



Evolution of postzygotic reproductive isolation in galliform birds: analysis of first and second hybrid generations and backcrosses

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The process of speciation is a crucial aspect of evolutionary biology. In this study, we analysed the patterns of evolution of postzygotic reproductive isolation in Galliformes using information on hybridization and genetic distance among species. Four main patterns arose: (1) hybrid inviability and sterility in F_1 hybrids increase as species diverge; (2) the presence of geographical overlap does not affect the evolution of postzygotic isolation; (3) the galliforms follow Haldane’s rule; (4) hybrid inviability is higher in F_2 than in F_1 hybrids, but does not appear to be increased in the backcrosses. This study contributes to the growing evidence suggesting that the patterns of evolution of postzygotic isolation and the process of speciation are shared among avian groups (and animals in general). In particular, our results support the notion of F_2 hybrid inviability as being key for the maintenance of species genetic integrity when prezygotic isolation barriers are overcome in closely related species, in which postzygotic isolation in the F_1 hybrid might still not be fully developed. To the contrary, hybrids from backcrosses did not show serious inviability problems (at least not more than F_1 hybrids), demonstrating that they could generate gene flow among bird species. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **110**, 528–542.

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INTRODUCTION

Understanding the process of speciation is crucial for the study of evolution. However, its causes are still among the most discussed topics in evolutionary biology (Coyne & Orr, 2004). There are two main reasons for this: (1) speciation is usually a slow process that cannot be studied as it happens; (2) rates and modes of speciation differ among taxa and depend on aspects related to geography, ecology, mating system and genetics. Therefore, comparative approaches using information from many groups and species are required to reach broad conclusions concerning the causes and modes of speciation and their relevance.

The Biological Species Concept defines species as groups of interbreeding natural populations that are

reproductively isolated from other such groups (Mayr, 1942). It simplifies the study of speciation to the analysis of the appearance of reproductive isolation mechanisms. Dobzhansky (1937) and Muller (1940, 1942) developed a simple model that explains how intrinsic postzygotic isolation mechanisms arise as geographically isolated lineages diverge and different alleles are fixed in complementary genes. This model, now widely accepted, considers two allopatric populations of a species that possess identical genotypes at two loci (one single species with genotype AA in one locus and BB in the other). An allele ‘a’ appears and is fixed in one population; the resulting aaBB genotype is perfectly viable and fertile (as is the intermediate genotype AaBB). In the other population, a mutation generates allele ‘b’, which is also fixed; AAbb is also viable and fertile (and so is the intermediate step AABb). The critical point is that, although the ‘b’ allele is compatible with ‘A’, it has not

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been 'tested' on an 'a' genetic background (and vice versa). Therefore, if the two populations meet and hybridize, the resulting AaBb hybrid may be inviable or sterile (Orr, 1995). This model has been the subject of much theoretical study in recent decades and has been formalized as the dominance theory (Orr, 1993, 1995; Turelli & Orr, 1995, 2000; Orr & Turelli, 1996, 2001; Turelli & Moyle, 2007; for a recent review, see Presgraves, 2010a).

The most comprehensive studies of the evolution of reproductive isolation in relation to species divergence were conducted in *Drosophila* (Coyne & Orr, 1989, 1997) and showed several relevant patterns. First, both prezygotic and postzygotic reproductive isolation were shown to increase with divergence in a manner consistent with the Dobzhansky–Muller model. In addition, prezygotic isolation mechanisms evolved faster than postzygotic ones between sympatric species, but not between allopatric species, an expected outcome of the fact that reinforcement cannot occur when populations are evolving in allopatry. Another relevant result was that hybrid sterility preceded inviability. Finally, the genus was shown to follow Haldane's rule: sterility or inviability appeared first in male hybrids (the heterogametic sex) and later in female ones [see Haldane, (1922) for the original study finding this pattern]. Similar studies conducted in Lepidoptera (Presgraves, 2002), fishes (Stelkens, Young & Seehausen, 2010), frogs (Sasa, Chippindale & Johnson, 1998; Malone & Fontenot, 2008) and birds (Price & Bouvier, 2002; Tubaro & Lijtmaer, 2002; Lijtmaer, Mahler & Tubaro, 2003) supported these general patterns. In particular, the appearance of Haldane's rule in most groups studied so far was one of the results that led to the revision of the Dobzhansky–Muller model and the incorporation of two of the most relevant novelties of the dominance theory: that the incompatible alleles of the complementary genes tend to be recessive and that these complementary genes occur many times in the sex chromosomes (Orr, 1993; Turelli & Orr, 1995, 2000; Orr & Turelli, 1996, 2001; Schilthuizen, Giesbers & Beukeboom, 2011).

As mentioned above, the study of different lineages of the tree of life is necessary to detect particular patterns of appearance of reproductive isolation mechanisms and to assess the generality of the Dobzhansky–Muller model. In the present study, we analyse the patterns of postzygotic isolation in Galliformes. The analysis of this group is relevant for various reasons. First, relatively few studies on this topic have been performed in birds (birds in general, Price & Bouvier, 2002; ducks, Tubaro & Lijtmaer, 2002; pigeons and doves, Lijtmaer *et al.*, 2003), and thus research in new avian groups could provide

relevant information. Moreover, different groups of birds could possess different predominant speciation mechanisms. For example, sexual selection is considered to have contributed to diversification in some groups, but is almost absent in others (in this regard, it should be mentioned that galliforms are a paradigmatic group for the study of sexual selection because males are usually characterized by conspicuous coloration and complex behavioural displays during courtship; Del Hoyo, Elliot & Sartagal, 1994). In particular, galliforms are an ideal group for this kind of analysis because they are commonly bred in captivity and, as a consequence, there is considerable information on the viability and sterility of hybrids, including data on the second hybrid generation and backcrosses, which have been much less studied in this context. In addition, and also because of the existence of controlled crosses in captivity, neither opportunities for hybridization nor differences in detectability of the hybrids can lead to biases in the data.

MATERIAL AND METHODS

CROSSES INCLUDED IN THE DATASET AND REPRODUCTIVE ISOLATION INDICES

We collected information from the literature on experimentally controlled crosses that were performed in captivity among different galliform species (Haig Thomas & Huxley, 1927; Shaklee & Knox, 1954; Lorenz, Asmundson & Wilson, 1956; Asmundson & Lorenz, 1957; Sandnes, 1957; Olsen, 1960; Wilcox & Clark, 1961; Lepori, 1964; Taibel, 1964, 1974; Maru & Ishijima, 1968; Morejohn, 1968; Higuchi, 1971; Johnsgard, 1973; Cink, 1975; Skjervold & Mjelstad, 1992; Deregnacourt, Guyomarc'h & Aebischer, 2002; Khosravinia, Narasimha Murthy & Kumar, 2005). These records include the number of laid eggs, fertilized eggs and hatched eggs in interspecific crosses, in crosses between first-generation (F_1) hybrids (which generate a second hybrid generation or F_2) and in backcrosses between hybrids and their parental species. We obtained hybridization data from 33 interspecific crosses involving 28 galliform species, 12 crosses between F_1 hybrids and 14 backcrosses. Of these crosses, we selected only those with at least 20 eggs to avoid including cases with results based on reduced offspring. As a consequence, our dataset included 24 interspecific crosses involving 22 species, nine crosses between F_1 hybrids and 13 backcrosses. All the species belong to the families Meleagrididae, Odontophoridae, Phasianidae and Tetraonidae. Tables 1–3 summarize the interspecific crosses, the crosses between F_1 hybrids and the backcrosses that were included in the study.

Table 1. Summary of the hybridization data from 24 interspecific crosses involving 22 species of Galliformes. Information includes the species involved in the crosses, the percentage of sequence divergence in cytochrome C oxidase (COI) and cytochrome b (cyt b), the percentage of unhatched eggs, the percentage of unfertilized eggs, the percentage of male hybrids and the presence or absence of geographical overlap between each pair of species

Interspecific crosses	Uncorrected percentage sequence divergence in COI/cyt b	Percentage of unhatched eggs (laid eggs)	Percentage of unfertilized eggs	Proportion of male hybrids (males/total)	Sympatric vs. allopatric pairs of species
<i>Coturnix coturnix</i> *	1.8/no data	58.7 (620)	41.6	51.5 (17/33)	Allopatric
<i>Chrysolophus amherstiae</i> †	2.5/2.1	55.3 (237)	No data	59.0 (59/100)	Allopatric
<i>Phasianus colchicus</i> †	3.7/3.8	89.3 (103)	87.4	60.0 (6/10)	Allopatric
<i>Meleagris ocellata</i> §	3.7/no data	81.1 (244)	72.1	No data	Allopatric
<i>Lophura nycthemera</i> †	No data/4.4	27.6 (29)	No data	60.0 (12/20)	Allopatric
<i>Colinus cristatus</i> ¶	5.2/2.2	92.8 (277)	72.6	No data	Allopatric
<i>Callipepla californica</i> †	6.4/5.9	59.6 (47)	44.7	No data	Allopatric
<i>Callipepla gambeli</i> **	6.4/no data	78.4 (37)	62.2	No data	Sympatric
<i>Colinus virginianus</i> †	8.1/8.6	73.1 (197)	46.7	No data	Sympatric
<i>Chrysolophus pictus</i> †	10.2/9.1	80.6 (31)	67.7	0 (0/1)	Sympatric
<i>Phasianus versicolor</i> †	11.0/9.1	88.3 (111)	85.6	69.2 (9/13)	Allopatric
<i>Phasianus colchicus</i> †	12.5/9.2	76.2 (42)	57.1	66.7 (4/6)	Sympatric
<i>Oreortyx pictus</i> †	13.0/10.4	91.2 (34)	79.4	No data	Sympatric
<i>Lophophorus impejanus</i> †	13.1/12.0	100 (27)	100	No data	Allopatric
<i>Gallus gallus</i> †	13.3/13.6	98.9 (2132)	47.9	100 (23/23)	Allopatric
<i>Gallus gallus</i> †	13.9/13.4	95 (4326)	82.0	50.0 (20/40)	Allopatric
<i>Tetrao urogallus</i> †	14.3/13.5	99.8 (1418)	99.4	No data	Allopatric
<i>Phasianus colchicus</i> †	14.9/13.8	93.6 (1445)	62.7	64.3 (45/70)	Sympatric
<i>Gallus gallus</i> †	15.0/13.3	99.4 (2682)	89.6	No data	Allopatric
<i>Gallus gallus</i> †	15.1/12.3	95.7 (93)	90.3	100 (1/1)	Allopatric
<i>Colinus nigrogularis</i> ††	No data	90.5 (42)	57.1	No data	Not used
<i>Colinus nigrogularis</i> ††	No data	55.4 (56)	46.4	No data	Not used
<i>Gallus gallus</i> †	No data	47 (115)	32.2	No data	Not used
<i>Lophura nycthemera</i> †	No data	100 (40)	100	No data	Not used

*COI and cyt b sequences publicly available but, because cyt b sequences in GenBank apparently belong to *C. japonica*, this gene was not used for the analyses involving genetic divergence in this cross.

†COI and cyt b sequences publicly available.

‡Cyt b sequences publicly available; COI sequences not available and tissue samples from the species not available. COI was not used for the analyses involving genetic divergence in this cross.

§Cyt b sequences not available; one COI sequence is part of a project in the Barcode of Life Data Systems (BOLD) that was still private when the analyses were performed and was kindly shared with us by Patricia Escalante Pliego from the Universidad Nacional Autónoma de México (the manager of the corresponding BOLD project).

¶COI sequences publicly available; cyt b sequences generated for the study.

**COI and cyt b sequences publicly available but, because cyt b has an insertion and a deletion, this gene was not used in analyses involving genetic divergence in this cross.

††Neither COI nor cyt b sequences publicly available; tissue samples from the species not available. Crosses including this species were not used in analyses involving genetic divergence.

‡‡COI and cyt b sequences publicly available, but *G. sonneratii* has two divergent lineages, and the articles with the information about this cross do not report to which lineage the individuals included in the cross belong. Therefore, this cross was not used in analyses including genetic divergence.

Table 2. Summary of the hybridization data from nine crosses of first-generation hybrids that generated a second hybrid generation. Information includes the hybrids involved in the crosses, the percentage of genetic divergence between cytochrome C oxidase (COI) and cytochrome b (cyt b) sequences (in the parental species), the percentage of unhatched eggs, the percentage of unfertilized eggs and the percentage of male hybrids

First-generation hybrids crossed to produce a second hybrid generation		Uncorrected percentage sequence divergence in COI/cyt b	Percentage of unhatched eggs (laid eggs)	Percentage of unfertilized eggs	Proportion of male hybrids (males/total)
<i>Coturnix coturnix-japonica</i>	×	1.8/no data	78.3 (277)	62.1	No data
<i>Chrysolophus amherstiae-Chrysolophus pictus</i>	×	2.5/2.1	58.4 (866)	51.3	70.8 (194/274)
<i>Phasianus versicolor-Phasianus colchicus</i>	×	3.7/3.8	72.8 (195)	65.1	80.4 (41/51)
<i>Lophura nycthemera-Lophura swinhoii</i>	×	No data/4.4	55.1 (158)	50.0	62.3 (33/53)
<i>Colinus cristatus-Colinus virginianus</i>	×	5.2/2.2	100 (22)	100	No data
<i>Callipepla californica-Callipepla squamata</i>	×	6.4/5.9	100 (266)	99.2	No data
<i>Callipepla gambelii-Callipepla squamata</i>	×	6.4/no data	100 (33)	78.8	No data
<i>Colinus virginianus-Callipepla squamata</i>	×	8.1/8.6	100 (370)	100	No data
<i>Gallus sonneratii-Gallus gallus</i>	×	No data	98.4 (321)	29.3	No data

The percentage of unhatched eggs was used as the measure of hybrid inviability in interspecific crosses, in crosses between F₁ hybrids and in backcrosses. Although, in some cases, the lack of egg hatching could be the consequence of prezygotic mechanisms (such as gametic incompatibility or differences in anatomical structures that prevent sperm transfer), these are considered to be much less prevalent than hybrid inviability (e.g. postcopulatory prezygotic mechanisms are thought to be less efficient in birds than in other taxa; Birkhead & Brillard, 2007).

In crosses between F₁ hybrids and in backcrosses, the percentage of unfertilized eggs reflects, at least partially, the level of sterility of F₁ hybrids. The presence or lack of fertilization of the eggs has been assessed in the sources of our data, mostly by checking the presence of the embryo 1 or 2 weeks after egg laying (Shaklee & Knox, 1954; Asmundson & Lorenz, 1957; Olsen, 1960; Cink, 1975; Skjervold & Mjelstad, 1992; Deregnacourt *et al.*, 2002) and thus eggs that are considered to be infertile can, in some cases, actually be the result of early inviability of the hybrids. Because hybrid sterility has been barely studied in birds in this context [Price & Bouvier (2002) is an exception], we consider that it is anyway worth analysing the results of these crosses with this non-ideal way of assessing egg fertilization.

We followed the taxonomic classification of Clements (2007) and made no differentiation among races or subspecies (i.e. crosses involving all races or subspecies of each species were pooled together). Approximately one-half of the crosses were reciprocal (i.e. females of species A crossed with males of species B, and vice versa) and the remaining were unidirectional. As is the rule in studies of postzygotic isolation, the percentages of fertilized and hatched eggs in both directions of each cross were generally different. These differences are the result of the existence of incompatibilities between autosomal loci and uniparentally inherited loci, such as those in the mitochondria or sex chromosomes (Turelli & Moyle, 2007). Because the actual level of this kind of asymmetry in each particular cross depends on stochastic effects (Turelli & Moyle, 2007), we pooled both directions of the cross in the cases in which reciprocal crosses had been performed.

GENETIC DISTANCE DATA

We used DNA sequence distance as a measure of divergence between species involved in the crosses to analyse the relationship between postzygotic reproductive isolation and species divergence. We chose subunit 1 of cytochrome C oxidase (COI) and cytochrome b (cyt b) because these genes

Table 3. Summary of the hybridization data from 13 crosses involving first-generation hybrids and one or both of their parental species. Information includes the hybrids involved in the crosses, the percentage of genetic divergence in cytochrome C oxidase (COI) and cytochrome b (cyt b) (belonging to the original parental species), the percentage of unhatched eggs, the percentage of unfertilized eggs and the percentage of male hybrids

Backcrossed hybrids	Uncorrected			Percentage of unfertilized eggs	Proportion of male hybrids (males/total)
	percentage sequence divergence in COI/cyt b	Percentage of unhatched eggs (laid eggs)	Percentage of unfertilized eggs		
<i>Callipepla californica</i> – <i>Callipepla gambelii</i>	×	1.6/no data	100 (32)	59.4	No data
<i>Coturnix coturnix</i> – <i>Coturnix japonica</i>	×	1.8/no data	73.5 (471)	55.8	No data
<i>Chrysolophus amherstiae</i> – <i>Chrysolophus pictus</i>	×	2.5/2.1	82.4 (381)	70.1	72.3 (34/47)
<i>Phasianus versicolor</i> – <i>Phasianus colchicus</i>	×	3.7/3.8	72.4 (58)	No data	68.8 (11/16)
<i>Meleagris ocellata</i> – <i>Meleagris gallopavo</i>	×	3.7/no data	35.1 (94)	25.5	No data
<i>Lophura nycthemera</i> – <i>Lophura swinhoii</i>	×	No data/4.4	86.5 (74)	81.1	69.2 (9/13)
<i>Colinus cristatus</i> – <i>Colinus virginianus</i>	×	5.2/2.2	50.0 (66)	24.2	No data
<i>Callipepla californica</i> – <i>Callipepla squamata</i>	×	6.4/5.9	66.7 (66)	18.2	No data
<i>Callipepla squamata</i> – <i>Callipepla gambelii</i>	×	6.4/no data	75.0 (32)	34.4	No data
<i>Colinus virginianus</i> – <i>Callipepla squamata</i>	×	8.1/8.6	96.7 (609)	75.0	No data
<i>Colinus virginianus</i> – <i>Callipepla gambelii</i>	×	8.9/no data	100 (28)	100	No data
<i>Lophura swinhoii</i> – <i>Syrnaticus reevesii</i>	×	No data/10.0	100 (128)	100	No data
<i>Gallus sonneratii</i> – <i>Gallus gallus</i>	×	No data	94.6 (56)	17.9	No data

have been sequenced for more avian species than any other loci. COI sequences were obtained from the Barcode of Life Data Systems (BOLD) and those of cyt b from GenBank. All available sequences longer than 500 bp in the case of COI and longer than 900 bp in the case of cyt b were used for the calculation of distances between the species pairs of each cross [the difference in the criterion for the minimum length accepted for each gene is a result of the standardization of the COI region used for DNA barcoding (see Hebert *et al.*, 2003): sequences superimpose almost completely in the case of COI, but a minimum length of 900 bp is needed to guarantee a minimum overlap of about 650 bp for cyt b].

Sequences from 20 of the 22 species of the dataset were available for COI and sequences from 16 species were available for cyt b (two more species actually had cyt b sequences in GenBank, but they were problematic and we decided not to use them; see Table 1 for details).

In addition, we were able to obtain cyt b sequences from two of the species with no publicly available data (using two tissue samples from *Callipepla squamata* and one from *Colinus cristatus*), thus totalling 18 species with cyt b sequences in our dataset. For this, DNA extraction was performed from muscle tissue following a glass fibre-based protocol developed by Ivanova, deWaard & Hebert (2006) adapted to individual spin columns (see Lijtmaer *et al.*, 2012). For polymerase chain reaction (PCR), we used a shorter version of primer L14841 and primer H16075 (Lougheed *et al.*, 2000) and employed the following amplification profile: 3 min at 94 °C; 40 cycles of 45 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C; final extension of 10 min at 72 °C. Sequencing was performed bidirectionally with the same primers at the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. GenBank accession numbers for these sequences are KC857649–KC857651.

Sequences for both genes were aligned using MEGA v.5.1 (Tamura *et al.*, 2011). Genetic distances between species were assessed using two different measures: uncorrected distance (percentage sequence divergence) and the distance obtained with the model selected by jModelTest2 (Darriba *et al.*, 2012). The latter was a three-parameter model for both genes: TPM2uf + G in the case of COI ($G = 0.1300$) and TPM2uf + I + G in the case of cyt b ($I = 0.5540$, $G = 1.4740$). To calculate the uncorrected distance, we used MEGA v.5.0 and, for the models selected by jModelTest2, we used PAUP (Swofford, 2001). The two measures gave identical results in all analyses, and so we report only those based on the percentage sequence divergence, which can be better compared with previous studies.

RELATIONSHIP BETWEEN POSTZYGOTIC REPRODUCTIVE ISOLATION AND DIVERGENCE

We analysed the relationship between postzygotic reproductive isolation and genetic divergence in interspecific crosses, in crosses between F_1 hybrids and in backcrosses through non-parametric correlations (Spearman correlations). This and all statistical analyses were performed using SPSS version 20 (IBM Corporation, 2011) and were two-tailed.

Interspecific crosses are evolutionarily independent only if they do not share branches in the tree that depicts their phylogenetic affinities. Therefore, it is not correct to consider every cross as an independent point of comparison and, because of this, we also performed this analysis using only those crosses that satisfied this requirement. We used the phylogeny developed by Zink & Blackwell (1998) and the most parsimonious cladogram obtained by the analysis of the combined dataset in Crowe *et al.* (2006) to identify independent crosses and to maximize the number of such crosses for the analysis. The maximum number of independent crosses that can be obtained from the dataset when using COI is eight, and there are three different possible combinations of crosses that allow this maximum to be obtained. With cyt b, the maximum number of independent crosses is seven and there are six possible combinations of such crosses. For both datasets, we performed Spearman correlations for all of the combinations to re-analyse the association between reproductive isolation and species divergence.

COMPARISON BETWEEN HYBRID GENERATIONS

In addition to the analysis of the patterns of postzygotic reproductive isolation in each hybrid generation, we also compared the percentage of unhatched eggs and the percentage of unfertilized eggs between F_1 and F_2 and between F_1 and backcrosses. For this, we considered all crosses of F_2 and backcrosses that had data on the percentage of unhatched or unfertilized eggs, and compared these indices with those of the same set of crosses in F_1 through a Wilcoxon matched pairs test. These comparisons included more crosses than the analyses of the association between reproductive isolation and divergence because the presence of DNA sequences is not needed (see the footnote of Table 1 for details).

RELATIONSHIP BETWEEN POSTZYGOTIC REPRODUCTIVE ISOLATION AND GEOGRAPHICAL OVERLAP

We analysed the relationship between the presence of geographical overlap between species and the evolution of reproductive isolation. We classified each pair

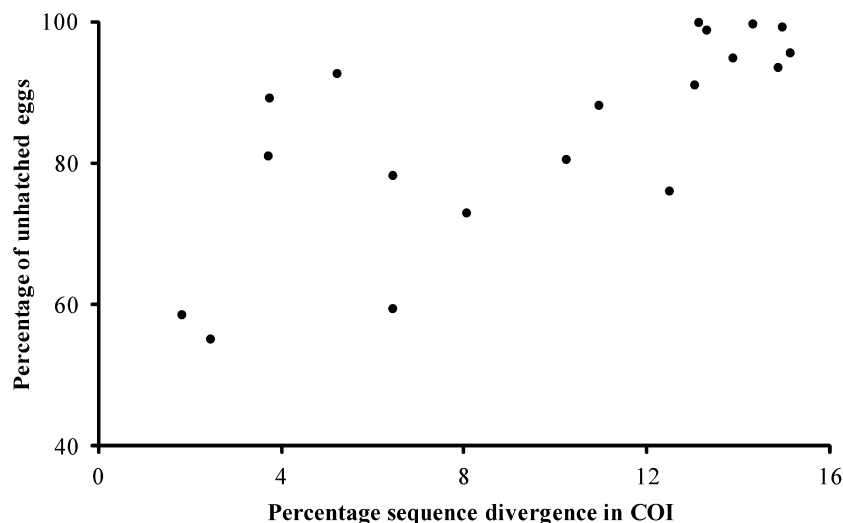


Figure 1. Percentage of unhatched eggs versus cytochrome C oxidase (COI) percentage sequence divergence in F_1 for 19 interspecific crosses. Inviability increases significantly as species diverge (Spearman $r = 0.77$; $P < 0.001$).

of species that had been crossed as allopatric or sympatric on the basis of the geographical distributions described in Del Hoyo *et al.* (1994) and Johnsgard (1999). A pair of species was considered to be sympatric if their breeding ranges overlapped, irrespective of the area of overlap involved. We then used a Mann–Whitney test to analyse differences in F_1 in hybrid inviability between sympatric and allopatric pairs of species. Only crosses from which we had DNA sequences from both species were included in this analysis to be able to control for differences between the two categories of geographical overlap in the divergence between the hybridizing pairs of species.

HALDANE'S RULE

The sex of the hybrids at the moment of hatching was reported for 11 of the 24 interspecific crosses of the dataset, for three of the nine crosses between F_1 hybrids and for three of the 13 backcrosses (see Tables 1–3). In a few additional cases, the sex was determined a few weeks after hatching, but we preferred not to include this information because it might contain post-hatching mortality and introduce noise into the dataset related to the comparison between the primary sex ratio in some hybrids and the secondary sex ratio in others, and may incorporate cases in which death might have been independent of inviability. This information allowed analysing if the galliforms follow Haldane's rule (i.e. if male hybrids were more common than female ones, given that females are the heterogametic sex in birds). To evaluate this, we considered, for each hybrid generation, all crosses with at least one sexed hybrid and

analysed the proportion of crosses with more males than females. We also analysed the existence of an association between the proportion of males and genetic distance through a Spearman correlation.

RESULTS

ANALYSIS OF POSTZYGOTIC ISOLATION IN THE FIRST HYBRID GENERATION

To analyse the association between postzygotic isolation and species divergence, we obtained data from 19 interspecific crosses involving 18 species when using COI to estimate species divergence and from 17 crosses involving 17 species when cyt b was employed. Table 1 summarizes these crosses, the percentage of COI and cyt b sequence divergence between hybridizing species, the percentages of unhatched and unfertilized eggs in each cross, the percentage of male hybrids and the presence or absence of geographical overlap between pairs of species.

We found a significant positive correlation between the percentage of unhatched eggs and sequence distance (COI: $N = 19$, Spearman $r = 0.77$, $P < 0.001$, Fig. 1; cyt b: $N = 17$, Spearman $r = 0.74$, $P = 0.001$, Supporting Information Fig. S1), showing that inviability increases with divergence. This result is supported by the analysis of the phylogenetically independent subset of crosses. With COI, the three combinations of eight independent crosses showed a significant positive correlation between the percentage of unhatched eggs and genetic divergence ($0.001 < P < 0.017$). For cyt b, the six combinations of seven independent crosses also showed this positive correlation and, even though in this case the correla-

tions were not statistically significant (possibly because this dataset included one cross less than that of COI), the presence of a positive correlation in the six combinations is significantly different from the expected outcome if the percentage of unhatched eggs and genetic distance were not correlated (binomial test, $P = 0.03$).

INFLUENCE OF GEOGRAPHICAL OVERLAP ON REPRODUCTIVE ISOLATION

We did not find significant differences in the percentage of unhatched eggs between sympatric and allopatric species pairs (mean percentage of unhatched eggs in crosses between allopatric species, 85.7%; mean percentage of unhatched eggs in crosses between sympatric species, 82.2%; Mann–Whitney $U = 25$; $N = 13$, 6; $P = 0.24$). It should be noted that this result is not influenced by differences in genetic distances between the sympatric and allopatric species pairs (mean percentage of COI divergence between allopatric species, 9.2%; mean percentage of divergence between sympatric species, 10.9%; Mann–Whitney $U = 35.5$; $N = 13$, 6; $P = 0.77$; the result was the same if cyt b was used instead of COI). This finding suggests that the evolutionary rate of postzygotic isolation in galliforms is not affected by the presence or absence of geographical overlap between species.

ANALYSIS OF THE SECOND HYBRID GENERATION AND THE BACKCROSSES

Our dataset includes data from nine crosses between F_1 hybrids that originate the F_2 generation and 13 backcrosses between hybrids of F_1 and one of their parental species. Using COI to quantify species divergence, we were able to analyse seven crosses between F_1 hybrids and 10 backcrosses to elucidate the relationship between reproductive isolation and genetic divergence. Using cyt b, the dataset includes six crosses between F_1 hybrids and seven backcrosses. Tables 2 and 3 summarize these crosses, the percentages of COI and cyt b sequence divergence between hybridizing species, the percentages of unhatched and unfertilized eggs in each cross and the percentage of male hybrids.

In F_2 , we found a significant positive correlation between the percentage of unhatched eggs and COI sequence divergence ($N = 7$; Spearman $r = 0.80$; $P = 0.03$; Fig. 2A) and between the percentage of unfertilized eggs and sequence divergence ($N = 7$; Spearman $r = 0.80$; $P = 0.03$; Fig. 2B). When divergence was estimated using cyt b, the relationships showed the same trends but, possibly because of the smaller number of crosses, they were not statistically significant (unhatched eggs: $N = 6$, Spearman

$r = 0.40$, $P = 0.44$; unfertilized eggs: $N = 6$; Spearman $r = 0.32$, $P = 0.54$, Supporting Information Fig. S2).

In the backcrosses, neither the relationship between the percentage of unhatched eggs and genetic divergence (COI: $N = 10$, Spearman $r = 0.09$, $P = 0.81$, Fig. 3A; cyt b: $N = 7$, Spearman $r = 0.64$, $P = 0.12$, Supporting Information Fig. S3A) nor that between the percentage of unfertilized eggs and genetic divergence (COI: $N = 9$, Spearman $r = 0.19$, $P = 0.62$, Fig. 3B; cyt b: $N = 6$, Spearman $r = 0.49$, $P = 0.33$, Fig. S3B) was significant.

The comparison of the indices of reproductive isolation between hybrid generations showed that the percentage of unhatched eggs was significantly higher in F_2 than in F_1 (mean percentage of unhatched eggs in F_2 , 84.8%, mean percentage of unhatched eggs in the same crosses of F_1 , 64.6%; $N = 9$, Wilcoxon $t = -2.31$, $P = 0.02$). The percentage of unfertilized eggs showed the same pattern, being higher in F_2 than in F_1 , but the difference was not statistically significant (mean percentage of unfertilized eggs in F_2 , 76.4%; mean percentage of unfertilized eggs for the same crosses in F_1 , 55.3%; $N = 7$, Wilcoxon $t = -1.52$, $P = 0.13$).

The comparisons between F_1 and the backcrosses did not show a significant difference in either the percentage of unhatched eggs (mean percentage of unhatched eggs in the backcrosses, 73.3%; mean percentage of unhatched eggs in the same crosses of F_1 , 66.3%; $N = 10$, Wilcoxon $t = -0.76$, $P = 0.44$) or in the percentage of unfertilized eggs (mean percentage of unfertilized eggs in the backcrosses, 35.9%; mean percentage of unfertilized eggs in the same crosses of F_1 , 53.1%; $N = 7$, Wilcoxon $t = -1.35$, $P = 0.18$).

HALDANE'S RULE

The analysis of the sex ratios of F_1 hybrids indicated that galliforms follow Haldane's rule: there were more male hybrids in nine of the crosses for which at least one hybrid had been sexed, the number of males and females was equal in one cross and in only one cross were there more females (see Table 1). This trend is significantly different from that expected if both sexes had equal probabilities of completing their development and hatching (binomial test, $P = 0.02$). We did not find a significant association between the proportion of male hybrids in F_1 and genetic divergence (COI: $N = 10$, Spearman $r = 0.50$, $P = 0.14$; cyt b: $N = 10$, Spearman $r = 0.41$, $P = 0.24$).

Although the data on sexed hybrids were much scarcer in F_2 and the backcrosses, their analyses also suggested that Haldane's rule is met in these generations: in both, the three crosses for which data on the sex of the hybrids were available had more males than females. As a result of the limited amount of

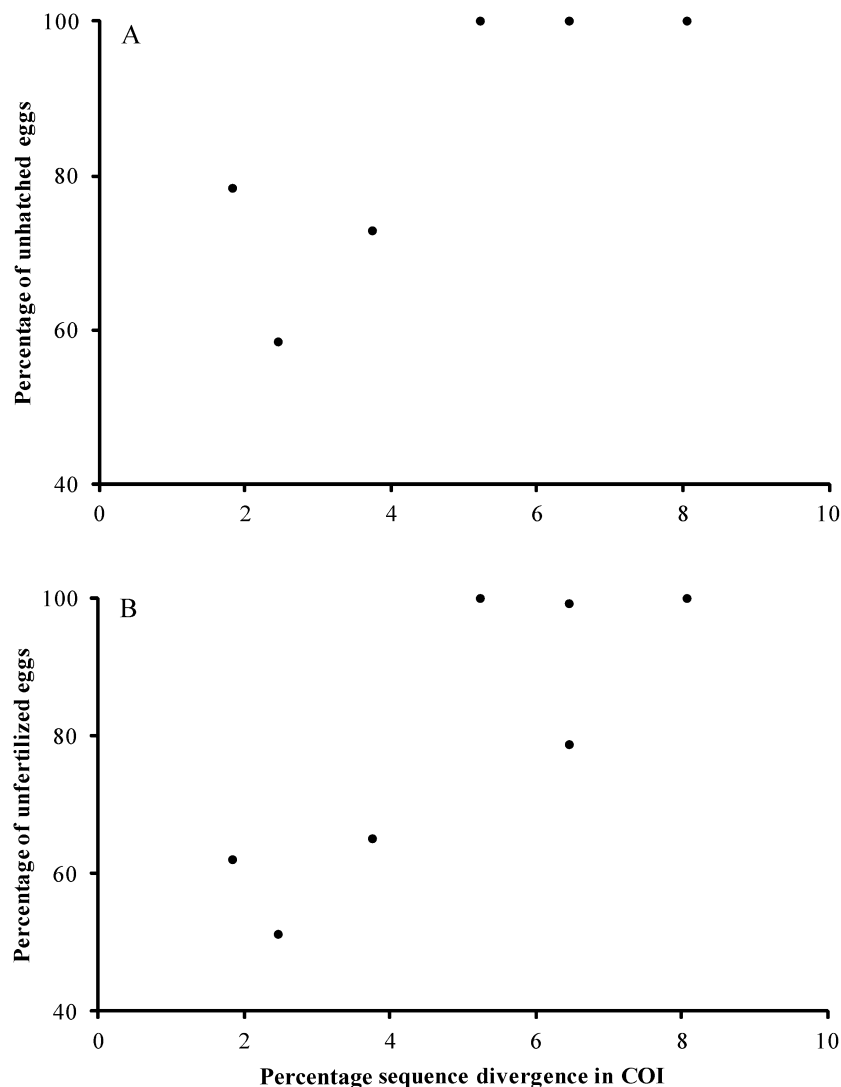


Figure 2. Postzygotic reproductive isolation versus cytochrome C oxidase (COI) percentage sequence divergence in F_2 . A, The percentage of unhatched eggs increases significantly as species diverge ($N = 7$, Spearman $r = 0.80$; $P = 0.03$). B, The percentage of unfertilized eggs increases significantly as species diverge ($N = 7$, Spearman $r = 0.80$; $P = 0.03$).

information, we were not able to analyse in these generations the association between the proportion of male hybrids and genetic divergence.

DISCUSSION

The analysis of hybridization in galliforms revealed patterns related to the increase in postzygotic isolation with species divergence, the lack of influence of geographical overlap on the appearance of inviability, the presence of Haldane's rule in the group and the differences among hybrid generations in the strength of their reproductive isolation. We discuss these findings below.

As predicted by the Dobzhansky–Muller model, both hybrid inviability and sterility gradually

increased with species divergence. The increase in inviability was evidenced by a positive and statistically significant association between the percentage of unhatched eggs and genetic distance between hybridizing species. This result is consistent with the observations in previous studies in several groups of organisms (*Drosophila*, Coyne & Orr, 1989, 1997; Lepidoptera, Presgraves, 2002; fishes, Stelkens *et al.*, 2010; anurans, Sasa *et al.*, 1998; Malone & Fontenot, 2008; birds, Price & Bouvier, 2002; ducks, Tubaro & Lijtmaer, 2002; pigeons and doves, Lijtmaer *et al.*, 2003).

A comparison with our study in pigeons and doves (Lijtmaer *et al.*, 2003) shows that, in galliforms, inviability appears to be higher in F_1 , and this is particularly clear in the case of species pairs with

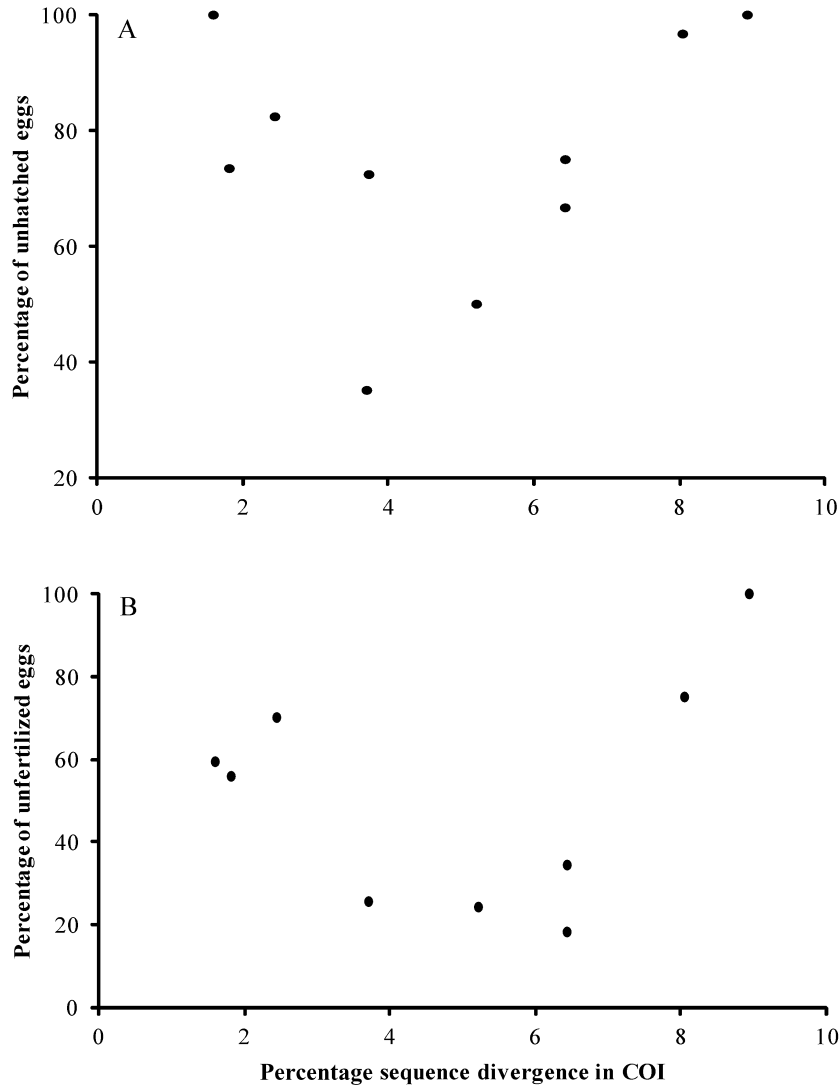


Figure 3. Postzygotic reproductive isolation versus cytochrome C oxidase (COI) percentage sequence divergence in the backcrosses. A, There is no significant correlation between the percentage of unhatched eggs and species divergence ($N = 10$, Spearman $r = 0.09$; $P = 0.81$). B, There is no significant correlation between the percentage of unfertilized eggs and species divergence ($N = 9$, Spearman $r = 0.19$; $P = 0.62$).

low genetic distance (see Supporting Information Fig. S4). For instance, if we consider all crosses for which we had cyt b data (because this is the locus used in the study of pigeons and doves), only in one cross in galliforms more than 50% of the eggs hatched (1 of 17 crosses, or 6%), whereas this figure is much higher in pigeons and doves (6 of 21 crosses, or 29%). This difference is found in spite of the fact that genetic differences between hybridizing species pairs are no higher in the case of galliforms (mean genetic distance in pigeons and doves, 9.4%; mean genetic distance in galliforms, 9.2%; Mann–Whitney $U = 178$; $N = 21$, 17; $P = 1$). Although it is speculative, one of the reasons for this difference

could be related to the fact that galliforms are precocial (more developed at hatching and relatively independent of their parents), whereas pigeons and doves are altricial (less developed and much more dependent on their parents). Therefore, inviability at later stages could occur inside the eggs in galliforms and be registered as hatching failure, but, in pigeons and doves, this mortality can occur after hatching and may not be registered in sources of data that only report eggs that failed to hatch. If this is a correct explanation, this highlights the need to consider the characteristics of each group in comparative approaches of the study of the evolution of reproductive isolation.

The increase in F_1 sterility with species divergence, in turn, can be inferred by the positive and significant correlation between the percentage of unfertilized eggs and genetic distance in crosses between F_1 hybrids. Although one should be cautious in the interpretation of this result, because of the possibility of early inviability in some of the eggs that are considered to be infertile, the pattern of increasing sterility with species divergence appears to be sound and consistent with previous studies in birds and other organisms (*Drosophila*, Coyne & Orr, 1989, 1997; Lepidoptera, Presgraves, 2002; fishes, Stelkens *et al.*, 2010; anurans, Sasa *et al.*, 1998; Malone & Fontenot, 2008; birds, Price & Bouvier, 2002).

The expected number of hybrid incompatibilities that cause postzygotic isolation should increase with the square of time since divergence ('snowball effect'; Orr, 1995; Orr & Turelli, 2001). However, this growth is related to the number of genetic incompatibilities between species, but is not necessarily reflected in the fitness problems faced by hybrids, because these are an unknown function of the amount of incompatibilities (Coyne & Orr, 2004: 275). Therefore, our findings are consistent with studies in other taxa which suggest that there is not an exponential but rather a linear relationship between reproductive isolation strength and species divergence (Coyne & Orr, 1989, 1997; Sasa *et al.*, 1998; Presgraves, 2002; Price & Bouvier, 2002; Tubaro & Lijtmaer, 2002; Lijtmaer *et al.*, 2003; Stelkens *et al.*, 2010; for discussions, see Gourbière & Mallet, 2010; Presgraves, 2010b). This pattern could be explained by the fact that, even if the number of genetic incompatibilities increases exponentially as species diverge, one or a few incompatibilities might be sufficient to sensibly decrease the viability or fertility of hybrids (see Turelli & Moyle, 2007) and, as a consequence, each new incompatibility has a smaller effect (Presgraves, 2010b).

In relation to the influence of the geographical overlap between species, we found no difference in inviability between sympatric and allopatric species pairs. This result is consistent with previous studies (Coyne & Orr, 1989, 1997; Lijtmaer *et al.*, 2003) and with the notion that prezygotic isolation, and not postzygotic isolation, should be affected by reinforcement (Dobzhansky, 1937).

Galliforms follow Haldane's rule, male hybrids being more common than female ones. This is consistent with previous studies on other bird groups (Price & Bouvier, 2002; Tubaro & Lijtmaer, 2002; Lijtmaer *et al.*, 2003) and with the expectations of dominance theory (Orr, 1993; Turelli & Orr, 1995, 2000; Orr & Turelli, 1996, 2001; Schilthuisen *et al.*, 2011). As expected, not only in F_1 hybrids were males more common than females, but also in F_2 hybrids

and the backcrosses, which have been much less studied in relation to this.

Contrary to our previous study in pigeons and doves, in which we found that the proportion of hybrid males in F_1 increased with species divergence, in this case we did not find a significant correlation. This could be a result of the relatively small number of crosses with data about the sex of the hybrids and the fact that we did not have this information in any of the crosses with intermediate genetic distance (COI divergence values for pairs with information about the sex of the hybrids were either less than 4% or more than 10%; see Table 1). However, a closer look at the data shows that the patterns could actually be different in these two groups. For instance, in galliforms, we found much variation in the percentage of males in distantly related crosses, including three of five crosses between species diverging by more than 12% with 50–70% males, whereas, in the case of pigeons and doves, in all crosses with more than 12% divergence, only male hybrids were viable. In their analysis spanning various groups of birds, Price & Bouvier (2002) also found various crosses between species with high divergence in which hybrids from both sexes had been reported. Future studies including other bird groups could allow the analysis of this in more detail.

The analysis of the F_2 hybrid generation revealed that there is a significant positive correlation between the percentage of unhatched eggs and genetic divergence and also a higher percentage of unhatched eggs in F_2 than in F_1 . This higher degree of inviability in F_2 is consistent with the results obtained in pigeons and doves (Lijtmaer *et al.*, 2003) and with predictions from the model developed by Turelli & Orr (2000), which states that hybrid inviability and sterility should increase in F_2 and backcrosses in comparison with F_1 . Specifically, although incompatibilities between autosomal complementary genes can only appear in heterozygosis in F_1 ($AaBb$; H_0 interactions according to the nomenclature used by Turelli & Orr, 2000), incompatibilities are expected to appear in homozygosis in F_2 , generating homozygous–heterozygous interactions ($aaBb$ or $Aabb$; H_1 interactions) and homozygous–homozygous interactions ($aabb$; H_2 interactions). Because the incompatibilities are partially recessive (Orr, 1993; Turelli & Orr, 1995, 2000), H_1 incompatibilities are much more severe than H_0 , and H_2 incompatibilities are even more problematic for hybrid viability and fertility. Because genes in the Z chromosome are expected to be involved in incompatibilities (see Turelli & Moyle, 2007), the negative effects in viability and fertility will be even stronger in F_2 females because of the presence of hemizygous–homozygous ($awbb$) and hemizygous–hemizygous ($awbw$) incompatibilities

(also highly deleterious, H_2 interactions). Supporting Information Tables S1–S3 show these different kinds of incompatibilities and their proportions in the different hybrid generations when both genes involved in an incompatibility are autosomal, when one of them is in the Z chromosome and when both are Z-linked, respectively. Although it is known that, even in the case of closely related species, genetic differentiation might involve many loci (McKinnon & Rundle, 2012; Parchman *et al.*, 2013), and that complex epistatic interactions involving more than two genes are common (see Presgraves, 2010a), this simpler analysis can be expanded to more interacting loci.

In their study including diverse avian taxa, Price & Bouvier (2002) concluded that many sympatric species show little or no intrinsic postzygotic isolation and suggested that species integrity is maintained by genetic incompatibilities that arise in F_2 and backcrosses. These hybrid generations, however, have been barely studied. Our results, as well as those of our previous analysis in pigeons and doves (Lijtmaer *et al.*, 2003), support this notion of postzygotic mechanisms in F_2 playing an important role in reproductive isolation. For a detailed analysis of the increase in fitness problems in F_2 hybrids, however, it would be required to better separate the effect of the inviability of F_2 and the sterility of F_1 hybrids. As a result of the aforementioned limitation in the way in which egg fertility has been assessed in hybrid crosses of captive galliforms, this fine differentiation was not possible. It should anyway be noted that the role of the low percentage of hatched eggs in crosses between F_1 hybrids in the maintenance of species genetic integrity is independent of the relative contribution of the sterility of F_1 hybrids versus the inviability of F_2 hybrids.

In contrast with F_2 , hybrids from backcrosses did not show a significant association between inviability and species divergence, and inviability was not higher than in F_1 hybrids. This lack of an increase in postzygotic isolation is consistent with findings in previous studies of hybridization in birds (Grant & Grant, 1992; Lijtmaer *et al.*, 2003; but see Wiley *et al.*, 2009), but differs from the expected outcome of the aforementioned model developed by Turelli & Orr (2000), which states that hybrids from backcrosses should face more postzygotic problems because of the appearance of the incompatible alleles in homozygosis.

One possible explanation for this difference between F_2 and backcrosses is that H_2 epistatic incompatibilities are much less frequent in hybrids from backcrosses than in F_2 and are restricted to females (see Tables S1–S3). In addition, combinations of alleles that are not incompatible and therefore do

not generate negative effects should be more frequent in hybrids from backcrosses than in F_2 hybrids.

In conclusion, the results of our analysis of the patterns of appearance of postzygotic reproductive isolation in F_1 hybrids in galliforms are consistent with those of previous studies in other groups of birds (and other organisms), showing that their inviability and sterility gradually increase as species diverge. In spite of this, Grant & Grant (1992) concluded that sympatric avian species hybridize frequently (registers of natural hybridization events involve as many as 10% of bird species), which is consistent with the results of Price & Bouvier (2002), who found that, in a diverse array of bird groups, intrageneric species pairs show little postzygotic reproductive isolation. This could be explained by a relatively slow rate of appearance of genetic incompatibilities in birds relative to other taxa (Lijtmaer *et al.*, 2003). Indeed, it is widely accepted that hybridization in birds is not more common because of the prevalence of prezygotic reproductive isolation mechanisms related to plumage coloration, vocalizations and complex behavioural displays during courtship (which, in many cases, could actually be a consequence of reinforcement as a result of the presence of postzygotic isolation; Price, 2008). Apart from studying F_1 hybrids, we were able to analyse other hybrid generations, and found evidence suggesting that, when these prezygotic isolation barriers are in fact overcome, F_2 hybrid inviability could be playing an important role in restricting gene flow between species and helping to maintain species genetic integrity (particularly in the case of closely related species, in which postzygotic isolation in F_1 might still not be fully developed). To the contrary, hybrids that result from backcrosses did not show serious inviability problems (at least not more than F_1 hybrids), demonstrating that this pathway of flow of genetic material could generate introgression of genes from one species into another, a phenomenon that has been found in several species of birds (e.g. Brumfield *et al.*, 2001; Weckstein *et al.*, 2001; Shapiro *et al.*, 2004; Rheindt & Edwards, 2011).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Percentage of unhatched eggs versus cyt b percentage sequence divergence in F₁ for the 17 interspecific crosses. Inviability increases significantly as the species diverge (Spearman $r = 0.74$; $P = 0.001$).

Figure S2. Postzygotic reproductive isolation versus cyt b percentage sequence divergence in F₂. A, The correlation between the percentage of unhatched eggs and species divergence is not statistically significant ($N = 6$, Spearman $r = 0.40$; $P = 0.44$). B, The correlation between the percentage of unfertilized eggs and species divergence is not statistically significant ($N = 6$, Spearman $r = 0.32$; $P = 0.54$).

Figure S3. Postzygotic reproductive isolation versus cyt b percentage sequence divergence in the backcrosses. A, The correlation between the percentage of unhatched eggs and species divergence is not statistically significant ($N = 7$, Spearman $r = 0.64$; $P = 0.12$). B, The correlation between the percentage of unfertilized eggs and species divergence is not statistically significant ($N = 6$, Spearman $r = 0.49$; $P = 0.33$).

Figure S4. Postzygotic reproductive isolation versus cyt b percentage sequence divergence for the 17 interspecific crosses in galliforms (circles; this study) and the 21 interspecific crosses in pigeons and doves (triangles; obtained from the dataset of Lijtmaer *et al.*, 2003).

Table S1. Negative epistatic interactions causing hybrid inviability and/or sterility in F_1 , F_2 and the backcrosses when both genes are autosomal.

Table S2. A, Negative epistatic interactions causing hybrid inviability and/or sterility in F_1 and F_2 when one of the genes is in the Z chromosome. B, Negative epistatic interactions causing hybrid inviability and/or sterility in the backcrosses when one of the genes is in the Z chromosome.

Table S3. A, Negative epistatic interactions causing hybrid inviability and/or sterility in F_1 and F_2 when both genes are in the Z chromosome. B, Negative epistatic interactions causing hybrid inviability and/or sterility in the backcrosses when both genes are in the Z chromosome.