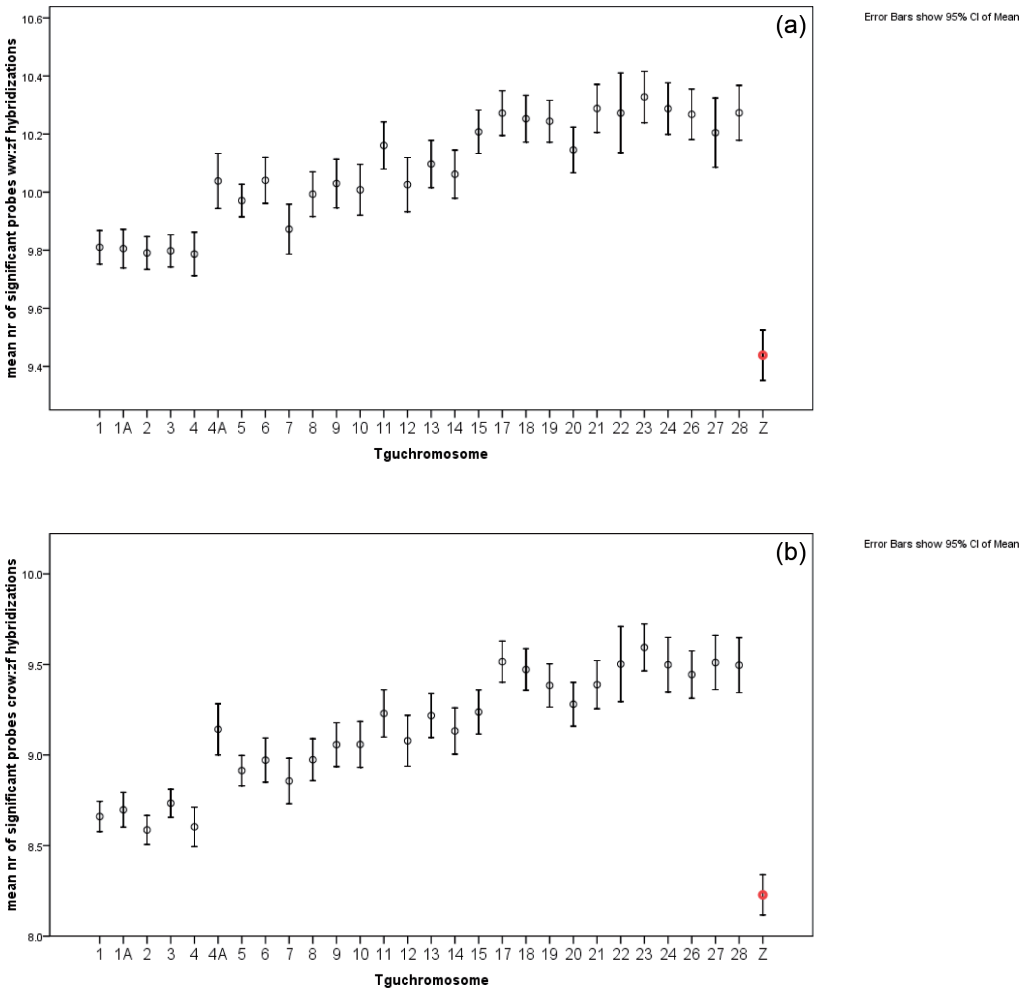

Figure 1. Mean number of significantly hybridizing probes in (a) the willow warbler : zebra finch hybridizations and (b) the crow : zebra finch hybridizations, for the different zebra finch chromosomes.

Results

Chromosomal location

There were significant differences between chromosomal categories in the number of significantly hybridizing probes in both the willow warbler to zebra finch hybridizations (ww:zf) and in the crow to zebra finch hybridizations (crow:zf; Figure 1a and 1b).

Z had a consistently lower number of significantly hybridizing probes (ww:zf: 9.44 ± 0.044; crow:zf: 8.23 ± 0.057) than autosomes (ww:zf: 10.0 ± 0.008, $F_{1,12904} = 260.5$, $p < 0.0001$; crow:zf: 9.00 ± 0.012, $F_{1,12904} = 230.2$, $p < 0.0001$; Figure 1 and 2).

Moreover, the macrochromosomes (Tgu 1, 1A, 2, 3, and 4, crow:zf: 8.66 ± 0.020; ww:zf: 9.80 ± 0.014) had a significantly lower number of hybridizing probes than the other autosomes (crow:zf: 9.10 ± 0.014, $F_{1,12032} = 499.2$, $p < 0.0001$; ww:zf: 10.1 ± 0.009, $F_{1,12032} = 372.8$, $p < 0.0001$; Figure 1a and b)

Ww:zf hybridizations and crow:zf hybridizations were highly correlated (between chromosomes: $r_{31} = 0.960$, $p < 0.0001$; between all ESTs: $r_{12906} = 0.812$, $p < 0.0001$; Figure 2).

The number of hybridizing probes was negatively correlated with chromosome size (ww:zf: $r_{31} = -0.755$, $p < 0.0001$; crow:zf: $r_{31} = -0.710$, $p < 0.0001$) with macrochromosomes having consistently lower numbers of hybridizing probes than microchromosomes (Figure 3a and b).

Gene expression levels

Gene expression was not significantly correlated with chromosome size (Figure 4; results not given). When the number of significantly hybridizing probes (in willow warbler and crow) is plotted against the gene expression level in the zebra finch it is obvious that it is not a simple positive relationship (Figure 5). Instead, the number of significant probes seems to be highest for ESTs with intermediate expression levels. In order to test if ESTs with high and low expression have a higher mean divergence than genes with medium expression levels we divided the dataset by expression level into 4 equally sized groups (Figure 6). For the ww:zf hybridizations, the group of ESTs with the highest expression level had a significantly lower number of hybridizing probes than the second lowest category on the autosomes (highest group: 9.44 ± 0.012; 2nd lowest group: 9.68 ± 0.018, $F_{1,9221} = 85.5$ $p < 0.0001$) and for the Z chromosome the same trend was clear even if the p-value fell just short of being significant (highest group: 8.89 ± 0.09; 2nd lowest group: 9.13 ± 0.09, $F_{1,549} = 3.7$ $p = 0.054$; Figure 6a). For the crow:zf hybridizations the group of highest expressed ESTs

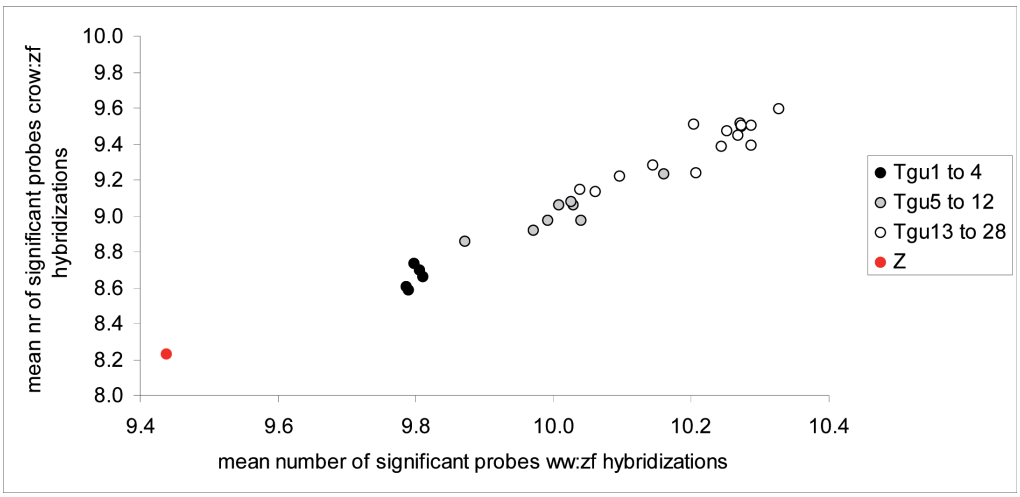

Figure 2. Correlation plot of willow warbler : zebra finch and crow : zebra finch hybridizations.

has a significantly lower number of hybridizing probes than the group with second lowest expression, and this was significant for both autosomal ESTs (highest group: 8.25 ± 0.024; 2nd lowest group: 8.71 ± 0.023, $F_{1,9221} = 176$, $p < 0.0001$) and Z-linked ESTs (highest group: 7.51 ± 0.11; 2nd lowest group: 7.90 ± 0.10, $F_{1,549} = 6.4$, $p = 0.012$; Figure 6b). Significance of comparisons between the other expression groups are indicated in figure 6 (details not given).

Discussion

This study explores data about the level of divergence in 12907 ESTs spread across the passerine genome. The very short probes (25 bp long) on the Lund-zfa array are sensitive to substitutions in the target sequences, making it possible to distinguish target DNA with zero to two substitutions from target DNA with three or more substitutions. The divergence level has thereby been esti-

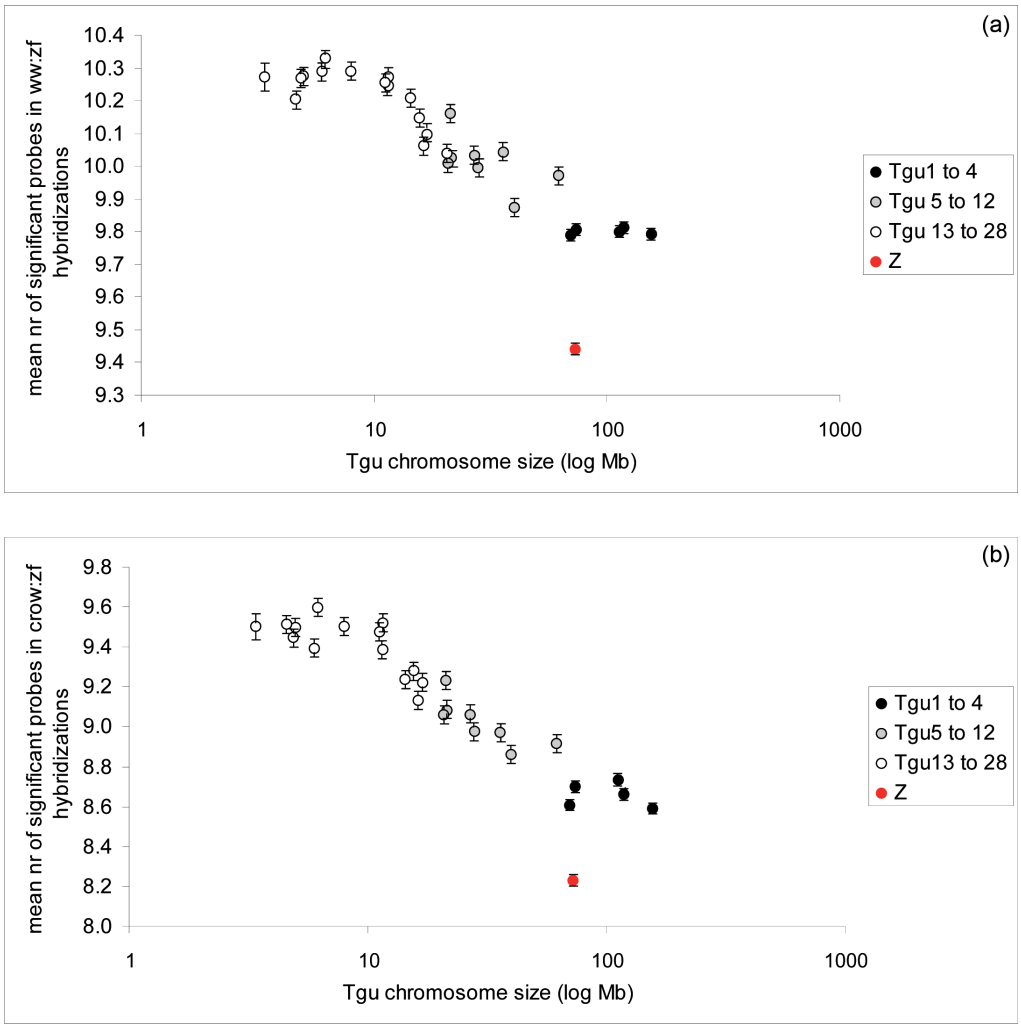

Figure 3. Mean number (+SE) of significantly hybridizing probes and how it relates to chromosome size in (a) the willow warbler : zebra finch hybridizations and (b) the crow : zebra finch hybridizations.

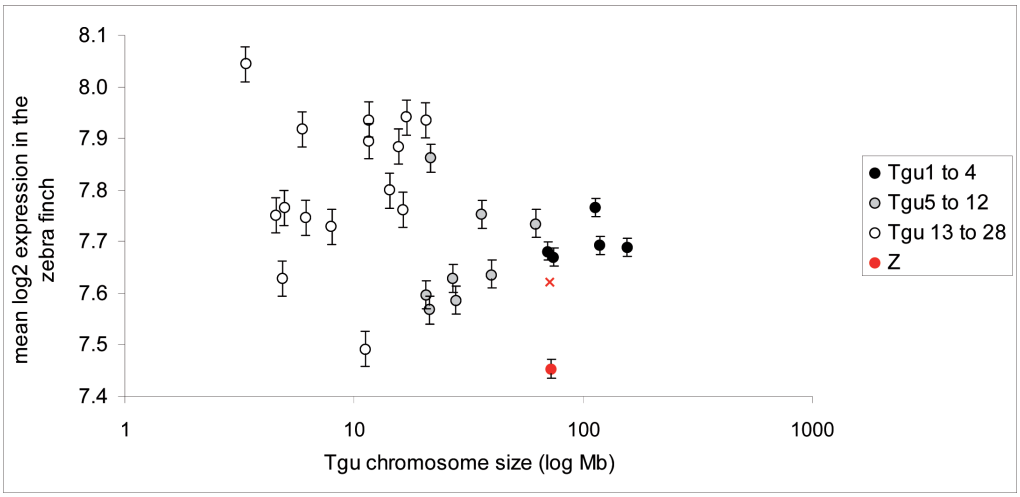

Figure 4. Gene expression as a mean (+SE) over 6 male and 6 female zebra finches and how it relates to chromosome size for the ESTs analysed in the present study. The red cross indicates the level of Z-linked gene expression as mean expression only for the 6 males.

mated in 11 regions of each EST included in this study. Our method does not directly quantify the number of substitutions but gives a relative measure of divergence, enabling us to rank ESTs from slowly to fast evolving. Access to information for such a high number of coding sequences gives us unique resolution when studying the evolution of avian chromosomes. Our results demonstrate the following patterns of gene divergence in the passerine genome:

A fast Z effect

It is clear from our results that the Z chromosome experiences the highest substitution rates in the passerine genome, leading to a lower number of functional probes compared to the autosomes (Figure 1 and 2). Hence, the fast-Z effect is thereby a major factor in birds (see also previous results in Axelsson et al. 2004; Mank et al. 2007). The pattern of fast Z-evolution is likely to be directly linked to recent results supporting that Z has played a large and substantial role in avian speciation (the so called large Z effect; for theoretical predictions see Coyne 1985; Dobzhansky 1974; Presgraves 2008; Templeton 1977). Z-linked genes are highly overrepresented amongst genes with adaptive evolution (Ellegren 2008), Z-chromosomes are likely to have played a larger role than X-chromosomes in sexual isolation (Qvarnstrom & Bailey 2009) and genes controlling male-specific plumage traits, species recognition and hybrid fitness have been found to be Z-linked in flycathcers (Saether *et al.* 2007).

A clear effect of chromosome size

Our results show a clear positive correlation between chromosome size and divergence rate (Figure 3). Previous results have shown that microchromosomes have high rates of recombination and substitution (Axelsson et al. 2005; ICGSC 2004). However, the International Chicken Genome Sequencing Consortium (ICGSC 2004) showed in comparisons between the chicken genome and mammalian genes that even if the substitution rate is higher on microchromosomes, the ratio of non-synonymous to synonymous substitutions (dN/dS) is actually lower than on macrochromosomes. Moreover, Axelsson et al. (Axelsson et al. 2005) compared sequences from the chicken and the turkey and showed that even if dS was higher in both introns and coding regions on microchromosomes than macrochromosomes, the dN/dS ratio was lower.

The differences in dN/dS are likely to be associated with the differences in recombination rates on avian chromosomes. The efficiency of selection covaries with recombination (Hill & Robertson 1996; Nordborg et al. 1996) and, hence, it is possible that deleterious mutations on the highly recombining microchromosomes are more easily purged (ICGSC 2004). Moreover, background selection and selective sweeps are more common in regions of lower recombination (Ellegren 2007). These processes lead to an increase in the number of fixed weakly deleterious alleles at loci that are linked to selected alleles (Charlesworth et al. 2003; Smith & Haigh 1974) and thereby to a potentially higher dN on the avian macrochromosomes which have a lower recombination rate.

It is clear from our results, which measure both synonymous and non-synonomous substitutions, that if dS is higher in coding regions on passerine microchromosomes than on macrochromosomes, then this effect is weak compared to the effects caused by purifying selection on microchromo-

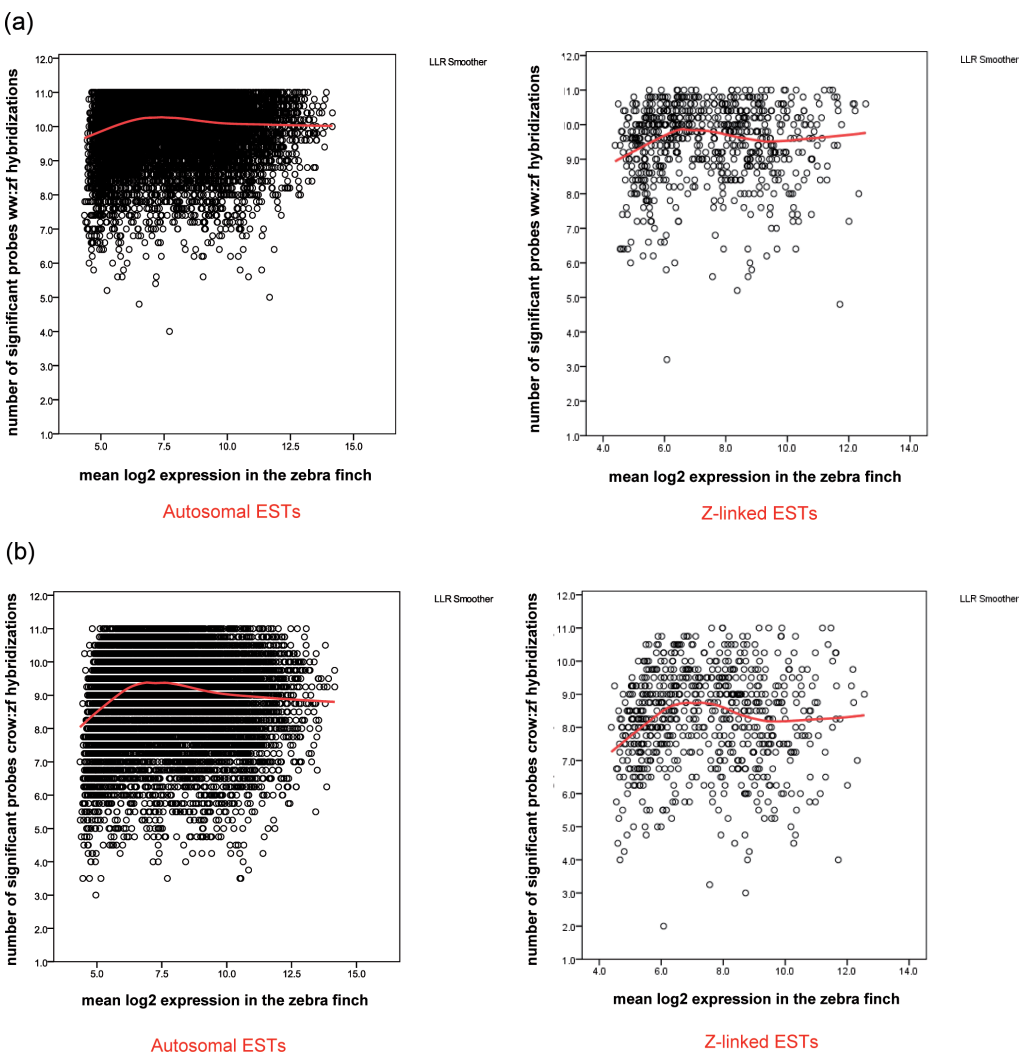

Figure 5. Mean number of significantly hybridizing probes and how it relates to gene expression in the zebra finch for (a) the willow warbler : zebra finch hybridizations and (b) the crow : zebra finch hybridizations.

somes and/or fixation of deleterious alleles on macrochromosomes.

Higher recombination is correlated with high GC content (ICGSC 2004) and comparisons between mouse and rat have shown that dN/dS is negatively correlated with GC content in mammals (Williams & Hurst 2000). Hence, the processes on the highly recombining GC-rich avian microchromosomes, which leads to a low dN/dS, are likely to be present in both birds and mammals.

Lastly, it has been hypothesized that coding sequences on microchromosomes might evolve slower due to the presence of more essential genes (Axelsson et al. 2005; Ellegren 2007). However, since chromosome size is directly linked to the level of divergence in our results this explanation

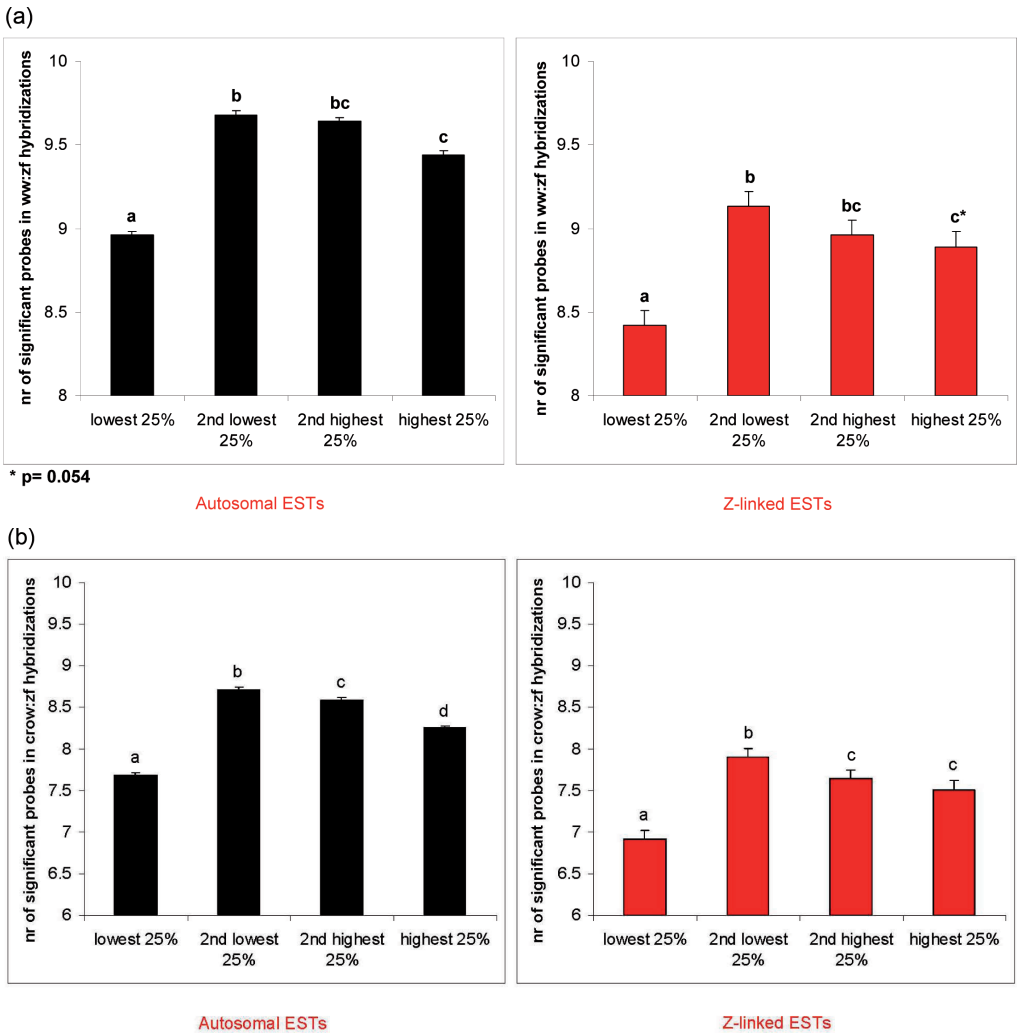

Figure 6. Mean number (+SE) of significantly hybridizing probes divided into quartiles by gene expression level for (a) the willow warbler : zebra finch hybridizations and (b) the crow : zebra finch hybridizations. P values from one way ANOVAs between the 2nd lowest and the highest group are included in results. The lowest group has a significantly lower number of hybridizing probes in all comparisons (all $p < 0.0001$), other p-values as indicated in figure, data not included here.

would require not only a higher number of essential genes on the smallest chromosomes but a general accumulation of essential genes with smaller chromosome size, a process which seems relatively unlikely.

An unexpected effect of gene expression level

We found that the degree of conservation is highest for ESTs with medium expression levels on both the Z-chromosome and the autosomes (Figure 5 and 6). This is somewhat different from previous results that have shown that sequence and protein evolution decrease with increasing gene expression (Duret & Mouchiroud 2000; Pal et al. 2001; Subramanian & Kumar 2004) . There are at least three different hypotheses which could explain this discrepancy:

1. We have studied divergence in 12220 autosomal and 687 Z-linked ESTs. This is more than double the sample size of any previous study and so it is therefore possible that these have not had enough statistical power to detect this pattern.
2. There might be a high degree of selection on highly expressed genes in birds and the pattern we have found could at least to some extent be bird-specific.
3. Our method rates ESTs from high to low levels of divergence, without any information regarding whether substitutions are synonymous or non-synonymous. We can therefore not distinguish between neutral changes or calculate dN/dS ratios. Most previous studies have focused on measures of dN/dS or protein evolution. It is possible that our simultaneous measurement of dN and dS causes the discrepancies to previous studies and that we would not have detected a higher degree of molecular evolution in highly expressed genes than previous studies had we been able to examine dN/dS for all our ESTs.

There are two reasons why we believe that the third hypothesis is unlikely.

First, a study in mammals has measured the rate of synonymous substitutions (dS) separately and found no significant correlation with gene expression even though dN/dS decreased with increasing gene expression (Duret & Mouchiroud 2000). Secondly, if dS is affected by the level of gene expression the expected pattern would be a lower divergence level of highly expressed genes and not to higher divergence as we observed. This is due to a process known as codon bias, where some codon triplets corresponding to a certain amino acid are more efficiently translated than others, leading to potential selection against certain synonymous substitutions as gene expression increases (Akashi 1994; Sharp et al. 1995).

Hence, if these patterns hold true for birds, i.e., if dS is either unaffected or lowered by increased gene expression, then our results have to be interpreted as a higher degree of directional selection at highly expressed genes or a higher degree of stabilizing selection at genes with medium expression levels.

Concluding remarks

It is becoming increasingly clear that the avian Z chromosome is a special case in terms of chromosomal evolution, experiencing very high substitution rates and strong selection (Axelsson et al. 2004; Ellegren 2008; Mank et al. 2007; Qvarnstrom & Bailey 2009 and present results). Future studies will elucidate to what extent this substantial fast Z effect (and large Z effect) is caused by 'neutral processes' like a male mutation bias and low population size and to what extent it is influenced by hemizygous exposure of recessive alleles and by a high degree of sexual selection in males. Furthermore, it is now clear that the chromosomal location of avian genes strongly influences their evolutionary rate in passerines as well as in the chicken. Coding regions on microchromosomes have lower dN/dS in comparisons between mammals and the chicken (ICGSC 2004), lower dN/dS in comparisons between the chicken and the turkey (Axelsson et al. 2005) and lower divergence rates than macrochromosomes in this study. It seems highly likely that this is to a large extent is caused by the high recombination on microchromosomes. It will be interesting to evaluate if the pattern of a high degree of conservation in genes with medium expression levels that we observed in passerines will be found also in other taxa as the number of large-scale studies increase. Future studies will also shed light on whether this pattern is caused by selection

on highly expressed genes or conservation of genes with medium expression levels.

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VI

Divergence of individual genes on the avian Z chromosome is determined by sex-bias and gene expression level

Sara Naurin, Martin Stervander, Bengt Hansson, Staffan Bensch and Dennis Hasselquist

Divergence of individual genes on the avian Z chromosome is determined by sex-bias and gene expression level

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Male-bias in gene expression is extensive on the avian Z chromosome and is most pronounced for genes with high expression levels. The high proportion of male-biased genes on the avian Z chromosome has been interpreted as inefficient dosage compensation, where females (being hemizygous; ZW) do not effectively compensate for the missing Z chromosome. If the extensive male-bias is due to inefficient compensation in females – rather than being shaped by particular properties of male-biased genes like a high degree of sexual selection or less evolutionary constraint – we would expect that male-biased and unbiased Z-linked genes have similar divergence levels. We investigated 12220 and 648 Z-linked ESTs in passerine birds and of 37 Z-linked orthologues between the zebra finch and the chicken, and found that (i) Z-linked ESTs diverge faster than autosomal ESTs regardless of bias, (ii) male-biased Z-linked ESTs evolved faster than unbiased Z-linked ESTs, (iii) unbiased ESTs were most conserved at intermediate expression levels, and (iv) male-biased ESTs diverged at the same rate regardless of gene expression level. These data imply that there are important differences between the evolutionary processes that have shaped male-biased and unbiased genes on the avian Z. Previous studies have shown that the Z chromosome in birds evolves rapidly (a 'fast Z' effect) and is highly implicated in avian adaptive evolution and speciation (a 'large Z' effect). Hence, the high degree of male-biased gene expression on the avian Z chromosome (comprising about one third of Z) is likely to have influeneced the rate of evolution of this chromosome, reinforcing the fast Z and the large Z effects.

Introduction

Genes on X chromosomes (in species with male heterogamety) and Z chromosomes (in species with female heterogamety) are expected to evolve faster than autosomal genes; a process known as the fast X or fast Z effect (Presgraves 2008; Vicoso & Charlesworth 2006). This is due to hemizygous exposure of recessive alleles in the heterogametic sex (XY males and ZW females) (Charlesworth et al. 1987) and the low population size of sex chromosomes (Charlesworth et al. 1987; Ellegren 2008). These particular X/Z chromosome characteristics will lead to a higher fixation rate of new mutations on these chromosomes compared to autosomes. Moreover, Z chromosomes

are present twice as often in males (ZZ) than in females (ZW). This is likely to increase the divergence rate of Z-linked genes due to a male-bias in mutation rate which is present in several taxa (Li et al. 2002; Makova & Li 2002) including birds (Axelsson et al. 2004; Ellegren & Fridolfsson 1997).

Fast Z evolution has been documented in birds by comparisons of 28 Z-linked introns in the chicken and the turkey (Axelsson et al. 2004), comparisons of 172 Z-linked genes between the chicken and the zebra finch (Mank et al. 2007b), and in studies of the divergence level between passerine bird species for 687 Z-linked EST sequences (Naurin et al. 2009c). This rapid evolution of the avian Z chromosome is likely to be linked to recent results demonstrating that the Z chromosome has had a substantial influence on avian adaptive evolution and speciation (a large Z effect see; Ellegren 2008; Qvarnstrom & Bailey 2009; Saether et al. 2007).

Recently it has been shown that Z chromosomes are not only evolving rapidly but also harbor many genes with a significant male-bias in gene expression. In all species studied so far (three birds and one insect species; Ellegren et al. 2007; Itoh et al. 2007; Melamed & Arnold 2007; Naurin et al. 2009b; Zha et al. 2009) the degree of male-biased gene expression is so extensive that it has been described as a general lack of dosage compensation (a process which occurs on X chromosomes resulting in equal gene expression in XY males and XX females; Charlesworth 1996; Mank 2009). In previous studies, we found that 39.5% (437 ESTs) of all Z-linked ESTs represented on a microarray for the zebra finch (*Taeniopygia guttata*) had significant male-bias in gene expression (Naurin et al. 2009a; Naurin et al. 2009b). Moreover, we showed that the male-bias increased with increasing gene expression level and that unbiased genes had lower general expression levels than male-biased genes on the avian Z chromosome (Naurin et al. 2009b).

Genes with sex-biased gene expression evolve faster than unbiased genes, with high rates of substitution and high divergence between species (Cutter & Ward 2005; Ellegren & Parsch 2007; Good & Nachman 2005; Khaitovich et al. 2005; Mank et al. 2007a; Reinke et al. 2004; Richards et al. 2005; Schultz et al. 2003; Torgerson et al. 2002; Zhang et al. 2004). Two hypotheses have been proposed to explain this:

a. A high degree of selection on sex-biased genes

Sex-biased gene expression is tightly linked to reproduction (Dauwalder et al. 2002; Drapeau et al. 2003; Kadener et al. 2006) and therefore subject to strong selection (Good & Nachman 2005; Jagadeeshan & Singh 2005; Khaitovich et al. 2005; Proschel et al. 2006; Swanson & Vacquier 2002; Torgerson et al. 2002) and several studies have supported a role for positive selection in the rapid evolution on male-biased genes (Nielsen et al. 2005; Proschel et al. 2006; Sawyer et al. 2007; Zhang & Parsch 2005).

b. Sex-biased genes might be more dispensable

Sex-biased genes have high expression variance and high divergence rates, characteristics typical of more dispensable genes (Ellegren & Parsch 2007; Mank & Ellegren 2009) and it is possible, therefore, that sex-biased genes are on average more dispensable (Mank & Ellegren 2009). If this is true sex-biased genes will face less effective purifying selection, leading to an increase in substitution rates.

Regardless of whether selection or relaxed constraint are most implicated in the rapid evolution of sex-biased genes, the finding that male-biased gene expression is associated with high expression levels (Naurin et al. 2009b) is unexpected. This is due to two separate aspects which should distinguish highly expressed genes from sex-biased genes:

First, it is well known that gene expression is an important determinant of sequence and protein evolution, but with a lower degree of sequence and protein divergence in genes with high expression levels than in genes with low expression levels (Drummond et al. 2005; Duret & Mouchiroud 2000; Pal et al. 2001; Subramanian & Kumar 2004). This might be due to highly expressed genes being less dispensable (Gu et al. 2003) and more likely to be found in protein-protein interactions (Bloom & Adami 2003).

Second, male-biased genes are known to have less codon bias than unbiased genes (Hambuch

& Parsch 2005; Whittle et al. 2007). Codon-bias occurs when particular codon triplets are favored and it leads to selection against synonymous substitutions (Akashi 1994). Hence, if codon bias is lower in male-biased genes than the number of fixed synonymous substitutions will be higher. However, the level of gene expression is positively correlated with the level of codon bias, most likely due to the fact that some codons are more efficiently translated than others (Akashi 1994). This will lead to more selection against synonymous substitutions in highly expressed genes (Akashi 1994).

Hence, it might have been expected that male-bias on the avian Z chromosome should be underrepresented among genes with high expression levels rather than the opposite (Naurin et al. 2009b).

Based on these findings there are three factors that might shape the evolution of male-biased genes on the avian Z chromosome:

1. Sex biased genes are known to evolve rapidly with high substitution rates and high divergence between species (Cutter & Ward 2005; Ellegren & Parsch 2007; Good & Nachman 2005; Khaitovich et al. 2005; Mank et al. 2007a; Reinke et al. 2004; Richards et al. 2005; Schultz et al. 2003; Torgerson et al. 2002; Zhang et al. 2004). This process should lead to higher divergence levels in male-biased than unbiased genes.
2. Highly expressed genes evolve slowly, a fact which might slow down the divergence of male-biased genes relative to the more low expressed unbiased genes.
3. It has been hypothesized that much of the male-bias on the avian Z chromosome could be due to incomplete dosage compensation in ZW-females (Ellegren et al. 2007; Itoh et al. 2007). If inefficient dosage compensation, rather than specific function in males, is the cause of the male-bias in gene expression then male-biased and unbiased Z-linked genes should have similar divergence rates.

To elucidate how the pattern of gene divergence on the avian Z chromosome is affected by the degree of male-bias and the level of gene expression, we studied divergence between different passerine bird species in 648 Z-linked ESTs (378 unbiased and 270 male-biased) and 12220 autosomal ESTs by Comparative Genome Hybridization (CGH) analyses. Each of these ESTs are represented by 11 probes (25 bp long) on our microarray (Naurin *et al.* 2008) and we have therefore analyzed 141548 markers in the coding parts of the passerine genome. The ESTs have been ranked as having 0 to 11 probes hybridizing significantly when the DNA of another species was hybridized to the zebra finch array. The probes are expected to yield non-significant signals if there are more than 2 substitutions between the microarray and the hybridized DNA (Naurin et al. 2008). We have analyzed hybridizations of DNA from the willow warbler (*Phylloscopus trochilus; Sylviidae*) and a basal passerine, the crow (*Corvus corvus; Corvidae*). We further studied sequence divergence in 37 Z-linked orthologues between the zebra finch (*Taeniopygia guttata*) and the chicken (*Gallus gallus*). We found a significant fast Z effect: Z-linked ESTs diverged faster than autosomal ESTs regardless of bias. Moreover, on the Z chromosome there was a higher degree of divergence in male-biased than unbiased ESTs and male-biased ESTs and orthologues evolve at the same rate regardless of their gene expression level, a fact that was not true for unbiased ESTs and othologues.

These findings suggest that the high degree of male-biased gene expression on the avian Z chromosome is likely to have influenced the rate of evolution on this chromosome, reinforcing the fast Z effect.

Methods

The Lund-zfa array

The Lund-zfa array is an Affymetrix custom array that consists of 22360 zebra finch ESTs, representing approximately 15800 genes (Naurin et al. 2008; Replogle et al. 2008). Each EST is represented by 11 probes (25 bp long) and the probes have been designed to have comparable GC content. Probes are expected to yield significant signals from hybridization with target cDNA (or target genomic DNA) if the match is perfect, if it includes one substitution or, less frequently, if

the target DNA has two substitutions compared to the microarray probe (Naurin et al. 2008). Sequences with three or more substitutions are expected to be washed away during Affymetrix's washing procedures (Naurin et al. 2008). All ESTs on the array have been BLASTed against the 3.2.4 build of the zebra finch genome (Naurin et al. 2009a) and hits in that BLAST was considered significant if the E-value was $\leq 10^{-20}$.

Samples and hybridizations

For descriptions of methods for extraction of DNA, labeling of DNA and hybridization of DNA to the array see Naurin *et. al.* (2008). After hybridizing DNA to the array, a background cutoff was calculated as mean hybridization signal plus two standard deviations in all empty features on the array (for details see; Naurin et al. 2008). All probes with signals ≤ the background cutoff were considered non-significant. Each EST was then ranked as having 0 to all of its 11 probes significantly hybridizing (i. e.; having a signal higher than the background cutoff) in the DNA sample hybridized to the microarray. ESTs were thereby ranked on a scale from 0 (very highly diverged) to 11 (very low divergence).

In this study, we used results from five hybridizations of willow warbler genomic DNA (containing DNA from 22 individuals) and four hybridizations of crow genomic DNA (containing DNA from 26 individuals) with the Lund-zfa array (for detailed descriptions of the samples see; Naurin et al. 2009c). Moreover, in order to exclude probes with technical problems and probes with introns in their sequence, we have only analyzed ESTs that had all 11 probes significantly hybridizing when zebra finch DNA was hybridized on the array (for details regarding the zebra finch hybridization see; Naurin et al. 2008). After these exclusions 12220 autosomal and 648 Z-linked ESTs (378 unbiased and 270 male-biased) remained and was included in further analyses.

Gene expression data

We have used gene expression data, and data on significant male-bias in gene expression, from hybridizations of samples of the telencephalon from 12 zebra finches to the Lund-zfa array, and all information regarding these hybridizations are available in a previous study (Naurin et al. 2009a). A mean signal over all 12 individuals (6 males and 6 females) has been used here and is labeled "expression level in the zebra finch" in our results. Hence, when the term gene expression level is used in this study it refers to the general level of gene expression in the brain of adult zebra finches.

Orthologues between the zebra finch and the chicken

37 othologous Z-linked genes between the zebra finch and the chicken have been used in this study. They were identified using gene expression data from Naurin et al. (2009a). ESTs with e-values $\leq e^{-20}$ when BLASTed against the zebra finch genome and with hits that were at least 300 bp long were chosen from this data set. Unbiased and male-biased ESTs were selected based on expression level (high expression or low expression) and BLASTed again against the 1.1 build of the zebra finch genome (www.ncbi.nlm.nih.gov/genome/guide/finch) and also against the chicken genome build 2.1 (ww.ncbi.nlm.nih.gov/genome/guide/chicken). If the best hits aligned within expressed regions of annotated othologous zebra finch and chicken genes the cds of the othologous genes were aligned using Geneious Pro 4.6 (Drummond et al. 2009) translation align. In case of multiple transcripts the most similar ones were chosen. Othologues were defined by Ensemble or, if no othologues were listed, when the translated zebra finch cds sequence rendered a BLASTX hit in the chicken genome with e-value $\leq e^{-10}$.

The number of synonymous (dS) and non-synonymous (dN) substitutions between the zebra finch and the chicken sequences were calculated in MEGA 4 (Tamura et al. 2007), followed by the calculation of dN/dS ratios.

Statistical analyses

Data was imported into SPSS Statistics, 17.0. One way ANOVAs were used to test if: (i) the number of significantly hybridizing probes differed between unbiased male-biased and autosomal ESTs, (ii) if the number of significantly hy-

bridizing probes was different between categories of ESTs with different expression levels, and (iii) if the number of substitutions differed between different categories of zebra finch: chicken orthologues. General Linear Models (GLM) were used to determine if the divergence level in unbiased and male-biased ESTs differed in relation to general gene expression level. Pearson's correlations were run to test if male-biased and unbiased Z-linked ESTs correlated with gene expression. When conducting post hoc tests between gene expression level groups after running ANOVAs, we Bonferroni-corrected p-values for multiple testing.

Results

Z-linked ESTs diverged faster than autosomal ESTs independent on if they were sex-biased or not. In crow to zebra finch hybridizations (crow:zf) the number of significantly hybridizing probes was lower for both unbiased and male-biased Z-linked ESTs (male-biased ESTs 8.00 ± 0.09; unbiased ESTs 8.36 ± 0.08) than for autosomal ESTs (8.99 ± 0.01; male-biased vs autosomal ESTs $F_{1,12490} = 149.8$, $p < 0.0001$; unbiased vs autosomal ESTs $F_{1,12598} = 94.5$, $p < 0.0001$). The same was true for willow warbler to zebra finch hybridizations (ww:zf) where the number of hybridizing probes in both unbiased and male-biased Z-linked ESTs (male-biased ESTs 9.34± 0.07; unbiased ESTs 9.48 ± 0.06) was significantly lower than the number in autosomal ESTs (9.99 ± 0.008; male-biased vs autosomal ESTs $F_{1,12490} = 142.4$, $p < 0.0001$; unbiased vs autosomal ESTs $F_{1,12598} = 129$, $p < 0.0001$).

Male-biased ESTs had a lower mean number of significantly hybridizing probes than unbiased ESTs in the crow:zf hybridizations (male-biased 8.00 ± 0.09; unbiased 8.36 ± 0.08; $F_{1,646} = 9.0$, $p = 0.003$; Figure 1). In the ww:zf hybridizations there was a non-significant trend in the same direction (male-biased: 9.34 ± 0.07; unbiased: 9.48 ± 0.06; $F_{1,646} = 2.46$, $p = 0.120$; Figure 1).

To study the divergence levels of Z-linked genes with comparable expression, we grouped the unbiased ESTs by expression level into four categories with 79 to 81 unbiased ESTs in each (from category 1= low expression to category 4 = high expression). Only category 3 and 4 of the unbiased ESTs overlapped with expression lev-

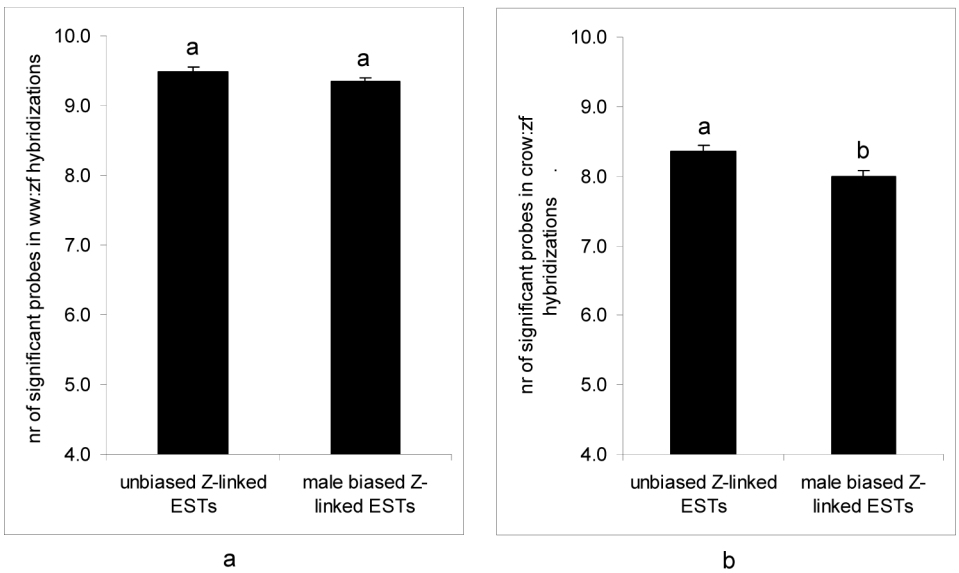

Figure 1. Mean number (+SE) of significantly hybridizing probes in unbiased and male-biased Z-linked ESTs for (a) willow warbler to zebra finch hybridizations (ww:zf) and (b) crow to zebra finch hybridizations (crow:zf).

els of male-biased genes so that sample size was high enough for comparisons (only 5 male-biased ESTs had expression so low that they belong to category 1 and only 27 fell into category 2). In category 3 (log 2 expression 6.0 to 7.79, with 76 male-biased ESTs and 80 unbiased ESTs), male-biased ESTs had a significantly lower number of hybridizing probes for ww:zf hybridizations (9.49 ± 0.123 vs 9.87 ± 0.104, $F_{1,154} = 5.76$, $p = 0.008$; Figure 2a) and the same was true for crow:zf hybridizations (8.81 ± 0.170 vs 8.96 ± 0.149, $F_{1, 154} = 11.96$, $p = 0.001$; Figure 2b). In category 4 (log 2 expression 7.80 to 12.55, with 165 male-biased and 81 unbiased ESTs), male-biased ESTs had a significantly lower number of hybridizing probes than unbiased ESTs in the crow:zf hybridizations (8.06 ± 0.117 vs 8.54 ± 0.183, $F_{1, 244} = 5.166$, $p = 0.029$; Figure 2b). However, in category 4 there was no significant difference between male-biased and unbiased ESTs in the ww:zf hybridizations (9.36 ± 0.091 vs 9.59 ± 0.330, $F_{1, 244} = 2.020$, $p = 0.156$; Figure 2a).

We tested if the divergence of unbiased and male-biased ESTs was related to the level of gene expression (Figure 3). In GLMs with the number of significant probes as dependent variable the following independent variables were added: mean level of gene expression (both as the original value and as the quadratic term), type (unbiased or male-biased) and the interactions between type and the two expression terms. In these GLMs, both interaction terms between gene expression type were significant (ww:zf: $p < 0.02$, Table 1; crow:zf: $p < 0.01$, Table 2). Unbiased ESTs were most conserved at intermediate expression levels. In contrast, the number of significant probes in male-biased ESTs did not vary with expression level (Figure 3). This was further confirmed using Pearson's correlations that failed to detect any significant correlation between the number of significant probes and gene expression in the male-biased ESTs (crow:zf $r_{1,273} = 0.048$, $p = 0.428$; ww:zf $r_{1,273} = 0.031$, $p = 0.611$). For the unbiased ESTs Pearson's correlations between the number of significant probes and gene expression revealed a significantly positive correlation (crow:zf $r_{1,378} = 0.137$, $p = 0.009$; ww:zf $r_{1,378} = 0.183$, $p < 0.0001$). However, the correlation between the number of significantly hybridizing probes in unbiased ESTs and gene expression cannot be explained simply by a linear relationship, because also the quadratic term was significant with the lowest divergence rate at intermediate gene expression levels (Figure 3; Table 3).

To further illustrate the difference between male-biased and unbiased ESTs in relation to gene expression level, we grouped the unbiased

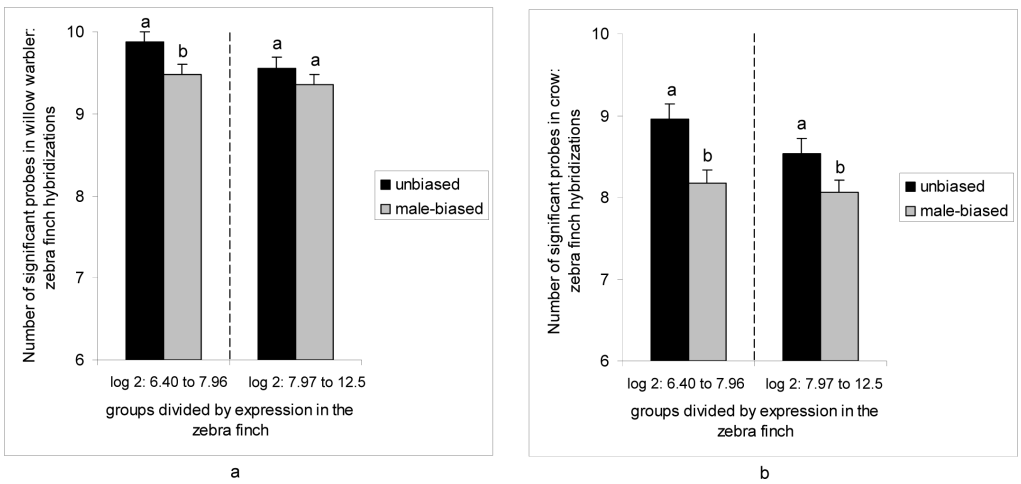

Figure 2. Male biased and unbiased ESTs grouped into categories of gene expression levels for (a) willow warbler to zebra finch hybridizations and (b) crow to zebra finch hybridizations. Log 2: 6.40 to 7.96 equals category 3 and log 2:7.97 to 12.5 equals category 4 as described in results. Columns represent mean number of significant probes (+SE)

and male-biased genes by gene expression into four gene expression categories containing similar number of ESTs (Figure 4). Male-biased ESTs did not differ significantly in the number of hybridizing probes between any of these categories, neither for the ww:zf hybridizations nor for the

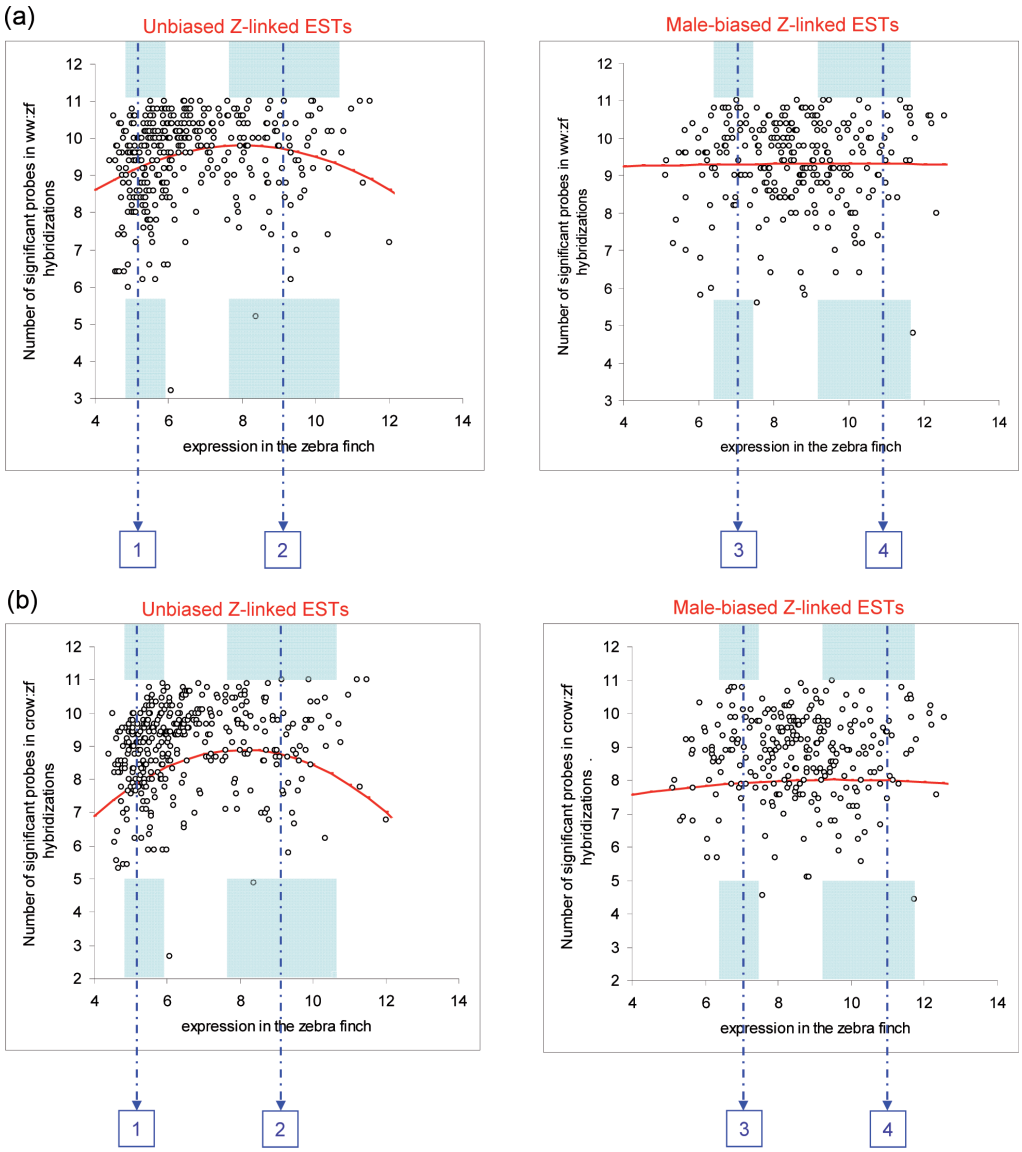

Figure 3. Association between number of significantly hybridizing probes and gene expression in the zebra finch for (a) willow warbler to zebra finch hybridizations and (b) crow to zebra finch hybridizations. Curves represent the association between number of significantly hybridizing probes and gene expression as given by the parameter estimates of the GLMs in Table 1 and 2. Arrows denote mean expression of the orthologues between the zebra finch and chicken used to calculate dN/dS: 1 = low expressed unbiased genes, 2 = high expressed unbiased genes, 3 = low expressed male-biased genes, 4 = high expressed male-biased genes. Gene expression of all orthologues fall within the shaded areas around the arrows.

Table 1. Output from a General Linear Model with number of significant probes in willow warbler : zebra finch hybridizations as dependent variable and with the following added as independent variables: mean leval of gene expression (both as the original value and as the quadratic term), type (unbiased or male-biased) and the interaction terms between type and the two expression terms. Sample sizes are 378 unbiased ESTs and 270 male-biased ESTs.

Tests of Between-Subjects Effects

Dependent Variable:number of significant probes in willow warbler : zebra finch hybridizations

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	29.354[a]	5	5.871	4.439	.001
Intercept	62.951	1	62.951	47.603	.000
gene expression in the zebra finch	8.918	1	8.918	6.744	.010
type	4.819	1	4.819	3.644	.057
type * gene expression in the zebra finch	6.363	1	6.363	4.812	.029
gene expression in the zebra finch quadratic	8.828	1	8.828	6.676	.010
type * gene expression in the zebra finch quadratic	6.814	1	6.814	5.152	.024
Error	849.001	642	1.322		
Total	58440.600	648			
Corrected Total	878.355	647			

a. R Squared = .033 (Adjusted R Squared = .026)

crow:zf hybridizations (Figure 4). For unbiased ESTs the group with second highest expression had a significantly higher number of hybridizing probes (ww:zf 9.42 ± 0.11; crow:zf 8.35 ± 0.14) than both the lowest expressed group (ww:zf 8.23 ± 0.14, $F_{1,291} = 50.45$, $p < 0.0001$; crow:zf 6.60 ± 0.14, $F_{1,291} = 76.6$, $p < 0.0001$) and the highest expressed group (ww:zf 8.97 ± 0.13, $F_{1,292} = 7.23$, $p = 0.008$; Figure 4a; crow:zf 7.69 ± 0.16, $F_{1,292} = 9.31$, $p = 0.002$; Figure 4b).

Analyses of dN, dS and dN/dS for the orthologues between the zebra finch and the chicken mirrored this pattern. Male-biased orthologues did not differ in any of these three measures between low and high expressed genes (Table 3; Figure 3). Low expressed unbiased genes had significantly higher dN and dN/dS than high expressed unbiased genes ($p < 0.0001$; Table3; Figure 3). dS differed significantly in comparisons between unbiased and male-biased orthologues, with a higher dS in male-biased genes (unbiased 0.413 ± 0.025; male-biased 0.565 ± 0.062; $F_{1,35} = 4.744$, $p = 0.025$). For dN there was no significant difference, although the numerical trend was of higher dN in male-biased than unbiased genes (unbiased 0.055 ± 0.013; male-biased 0.073 ± 0.017; $F_{1,35} = 0.738$, $p = 0.396$).

Discussion

The short probes (25 bp long) on the Lund-zfa array are sensitive to substitutions in the target sequences, making it possible to distinguish target DNA with 0 – 2 substitutions from target DNA with ≥ 3 substitutions. We have therefore been able to estimate gene divergence level in 11 regions of each EST included in this study. Our method does not directly quantify the number of substitutions, however, it gives a relative measure of divergence enabling us to rank ESTs from slowly to fast evolving.

We found a clear 'fast Z' effect in our passerine bird data, with higher genetic divergence (measured as a lower number of significant probes) in both unbiased and male-biased Z-linked ESTs

Table 2. Output from a General Linear Model with number of significant probes in crow: zebra finch hybridizations as dependent variable and with the following added as independent variables: mean leval of gene expression (both as the original value and as the quadratic term), type (unbiased or male-biased) and the interaction terms between type and the two expression terms. Sample sizes are 378 unbiased ESTs and 270 male-biased ESTs.

Tests of Between-Subjects Effects

Dependent Variable:number of significant probes in crow : zebra finch hybridizations

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	93.050[a]	5	18.610	8.889	.000
Intercept	18.191	1	18.191	8.689	.003
gene expression in the zebra finch	27.852	1	27.852	13.303	.000
type	9.682	1	9.682	4.625	.032
type * gene expression in the zebra finch	13.930	1	13.930	6.653	.010
gene expression in the zebra finch quadratic	26.934	1	26.934	12.865	.000
type * gene expression in the zebra finch quadratic	15.129	1	15.129	7.226	.007
Error	1344.114	642	2.094		
Total	45117.813	648			
Corrected Total	1437.164	647			

a. R Squared = .065 (Adjusted R Squared = .057)

than in autosomal ESTs. These data imply that the avian Z chromosome is evolving rapidly and this is true regardless of sex-bias and gene expression level. This 'fast-Z' effect is likely due to hemizygous exposure of recessive alleles in ZW-females (Charlesworth et al. 1987) and to the lower general population size of Z compared to autosomes (Charlesworth et al. 1987; Ellegren 2008), two processes that lead to a higher fixation rate for mutations on Z.

We also found that male-biased ESTs have diverged faster than unbiased ESTs on the Z chromosome (Figure 1 and 2). Moreover, the levels of divergence of unbiased and male-biased ESTs were differentially affected by their gene expression levels. Unbiased ESTs showed a significantly higher degree of conservation at intermediate expression levels, a pattern we have previously shown for autosomal ESTs (Naurin et al. 2009c). In contrast, male-biased genes had similar divergence rates regardless of expression level (Figure 3 and 4).

The generally fast evolution of male-biased genes observed in the present study can be explained by two (not mutually exclusive) processes:

First, male-biased genes could have faced a high degree of positive selection, either through sexual selection on males or through sexually antagonistic selection (Ellegren & Parsch 2007). In line with this other studies have found support for positive selection playing a role in the rapid evolution of male-biased genes (Nielsen et al. 2005; Proschel et al. 2006; Sawyer et al. 2007; Zhang & Parsch 2005).

Secondly, it has been hypothesized that sex-biased genes are on average more dispensable (Mank & Ellegren 2009). If this is true, purifying selection might be less effective in male-biased Z-linked genes, leading to relaxed constraint and hence a higher degree of sequence evolution.

These two mechanisms (selection and relaxed constraint) are interlinked since less dispensable genes are also more likely to evolve through positive selection to a state where both sexes reach their specific optimum (through sex-biased expression) (Ellegren & Parsch 2007; Mank & Ellegren 2009).

One factor that argues against the decisive importance of the second mechanism is that it would require that up to a third of the Z chromosome harbor dispensable genes. Moreover, if male-biased genes in general were less essential we would perhaps not expect that these genes were also highly expressed, a pattern we have found in a previous study (Naurin et al. 2009b).

The results presented in this study and some other recent studies of birds allow us to make the following tentative conclusions regarding the degree of evolution of male-biased Z-linked genes:

Male-biased Z-linked genes diverge on average faster than unbiased Z-linked and autosomal genes

In this study male-biased Z-linked ESTs have a lower number of significantly hybridizing probes compared to unbiased Z-linked and autosomal

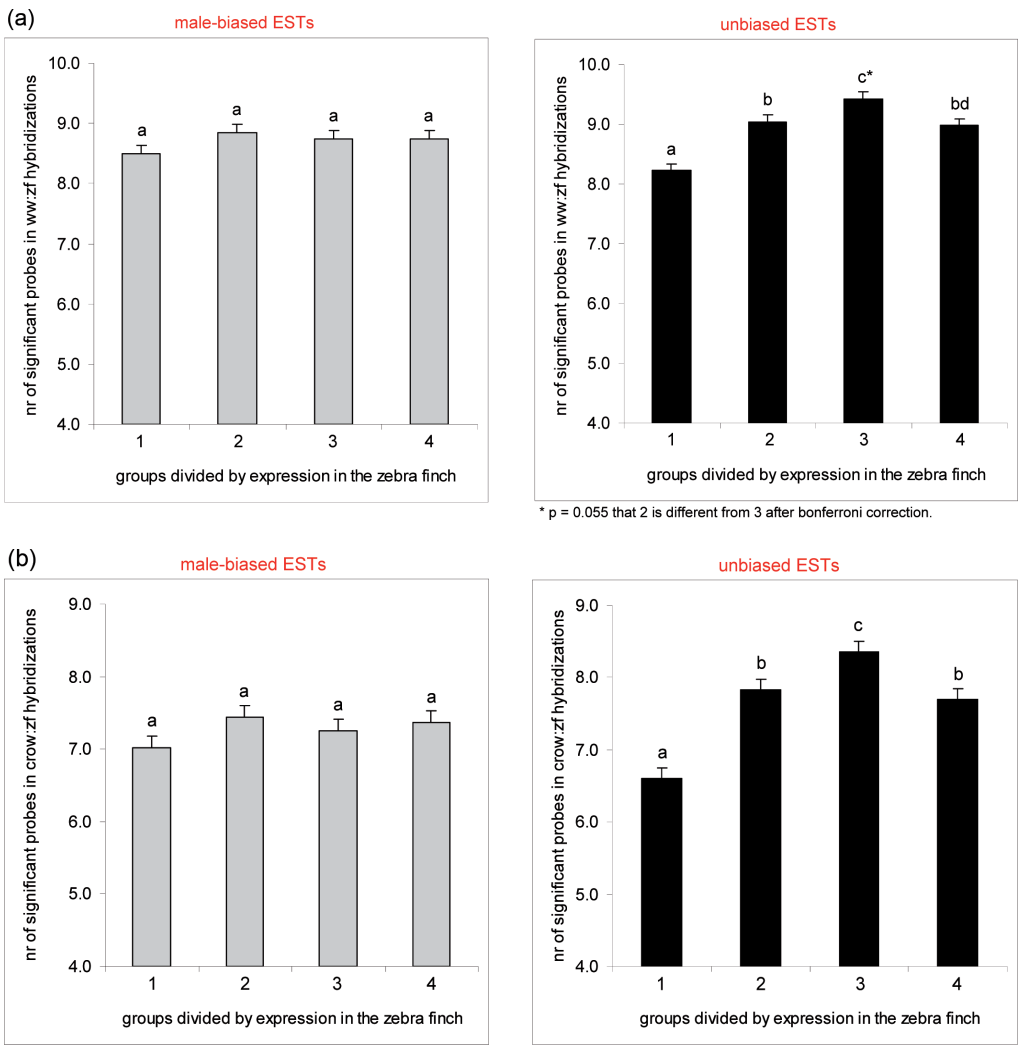

Figure 4. Mean number (+SE) of significantly hybridizing probes grouped by four categories of gene expression levels for (a) willow warbler to zebra finch hybridizations and (b) crow to zebra finch hybridizations.

Table 3. Descriptives and One-way ANOVAs for the 37 Z-linked orthologues between the zebra finch and the chicken.

	unbiased genes, low expression	unbiased genes, high expression	One-way ANOVA unbiased high versus unbiased low	male-biased genes, low expression	male-biased genes, high expression	One-way ANOVA male-biased high versus male-biased low
dS	0.387 ± 0.029	0.428 ± 0.036	$F_{1, 17} = 0.600$, p = 0.449	0.376 ± 0.044	0.302 ± 0.048	$F_{1, 16} = 1.246$, p = 0.279
dN	0.097 ± 0.029	0.029 ± 0.004	$F_{1, 17} = 9.120$, p = 0.008	0.078 ± 0.028	0.065 ± 0.013	$F_{1, 16} = 3.516$, p = 0.077
dN/dS	0.258 ± 0.073	0.073 ± 0.010	$F_{1, 17} = 10.659$, p = 0.005	0.117 ± 0.020	0.117 ± 0.026	$F_{1, 16} = 2.667$, p = 0.120
number of orthologues	7	12		8	10	

ESTs. They are thereby highly affected by the processes that causes a generally high divergence rate of sex-biased genes, just as autosomal sex-biased genes in birds have previously been shown to be (Mank et al. 2007a).

Gene expression level does not determine divergence rate for male-biased Z-linked genes

Male-bias in gene expression on the avian Z chromosome increases with increasing gene expression level (Melamed & Arnold 2007; Naurin et al. 2009b). Highly expressed genes have been found to evolve slowly (Drummond et al. 2005; Duret & Mouchiroud 2000; Pal et al. 2001; Subramanian & Kumar 2004). However, for the Z chromosome in passerine birds we found no pronounced differences in the level of divergence between male-biased ESTs of different expression categories, indicating that they are subject to similar selection pressures regardless of their gene expression level. This sets them apart from unbiased Z-linked ESTs and autosomal ESTs which are more conserved at intermediate expression levels (present result and Naurin et al. 2009c).

Male-biased and unbiased Z-linked genes are subject to different selection pressures

The extensive male-bias in gene expression on the avian Z chromosome (Ellegren et al. 2007; Itoh et al. 2007; Melamed & Arnold 2007; Naurin et al. 2009b) is not mirrored by an extensive female-bias on X chromosomes in XY-species like mammals and *Drosophila* (Charlesworth 1996; Mank 2009). This has been interpreted as a lack of dosage compensation in female birds (see above and Mank 2009). If the extensive male-bias simply reflects a lower Z-linked expression in ZW females compared to ZZ males (rather than genes with specific male function) male-biased genes on Z should not be expected to evolve faster than unbiased genes, in particular not if they have high or intermediate expression levels. In contrast, we have shown that male-biased ESTs evolve more rapidly than unbiased ESTs and their rate of divergence does not relate to gene expression level, which make these genes different from unbiased

genes on Z. This implies that male-biased and unbiased Z-linked genes are subject to different selection pressures.

Our results indicate that the male-bias in gene expression of many Z-linked genes has strongly influenced the evolution of the avian Z chromosome. Male-biased genes tend to evolve at the same rate regardless of expression level and their rapid gene divergence rates indicates that genes with male-bias in gene expression often have male specific functions. It seems likely, therefore, that most of the male-biased genes on the avian Z chromosome are not male-biased simply due to an effect of the double dose of the Z chromosome in males. Rather, many of the male-biased genes on the avian Z chromosome are likely to be male-biased because their high substitution rates (caused by the fast Z effect and the rapid evolution of male-biased genes) has led to optimization of these genes in males, perhaps forcing females to down-regulate their gene expression in the process.

Future studies will determine to what extent the rapid sequence evolution of male-biased genes can be attributed to synonymous substitutions and to what extent it is a result of selection for amino acid changes in male-biased genes or selection against such changes in unbiased genes. Future studies will also shed light on whether or not the high conservation in unbiased Z-linked genes with intermediate expression levels (a pattern we have previously demonstrated for autosomal genes in passerine birds; Naurin et al. 2009c) is general or bird specific.

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VII

On sex chromosome evolution: trade-offs between gene dose and sexual antagonism

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On sex chromosome evolution: trade-offs between gene dose and sexual antagonism

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Recent results have demonstrated extensive male-bias in gene expression on Z chromosomes in species with ZW sex-determination. This has led to claims that ZW-females have inefficient dosage compensation, a process which increases gene expression in XY-males in species with XY-sex determination (XY-males have expression which it is equal to that in XX-females for most X-linked genes). However, we argue that the extensive male-bias on Z chromosomes cannot be explained simply by inefficient dosage compensation in ZW-females. This is made clear by results demonstrating that: (i) ZW-females do dosage compensate to quite a high extent, (ii) male-biased and unbiased Z-linked genes have different expression levels, (iii) male-biased and unbiased Z-linked genes have different levels of divergence, and (iv) male-biased and unbiased Z-linked genes belong to different functional categories. We propose that two processes have interacted and caused the extensive male-bias in gene expression on Z chromosomes. First, Z chromosomes are more conductive to certain types of sexual selection than X chromosomes and such sexually selected alleles are likely to be antagonistic and detrimental to females. Secondly, a male-bias in mutation rate, known from many taxa, is likely to have lead to a scenario where: (i) there was an early onset of selection for dosage compensation on X, (ii) X-homologues were largely unchanged as XY-males were selected for compensation, (iii) the onset of dosage compensation on Z was delayed compared to that on X, and (iv) when ZW-females were selected for dosage compensation Z had evolved rapidly while being particularly conductive to sexual selection in males. Hence, ZW-females likely faced a trade off between retaining gene expression dose and avoiding expression of traits which compromised female function. A higher conductivity to sexual selection and male-biased mutation rates are thereby proximate mechanisms which can explain why the male-bias on Z chromosomes is not mirrored by an equally extensive female-bias on X chromosomes.

An extensive male-bias on Z chromosomes

Many eukaryotes have sexes with different chromosomal constitutions, and heteromorphic X/Y and Z/W sex chromosomes have evolved independently many times (Charlesworth 1996; Rice 1996a). Sex chromosomes are not only the vehicles by which sex is genetically determined but also harbor a large proportion of all genes with sex-biased gene expression (Ellegren & Parsch 2007), are particularly conductive to accumulation of sexual antagonism (Charlesworth et al. 1987; Rice 1984) are expected to evolve rapidly (Presgraves 2008; Vicoso & Charlesworth 2006) and should be highly implicated in adaptive evolution and speciation (Coyne 1985; Dobzhansky 1974; Presgraves 2008; Templeton 1977).

Glossary Box

Sex chromosomes:
Originally homologous pairs of autosomes, X/Y or Z/W are today harboring the genes that determine sex (see Box 1). Y is male-determining while W is female-determining.

XY-systems:
Species with X and Y sex chromosomes, where females are the homogametic sex (XX) and males are the heterogametic sex (XY). The Y chromosome is only present in males while the X chromosome is present two thirds of time in females. XY sex determining systems include for example mammals, *Drosophila* and *C. elegans*.

ZW-systems:
Species with Z and W sex chromosomes, where males are the homogametic sex (ZZ) and females are the heterogametic sex (ZW). The W chromosome is only present in females while the Z chromosome (the equivalent of X chromosomes) is present two thirds of the time in males. ZW sex determining systems include for example birds, reptiles and many insect- and fish-species.

Homogametic sex:
The sex that carries two copies of the X or Z chromosome (XX-females and ZZ-males).

Heterogametic sex:
The sex that is determined by the Y or W chromosome and also carries one copy of the X or Z chromosome (XY-males and ZW-females).

Dosage compensation:
A mechanism that alters gene expression in response to a change in gene copy number so that both sexes have X (or Z) chromosome-linked gene expression that is balanced with autosomal gene expression, regardless of whether or not they have one or two copies of the X (or Z) chromosome.

Sex-biased gene expression:
Transcription from genes that has a higher activity per copy in one sex than in the other, or transcription from genes that have a higher dose (gene copy number) in one sex than the other but lacks dosage compensation.

Sexually antagonistic genes:
Genes that are beneficial to one sex but detrimental to the other. Fixation of such genes should be facilitated by X (or Z) chromosome-linkage due to the sex-biased transmission pattern of those chromosomes, where one sex has one more copy than the other sex (see Box 2).

Homologous genes:
In a diploid organism each chromosome is present in one paternal and one maternal copy. Each gene is thereby present in two homologous copies, one on each of the two chromosomes.

Large X effect:
The hypothesis that the X chromosome plays a disproportionally large role in adaptive evolution and speciation.

Large Z effect:
The hypothesis that the Z chromosome plays a disproportionally large role in adaptive evolution and speciation.

Male-biased mutation rate:
Due to a higher number of meiotic cell divisions in the male- than in the female germline, any given gene will have a higher mutation rate when it is present in a male. This will have a large effect on sex-chromosomes due to the fact that they reside more often in one sex than the other.

Sex chromosomes of species with male heterogamety (XY-systems) and female heterogamety (ZW-systems) share many characteristics and are believed to have evolved in similar ways (Box 1). Yet, the two systems demonstrate clear differences in the regulation of sex-linked genes. Noticeably the Z chromosomes studied so far (in three bird and one insect species Ellegren et al. 2007; Itoh et al. 2007; Naurin et al. 2009a; Zha et al. 2009) have extensive male-bias in gene expression, comprising up to a third of the genes on the chromosome. No such extensive sex-bias in gene expression has been found on the X chromosomes of mammals, *Drosophila* or *C. elegans* (Charlesworth 1996; Mank 2009). It is highly unlikely that this is due to more essential genes on X chromosomes, as both the different Z chromosomes (in birds and in the silkworm) and the different X chromosomes (in mammals, *Drosophila*, and *C. elegans*) are of different evolutionary origin. So far no conclusive explanation for what could be causing this discrepancy between Z chromosomes and X chromosomes has been suggested.

Male-bias on Z as a result of inefficient dosage compensation

In the ancestral state of sex chromosomes, each gene had a copy on both the X and the Y chromosome (Bergero & Charlesworth 2008; Charlesworth & Charlesworth 2000; Box 1). During the evolution of sex chromosomes, most genes on the Y chromosome were lost and for these genes only one gene copy remain in XY-males, the one on the X chromosome. For most X-linked genes in XY-males, however, expression is not lower than

Box 1. The evolution of sex chromosomes

The sex chromosomes in XY and ZW sex determining systems have evolved in much the same way. X/Y and Z/W are homologous pairs of chromosomes and have evolved from ancestral pairs of autosomes (Fridolfsson et al. 1998; Rice 1984). Sex was environmentally determined until a mutation on one of the pair of autosomes gave rise to a sex determining allele. In the case of XY-systems this allele gave rise to males whereas in ZW-systems it determined females. The sex limited distribution of this allele on the ancestral pair of autosomes had large implications for the surrounding region. Genes closely linked to the allele were then also predominantly inherited by the sex it determined. Consider the following scenario put forward by Rice (Rice 1996b): A mutation takes place one map unit (cM) away from the sex determining locus on the Y chromosome. In 99% of the time this new mutation will cosegregate with the sex determining allele and it will therefore be present in females only 1% of the time. A mutation that takes place at a locus present in males 99% of the time will remain in the population even if it is harmful to females the 1% of the time they inherit it. Hence, mutations detrimental to females could aggregate in close linkage to the sex determining locus on Y and thereby lead to selection against recombination between Y and X in this region. Such recombination could lead to inviable phenotypes due to expression of female detrimental mutations in females. Once recombination ceased in a certain region that part of Y will be totally male-limited and the regions close to it will again accumulate sexually antagonistic variation. In this fashion selection against recombination will spread along the sex-chromosomes until recombination ceases almost entirely (Bergero & Charlesworth 2008). Y (and W) will then not experience any recombination, while X (and Z) still recombine in the homogametic sex (XX or ZZ). The complete lack of recombination has, for most Y and W chromosomes, led to a subsequent degradation and silencing of Y- and W-homologues (for a review of the processed leading to Y and W degradation see: Bachtrog 2006). This degradation has led to the small and gene-poor Y and W chromosomes found in many species today. At this stage, the homogametic sex (XX or ZZ) has two copies of each gene while the heterogametic sex (XY or ZW) have only one copy of most genes, present on their single X or Z chromosome.

it is in XX-females (Birchler et al. 2006; Marin et al. 2000). Moreover, mean gene expression on the single X chromosome in males is similar to that on autosomes (Gupta et al. 2006; Nguyen & Disteche 2006). The net expression of sex-linked genes in males has been retained through a process called dosage compensation, resulting in increased gene expression on the single copy of X in males (Mank 2009; Marin et al. 2000). Dosage compensation is regarded as a vital part of sex chromosome evolution necessary for survival (Mank 2009). Aneuploidy, i.e. having extra or fewer copies of parts of the genome, is generally lethal (due to copy number effects on the expression of genes involved in genetic networks see; Jeong et al. 2001; Lindsley et al. 1972; Lu & Oliver 2001; Pauli et al. 1993; Rosenbusch 2004) and chromosome-wide dosage compensation occurs through different mechanisms in XY-systems as diverse as mammals, *Drosophila* and *C. elegans* (Mank 2009; Marin et al. 2000).

Recent results from ZW sex determining systems demonstrating extensive male-biased gene expression on Z chromosomes (Ellegren et al. 2007; Itoh et al. 2007; Naurin et al. 2009a; Zha et al. 2009) indicate that dosage compensation which leads to completely balanced gene expression between the sexes is not essential. Female chicken (*Gallus gallus*), zebra finches (*Taenopygia guttata*) and common whitethroats (*Sylvia communis*) have lower gene expression than males for many Z-linked genes (Ellegren et al. 2007; Itoh et al. 2007; Naurin et al. 2009a). Furthermore, extensive male-bias in gene expression has been confirmed on the Z chromosome of another female-heterogametic species, the silkworm (*Bombyx mori*; Zha et al. 2009). This extensive male-bias in gene expression on Z chromosomes has been interpreted as inefficient dosage compensation in ZW-females (Ellegren et al. 2007; Itoh et al 2007). In the light of the detrimental effects of aneuploidy, questions have been posed regarding how females cope with this potential disruption of gene networks (Birchler 2009; Ellegren et al. 2007; Mank 2009). However, we argue that females have been selected to balance increased doses against female-detrimental effects of male-biased genes. Several recent results indicate this interpretation is more likely than an inability of females to achieve a dose as high as males.

First, two separate studies have demonstrated that the ratios of Z-linked to autosomal gene expression (Z:A ratios) in female birds fall within the range of X:A ratios as demonstrated for mammals (Itoh et al. 2007; Naurin et al. 2009a). Hence, even if ZW-females have lower gene expression than ZZ-males for many Z-linked genes they are likely to have achieved enough dosage compensation to balance their gene networks.

Secondly, male-biased and unbiased genes differ with respect to their gene expression levels both in birds and in the silkworm (Melamed & Arnold 2007; Naurin et al. 2009a).

Thirdly, male-biased genes on the avian Z chromosome evolve faster than unbiased genes and unlike unbiased Z-linked genes and autosomal genes they evolve at the same rate regardless of their gene expression level (Naurin et al. 2009c).

Finally, both in the silkworm and in the chicken unbiased and male-biased genes have different functions and in the silkworm male-biased genes are likely to be controlling sexual differentiation (Melamed & Arnold 2007; Zha et al. 2009).

Hence, even if the male-bias on Z chromosomes truly reflects differences in the degree of dosage compensation in ZW-females and in XY-males we argue that that this difference has been caused by particular properties of the male-biased Z-linked genes and thereby that the lower level of gene expression in ZW-females is adaptive. This means that the differences between Z and X chromosomes are likely to originate in some aspect of sex chromosome evolution where ZW-females and XY-males have faced different trade-offs regarding the regulation of sex-linked genes.

Differences between XY and ZW systems which could explain the male-bias on Z

If it is adaptive for ZW-females to keep the gene expression level for some Z-linked genes at lower levels than males then there must be some property of these genes that compromises female function when expression reaches a critical level.

There are two properties of Z chromosomes that sets them apart from X chromosomes and which might have played a crucial role in the evolution of Z-linked genes leading to such a scenario: (1) Z chromosomes are more conductive to sexual selection than X chromosomes and (2) Z/W and X/Y chromosomes will be differentially affected by a male-bias in mutation rate.

1. Conductivity to sexual selection

Modelling has demonstrated that Z chromosomes are more conductive to certain types of sexual selection (Albert & Otto 2005; Hall & Kirkpatrick 2006; Reeve & Pfennig 2003; Servedio & Saetre 2003). Albert and Otto (2005) studied how female preferences coevolved with sexually antagonistic traits in both XY- and ZW-systems. They found that sexually antagonistic selection was always resolved in favour of females in XY-species but in favour of males in ZW-species when the preference was Z-linked. Hence, ZW-systems are more likely to evolve Z-linked costly male traits coupled with Z-linked alleles for female preference (Albert & Otto 2005). Moreover, Reeve and Pfenning (2003) showed that rare alleles coding for male traits and female preferences were better protected against random loss when Z-linked than when autosomal or X-linked. Lastly, mate recognition often involve secondary sexual characters in males which are associated with a female preference. Servedio and Saetre (2003) showed that genes coding for hybrid incompatabilty (postzygotic isolation) will hitchhike along with mate recognition traits (prezygotic isolation) in a positive feedback loop where both are strengthened. Z-linkage of trait, preference and incompabilities causes greater reinforcement than autosomal linkage (Lemmon & Kirkpatrick 2006; Servedio & Saetre 2003). There is empirical evidence of Z-linkage of male traits and female preferences in birds (Ohno 1967; Saether et al. 2007).

2. The effect of a male mutation bias

Z/W chromosomes and X/Y chromosomes will be differentially affected by a male-bias in mutation rate, a phenomenon likely to be caused by more cell divisions in the male germ line (Li et al. 2002; Makova & Li 2002). Male mutation bias is present in several taxa (Li et al. 2002; Makova & Li 2002) including birds (e. g. Axelsson et al. 2004; Ellegren & Fridolfsson 1997). Due to their sex-biased transmission pattern sex chromosomes spend different amounts of time in males so that; Y is 100% in males, Z will be present in males 66% of the time, X will be present in males 33% of the time while W will only reside in females. Hence, a male mutation bias will affect mutation rate on the sex chromosomes in a way so that Y will mutate faster than W chromosomes while Z will mutate faster than X chromosomes.

Sexual antagonism and the differences between Z and X chromosomes

Both X and Z chromosomes are expected to accumulate sexually antagonistic mutations, beneficial to one sex and detrimental to the other, and such antagonism can be resolved by sex-biased gene expression (Box 2). This accumulation of sexual antagonism is caused by the sex-bias in transmission pattern of Z and X chromosomes where one sex will always have one copy more than the other (Charlesworth et al. 1987; Rice 1984).

The two differences between X/Y and Z/W above will influence the extent to which sexually antagonistic mutations are fixed on Z and X chromosomes:

(1) The higher conductivity of Z than of X to certain types of sexual selection should lead to more antagonism on Z. Traits subject to sexual selection will often be sexually antagonistic, for example traits which will increase reproductive success in males while only inducing a cost in females (Rice 1984; Rice & Chippindale 2001; Seger & Trivers 1986).

(2) Male-biased mutation rates will lead to a higher general mutation rate on Z (2/3 of the time in males) compared to X (1/3 of the time in males) and thereby to more raw material for sexual selection and sexually antagonistic selection on Z than on X chromosomes.

Hence, we argue that it is likely that these differences between Z and X have led to more male-biased gene expression on Z chromosomes.

Male-biased mutation and the rate of evolution on sex chromosomes in ZW- and XY-systems

Early in the process of evolution of sex chromosomes, after repression of recombination but before the degradation of Y and W (Box 1) the relationship between the rate of evolution on the different sex chromosomes should have been as follows:

Y > Z > X > W

That is, Y (100% in the faster mutating males) will evolve fastest followed by Z (66% in males), X (33% in males) and lastly W (0% in males). Substitution rates on autosomal genes in both systems (50% in males) will fall between Z-linked and X-linked genes. Hence, during the initial stages of sex chromosome evolution male-biased mutation will have caused two important differences between XY- and ZW-systems:

1. Z chromosomes evolved on average faster than X chromosomes while Y chromosomes degraded at a higher pace than W chromosomes.
2. The male limited Y degraded fast while X evolved slowly in XY-systems. In ZW-systems, Z evolved rapidly while the female limited W degraded slowly

Degeneration of non-recombining chromosomes is inevitable (e. g. Bachtrog 2006; Charlesworth & Charlesworth 2000; Box 1) and today Y and W chromosomes have degraded in all species where dosage compensation has been studied. This means that genes which remain on the small Y and W chromosomes are highly linked and selection at one locus will lead to fixation of alleles in other loci and thereby to a rapid evolutionary rate on W as well as on Y chromosomes (Berlin & Ellegren 2006). However, because of the male mutation bias there will continue to be a higher rate of evolution on Y than on W and on Z than on X and the current rate of evolution on the different sex chromosomes will be as follows:

Y > W > Z > X (autosomal genes in both systems will still fall between Z-linked and X-linked genes).

Hence, if there is a male mutation bias Z evolves faster than X and Y degrades faster than W at all stages of sex chromosome evolution. The differential effect of male-biased mutation rates on Z and X chromosomes is crucial since it will have affected dosage compensation and the degree of sex-biased gene expression in two ways: (i) it led to an important difference in the timing of selection for dosage compensation on Z and X, and (ii) it led to a scenario where Z-linked genes had evolved to a higher extent than X-linked genes when selection for dosage compensation took place.

XY-males would have faced rapid loss of gene functionality on the Y chromosome. However, the X chromosome evolved slowly, slower than autosomal loci, indicating that it had largely retained original function when up-regulation in order to achieve original dose was selected in XY-males.

Hence, male mutation bias likely led to an early onset of selection for dosage compensation on X and X-homologues would have remained largely unchanged when it occurred.

Due to the slow degradation of W in the initial stages of sex chromosome evolution ZW-females would have retained functionality on the W chromosome for a longer time than XY-males did on Y. It would have taken longer before degradation on W reached large scale proportions. During this longer period of time the evolution on the Z chromosome was affected by three important processes: (1) Z was present in the faster mutating males 66% of the time and was thereby evolving rapidly, (ii) Z was more conductive than X to accumulation of certain types of sexually selected mutations, and (iii) Z-linked genes likely faced more effective selection pressures in males compared to females. More effective selection in males would have been a result of the double doses of gene expression for Z-linked genes in males compared to females. As long as W was functional 50% of the female gene expression would have been W-linked.

Later in the process, when W reached a state where degradation progressed more rapidly due to shrinking size and increased linkage, ZW-females faced a scenario where dosage compensation of Z (to the level of expression in males) would have led to up-regulation of Z-linked homologues which had diverged from their original state in such a way that they might not have been suitable for high expression in females.

Hence, male mutation bias likely led to a delay in the timing of selection for dosage compensation on Z compared to that on X and to a fast male-driven evolution of Z-linked genes.

In the light of these facts we argue that:

1. The extensive male-bias on Z chromosomes cannot be explained simply by a lack of efficient dosage compensation in females, rather, the lower gene expression in females than males is likely to be adaptive.
2. The male-bias in mutation rate has likely caused important differences in the evolutionary rate on X, Y, Z and W chromosomes. Together with the higher conductivity of Z chromosomes to accumulate sexually selected male traits this most likely led towards optimization of many Z-linked genes for male reproductive success and male function.
3. As dosage compensation was selected on the Z chromosome, ZW-females were selected to balance gene dose with the potential loss of female function and fitness (caused by high expression of genes adapted to optimize male function and fitness). ZW-females have thereby faced different trade-offs than XY-males as dosage compensation evolved.

Based on the hypothesis we have outlined above the following predictions can be made; (i) there should be evidence of male-mutation bias on Z chromosomes, (ii) Z-linked genes should evolve rapidly, (iii) Z-linked genes should have influenced adaptive evolution and speciation, and (iv) there should be evidence for female-detrimental sexual antagonism on Z chromosomes. We will now proceed to review the empirical evidence for these conclusions on the avian sex chromosomes, the only Z and W chromosomes to date which have been extensively studied. When possible, we will compare with evidence from studies on X chromosomes.

Empirical evidence of male-biased mutation rates in ZW-birds

Male mutation bias in birds have been demonstrated by comparisons between the evolutionary rate in neutral sequences in the Z-linked and the W-linked copy of the CHD1 gene (Ellegren & Fridolfsson 1997; Fridolfsson & Ellegren 2000; Garcia-Moreno & Mindell 2000; Kahn & Quinn 1999) and the ATP5A gene (Carmichael et al. 2000) and was estimated to be between 1.8 – 6.5. This is to be compared to a male mutation bias of 4 – 6 in primates (Li et al. 1996; Miyata et al. 1987; Pamilio & Bianchi 1993; Shimmin et al. 1993; Wolfe & Sharp 1993) and of about 2 in rodents (Chang & Li 1995; Chang et al. 1994). Moreover, Axelsson et al. (2004) showed in comparisons of 33 autosomal, 28 Z-linked and 14 W-linked introns between the chicken and the turkey that the mean intronic divergence on the Z chromosome was 12%, compared to 8% in autosomal introns and only 5% in W linked introns. They concluded that the male-mutation bias was sufficient to explain observed differences in sequence divergence of autosomal, Z-linked and W-linked introns in the chicken to turkey comparison.

Overall, results regarding male-mutation bias in birds so far suggest that it is similar to that seen in small to medium sized mammals (Axelsson et al. 2004; Ellegren 2007), which is not surprising given the fact that generation times is shorter in birds than in for example primates and that there is a positive correlation between generation time and excess of male mutation (Ellegren 2007), an association which has also been found in birds (Bartosch-Härlid et al. 2003).

Interestingly, avian species with a high degree of sperm production due to sexual selection also have a more pronounced excess of male mutations (Bartosch-Härlid et al. 2003) indicating that male mutation bias and sexual selection is interlinked in birds, and that they might have reinforced each other as the Z chromosome evolved.

Empirical evidence of rapid evolution on the avian Z chromosome

Apart from the study by Axelsson et al. (2004), a rapid evolution of the Z chromosome has been documented in birds by comparisons of 172 Z-linked genes between the chicken and the zebra finch (Mank et al. 2007), and in studies of the

divergence level between passerine bird species for 687 Z-linked EST sequences (Naurin et al. 2009b). The avian Z chromosome has thereby been repeatedly shown to evolve faster than the autosomes in the avian genome.

Genes on X and Z chromosomes are expected to evolve faster than autosomal genes; a process known as the fast X or fast Z effect (Presgraves 2008; Vicoso & Charlesworth 2006). This is due to hemizygous exposure of recessive alleles in the heterogametic sex (XY males and ZW females; Charlesworth et al. 1987) and the low population size of sex chromosomes (Charlesworth et al. 1987; Ellegren 2008). These processes will lead to a higher fixation rate of new mutations on X and Z chromosomes as compared to autosomes.

However, the fact that neutral sequences on Z evolve faster than on W (Carmichael et al. 2000; Ellegren & Fridolfsson 1997; Fridolfsson & Ellegren 2000; Garcia-Moreno & Mindell 2000; Kahn & Quinn 1999) demonstrates that the rapid evolution of Z is not caused entirely by hemizygous exposure and low populations size, but that male mutation bias also plays an important role. Moreover, W chromosomes degrade more slowly than Y chromosomes (Ellegren 2007) and theoretical modelling has shown that Z will evolve faster than X if there is a male mutation bias (Kirkpatrick & Hall 2004) further indicating that the mutation bias is likely to drive some of the differences in rate of evolution on X/Y and Z/W.

Empirical evidence regarding the role of the Z chromosome in adaptive evolution and speciation

Theory predicts that both X chromosomes and Z chromosomes should play a disproportionally large role in adaptive evolution, a process known as the large X, or large Z effect (Coyne 1985; Dobzhansky 1974; Presgraves 2008; Templeton 1977).

Several recent results indicate that the large Z effect might be more pronounced than the large X effect: (i) in a study of avian-mammalian orthologues avian Z-linked genes were highly overrepresented among orthologues with an accelerated rate of functional evolution, whereas there was no excess of X-linkage among the genes with accelerated functional evolution in mammals (Ellegren 2008), (ii) genes involved in hybrid sterility seem to have aggregated on X as well as on Z, but indications are that genes involved in sexual isolation are disproportionally Z-linked only (Qvarnstrom & Bailey 2009), (iii) genes controlling male-specific plumage traits, species recognition and hybrid fitness have been found to be Z-linked in flycatchers (Saether et al. 2007), and (iv) in a large screen of about 10 000 orthologues between rhesus macaque, chimpanzee and human, selected genes were not overrepresented on the X chromosome (Rhesus Macaque Genome Sequencing and Analysis Consortium 2007).

Empirical evidence of sexual antagonism detrimental to females on the avian Z chromosome

Almost 25 years ago Rice (Rice 1984) and Charlesworth et al (Charlesworth et al. 1987) refined the theory of aggregation of antagonism on sex chromosomes (Box 2), and extensive research on X chromosomes has been undertaken since then. Nevertheless, antagonistic genes detrimental to the heterogametic sex has not been readily found on X , and almost all genes on X have equal expression in both sexes indicating that antagonism is unlikely to be wide-spread. It is interesting, therefore, that antagonistic genes detrimental to females were recently found to be overrepresented on the comparatively unknown avian Z chromosome recently in chicken (Mank & Ellegren 2009b). To some extent a lack of antagonism on X might be explained by certain cellular processes, like meiotic sex-chromosome inactivation, of which there is no evidence of in birds (Hense et al. 2007; Turner 2007) and it is therefore difficult to directly compare levels of antagonism. However, even though there is antagonism on X (Connallon & Knowles 2005; Gibson et al. 2002; Gurbich & Bachtrog 2008; Khil et al. 2004) the highly compensated state of this chromosome indicates that it might harbor less potential for antagonism

Box 2. Sexual antagonism accumulates on X and Z chromosomes

Selection on any gene may be sexually antagonistic, i.e. have different directions in males and females. Antagonistic mutations should benefit from sex linkage because of the uneven dose of sex chromosomes in the homo- and heterogametic sex (Charlesworth et al. 1987; Rice 1984). The probability that a mutation will go to fixation on the X or Z chromosome depends on the dominance coefficient of the mutation (Ellegren & Parsch 2007; Rice 1984).

Let us consider the avian Z chromosome, a chromosome that is present two thirds of the time in males (ZZ) and only one third of the time in females (ZW). When a Z chromosome is present in a female, all alleles, regardless of whether they are dominant or recessive, will be expressed. Two major conclusions regarding the fate of a Z-linked antagonistic mutation can be drawn (Rice 1984):

1. Dominant Z-linked mutations will be selected two thirds of the time in males and will therefore remain in the population even if they are harmful to females.
2. Recessive Z-linked mutations will always be expressed in females and if such a mutation is beneficial to females it will remain in the population regardless of its effect on males.

Because Z-linked antagonistic mutations cannot be eliminated from the gene pool by the sex that carries the cost, such genes will instead face selection for down-regulation or silencing in the cost-carrying sex (Ellegren & Parsch 2007). This should lead to an increase of genes with sex-bias in gene expression on the Z chromosome (and on X in XY-systems).

Selection experiments have shown that antagonistic variation can accumulate rapidly in parts of the genome that are suddenly restricted to one sex, leading to substantial fitness-loss for the other sex (Rice 1992; Rice 1996b). Hence, once a species have evolved two separate sex-chromosomes, the X or Z chromosome is expected to accumulate antagonistic variation and this should be seen as a non-random distribution of antagonism in the genome, with the sex chromosomes being enriched for such variation.

than the sex-biased Z (Itoh et al. 2007; Mank & Ellegren 2009a, Naurin et al. 2009a).

Additional predictions regarding dosage compensation on Z chromosomes

Based on our hypothesis, two additional predictions can be made: (1) the degree of compensation on single Z-linked genes can be expected to vary between age groups, and (2) more essential genes on the Z chromosome are likely to be more dosage compensated.

1. *Variation between age groups*. If a gene is antagonistic, females should be selected to balance the detrimental effect of lower dose against the detrimental effect of antagonism. This should lead to a fluctuating level of compensation on single genes over the lifetime of a female, since the importance of dose versus antagonism is likely to vary depending on whether a female is a developing embryo, a breeding or a non-breeding adult. In line with this, the level of compensation between the sexes on single genes in birds does vary between age groups (Itoh et al. 2007; Mank & Ellegren 2009a).
2. *More compensation on essential genes on the Z chromosome*. For genes that are particularly important and dose-sensitive, selection should favour a high degree of compensation, leading to the conclusion that essential genes on Z should have higher relative levels of dosage compensation. Moreover, highly essential genes should be less conductive to antagonism as they are often involved in several different pathways and

networks (Jeong et al. 2001). Even if a particular newly mutated allele of a Z-linked gene is beneficial to males in one pathway it might be detrimental in another, and the probability that the overall effect is positive should be lower than for alleles at non-networking genes. In line with this reasoning, there is some evidence of more compensation on essential genes on Z chromosome in chicken (Ellegren et al. 2007), but studies on more species are needed in order to truly answer this question.

Concluding remarks

We conclude that empirical data supports a male-biased mutation rate in birds (e. g. Axelsson et al. 2004; Ellegren & Fridolfsson 1997), a rapid evolution of the avian Z chromosome (Axelsson et al. 2004; Mank et al. 2007; Naurin et al. 2009b), a large and important role of the avian Z chromosome in adaptive evolution and speciation (Ellegren 2008; Qvarnstrom & Bailey 2009; Saether et al. 2007) and, the presence of sexually antagonistic genes detrimental to females on the avian Z chromosome (Mank & Ellegren 2009b). Moreover we conclude that the avian Z chromosome seems to have played a larger role in adaptive evolution than X chromosomes (Ellegren 2008; Qvarnstrom & Bailey 2009; Rhesus Macaque Genome Sequencing and Analysis Consortium 2007). X chromosomes have been studied for decades in terms of sex chromosome evolution, sexual antagonism and dosage compensation and in comparison avian genomics is a very young field of science. It is therefore intriguing, and in favour of our hypothesis, that rapid evolution, adaptive evolution and sexual antagonism has been so readily found on the avian Z chromosome, while these processes have been rather elusive on X chromosomes (e. g. Presgraves 2008).

The extensive male-bias on Z chromosomes cannot be explained simply as an inability of ZW-females to dosage compensate as efficiently as XY-males. This is made clear by results demonstrating that (i) ZW-females do achieve quite high levels of dosage compensation (Itoh et al. 2007; Naurin et al. 2009a) (ii) that male-biased and unbiased genes on the Z chromosome have different gene expression levels (Melamed & Arnold 2007; Naurin et al. 2009a; Zha et al. 2009), (iii) that male-biased and unbiased genes have different levels of divergence (Naurin et al. 2009c), and (iv) that male-biased and unbiased genes belong to different functional categories (Melamed & Arnold 2007; Zha et al. 2009). Hence, male-biased genes on Z chromosomes are likely to have male-specific functions, and ZW-females are thereby likely to have faced a trade off between retaining gene expression dose for these genes and avoiding expression of traits which compromise female function. Male-biased mutation rates and higher conductivity to sexual selection on Z chromosomes are proximate mechanisms which can explain why the extensive male-bias on Z chromosomes is not mirrored by a female-bias on extensive X chromosomes.

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The following is a list of Doctoral theses (Lund University, Sweden) from the Department of Animal Ecology (nos. 1–78, from no. 79 and onwards denoted by (A)) and Theoretical Ecology (T). (E) refers to Doctoral theses from the Department of Chemical Ecology/ Ecotoxicology during the years 1988–1995.

1. CHARLOTTE HOLMQVIST. Problem on marine-glacial relicts on account on the genus *Mysis*. 6 May 1959.
2. HANS KAURI. Die Rassenbildung bei europäischen *Rana*-Arten und die Gültigkeit der Klimaregeln. 9 May 1959.
3. PER DALENIUS. Studies on the Oribatei (Acari) of the Torneträsk territory in Swedish Lapland. 14 May 1963.
4. INGEMAR AHLÉN. Studies on the history of distribution, taxonomy and ecology of the Red Deer in Scandinavia. 21 May 1965.
5. STAFFAN ULFSTRAND. Bentic animal communities of river Vindelälven in Swedish Lapland. 8 May 1968.
6. SAM ERLINGE. Food habits, home range and territoriality of the otter *Lutra lutra* L. 6 May 1969.
7. GUNNAR MARKGREN. Reproduction of moose in Sweden. 17 May 1969.
8. ARNE BERGENGREN. On genetics, evolution and history of the heath-hare, a distinct population of the arctic hare, *Lepus timidus* L. 17 October 1969.
9. HÅKAN HALLANDER. Habitats and habitat selection in the wolf spiders *Pardosa chelata* (O.F. Müller) and *P. pullata* (Clerck). 20 March 1970.
10. ULF SCHELLER. The Pauropoda of Ceylon. 29 May 1970.
11. LEIF NILSSON. Non-breeding ecology of diving ducks in southernmost Sweden. 2 December 1970.
12. RUNE GERELL. Distributional history, food habits, diel behaviour, territoriality, and population fluctuations of the mink *Mustela vison* Schreber in Sweden. 30 March 1971.
13. INGRID HANSSON. Skull nematodes in mustelids. 3 June 1971.
14. STURE ABRAHAMSSON. Population ecology and relation to environmental factors of *Astacus astacus* Linné and *Pacifastacus leniusculus* Dana. 3 June 1971.
15. LENNART HANSSON. Food conditions and population dynamics of Scandinavian granivorous and herbivorous rodents. 26 November 1971.
16. SVEN-AXEL BENGTSON. Ecological segregation, reproduction and fluctuations in the size of duck populations in Iceland. 21 April 1972.
17. STEN ANDREASSON. Distribution, habitat selection, food and diel activity of Swedish freshwater sculpins (*Cottus* L.). 5 May 1972.
18. KERSTIN SVAHN. Coccidian blood parasites in Lacertids. 17 May 1972.
19. RUTGER ROSENBERG. Macrofaunal recovery in a Swedish fjord following the closure of a sulphite pulp mill. 13 April 1973.
20. SVEN ALMQVIST. Habitat selection and spatial distribution of spiders in coastal sand dunes. 25 May 1973.
21. TORSTEN MALMBERG. Population fluctuations and pesticide influence in the rook *Corvus frugilegus* L., in Scania, Sweden 1955-1970. 25 May 1973.
22. ANDERS SÖDERGREN. Transport, distribution, and degradation of organochlorine residues in limnic ecosystems (defended at the Dept of Limnology). 23 May 1973.
23. BERITH PERSSON. Effects of organochlorine residues on the whitethroat *Sylvia communis* Lath.. 7 December 1973.
24. PLUTARCO CALA. The ecology of the ide *Idus idus* (L.) in the river Kävlingeån, South Sweden. 23 May 1975.
25. ÅKE GRANMO. Effects of surface active agents on marine mussels and fish. 26 May 1975.
26. BO W. SVENSSON. Population ecology of adult *Potamophylax cingulatus* (Steph.) and other Trichoptera at a South Swedish stream. 15 October 1975.
27. STEN NORDSTRÖM. Associations, activity, and growth in lumbricids in southern Sweden. 6 April 1976.
28. STEN RUNDGREN. Environment and lumbricid populations in southern Sweden. 8 April 1976.
29. CHRISTIAN OTTO. Energetics, dynamics and habitat adaptation in a larval population of *Potamophylax cingulatus* (Steph.) (Trichoptera). 9 April 1976.

30. JAN LÖFQVIST. The alarm-defence system in formicine ants. 21 May 1976.
31. LARS HAGERMAN. Respiration, activity and salt balance in the shrimp *Crangon vulgaris* (Fabr.). 22 October 1976.
32. THOMAS ALERSTAM. Bird migration in relation to wind and topography. 29 October 1976.
33. LARS M NILSSON. Energetics and population dynamics of *Gammarus pulex* L. Amphipoda. 20 December 1977.
34. ANDERS NILSSON. Ticks and their small mammal hosts. 24 May 1978.
35. SÖREN SVENSSON. Fågelinventeringar - metoder och tillämpningar. (Bird censuses - methods and applications.) 23 May 1979.
36. BO FRYLESTAM. Population ecology of the European hare in southern Sweden. 1 June 1979.
37. SVEN G. NILSSON. Biologiska samhällen i heterogena miljöer: En studie på fastland och öar. (Biological communities in heterogeneous habitats: A study on the mainland and islands.) 12 October 1979.
38. BJÖRN SVENSSON. The association between *Epoicocladius flavens* (Chironomidae) and *Ephemera danica* (Ephemeroptera). 26 October 1979.
39. GÖRAN HÖGSTEDT. The effect of territory quality, amount of food and interspecific competition on reproductive output and adult survival in the magpie *Pica pica*; an experimental study. 29 February 1980.
40. JON LOMAN. Social organization and reproductive ecology in a population of the hooded crow *Corvus cornix.* 9 April 1980.
41. GÖRGEN GÖRANSSON. Dynamics, reproduction and social organization in pheasant *Phasianus colchicus* populations in South Scandinavia. 26 September 1980.
42. TORSTEN DAHLGREN. The effects of population density and food quality on reproductive output in the female guppy, *Poecilia reticulata* (Peters). 27 February 1981.
43. AUGUSTINE KORLI KORHEINA. Environments and co-existence of *Idotea* species in the southern Baltic. 15 May 1981.
44. INGVAR NILSSON. Ecological aspects on birds of prey, especially long-eared owl and tawny owl. 9 October 1981.
45. TORBJÖRN von SCHANTZ. Evolution of group living, and the importance of food and social organization in population regulation; a study on the red fox (*Vulpes vulpes*). 23 October 1981.
46. OLOF LIBERG. Predation and social behaviour in a population of domestic cat. An evolutionary perspective. 11 December 1981.
47. BJÖRN MALMQVIST. The feeding, breeding and population ecology of the brook lamprey (*Lampetra planeri*). 12 March 1982.
48. INGVAR WÄREBORN. Environments and molluscs in a non-calcareous forest area in southern Sweden. 19 March 1982.
49. MAGNUS SYLVÉN. Reproduction and survival in common buzzards (*Buteo buteo*) illustrated by the seasonal allocation of energy expenses. 26 March 1982.
50. LARS-ERIC PERSSON. Structures and changes in soft bottom communities in the southern Baltic. 23 April 1982.
51. GÖRAN BENGTSSON. Ecological significance of amino acids and metal ions, a microanalytical approach. 24 May 1982.
52. JAN HERRMANN. Food, reproduction and population ecology of *Dendrocoelum lacteum* (Turbellaria) in South Sweden. 10 December 1982.
53. BO EBENMAN. Competition and differences in niches and morphology between individuals, sexes and age classes in animal populations, with special reference to passerine birds. 8 April 1983.
54. HANS KÄLLANDER. Aspects of the breeding biology, migratory movements, winter survival, and population fluctuations in the great tit *Parus major* and the blue tit *P. caeruleus*. 29 April 1983.
55. JOHNNY KARLSSON. Breeding of the starling (*Sturnus vulgaris*). 6 May 1983.
56. CARITA BRINCK. Scent marking in mustelids and bank voles, analyses of chemical compounds and their behavioural significance. 17 May 1983.
57. PER SJÖSTRÖM. Hunting, spacing and antipredatory behaviour in nymphs of *Dinocras cephalotes* (Plecoptera). 1 June 1983.
58. INGE HOFFMEYER. Interspecific behavioural niche separation in wood mice (*Apodemus flavicollis* and *A. sylvaticus*) and scent marking relative to social dominance in bank voles (*Clethrionomys glareolus*). 9 December 1983.
59. CHRISTER LÖFSTEDT. Sex pheromone communication in the turnip moth *Agrotis segetum*. 30 November 1984.

60. HANS KRISTIANSSON. Ecology of a hedgehog *Erinaceus europaeus* population in southern Sweden. 7 December 1984.
61. CHRISTER BRÖNMARK. Freshwater molluscs: Distribution patterns, predation and interactions with macrophytes. 19 April 1985.
62. FREDRIK SCHLYTER. Aggregation pheromone system in the spruce bark beetle *Ips typographus*. 26 April 1985.
63. LARS LUNDQVIST. Life tactics and distribution of small ectoparasites (Anoplura, Siphonaptera and Acari) in northernmost Fennoscandia. 10 May 1985.
64. PEHR H ENCKELL. Island life: Agency of Man upon dispersal, distribution, and genetic variation in Faroese populations of terrestrial invertebrates. 3 June 1985.
65. SIGFRID LUNDBERG. Five theoretical excursions into evolutionary ecology: on coevolution, pheromone communication, clutch size and bird migration. 7 November 1985.
66. MIKAEL SANDELL. Ecology and behaviour of the stoat *Mustela erminea* and a theory on delayed implantation. 8 November 1985.
67. THOMAS JONASSON. Resistance to frit fly attack in oat seedlings, and ecological approach to a plant breeding problem. 13 November 1985.
68. ANDERS TUNLID. Chemical signatures in studies of bacterial communities. Highly sensitive and selective analyses by gas chromatography and mass spectrometry. 3 October 1986.
69. BOEL JEPPSSON. Behavioural ecology of the water vole, *Arvicola terrestris*, and its implication to theories of microtine ecology. 27 May 1987.
70. TORSTEN GUNNARSSON. Soil arthropods and their food: choice, use and consequences. 2 June 1987.
71. THOMAS MADSEN. Natural and sexual selection in grass snakes, *Natrix natrix*, and adders, *Vipera berus*. 4 September 1987.
72. JENS DAHLGREN. Partridge activity, growth rate and survival: Dependence on insect abundance. 4 December 1987.
73. SCOTT GILBERT. Factors limiting growth of sympatric *Peromyscus* and *Clethrionomys* populations in northern Canada. 11 December 1987.
74. OLLE ANDERBRANT. Reproduction and competition in the spruce bark beetle *Ips typographus*. 8 April 1988.
75. EINAR B. OLAFSSON. Dynamics in deposit-feeding and suspension-feeding populations of the bivalve *Macoma baltica*; an experimental study. 29 April 1988.
76. JAN-ÅKE NILSSON. Causes and consequences of dispersal in marsh tits, time as a fitness factor in establishment. 11 May 1988.
77. PAUL ERIC JÖNSSON. Ecology of the southern Dunlin *Calidris alpina schinzii.* 13 May 1988.
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