The genomic basis of hybrid male sterility in *Ficedula* flycatchers

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Abstract

Identifying genes involved in genetic incompatibilities causing hybrid sterility or inviability is a long-standing challenge in speciation research, especially in studies based on natural hybrid zones. Here we present the first high-probability candidate genes for hybrid male sterility in birds by using a combination of whole genome sequence data, histology sections of testis and single cell transcriptomics of testis samples from male pied-, collared-, and hybrid flycatchers. We reveal failure of meiosis in hybrid males and propose candidate genes involved in genetic incompatibilities causing this failure. Based on identification of genes with non-synonymous fixed differences between the two species and revealing miss-expression patterns of these genes across the various stages of hybrid male spermatogenesis we conclude aberrant chromosome segregation and/or faulty chromatin packing. A lower proportion of spermatids produced by hybrid males implies that a proportion of the aberrant spermatids undergo apoptosis. Finally, we report an overrepresentation of Z-linkage of the revealed candidate incompatibility genes. Our results challenge the assumption that speciation processes are driven by fast evolving genes by showing that a few changes in genes with highly conserved and central functions may quickly ensure reproductive isolation through post-zygotic isolation.

Introduction

In sexually reproducing organisms, evolutionary divergence that causes reproductive isolation is necessary to achieve speciation. Although there are several types of reproductive barriers that can generate and maintain genetic integrity of emerging new species, post zygotic barriers are considered to be more permanent, especially intrinsic postzygotic isolation in the form of hybrid sterility or inviability (Muller, 1942; Coyne & Orr, 2004; Coughlan & Matute, 2020). However, even if there is an extensive theoretical framework outlining possible explanations for how intrinsic postzygotic can arise, these ideas remain largely empirically untested. The molecular basis for specific genomic clashes still remains poorly known, with the exception of a few model systems. We therefore need to reveal the underlying genetics in a broader range of organisms if we want to understand the evolutionary forces driving speciation.

The well-established Bateson-Dobzhansky-Muller model (Bateson, 1909; Dobzhansky, 1936; Muller, 1942) proposes that hybrid intrinsic dysfunction is caused by negative epistatic interactions between two or more loci (i.e. BDMI's). These loci have become incompatible with each other following divergence in different allopatric populations and cause a lethal or underperforming hybrid phenotype when these populations interbreed at secondary contact. The common observation of greater intrinsic fitness reduction of hybrids belonging to the heterogametic sex, known as Haldane's rule, implies a particular role of sex-linked genes in causing genetic incompatibilities (Coyne & Orr, 2004). The main hypotheses outlined to explain Haldane's rule suggests a higher exposure of recessive incompatible alleles when hemizygous (Muller, 1940) . This idea holds even when incompatibility genes are randomly distributed across the genome. Alternatively, there may be a relatively faster accumulation of

incompatibilities on the sex-chromosomes as compared to the autosomes due to an overall relatively faster evolutionary divergence of sex-linked genes "faster- X", (Charlesworth et al., 1987). In addition, genes that are especially crucial for functional reproduction may be located on the sex-chromosomes including potentially troublemaking genes such as selfish meiotic-drive alleles that can escape the control of the co-evolved suppressors in hybrids (Pomiankowski & Hurst, 1993). Finally, some additional ideas have been proposed that do not focus on sex chromosomes and cannot explain Haldane's rule in species where females constitute the heterogametic sex. One such idea is that male reproductive traits in general may evolve faster than female reproductive traits due to more intense reproductive competition among males (Wu & Davis, 1993; Wu et al., 1996).

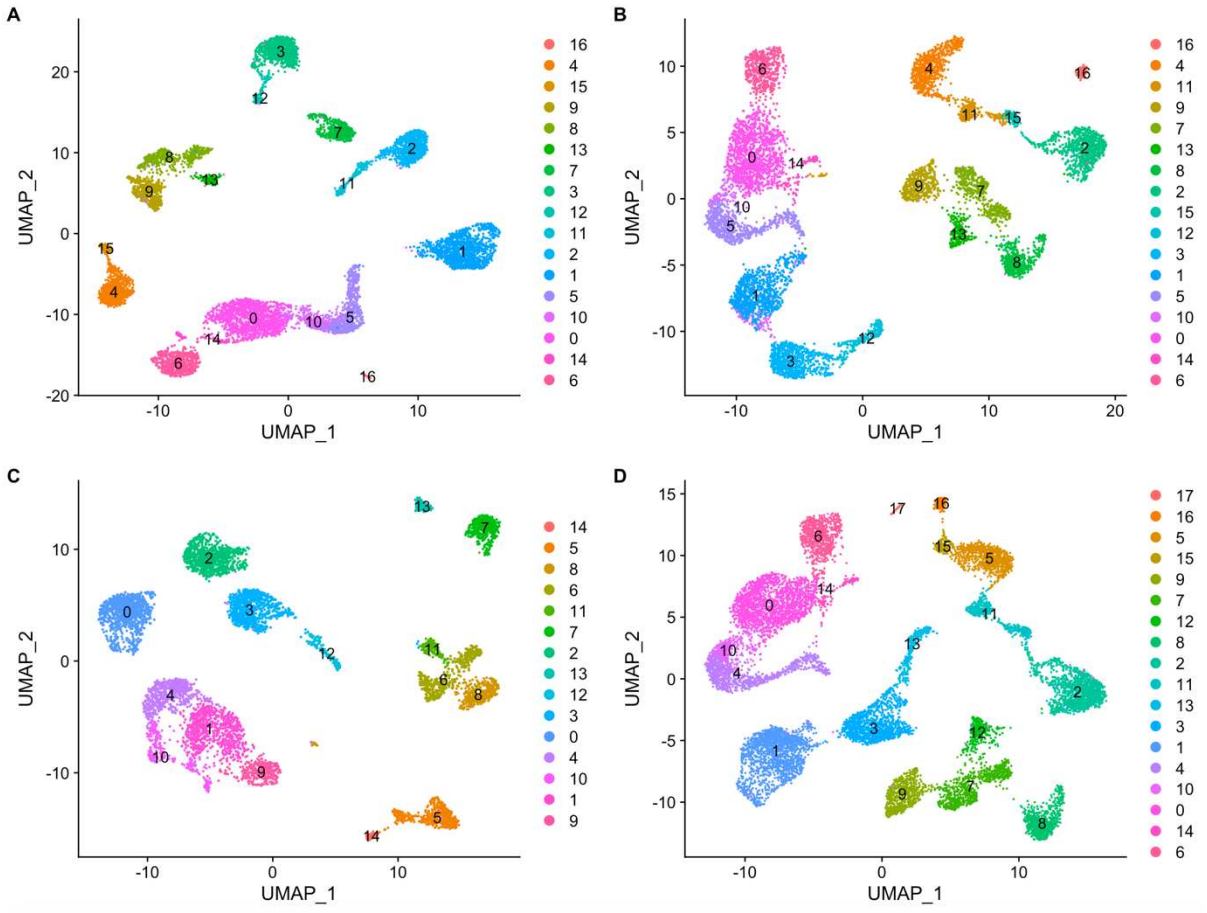
Few examples of Bateson-Dobzhansky-Muller incompatibilities (BDMIs) have been identified and most of them have been established by empirical work using model organisms such as drosophila and mouse (Presgraves, 2010; Maheshwari & Barbash, 2011). Based on these studies there is strong support for a non-random expression of intrinsic isolation on the X chromosome, but few studies control for recessively of hybrid dysfunction reviewed by (Qvarnström & Bailey, 2009; Charlesworth et al., 2018). However, the majority of the studies that have detected BDMIs have focused on species pairs than have diverged 2 or 3 million years ago and therefore may not naturally hybridize any more (Dobzhansky, 1936; Sawamura, 2000; Mallet, 2006; Presgraves & Meiklejohn, 2021). In order to obtain a comprehensive understanding on the role of BDMIs in speciation it is important to reveal the molecular basis to hybrid dysfunction in natural populations of closely related species that hybridize. Only two genes driving hybrid incompatibilities have been identified in vertebrates, *prdm9* for mouse (Mihola et al., 2009) causing hybrid sterility and *xmrk* in sword tail fish causing hybrid inviability (Gordon, 1937; Powell et al., 2020). There are, to our

knowledge, no previous identified candidate genes for hybrid incompatibilities in birds where males constitute the homogametic sex. Male gametogenesis is a complicated process that involves many stages of development that could potentially fail in hybrids. Since this process also is encoded by a large percentage of the genome, the number of potential diverged genomic sites that may cause hybrid male sterility is high. Identifying high-probability BMDI candidates for hybrid male sterility therefore requires establishing high-resolution genotype-phenotype connections.

In this study, we use naturally hybridizing *Ficedula* flycatchers to shed novel light on the evolution of hybrid male sterility in a study system with well-developed genomic tools (Qvarnström et al., 2010, 2016). Collared and pied flycatchers are closely related species that diverged less than ~1 million years ago (Ellegren et al., 2012). The males differ in important sexually selected traits such as plumage coloration and song vocalization but mixed-species pairing regularly occur at natural hybrid zones. The most recently formed hybrid zone is found on the Baltic Island Öland where collared flycatchers colonized the island during the 1960s. Hybrids between collared and pied flycatchers display several intermediate phenotypic including plumage (Alatalo et al., 1982; Svedin et al., 2008) and song (Gelter, 1987; Qvarnström et al., 2010). Hybrid males also show clear signs of intrinsic incompatibilities in terms of higher metabolic rate (McFarlane et al., 2016), and malformed sperm (Alund et al., 2013). Mugal et al., 2020 found an intermediate gene expression of testis genes in bulk samples from hybrids indicating no major incompatibilities between collared and pied flycatchers. However, hybrids may have a different testis cell composition than the pure species (Hunnicutt et al., 2022) and we therefore recently characterized the spermatogenesis of *Ficedula* flycatchers at a single cell level to reveal species differences in gene expression at specific stages of the process (Segami et al., 2022). We found that most

126 differentially expressed (DE) genes were active after meiosis and typically coding for cell
127 respiratory or motility pathways at a late stage of spermatogenesis. However, we also
128 detected DE genes during meiosis. Among these DE genes there was a tendency for
129 enrichment of Z-linked genes suggesting fast Z evolution.
130
131 Here, we aim at identifying the molecular basis of sterility in *Ficedula* male hybrids by
132 characterizing the hybrid spermatogenesis transcriptome at a single cell level. Using single-
133 cell RNA sequencing of testis cell suspensions of two hybrids, three Collareds and three Pied
134 flycatchers and markers developed by (Segami et al., 2022) and histology sections we first
135 aim to investigate whether hybrid males go through all "normal" stages of spermatogenesis
136 by identifying if hybrids have all testis cell types found in the parental species. Secondly, we
137 will investigate whether hybrid expression patterns are different from the parental species in
138 any particular stage of spermatogenesis as an indication of a possible failure. Finally, we
139 identified non-synonymous fixed differences between the two species and analyzed their role
140 across the various stages of spermatogenesis and connect this information with possible miss-
141 expression patterns.
142
143 **Results**
144
145 We generated scRNA data from testis cells suspensions obtained from 8 sacrificed male birds
146 (3 Pied flycatchers, 3 collared flycatchers and 2 F1 hybrids, both with a pied mother, see
147 Table S1). We created a consensus clustering using the 6 pure species samples to use as a
148 baseline for all our consecutive analysis. The identities of the obtained 17 different cell
149 clusters (Figure 1A) were verified using the characterized flycatcher markers for
150 spermatogenesis populations developed by Segami et al 2022 (Figure S1). Our new clusters

151 corresponded to all the previously identified testis cell clusters in flycatcher spermatogenesis
152 except for one of the somatic cell clusters that was not found (Segami et al 2022).
153



154

155 ***Figure 1. UMAP plot for different clustering comparisons.*** *(A) Clustering of pure spercies'*
156 *testis cells samples, 3 collared flycatchers and 3 pied flycatchers. (B) Clustering of Collared*
157 *flycatcher testis cell samples and hybrid samples, 3 collared flycatchers and 2 hybrid*
158 *flycatchers. (C) Clustering of Pied flycatcher testis cell samples, 3 pied flycatchers and 2*
159 *hybrid flycatchers. (D) Clustering of all samples together. All the clusterings are highly*
160 *consistent and vary only in the number of somatic cell cluster or in the case of panel C for the*
161 *amount of clusters identified in the post meiotic stages, 5 spermatid clusters instead of the 6*
162 *spermatid clusters found in all the other clusterings.*
163

Using the pure species reference clustering, we made projections of our 2 hybrid samples to examine equivalences with all pure species testis cell clusters (Figure 2). We find that male hybrids have all the testis cell clusters that pure species males have. However, there was a significant lower proportion of cells belonging to the spermatid clusters in hybrids (Table 1). This finding matches the histological observations of a lower overall number of spermatids produced in hybrids (Figure 3). Histology also shows an abnormal head phenotype of the developing spermatids as well as a lack of structural bundle organization of hybrid sperm cells that is observed in the samples from pure species males (Figure 3).

Table 1. General linear model with binomial response of spermatid cells and non-spermatid cells. *We use species of our 8 individuals as explanatory variable.*

Coefficients	Estimate	Std. Error	Z value	P value	Confidence Intervals
Intercept	-0.37	0.03	-14.5	**< 0.01**	**-0.43 - -0.32**
Hybrid	-0.4	0.05	-8.77	**< 0.01**	**-0.49 - -0.31**
Pied	0.11	0.04	2.73	**<0.01**	**0.03 – 0.19**

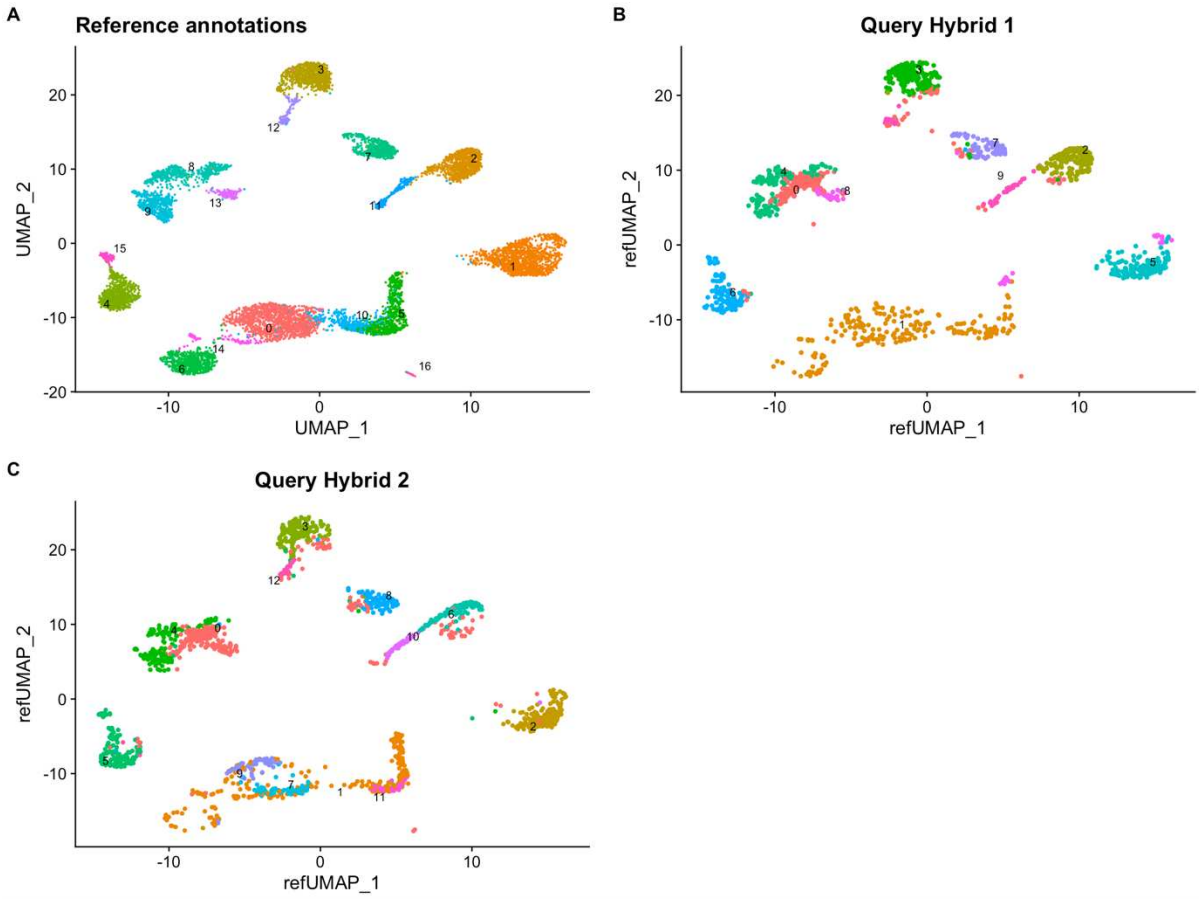


Figure 2. UMAP of hybrid samples mapped to the reference clustering of the pure species. *Hybrid testis cells map to all stages of spermatogenesis found in the pure species, however there is a significant proportional underrepresentation of the post meiotic clusters.*

In order to make differential gene expression comparisons, we did three additional clustering including our hybrid samples in the following groups: collareds and hybrids, pieds and hybrids, all individuals (Figure 1B -D). We decided to perform all comparisons in case hybrids would complicate clustering due to their testis cell populations being too deviating. We found consistency across all clusters indicating that hybrids do not bias the clustering process (Figure 1). Because the IDs for equivalent cell clusters differ in the different clusterings, we assigned a common name to equivalent clusters (Figure 4).

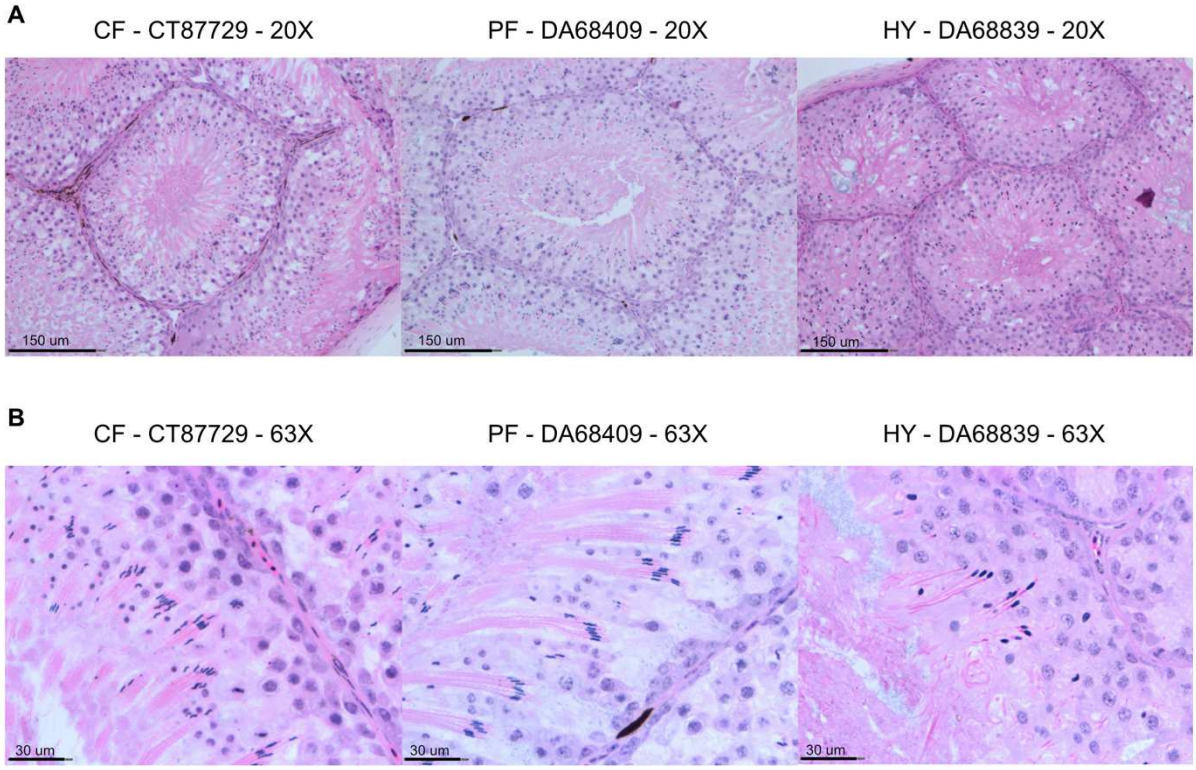


Figure 3. Histology sections of testis samples of Collared, Pied and Hybrids with hematoxylin and eosin staining. *(A) Transversal cut of seminiferous tubuli. (B) Detail of spermatid bundles maturing towards the lumen of the tubuli. The spermatid heads have an abnormal phenotype in the hybrid and the bundle of spermatids structure is lacking. Hybrids also show less quantity of spermatids with respect to the pure species.*

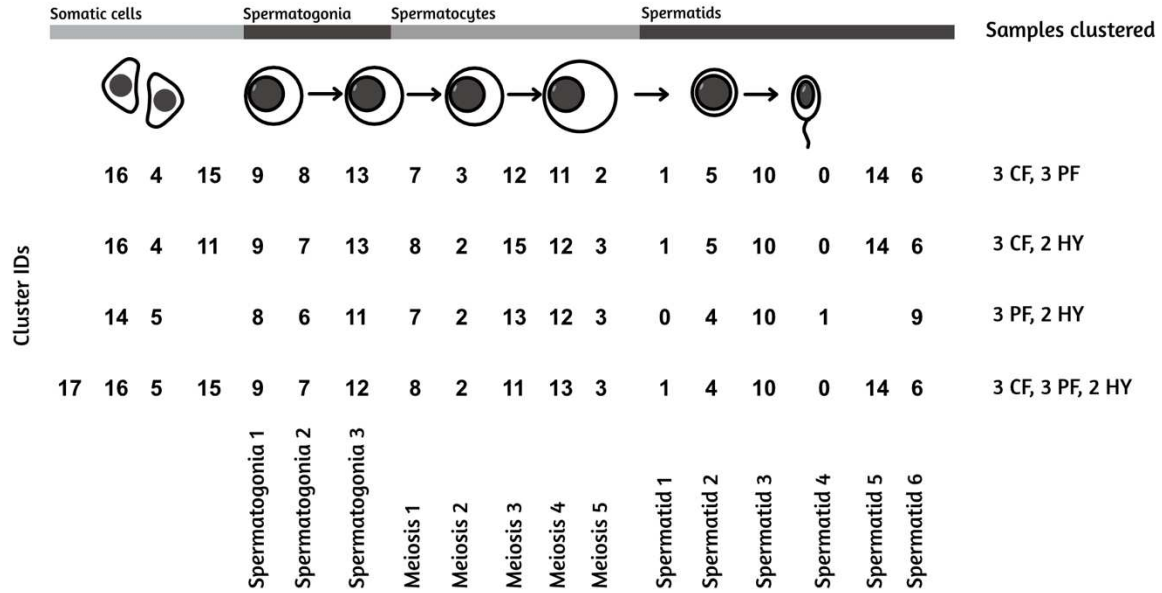


Figure 4. Stages of spermatogenesis and cluster IDs for the different clustering combinations.

Differential expression (DE) analysis between collared and pied, was also consistent with previous patterns found by (Segami et al 2022), most differences are found in the post-meiotic clusters, some DE genes are also found in meiosis clusters and almost no DE are found in the earliest stages of spermatogenesis (Figure 5A). The 3 comparisons of expression patterns against hybrids (i.e. collared against hybrids, pied against hybrids and pure species against hybrids) were consistent with each other. There was a slightly lower proportion of DE genes during the meiosis stages observed in the three contrasts including hybrids (Figure 5, C-D) as compared to the contrast between pied and collared flycatchers (Figure 5 A). An analysis of the hybrid inheritance patterns of gene expression shows differences between the different stages of spermatogenesis indicating that miss-expression, additive expression or a more dominant parental species expression vary along the timeline (Figure 6). For example, visual inspection suggests that “Meiosis 4” shows the strongest tendency for miss-expression (both over-dominant and under-dominant) with a narrow shape of the ellipse occupying the

214 right top and left bottom areas of the plot. Statistical analysis is needed to assess its

215 significance.

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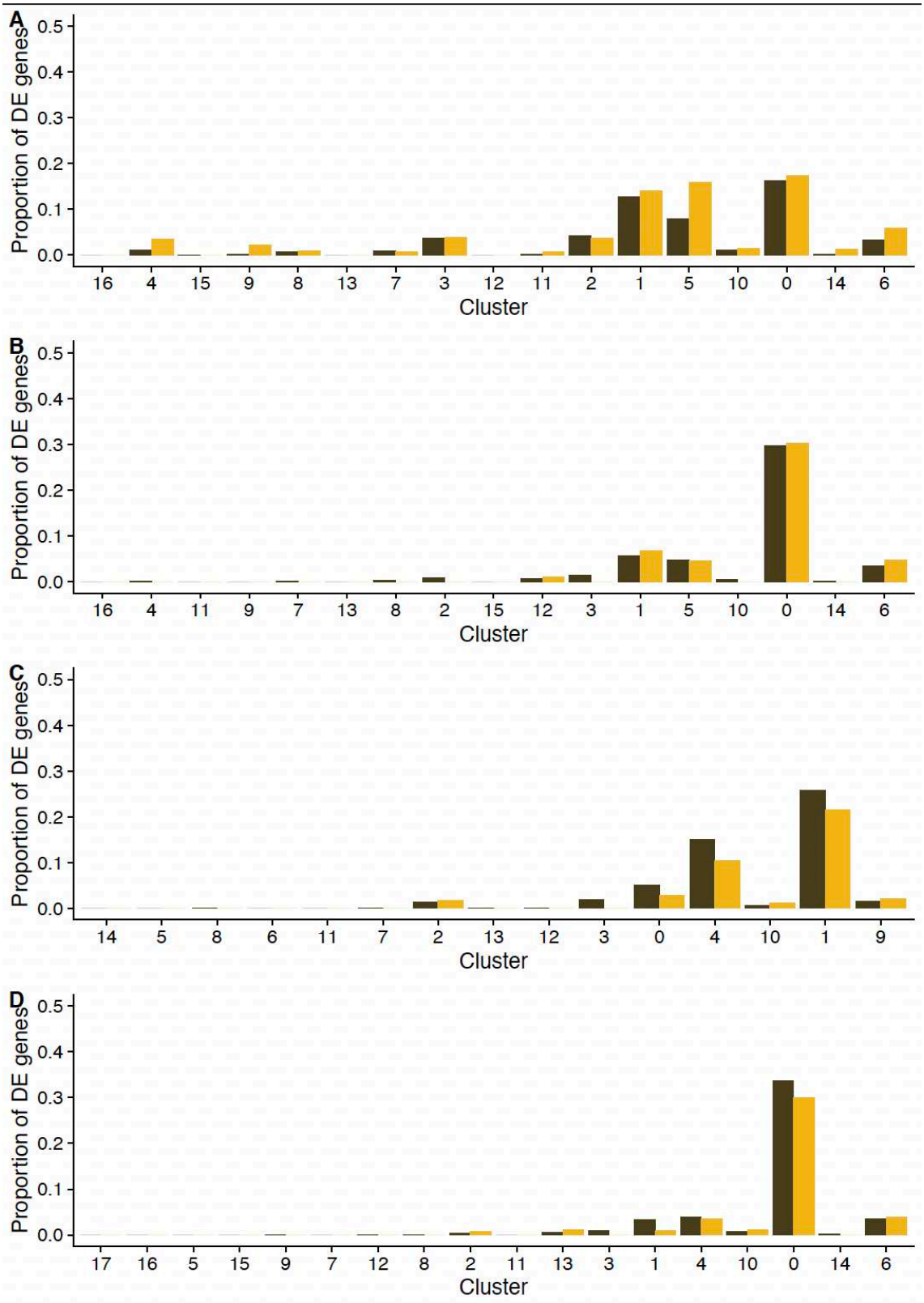

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Figure 5. Proportion of DE genes along the timeline of cell clusters of spermatogenesis for different pairwise comparisons. *(A) Collared against pied flycatcher. (B) Collared flycatcher against hybrids. (C) Pied flycatcher against hybrids. (D) Collared and pied flycatchers combined against hybrids. The general pattern of more similar gene expression at the first stage of spermatogenesis is consistent across the four comparisons. The differential expression genes start to be found in meiosis clusters and in higher proportion at the post-meiosis stages. All pairwise comparisons including hybrids show a higher proportion of DE genes than the collared vs pied comparison in one of the post meiotic clusters.*

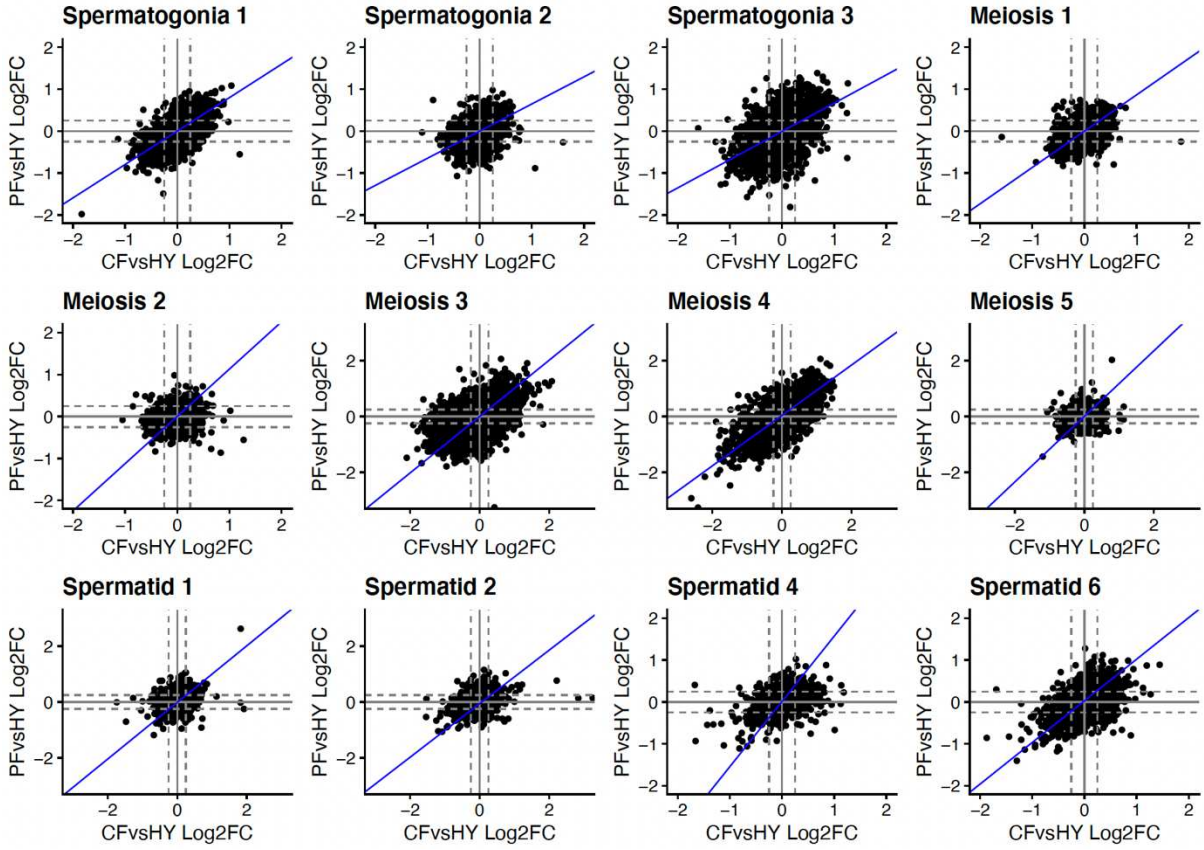

Figure 6. Inheritance patterns of inheritance in different stages of spermatogenesis in F1 Ficedula hybrids. *Each dot represents a single gene, the grey dashed lines represent the 0.25 log2-fold change threshold, and the blue regression line is the major axis of variation in*

log2-fold changes. The inheritance patterns vary on the different stages of spermatogenesis, suggested by the change of the shape of the ellipsis.

We identified 303 genes with fixed non-synonymous differences between collared and pied flycatchers across the whole genome (Table S2). These genes are significantly enriched on the Z chromosome (hypergeometric test: 65,590, 21908,241, p = 2.914911e-48). Gene Ontology (GO) analysis suggest that several of these genes belong to gene networks involved in regulating several processes associated with meiosis, including DNA replication, nuclear division, chromatin remodeling and packaging (Figure 7, Table 2). Moreover, several of the genes included in the identified networks are located on the Z chromosome. We found that 71 genes with non-synonymous fixed differences between collared and pied flycatchers were expressed in the testis cell clusters (Figure 8). Interestingly, there is a clear pattern showing that they are mainly expressed in pre-meiotic and meiotic clusters, especially during Spermatogonia 1, Spermatogonia2 and Meiosis 3. By contrast, we find very few genes with fixed differences between the two species that were expressed in the spermatid clusters.

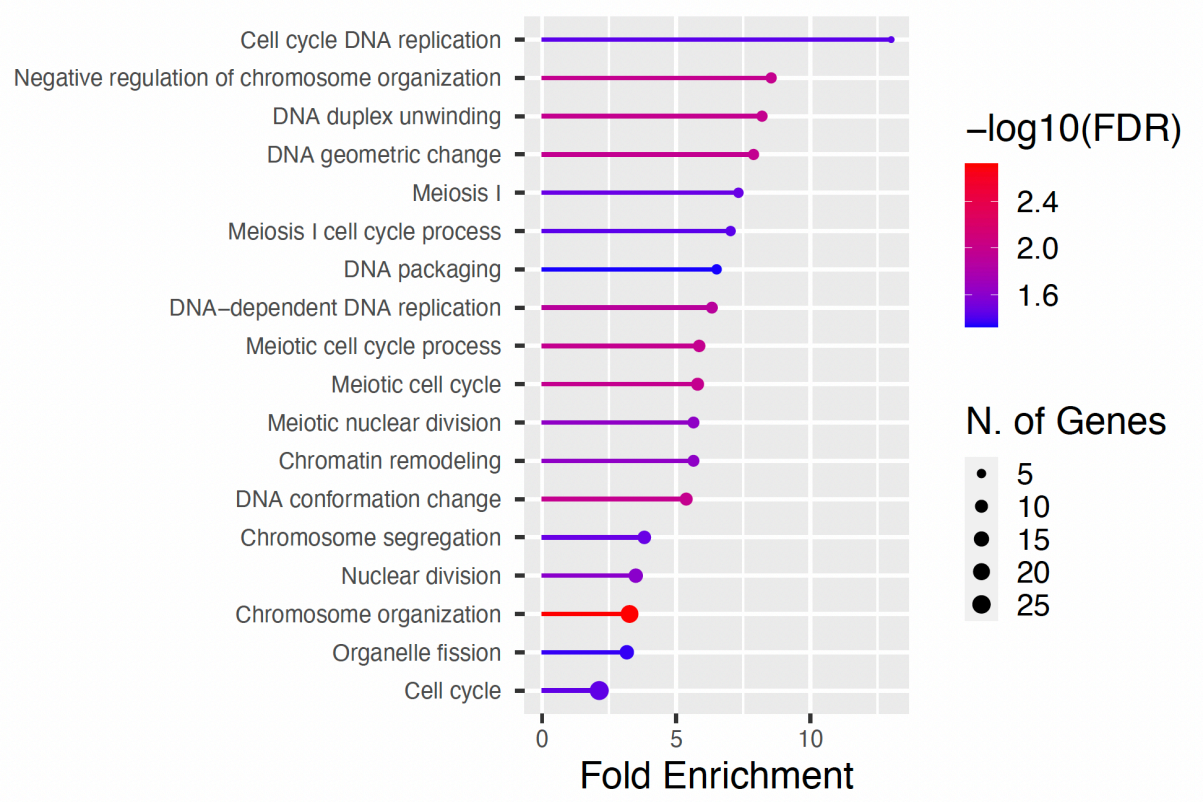

Figure 7. Significant GO terms for fixed non-synonymous differences between collared and pied flycatchers.

Table 2. Non redundant GO terms of fixed differences analysis.

TermID	*Name*	*Frequency*
GO:0051276	*"chromosome organization"*	*1.89362971*
GO:2001251	*"negative regulation of chromosome organization"*	*0.04845581*
GO:1903046	*"meiotic cell cycle process"*	*0.14581488*
GO:0007059	*"chromosome segregation"*	*0.44459536*
GO:0006261	*"DNA-dependent DNA replication"*	*0.55333922*
GO:0007049	*"cell cycle"*	*1.72238547*
GO:0000280	*"nuclear division"*	*0.23301961*
GO:0048285	*"organelle fission"*	*0.27408856*

GO:0044786	*"cell cycle DNA replication"*	*0.02981259*

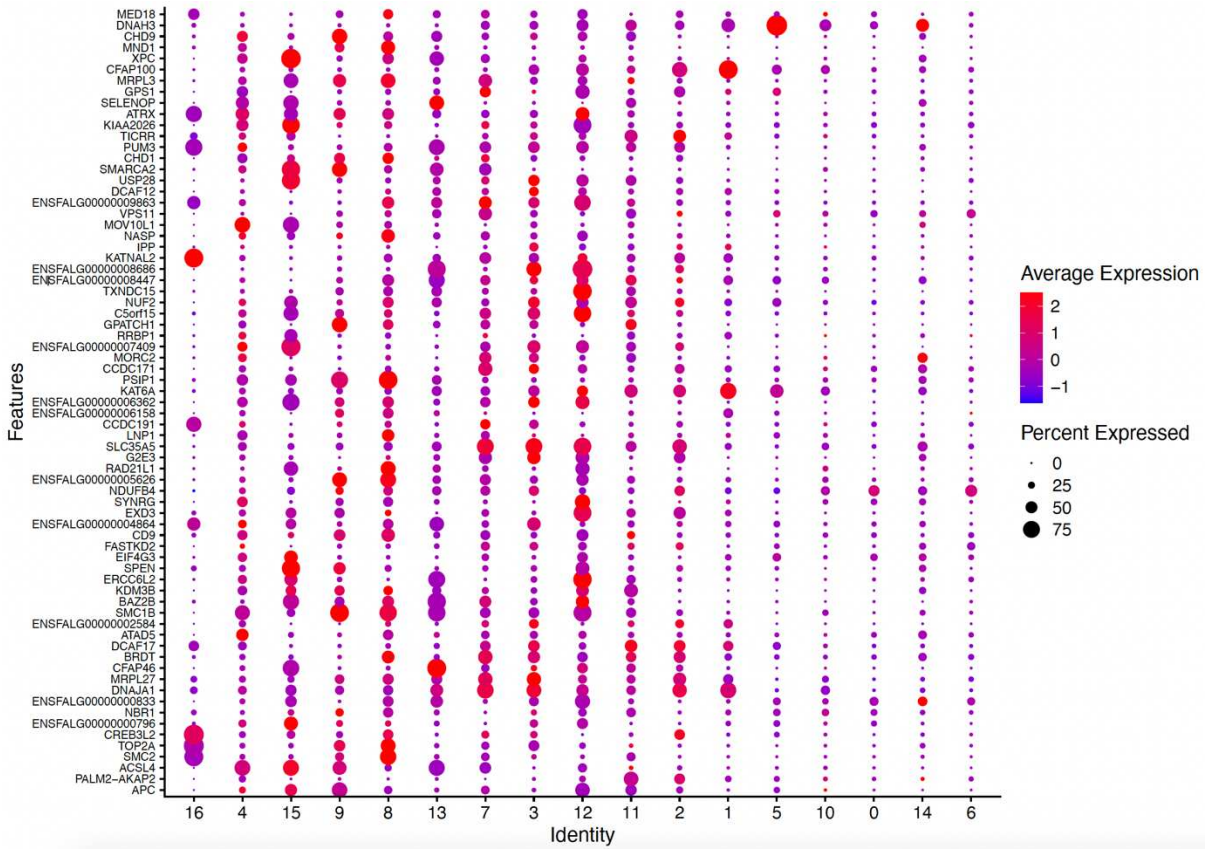

Figure 8. Average gene expression of genes with fixed non-synonymous differences between Collared and Pied flycatcher across the spermatogenesis stages. *There is a clear pattern of absence of the fixed differences in genes expressed in spermatid clusters (1, 5, 10, 0, 14, 6). Spermatogonia and the meiosis cell clusters have the highest expression of genes with non-synonymous differences, particularly cluster 12 belonging to meiosis seems to be enriched.*

A STRING analysis based on the 71 genes with non-synonymous differences that were expressed in our testis cell clusters revealed two major networks of interacting genes (Figure 9). Of these interacting genes, 3 were also DE between collared and pied flycatchers: CHD1,

PSIP1 and SMC1B. CDH1 is a transcription factor associated with chromatin remodeling, PSIP1 a transcriptional co-activator involved in stem cell differentiation and SMC1B is a meiosis specific component of cohesion complex involved in chromatid movement and organization (Table 3).

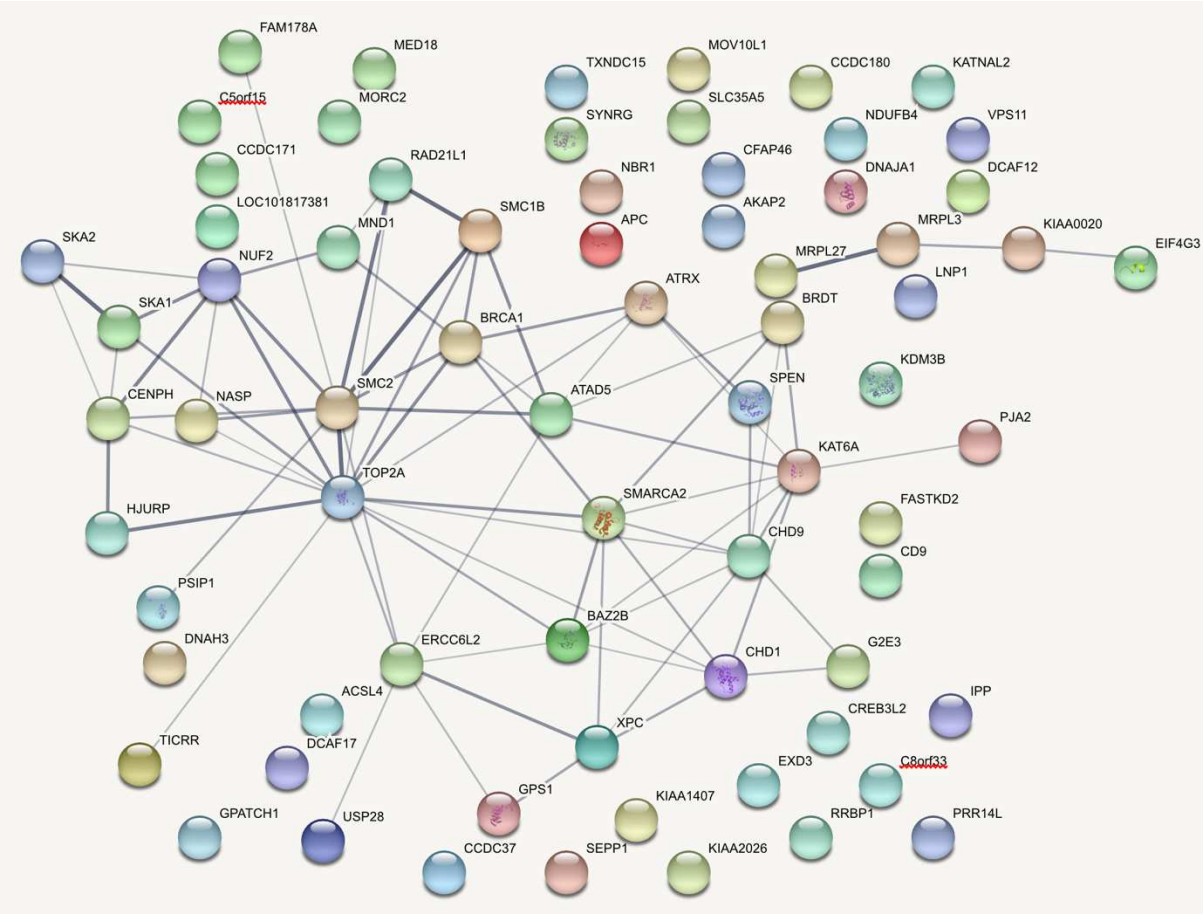

Figure 9. STRING Interacting gene networks for genes with non-synonymous fixed differences that are expressed in our spermatogenesis cell populations.

Table 3. Genes with non-synonymous fixed differences between collared and pied flycatchers that are also DE.

Gene name	avg_log2FC	p_val_adj	cluster	Location	Stage	Interactive
DNAJA1	0.34508428	0.00695606	0	ChrZ	Spermatid 4	F

BRDT	0.18172319	0.03961799	1	Chr8	Spermatid 1	F
ENSFALG00000002584	-0.9758906	1.28E-12	1	ChrZ	Spermatid 1	F
SMC1B	0.47704003	1.74E-06	0	Chr1A	Spermatid 4	T
SMC1B	0.47439395	0.04670332	2	Chr1A	Meiosis 5	T
FASTKD2	0.39990957	0.0190364	5	Chr7	Spermatid 2	F
CD9	0.3525327	0.02517891	5	Chr1A	Spermatid 2	F
EXD3	0.48889256	0.03627111	2	Chr17	Meiosis 5	F
ENSFALG00000005626	0.28364684	0.04608302	0	Chr19	Spermatid 4	F
SLC35A5	0.47537443	1.38E-07	1	Chr1	Spermatid 1	F
SLC35A5	0.61874925	0.03077243	8	Chr1	Spermatogonia 2	F
SLC35A5	0.38004429	0.00117804	5	Chr1	Spermatid 2	F
PSIP1	0.19808177	0.02319633	0	NA	Spermatid 4	T
C5orf15	0.25793601	0.00053715	1	Chr13	Spermatid 1	F
KATNAL2	0.71994615	1.68E-18	1	ChrZ	Spermatid 1	F
KATNAL2	0.48924288	0.00379985	3	ChrZ	Meiosis 2	F
KATNAL2	0.74953236	3.21E-10	0	ChrZ	Spermatid 4	F
KATNAL2	0.82509373	1.89E-12	5	ChrZ	Spermatid 2	F
VPS11	0.34249787	0.0019175	1	Chr24	Spermatid 1	F
CHD1	-1.1969406	0.0002026	4	NA	Somatic	T

We also found that some of the non-interacting genes were DE (Table 3). Of these, EXD3, SLC35A5 and KATNAL2 are DE in spermatogonia and meiosis cell clusters. EXD3 is an exonuclease, SLC35A5 is transmembrane sugar transporter and KATNAL2 is a katanin microtubule-severing protein.

Discussion

Identifying genes involved in genetic incompatibilities causing hybrid sterility or inviability is a long-standing challenge in speciation research. Here we present several lines of evidence implying that hybrid male sterility in *Ficedula* flycatchers is associated with a failure of meiosis and we propose candidate genes involved in genetic incompatibilities causing this failure. We conclude that dysfunctional meiosis most likely leads to aberrant chromosome segregation and/or faulty chromatin packing, similarly to what has been found in mouse (Mihola et al., 2009) and drosophila (Kanippayoor et al., 2020). This conclusion is based on combined evidence from whole genome DNA re-sequencing data, single cell transcriptomics of testis samples and testis histology sections. STRING and GO analysis show that genes with non-synonymous differences between the two species of flycatchers are part of gene networks involved in key processes of meiosis. A lower proportion of spermatids produced by hybrid males as revealed by histology and single cell data implies that a proportion of the aberrant spermatids undergo apoptosis.

Based on the histological observations of a lower overall number of spermatids, all with an abnormal head phenotype and lacking the structural bundle organization in the testis of hybrid flycatchers (Figure 3), we were surprised to find that all main cell clusters were present in their testis samples. However, many genes with non-synonymous fixed differences between collared and pied flycatchers were expressed during, the early and most fundamental developmental stage of spermatogenesis (i.e. during Spermatogonia 1, Spermatogonia2 and Meiosis 3). Moreover, we revealed two major networks of interacting genes (Figure 10) where 3 genes stand-out by also being DE between collared and pied flycatchers: CHD1, PSIP1 and SMC1B. In addition, we detected three additional genes with fixed-differences

that were DE in spermatogonia and meiosis cell clusters: EXD3, SLC35A5 and KATNAL2. Based on the known central functions of all these genes (see Table 3) failure during meiosis is a highly likely explanation for dysfunction of spermatogenesis in hybrid *Ficedula* flycatchers.

Two of the BDMI candidate genes for hybrid sterility in flycatchers have been described before as having a crucial role during spermatogenesis and/or for maintaining fertility. SCMB1 is required for sister chromatid cohesion and DNA recombination in mouse and deficient SCMB1 mice of both sexes show sterility associated to pachytene in males and meiosis II failure in females (Revenkova et al., 2004). This roughly correspond with the stage where we observe meiosis failure in flycatchers, and although this study focuses only on male gametogenesis, we know that female F1 hybrids are also sterile as they produce eggs that do not hatch(Svedin et al., 2008). It is also shown that a spontaneous mutation in the SCMB1 mice gene caused sterility in both sexes (Takabayashi et al., 2009). In addition, SCMB1 also causes sterility in both sexes of zebrafish impairing correct synapsis (Islam et al., 2021). Thus, although SCMB1 has a slightly different role among vertebrates, this gene is essential for correct meiosis. One of the most interesting things is that in all these cases there was an essential role of SCMB1 for fertility but not for sperm development, i.e. spermatogenesis was not arrested but sperm cells were not fertile, this aligns perfectly with our observations in flycatchers. KATNAL2 has several essential functions related to microtubule dynamics, cytokinesis and ciliogenesis. Knockouts of this gene results in aberrant phenotypes of cells showing multinuclearity and abnormal sperm heads in mice (Ververis et al., 2016; Houston et al., 2020). This gene also has a critical role during spermiogenesis as it is involved in sperm tail growth, sperm head shaping, acrosome attachment and sperm release to the lumen of seminiferous tubuli (Dunleavy et al., 2017). The defects described for mice caused by

334 mutations or knockout of KATNAL2 fit well with our observation of abnormal head shape in
335 the histology samples of the testis of hybrid flycatchers.
336
337 Our results may appear contractionary in the light of the first developmental stages of
338 spermatogenesis (i.e. mitosis and meiosis) being known to be strongly evolutionary
339 constrained both in mammals (Larson et al., 2018; Kopania et al., 2022) and in birds (Segami
340 et al 2022). Most of the known examples of genes associated with hybrid sterility in animals
341 are, in fact, expressed during either the pre- or post- meiotic stages of gametogenesis (Coyne
342 & Orr, 2004). However, most of these examples come from studies based on drosophila
343 species that usually have diverged for several millions of years. The only previously known
344 example of a specific gene causing hybrid sterility in vertebrates is based on studies of a
345 natural hybrid zone between two closely related species of mouse. In that case, *prdm9* was
346 shown to cause hybrid sterility through disrupted meiosis (Mihola et al., 2009) . One possible
347 explanation to the findings in flycatchers and mice is that disruption of meiosis in hybrids
348 quickly leads to reproductive isolation, which in turn leads to accumulation of genetic
349 differences in faster evolving regions of the genome that may rather be a consequence than a
350 cause of speciation.
351
352 In agreement with both theoretical expectations and empirical findings based on study
353 species with XY sex-determination systems, our findings imply an important role of Z-linked
354 BDMIs in speciation. The genes expressed during spermatogenesis that contain non-
355 synonymous differences between the two flycatcher species are enriched on the Z
356 chromosome and three of our BDMI candidate genes are Z-linked. Two additional detected
357 candidate genes, CHD1 and PSIP1 are located on a scaffold that remains unassigned in the
358 flycatchers but are known to be located on the Z chromosome in the zebra finch. Since birds

359 have a relatively stable karyotypes which also tend to keep gene order across species
360 (Ellegren, 2010), we consider it highly likely that these genes are Z-linked also in
361 flycatchers. This remains to be confirmed once a better assembly of the flycatcher genome is
362 available.
363
364 The latest evidence for fast Z, not only in flycatchers (Ellegren et al., 2012; Mugal et al.,
365 2020) but also in birds in general, points to drift rather than selection (Mank et al., 2010) as a
366 main driving evolutionary force. However, since drift is a very slow evolutionary process, we
367 consider drift unlikely to explain species divergence in a highly evolutionary conserved
368 function such as meiosis. Instead, selection may have acted on standing genetic variation at
369 the very early stages of the split between the two flycatchers resulting in comparatively fast
370 lineage sorting of ancestral allelic variation in these particular genes.
371
372 Our findings correspond well with what has been previously described in model species with
373 XY- sex determination systems. The two proteins encoded by SCMB1 and KATNAL2 are
374 with a high probability central for causing genetic incompatibilities in F1 *Ficedula* hybrids.
375 Since a handful of incompatibilities, rather than a simply two allele single incompatibility
376 contribute to sterility in hybrid flycatchers, several incompatibilities may have arisen in a
377 snowballing fashion. Although milder incompatibilities between fast evolving alleles are
378 predicted to arise first during genetic divergence our results points in the opposite direction
379 with the most serious incompatibilities affecting central functions having arisen first. This
380 could be due to meiosis being a particularly sensitive process given the complicated networks
381 of genes. One change could then cause selection favoring compensatory changes in
382 epistatically interacting genes thereby unleashing snowballing of incompatibilities.
383

384 This study is to our knowledge the first one to propose candidate BDMI genes causing hybrid
385 sterility in birds and together with (Rosser et al., 2022) the second study to propose candidate
386 genes causing hybrid sterility in ZW systems. We revealed evidence for disrupted meiosis
387 during spermatogenesis and an overrepresentation of Z-linkage of the targeted candidate
388 incompatibility genes. Our results challenge the assumption that speciation processes are
389 driven by fast evolving genes by showing that changes in genes with highly conserved
390 functions may, when slightly modified, be likely to quickly cause speciation by ensuring
391 reproductive isolation at secondary contact.
392
393 **Methods**
394 **Samples and sequencing**
395 Three collared flycatchers, three pied flycatcher and two F1 hybrids with a pied mother were
396 caught at the start of the reproductive season in the monitored populations of Öland
397 (57°100N, 16°580E) (Anna Qvarnström et al., 2010), Sweden in May 2020. The birds were
398 kept in outdoor aviaries until all birds were caught before being sacrificed by cervical
399 dislocation and immediately dissected. The testes were cleaned from other tissues and cut in
400 halves on a cold petri dish with PBD BSA buffer. A half of testis was mechanically
401 disassociated in 3ml of PBD BSA buffer using a gentleMACS Dissociator (Miltenyi Biotech,
402 Bergisch Gladbach, Germany) as described in (Segami et al 2022). We verified that cell
403 viability was over 80% by examining the cell suspensions under the microscope with
404 propidium iodide and Hoechst staining. Then the cell suspension concentration was
405 determined with a Neubauer chamber and diluted to achieve 1x10^6 cell/ml. The final cell
406 suspensions were delivered to the sequencing platform for library preparations with 10X
407 genomics Chromium Single Cell 3' v3 kit for scRNAseq. All the libraries were sequenced in
408 two lanes of NovaSeq S1 flow cells.

409

410 **Data pre-processing**

411 We used the 10x Genomics Cell Ranger v. 6.0.0 (Zheng et al., 2017) pipeline, we created a
412 custom reference for cell counting based on the public genome for *Ficedula albicollis* (v. 1.4)
413 and the public annotation from *Ensembl* (v. 1.4). After obtaining count matrixes for every
414 gene on each cell for our 8 samples, we generated .loom files for each individual using the
415 command run10X from the python package Velocyto v. 0.17.17 (La Manno et al., 2018).
416 Finally, we exported all the loom files to Seurat v. 4.1.0 (Stuart et al., 2019) for downstream
417 analysis.

418

419 **Filtering, normalization, and clustering**

420 We converted all the loom files to Seurat objects and merged the objects in the following
421 combinations: only pure species (6 individuals), Collareds and hybrids (5 individuals), Pied
422 and hybrids (5 individuals), all individuals (8 individuals). We excluded all cells with less
423 than 200 features (expressed genes), more than 2500 and less than 5% of mitochondrial
424 genes. For normalization, variance stabilization and integration of the different samples in a
425 single object we used the SCTransform v2 (Choudhary & Satija, 2022) command together
426 with anchor integration as suggested on the Seurat documentation. Then we performed
427 standard clustering analysis for all the combinations with the following parameters, we run
428 UMAP (Uniform Manifold Approximation and Projection) with 20 dimentions and min.dist
429 = 0.6. Finally, we found clusters using a resolution of 0.5. For all the 4 clusterings we found
430 an heterogeneous cluster with no good markers that we excluded and reclustered the
431 remaining cells again. After obtaining all our final clusterings we established the identity of
432 all clusters using the flycatcher gene markers characterized by (Segami et al 2022). We then
433 established the equivalences of the clusters across our different clusterings (Figure 4).

434

435 **Histology**

436 Whole testes were preserved in formalin for 3 collareds, 3 pieds and 2 hybrids. Histology
437 sections were done for each sample fixed to slides and then stained with hematoxylin and
438 eosin. Pictures were taken using an optical microscope with magnification 65X and 20X.

439

440 **Hybrid cell clusters analysis**

441 In order to test if our hybrid samples contained all the cell clusters present in the
442 spermatogenesis of pure species, we performed a unimodal UMAP projection of each hybrid
443 sample on the reference clustering of the pure species. We visually observed in the clustering
444 analysis that the proportion of spermatids was lower in both hybrids and therefore decided to
445 test whether the proportion of spermatids was significantly different than the proportion of
446 spermatids found in pure species males. For this purpose, we clustered all our samples
447 individually and identified the proportion of total cells that corresponded to spermatids. Then
448 we performed a binomial general linear model with number of spermatid cells and non-
449 spermatid cells as response variable and species as explanatory variable.

450

451 **Differential expression analysis**

452 We performed DE analysis for all our clusterings comparing collared vs. pied, collared vs.
453 hybrid, pied vs. hybrid and pure species vs. hybrid. The Seurat function FindMarkers was
454 used to perform a Wilcoxon rank sum test to calculate average fold change and identify DE
455 genes. We used the normalized counts from RNA slot of our Seurat objects and did not use a
456 minimum threshold for fold change. To establish if a gene was significantly DE, we used the
457 adjusted p-value with a 0.05 threshold. In order to explore the hybrid gene expression
458 inheritance patterns for each cluster, we plotted the obtained fold changes for the

459 comparisons of collared vs. hybrid and pied vs. hybrid for each gene. We then calculated the
460 major variation regression line using the r package lmodel2.
461
462 **Identification of non-synonymous fixed differences**
463 Fixed differences between collared flycatcher and pied flycatcher were identified based on
464 polymorphism data for 19 collared and 19 pied flycatchers from the island of Öland retrieved
465 from (Burri et al., 2015). We excluded polymorphic sites with more than two alleles at a
466 specific site and further restricted the data to sites that were covered by at least 1 read across
467 all 38 individuals. Sites that were classified as fixed differences if all collared and pied
468 individuals showed a different allele. Fixed differences were annotated using SnpEff version
469 4.3T (Cingolani et al., 2012) based on Ensembl gene annotation v 73 (Uebbing et al., 2016),
470 which resulted in a set of 303 non-synonymous fixed differences across 21908 genes.
471
472 **Gene Ontology and STRING Analysis**
473 We did Gene Ontology enrichment analysis for biological processes on the set of genes with
474 non-synonymous fixed differences and on the subset of these genes that were expressed in
475 our testis cell clusters using the web tool ShinyGO v.075 (Ge et al., 2020). To prescind of the
476 redundant and/or nested GO terms we used the web tool REVIGO (Supek et al., 2011). To
477 identify the interacting gene networks we performed STRING (Szklarczyk et al., 2019)
478 analysis using their web tool v.11.5 on the same sets of genes.
479
480 **Author Contributions**
481 AQ and JCS conceived the study. AQ, JCS and CC collected the samples. JCS sacrificed the
482 birds. CC performed the dissections. JCS, CC and CB carried out the cell suspension
483 protocol. MSz performed the histology. JCS and MS performed the single cell clustering and

484 bioinformatics analysis. CFM performed bioinformatics analysis and identified the fixed
485 differences. JCS, MS and CFM performed and discussed the GO analysis and all the
486 statistical analysis. JCS, MS, CFM and AQ discussed and interpreted all the results. JCS and
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488
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505
506 **Ethical permits**
507 Permit for keeping flycatchers in aviaries and sacrificing maximum 17 flycatchers per year.

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Bibliography

Alatalo, R. V., Gustafsson, L., & Lundberg, A. (1982). Hybridization and breeding success of collared and pied flycatchers on the island of Gotland. *Auk*, *99*(2).

Alund, M., Immler, S., Rice, A. M., & Qvarnstrom, A. (2013). Low fertility of wild hybrid male flycatchers despite recent divergence. *Biol. Lett.*, *9*(3), 20130169. https://doi.org/10.1098/rsbl.2013.0169

Bateson, W. (1909). Heredity and variation in modern lights. In *Seward AC (ed) Darwin and modern science.* (pp. 85–101). Cambridge University Press. https://doi.org/10.1017/CBO9780511693953.006

Burri, R., Nater, A., Kawakami, T., Mugal, C. F., Olason, P. I., Smeds, L., Suh, A., Dutoit, L., Bureš, S., Garamszegi, L. Z., Hogner, S., Moreno, J., Qvarnström, A., Ružić, M., Sæther, S. A., Sætre, G. P., Török, J., & Ellegren, H. (2015). Linked selection and recombination rate variation drive the evolution of the genomic landscape of differentiation across the speciation continuum of Ficedula flycatchers. *Genome Res.*, *25*(11), 1656–1665. https://doi.org/10.1101/GR.196485.115

Charlesworth, B., Coyne, J. A., & Barton, N. H. (1987). The relative rates of evolution of sex chromosomes and autosomes. *Am. Nat.*, *130*(1), 113–146. https://doi.org/10.1086/284701

Charlesworth, Brian, Campos, J. L., & Jackson, B. C. (2018). Faster-X evolution: Theory and evidence from Drosophila. *Mol. Ecol.*, *27*(19). https://doi.org/10.1111/mec.14534

Choudhary, S., & Satija, R. (2022). Comparison and evaluation of statistical error models for

scRNA-seq. *Genome Biol.*, *23*(1), 1–20. https://doi.org/10.1186/S13059-021-02584-9/FIGURES/4

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., Land, S. J., Lu, X., & Ruden, D. M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly (Austin).*, *6*(2), 80–92. https://doi.org/10.4161/fly.19695

Coughlan, J. M., & Matute, D. R. (2020). The importance of intrinsic postzygotic barriers throughout the speciation process. *Philos. Trans. R. Soc. B Biol. Sci.*, *375*(1806), 20190533. https://doi.org/10.1098/rstb.2019.0533

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sinauer Associates. https://books.google.se/books/about/Speciation.html?id=2Y9rQgAACAAJ&redir_esc=y

Dobzhansky, T. (1936). Studies on Hybrid Sterility. II. Localization of Sterility Factors in Drosophila Pseudoobscura Hybrids. *Genetics*, *21*(2), 113–135. https://doi.org/10.1093/genetics/21.2.113

Dunleavy, J. E. M., Okuda, H., O'Connor, A. E., Merriner, D. J., O'Donnell, L., Jamsai, D., Bergmann, M., & O'Bryan, M. K. (2017). Katanin-like 2 (KATNAL2) functions in multiple aspects of haploid male germ cell development in the mouse. *PLOS Genet.*, *13*(11), e1007078. https://doi.org/10.1371/JOURNAL.PGEN.1007078

Ellegren, H. (2010). Evolutionary stasis: the stable chromosomes of birds. *Trends Ecol. Evol.*, *25*(5), 283–291. https://doi.org/10.1016/J.TREE.2009.12.004

Ellegren, H., Smeds, L., Burri, R., Olason, P. I., Backström, N., Kawakami, T., Künstner, A., Mäkinen, H., Nadachowska-Brzyska, K., Qvarnström, A., Uebbing, S., & Wolf, J. B. W. (2012). The genomic landscape of species divergence in Ficedula flycatchers. *Nature*, *491*(7426), 756–760. https://doi.org/10.1038/nature11584

556 Ge, S. X., Jung, D., & Yao, R. (2020). ShinyGO: a graphical gene-set enrichment tool for
557 animals and plants. *Bioinformatics*, *36*(8), 2628–2629.
558 https://doi.org/10.1093/bioinformatics/btz931
559 Gelter, H. P. (1987). Song Differences between the Pied Flycatcher Ficedula hypoleuca, the
560 Collared Flycatcher F. albicollis, and Their Hybrids. *Ornis Scand.*, *18*(3).
561 https://doi.org/10.2307/3676768
562 Gordon, M. (1937). Heritable color variations in the mexican swordtail-fish: Aquarium
563 species as the drosophila of fish genetics. *J. Hered.*, *28*(6).
564 https://doi.org/10.1093/oxfordjournals.jhered.a104368
565 Houston, B. J., Conrad, D. F., & O'Bryan, M. K. (2020). A framework for high-resolution
566 phenotyping of candidate male infertility mutants: from human to mouse. *Hum. Genet.*
567 *2020 1401*, *140*(1), 155–182. https://doi.org/10.1007/S00439-020-02159-X
568 Hunnicutt, K. E., Good, J. M., & Larson, E. L. (2022). Unraveling patterns of disrupted gene
569 expression across a complex tissue. *Evolution (N. Y).*, *76*(2), 275–291.
570 https://doi.org/10.1111/evo.14420
571 Islam, K. N., Modi, M. M., & Siegfried, K. R. (2021). The Zebrafish Meiotic Cohesin Complex
572 Protein Smc1b Is Required for Key Events in Meiotic Prophase I. *Front. Cell Dev. Biol.*, *9*.
573 https://doi.org/10.3389/FCELL.2021.714245/FULL
574 Kanippayoor, R. L., Alpern, J. H. M., & Moehring, A. J. (2020). A common suite of cellular
575 abnormalities and spermatogenetic errors in sterile hybrid males in *Drosophila*. *Proc. R.*
576 *Soc. B Biol. Sci.*, *287*(1919), 20192291. https://doi.org/10.1098/rspb.2019.2291
577 Kopania, E. E. K., Larson, E. L., Callahan, C., Keeble, S., & Good, J. M. (2022). Molecular
578 Evolution across Mouse Spermatogenesis. *Mol. Biol. Evol.*, *39*(2).
579 https://doi.org/10.1093/molbev/msac023

La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., Lidschreiber, K., Kastriti, M. E., Lönnerberg, P., Furlan, A., Fan, J., Borm, L. E., Liu, Z., van Bruggen, D., Guo, J., He, X., Barker, R., Sundström, E., Castelo-Branco, G., … Kharchenko, P. V. (2018). RNA velocity of single cells. *Nature*, *560*(7719), 494–498. https://doi.org/10.1038/s41586-018-0414-6

Larson, E. L., Kopania, E. E. K., & Good, J. M. (2018). Spermatogenesis and the Evolution of Mammalian Sex Chromosomes. In *Trends in Genetics* (Vol. 34, Issue 9, pp. 722–732). https://doi.org/10.1016/j.tig.2018.06.003

Maheshwari, S., & Barbash, D. A. (2011). The Genetics of Hybrid Incompatibilities. *Http://Dx.Doi.Org/10.1146/Annurev-Genet-110410-132514*, *45*, 331–355. https://doi.org/10.1146/ANNUREV-GENET-110410-132514

Mallet, J. (2006). What does Drosophila genetics tell us about speciation? *Trends Ecol. Evol.*, *21*(7), 386–393. https://doi.org/10.1016/J.TREE.2006.05.004

Mank, J. E., Nam, K., & Ellegren, H. (2010). Faster-Z Evolution Is Predominantly Due to Genetic Drift. *Mol. Biol. Evol.*, *27*(3), 661–670. https://doi.org/10.1093/molbev/msp282

McFarlane, S. E., Sirkiä, P. M., Ålund, M., & Qvarnström, A. (2016). Hybrid dysfunction expressed as elevated metabolic rate in male ficedula flycatchers. *PLoS One*, *11*(9), e0161547. https://doi.org/10.1371/journal.pone.0161547

Mihola, O., Trachtulec, Z., Vlcek, C., Schimenti, J. C., & Forejt, J. (2009). A mouse speciation gene encodes a meiotic histone H3 methyltransferase. *Science (80-.).*, *323*(5912). https://doi.org/10.1126/science.1163601

Mugal, C. F., Wang, M., Backström, N., Wheatcroft, D., Ålund, M., Sémon, M., McFarlane, S. E., Dutoit, L., Qvarnström, A., & Ellegren, H. (2020). Tissue-specific patterns of regulatory changes underlying gene expression differences among Ficedula flycatchers

and their naturally occurring F1 hybrids. *Genome Res.*, *31*(12), 1727–1739. https://doi.org/10.1101/gr.254508.119

Muller, H. J. (1940). An analysis of the process of structural change in chromosomes of Drosophila. *J. Genet.*, *40*(1–2). https://doi.org/10.1007/BF02982481

Muller, HJ J. (1942). Isolating mechanisms, evolution and temperature. In *Biol. Symp* (Vol. 6).

Pomiankowski, A., & Hurst, L. D. (1993). Genomic conflicts underlying Haldane's rule [2]. In *Genetics* (Vol. 133, Issue 2, pp. 425–432). https://doi.org/10.1093/genetics/133.2.425

Powell, D. L., García-Olazábal, M., Keegan, M., Reilly, P., Du, K., Díaz-Loyo, A. P., Banerjee, S., Blakkan, D., Reich, D., Andolfatto, P., Rosenthal, G. G., Schartl, M., & Schumer, M. (2020). Natural hybridization reveals incompatible alleles that cause melanoma in swordtail fish. *Science (80-.).*, *368*(6492). https://doi.org/10.1126/science.aba5216

Presgraves, D. C. (2010). The molecular evolutionary basis of species formation. *Nat. Rev. Genet.*, *11*(3), 175–180. https://doi.org/10.1038/nrg2718

Presgraves, D. C., & Meiklejohn, C. D. (2021). Hybrid Sterility, Genetic Conflict and Complex Speciation: Lessons From the Drosophila simulans Clade Species. *Front. Genet.*, *12*, 669045. https://doi.org/10.3389/fgene.2021.669045

Qvarnström, A., & Bailey, R. I. (2009). Speciation through evolution of sex-linked genes. In *Heredity* (Vol. 102, Issue 1, pp. 4–15). https://doi.org/10.1038/hdy.2008.93

Qvarnström, Anna, Ålund, M., Mcfarlane, S. E., & Sirkiä, P. M. (2016). Climate adaptation and speciation: particular focus on reproductive barriers in Ficedula flycatchers. *Evol. Appl.*, *9*(1), 119–134. https://doi.org/10.1111/EVA.12276

Qvarnström, Anna, Rice, A. M., & Ellegren, H. (2010). Speciation in Ficedula flycatchers. *Philos. Trans. R. Soc. B Biol. Sci.*, *365*(1547), 1841–1852. https://doi.org/10.1098/rstb.2009.0306

628 Revenkova, E., Eijpe, M., Heyting, C., Hodges, C. A., Hunt, P. A., Liebe, B., Scherthan, H., &
629 Jessberger, R. (2004). Cohesin SMC1β is required for meiotic chromosome dynamics,
630 sister chromatid cohesion and DNA recombination. *Nat. Cell Biol. 2004 66*, *6*(6), 555–
631 562. https://doi.org/10.1038/ncb1135
632 Rosser, N., Edelman, N. B., Queste, L. M., Nelson, M., Seixas, F., Dasmahapatra, K. K., &
633 Mallet, J. (2022). Complex basis of hybrid female sterility and Haldane's rule in
634 Heliconius butterflies: Z-linkage and epistasis. *Mol. Ecol.*, *31*(3), 959–977.
635 https://doi.org/10.1111/MEC.16272
636 Sawamura, K. (2000). Genetics of hybrid inviability and sterility in *Drosophila* : The
637 *Drosophila melanogaster* – *Drosophila simulans* case. *Plant Species Biol.*, *15*(3), 237–
638 247. https://doi.org/10.1111/j.1442-1984.2000.00043.x
639 Segami, J. C., Semon, M., Cunha, C., Bergin, C., Mugal, C. F., & Qvarnström, A. (2022). Single-
640 Cell Transcriptomics reveals relaxed evolutionary constraint of spermatogenesis in two
641 passerine birds as compared to mammals. *BioRxiv*, 2022.01.22.477241.
642 https://doi.org/10.1101/2022.01.22.477241
643 Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., Hao, Y.,
644 Stoeckius, M., Smibert, P., & Satija, R. (2019). Comprehensive Integration of Single-Cell
645 Data. *Cell*, *177*(7), 1888-1902.e21. https://doi.org/10.1016/j.cell.2019.05.031
646 Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO Summarizes and Visualizes
647 Long Lists of Gene Ontology Terms. *PLoS One*, *6*(7), e21800.
648 https://doi.org/10.1371/journal.pone.0021800
649 Svedin, N., Wiley, C., Veen, T., Gustafsson, L., & Qvarnström, A. (2008). Natural and sexual
650 selection against hybrid flycatchers. *Proc. R. Soc. B Biol. Sci.*, *275*(1635), 735–744.
651 https://doi.org/10.1098/rspb.2007.0967

652 Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M.,
653 Doncheva, N. T., Morris, J. H., Bork, P., Jensen, L. J., & Von Mering, C. (2019). STRING
654 v11: protein-protein association networks with increased coverage, supporting
655 functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.*, *47*(D1),
656 D607–D613. https://doi.org/10.1093/NAR/GKY1131
657 Takabayashi, S., Yamauchi, Y., Tsume, M., Noguchi, M., & Katoh, H. (2009). A Spontaneous
658 Smc1b Mutation Causes Cohesin Protein Dysfunction and Sterility in Mice:
659 *Https://Doi.Org/10.3181/0808-RM-244*, *234*(8), 994–1001.
660 https://doi.org/10.3181/0808-RM-244
661 Uebbing, S., Künstner, A., Mäkinen, H., Backström, N., Bolivar, P., Burri, R., Dutoit, L., Mugal,
662 C. F., Nater, A., Aken, B., Flicek, P., Martin, F. J., Searle, S. M. J., & Ellegren, H. (2016).
663 Divergence in gene expression within and between two closely related flycatcher
664 species. *Mol. Ecol.*, *25*(9), 2015–2028. https://doi.org/10.1111/mec.13596
665 Ververis, A., Christodoulou, A., Christoforou, M., Kamilari, C., Lederer, C. W., & Santama, N.
666 (2016). A novel family of katanin-like 2 protein isoforms (KATNAL2), interacting with
667 nucleotide-binding proteins Nubp1 and Nubp2, are key regulators of different MT-
668 based processes in mammalian cells. *Cell. Mol. Life Sci.*, *73*(1), 163–184.
669 https://doi.org/10.1007/S00018-015-1980-5/FIGURES/7
670 Wu, C.-I., & Davis, A. W. (1993). Evolution of Postmating Reproductive Isolation: The
671 Composite Nature of Haldane's Rule and Its Genetic Bases. *Am. Nat.*, *142*(2), 187–212.
672 https://doi.org/10.1086/285534
673 Wu, C. I., Johnson, N. A., & Palopoli, M. F. (1996). Haldane's rule and its legacy: Why are
674 there so many sterile males? In *Trends in Ecology and Evolution* (Vol. 11, Issue 7).
675 https://doi.org/10.1016/0169-5347(96)10033-1

676 Zheng, G. X. Y., Terry, J. M., Belgrader, P., Ryvkin, P., Bent, Z. W., Wilson, R., Ziraldo, S. B.,
677 Wheeler, T. D., McDermott, G. P., Zhu, J., Gregory, M. T., Shuga, J., Montesclaros, L.,
678 Underwood, J. G., Masquelier, D. A., Nishimura, S. Y., Schnall-Levin, M., Wyatt, P. W.,
679 Hindson, C. M., … Bielas, J. H. (2017). Massively parallel digital transcriptional profiling
680 of single cells. *Nat. Commun.*, *8*(1), 14049. https://doi.org/10.1038/ncomms14049

681