THE ROYAL SOCIETY

Received 4 July 2002
Accepted 16 September 2002
Published online 19 November 2002

Sex chromosome evolution and speciation in *Ficedula* flycatchers

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Speciation is the combination of evolutionary processes that leads to the reproductive isolation of different populations. We investigate the significance of sex-chromosome evolution on the development of post- and prezygotic isolation in two naturally hybridizing *Ficedula* flycatcher species. Applying a tag-array-based mini-sequencing assay to genotype single nucleotide polymorphisms (SNPs) and interspecific substitutions, we demonstrate rather extensive hybridization and backcrossing in sympatry. However, gene flow across the partial postzygotic barrier (introgression) is almost exclusively restricted to autosomal loci, suggesting strong selection against introgression of sex-linked genes. In addition to this partial postzygotic barrier, character displacement of male plumage characteristics has previously been shown to reinforce prezygotic isolation in these birds. We show that male plumage traits involved in reinforcing prezygotic isolation are sex linked. These results suggest a major role of sex-chromosome evolution in mediating post- and prezygotic barriers to gene flow and point to a causal link in the development of the two forms of reproductive isolation.

Keywords: introgressive hybridization; reinforcement; reproductive isolation; single nucleotide polymorphism; Z-chromosome

1. INTRODUCTION

When differentiated populations come into secondary contact, they may have diverged to such an extent that hybrid offspring have reduced, but still non-zero fitness. Yet, the sexual signals of the two populations may be sufficiently similar for heterospecific mating to occur. The theory of reinforcement suggests, in such cases, that natural selection against the production of unfit hybrids may reinforce premating isolation, eventually completing the speciation process (Dobszhansky 1940). Although intuitively appealing, the theory of reinforcement has been much debated (Felsenstein 1981; Butlin 1987; Sanderson 1989; Liou & Price 1994; Servedio 2000). An important objection has been that, when hybrids are partially fertile, recombination would tend to break down the association between alleles affecting hybrid fitness and alleles promoting prezygotic isolation (Felsenstein 1981; Sanderson 1989). However, reinforcement is much more probable if the two classes of genes (i.e. those affecting hybrid fitness and mate recognition) are physically linked so that recombination is reduced or absent (Felsenstein 1981; Noor *et al.* 2001). Comparative evidence suggests that the genes controlling sex and reproduction-related traits are disproportionally abundant on the macro sex chromosome (X or Z) in various organisms (Reinhold 1998; Hurst & Randerson 1999; Saifi & Chandra 1999; Ritchie 2000; Noor *et al.* 2001; Wang *et al.* 2001). Such traits would include characters associated with postzygotic isolation, such as hybrid sterility, as well as with prezygotic isolation, such as mate preferences and secondary sexual traits used in mate recognition. Accordingly, sex-chromosome evolution may be important in the development of both post- and prezygotic isolation, a fact that has often been neglected in discussions of the reinforcement hypothesis (but see Noor *et al.* 2001).

The breeding distribution of the pied flycatcher (*Ficedula hypoleuca*) and the collared flycatcher (*F. albicollis*) overlaps in Central Europe and on the islands of Gotland and Öland in the Baltic Sea. Apparently, the two species became isolated in southern refuges during the Pleistocene glaciations, and came into secondary contact following the northward expansion of deciduous forests after the last glaciation period (Sætre *et al.* 2001*a*). In these areas of secondary contact, hybridization occurs at a moderate frequency (Sætre *et al.* 1999; Veen *et al.* 2001). In a previous study of a flycatcher, hybrid zone evidence for reinforcement of prezygotic isolation was found, mediated by divergent selection on male plumage characteristics (Sætre *et al.* 1997). Estimates based on hatching success suggest that hybrid fertility follows Haldane's rule (Haldane 1922) in these birds; female hybrids are usually sterile, whereas apparently only a proportion of male hybrids are sterile (Sætre *et al.* 1999; Veen *et al.* 2001).

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We investigate the significance of sex-chromosome evolution on the development of post- and prezygotic isolation in these naturally hybridizing flycatcher species. Applying a tag-array-based mini-sequencing assay to genotype single nucleotide polymorphisms (SNPs) and interspecific substitutions, we investigate the pattern of backcrossing and introgression among sympatric flycatchers. Genes involved in postzygotic isolation and loci linked to such genes are assumed to introgress at a lower rate than loci not affecting hybrid fitness (see Wu 2001). Specifically, we look for reduced rates of introgression at Z-linked genes. We also investigate whether male plumage traits involved in reinforcing prezygotic isolation are sex linked by comparing the phenotypes and genotypes of individuals from sympatry.

2. MATERIAL AND METHODS

Birds were captured on breeding sites. Allopatric collared flycatchers are from Abruzzo, Italy and Breclav, Czech Republic. Allopatric pied flycatchers are from near Madrid, Spain, Lingen, Germany and Oslo, Norway. Sympatric birds are from the Jeseník Mountains, Czech Republic, and Gotland and Öland, Sweden. Measures of plumage and other morphological traits, as well as blood sampling, were performed as previously described (Sætre *et al.* 2001*b*). The fertility of sympatric birds was estimated from hatching success (Sætre *et al.* 1999).

We applied sequence analysis of allopatric representatives of the two species in order to find appropriate autosomal and sex-linked (Z-chromosome) markers for studying hybridization and backcrossing in sympatry. Sequences were obtained from 30 autosomal loci and from four Z-linked genes (Sætre *et al.* 2001*a*; Primmer *et al.* 2002). Previously unpublished sequences are from the autosomal *B-CK* gene and the Z-linked genes *ALDOB*, *GHR* and *PRLR* (accession nos: AY154292–5 and AY154320–57). Autosomal or Z-linked inheritance of the various loci was assumed based on GenBank information on homologues loci in chicken and confirmed by genotyping flycatchers with known sex. Between four and eight pied and collared flycatchers were sequenced at each locus to identify SNPs and potential interspecific substitutions. For phylogenetic analysis, sequences from two semi-collared flycatchers (*F. semitorquata*), five Atlas flycatchers (*F. speculigera*) and two red-breasted flycatchers (*F. parva*) (Sætre *et al.* 2001*a*) were also obtained. Phylogenetic reconstructions were performed using programs implemented in the software PHYLIP (Felsenstein 1993).

Based on the sequence analysis, we chose 10 autosomal and 10 Z-linked nucleotide sites for large-scale genotyping on a microarray platform. Two classes of markers were sought: fixed substitutions and SNPs with great differences in allele frequencies between the two species. Fixed substitutions and SNPs from the following loci were chosen for large-scale genotyping; autosomal loci: *ALASY*, *B-CK*, *Fa45B*, *Fh45*, *FhU4*, *laminA*, *OrDe*, *rhodopsin* (2), and *TGFB2*; Z-linked loci: *ALDOB* (4), *CHD1Z* (1), *GHR* (3) and *PRLR* (2). In addition, we used the autosomal microsatellite locus *FhU1* (previously shown to be a species-specific marker; Sætre *et al.* 2001*a*), and a mitochondrial (mt) control region fragment that harbours a species-specific indel (Sætre & Moum 2000). The latter loci were genotyped using polyacrylamide gel electrophoresis and silver staining (Sætre *et al.* 2001*a*). Because no species differences have been found on the W-chromosome in these birds (Montell *et al.* 2001), we used mtDNA genotype to assess the sex-chromosome genotype of females. Mitochondrial DNA, like W, shows clonal inheritance through the female germ-line and would consequently pass from mother to daughter in parallel with W (Berlin & Ellegren 2001). We investigated hybridization and backcrossing by comparing genotypes of sympatric birds ($n = 302$) with those of allopatric pied flycatchers ($n = 106$) and collared flycatchers ($n = 51$) applying the model-based clustering method of Pritchard *et al.* (2000).

The microarrays were prepared using an isothiocyanate surface for attaching 22 3′ NH2-modified tag oligonucleotides (Interactiva Biotechnologie GmbH) from the Affymetrix GeneChip Tag Array collection as previously described (Lindroos *et al.* 2001). The oligonucleotides were printed as duplicate spots to form 36 subarrays per slide using a ProSys 5510A instrument (Cartesian Technologies, Inc.) with four Stealth Micro Spotting Pins (SMP3) (TeleChem International). A Cy3-labelled oligonucleotide was included in each array as a spotting control and one tag sequence on the array was used as a hybridization control.

Four separate aliquots of pooled PCR products from each sample were treated with exonuclease I and shrimp alkaline phosphatase (USB Corporation). Each mini-sequencing reaction mixture contained this enzyme-treated PCR product, all the 20 tagged mini-sequencing primers, one of the four TAMRA-labelled ddNTPs (NEN™, Life Science Products), the other three unlabelled ddNTPs (Amersham Pharmacia Biotech) Triton-X, Tris–HCl and Thermo Sequenase™ DNA Polymerase (Amersham Pharmacia Biotech). The mini-sequencing reactions were performed in a programmable thermal controller for 99 cycles of 95 °C and 55 °C for 20 s each.

The arrays were preheated to 42 °C in a custom-made reaction rack equipped with a silicon rubber grid forming 36 separate reaction chambers on each slide (Pastinen *et al.* 2000). Four hybridization reactions, corresponding to the mini-sequencing reactions were performed for each sample. The hybridization mixtures, containing mini-sequencing reaction product and a TAMRA-labelled hybridization control oligonucleotide in NaCl and sodium citrate, were added to each reaction chamber on the microscope slide. Four negative control reactions without mini-sequencing reaction product were included on each slide. The hybridization time was 2.5–3 h at 42 °C after which the slides were rinsed with NaCl and sodium citrate at decreasing concentrations and spin dried.

The fluorescence signals were detected and quantified using a Scan Array 5000 instrument (Packard BioScience Ltd) (Lindroos *et al.* 2001). The genotypes were determined with a Microsoft Excel macro accepting signal intensities 300% stronger than background as true signals, followed by manual inspection. Accuracy of genotyping was checked by single-strand conformation polymorphism analysis of three loci (all individuals) and control sequencing of 8–20 individuals for the remaining loci. In all cases, 100% matching genotypes were obtained.

Assignment tests were calculated applying the model-based cluster method of Pritchard *et al.* (2000), as implemented in the software STRUCTURE. Optimization of estimates of relative amount of pied and collared flycatcher alleles in sympatric birds was carried out by simulations in which 1400 pure, hybrid, B_1 and B_2 (first and second generation backcrosses, respectively) genotypes were generated by random sampling from the observed allele frequencies of allopatric birds. Simulated genotypes were analysed using different options available in the STRUCTURE software. The approach of using 100 000 replicates

and a burn-in time of 50 000 steps in which sympatric birds were introduced for classification singularly together with all the allopatric birds, without pre-assigning birds to species, yielded the best fit between the observed and expected estimate (linear regression: root mean square error: 0.09, $r^2 = 0.95$). We chose to present assignment probabilities as estimates of relative amount of pied and collared flycatcher genomic DNA in individual sympatric birds rather than grouping them into hybrid and backcross classes. This was to avoid arbitrary classifications resulting from repeated events of hybridization and backcrossing. Comparisons of morphological traits were carried out using the GLM-procedure as implemented in the software SAS (SAS Institute, Inc. 1996).

3. RESULTS

The autosomal loci were found to be highly polymorphic in these birds (Sætre *et al.* 2001*a*; Primmer *et al.* 2002; present study). At 30 loci, comprising *ca*. 9.6 kb of sequence, we found a total of 93 sites polymorphic in one or both species (SNPs) but only two fixed substitutions. By contrast, at four Z-linked loci comprising *ca*. 2.5 kb of sequence, only eight SNPs were found but 11 fixations. The ratio of fixations (F) to polymorphisms (P) is significantly higher at these Z-linked loci compared with autosomal loci (95% CI for F/P-ratio of the autosomal loci: (0, 0.061), for the Z-linked loci (0.78, ∞); confidence intervals were estimated using 1000 bootstraps, sampling with replacement, under the assumption that loci (genes) evolve independently). The two flycatcher species appear more differentiated than expected at the four Z-linked genes compared with phylogenies estimated using mitochondrial and autosomal gene sequences (figure 1).

Eight of the 10 Z-linked sites chosen for large-scale genotyping were species-specific substitutions (one to three substitutions from each of the four genes). Among sympatric birds only pure or non-recombinant genotypes were observed at Z; that is, two female genotypes: $C_1C_2C_3C_4$ and $P_1P_2P_3P_4$ and three male genotypes: $C_1C_2C_3C_4/C_1C_2C_3C_4$, $P_1P_2P_3P_4/P_1P_2P_3P_4$ and $C_1C_2C_3C_4/P_1P_2P_3P_4$, where C and P denote the two species and the numbers denote genes 1–4. Thus, we find no cases where a Z-chromosome has collared alleles at some locus but pied alleles at others. The other two Z-markers were from different genes (*GHR* and *ALDOB*) and were polymorphic in collared flycatchers. All combinations of the four alleles were found among collared flycatcher females, in proportions not significantly different from the expectation for unlinked genes (goodness of fit: $\chi^2 = 0.36$, $n = 73$, $p = 0.95$). Thus, intraspecific recombination occurs on the Z-chromosome.

With respect to allopatric birds, three of the 11 autosomal markers used in large-scale genotyping were species specific (two interspecific substitutions: *ALASY* and *FhU4*; and one microsatellite with non-overlapping allele frequencies: *FhU1*). Furthermore, one marker was monomorphic in collared flycatchers but polymorphic in pied flycatchers (*laminA*), three were monomorphic in pied flycatchers but polymorphic in collared flycatchers (*rhodopsin1*, *rhodopsin2* and *B-CK*) and four were polymorphic in both species (*Fa45B*, *Fh45*, *OrDe* and *TGFB2*). However, although most loci exhibited some intraspecific polymorphism in one or both species, the allele frequency differences were substantial in each case. Thus, perfect assignment to correct species of allopatric birds was obtained with respect to the autosomal loci (95% CI for assignment probabilities to correct species: (0.998, 0.999)). In sympatry, however, an array of backcrossed genotypes was observed at these loci (figure 2).

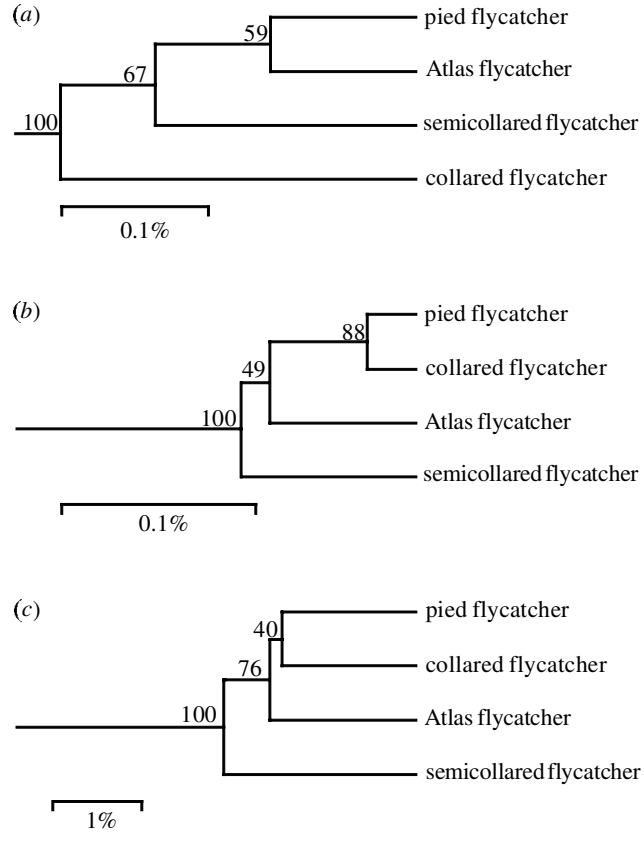

Figure 1. (*a*) Phylogenetic relations among *Ficedula* flycatchers based on DNA sequences from four genes on the Z-chromosome. Phylogenetic reconstructions based on (*b*) autosomal and (*c*) mitochondrial DNA sequences are shown for comparison (from Sætre *et al.* 2001*a*). Fitch–Margoliash trees are presented with genetic distances drawn to scale. The scales refer to Jukes–Cantor distances (%). Values at nodes represent bootstrap replication scores (%) based on 1000 resamplings. The trees are drawn to approximately similar relative scales (with respect to genetic distances between pied and Atlas flycatchers) to facilitate comparisons of relative rates of evolution. The red-breasted flycatcher was used for outgroup rooting (not included in the tree).

Fitness estimates from the field (hatching success) show that all seven females with a hybrid sex-chromosome genotype were sterile, whereas three of 11 males heterozygous at Z were sterile ($\chi^2 = 6.5$, d.f. = 1, $p = 0.011$). All other birds were apparently fertile. In no cases are Z-linked genes (or mtDNA) from one of the species found to cross the B_1 stage of the other species (figure 2). Thus, our results show that Z-linked genes, or the Z-chromosome, have a major influence on hybrid fertility.

Introgression of autosomal alleles into the two species was strikingly asymmetric (figure 2). Excluding hybrids and other birds heterozygous at the sex chromosomes, only one of 124 pied flycatchers (0.8%) had an introgressed allele (defined as an allele not found among allopatric conspecifics but occurring in sympatric

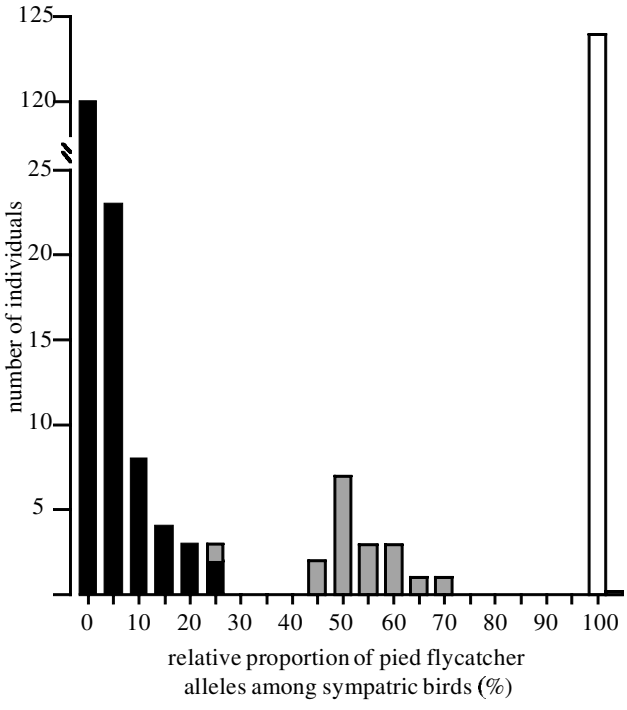

Figure 2. Frequency distribution of genotypes among sympatric flycatchers (to nearest 5%). The scale on the *x*-axis can be interpreted as the relative amount of autosomal pied and collared flycatcher alleles in the birds, as estimated from assignment probabilities to species (here shown with respect to the pied flycatcher). Note that all allopatric collared and pied flycatchers were assigned as having respectively 0% and 100% autosomal pied flycatcher alleles (not included in the figure). The shades of the bars refer to genotype of the sex chromosomes. Black bars, collared flycatcher genotype; grey bars, hybrid genotype; open bars, pied flycatcher genotype.

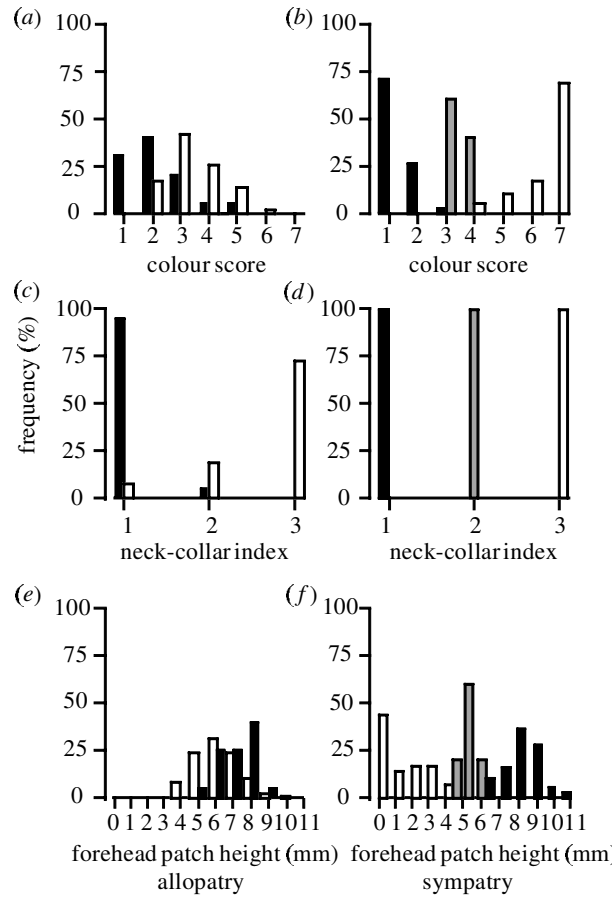

Figure 3. Frequency distribution of three sex-limited male plumage characteristics among allopatric (*a*,*c*,*e*) and sympatric (*b*,*d*,*f*) pied and collared flycatchers and their hybrids. Black bars, collared flycatchers; grey bars, hybrids (and backcrosses heterozygous at Z-linked genes); open bars, pied flycatchers. Allopatric collared flycatchers (from Italy) have, on average, a darker plumage colour (colour score 1, black head and back; 7, greyish brown) ($F_{1,195} = 33.0$, $p < 0.0001$), a larger neck-collar (neck collar index 1, a complete white collar; 3, no collar) ($F_{1,501} = 44.5$, $p < 0.0001$) and a higher (larger) white forehead patch ($F_{1,461} = 13.5$, $p < 0.0003$) than allopatric pied flycatchers (from Spain). These traits show character displacement since the differences are accentuated in sympatry (Jeseník Mountains, Czech Republic) to the extent that there is no longer overlap between the species. Males that are heterozygous at Z-linked genes have an intermediate phenotype.

heterospecifics). By contrast, 58 out of 160 collared flycatchers (36%) had at least one introgressed allele ($\chi^2 = 51.2$, d.f. = 1, $p < 0.0001$).

Character displacement is evident for three sex-limited male plumage characteristics: plumage colour, neck-collar size and forehead patch height (figure 3). We tested whether these traits may be linked to the Z-chromosome by comparing the phenotypes and genotypes of birds from the sympatric populations (table 1). For comparison, we performed similar tests on two phenotypic traits which are not sex limited and show no signs of character displacement, but for which there are clear differences between the species: width of white wing patch and wing length. All males heterozygous at the Z-linked genes (including two individuals more likely to be B_1s rather than F_1 hybrids) had an intermediate phenotype with respect to the three sex-limited traits (figure 3). Applying factorial ANCOVA we found that the Z-chromosome (but not autosomal genotype) has a significant influence on the expression of the three sex-limited traits. By contrast, autosomal genotype (but not the Z-chromosome) has a significant influence on the expression of the two traits that are found in both sexes (table 1).

4. DISCUSSION

Sequence comparisons showed that the autosomal loci exhibited a high frequency of intraspecific polymorphism but a low frequency of interspecific substitutions. By contrast, the Z-linked loci showed an opposite pattern, i.e. little polymorphism but a high frequency of substitutions. The effective population size of a Z-linked locus is ideally three-quarters that of an autosomal locus since the female only has one copy of the chromosome. Thus, a reduced level of polymorphism is to be expected at Z-linked loci, the magnitude increasing with decreasing effective population sizes. Current effective population sizes of these species are large and previous analysis of microsatellite and mtDNA markers do not indicate severe historical

Table 1. Results of variance analysis testing for Z-linked and autosomal inheritance of morphological traits.
(Type 1: traits expressed in males only; type 2: traits expressed in both sexes. In the analysis of genetic contributions on response variables we controlled for factors known to affect the traits, age (first year or older), sex and locality (Jeseník Mountains, Gotland or Öland). The significant interactions between Z and locality are caused by large phenotypic differences between the pied, but not collared, flycatcher populations. Czech pied flycatchers (and hybrids) have lighter plumage colours and smaller forehead patches than Swedish birds. Non-significant interaction terms were excluded from the analysis. (n.d.f., nominator degrees of freedom; d.d.f., denominator degrees of freedom.))

response variable	type	source	n.d.f.	d.d.f.	MS	F	p	r^2 model
neck-collar	1	Z	2	88	3.9	39.0	0.0001	0.99
		autosome	1	88	0.008	0	0.78	
		age	1	88	1.3	12.7	0.0006	
		locality	2	88	0	0.03	0.99	
colour score	1	Z	2	108	3.8	7.3	0.001	0.88
		autosome	1	108	0.4	0.9	0.36	
		age	1	108	4.4	8.5	0.004	
		locality	2	108	16.5	31.9	0.0001	
		Z∗locality	3	108	17.2	33.3	0.0001	
forehead patch	1	Z	2	108	37.6	3.6	0.03	0.86
		autosome	1	108	0.07	0.01	0.93	
		age	1	108	96.7	9.3	0.003	
		locality	2	108	96.1	9.2	0.0002	
		Z∗locality	3	108	92.6	8.9	0.0001	
wing-patch	2	Z	2	196	4.55	1.3	0.27	0.66
		autosome	1	196	58.5	17.0	0.0001	
		age	1	196	152.4	44.3	0.0001	
		locality	2	196	23.7	6.9	0.001	
		sex	1	196	218.8	63.6	0.0001	
wing length	2	Z	2	202	3.9	1.5	0.22	0.59
		autosome	1	202	36.6	14.6	0.0002	
		age	1	202	35.2	14.0	0.0002	
		locality	2	202	28.4	11.3	0.0001	
		sex	1	202	122.7	48.8	0.0001	

bottleneck events (Haavie *et al.* 2000; Sætre *et al.* 2001*a*). Thus, in the case of these species the difference in ratio of inter- and intra-specific polymorphism at Z-linked and autosomal loci appears to be far greater than could be ascribed to differences in effective population size. We suggest that a probable explanation for the observed pattern is sweeps of selection in one or both species at Z-linked genes. This would lead to fixations and loss of polymorphism at the sites under selection and at linked sites (Aguadé *et al.* 1989). More frequent selective sweeps are to be expected at the Z-chromosome due to a reduced rate of recombination (which only occurs in the homogametic sex) and because recessive alleles with a bearing on fitness are not masked by dominance in females (Charlesworth *et al.* 1987; Begun & Whitley 2000). The selective sweep hypothesis is also apparently supported by phylogenetic comparisons. Although the bootstrap support for the phylogenetic trees was relatively low, the comparisons indicate that the pied and the collared flycatcher are more differentiated at Z-linked loci than expected from phylogenies estimated from autosomal and mtDNA loci. The substantial evolutionary divergence of Z-linked genes is likely to have had significant effects on the development of barriers to gene flow (see below).

Among sympatric birds, only pure or non-recombinant genotypes were observed at Z. We find no cases where a Z-chromosome has collared alleles at some loci but pied alleles at others. The other two Z-markers from different genes (*GHR* and *ALDOB*) were polymorphic in collared flycatchers. All combinations of the four alleles were found among collared flycatcher females, in proportions not significantly different from the expectation for unlinked genes. Thus, intraspecific recombination occurs. Accordingly, the Z-chromosome genotype results imply either (i) that hybrids have a fitness of zero, (ii) that interspecific recombination of the Z-chromosome is prohibited, for example, due to a chromosomal rearrangement in one of the species (Rieseberg 2001; Noor *et al.* 2001), or (iii) that recombinant genotypes are efficiently removed from the mixed populations by selection.

The results from the autosomal loci, however, enable us to reject the first of the three proposals suggested above; hybrids do contribute to the gene pool. In sympatry, an array of backcrossed genotypes was observed at the autosomal loci. Fitness estimates from the field (hatching success) show that all seven females with a hybrid sex-chromosome genotype were sterile, whereas three of 11 males heterozygous at Z were sterile. All other birds were apparently fertile. In no cases are Z-linked genes (or mtDNA) from one of the species found to cross the B_1-stage of the other species. Thus, whether interspecific recombination occurs or not, our results show that Z-

linked genes, or the Z-chromosome, have a major influence on hybrid fertility. Further studies are needed to investigate whether the apparent absence of recombinant genotypes at our Z-markers is also influenced by a chromosomal rearrangement.

Muller–Dobzhansky incompatibilities (epistasis) between Z-linked genes and other genes (e.g. autosomals) can explain the observed pattern of introgression and fitness of the various hybrids and backcrosses (Coyne & Orr 1998). Females are hemizygous for Z-linked genes. Thus, if the Z-linked gene(s) affecting fertility in hybrids and backcrosses are partially recessive (Turelli & Orr 2000), the incompatibilities would affect females more severely than males. Unfortunately, however, the low number of autosomal loci available in this study makes it impossible to infer further details regarding the genetic architecture of hybrid sterility. The likelihood of identifying autosomal genomic regions associated with hybrid incompatibilities is low when only 10 independent loci are at hand.

Introgression of autosomal alleles into the two species was strikingly asymmetric. Alleles of collared flycatcher origin were almost completely absent among pied flycatchers whereas a large proportion of the collared flycatchers had at least one allele of pied flycatcher origin. One possible explanation for this pattern is that some genetic or epigenetic mechanism renders introgression from collared to pied flycatchers less likely than vice versa, such as meiotic drive (Hurst & Schilthuizen 1998) or an asymmetry in genetic incompatibilities with respect to the pied and the collared flycatcher genomes. Alternatively, asymmetric introgression could result if pied flycatcher females were more selective than collared flycatcher females. The latter proposal is not supported by empirical data, however (Veen *et al.* 2001). Finally, the observed pattern could simply be a result of the demography of these birds. In the three hybrid zones investigated here, the collared flycatcher is the numerically dominant species. Hence, events of backcrossing are more likely to involve a collared than a pied flycatcher (Veen *et al.* 2001). In addition, two of the hybrid zones are on islands where collared flycatchers are geographically isolated from continental populations, whereas gene flow of pied flycatchers would occur from the surrounding mainland (Lundberg & Alatalo 1992; Sætre *et al.* 1999; Veen *et al.* 2001). The two latter factors render introgression from pied to collared flycatchers more likely than vice versa.

Correlative evidence suggested that the expression of three male-specific plumage traits, previously shown to be involved in reinforcement of prezygotic isolation (Sætre *et al.* 1997), are influenced by genes on the Z-chromosome. By contrast, apparently only autosomal genes influence the expression of two other morphological traits that are found in both sexes, which do not show signs of character displacement but in which there are clear species differences. We note, however, that the power of the above analysis depends on different unknown factors. First, this is a very coarse-grained linkage mapping analysis with only two categories (Z or autosomal). Second, the likelihood of detecting an autosomal influence of a given trait depends both on the number of genes affecting the trait and their pattern of inheritance (e.g. dominance). Clearly, correct mapping (especially in the case of autosomal inheritance) is most likely if many independent loci having an additive genetic effect on the trait are involved. Finally, another potential problem is collinearity between autosomal and Z-genotype. As evident from figure 2, a significant proportion of the sympatric birds consists of individuals with pure genotypes at Z and autosomes and individuals with effectively hybrid genotypes at both sets of chromosomes. Nevertheless, the highly significant effects revealed suggest that genes on the Z-chromosome influence the male-specific traits involved in reinforcement. Indeed, only males heterozygous at Z (including birds more likely to be B_1 rather than F_1 hybrids) had an intermediate, hybrid appearance. Moreover, birds with a high proportion of autosomal alleles of pied flycatcher origin but with collared flycatcher genotype at Z were indistinguishable from pure collared flycatchers at these traits.

Our results suggest that Z-linked genes have a major influence on hybrid sterility, and indicate that traits associated with species recognition may be linked to the same chromosome. Linkage between genes affecting hybrid fertility and traits used in species recognition can explain why reinforcement operates despite rather extensive introgression and recombination of autosomal genes. This conclusion also points to a plausible causal link between the development of post- and prezygotic isolation. Divergent selection on sex-limited traits, confined to the sex chromosome, may affect postzygotic isolation through effects on hybrid fertility as well as prezygotic isolation through effects on secondary sexual characteristics, i.e. traits involved in mate choice and species recognition. We suggest that these conclusions may be generally applicable to organisms with sex chromosomes. Evidence is accumulating that genes affecting hybrid fitness and sexually selected traits are often sex linked (Coyne & Orr 1998; Reinhold 1998; Hurst & Randerson 1999; Saifi & Chandra 1999; Ritchie 2000; Turelli & Orr 2000; Noor *et al.* 2001; Wang *et al.* 2001). Thus, reinforcement may be a more likely outcome than predicted from existing models (Felsenstein 1981; Butlin 1989; Sanderson 1989; Liou & Price 1994; Servedio 2000). We suggest that postzygotic and prezygotic barriers to gene flow may have a common evolutionary origin and that the sex chromosome(s) is the main arena where gene flow is brought to a halt.

The authors thank G. Kärf, K. Larsson, M. Lindersson, R. Figueroa and P. Nádvornik for help in the laboratory, Centro Studi Ecologici Appenninici, C. Berg, S. Bures, M. Král, T. Lubjuhn, J. Potti and J. Moreno for field assistance, morphological data or blood samples, K. Räsänen and N. Smith for help with programming and data analyses and J. T. Lifjeld, L. H. Rieseberg, S.-A. Sæther, M. R. Servedio and two anonymous referees for comments on the manuscript. Financial support was received from the Swedish Research Council, the Norwegian Research Council, O. & L. Lamms Memorial Foundation, Uddenberg-Nordingska Foundation, Wenner-Gren Foundation, K. & A. Wallenberg Foundation, The Royal Society, the University of Helsinki and The Finnish Academy.

REFERENCES

Aguadé, M., Miyashita, N. & Langley, C. H. 1989 Reduced variation on the *yellow-achaete-scute* region in natural populations of *Drosophila melanogaster*. *Genetics* **122**, 607–615.

Begun, D. J. & Whitley, P. 2000 Reduced X-linked nucleotide polymorphism in *Drosophila simulans*. *Proc. Natl Acad. Sci. USA* **97**, 5960–5965.

Berlin, S. & Ellegren, H. 2001 Clonal inheritance of avian mitochondrial DNA. *Nature* **413**, 37–38.

Butlin, R. 1987 Speciation by reinforcement. *Trends Ecol. Evol.* **2**, 8–13.

Butlin, R. 1989 Reinforcement of premating isolation. In *Speciation and its consequences* (ed. D. Otte & J. A. Endler), pp. 158–179. Sunderland, MA: Sinauer.

Charlesworth, B., Coyne, J. A. & Barton, N. H. 1987 The relative rates of evolution of sex chromosomes and autosomes. *Am. Nat.* **130**, 113–146.

Coyne, J. A. & Orr, H. A. 1998 The evolutionary genetics of speciation. *Phil. Trans. R. Soc. Lond.* B **353**, 287–305. (DOI 10.1098/rstb.1998.0210.)

Dobszhansky, T. 1940 Speciation as a stage in evolutionary divergence. *Am. Nat.* **74**, 312–321.

Felsenstein, J. 1981 Scepticism towards Santa Rosalia, or why are there so few kinds of animals. *Evolution* **35**, 124–138.

Felsenstein, J. 1993. *Phylip: phylogeny inference package*, version 3.5.c. Seattle, WA: University of Washington.

Haavie, J., Sætre, G.-P. & Moum, T. 2000 Discrepancies in population differentiation at microsatellites, mitochondrial DNA and male plumage colour in the pied flycatcher—inferring evolutionary processes. *Mol. Ecol.* **9**, 1137–1148.

Haldane, J. S. B. 1922 Sex-ratio and unisexual hybrid sterility in animals. *J. Genet.* **12**, 101–109.

Hurst, L. D. & Randerson, J. P. 1999 An eXceptional chromosome. *Trends Genet.* **15**, 383–385.

Hurst, G. D. D. & Schilthuizen, M. 1998 Selfish genetic elements and speciation. *Heredity* **80**, 2–8.

Lindroos, K., Liljedahl, U., Raito, M. & Syvänen, A. C. 2001 Minisequencing on oligonucleotide microarrays: comparison of immobilisation chemistries. *Nucleic Acids Res.* **29**, E69-9.

Liou, L. W. & Price, T. D. 1994 Speciation by reinforcement of premating isolation. *Evolution* **48**, 1451–1459.

Lundberg, A. & Alatalo, R. V. 1992 *The pied flycatcher*. London: T. & A. D. Poyser.

Montell, H., Fridolfsson, A.-K. & Ellegren, H. 2001 Contrasting levels of nucleotide diversity on the avian Z and W. *Mol. Biol. Evol.* **18**, 2010–2016.

Noor, M. A. F., Grams, K. L., Bertucci, L. A. & Reiland, J. 2001 Chromosomal inversions and the reproductive isolation of species. *Proc. Natl Acad. Sci. USA* **98**, 12 084-12 088.

Pastinen, T., Raito, M., Lindroos, K., Tainola, P., Peltonen, L. & Syvänen, A. C. 2000 A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays. *Genome Res.* **10**, 1031–1042.

Primmer, C. R., Borge, T., Lindell, J. & Sætre, G.-P. 2002 Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. *Mol. Ecol.* **11**, 603–612.

Pritchard, J. K., Stephens, M. & Donelly, P. 2000 Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.

Reinhold, K. 1998 Sex linkage among genes controlling sexually selected traits. *Behav. Ecol. Sociobiol.* **44**, 1–7.

Rieseberg, L. H. 2001 Chromosomal rearrangement and speciation. *Trends Ecol. Evol.* **16**, 351–358.

Ritchie, M. G. 2000 The inheritance of female preference functions in a mate recognition system. *Proc. R. Soc. Lond.* B **267**, 327–332. (DOI 10.1098/rspb.2000.1004.)

Sætre, G.-P. & Moum, T. 2000 A simple molecular method for species identification of pied and collared flycatchers. *Hereditas* **132**, 171–172.

Sætre, G.-P., Moum, T., Bures, S., Král, M., Adamjan, M. & Moreno, J. 1997 A sexually selected character displacement in flycatchers reinforces premating isolation. *Nature* **387**, 589–592.

Sætre, G.-P., Král, M., Bures, S. & Ims, R. A. 1999 Dynamics of a clinal hybrid zone and a comparison with island hybrid zones of flycatchers. *J. Zool. Lond.* **247**, 53–64.

Sætre, G.-P., Borge, T., Lindell, J., Moum, T., Primmer, C. R., Sheldon, B. C., Haavie, J., Johnsen, A. & Ellegren, H. 2001*a* Speciation, introgressive hybridization and nonlinear rate of molecular evolution in flycatchers. *Mol. Ecol.* **10**, 737–749.

Sætre, G.-P., Borge, T. & Moum, T. 2001*b* A new bird species? The taxonomic status of 'the Atlas flycatcher' assessed from DNA sequence analysis. *Ibis* **143**, 494–497.

Saifi, G. M. & Chandra, H. S. 1999 An apparent excess of sex and reproduction related genes on the human X chromosome. *Proc. R. Soc. Lond.* B **266**, 203–209. (DOI 10.1098/rspb.1999.0623.)

Sanderson, N. 1989 Can gene flow prevent reinforcement? *Evolution* **43**, 1223–1235.

SAS Institute, Inc. 1996 *Sas propriety software release 6.12*. Cary, NC: SAS Institute, Inc.

Servedio, M. R. 2000 Reinforcement and the genetics of nonrandom mating. *Evolution* **54**, 21–29.

Turelli, M. & Orr, H. A. 2000 Dominance, epistasis and the genetics of postzygotic isolation. *Genetics* **154**, 1663–1679.

Veen, T., Borge, T., Griffith, S. C., Sætre, G.-P., Bures, S., Gustafsson, L. & Sheldon, B. C. 2001 Hybridization and adaptive mate choice in flycatchers. *Nature* **411**, 45–50.

Wang, P. J., McCarrey, J. R., Yang, F. & Page, D. C. 2001 An abundance of X-linked genes expressed in spermatogonia. *Nat. Genet.* **27**, 422–426.

Wu, C.-I. 2001 The genic view of the process of speciation. *J. Evol. Biol.* **14**, 851–865.

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