INVITED REVIEW

Ecology and genetics of speciation in *Ficedula* flycatchers

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Abstract

Birds have for long been popular study objects in speciation research. Being easy to observe in the field, they have traditionally been particularly important in studies of behavioural and ecological factors in speciation, whereas the genetic aspects of the process have been studied in other organisms, such as *Drosophila*. More recently, however, a stronger genetic focus has been placed on speciation research also in birds. Here, we review ecological, behavioural and genetic studies on speciation in the pied flycatcher (*Ficedula hypoleuca*) and the collared flycatcher (*Ficedula albicollis*). These well-studied birds provide among the few proposed examples of the process of reinforcement of premating isolation, and the evidence for reinforcement is strong. They are further characterized by having strong intrinsic postzygotic barriers (female hybrid sterility), yet the two species appear to be very similar ecologically. This is in stark contrast to another well-studied bird complex, Darwin's finches, in which the species differ vastly in ecologically important traits but have no developmental problems arising from genetic incompatibilities, and where no evidence for reinforcement is found. In the flycatchers, sex chromosome linkage of genes affecting traits associated with both pre- and postzygotic barriers to gene exchange is likely to facilitate reinforcement. We discuss whether such sex-linkage may be common in birds. The contrast between flycatchers and Darwin's finches indicate that speciation in birds cannot always be understood mainly as a result of divergent natural selection ('ecological speciation'), and generalizations from one system may lead us astray. We discuss to what extent insight from research on the flycatchers may point to fruitful avenues for future research on bird speciation and specifically call for a more systematic effort to simultaneously investigate ecology, behaviour and genetics of birds caught in the process of speciation.

Keywords: adaptive radiation, Darwin's finches, flycatchers, hybridization, introgression, postzygotic isolation, premating isolation, reinforcement

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Introduction

For reasons that may not always be entirely obvious, certain organisms become the target of intensive empirical research in specific fields of biology. Examples of such model organisms are particularly evident in genomics, including *Drosophila* (Adams *et al.* 2000),

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Arabidopsis (The Arabidopsis Genome Initiative 2000) and our own species (Lander *et al.* 2001; Venter *et al.* 2001). However, also in other fields, such as ecology and evolution, well-studied organisms are important in our efforts to understand the living world. For example, what would we have known about predator–prey interactions without the lynx–snowshoe hare system (e.g. Elton & Nicholson 1942; Stenseth *et al.* 1999; Krebs *et al.* 2001), about sympatric speciation without cichlid fishes (e.g. Turner *et al.* 2001; Kocher 2004; Barluenga *et al.* 2006) or about ecological adaptive radiations

without Darwin's finches (Geospizinae, e.g. Lack 1947; Grant 1999; Grant & Grant 2002, 2008)? By focusing on selected empirical systems, we gain from the accumulation of specific knowledge and get to sharpen the precision of and dig deeper into the questions of interest. These model systems are important for the scientific progress in biological research. One potential danger, however, is to generalize insights from research on model organisms too eagerly.

Within the field of bird speciation, Darwin's finches have arguably been the most prominent model system ever since David Lack's classical publications (e.g. Lack 1947). These finches exemplify important concepts and processes such as the significance of ecological adaptation, niche differentiation, ecological character displacement and adaptive radiation (see e.g. Grant & Grant 2008 and Price 2008, for recent reviews). However, when comparing speciation research on birds with that on other important model systems, such as *Drosophila* (e.g. Coyne & Orr 2004), Price (2008) holds that birds have been particularly important for investigating the ecological aspects of speciation, whereas the genetic aspects, at least the genetics of hybrid incompatibilities, have been better addressed in other organisms. In other words, research on the genetics of speciation has lagged behind in avian studies.

Here, we review behavioural, ecological and genetic research related to speciation in the pied flycatcher (*Ficedula hypoleuca*) and the collared flycatcher (*Ficedula albicollis*). These birds have been popular study objects for behavioural and ecological questions for decades (see e.g. Lundberg & Alatalo 1992 for an early review). Due to their locally high abundance and habit of preferring artificial nest boxes as breeding sites, it is relatively easy to obtain good estimates and reasonable sample sizes for important fitness components such as survival and reproductive success in these birds (e.g. Slagsvold 1986; Gustafsson & Sutherland 1988). Such data, often in combination with clever field experiments, have been used to investigate a wide range of phenomena, including mate choice (Dale *et al.* 1992), life history trade-offs (Pärt *et al.* 1992), natural selection on specific traits (Merilä *et al.* 1998) and responses to climate change (Both *et al.* 2006). In recent years, the flycatchers have also become the target of genetic and genomic investigations (e.g. Primmer *et al.* 2002; Borge *et al.* 2005a; Backström *et al.* 2006a,b, 2008; Buggiotti *et al.* 2008). Speciation research on the flycatchers builds on the combined legacy of a well-studied ecology and recent genetic advances. Below we review this body of research and discuss how insight from the flycatcher system compares with other model systems of bird speciation, in particular Darwin's finches.

Phylogeny and evolutionary history

The pied and the collared flycatcher are part of a species complex that also includes the Atlas flycatcher (*Ficedula speculigera*) and the semicollared flycatcher (*Ficedula semitorquata*; Sætre *et al.* 2001a,b). Whereas females of these species have a brownish plumage, males are generally conspicuously coloured in black and white and the species complex can be referred to as the black-and-white flycatchers. The *Ficedula* genus (Muscicapidae) comprises some 31 species of forest dwelling passerines. According to a phylogenetic study by Outlaw & Voelker (2006), the closest relatives of the black-and-white flycatchers are the red-breasted flycatcher (*Ficedula parva*) and the taiga flycatcher (*Ficedula albicilla*). The *Ficedula* genus is mainly distributed throughout South-East Asia, whereas the black-and-white flycatchers constitute a western radiation (Sætre *et al.* 2001b; Outlaw & Voelker 2006).

The taxonomy and evolutionary relationship between the various black-and-white flycatchers was much debated during the premolecular era (Lundberg & Alatalo 1992), but there is now strong genetic evidence that an ancestral species radiated into four distinct lineages *c.* 1.5–2 Ma (Fig. 1, Sætre *et al.* 2001b, 2003), coinciding approximately in time with the cooling climate at the Pliocene–Pleistocene transition. The most easterly distributed semicollared flycatcher first split off from the others, reflecting an initial east–west bifurcation event, followed basically by a trifurcation of the western species (Fig. 1). Their current distribution is depicted in Fig. 2. The Atlas flycatcher breeds in Northwest Africa and the semicollared flycatcher in Southeastern Europe, Asia Minor, Caucasus and northern Iran. These two species are strictly allopatric. The pied and collared flycatchers are widely distributed across Europe and have overlapping breeding ranges in Central and Eastern Europe as well as on the Baltic Isles of Gotland and Öland off the east coast of Sweden (Fig. 2).

Based on their phylogeny and current distribution, it has been suggested that the four flycatcher lineages (species) survived the last glaciation period in four different refugia (Sætre *et al.* 2001a): North Africa (the Atlas flycatcher), around Caucasus (the semicollared flycatcher), Italy (the collared flycatcher) and the Iberian Peninsula (the pied flycatcher). The latter two have expanded north and eastwards after the last glaciation and eventually came into secondary contact in Central and Eastern Europe (Sætre *et al.* 2001a, 2003), and very recently also on Gotland and Öland (Lundberg & Alatalo 1992). Indeed, collared flycatchers colonized Öland as recently as the late 1950s and Gotland somewhat earlier (but probably no more than *c.* 150 years ago: Lundberg & Alatalo 1992). It is unclear whether and what
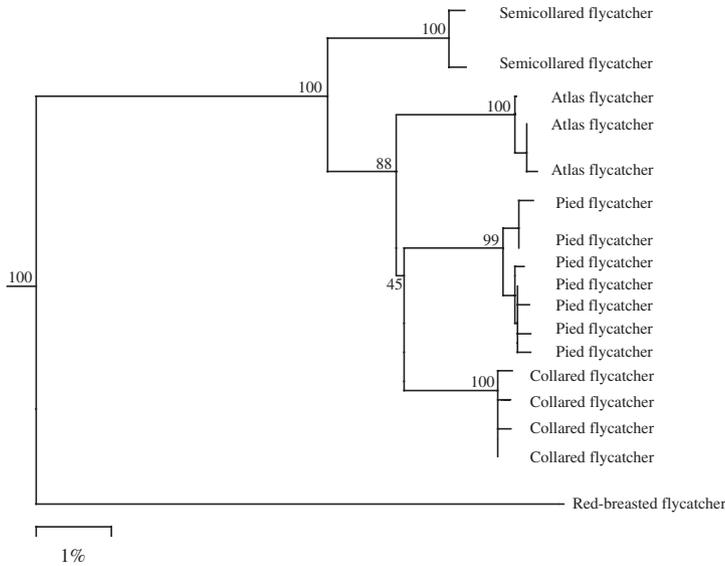
Fig. 1 Phylogenetic relations among the four black-and-white *Ficedula* flycatcher species, using the red-breasted flycatcher *Ficedula parva* for outgroup rooting, based on mitochondrial ND6 sequences according to a neighbour-joining analysis. Values at the nodes represent bootstrap replication scores (from Sætre *et al.* 2001b).
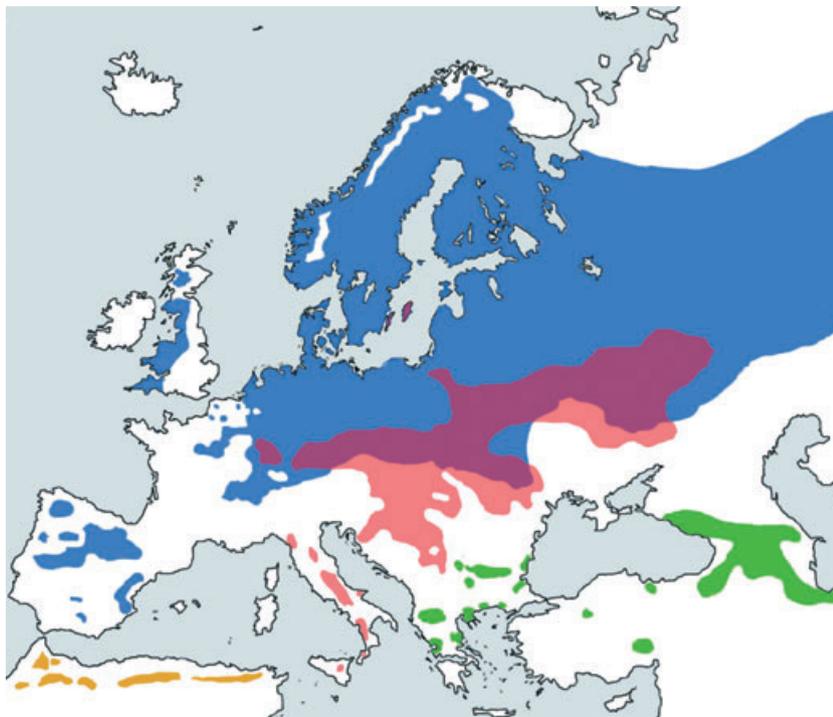
Fig. 2 Breeding distribution of the four black-and-white *Ficedula* flycatcher species: Atlas flycatcher *Ficedula speculigera* (yellow), collared flycatcher *Ficedula albicollis* (red), pied flycatcher *Ficedula hypoleuca* (blue) and semicollared flycatcher *Ficedula semitorquata* (green). The areas of distributional overlap between the pied and collared flycatcher are indicated in purple. The map is based on Cramp & Perrins (1993) as well as personal observations and information from colleagues.

flycatcher species bred in the Balkan Peninsula during the last glaciation. The semicollared flycatcher may have expanded westwards from the Caucasus after the last glaciation. It now occurs close to the distribution of collared flycatcher in the Central Balkans, but, to our knowledge, no sympatric populations have been found.

Forest-dwelling organisms, in particular insect-eaters such as flycatchers, must have been greatly affected by the climatic fluctuations during the Pleistocene. During cold periods forests were restricted to isolated patches

in the Southwestern Palearctic, including the Iberian Peninsula, Italy, Balkans, and south and east of the Caucasus Mountains (e.g. Hewitt 2000). Hence, prolonged periods of geographic isolation have limited or hindered gene flow among lineages isolated in different forest refugia, allowing taxonomic divergence. By combining data from phylogenetic reconstructions with current geographic distribution, molecular ecologists can suggest likely scenarios for the recent demographic history of extant taxa, as well as likely locations of refugia

during the last glaciation and routes of recolonization after the ice sheet retracted (reviewed in Hewitt 2000). However, for many investigated taxa, including the flycatchers, lineage splitting clearly predates the onset of the last glaciation. Reconstructing the phylogeography of such lineages is therefore difficult. The lineages may have experienced past episodes of secondary contact, e.g. during warm interglacial periods, and these may have affected their evolution. Comparisons of divergence and polymorphism of loci from different parts of the genome may, however, shed light on a complex demographic and evolutionary history (e.g. Wakeley & Hey 1997; Hey & Machado 2003). Episodes of introgression, e.g. during phases of secondary contact in past interglacial periods, or of selection, is likely to only affect parts of the genome whereas demographic processes such as historic changes in population size will have genome-wide effects. Indeed, using multilocus sequence analysis, Borge et al. (2005a) found results consistent both with historical introgression events as well as signs of selection in the genomes of the pied and collared flycatcher.

Ecological divergence and competition in sympatry

Even though there may have been repeated episodes of contact in the past, the genomes of the four flycatcher species are clearly divergent (Sætre et al. 2001a, 2003; Borge et al. 2005a) and combined with their current distribution, this suggests a long history of restricted gene flow in allopatry. If the environments of the two species differed, this may have led to local adaptation and divergence in ecological resource-exploiting traits. Such traits may then have further diverged in sympatry due to ecological interactions through a process of character displacement, which resulted in reduced interspecific competition and facilitated coexistence. However, there is little to suggest that such a process has been important in the speciation of the flycatchers. Some differences in plumage and vocalization are apparent (e.g. Sætre et al. 2001b), possibly reflecting divergent sexual selection, but the species appear to be very similar ecologically.

Pending further research on the Atlas and semicollared flycatchers, we now narrow our focus to the better-studied pied and collared flycatchers. The diet of both species varies geographically and largely reflects the local abundance of different insect species. The feeding techniques (Alerstam et al. 1978) and diet (Bureš 1995; Wiley et al. 2007) of the two species overlap to a large extent when in the same habitat and area. Both species breed in deciduous forests, but the pied flycatcher also occurs in mixed and coniferous forests

over large parts of its distribution (Lundberg & Alatalo 1992; Veen et al. 2010). In the absence of the collared flycatcher, the pied flycatcher has higher reproductive success in the kind of deciduous forests preferred by the collared flycatcher (Lundberg & Alatalo 1992). In sympatric areas in Central Europe, there is segregation by habitat and altitude between the two species (Sætre et al. 1999a). This habitat segregation appears to be partly due to interference competition over nest holes, where the collared flycatcher is socially dominant (Löhrl 1955; Lundberg & Alatalo 1992). On the other hand, individuals of the two species prefer their natal breeding habitat and have habitat-specific foraging skills (Adamík & Bureš 2007), possibly acquired through learning.

Existence of interspecific competition is illustrated by the last decades' expansion of collared flycatchers on the island of Öland into deciduous woodlands where only pied flycatchers were breeding previously. Pieds are now becoming rare in these areas (Qvarnström et al. 2009). A similar relative decrease in pied flycatchers has been observed in the Czech Republic in recent years (M. Král pers. comm.). Indeed, a time series analysis of the Czech population provides evidence for interspecific competition: the density of collared flycatchers is negatively affecting the breeding density of pied flycatchers whereas collared flycatchers are mainly affected by variation in annual breeding conditions (Sætre et al. 1999b). It is thus tempting to speculate that the recent decline of pied flycatchers in the two mixed populations is linked to recent climate change, in which improved breeding conditions for the collared flycatcher has led to stronger negative effects of interspecific competition on the pied flycatcher. Walankiewicz et al. (1997), in contrast, found breeding densities of the two species to be positively correlated in Białowieża, Poland, although this may be confounded by an increase in density of both species over time in their study area. Finally, former mixed populations in which the collared flycatcher has been replaced entirely by the pied flycatcher are also found, e.g. in Germany (M. Král, pers. comm.).

In addition to being more affected by annual variation in breeding conditions in investigated mixed-species populations, the collared is also more sensitive to variation in conditions within the breeding season, and its reproductive success declines more rapidly late in the season than that of the pied flycatchers (Wiley et al. 2007). When offspring are cross-fostered between nests of the two species, however, collared flycatcher offspring reared by pied flycatcher foster-parents do very well also late in the season (Qvarnström et al. 2005). The collared flycatcher lays a smaller clutch, has a faster growth rate, and offspring that beg more intensively for food (Qvarnström et al. 2007), which has been

suggested to reflect adaptations to better cope with environmental variation (Qvarnström *et al.* 2009). Alternatively, these differences mirror adaptations to slightly different environments in the two flycatchers in which the mixed populations tend to occur in marginal habitat for the collared flycatcher.

Admittedly, we do not yet fully understand how competition and adaptation to different environments have interplayed to shape the population dynamics and co-existence of the two species throughout their breeding range. Our conclusion is that they are two ecologically quite similar species, but that the pied flycatcher is able to breed successfully over a wider range of conditions than the collared flycatcher, which in turn is socially dominant over pieds in direct interference competition over nest holes. Given that they also hybridize to some extent in sympatry, what is keeping them apart and how did such barriers evolve?

Hybrid zones and the study of reproductive barriers

Barriers against reproduction between divergent taxa are often assumed to evolve as an indirect effect of divergence whilst in geographic isolation (Coyne & Orr 2004). Although much has been learned about divergence and adaptation relevant for speciation from studies of allopatric model systems (see e.g. Price 2008, for a review), hybrid zones have arguably been the most prominent empirical model systems in speciation research. Hybrid zones are important, in part because they can be seen as representing evolutionary 'snap shots' on the road towards reproductive isolation (e.g. Barton & Hewitt 1985, 1989; Via 2009). Comparative studies of hybrid zones may thus yield information on the speciation sequence, such as the order at which various pre- and postzygotic barriers to gene exchange evolve (Price & Bouvier 2002; Price 2008). Birds generally appear to evolve intrinsic postzygotic barriers quite late in the speciation sequence compared with, e.g. mammals (Price & Bouvier 2002; Coyne & Orr 2004; Fitzpatrick 2004); such barriers may even not be evident before long after speciation is complete and may thus not have contributed to speciation. However, the pied and collared flycatchers have evolved unusually strong intrinsic postzygotic barriers given their rather recent split (Price & Bouvier 2002). Yet, a number of factors contribute to reduce gene flow between the two species (Table 1).

Premating barriers

A premating barrier is any behaviour that causes assortative mating between (incipient) species. In an

Table 1 A classification of barriers to gene flow between pied and collared flycatchers

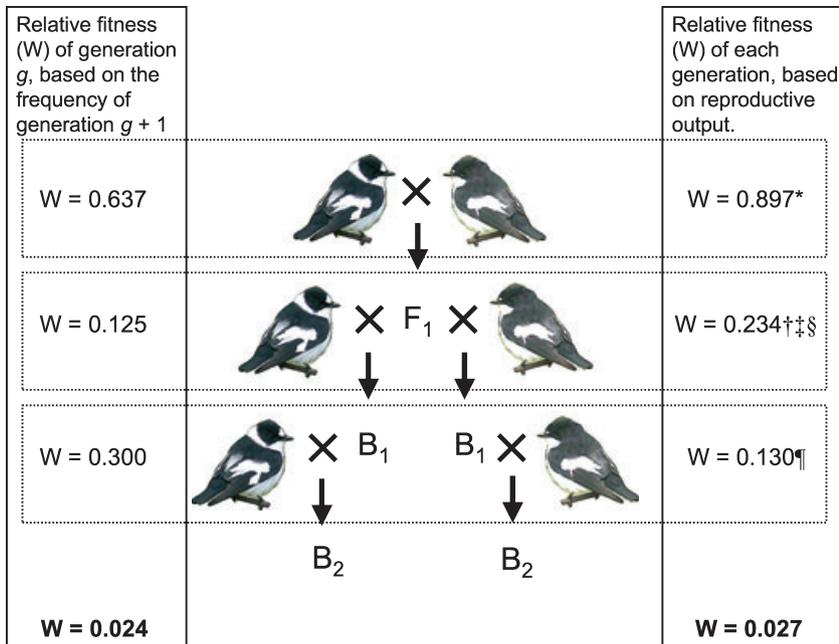| |
|---|
| Premating barriers |
| Behavioural isolation (species-assortative female mate preferences for species-specific male plumage and song characteristics) |
| Ecological isolation |
| Habitat isolation (partial habitat segregation in sympatry) |
| Temporal isolation (collared flycatchers start breeding on average slightly earlier than pied flycatchers) |
| Postmating, prezygotic barriers |
| *Mechanical isolation (no evidence)* |
| Copulatory behavioural isolation (females in heterospecific pairs have extra-pair copulations with conspecific males) |
| Gametic isolation (possible conspecific sperm precedence) |
| Postzygotic barriers |
| Extrinsic |
| *Ecological inviability (no evidence)* |
| Behavioural sterility (male hybrids are less successful in obtaining mates and those that obtain mates are often cuckolded) |
| Intrinsic |
| *Hybrid inviability (no evidence)* |
| Hybrid sterility (female hybrids are sterile, male hybrids have reduced fertility and also first and second generation backcrosses have low fertility) |

Modified from Coyne & Orr (2004). Potential barriers in animals, but not found among these flycatchers are shown in italic.

intraspecific context, mate choice may be caused by direct preferences for specific traits in a potential mate or by indirect mechanisms such as being at a time or place often frequented by certain members of the opposite sex (Wiley & Poston 1996; Widemo & Sæther 1999). Whether mate choice is direct or indirect has evolutionary consequences because different kinds of traits are likely to be affected (Sæther *et al.* 2005). Assortative mating between species is likewise a pattern that may be caused directly by different preferences for species-specific traits in potential mates or indirectly by mechanisms such as different microhabitat preferences or segregation in timing of breeding. Whether assortative mating is direct or indirect may have important implications for speciation, such as for divergent evolution through reinforcement. For example, preferences based on habitat or timing of breeding may be under stronger ecological constraints than preferences based on arbitrary secondary sexual traits, and hence be less likely to evolve.

In sympatric areas of pied and collared flycatchers, the frequency of heterospecific pairs is lower than expected from random mating (Alatalo *et al.* 1982;. Sætre *et al.* 1999a; Veen *et al.* 2001). This pattern is in part due to direct assortative mate preferences among

Relative fitness (W) of generation *g*, based on the frequency of generation *g* + 1		Relative fitness (W) of each generation, based on reproductive output.
W = 0.637		W = 0.897*
W = 0.125		W = 0.234†‡§
W = 0.300		W = 0.130¶
W = 0.024		**W = 0.027**

Fig. 3 Fitness of various hybrid generations relative to nonhybrids. The product (shown in bold) of the estimates for each generation gives the fraction of grand-offspring produced by heterospecific pairing as compared to mating with conspecifics. Estimates within the left column are derived from frequencies of the different hybrid generations found breeding in the population according to Wiley *et al.* 2009; whereas the right column contains data on relative reproductive output obtained from various published sources (*Veen *et al.* 2007; †Svedin *et al.* 2008; ‡Veen *et al.* 2001; §Gelter *et al.* 1992; and ¶Wiley *et al.* 2009). The figure is taken from Wiley *et al.* (2009).

females. In aviary trials, female pied and collared flycatchers chose to build a nest with the conspecific male when given the choice between a conspecific and a heterospecific (Sætre *et al.* 1997a). Male flycatchers, in contrast, do not seem to discriminate between females of the two species and will court both conspecifics and heterospecifics with a similar intensity (Sætre *et al.* 1997b).

Although direct preferences are involved, other mechanisms also contribute to assortative mating (Table 1). Collared flycatchers breed slightly earlier than pieds on average, but with extensive overlap in breeding phenology (Alatalo *et al.* 1990; Sætre *et al.* 1999a; Qvarnström *et al.* 2009). Moreover, the two species occur in different relative densities in different forest types (Sætre *et al.* 1999a; Veen *et al.* 2010) and have corresponding differences in habitat preferences (Adamík & Bureš 2007). Thus, at a larger scale that takes into account the partial habitat segregation of the two species, assortative mating is stronger than it appears at the local scale. Both microhabitat preferences and preferences for species-specific sexual traits appear to be important among flycatchers for overall assortative mating and thus for reducing gene flow (Table 1). Nevertheless, hybridization does occur, and between 2% and 7% of breeding flycatchers are hybrids according to estimates from different mixed-species populations (Alatalo *et al.* 1990; Sætre *et al.* 1999a; Veen *et al.* 2001).

Postzygotic barriers

Alatalo *et al.* (1990) observed that (putative) female flycatcher hybrids often fail to hatch their clutches, whereas eggs in nests attended by male hybrids often hatch normally. As extra-pair paternity was suspected in these birds, Gelter *et al.* (1992) used DNA fingerprinting to investigate if male hybrids were indeed fertile: six of seven male hybrids sired own offspring, but 22% of the young had extra-pair fathers. This pattern has later been confirmed by genetic studies using high-resolution genetic markers to identify hybrids and backcrosses using much larger sample sizes: not only do female hybrids appear invariably to be sterile but also males have somewhat reduced fertility (Table 1, Sætre *et al.* 1999a; Veen *et al.* 2001; Wiley *et al.* 2009). This pattern is in accordance with Haldane's (1922) rule, i.e. more severe fitness reduction in hybrids of the heterogametic sex (females in birds) than in the homogametic sex.

Although the intrinsic postzygotic barriers are evidently not absolute as male hybrids are (partially) fertile, extrinsic postzygotic barriers may further reduce gene flow between the species. Moreover, intrinsic barriers may persist beyond the hybrid generation and diminish the genetic contribution of hybridization much more than is apparent from surveying the population. This is exactly what Wiley *et al.* (2009) demonstrated in a recent analysis (Fig. 3). Using high-resolution species-specific SNPs, these authors compared the relative fitness of pure-bred pairs and hybridization events beyond the hybrid generation and into to the first and second backcross generation. The accumulated fitness loss of heterospecific pairing was dramatic: in terms of number of great grand-offspring produced, heterospecific pairing has <3% of the fitness of conspecific pairing (Fig. 3). This was due both to female sterility persisting beyond the F1 generation and that male backcrosses

sired a low proportion of the offspring produced in their nests (Table 1).

In principle, the observed fitness reduction may be due to sexual selection against hybrids and backcrosses, intrinsic incompatibilities (partial sterility beyond the hybrid generation) and lower survival (natural selection against hybrids). Wiley *et al.* (2009) demonstrated that the reduction in relative fitness was at least partially due to intrinsic barriers, as not only hybrids but also identified backcrosses had reduced fertility. This was especially apparent in females, but again also in males. Other studies have found that sexual selection against hybrids and backcrosses is also involved: Svedin *et al.* (2008) found evidence for lower mating success of hybrid males from long-term field data; hybrid males known to be alive were less often found breeding than pure males of either species (Table 1). Genetically pure pied and collared flycatcher males but with an intermediate, hybrid-like plumage experienced a similar reduction in mating success as male hybrids, suggesting a direct role of sexual selection on male plumage traits (Svedin *et al.* 2008). No evidence for reduced survival of hybrids or other sources of ecological postmating isolation has been found although it has been looked for (Veen *et al.* 2007), probably reflecting the ecological similarity of the two species.

Comparing the estimates of fitness consequences of heterospecific pairing from the early publications such as Alatalo *et al.* (1990), via Sætre *et al.* (1999a), Veen *et al.* (2001), Svedin *et al.* (2008) and finally to Wiley *et al.* (2009), more and stronger barriers against gene flow have now been identified in the flycatchers. Clearly, reproductive isolation is easily underestimated from field surveys of heterospecific pairs and hatching success of pairs involving a hybrid: there is much less gene flow between the two species than expected from the frequency of heterospecific pairs. Detailed field studies and experiments, aided by species-diagnostic genetic markers have been necessary for accurate estimates of reproductive isolation in the flycatchers (Wiley *et al.* 2009).

Postmating, prezygotic barriers vs. possible benefits of heterospecific pairing

Males of both species as well as female pied flycatchers all experience very low fitness returns from heterospecific pairing (Veen *et al.* 2001; Wiley *et al.* 2009). However, for collared flycatcher females that pair up with heterospecifics, some of the costs are reduced by extra-pair fertilization from conspecific males (Table 1, Veen *et al.* 2001). Some direct benefits of heterospecific pairing have also been suggested for collared flycatcher females (Wiley *et al.* 2007). Late in the breeding season,

territories occupied by pied flycatcher males do not suffer the same temporal decline in quality as typical collared flycatcher territories do. Accordingly, a female collared flycatchers paired with a male pied flycatcher will enjoy higher survival of her offspring, and some of these will not be hybrids due to extra-pair copulations with conspecific males, possibly aided by conspecific sperm precedence (Table 1, Veen *et al.* 2001; Wiley *et al.* 2007). However, it is highly doubtful that these benefits and reduced costs can offset the dramatic fitness loss of producing hybrid offspring, and heterospecific pairing should in our view not be considered an adaptation in flycatchers. The extra-pair offspring in heterospecific pairs must have twice the fitness of other collared flycatcher offspring or heterospecific pairs must produce twice as many offspring as normal pairs if heterospecific pairing is to be adaptive for collared flycatcher females (Wiley *et al.* 2009). There is no evidence that this is the case. We therefore believe that heterospecific pairing is maladaptive also for collared flycatcher females (or, at best, a best-of-a-bad job situation if no conspecific male is available). Heterospecific pairing may be explained by imperfect species recognition (i.e., some heterospecific males are acceptable to at least some females) in combination with constraints on the mate sampling activity of females and limited availability of conspecific mates (Sætre *et al.* 1999a; Wiley *et al.* 2009). It is well known that limited availability of conspecific mates for the rarer of two hybridizing species (Randler 2002) is related to frequency of hybridization. In the investigated flycatcher populations, the rarer species is normally the pied flycatcher. However, the frequency of heterospecific pairing involving female collared flycatchers increases over the breeding season (Veen *et al.* 2001), suggesting a seasonal decline in availability of conspecific mates also for the more numerous species.

Imperfect species recognition

The males chosen as mates by heterospecific females are not a random sample of flycatcher males. We can envisage a continuum in male attractiveness to a female where most heterospecific males are at the lower extreme. Some of the heterospecific males will be more attractive, i.e. those males that have sexual signals more similar to the conspecific males. We may thus expect such males to be more often involved in heterospecific mating. Indeed this seems to be the case. In the collared flycatcher, there is delayed plumage maturation, where the first year plumage of males more resembles the plumage of adult pied flycatchers. Collared flycatcher males mated to female pied flycatchers are often young, and when experimentally given a choice between young

and adult collared flycatcher males, pied flycatcher females prefer the young males that are more similar in plumage to conspecific males (Wiley *et al.* 2005).

A parallel situation is found in male pied flycatchers. Here, it is the song that may resemble the other species. Many pied flycatcher males in sympatry learn elements of the song of the collared flycatcher. Singing this 'mixed song' (Alatalo *et al.* 1990; Haavie *et al.* 2004) is associated with a 30% probability of mating with a heterospecific female on Öland (Qvarnström *et al.* 2006). In fact, no males singing the normal allopatric pied flycatcher song (35% of pieds in sympatry on Öland) have been found to hybridize with collared flycatcher females. The incidence of mixed singing very accurately predicts the frequency of heterospecific pairing among male pied flycatchers on Öland (Qvarnström *et al.* 2006).

Sexual signals (plumage and song) affect hybridization risk in the two species, and because hybridization is costly, we may expect selection on such traits to act differently in sympatry than it does in allopatry. Indeed, males with phenotypes that are within the acceptable range of heterospecific females may have been selected against in sympatry and preferences among females shifted away from heterospecific males through the process of reinforcement of premating barriers.

Signal divergence in sympatric flycatchers and the theory of reinforcement

The pied and collared flycatchers are famous for providing one of relatively few empirical examples of reinforcement of prezygotic isolation. When hybrids have low fitness, then natural selection may favour traits that reduce hybridization risk (Dobzhansky 1940; Butlin 1987; Servedio & Noor 2003). Such reinforcement may result in a sympatric character displacement of traits used in mate choice and the preferences for those traits (Servedio & Noor 2003).

Drost (1936) quantified plumage colour variation among male pied flycatchers and appears to be the first to report a geographic pattern in colour variation in the species, males being light, brown-coloured in Central and Eastern Europe but darker elsewhere. Many hypotheses have been put forward to explain this geographic pattern over the years, such as variation in the strength of selection for crypsis against predators (von Haartman 1985). That idea was early dismissed, however, because of lack of evidence (e.g. Lundberg & Alatalo 1992). Rather, the occurrence of light-coloured populations coincides quite well with the area of distributional overlap with the collared flycatcher, with increasingly dark-coloured populations being found at increasing distances from sympatry (Lundberg & Alatalo 1992; Røskaft & Järvi 1992; Sætre *et al.* 1993; Huhta *et al.* 1997; Haavie *et al.* 2000; Lehtonen *et al.* 2009), indicating that interaction between the two species has caused the colour change.

There is strong evidence for reinforcement among sympatric flycatchers (Sætre *et al.* 1997a, 2003; Haavie *et al.* 2004; Sæther *et al.* 2007). First, as explained above, the dramatic fitness consequences of heterospecific mating in sympatric flycatchers causes strong selection against hybridization (Wiley *et al.* 2009). Second, female pied flycatcher mate preferences for male plumage colour are displaced in sympatry accompanied by a displacement in the male trait (Sætre *et al.* 1997a). The diverged preferences are not simply a consequence of females being imprinted on the plumage characteristics of local males, but are genetically inherited (Sæther *et al.* 2007). The resulting colour-change from a dark black to a light brown dorsal plumage makes pied flycatchers more different from black-and-white collared flycatcher males. In accordance with reinforcement, there is also evidence of character displacement in plumage characteristics among collared flycatcher males, males being darker and having larger patches of white on the forehead and the neck in sympatry than in allopatry (Sætre *et al.* 2003). Sympatric female collared flycatchers prefer these traits (Sætre *et al.* 1997a) and although allopatric collareds appear more or less to prefer the same traits (e.g. Hegyi *et al.* 2007), it seems reasonable that the accentuation of the male traits in sympatry is caused by stronger female preferences. Finally, experimental evidence shows that female flycatchers of both species are significantly less likely to choose a heterospecific male when given a choice between a male pied and a collared flycatcher with divergent plumage traits (as found in sympatry) than when choosing among males with more similar plumage traits (as found in allopatry) (Sætre *et al.* 1997a). In short, a sympatric character divergence in genetically determined female mate preferences has apparently produced a corresponding character displacement in male plumage traits that reduces maladaptive hybridization (Sætre *et al.* 1997a; Sæther *et al.* 2007).

Plumage characteristics function as signals both between and within the sexes. A brown-coloured male pied flycatcher resembles a female. Both pied and collared flycatcher males will actually court such a female-like male pied intruder, apparently mistaking him for a prospecting female, but will attack a black-and-white coloured one (Slagsvold & Sætre 1991; Sætre *et al.* 1993; Sætre & Slagsvold 1996). Avoiding aggression from collared flycatcher males by such (apparent) female mimicry could be adaptive, enabling pied flycatchers to establish breeding territories in high-quality habitats

Table 2 Observed character changes (displaced character) in the Central European flycatcher hybrid zone and predictions from two hypotheses: reinforcement of premating isolation (reinforcement) and female mimicry to avoid interspecific aggression (female mimicry)

Displaced character	Reinforcement	Female mimicry
Male pied plumage colour*,†	Consistent	Consistent
Male collared plumage traits‡	Consistent	Not predicted
Female pied preference for male colour*,§	Consistent	Not predicted
Degree of heterospecific song copying in pieds¶	Consistent	Not predicted
Song characters in collareds¶	Consistent	Not predicted

*Sætre *et al.* 1997a; †Sætre *et al.* 1993; ‡Sætre *et al.* 2003; §Sæther *et al.* 2007; ¶Haavie *et al.* 2004.

among socially dominant collared flycatchers (Král *et al.* 1988; Sætre *et al.* 1993; Alatalo *et al.* 1994). Experimental evidence shows that brown-coloured pied flycatchers are able to maintain a territory closer to neighbouring collared flycatchers than are dark-coloured ones (Alatalo *et al.* 1994). It is difficult, however, to estimate potential fitness effects of the resulting reduction in interspecific aggression. To the extent that avoidance of aggression may help pied flycatchers get access to high-quality habitats, breeding among collared flycatchers is associated with an increased risk of hybridization.

Female mimicry has been considered an alternative explanation to reinforcement in the flycatchers by some authors (Coyne & Orr 2004; Price 2008). However, female mimicry cannot explain the range of character changes that have occurred in sympatry: These changes are instead all in agreement with predictions from reinforcement (Table 2).

Like plumage traits, bird song often has a dual function in mate attraction and as an aggressive signal among males (Catchpole & Slater 2008), but appears mainly to function in mate attraction in flycatchers (Eriksson & Wallin 1986; Lundberg & Alatalo 1992). However, the pattern of geographic variation in song is more complex than that of plumage traits (Haavie *et al.* 2004). As mentioned above, some male pied flycatchers copy song elements from the collared flycatcher and sing a mixed song in sympatry (Alatalo *et al.* 1990; Haavie *et al.* 2004). Such mixed singing significantly increases hybridization risk suggesting that it is a strongly maladaptive behaviour (Qvarnström *et al.* 2006). Mixed song is likely to be a consequence of a behaviour that is adaptive among allopatric birds. Male

pied flycatchers normally increase their repertoire size by incorporating sound elements from their auditory environment, typically from conspecific males, and females prefer to mate with males with larger repertoires (Lampe & Sætre 1995). In sympatry, this strategy apparently backfires.

Interestingly, however, there is evidence for reinforcement in song despite trait convergence due to learning. First, sympatric collared flycatchers have diverged in the same song characteristics as the pied flycatchers copy. Hence, the relative species difference in song is maintained in sympatry (Haavie *et al.* 2004). Second, the incidence of mixed singing among pied flycatchers appears to be reduced over time. In the presumably old Central European hybrid zone, only ~10% of the male pied flycatchers sing a mixed song compared with 65% in the younger hybrid zone of Öland, and song similarity to collared flycatchers is also greater among the mixed singers on Öland (Haavie *et al.* 2004). Hence, reinforcement appears to have reduced the incidence of maladaptive song copying in Central Europe, and also affected song characteristics among sympatric collared flycatchers in such a way as to reduce hybridization risk.

Prezygotic barriers to gene flow appear more strongly developed among pied flycatchers in the hybrid zone of Central Europe compared with the islands of Öland and Gotland. As explained above, the incidence of mixed singing is reduced (Haavie *et al.* 2004) and also the occurrence of light-coloured males is increased in the former zone (Sætre *et al.* 1999a). As expected from stronger prezygotic barriers, the level of introgression is also lower in Central Europe than on the islands (Borge *et al.* 2005b). These differences may be explained by the different ages of the hybrid zones (that is, more time for reinforcement of prezygotic isolation to have occurred in the older, Central European hybrid zone). Alternatively, or in addition, the difference may be due to higher levels of gene flow of allopatric pied flycatchers (i.e. birds without specific adaptations against hybridization) to the islands than to the hybrid zone of Central Europe (Sætre *et al.* 1999a; Borge *et al.* 2005b).

The role of the Z-sex chromosomes in flycatcher speciation

Since the theory of reinforcement was introduced (Dobzhansky 1940), its popularity has varied dramatically (Servedio & Noor 2003). After initial applause, reinforcement was for some years deemed highly unlikely due to an influential theoretical paper (Felsenstein 1981) that pinpointed severe problems with the theory (Servedio & Noor 2003). Felsenstein (1981) demonstrated that reinforcement is sensitive to recombination

in hybridizing taxa because species-specific genes affecting hybrid fitness and those promoting assortative mating would become dissociated. However, convincing empirical examples of reinforcement appeared from the 1990s (e.g. Coyne & Orr 1989, 1997; Noor 1995; Sætre *et al.* 1997a; Rundle & Schluter 1998; Nosil *et al.* 2003; Pfennig 2003; Jaenike *et al.* 2006) resulting in renewed interest in the process also among theoreticians (e.g. Servedio 2000; Kirkpatrick & Ravigné 2002; Servedio & Sætre 2003; Telschow *et al.* 2005; Hall & Kirkpatrick 2006; Lemmon & Kirkpatrick 2006; Yukilevich & True 2006; Servedio *et al.* 2009). A common theme in many of these studies is to investigate the facilitating effect on the reinforcement process of mechanisms that would reduce recombination between genes controlling traits associated with pre- and postzygotic isolation.

For empiricists interested in reinforcement and the potential role of physical linkage to aid the process, the sex chromosome is a natural place to start when searching for such linked 'speciation genes'. This is because intrinsic hybrid incompatibilities almost invariably are more severe in the heterogametic sex in organisms with sex chromosomes (i.e. Haldane's rule – Haldane 1922). Coyne & Orr (2004) conclude that Haldane's rule is explained by the dominance theory, originally proposed by Muller (1940), that hybrid incompatibility involves (partially) recessive X/Z-linked alleles being masked in the homogametic sex. Other factors may, however add to the sex difference in hybrid fitness (Coyne & Orr 2004). In birds, there is evidence for an accelerated rate of adaptive divergence of Z-linked genes compared with autosomal genes (Ellegren 2009). Hence, incompatibilities between incipient bird species may also accumulate faster on the Z than on other chromosomes as an indirect consequence of this difference in the rate of adaptive divergence.

Besides confirming to Haldane's rule (females are sterile, males are partially fertile), there is additional evidence that some Z-linked genes have a large effect on postzygotic isolation between pied and collared flycatchers: there is much less introgression of Z-linked than of autosomal genes occurring in flycatcher hybrid zones and also limited interspecific recombination of Z chromosomes (Sætre *et al.* 2003; Borge *et al.* 2005b). Using multilocus sequence analysis of allopatric pied and collared flycatchers (from Spain and Italy respectively), Borge *et al.* (2005a) reported significantly fewer shared polymorphisms and more fixed differences on Z-linked genes than on autosomal ones, beyond what could be ascribed to differences in effective population size of these chromosomes. This pattern may reflect past episodes of secondary contact with different levels of Z-linked and autosomal introgression, similar to that seen in current hybrid zones. Such episodes of

secondary contact are likely to have occurred in the past, for instance during warm, interglacial periods. Alternatively, or in addition, the pattern may reflect the large Z-effect (Ellegren 2009), i.e. an accelerated rate of adaptive divergence on the Z chromosome accompanied with selective sweeps that reduce variation around the sites under selection through hitchhiking (Borge *et al.* 2005a).

Interestingly, also traits involved in premating isolation appear to be affected by Z-linked genes in the flycatchers. Using species-informative SNP markers to genotype sympatric flycatchers, including hybrids and various backcrosses, Sætre *et al.* (2003) found positive correlations between male plumage characteristics and Z-genotype. Moreover, preliminary results indicate an association between pied flycatcher plumage colour variation and different splice variants of a Z-linked gene, tyrosinase-related protein 1 (TYRP1), which is involved in the melanin synthesis pathway in vertebrates (Buggiotti 2007). Species assortative mate preference in females also appears to be caused by Z-linked genes. Using sex- and species-specific genetic markers, Sæther *et al.* (2007) first demonstrated that female F_1-hybrids chose a mate of the same species as their father. Such paternally inherited mate preferences would imply either that females learn to prefer the phenotype of their father through sexual imprinting (see e.g. ten Cate & Vos 1999, for a review), or if genetically determined, that the preference is caused by some gene on the Z-chromosome (female birds inherit a Z-chromosome from their father and a W from their mother). By investigating the mating pattern of female pied and collared flycatchers reared by a heterospecific foster-father (from cross-fostering experiments as well as from cases where females in a heterospecific pair had female offspring sired by a conspecific extra-pair male), Sæther *et al.* (2007) found no effect of the rearing condition. Female pied and collared flycatchers reared by a heterospecific foster-father chose conspecific mates at the same rate as other genetically pure females did, suggesting that mate preferences are largely genetically determined and Z-linked according to the pattern of paternal inheritance. Physical linkage on the Z-chromosome of genes affecting postzygotic barriers and those affecting the male and female components of premating isolation is likely to reduce recombination, and hence, facilitate reinforcement (see e.g. Servedio & Sætre 2003; Hall & Kirkpatrick 2006; Servedio *et al.* 2009).

Speciation in flycatchers vs. other birds

Reinforcement apparently characterizes the evolutionary history of the pied and the collared flycatcher. Why then is the evidence for reinforcement so scarce in other

birds? For example, no evidence for reinforcement is found in the well-studied Darwin's finches, either in plumage or in song (Grant & Grant 2008). Although other examples of character displacement exist in birds, few entirely convincing examples that reinforcement is involved are available (Price 2008). Two main explanations seem possible: either the evidence for reinforcement is hidden from view in most other birds, or rare circumstances facilitating reinforcement are met in flycatchers.

Evidence for reinforcement is classically found by comparing differences among sympatric and allopatric pairs of species or populations (Coyne & Orr 1989). There are several potential problems with this approach that may lead to false rejection of reinforcement. We briefly mention two difficulties. First, the decision of what species pairs to compare in the first place may exclude species that have diverged so much phenotypically as to no longer been recognized as sister species. Effective reinforcement may hence from the very outset disqualify the cases where the process has been most prominent. We clearly need phylogenetic comparative analyses that use divergence in genetic markers, and not phenotypic similarity, to assign species pairs. Second, the degree of overlap in geographic distribution may change over time. This may create biases that will erase the evidence of reinforcement. Current distribution may be a poor guide to past events.

The other possibility is that reinforcement is indeed rare, but that it has been facilitated in flycatchers due to special circumstances that are rarely met in other systems. In general, both ecological and genetic differences among populations may increase the chances of speciation (Rundell & Price 2009). The relative importance of ecological and nonecological divergence in speciation has been much debated during the last decade (e.g. Schluter 2000; Rundell & Price 2009) and it seems to vary between taxa (e.g. Turner *et al.* 2001; Svensson *et al.* 2006; Grant & Grant 2008). Following Price (2008), we may visualize this in a two-dimensional space, where both divergent selection pressures and accumulation over time of (nonadaptive) genetic differences in geographic isolation may contribute to reproductive isolation (Fig. 4).

In contrast to the ecologically very different species of Darwin's finches that have undergone divergent selection and adapted to different environments (Grant & Grant 2008), the *Ficedula* flycatchers are ecologically very similar and overlap to a large extent in habitat and food. Genetic differences have evolved among the flycatchers to the extent that there are strong intrinsic incompatibilities leading to hybrid sterility in females and reduced fertility also in males. Again, this is in contrast to Darwin's finches where
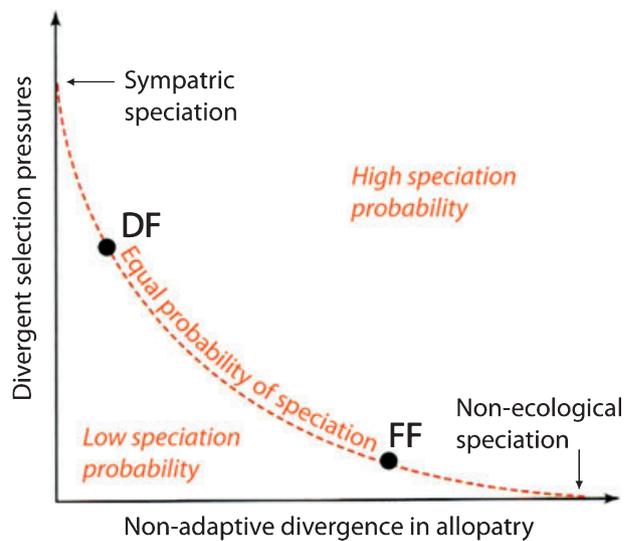

Fig. 4 A conceptual model of speciation in birds. Reproductive isolation can develop between two populations either due to strong divergent natural selection in different environments or due to build-up of incompatibilities in spatial separation over time. One can imagine the strength of these two factors as continuous (sympatric speciation and nonecological speciation are extreme combinations of the two dimensions). Further, one can envisage that there exists a range of combinations that leads to the same probability of speciation. The contour of one such curve of equal probability of speciation is shown; areas below and above the curve will have relatively lower and higher probabilities respectively (modified after Price 2008). Qualitative differences between two well-studied avian radiations are indicated. Speciation in *Ficedula* flycatchers (FF) appears mostly driven by genetic incompatibilities evolved in allopatry with only little ecological divergence. Speciation in Darwin's finches (DF), on the contrary, appears more driven by divergent selection and local adaptation with no evidence of intrinsic incompatibilities. DF can be understood as an adaptive radiation (Schluter 2000) whereas FF might be better described as a nonadaptive radiation as argued here.

reproductive isolation is mainly due to strong premating barriers (Grant 1999; Grant & Grant 2008). When these occasionally break down (due to misimprinting), the resulting hybrids may or may not have as high fitness as pure-bred birds conditional on environmental circumstances, and there is no evidence for intrinsic genetic incompatibilities (Grant & Grant 2008 and references therein).

It thus appears that flycatchers and Darwin's finches occupy well-separated points in the ecological vs. nonecological speciation space (Fig. 4). Darwin's finches are genetically very similar but highly divergent ecologically, whereas flycatchers are genetically highly incompatible but ecologically very similar. This difference in the importance of ecological and nonecological reproductive isolation may have made the flycatchers more

prone to speciation through reinforcement because of more opportunities for hybridization and/or because of stronger penalties from hybridizing.

Another feature of the flycatcher system that we have highlighted is that key components of reproductive isolation all appear to be linked to the Z chromosome. This involves the intrinsic incompatibilities, the male colour variation and the female species-assortative mating preferences. Such physical linkage of pre- and postmating barriers to the same chromosome facilitates reinforcement as it reduces the effect of recombination to break up the genetic associations necessary for a response to selection against hybridization (Servedio & Sætre 2003; Sæther et al. 2007).

Only a little is known about the genetics of reproductive isolation in other birds (Price 2008). As far as intrinsic incompatibilities follows Haldane's rule in birds, genes on the Z are probably involved (Coyne & Orr 2004). However, such barriers seem in general to evolve slowly in birds compared with, e.g. mammals (Price & Bouvier 2002; Fitzpatrick 2004). Whether there are intrinsic genetic incompatibilities between all four species in the black-and-white flycatcher radiation is not known, but it would be illuminating to get information on what combination of parental species give rise to sterile female hybrids. This would answer whether incompatibilities have arisen repeatedly or involve only a single event in one of the species. In lieu of such data, identifying the actual mutations or gene rearrangements involved may be possible and may indeed be cross-validated by predicting the outcome of future such crosses.

Generally, extrinsic ecological postzygotic barriers in birds may evolve much faster than intrinsic incompatibilities (Price 2008), but may not be particularly likely to involve the Z chromosome (Qvarnström & Bailey 2009).

The genetics of traits affecting assortative mating is poorly investigated, but sexually selected traits appear more likely to be Z-linked than traits affecting ecological aspects of assortative mating (see e.g. Servedio & Sætre 2003; Ellegren 2009; Qvarnström & Bailey 2009). Examples of Z-linked plumage variation are known from some other birds. These include the head colour polymorphism in Gouldian finches *Erythrura gouldiae* (Southern 1945) and colour variants in chicken *Gallus gallus* and Japanese quails *Coturnix japonica* (Minvielle et al. 2000; Gunnarsson et al. 2007). In the case of the Gouldian Finch, the colour polymorphism is also known to be the basis for strong assortative mate choice (Pryke & Griffith 2007) and indeed Z-linked incompatibilities exist between the morphs (Pryke & Griffith 2009).

Female preferences for male secondary sexual traits may, on the contrary, often not be Z-linked or even genetically determined in birds, but may instead be learned through sexual imprinting (ten Cate & Vos 1999). However, a similar facilitation of reinforcement may be obtained if the females learn their preferences by sexual imprinting on their fathers (Servedio et al. 2009). Z-linkage of assortative mating preferences is thus not a necessary component of reinforcement – sexual imprinting on the Z-linked traits of males is just as effective in reinforcing premating barriers. As sexual imprinting seems common in birds (ten Cate & Vos 1999), the lack of evidence (to date) for Z-linked preferences in other birds than flycatchers may not be critical for or against reinforcement.

Clearly, we are in need of more data on the genetics of both pre- and postzygotic barriers to gene flow in incipient bird species experiencing secondary contact. We also need more information on whether plumage variation is frequently influenced by Z-linked genes and if Z-linked assortative mating preferences are widespread. We do not know how commonly intrinsic postzygotic barriers play any role in speciation in birds, and information on the genetics of extrinsic postzygotic barriers is totally lacking.

Concluding remarks

In summary, the four Western Palearctic *Ficedula* flycatchers do not represent a classical 'adaptive radiation' in that ecological differences and divergent selection are probably not the main reasons behind the speciation. Rather, prolonged periods of allopatric isolation that have resulted in intrinsic genetic barriers to gene flow (female hybrid sterility at least among the two species studied in detail) and reinforcement of premating barriers seem to have been instrumental. The ecological similarity but genetic divergence may, coupled with the Z-linkage of components of reproductive isolation, explain why we find evidence for reinforcement of reproductive barriers in these birds and not in birds that are genetically weakly differentiated but under stronger divergent selection due to more pronounced ecological differences. Although flycatchers have clear limitations as a model system in that, for example, laboratory breeding over several generations is not feasible, it has proved to be a fruitful system for field-based experimental and genetic studies of speciation. As such, it has offered a perspective on the process of speciation in birds that is different from that of ecological speciation as found in Darwin's finches.

As new genomic tools are now becoming accessible for large-scale studies also in nonmodel organisms, we foresee continued fruitful avenues for research in the flycatcher model system. Biological research is currently characterized by conspicuous technology optimism. In

our view, however, traditional observational and experimental approaches for studying behaviour and ecology will remain paramount in speciation research also in the genomic era: We call for a systematic effort to simultaneously investigate ecology, behaviour and genomics of birds caught in the process of speciation.

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