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, mito-nuclear 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

76 hybrids in several taxa, and the underlying regulatory divergence for transgressive expression
77 has been associated with divergence in *cis*- and *trans*-regulatory elements in the parental
78 species (Ranz, et al. 2004; Haerty and Singh 2006; Rockman and Kruglyak 2006; Malone and
79 Michalak 2008; Renaut, et al. 2009). In post-F1 generation hybrids, transcriptomes show a
80 higher level of transgressive expression due to uncoupling of regulatory elements in the
81 process of recombination. In the lake white fish *Coregonus clupeaformis*, the F2 hybrids
82 showed a higher level of nonadditive inheritance in gene expression compared to F1 hybrids
83 (Renaut, et al. 2009). An additional line of evidence of a non-linear relationship between
84 genetic composition and gene expression comes from recent studies on allopolyploid plant
85 taxa. Both preferential expression of one of the parental genomes (Edger, et al. 2017), and
86 tissue dependent expression of this dominance (Kryvokhyzha, et al. 2019) have been
87 documented in allopolyploids. Jointly, the transgressive expression in experimental hybrids
88 and non-linear relationship between genomic composition and gene expression similarity,
89 suggest that gene expression will not be proportional to the parental contributions to the
90 genome. Whether transgressive patterns of gene expression are found in old, stabilized
91 homoploid hybrid taxa is therefore important for understanding the potential of hybridization
92 to produce novel variation.
93
94 Gene expression studies in hybrids resulting from strongly divergent taxa has shown an
95 extensive level of transgressive expression arising from uncoupling of co-evolved *cis*- and
96 *trans*-regulatory elements in the hybrids (Haerty and Singh 2006; McManus, et al. 2010;
97 Coolon, et al. 2014). However, transgressive expression patterns in the hybrid of more
98 recently diverged species pairs can either arise due to polymorphic incompatible loci (Cutter
99 2012) or physiological response to hybrid dysfunction (Barreto, et al. 2015). In
100 interpopulation hybrids at F2 or later generation of copepod species, *Tigriopus californicus*,

101 gene expression showed transgressive inheritance only in about 1.2% of the transcriptome
102 and was linked to physiological response in hybrids (Barreto, et al. 2015). Additionally,
103 recent study on F1 hybrids between the pied and collared flycatchers with few fixed
104 differences and large number of shared polymorphisms showed a tissue-specific pattern of
105 mis-expression. Testis had the highest divergence between the parental species but the lowest
106 level of mis-expression in F1 hybrids (Mugal, et al. 2020). To which extent this pattern holds
107 up across species, and how sorting of segregating polymorphisms may result in novel patterns
108 of gene expression remains a challenge for researchers to address.
109
110 Here, we leverage a unique study system to address how gene expression evolves. We
111 investigate gene expression in the homoploid hybrid Italian sparrow (*Passer italiae*) (Fig. 1),
112 through combining data from the old, stabilized hybrid taxon, the parental species and
113 experimental F1-hybrids. The Italian sparrow has arisen from hybridization between the
114 house sparrow (*P. domesticus*) and Spanish sparrow (*P. hispaniolensis*) (Hermansen, et al.
115 2011; Hermansen, et al. 2014; Trier, et al. 2014; Elgvin, et al. 2017). The parent species
116 diverged approximately 0.85 million years ago (Ravinet, et al. 2018) and the Italian sparrow
117 originated within ca. 5800 years ago, most likely as the house sparrow expanded its range
118 into Europe and hybridized with the Spanish sparrow (Ravinet, et al. 2018). This system
119 provides a unique possibility to test to what extend an old, stabilized homoploid hybrid
120 species has diverged in gene expression from the parent species, and how gene expression
121 has evolved after hybridization. In this study, we compared the gene expression profiles of
122 the gonads of the Italian sparrow to gene expression of the parental species and lab bred
123 experimental F1 hybrids to address a) if Italian sparrows exhibit transgressive gene
124 expression compared to the parent species and b) whether inheritance mode and
125 misexpression in F1s predict which genes are transgressively expressed in Italian sparrows.

126 # Results

127

128 **Differential expression between the parental species**

129 Gene expression divergence in gonads between the parental species was strongly asymmetric
130 with testis showing a more conserved pattern of expression compared to ovary (Table 1). In
131 testis, 135 genes (1.16%) and in ovary, 1382 genes (11.65%) were significantly differentially
132 expressed. Of the differentially expressed genes, 60.7% of genes in testis and 69.3% of genes
133 in ovary were upregulated in the Spanish sparrow. A significantly higher proportion of genes
134 that were differentially expressed in testis were located on the Z chromosome (24 genes,
135 hypergeometric test, *P*-value < 1.82-08). We found no evidence for functional enrichment
136 among differentially expressed genes in parental species for neither testis nor ovary.

137

138 **Differential expression in Italian sparrows compared to the parental species**

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

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

152

153 Genes that were differentially expressed in Italian sparrow testis were enriched for similar
154 biological processes in comparison to both parent species. Primarily, functions involving
155 protein synthesis and mitochondrial gene expression and function were overrepresented. In
156 the comparison to house sparrow, 27 significant GO categories were found (Table S4), with
157 top biological processes involved in viral transcription, SRP-dependent co-translational
158 protein targeting to membrane, translation, ribosome and mitochondrial respiratory chain
159 complex I assembly. In comparison to the Spanish sparrow, the 15 enriched GO terms
160 reflected similar biological processes, with ribosome as the top GO term (Table S5). In ovary,
161 6 GO terms were detected in the comparison to house sparrow with sodium ion
162 transmembrane transporter activity as the top term (Table S6) and 1 GO term, negative
163 regulation of protein phosphorylation detected in the comparison to Spanish sparrow (Table
164 S7).

165

166 **Differential expression in F1 hybrids compared to the parental species**

167 Both testis and ovary showed a more conserved pattern of expression between the F1 hybrid
168 and the house sparrow than between F1 hybrids and the Spanish sparrow (Table 1, Figs. 2A-
169 3A). The overall magnitude of differences to the parent species was much lower than for the
170 Italian sparrow. In testis, 106 genes (0.9%) and 263 genes (2.25%) were differentially
171 expressed compared to house and Spanish sparrows, respectively. In ovary, 30 genes (0.25%)
172 and 140 genes (1.15%) showed a significant difference in expression compared to the house
173 and Spanish sparrows, respectively. Similar to the Italian sparrow, the pattern of up- and
174 down-regulation was consistent in both testis and ovary in the comparison to each of the
175 parental species (Figs. 2B-3B). A larger number of genes were up-regulated in F1 hybrid in

176 the comparison to house sparrow and down-regulated in the comparison to Spanish sparrow
177 (Chi-squared test *P*-value = 1.96-05 in testis and 0.01 in ovary). As in the Italian sparrow,
178 there was an over-representation of Z-linked genes among those differentially expressed
179 between F1 hybrids and Spanish sparrows (31 of the 263 differentially expressed genes,
180 hypergeometric test, *P*-value = 2.42-06). The enriched functions were different to those
181 enriched for Italian sparrows. Testis genes differentially expressed in F1 showed functional
182 enrichment for primarily collagen catabolic process. In the comparison to house sparrow, 46
183 GO terms were significantly over-represented. Among these GO terms, 20 GO terms had an
184 Padj < 0.01, whereas there were few significant GO terms compared to Spanish sparrows
185 with top GO term common with the comparison to house sparrow (Tables S8 and S9). The
186 top 5 GO terms were enriched in proteinaceous extracellular matrix, extracellular matrix
187 structural constituent, extracellular vesicle, collagen trimer and collagen catabolic process. In
188 ovary, while there was no functionally significant term associated with differentially
189 expressed genes in F1 compared to the house sparrow, there were 28 significant GO terms in
190 the comparison to Spanish sparrow with the top category to be response to vitamin D (Table
191 S10).
192
193 **Classification of inheritance patterns of gene expression**
194 Gene expression was generally conserved in both Italian sparrow and F1 hybrid (Table 2),
195 but in the testis of Italian sparrow, 2611 genes (22.71%) showed a non-conserved pattern of
196 inheritance with transgressive expression and house-dominant being the two largest
197 categories (Fig. 4C). Transgressively expressed genes in Italian sparrow comprised 22% of
198 the testis transcriptome tested for inheritance. In contrast, in F1 testis only 0.37% of the genes
199 were transgressively expressed (Fig. 4D). The majority of the genes with non-conserved
200 pattern of inheritance in Italian sparrow ovary, over 96% of the genes, had a house-dominant

201 pattern of expression (Fig. 4E). In contrast to the high incidence of transgressive expression
202 observed in Italian sparrow testis, only 3 genes (0.028%) were transgressively expressed in
203 ovaries. Gene expression in F1 ovary was more conserved compared to that of the Italian
204 sparrow. Out of 376 genes with non-conserved pattern of inheritance, the two largest
205 categories of inheritance were additive and house-dominant inheritance pattern (Fig. 4F).
206
207 Overdominant genes in Italian sparrow testis were enriched for functional categories involved
208 in protein synthesis, mitochondrial protein complex and gene expression and binding of
209 sperm to zona pellucida (Table S11). We found a gene network with significant protein-
210 protein interaction (PPI Enrichment: 1.0E-16) among the overdominant genes in Italian
211 sparrow (Fig. 5, Fig. S4). A different set of genes were underdominant in Italian sparrow,
212 with functions including regulation of cellular component size and negative regulation of
213 neuron projection development. Overdominant genes in F1 testis were functionally enriched
214 for different GO-terms, including collagen catabolic process and proteinaceous extracellular
215 matrix (Table S12). The only significant GO term for underdominant genes in F1 testis was
216 voltage-gated potassium channel complex.
217
218 **Comparison of gene expression divergence in respect to parent species between Italian**
219 **sparrows and F1**
220 To evaluate how gene expression evolves over time asking whether mis-expression in early
221 generation hybrids predicts differential expression in Italian sparrows, we checked if the
222 same genes or gene functions that were differentially expressed in comparison to parental
223 species in F1 hybrids also were differentially expressed in the Italian sparrow (Figs. 2C-3C).
224 In testis, 1.28% of the genes that were differentially expressed in Italian sparrows compared
225 to house sparrow were also differentially expressed in experimental F1 hybrids, whereas in

226 comparison to Spanish sparrows it was 3.9%. In ovary, only 1 gene (2%) was differentially
227 expressed in F1 and Italian sparrow compared to house sparrow, whereas 5.9% were
228 differentially expressed in F1 hybrids and Italian sparrows in comparison to Spanish
229 sparrows. The majority of the commonly differentially expressed genes were differentially
230 expressed in the same direction (i.e., up- or down-regulated) in both testis and ovary, both in
231 F1 hybrids and Italian sparrows. Testis genes commonly differentially expressed in F1 hybrid
232 and Italian sparrow compared to house sparrow were enriched in extracellular vesicle, insulin
233 like growth factor binding, extracellular matrix structural constituent and extracellular matrix
234 component. In the comparison to Spanish sparrow testis, only one significant GO term,
235 cAMP binding was detected.
236
237 **Mode of regulatory divergence and prediction of transgressive expression in Italian**
238 **sparrows**
239 To address if the regulatory basis can predict transgressive gene expression in hybrid species,
240 we classified genes dependent on their regulatory basis (Table 3). We expect genes under *cis-*
241 *trans* compensatory regulation and trans-regulated genes to more easily produce transgressive
242 patterns of gene expression in later generation hybrids than *cis*-regulated genes, as
243 recombination may de-couple *cis*- and *trans*- or different trans-elements in the sorting of
244 parental variation in the hybrid lineage. Therefore, we checked the percentage of genes
245 transgressively expressed in the Italian sparrow depending on the mode of regulatory
246 divergence. We found that 16/130 genes (12%) of the *trans*-regulated testis genes were
247 transgressively expressed in Italian sparrow, and 28/159 genes (18%) of the genes under *cis-*
248 trans compensatory regulation were transgressively expressed in Italian sparrows. Finally, we
249 tested whether genes with a non-additive inheritance, that were transgressively expressed in
250 F1 hybrids, were more likely to be transgressively expressed in the Italian sparrow. We found

251 that 20 out of 45 genes that were transgressively expressed in the F1 hybrid (44.4%) also
252 were transgressively expressed in Italian sparrow testis.
253
254 **Discussion**
255 In this study, we aimed to document the evolution of gene expression in the gonads of a
256 homoploid hybrid species, the Italian sparrow. An extensive level of transgressive expression
257 was found in Italian sparrow testis, with an over-expression of genes involved in functions
258 related to mitochondrial respiratory chain and, cytosolic and mitochondrial ribosomal
259 proteins, as well as proteins involved in protein folding and the proteasome complex. In
260 contrast to testis, the gene expression pattern in the ovary was asymmetric compared to
261 parental species, with a house-dominant expression in the Italian sparrow. Experimental F1
262 hybrids showed a much more conserved pattern of gene expression in both testis and ovary.
263 In the following sections, we discuss the potential implications of our results and consider the
264 limitations of these data.
265
266 **Extensive transgressive expression in Italian sparrow testis**
267 About 22% of testis transcriptome in Italian sparrow showed transgressive expression pattern,
268 while this level was less than 1% in the F1 hybrid. Transgressive gene expression, despite the
269 fact that gene expression is conserved between parent species, is expected to occur in later
270 generation hybrids due to the uncoupling of co-evolved *cis*- and *trans*- regulatory elements
271 through recombination (Rieseberg, et al. 2003; Mallet 2007). There are, however, few studies
272 of gene expression in post F1-hybrids. In Lake whitefish, F2 hybrids showed higher
273 misexpression and percentage of genes with nonadditive inheritance compared to F1 hybrids
274 (Renaut, et al. 2009). Transgressive expression is commonly referred to "mis-expression" in
275 early generation hybrids while Italian sparrow is a successful hybrid taxon likely formed

276 within the last 5.800 years (Ravinet, et al. 2018). Here, we show that transgressive
277 expression is found not only as mis-expression in early generation hybrids but remains in
278 well-functioning homoploid hybrid taxa long after hybridization. Hence, evolution of the
279 regulation of gene expression following hybridization may produce novel expression
280 phenotypes from intermediate genomes. In hybrid lineages, transgressive segregation, which
281 leads to extreme phenotypes, has been considered as a major mechanism by which extreme or
282 novel adaptations observed in new hybrid ecotypes or species arise (Rieseberg, et al. 1999),
283 but sorting of regulatory elements have not formally been suggested to contribute to this. This
284 research suggests that break-up of co-evolved regulatory elements, resulting in transgressive
285 patterns of gene expression, partly can explain the generation of transgressive phenotypes in
286 hybrid taxa.
287
288 **Transgressive expression arising in spite of low parental divergence**
289 Hybrid incompatibilities arisen due to bringing together novel genetic combinations have
290 been mainly considered when the parental species are fixed for alleles potentially
291 contributing to reproductive isolation (Cutter 2012). In recently diverged species,
292 reproductive incompatibility may arise due to polymorphic loci within each lineage before
293 fixation for the incompatibility alleles (Cutter 2012). Therefore, if transgressive expression in
294 the Italian sparrow is driven by bringing together *cis*- and *trans*-regulatory variants not yet
295 co-adapted, this must either be by bringing together polymorphic loci in each of the parental
296 species or novel mutations that have occurred in Italian sparrow since isolation from the
297 parental species. Each of these hypotheses can be evaluated in future work by studying the F1
298 and F2 hybrids and the direct parents of the cross in order to find *cis* and *trans*-regulatory
299 variations. Interestingly, during ca. 5.800 years of evolution, Italian sparrows have achieved a
300 26 times stronger divergence in gene expression the parents have accumulated during 0.68

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

302

303 **Tissue- and parent specific patterns of differentiation in gene expression**

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

318

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

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

326 in eukaryotes. RNA binding proteins play an important role in post-translational regulation of
327 gene expression and spermatogenesis and appear to be key regulatory factors that ensure
328 male fertility (Paronetto and Sette 2010; Phillips, et al. 2019). Several genes coding for
329 subunits of T-complex protein Ring Complex (TRiC) (*CCT4, CCT5, CCT6A, TCP1*) were
330 also among the overdominant genes. The TRiC complex aids in the folding of about 10% of
331 the proteome. Subunits of TRiC complex has shown to be required for spermatogenesis
332 (Counts, et al. 2017) and have shown evidence for positive selection among the seminal fluid
333 genes between house and Spanish sparrows (Rowe, et al. 2020). Another category of gene
334 networks which were upregulated in Italian sparrow included genes encoding for components
335 of all mitochondrial respiratory chain enzymatic complexes. Additionally, genes encoding for
336 chaperone proteins involved in the assembly of these enzymatic complexes such as
337 *TIMMDC1* were also over-expressed.
338
339 In Italian sparrow testis, mito-nuclear genes have been shown to be important in generating
340 reproductive isolation in the Italian sparrow (Trier, et al. 2014), and mito-nuclear sites have
341 been under selection for inheritance of the house sparrow allele in Italian sparrows
342 (Runemark, Trier, et al. 2018). Although the mitochondrion has its own genome, more than
343 98% of its proteins are encoded in the nuclear genes and its correct function requires a
344 coordinated expression of both nuclear and mitochondrial genomes (Ryan and Hoogenraad
345 2007). Therefore, the upregulation of genes involved in respiratory chains and protein
346 synthesis corroborate the evidence for a strong effect of hybridization on genes associated
347 with metabolism in other bird taxa (Wagner, et al. 2020). Overexpression of cytosolic
348 ribonucleoproteins and mitochondrial respiratory chain enzymes has also been observed in
349 later generation hybrids (F3+) in copepod species *Tigriopus californicus* and has been linked
350 to cellular responses to physiological dysfunction (Barreto, et al. 2015). Our study thus

351 contributes to the growing body of evidence of mito-nuclear interactions as important
352 reproductive barriers (Hill 2017), and provides a unique insight into altered patterns of gene
353 expression as a possible resolution to the conflict.
354
355 **Conclusion**
356 This study presents the first evidence for transgressive gene expression in a wild homoploid
357 hybrid species. The strongly divergent gene expression profile in Italian sparrow testis
358 highlights the potential of intermediate hybrid genomes to rapidly evolve through altered
359 regulation of gene expression. Comparing patterns of gene expression in wild homoploid
360 hybrid species to those seen in experimental F1 hybrids, we document rapid evolution of
361 gene expression, especially for networks of genes involved in mitochondrial respiratory chain
362 enzymes and protein synthesis. The strongly divergent testis transcriptome was unexpected
363 given the low parental divergence, highlighting that alleles contributing to reproductive
364 isolation may be polymorphic rather than fixed. In contrast, Italian sparrow ovary had a
365 transcriptome similar to that of the house sparrow. Future studies should therefore evaluate
366 the current results in more tissues. Using pedigrees of parents and hybrid individuals would
367 also improve the power to identify the regulatory underpinnings of gene expression. Finally,
368 examining the link between transgressive gene expression and transgressive hybrid
369 phenotypes is likely to significantly increase our understanding of how novel variation arise
370 from hybridization.
371
372 **Materials and methods**
373
374 **Sampling, RNA extraction, library preparation, and sequencing**
375 We compared the gene expression of Italian sparrows to its two parent species, house and
376 Spanish sparrow and their experimental F1-hybrids. As the species differ slightly in

377 phenology, birds were sampled during their peak reproductive periods at their respective
378 locations. Spanish sparrows and F1 hybrids were sampled near Olivenza, Spain (38º 40'
379 56"N, 7º 11' 17"W) in March 2016 and 2017, house sparrows in Oslo, Norway (59º 55' 2"N,
380 10º 46' 11"E) in May 2016 and Italian sparrows from Montanari in the Gargano peninsula
381 (41º 54' 36.8"N, 15º 51' 13.0"E) in May 2017 (Fig 1). Experimental F1 hybrids were bred in
382 outdoor cages using house sparrow females and Spanish sparrow males. All other birds were
383 trapped using mist nets outside or within cages. Birds were immediately euthanized by
384 cervical dislocation, and gonads (ovary and left testis) were harvested directly after death.
385 Sampling was performed between 10:00 and 16:00, with individuals from each of the species
386 being sampled over most of the day, in order to avoid biases in sampling time. The gonads
387 were stored in RNAlater, and immediately stored at -80 degrees, with extractions later
388 performed at the University of Oslo using the Qiagen miRNeasy micro kit. Samples were
389 prepped with TruSeq® Stranded mRNA library prep from Illumina on an automated Perkin
390 Elmer Sciclone NGSx liquid handler and sequenced in the Norwegian Sequencing Center in
391 Oslo, as paired-end (2x150 bp) on the Illumina Hiseq4000. The number of reads obtained per
392 each sample is reported in Table S1. As F1 hybrids were bred in an area where house and
393 Spanish sparrows are sympatric (Olivenza, Spain; see Fig 1) and correct identification of
394 females can be challenging, we performed a post-hoc genomic analysis based on
395 mitochondrial and genomic DNA to ensure that only individuals that clustered with the
396 expected species were included in the analyses (Supplementary materials and methods, Figs.
397 S1-S2).

398

399 **Read mapping, quality check and counting**

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

402 5, Spanish sparrow = 3, F1 hybrid = 8 and Italian sparrow = 5) to the house sparrow reference
403 genome using STAR2 v2.7.2b (Dobin, et al. 2013) with default parameters and the house
404 sparrow gene annotation as reported in (Elgvin, et al. 2017). Over 90% of the reads mapped
405 successfully (Table S1). The house sparrow general feature format (GFF) file contained
406 14,734 annotated genes of which 92.52% were anchored to chromosomes and 7.48% were
407 located on unanchored scaffolds. Of the genes anchored to chromosomes, 12,595 genes were
408 annotated on autosomes and 598 genes were on Z chromosome (Table S2). To ensure
409 homogeneity in quality across our samples, we used RSeQC (Wang, et al. 2012) to measure
410 RNA integrity at the transcriptome level for each sample by calculating Transcript Integrity
411 Number (TIN). The median TIN scores across samples ranged between 73.7 and 85.9 (Table
412 S3). We counted the number of reads mapping to the house sparrow gene features using
413 HTSeq v0.9.1 (Anders, et al. 2015). Read counting was performed for all reads with
414 minimum quality score of 30 and it was configured to handle reverse-stranded sequencing
415 data, with the parameter controlling for overlapping gene features set to union. We created a
416 dendrogram to show sample relationships. The main split was clearly between tissue types,
417 i.e. testis and ovary (Figure S3).

418

419 **Differential gene expression analysis**

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

427 expressed if they showed False Discovery Rate (FDR) Padj < 0.05 and shrunken LFC > 0.32.

428

429 **Classification of gene expression inheritance in experimental F1 hybrids and Italian**

430 **sparrows**

431 The mode of inheritance for differentially expressed genes was determined following

432 (McManus, et al. 2010). We normalized gene expression using median of ratios method in

433 DESeq2 (Love, et al. 2014). Genes whose total expression in hybrids deviated significantly

434 more than 1.25-fold (LFC > 0.32) from that of either parent were considered to have non-

435 conserved inheritance. These genes were classified as having additive, dominant, under-

436 dominant, or over-dominant inheritance, based on the magnitude of the difference between

437 total expression in the Italian sparrow / experimental F1 hybrid and in each parental species.

438 Genes for which expression in the Italian sparrow / experimental F1 hybrid was less than for

439 house sparrows and greater than for Spanish sparrow (or vice versa) were classified as

440 additive; genes for which expression in Italian sparrow / experimental F1 hybrid was similar

441 to one of the parents were classified as dominant; and genes for which expression in Italian

442 sparrow / experimental F1 hybrid was either greater than or less than both parent species

443 were classified as over-dominant and under-dominant, respectively (Figs. 4A-4B).

444

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

446 Conserved expression level between parents controlled by compensatory variation in parental

447 species is often associated with misregulation in their hybrids (Denver, et al. 2005; Landry, et

448 al. 2005; Goncalves, et al. 2012).-By using sites in experimental F_1 hybrids that contain both

449 parental alleles, we measured allele specific expression, and compared that to expression

450 levels in the parental species to infer whether the divergence is due to *cis*-, *trans*- or

451 compensatory evolution. A typical approach for the study of ASE is to select the sites for

452 which the parental species are fixed for two different alleles and study the ASE in
453 heterozygote hybrids (Wittkopp, et al. 2004). House and Spanish sparrows diverged only
454 about 0.85 MYA, with many polymorphisms still shared between the two species. We
455 therefore took an approach similar to the one used in other studies with species pairs
456 containing high number of shared polymorphisms and few fixed differences (Mugal, et al.
457 2020).
458
459 We performed the SNP calling and filtering as described in the section "Genetic
460 Characterization of Samples" (Supplementary Materials and Methods). We additionally
461 filtered the sites for DP of minimum 10, base quality of 20 and mapping quality of 60. We
462 used the R package MBASED (Mayba, et al. 2014) in the unphased mode to identify genes
463 with allele-specific expression. We filtered the MBASED results for the FDR < 0.05 and
464 major allele frequency of minimum 0.7. To balance the power, we chose 5 individuals for
465 each house, Spanish, F1 and Italian sparrow in testis and 3 individuals of each group for
466 ovary. To identify allele-specific expression at the population level, we required genes to
467 show significant allele-specific expression in at least two individuals. Following (McGirr and
468 Martin 2019), we divided the regulatory categories as follow: Cis-regulatory divergence was
469 attributed to genes that are differentially expressed between parents and show allele-specific
470 expression in hybrids. Trans-regulatory divergence was assigned to genes that are
471 differentially expressed between parents and do not show allele-specific expression in
472 hybrids. Putative compensatory regulatory variation was determined if a gene 1) did not show
473 differential expression between parents, 2) showed significant allele-specific expression in
474 hybrids, and 3) did not show allele-specific expression in parents.
475
476 **Functional Annotation and Enrichment Analysis**
477 Gene ontology (GO) analysis was done for differentially expressed genes in each pairwise

478 comparison. GO functional annotations and gene descriptions were obtained for protein
479 sequences from the entire house sparrow protein set as described in (Rowe, et al. 2020) using
480 PANNZER (Koskinen, et al. 2015). Functional enrichment of GO terms present in the set of
481 differentially expressed genes relative to the background consisting of all genes expressed
482 and tested for differential expression was performed using the clusterProfiler (Yu, et al. 2012)
483 and significant enrichment was determined at FDR < 0.05. Interactions among proteins with
484 significant GO terms were predicted using STRING-v11 (Szklarczyk, et al. 2019) using
485 "Experiment", "Databases" and "Co-expression" as interaction sources. We set the minimum
486 required interaction score to the highest confidence (0.9) and used only the query proteins to
487 build the interaction network after removing the disconnected nodes in the network. The
488 obtained network was then exported to Cytoscape (Shannon, et al. 2003) and major
489 representations of biological processes were detected using ClueGO (Bindea, et al. 2009)
490 with Padj< 0.01.
491
492 **Animal Ethics**
493 In Spain, all trapping and sampling of birds was conducted in accordance with Spanish
494 Animal Protection Regulation RD53/2013, and all methods were approved by the
495 Institutional Commission of Bioethics at the University of Extremadura (CBUE 49/2011).
496 Birds were sampled in Oslo with permission from Miljø-direktoratet (2016/2225). In Italy, all
497 birds were sampled with permission from ISPRA (protocol number 12404) and with
498 permission (no. 305) from regione Puglia. All samples taken from Italy to Oslo in accordance
499 with the Nagoya protocols.
500
501 **Scripts for the analyses**
502 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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528 **Figure legends**

529

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

535

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

544

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

553 Fig. 4. Inheritance pattern of gene expression in testis and ovary in Italian sparrows and
554 experimental F1 hybrids. A and B: Schematic figures representing the classification of
555 inheritance patterns. C and D: Scatter plots showing shrunken log2 fold change (LFC) in
556 testis between the Italian sparrow and experimental F1 hybrid with the house sparrow on the
557 *x*-axis and with Spanish sparrow on the *y*-axis, respectively. E and F: Scatter plots show
558 shrunken log2 fold change (LFC) in ovary between the Italian sparrow and experimental F1
559 hybrid with the house sparrow on the *x*-axis and with Spanish sparrow on the *y*-axis,
560 respectively. Grey points in each graph depicts the total number of genes studied for gene
561 expression with those colored representing the ones significantly different from parental
562 species to be divided into each of the inheritance categories (Conserved: grey, additive: pink,
563 house dominant: blue, Spain dominant: red, transgressive (over-dominant and under-
564 dominant: yellow). Grey dotted lines indicate the fold-change threshold of 1.25 used for
565 classification of inheritance pattern. Regression line represents the major variation in log2
566 fold change. F1h: experimental F1 hybrid – house sparrow; Ih: Italian sparrow – house
567 sparrow; F1S: experimental F1 hybrid – Spanish sparrow; IS: Italian sparrow – Spanish
568 sparrow.
569
570 Fig. 5. Gene ontology network represented in overdominant genes in testis of Italian sparrow.
571 Different colors depicts different significant biological processes. Circle size represents
572 adjusted *P*-value for each node with all Padj < 0.01.
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578 **Table 1.** Number of differentially expressed genes and log2 fold change (LFC) with Padj<

579 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

580 * LFC: Log Fold Change

581 **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%)

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588 **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

589 * Compensatory evolution

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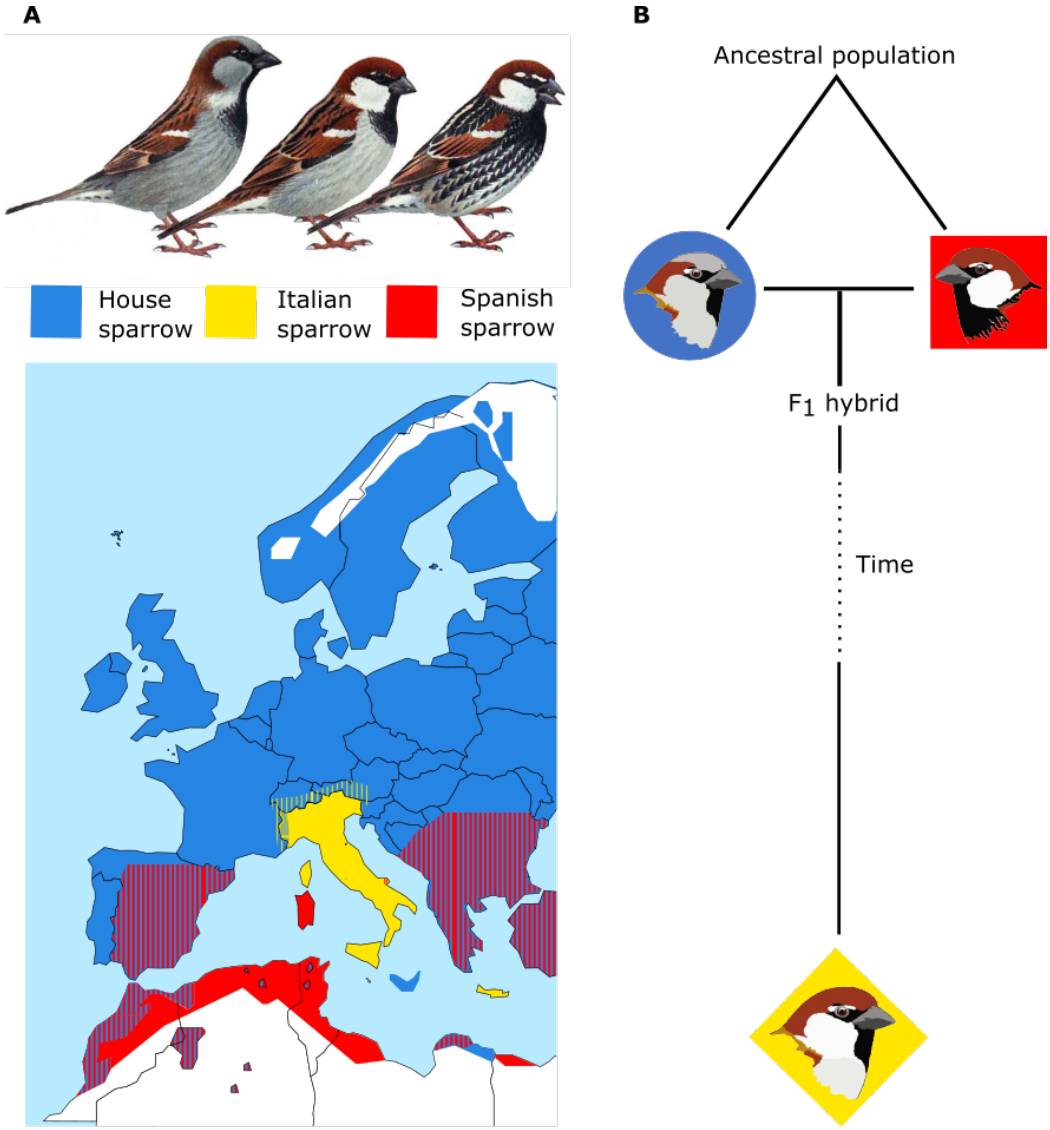
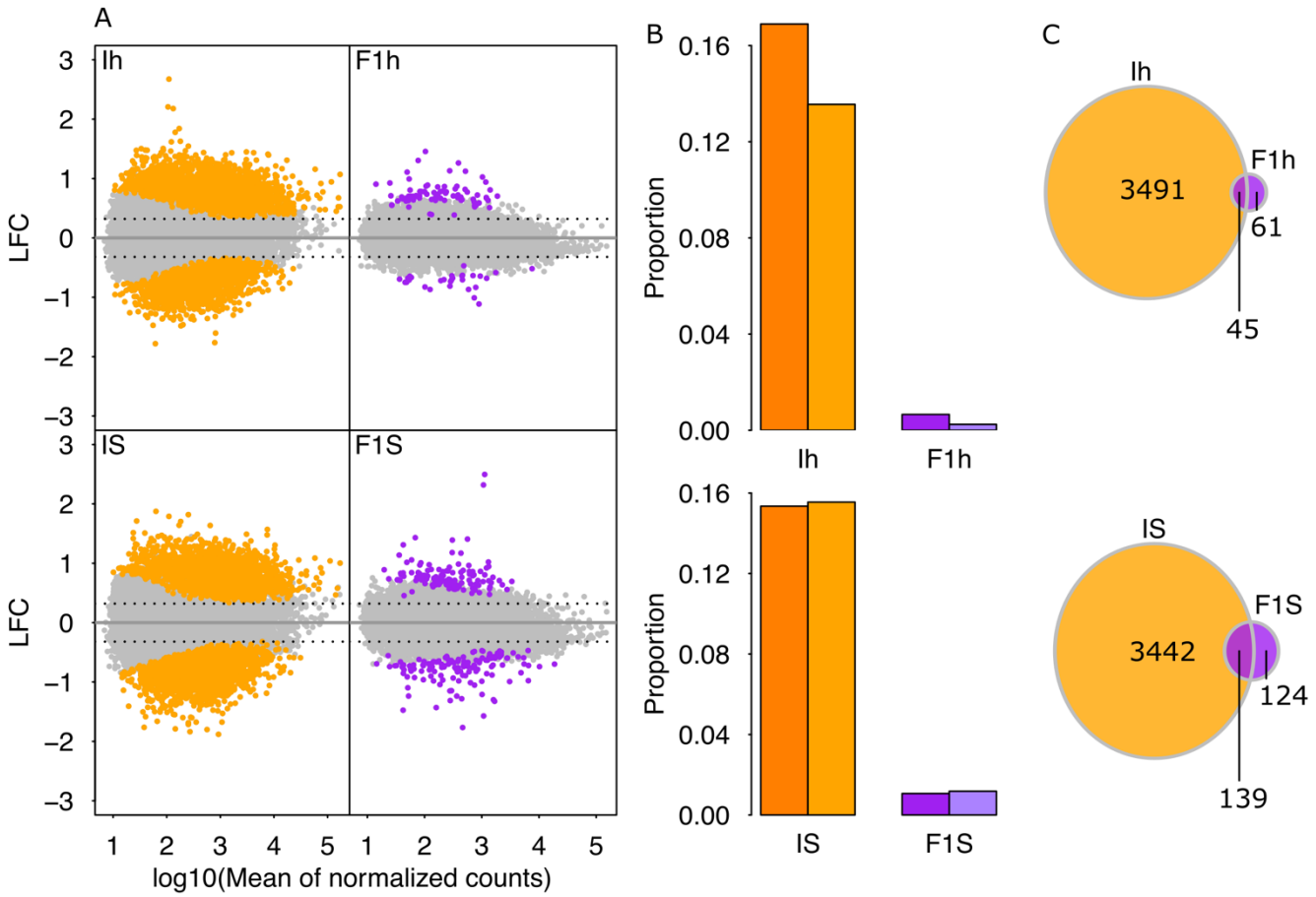

A
House sparrow
Italian sparrow
Spanish sparrow
B
Ancestral population
F1 hybrid
Time


A
Ih
F1h
IS
F1S
LFC
log10(Mean of normalized counts)
B
Proportion
Ih
F1h
IS
F1S
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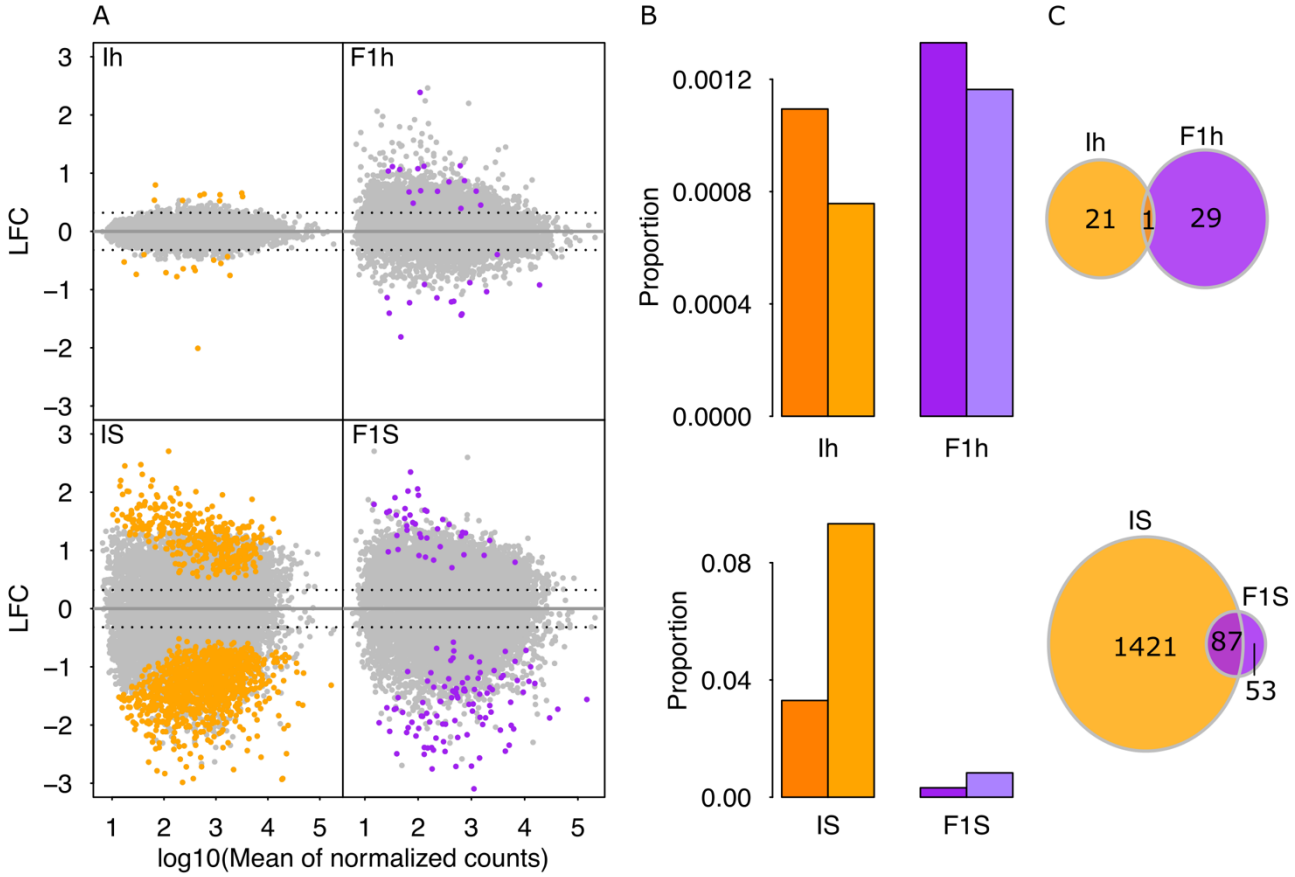
F1S
3442
124
139


A
B
C
Ih
F1h
IS
F1S
LFC
log10(Mean of normalized counts)
Proportion
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0.04
0.00
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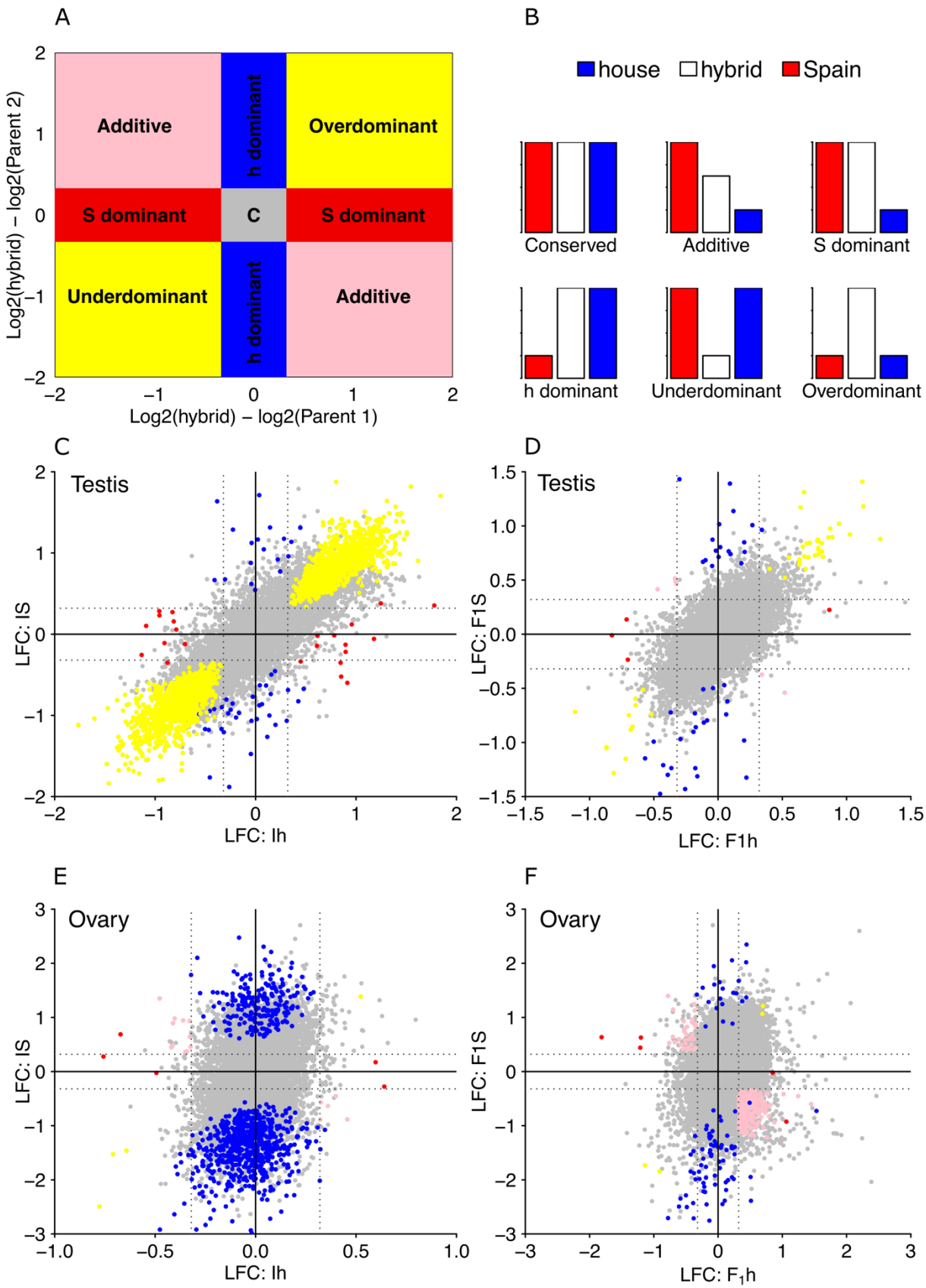
1
29
1421
87
53


A
Additive
h dominant
Overdominant
S dominant
C
S dominant
Underdominant
h dominant
Additive
Log2(hybrid) – log2(Parent 2)
Log2(hybrid) – log2(Parent 1)
B
house
hybrid
Spain
Conserved
Additive
S dominant
h dominant
Underdominant
Overdominant
C
Testis
LFC: IS
LFC: Ih
D
Testis
LFC: F1S
LFC: F1h
E
Ovary
LFC: IS
LFC: Ih
F
Ovary
LFC: F1S
LFC: F1h

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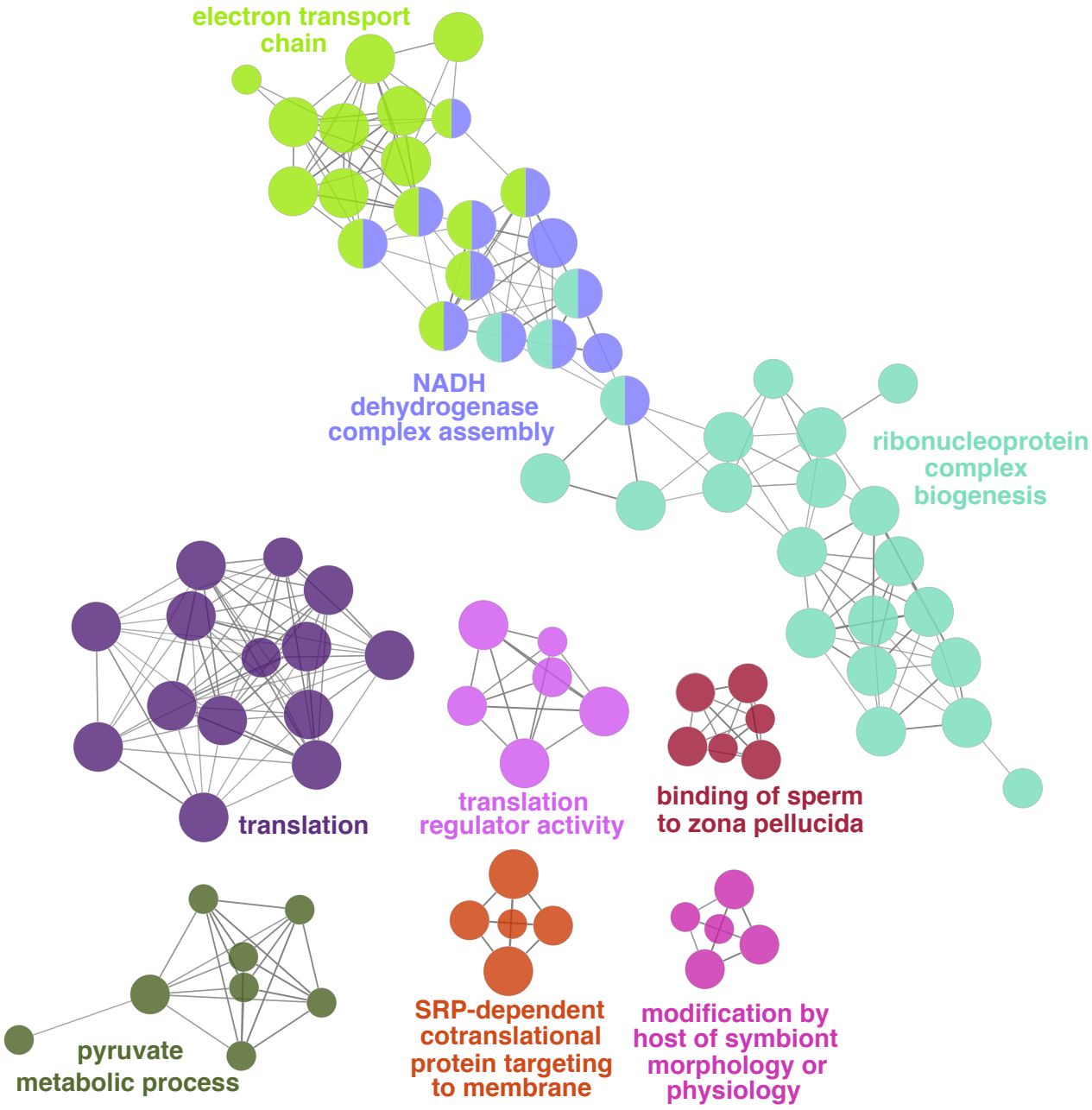
781


electron transport chain
NADH dehydrogenase complex assembly
ribonucleoprotein complex biogenesis
translation
translation regulator activity
binding of sperm to zona pellucida
pyruvate metabolic process
SRP-dependent cotranslational protein targeting to membrane
modification by host of symbiont morphology or physiology