Supporting Online Material for

Sex Chromosome–Linked Species Recognition and Evolution of Reproductive Isolation in Flycatchers

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Supporting Online Material

"Sex chromosome-linked species recognition and evolution of reproductive isolation in flycatchers" (Sæther *et al*.)

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Materials and Methods

We tested whether the female species recognition that causes assortative mating in flycatchers is due to 1) autosomally inherited preferences, 2) sexual imprinting, or 3) sex-linked preferences. We did this in two steps, both involving testing mutually exclusive pair-wise predictions that enabled us to produce a unique combination of predictions for each of the three scenarios. First, we distinguished between autosomal inheritance versus paternal determination (i.e., 1 vs. 2 or 3) by examining mate choice of hybrid females differing in their parental combination. Then, we distinguished between sexual imprinting and genetic inheritance (i.e., 2 vs. 1 or 3) by cross-fostering offspring between the two species and identifying their subsequent mate choice.

Mate choice of hybrids

The fieldwork was conducted in Northern Moravia, Czech Republic from 1985-2005, and on the islands of Öland and Gotland, closely situated in the Baltic Sea, Sweden, from 1980-2005. We caught females during incubation and males when feeding nestlings. Since female flycatcher hybrids are sterile the nests are eventually abandoned when the eggs do not hatch, and the mates of female hybrids are therefore difficult to catch. This problem was solved by temporarily introducing nestlings from a nest-box nearby so we could catch the male when feeding the foster-nestlings. The paternal and maternal species identity of female hybrids was established with DNA analysis (see below). We genotyped a total of 31 female F_1 hybrids with male partners of known species identity (14 from Öland, 9 from the Czech population, and 8 from Gotland). The three areas of sympatry are qualitatively similar in many respects. In all areas the collared flycatcher outnumbers the pied flycatcher (the proportion of Collared to Pied is about 0.70 on Öland, 0.85 in the Czech study areas and 0.96 on Gotland); F_1 hybrids constitute a relatively low percentage of the breeding population (about 4% on Öland, 3% in the Czech population and 2% on Gotland) and heterospecific pairing is rare compared to within-species pairing (e.g. Sætre *et al.* 1999; Veen *et al.* 2001). Therefore, we pooled data on female hybrids from the three areas, but checked if patterns held within the Czech and the Swedish hybrid zones.

We also genetically identified the paternal species of 42 male hybrids from the same areas as the female hybrids and identified the species of their partner based on morphology and vocalizations. If assortative mating is due to paternally determined mate preferences, there should be a relationship between the parental combination of female hybrids and the species of their partner, but not for male hybrids. We expect absence of such a relation in male hybrids for two separate reasons. First, male birds inherit a Z chromosome from each parent, and the parental combination of male hybrids should hence not matter if preferences were genetically inherited at the Z. Second, empirical data suggest that flycatcher males, unlike females, do not discriminate against mating with heterospecifics (Sætre *et al.* 1997b), and male hybrids are consequently expected to mate independently of their parental combination. Therefore, the mating pattern of male hybrids provides a nice test of whether mating of female hybrids according to their parental combination could be due to something else than paternally determined mate preferences (e.g. biased occurrence of certain hybrids in relation to the frequency of the two parental species).

Genetic identification of hybrids and their parental species combination

Females of suspected hybrid identity on basis of plumage and/or whole clutches of unhatched eggs were genotyped for species-specific markers. Since mitochondria are inherited through the female germ-line, the species identity of a hybrid's mother was determined using a species-specific mtDNA marker. Similarly, since females are the heterogametic sex (ZW) in birds, the species identity of a hybrid female's father was determined with a species-specific marker on the Z chromosome. In addition, F1 hybrid status was also confirmed for the majority of birds using autosomal species-informative SNPs and species-specific substitutions, altogether 34 autosomal markers from 23 different genes (Borge *et al*. 2005a). The two methods always agreed about F1 hybrid status.

Male hybrids are easier to identify as such based on plumage, but to be sure to exclude later generation backcrosses or aberrant individuals of either species, we identified suspected male F1 hybrids as such by autosomal species-specific substitutions and SNPs, in the same way as described above for females (Borge *et al*. 2005a). The species of their mother was then identified by a species-specific mtDNA marker as for female hybrids.

25 μL blood was collected from each individual by puncturing the brachial vein and the blood was suspended in 1mL Queen's lysis buffer (Seutin *et al.* 1991) or in ethanol. DNA was extracted from the blood samples by overnight incubation at 45°C with 50 μL of Proteinase K solution (10 mg/mL) and 50 μL of 10% SDS. Each sample was extracted with two rounds of standard phenol/chloroform treatment before DNA was recovered by ethanol precipitation, dried, and re-dissolved in 500-1000 μL TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5).

We amplified a stretch of mtDNA containing a 32 bp species-specific indel as described in Sætre & Moum (2000). We used 10 μL PCR reactions containing 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.32 μM of each primer, 1 μg of Bovine Serum Albumine (BSA), 0.3 units of HotStar DNA polymerase (Qiagen), 1X PCR Buffer (Qiagen) and 20ng DNA. On a PTC 225 (MJ Research), 35 cycles of amplification with 94°C for 30 seconds (s), 60°C for 30 s and 72°C for one minute (min) were preceded by 15 min pre-denaturation at 95°C and followed by a prolonged 10 min extension step at 72°C. The species-specific PCR fragments were compared on a 4% agarose gel fixed in ethidium-bromide and the species identity determined based on fragment length comparisons of reference samples (see Sætre & Moum 2000 for further detail).

We used the Z-linked locus *CHDZ-20* that has several nucleotide positions with a fixed difference between the species (Borge *et al*. 2005). The primers *CHDZ-20F*: 5´-GAA GAG AGC TGA AAC TCG G-3 and *CHDZ-20R*: 5´-TCA TCT TCA TCC ATA TTG G-3´were used with the same reaction mix and PCR protocol as described above except that the fragment was amplified with two amplification steps and with different annealing temperatures: first 5 cycles, then 30 cycles with annealing temperatures of 58°C and 50°C, respectively. The fragments were purified using ExoSAP-IT (Amersham Biosciences) and direct sequenced using original PCR primers with the DYEnamic cycle sequencing kit (Amersham Biosciences), and analyzed on a MegaBACE 1000 (Amersham Biosciences) instrument. Sequences from both directions were aligned and edited in Sequencher 4.1 (Gene Codes).

Mate choice of pure female recruits from mixed species pairs (naturally cross-fostered extra-pair recruits)

To separate the effects on mate choice of genetically inherited species recognition and preferences learned by sexual imprinting, we analyzed the mate choice of females reared by heterospecific foster fathers on the Swedish island of Gotland in the Baltic Sea (1980-2005). We assumed that female hybrid flycatchers are sterile, strongly supported by previous genetic studies (e.g., Gelter *et al.* 1992, Veen *et al*. 2001). Female recruits that did not show evidence of reduced fertility (i.e., had eggs that hatched) and that came from nests of mixed pairs were thus identified as offspring resulting from conspecific extra-pair copulations. In pairs with a male Pied and a female Collared, on average 56 % of offspring are fathered by conspecific extra-pair males, as shown using molecular markers (Veen *et al.* 2001).

Cross-fostering experiment

To further test if females are sexually imprinted on heterospecific males, we conducted cross-fostering experiments (on Öland) by reciprocal transfer of half the brood between nests of the two species. Nests were matched for clutch size (± 1 egg) and laying date (same date). Chicks were transferred when 3 days old, temporarily identified by toe nail clipping and later permanently identified by numbered leg rings. Since we wanted to track the origin of individual offspring, we did not transfer eggs and assumed instead that any sexual imprinting effects would be negligible before age 3 days old. This assumption is supported by the fact that flycatcher offspring do not fully open their eyes until 7 days old (Creutz 1955). In total, 361 offspring cross-fostered during 2002-2004 (from 138 experimental broods) survived to fledging. (A larger number was initially cross-fostered, but predation rates before fledging were sometimes extensive.) Data on the species of the breeding partner of females that had been cross-fostered were gathered in 2003-2005.

Null-model expectations of how preferences translate into mate choice: assortative mating of recruits from pure pairs

Because of constraints of mate availability and other factors, female flycatchers are not expected to always mate with a male of the preferred species. We assumed that a female having a preference for a certain species would have a probability of mating with a male of that species equal to the proportion of females of the preferred species that mated conspecifically. For example, if a female prefers Collared males and 95% of Collared females mate with Collared males, then we assume that she has a 95% probability of mating with a Collared male, irrespective of whether she is herself a Pied or a Collared. Male flycatchers do not seem to discriminate against heterospecific females (Sætre *et al*. 1997b), so the species of a female *per se* should not influence whether or not a female actually obtains a mate of the preferred species. The overall pattern of assortative mating in each sympatric population may thus be used as a measure of the expected mate choice under conspecific mate preference. We therefore calculated, in each population, the proportion of females of either species mated to Collared males, Pied males and hybrid males. As the expected mate choice pattern of cross-fostered Collared females under genetic inheritance of species recognition, we used the overall mating pattern of Collared females in the population (proportion of breeding females paired to Collared males).

Similarly, we used the mating pattern of females of the foster father species (proportion of Pied females paired with Pied males) as the alternative mating pattern, expected under imprinted preferences. Expected mate choice of cross-fostered Pied females was simply the reverse of the expectations for the cross-fostered Collared females.

However, the overall mating pattern in the population might provide a biased expectation of how preferences translate into mate choice due to several potential pitfalls. First, immigrants from allopatric populations could potentially have different preferences than local recruits (and non-local recruits are more common among pied than collared flycatchers). Second, in the complete data-set, some pure females may have been raised by a heterospecific foster father (due to conspecific extra-pair copulations by females in mixed pairs) and could potentially have developed preferences for heterospecific males due to sexual imprinting (although our results show that imprinting was not important). And last, misidentification of female species could occasionally occur (Pied and Collared females look very similar, and Pied females mated to males of the numerically more common Collared may in particular have been overlooked). To avoid these potential biases in expected values, we also calculated expectations based on the mate choice of a restricted sample of local female recruits from known pure pairs (excluding cross-fostered recruits). This should provide a more precise estimate of the probability that a female with a certain preference actually obtains a mate according to the preference, but we present results from both approaches since the sample sizes were sometimes small for the females of known pedigree.

Since the proportion of the two species and the overall mating patterns differed somewhat among populations, the expected mating patterns were calculated specific to the population where the particular mating pattern of females reared by heterospecific fathers was collected (Gotland for naturally cross-fostered extra-pair recruits, and Öland for experimentally cross-fostered recruits).

Supporting Online Text

Reduced recombination between loci located at the Z chromosome

We base our assumption that physical linkage at the Z chromosome also imply genetic linkage (reduced recombination) on four lines of evidence: 1) there are reduced interspecific recombination rates at the Z chromosome compared to autosomes for crosses between these two particular species (i.e., the Z is sheltered against gene flow). 2) There are reduced intraspecific recombination rates at the Z in one of the species and in birds in general, as predicted by theoretical arguments concerning 3) the Z chromosome in particular and 4) physical linkage in general. These facts all imply that loci situated on the Z chromosome are genetically linked in our system, and taken together the evidence for reduced recombination is very strong. We outline each of these four arguments in more detail below.

1. From genetic analyses of flycatcher hybrids and backcrosses (Sætre *et al.* 2003) - using species-specific markers located at the Z chromosome and autosomes - we have strong evidence that there is very little or no recombination at the Z chromosome between

the two species (whereas autosomes recombine between species, and there is some recombination at the Z within species). This clearly demonstrates that species-specific alleles at loci physically linked at the Z sex chromosome will also be genetically linked because there is no (or very little) introgression of genes on the sex chromosomes between the two gene pools - in contrast to the situation for autosomes. There is no reason to believe that this is unique to flycatchers, but no information is, to our knowledge, available from other organisms with a ZW sex chromosome system.

2. Empirical results from mapping of the chicken genome shows that recombination rates at the Z is only about 25-50 % of that found on autosomes (see e.g. Sundström *et al.* 2004). Pedigree-based linkage-mapping in one of our species shows that the Z chromosome has even lower recombination rates in flycatchers than in the chicken (Backström *et al.* 2006a), and that the Z exhibit high levels of long-range linkage disequilibrium between markers (at least up to a distance of 500kb; Backström *et al.* 2006b).

3. Because females only have one Z, any two loci at the Z may only recombine in males (outside the pseudo-autosomal region). From this consideration alone, two Z-linked loci will therefore be expected to have only half the recombination rate of two loci at an autosomal chromosome.

4. Unless the probability for crossover is 1 (complete non-linkage), two loci on the same chromosome will have a reduced recombination rate compared to if they were at different chromosomes.

There does not have to be complete genetic linkage (zero crossover probability) for our finding of a physical linkage between these genes to be beneficial for speciation (Felsenstein 1981). Any crossover probability less than one (partial linkage) would facilitate reinforcement (Servedio 2000). Compared to a situation where these loci are located at different chromosomes, our finding of a physical linkage therefore already suggests that there is likely to be some genetic linkage, and more probably so because we find not autosomal linkage, but Z-linkage. Moreover, genetic linkage for the particular situation we are analyzing is directly established by the finding that sex chromosomes do not recombine between the two species (Sætre *et al.* 2003).

Implications for the flycatcher system

Although *a priori* unlikely (at least in the flycatcher system), the design of our study also allowed us to exclude maternally determined species recognition (W-linked, or learned by sexual imprinting on the mother). Maternal species recognition predicts that female hybrids should mate with males belonging to the same species as their mother, opposite to what was found. We also exclude the possibility of random mating because assortative mating is very strong in the hybrid zones (see expected patterns in Fig. 2 in the main text of this study, Alatalo *et al*. 1990, Veen *et al*. 2001). Furthermore, the results support Z-linkage in both flycatcher species.

Most female hybrids mated according to the paternal species, but a few did not. This is not surprising even under strict paternal determination of partner preferences given that 2-7% of Collared females and 15-30 % of Pied females in sympatry are involved in heterospecific mating (Alatalo *et al.* 1982, Sætre *et al.* 1999, Veen *et al.* 2001, this study). We have no strong evidence that flycatcher females that mate with heterospecific males actually have a preference for such males over conspecifics, since most of these females

mate with conspecific males at other breeding attempts (Qvarnström *et al.* 2006a). Instead, mate availability, time and available territory quality may constrain the mate choice of females. Hence, a particular preference is not expected to always translate into a particular choice of mate (see calculation of expected values above).

Regarding the cross-fostering experiment, the final sample sizes became quite small due to the low return rate of females (only 9 females returned as breeding adults of the estimated 361/2 = 180.5 female fledglings i.e., 5 %). However, the mating patterns obtained are very unlikely to have occurred under sexual imprinting on the foster father since all cross-fostered female Collared recruits ($N = 7$) mated with Collared males (compared to 16% or less on Öland expected from imprinting, binomial $P < 0.001$) and the two female Pied recruits both mated with Pied males (compared to 3% or less expected from imprinting, binomial $P < 0.001$). We would have expected the usual mating patterns to become reversed under sexual imprinting, but they were clearly not.

Our results suggest that a previously reported divergence of mate preferences in female flycatchers in sympatry compared to allopatry (Sætre *et al*. 1997a) has a genetic basis and is not simply an accidental side-effect of a change in male plumage followed by sexual imprinting on different-looking males. This is important to establish since there is otherwise a danger of circularity if one argues that male plumage has changed in sympatry in response to reinforced female preferences. We still do not know the ultimate reason why male plumage and female preferences have changed in sympatry, but our results are consistent with reinforcement due to selection against hybridization, facilitated by physical linkage among the genes involved in reproductive isolation.

Recent work in the collared flycatcher has found low heritability of a female preference for males with larger forehead patches and low genetic correlation between this preference and the preferred trait (Qvarnström *et al*. 2006b). This finding is not in contradiction to our result of a genetic determination of preferences for species-assortative mating since only within-species variation in a mate preference was considered in that study. Entirely different evolutionary dynamics may be involved in generating genetic correlations between preferences and preferred traits within as compared to among species (see also Blows 1999).

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