Extensive transgressive gene expression in a homoploid hybrid species

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Abstract

Hybridization is increasingly recognized as an important evolutionary force contributing novel variation for selection to act on. While mis-expression in F1 hybrids is well documented, how gene expression evolves in stabilized hybrid taxa and contributes novel variation remains an open question, especially for hybrid species without an increase in ploidy. As gene expression evolves in a stabilizing manner, break-up of co-evolved *cis*- and *trans*-regulatory elements could lead to transgressive patterns of gene expression in hybrids. Here, we address to what extent gonad gene expression has evolved in an old homoploid hybrid, the Italian sparrow *Passer italiae*. Through comparing the gene expression of parental species and F1 hybrids to that of the Italian sparrow, we find evidence for strongly transgressive expression in the Italian sparrow, with 22% of the testis genes exhibiting expression patterns outside the range of both parent species, compared to only 0.37% in the F1s. In contrast, Italian sparrow ovary expression was similar to that of one parent species, the house sparrow (*P. domesticus*). Moreover, the Italian sparrow testis transcriptome is 26 times as diverged from those of the parent species as the parental transcriptomes are from each other, despite being genetically intermediate. This highlights the potential for regulation of gene expression to produce novel variation following hybridization. Genes involved in mitochondrial respiratory chain complexes and protein synthesis are enriched in the subset that is over-dominantly expressed in Italian sparrow testis, suggesting that selection on key functions have molded the hybrid Italian sparrow transcriptome.

Keywords: Hybridization, gene expression, transgressive expression, testis, mitochondrial genes

Introduction

Hybridization is an important evolutionary process, contributing to novel genetic variation, that natural selection can act on (Mallet 2005; Abbott, et al. 2013; Runemark, et al. 2019; Taylor and Larson 2019). While divergence in parental genomes brings together novel allelic combinations in the hybrid, which may lead to a reduction in hybrid fitness (Dobzhansky 1936; Muller 1942), it is now clear that hybridization is frequent (Marques, et al. 2019; Runemark, et al. 2019; Taylor and Larson 2019). Hence, we can no longer study hybridization as an isolated phenomenon, and the processes at play in hybrids should be integrated in our general understanding of how novel variation can arise. While there is ample evidence for admixed hybrid genomes (Runemark, et al. 2019) and transgressive hybrid phenotypes (Rieseberg, et al. 1999), the effect of hybridization on gene expression is not well understood. Divergence in regulation of gene expression is thought to be the basis for much of phenotypic evolution (Rest, et al. 2013; Hodgins-Davis, et al. 2015). In spite of this, we lack an understanding of how gene expression contributes to novel phenotypes and adaptation in old, stabilized hybrid taxa, in particular for homoploid hybrid species, with no increase in ploidy level.

Gene expression evolves in a stabilizing manner where regulatory elements accumulate compensatory mutations that keep gene expression at an optimum level for physiological functions (Gilad, et al. 2006; Coolon, et al. 2014; Hodgins-Davis, et al. 2015). The compensatory evolution means that as hybridization breaks up co-inheritance of regulatory elements, hybrids may experience novel combinations (Landry, et al. 2005; Renaut, et al. 2009), potentially leading to transgressive expression patterns, that transcends the range of parental expression profiles. Indeed, transgressive gene expression has been reported in F1

hybrids in several taxa, and the underlying regulatory divergence for transgressive expression has been associated with divergence in *cis*- and *trans*-regulatory elements in the parental species (Ranz, et al. 2004; Haerty and Singh 2006; Rockman and Kruglyak 2006; Malone and Michalak 2008; Renaut, et al. 2009). In post-F1 generation hybrids, transcriptomes show a higher level of transgressive expression due to uncoupling of regulatory elements in the process of recombination. In the lake white fish *Coregonus clupeaformis*, the F2 hybrids showed a higher level of nonadditive inheritance in gene expression compared to F1 hybrids (Renaut, et al. 2009). An additional line of evidence of a non-linear relationship between genetic composition and gene expression comes from recent studies on allopolyploid plant taxa. Both preferential expression of one of the parental genomes (Edger, et al. 2017), and tissue dependent expression of this dominance (Kryvokhyzha, et al. 2019) have been documented in allopolyploids. Jointly, the transgressive expression in experimental hybrids and non-linear relationship between genomic composition and gene expression similarity, suggest that gene expression will not be proportional to the parental contributions to the genome. Whether transgressive patterns of gene expression are found in old, stabilized homoploid hybrid taxa is therefore important for understanding the potential of hybridization to produce novel variation.

Gene expression studies in hybrids resulting from strongly divergent taxa has shown an extensive level of transgressive expression arising from uncoupling of co-evolved *cis*- and *trans*-regulatory elements in the hybrids (Haerty and Singh 2006; McManus, et al. 2010; Coolon, et al. 2014). However, transgressive expression patterns in the hybrid of more recently diverged species pairs can either arise due to polymorphic incompatible loci (Cutter 2012) or physiological response to hybrid dysfunction (Barreto, et al. 2015). In interpopulation hybrids at F2 or later generation of copepod species, *Tigriopus californicus*,

gene expression showed transgressive inheritance only in about 1.2% of the transcriptome and was linked to physiological response in hybrids (Barreto, et al. 2015). Additionally, recent study on F1 hybrids between the pied and collared flycatchers with few fixed differences and large number of shared polymorphisms showed a tissue-specific pattern of mis-expression. Testis had the highest divergence between the parental species but the lowest level of mis-expression in F1 hybrids (Mugal, et al. 2020). To which extent this pattern holds up across species, and how sorting of segregating polymorphisms may result in novel patterns of gene expression remains a challenge for researchers to address.

Here, we leverage a unique study system to address how gene expression evolves. We investigate gene expression in the homoploid hybrid Italian sparrow (*Passer italiae*) (Fig. 1), through combining data from the old, stabilized hybrid taxon, the parental species and experimental F1-hybrids. The Italian sparrow has arisen from hybridization between the house sparrow (*P. domesticus*) and Spanish sparrow (*P. hispaniolensis*) (Hermansen, et al. 2011; Hermansen, et al. 2014; Trier, et al. 2014; Elgvin, et al. 2017). The parent species diverged approximately 0.85 million years ago (Ravinet, et al. 2018) and the Italian sparrow originated within ca. 5800 years ago, most likely as the house sparrow expanded its range into Europe and hybridized with the Spanish sparrow (Ravinet, et al. 2018). This system provides a unique possibility to test to what extent an old, stabilized homoploid hybrid species has diverged in gene expression from the parent species, and how gene expression has evolved after hybridization. In this study, we compared the gene expression profiles of the gonads of the Italian sparrow to gene expression of the parental species and lab bred experimental F1 hybrids to address a) if Italian sparrows exhibit transgressive gene expression compared to the parent species and b) whether inheritance mode and misexpression in F1s predict which genes are transgressively expressed in Italian sparrows.

Results

Differential expression between the parental species

Gene expression divergence in gonads between the parental species was strongly asymmetric with testis showing a more conserved pattern of expression compared to ovary (Table 1). In testis, 135 genes (1.16%) and in ovary, 1382 genes (11.65%) were significantly differentially expressed. Of the differentially expressed genes, 60.7% of genes in testis and 69.3% of genes in ovary were upregulated in the Spanish sparrow. A significantly higher proportion of genes that were differentially expressed in testis were located on the Z chromosome (24 genes, hypergeometric test, P -value $< 1.82 \cdot 10^{-8}$). We found no evidence for functional enrichment among differentially expressed genes in parental species for neither testis nor ovary.

Differential expression in Italian sparrows compared to the parental species

In contrast to its genome composition, intermediate of the parent species, the Italian sparrow testis gene expression differed more in comparison to the parental species than the parent species differed to each other (Table 1). About one-third of the testis transcriptome was differentially expressed compared to both parental species, whereas the ovary expression was similar to that of the house sparrow (Table 1, Figs. 2A-3A). In testis, 3536 genes (30.45%) and 3581 genes (30.9%) were differentially expressed in comparison to house and Spanish sparrow, respectively. In ovary, only 22 genes (0.18%) differed from the house sparrow whereas 1508 genes (12.63%) were differentially expressed compared to the Spanish sparrow. Significant over-representation of Z-linked genes among the differentially expressed genes was detected in testis but only in the comparison to Spanish sparrow (196 of the 3581 genes, hypergeometric test, p value = 0.006). Most genes in both testis and ovary were up-regulated compared to house and down-regulated compared to Spanish sparrow (Chi-squared

test p value = $1.05e-06$ in testis and 0.001 in ovary) (Figs. 2B-3B).

Genes that were differentially expressed in Italian sparrow testis were enriched for similar biological processes in comparison to both parent species. Primarily, functions involving protein synthesis and mitochondrial gene expression and function were overrepresented. In the comparison to house sparrow, 27 significant GO categories were found (Table S4), with top biological processes involved in viral transcription, SRP-dependent co-translational protein targeting to membrane, translation, ribosome and mitochondrial respiratory chain complex I assembly. In comparison to the Spanish sparrow, the 15 enriched GO terms reflected similar biological processes, with ribosome as the top GO term (Table S5). In ovary, 6 GO terms were detected in the comparison to house sparrow with sodium ion transmembrane transporter activity as the top term (Table S6) and 1 GO term, negative regulation of protein phosphorylation detected in the comparison to Spanish sparrow (Table S7).

Differential expression in F1 hybrids compared to the parental species

Both testis and ovary showed a more conserved pattern of expression between the F1 hybrid and the house sparrow than between F1 hybrids and the Spanish sparrow (Table 1, Figs. 2A-3A). The overall magnitude of differences to the parent species was much lower than for the Italian sparrow. In testis, 106 genes (0.9%) and 263 genes (2.25%) were differentially expressed compared to house and Spanish sparrows, respectively. In ovary, 30 genes (0.25%) and 140 genes (1.15%) showed a significant difference in expression compared to the house and Spanish sparrows, respectively. Similar to the Italian sparrow, the pattern of up- and down-regulation was consistent in both testis and ovary in the comparison to each of the parental species (Figs. 2B-3B). A larger number of genes were up-regulated in F1 hybrid in

the comparison to house sparrow and down-regulated in the comparison to Spanish sparrow (Chi-squared test P -value = 1.96-05 in testis and 0.01 in ovary). As in the Italian sparrow, there was an over-representation of Z-linked genes among those differentially expressed between F1 hybrids and Spanish sparrows (31 of the 263 differentially expressed genes, hypergeometric test, P -value = 2.42-06). The enriched functions were different to those enriched for Italian sparrows. Testis genes differentially expressed in F1 showed functional enrichment for primarily collagen catabolic process. In the comparison to house sparrow, 46 GO terms were significantly over-represented. Among these GO terms, 20 GO terms had an $\text{Padj} < 0.01$, whereas there were few significant GO terms compared to Spanish sparrows with top GO term common with the comparison to house sparrow (Tables S8 and S9). The top 5 GO terms were enriched in proteinaceous extracellular matrix, extracellular matrix structural constituent, extracellular vesicle, collagen trimer and collagen catabolic process. In ovary, while there was no functionally significant term associated with differentially expressed genes in F1 compared to the house sparrow, there were 28 significant GO terms in the comparison to Spanish sparrow with the top category to be response to vitamin D (Table S10).

Classification of inheritance patterns of gene expression

Gene expression was generally conserved in both Italian sparrow and F1 hybrid (Table 2), but in the testis of Italian sparrow, 2611 genes (22.71%) showed a non-conserved pattern of inheritance with transgressive expression and house-dominant being the two largest categories (Fig. 4C). Transgressively expressed genes in Italian sparrow comprised 22% of the testis transcriptome tested for inheritance. In contrast, in F1 testis only 0.37% of the genes were transgressively expressed (Fig. 4D). The majority of the genes with non-conserved pattern of inheritance in Italian sparrow ovary, over 96% of the genes, had a house-dominant

pattern of expression (Fig. 4E). In contrast to the high incidence of transgressive expression observed in Italian sparrow testis, only 3 genes (0.028%) were transgressively expressed in ovaries. Gene expression in F1 ovary was more conserved compared to that of the Italian sparrow. Out of 376 genes with non-conserved pattern of inheritance, the two largest categories of inheritance were additive and house-dominant inheritance pattern (Fig. 4F).

Overdominant genes in Italian sparrow testis were enriched for functional categories involved in protein synthesis, mitochondrial protein complex and gene expression and binding of sperm to zona pellucida (Table S11). We found a gene network with significant protein-protein interaction (PPI Enrichment: $1.0E-16$) among the overdominant genes in Italian sparrow (Fig. 5, Fig. S4). A different set of genes were underdominant in Italian sparrow, with functions including regulation of cellular component size and negative regulation of neuron projection development. Overdominant genes in F1 testis were functionally enriched for different GO-terms, including collagen catabolic process and proteinaceous extracellular matrix (Table S12). The only significant GO term for underdominant genes in F1 testis was voltage-gated potassium channel complex.

Comparison of gene expression divergence in respect to parent species between Italian sparrows and F1

To evaluate how gene expression evolves over time asking whether mis-expression in early generation hybrids predicts differential expression in Italian sparrows, we checked if the same genes or gene functions that were differentially expressed in comparison to parental species in F1 hybrids also were differentially expressed in the Italian sparrow (Figs. 2C-3C). In testis, 1.28% of the genes that were differentially expressed in Italian sparrows compared to house sparrow were also differentially expressed in experimental F1 hybrids, whereas in

comparison to Spanish sparrows it was 3.9%. In ovary, only 1 gene (2%) was differentially expressed in F1 and Italian sparrow compared to house sparrow, whereas 5.9% were differentially expressed in F1 hybrids and Italian sparrows in comparison to Spanish sparrows. The majority of the commonly differentially expressed genes were differentially expressed in the same direction (i.e., up- or down-regulated) in both testis and ovary, both in F1 hybrids and Italian sparrows. Testis genes commonly differentially expressed in F1 hybrid and Italian sparrow compared to house sparrow were enriched in extracellular vesicle, insulin like growth factor binding, extracellular matrix structural constituent and extracellular matrix component. In the comparison to Spanish sparrow testis, only one significant GO term, cAMP binding was detected.

Mode of regulatory divergence and prediction of transgressive expression in Italian sparrows

To address if the regulatory basis can predict transgressive gene expression in hybrid species, we classified genes dependent on their regulatory basis (Table 3). We expect genes under *cis-trans* compensatory regulation and trans-regulated genes to more easily produce transgressive patterns of gene expression in later generation hybrids than *cis*-regulated genes, as recombination may de-couple *cis*- and *trans*- or different trans-elements in the sorting of parental variation in the hybrid lineage. Therefore, we checked the percentage of genes transgressively expressed in the Italian sparrow depending on the mode of regulatory divergence. We found that 16/130 genes (12%) of the *trans*-regulated testis genes were transgressively expressed in Italian sparrow, and 28/159 genes (18%) of the genes under *cis-trans* compensatory regulation were transgressively expressed in Italian sparrows. Finally, we tested whether genes with a non-additive inheritance, that were transgressively expressed in F1 hybrids, were more likely to be transgressively expressed in the Italian sparrow. We found

that 20 out of 45 genes that were transgressively expressed in the F1 hybrid (44.4%) also were transgressively expressed in Italian sparrow testis.

Discussion

In this study, we aimed to document the evolution of gene expression in the gonads of a homoploid hybrid species, the Italian sparrow. An extensive level of transgressive expression was found in Italian sparrow testis, with an over-expression of genes involved in functions related to mitochondrial respiratory chain and, cytosolic and mitochondrial ribosomal proteins, as well as proteins involved in protein folding and the proteasome complex. In contrast to testis, the gene expression pattern in the ovary was asymmetric compared to parental species, with a house-dominant expression in the Italian sparrow. Experimental F1 hybrids showed a much more conserved pattern of gene expression in both testis and ovary. In the following sections, we discuss the potential implications of our results and consider the limitations of these data.

Extensive transgressive expression in Italian sparrow testis

About 22% of testis transcriptome in Italian sparrow showed transgressive expression pattern, while this level was less than 1% in the F1 hybrid. Transgressive gene expression, despite the fact that gene expression is conserved between parent species, is expected to occur in later generation hybrids due to the uncoupling of co-evolved *cis*- and *trans*- regulatory elements through recombination (Rieseberg, et al. 2003; Mallet 2007). There are, however, few studies of gene expression in post F1-hybrids. In Lake whitefish, F2 hybrids showed higher misexpression and percentage of genes with nonadditive inheritance compared to F1 hybrids (Renaut, et al. 2009). Transgressive expression is commonly referred to “mis-expression” in early generation hybrids while Italian sparrow is a successful hybrid taxon likely formed

within the last 5.800 years (Ravinet, et al. 2018). Here, we show that transgressive expression is found not only as mis-expression in early generation hybrids but remains in well-functioning homoploid hybrid taxa long after hybridization. Hence, evolution of the regulation of gene expression following hybridization may produce novel expression phenotypes from intermediate genomes. In hybrid lineages, transgressive segregation, which leads to extreme phenotypes, has been considered as a major mechanism by which extreme or novel adaptations observed in new hybrid ecotypes or species arise (Rieseberg, et al. 1999), but sorting of regulatory elements have not formally been suggested to contribute to this. This research suggests that break-up of co-evolved regulatory elements, resulting in transgressive patterns of gene expression, partly can explain the generation of transgressive phenotypes in hybrid taxa.

Transgressive expression arising in spite of low parental divergence

Hybrid incompatibilities arisen due to bringing together novel genetic combinations have been mainly considered when the parental species are fixed for alleles potentially contributing to reproductive isolation (Cutter 2012). In recently diverged species, reproductive incompatibility may arise due to polymorphic loci within each lineage before fixation for the incompatibility alleles (Cutter 2012). Therefore, if transgressive expression in the Italian sparrow is driven by bringing together *cis*- and *trans*-regulatory variants not yet co-adapted, this must either be by bringing together polymorphic loci in each of the parental species or novel mutations that have occurred in Italian sparrow since isolation from the parental species. Each of these hypotheses can be evaluated in future work by studying the F1 and F2 hybrids and the direct parents of the cross in order to find *cis* and *trans*-regulatory variations. Interestingly, during ca. 5.800 years of evolution, Italian sparrows have achieved a 26 times stronger divergence in gene expression the parents have accumulated during 0.68

million years, in spite of them being genomically intermediate (Elgvin, et al. 2017)

Tissue- and parent specific patterns of differentiation in gene expression

We find that the degree of difference in gene expression is tissue- and parent specific. We generally observed fewer differentially expressed genes relative to house sparrow than relative to the Spanish sparrow, both for Italian sparrows and F1 hybrids, and especially for ovary. This is likely partly explained by the fact that both Italian sparrows and F1 hybrids have house sparrow mitochondria. Mito-nuclear interactions can strongly affect gene expression, and even result in misexpression (Runemark, Eroukhmanoff, et al. 2018). An additional explanation for a more conserved expression pattern in ovary could be a contribution of the W chromosome. While we can assume that F1 hybrids examined in this study inherit house sparrow W chromosomes, there is not yet an assembly of the W chromosome of any of the three sparrow species. Investigating how the W chromosome affects gene expression is an interesting future line of inquiry. Finally, we cannot exclude that a technical artefact contributes to the house-dominant pattern of expression. More specifically, only 3 Spanish sparrows were used in the analysis of ovary transcriptome, this finding may be driven by large within-species variance in ovarian gene expression.

Enrichment of over-dominant genes in Italian sparrow for translation and mitochondrial function

A large set of genes involved in protein synthesis were over-expressed in Italian sparrow testis compared to both parents. These include a set of ribonucleoproteins present in both cytosolic and mitochondrial ribosomes, eukaryote translation initiation factor such as *EIF1AY* required for maximal rate of protein synthesis and SRP (signal recognition particle), a ribonucleoprotein that recognizes and targets specific proteins to the endoplasmic reticulum

in eukaryotes. RNA binding proteins play an important role in post-translational regulation of gene expression and spermatogenesis and appear to be key regulatory factors that ensure male fertility (Paronetto and Sette 2010; Phillips, et al. 2019). Several genes coding for subunits of T-complex protein Ring Complex (TRiC) (*CCT4*, *CCT5*, *CCT6A*, *TCPI*) were also among the overdominant genes. The TRiC complex aids in the folding of about 10% of the proteome. Subunits of TRiC complex has shown to be required for spermatogenesis (Counts, et al. 2017) and have shown evidence for positive selection among the seminal fluid genes between house and Spanish sparrows (Rowe, et al. 2020). Another category of gene networks which were upregulated in Italian sparrow included genes encoding for components of all mitochondrial respiratory chain enzymatic complexes. Additionally, genes encoding for chaperone proteins involved in the assembly of these enzymatic complexes such as *TIMMDC1* were also over-expressed.

In Italian sparrow testis, mito-nuclear genes have been shown to be important in generating reproductive isolation in the Italian sparrow (Trier, et al. 2014), and mito-nuclear sites have been under selection for inheritance of the house sparrow allele in Italian sparrows (Runemark, Trier, et al. 2018). Although the mitochondrion has its own genome, more than 98% of its proteins are encoded in the nuclear genes and its correct function requires a coordinated expression of both nuclear and mitochondrial genomes (Ryan and Hoogenraad 2007). Therefore, the upregulation of genes involved in respiratory chains and protein synthesis corroborate the evidence for a strong effect of hybridization on genes associated with metabolism in other bird taxa (Wagner, et al. 2020). Overexpression of cytosolic ribonucleoproteins and mitochondrial respiratory chain enzymes has also been observed in later generation hybrids (F3+) in copepod species *Tigriopus californicus* and has been linked to cellular responses to physiological dysfunction (Barreto, et al. 2015). Our study thus

contributes to the growing body of evidence of mito-nuclear interactions as important reproductive barriers (Hill 2017), and provides a unique insight into altered patterns of gene expression as a possible resolution to the conflict.

Conclusion

This study presents the first evidence for transgressive gene expression in a wild homoploid hybrid species. The strongly divergent gene expression profile in Italian sparrow testis highlights the potential of intermediate hybrid genomes to rapidly evolve through altered regulation of gene expression. Comparing patterns of gene expression in wild homoploid hybrid species to those seen in experimental F1 hybrids, we document rapid evolution of gene expression, especially for networks of genes involved in mitochondrial respiratory chain enzymes and protein synthesis. The strongly divergent testis transcriptome was unexpected given the low parental divergence, highlighting that alleles contributing to reproductive isolation may be polymorphic rather than fixed. In contrast, Italian sparrow ovary had a transcriptome similar to that of the house sparrow. Future studies should therefore evaluate the current results in more tissues. Using pedigrees of parents and hybrid individuals would also improve the power to identify the regulatory underpinnings of gene expression. Finally, examining the link between transgressive gene expression and transgressive hybrid phenotypes is likely to significantly increase our understanding of how novel variation arise from hybridization.

Materials and methods

Sampling, RNA extraction, library preparation, and sequencing

We compared the gene expression of Italian sparrows to its two parent species, house and Spanish sparrow and their experimental F1-hybrids. As the species differ slightly in

phenology, birds were sampled during their peak reproductive periods at their respective locations. Spanish sparrows and F1 hybrids were sampled near Olivenza, Spain (38° 40' 56"N, 7° 11' 17"W) in March 2016 and 2017, house sparrows in Oslo, Norway (59° 55' 2"N, 10° 46' 11"E) in May 2016 and Italian sparrows from Montanari in the Gargano peninsula (41° 54' 36.8"N, 15° 51' 13.0"E) in May 2017 (Fig 1). Experimental F1 hybrids were bred in outdoor cages using house sparrow females and Spanish sparrow males. All other birds were trapped using mist nets outside or within cages. Birds were immediately euthanized by cervical dislocation, and gonads (ovary and left testis) were harvested directly after death. Sampling was performed between 10:00 and 16:00, with individuals from each of the species being sampled over most of the day, in order to avoid biases in sampling time. The gonads were stored in RNAlater, and immediately stored at -80 degrees, with extractions later performed at the University of Oslo using the Qiagen miRNeasy micro kit. Samples were prepped with TruSeq® Stranded mRNA library prep from Illumina on an automated Perkin Elmer Sciclone NGSx liquid handler and sequenced in the Norwegian Sequencing Center in Oslo, as paired-end (2x150 bp) on the Illumina Hiseq4000. The number of reads obtained per each sample is reported in Table S1. As F1 hybrids were bred in an area where house and Spanish sparrows are sympatric (Olivenza, Spain; see Fig 1) and correct identification of females can be challenging, we performed a post-hoc genomic analysis based on mitochondrial and genomic DNA to ensure that only individuals that clustered with the expected species were included in the analyses (Supplementary materials and methods, Figs. S1-S2).

Read mapping, quality check and counting

We mapped reads from samples of all four groups (testis sample size: house sparrow = 5, Spanish sparrow = 5, F1 hybrid = 5, Italian sparrow = 5; ovary sample size: house sparrow =

5, Spanish sparrow = 3, F1 hybrid = 8 and Italian sparrow = 5) to the house sparrow reference genome using STAR2 v2.7.2b (Dobin, et al. 2013) with default parameters and the house sparrow gene annotation as reported in (Elgvin, et al. 2017). Over 90% of the reads mapped successfully (Table S1). The house sparrow general feature format (GFF) file contained 14,734 annotated genes of which 92.52% were anchored to chromosomes and 7.48% were located on unanchored scaffolds. Of the genes anchored to chromosomes, 12,595 genes were annotated on autosomes and 598 genes were on Z chromosome (Table S2). To ensure homogeneity in quality across our samples, we used RSeQC (Wang, et al. 2012) to measure RNA integrity at the transcriptome level for each sample by calculating Transcript Integrity Number (TIN). The median TIN scores across samples ranged between 73.7 and 85.9 (Table S3). We counted the number of reads mapping to the house sparrow gene features using HTSeq v0.9.1 (Anders, et al. 2015). Read counting was performed for all reads with minimum quality score of 30 and it was configured to handle reverse-stranded sequencing data, with the parameter controlling for overlapping gene features set to union. We created a dendrogram to show sample relationships. The main split was clearly between tissue types, i.e. testis and ovary (Figure S3).

Differential gene expression analysis

We analyzed the raw read counts obtained from htseq-count using the R package DESeq2 (Anders and Huber 2010; Love, et al. 2014). We first tested genes for differential expression between house and Spanish samples. We then compared each of the parents to the experimental F1 hybrid and the Italian sparrow. We pre-filtered the data to a minimum of total read counts of 10 across at least half of the samples in each pairwise comparison. To generate more accurate estimates of log2 fold change (LFC) for genes with low count number or large dispersion, we shrank LFC estimates. We considered genes to be differentially

expressed if they showed False Discovery Rate (FDR) $P_{adj} < 0.05$ and shrunken LFC > 0.32 .

Classification of gene expression inheritance in experimental F1 hybrids and Italian sparrows

The mode of inheritance for differentially expressed genes was determined following (McManus, et al. 2010). We normalized gene expression using median of ratios method in DESeq2 (Love, et al. 2014). Genes whose total expression in hybrids deviated significantly more than 1.25-fold (LFC > 0.32) from that of either parent were considered to have non-conserved inheritance. These genes were classified as having additive, dominant, under-dominant, or over-dominant inheritance, based on the magnitude of the difference between total expression in the Italian sparrow / experimental F1 hybrid and in each parental species. Genes for which expression in the Italian sparrow / experimental F1 hybrid was less than for house sparrows and greater than for Spanish sparrow (or vice versa) were classified as additive; genes for which expression in Italian sparrow / experimental F1 hybrid was similar to one of the parents were classified as dominant; and genes for which expression in Italian sparrow / experimental F1 hybrid was either greater than or less than both parent species were classified as over-dominant and under-dominant, respectively (Figs. 4A-4B).

Allele-specific expression and *cis*- and *trans*- regulatory divergence assignment

Conserved expression level between parents controlled by compensatory variation in parental species is often associated with misregulation in their hybrids (Denver, et al. 2005; Landry, et al. 2005; Goncalves, et al. 2012).-By using sites in experimental F₁ hybrids that contain both parental alleles, we measured allele specific expression, and compared that to expression levels in the parental species to infer whether the divergence is due to *cis*-, *trans*- or compensatory evolution. A typical approach for the study of ASE is to select the sites for

which the parental species are fixed for two different alleles and study the ASE in heterozygote hybrids (Wittkopp, et al. 2004). House and Spanish sparrows diverged only about 0.85 MYA, with many polymorphisms still shared between the two species. We therefore took an approach similar to the one used in other studies with species pairs containing high number of shared polymorphisms and few fixed differences (Mugal, et al. 2020).

We performed the SNP calling and filtering as described in the section “Genetic Characterization of Samples” (Supplementary Materials and Methods). We additionally filtered the sites for DP of minimum 10, base quality of 20 and mapping quality of 60. We used the R package MBASED (Mayba, et al. 2014) in the unphased mode to identify genes with allele-specific expression. We filtered the MBASED results for the FDR < 0.05 and major allele frequency of minimum 0.7. To balance the power, we chose 5 individuals for each house, Spanish, F1 and Italian sparrow in testis and 3 individuals of each group for ovary. To identify allele-specific expression at the population level, we required genes to show significant allele-specific expression in at least two individuals. Following (McGirr and Martin 2019), we divided the regulatory categories as follow: Cis-regulatory divergence was attributed to genes that are differentially expressed between parents and show allele-specific expression in hybrids. Trans-regulatory divergence was assigned to genes that are differentially expressed between parents and do not show allele-specific expression in hybrids. Putative compensatory regulatory variation was determined if a gene 1) did not show differential expression between parents, 2) showed significant allele-specific expression in hybrids, and 3) did not show allele-specific expression in parents.

Functional Annotation and Enrichment Analysis

Gene ontology (GO) analysis was done for differentially expressed genes in each pairwise

comparison. GO functional annotations and gene descriptions were obtained for protein sequences from the entire house sparrow protein set as described in (Rowe, et al. 2020) using PANNZER (Koskinen, et al. 2015). Functional enrichment of GO terms present in the set of differentially expressed genes relative to the background consisting of all genes expressed and tested for differential expression was performed using the clusterProfiler (Yu, et al. 2012) and significant enrichment was determined at $FDR < 0.05$. Interactions among proteins with significant GO terms were predicted using STRING-v11 (Szklarczyk, et al. 2019) using “Experiment”, “Databases” and “Co-expression” as interaction sources. We set the minimum required interaction score to the highest confidence (0.9) and used only the query proteins to build the interaction network after removing the disconnected nodes in the network. The obtained network was then exported to Cytoscape (Shannon, et al. 2003) and major representations of biological processes were detected using ClueGO (Bindea, et al. 2009) with $Padj < 0.01$.

Animal Ethics

In Spain, all trapping and sampling of birds was conducted in accordance with Spanish Animal Protection Regulation RD53/2013, and all methods were approved by the Institutional Commission of Bioethics at the University of Extremadura (CBUE 49/2011). Birds were sampled in Oslo with permission from Miljø-direktoratet (2016/2225). In Italy, all birds were sampled with permission from ISPRA (protocol number 12404) and with permission (no. 305) from regione Puglia. All samples taken from Italy to Oslo in accordance with the Nagoya protocols.

Scripts for the analyses

All scripts written for the analyses presented in this manuscript are deposited under

github.com/Homap/Expression_sparrow

Author contributions

A.R. and H.P.Y. conceived of the study. A.M. raised the hybrids and M. Ro., M. Ra., A.R., F.E, C.O. and G-P. S. performed field work and sampling, C.O. performed the molecular lab work, and H.P.Y. designed and performed the bioinformatical analysis based on discussions with A.R and M. Ra. H.P.Y. drafted the manuscript, with revisions based on the input from A.R., M.Ra and M.Ro.

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Figure legends

Fig. 1. Species distribution and study design. (A) Top: Illustrations of male plumage patterns in house, Italian, and Spanish sparrows modified from (Svensson, et al. 1999). Bottom: A distribution map of house, Italian, and Spanish sparrows throughout Europe and northern Africa (Summers-Smith). This picture is modified from (Elgvin, et al. 2017). (B) Pedigree of the F1 hybrids with house sparrow mother and Spanish sparrow fathers.

Fig. 2. Gene expression in Italian sparrow and experimental F1 hybrid in comparison with parental species for testis. A) Log2 fold change (LFC) as a function of mean of normalized counts. B) Proportion of up- and down-regulated genes. Dark color: up-regulated, light color: down-regulated. C) Venn diagram depicting the intersection between differentially expressed genes between the experimental F1 hybrid and Italian sparrow with each of the parental species. F1h: experimental F1 hybrid – house sparrow; Ih: Italian sparrow – house sparrow; F1S: experimental F1 hybrid – Spanish sparrow; IS: Italian sparrow – Spanish sparrow. Purple: F1 hybrids, Orange: Italian sparrows.

Fig. 3. Gene expression in Italian sparrow and experimental F1 hybrids in comparison with parental species for ovary. A) Log2 fold change (LFC) as a function of mean of normalized counts. B) Proportion of up- and down-regulated genes. Dark color: up-regulated, light color: down-regulated. C) Venn diagram depicting the intersection between differentially expressed genes between the experimental F1 hybrid and Italian sparrow with each of the parental species. F1h: experimental F1 hybrid – house sparrow; Ih: Italian sparrow – house sparrow; F1S: experimental F1 hybrid – Spanish sparrow; IS: Italian sparrow – Spanish sparrow. Purple: F1 hybrids Orange: Italian sparrows.

Fig. 4. Inheritance pattern of gene expression in testis and ovary in Italian sparrows and experimental F1 hybrids. A and B: Schematic figures representing the classification of inheritance patterns. C and D: Scatter plots showing shrunken log2 fold change (LFC) in testis between the Italian sparrow and experimental F1 hybrid with the house sparrow on the *x*-axis and with Spanish sparrow on the *y*-axis, respectively. E and F: Scatter plots show shrunken log2 fold change (LFC) in ovary between the Italian sparrow and experimental F1 hybrid with the house sparrow on the *x*-axis and with Spanish sparrow on the *y*-axis, respectively. Grey points in each graph depicts the total number of genes studied for gene expression with those colored representing the ones significantly different from parental species to be divided into each of the inheritance categories (Conserved: grey, additive: pink, house dominant: blue, Spain dominant: red, transgressive (over-dominant and under-dominant: yellow). Grey dotted lines indicate the fold-change threshold of 1.25 used for classification of inheritance pattern. Regression line represents the major variation in log2 fold change. F1h: experimental F1 hybrid – house sparrow; Ih: Italian sparrow – house sparrow; F1S: experimental F1 hybrid – Spanish sparrow; IS: Italian sparrow – Spanish sparrow.

Fig. 5. Gene ontology network represented in overdominant genes in testis of Italian sparrow. Different colors depicts different significant biological processes. Circle size represents adjusted *P*-value for each node with all $P_{adj} < 0.01$.

Table 1. Number of differentially expressed genes and log2 fold change (LFC) with $P_{adj} < 0.05$ and $LFC^* > 0.32$.

	Testis			Ovary		
Comparison	Significant	LFC > 0	LFC < 0	Significant	LFC > 0	LFC < 0
Spain - House	135	82	53	1382	958	424
F ₁ - House	106	77	29	30	16	14
F ₁ - Spain	263	125	138	140	39	101
Italy - House	3536	1962	1574	22	13	9
Italy - Spain	3581	1779	1802	1508	394	1114

* LFC: Log Fold Change

Table 2. Number and percentage of genes in each inheritance category.

	Testis		Ovary	
Inheritance	F ₁	Italy	F ₁	Italy
Conserved	11515 (99.14%)	8883 (77.30%)	11600 (96.9%)	10889 (92.26%)
Additive	6 (0.05%)	2 (0.02%)	272 (2.27%)	19 (0.16%)
house dominant	45 (0.39%)	54 (0.47%)	94 (0.78%)	895 (7.58%)
Spain dominant	4 (0.03%)	25 (0.22%)	6 (0.05%)	7 (0.06%)
Underdominant	12 (0.1%)	1205 (10.47%)	2 (0.17%)	3 (0.02%)
Overdominant	33 (0.27%)	1325 (11.53%)	2 (0.17%)	1 (0.008%)

Table 3. Counts of genes with ASE and *cis*- , *trans*- and CE*-evolution

	Testis				Ovary			
Comparison	ASE	cis	trans	CE	ASE	cis	trans	CE
House	261	-	-	-	72	-	-	-
Spain	456	-	-	-	118	-	-	-
F1	164	5	130	159	54	9	1373	45

* Compensatory evolution

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