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Research article

Little qualitative RNA misexpression in sterile male F_1 hybrids of *Drosophila pseudoobscura* and *D. persimilis*

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Abstract

Background: Although the genetics of hybrid sterility has been the subject of evolutionary studies for over sixty years, no one has shown the reason(s) why alleles that operate normally within species fail to function in another genetic background. Several lines of evidence suggest that failures in normal gene transcription contribute to hybrid dysfunctions, but genome-wide studies of gene expression in pure-species and hybrids have not been undertaken. Here, we study genome-wide patterns of expression in *Drosophila pseudoobscura*, *D. persimilis*, and their sterile F_1 hybrid males using differential display.

Results: Over five thousand amplifications were analyzed, and 3312 were present in amplifications from both of the pure species. Of these, 28 (0.5%) were not present in amplifications from adult F_1 hybrid males. Using product-specific primers, we were able to confirm one of nine of the transcripts putatively misexpressed in hybrids. This transcript was shown to be male-specific, but without detectable homology to *D. melanogaster* sequence.

Conclusion: We tentatively conclude that hybrid sterility can evolve without widespread, qualitative misexpression of transcripts in species hybrids. We suggest that, if more misexpression exists in sterile hybrids, it is likely to be quantitative, tissue-specific, and/ or limited to earlier developmental stages. Although several caveats apply, this study was a first attempt to determine the mechanistic basis of hybrid sterility, and one potential candidate gene has been identified for further study.

Background

Understanding the mechanistic basis for speciation, and hybrid dysfunctions in particular, has been a central but elusive goal of evolutionary biology since the fusion of Darwinian theory with Mendelian genetics. Hybrid dysfunctions like sterility and inviability result from detrimental gene interactions in individuals of mixed-species genetic ancestry [1–3]. Several types of negative gene interactions can account for these dysfunctions: for example,

ple, changes in the gene products (proteins) themselves could fail to interact properly thereby producing unfit phenotypes, other post-transcriptional processes such as mRNA splicing or stability may be disrupted, or genes may be inappropriately over- or undertranscribed in hybrids relative to pure species.

Several lines of reasoning suggest that normal gene transcription may be disrupted in hybrids and suggest that

such disruptions can contribute to hybrid dysfunctions including sterility. First, the overexpression of ONC-*Xmrk* (*Xiphophorus melanoma receptor* oncogene) causes hybrid melanoma formation, and often lethality, in crosses between *Xiphophorus* species [4–6]. Second, hybrid morphological anomalies in crosses of *Drosophila melanogaster* and *D. simulans* are associated with disruptions in the regulation of *Achaete-Scute*[7]. Third, one might predict that regulatory changes between species can cause dysfunctions in hybrids because such changes have been implicated in a wide variety of striking differences between taxa [e.g., [8–11]]. Finally, a recent theoretical model suggests that binding strength of regulator proteins to promoter regions can provide biologically plausible postmating isolation, such as hybrid sterility [12].

Although disruptions in gene expression may sometimes be associated with hybrid sterility [13,14], little information exists on how gene expression is altered across the genome in hybrids. To address whether qualitative failures in gene expression may be associated with hybrid sterility, we examined genome-wide patterns of expression in adult male *Drosophila pseudoobscura*, *D. persimilis*, and their sterile F_1 hybrid males using differential display [15–17]. Differential display involves PCR amplification from cDNA with a short primer. Multiple cDNA fragments are amplified with each reaction, and differences in amplification between strains reflect either sequence differences, or more interestingly, differences in RNA expression. *D. pseudoobscura* and *D. persimilis* have been studied extensively with regard to the genetic architecture of hybrid male sterility [3,18–21]. In their hybrids, the second meiotic division never occurs, and the spermatids that are formed in the first division degenerate. We focused on males, as the heterogametic sex is generally more likely to exhibit sterility and other hybrid defects than the homogametic sex [1,22].

Results and Discussion
In total, 5644 amplifications were evaluated using differential display PCR (DD-PCR), and 3312 amplifications were detected in all *D. pseudoobscura* and *D. persimilis* samples. Of these amplifications, only 28 were present in the two pure species but absent in the hybrids (Table 1). This figure may represent an overestimate of the extent of qualitative misexpression as well, since in all of these cases, the amplifications from the pure species males were very weak. As such, the lack of amplification from the F_1 hybrids likely represents either slight quantitative differences in expression between samples or variance in PCR rather than qualitative differences in expression in some of these cases. Fifty-seven amplifications were observed in DD-PCRs from hybrids but absent from those of pure species, and again, these amplifications were uniformly very weak.

Table 1: Differential display PCR amplifications

Band presence	Number of bands	Percent of total
D. persimilis (*per*) only	392	6.9%
D. pseudoobscura (*ps*) only	157	2.8%
F_1 hybrids only	57	1.0%
per and *ps*	28	0.5%
per and F_1 hybrids	655	11.6%
ps and F_1 hybrids	1071	19.0%
all three classes	3284	58.2%
total	5644	

We sought to confirm the putative hybrid misexpression of several of these transcripts using product-specific primers. As DD-PCR reactions amplify fragments from many transcripts simultaneously, the variance in PCR may be high, so one must develop product-specific primers to examine the expression of each putatively misexpressed transcript individually. Nine of the transcripts putatively misexpressed in hybrids were assayed via semiquantitative PCR (sqPCR). These were among the products with the most intense DD-PCR amplifications in pure-species samples and no or virtually no amplification in hybrids. We failed to confirm differential expression between pure-species and hybrids in eight of these nine products using sqPCR: amplifications from F_1 hybrid cDNA were comparable in intensity to amplifications from cDNA of one or both of the pure species, when scored by Intelligent Quantifier. Only one product putatively misexpressed in hybrid males (GenBank Accession AF510848) was confirmed repeatedly by our sqPCR assays: amplification from F_1 hybrid cDNA was comparable in intensity to amplification from 1/10 dilution pure-species cDNA. In contrast, amplifications of our control transcript were comparable across all samples. This comparison was repeated at least four times with independent RNA samples that were used to evaluate several of the other putatively misexpressed transcripts. The misexpressed product appears to be male-specific, as we were unable to amplify it from cDNA of either pure-species or F_1 hybrid females (Figure 1). No substantial sequence homology was noted by BLAST [23] search, perhaps because the isolated sequence likely comes from relatively unconstrained 3'-untranslated region of the gene.

Given the large number of products assayed by our DD-PCRs, we expected to identify some false positives that would not be confirmed by sqPCR. Assuming three repli-
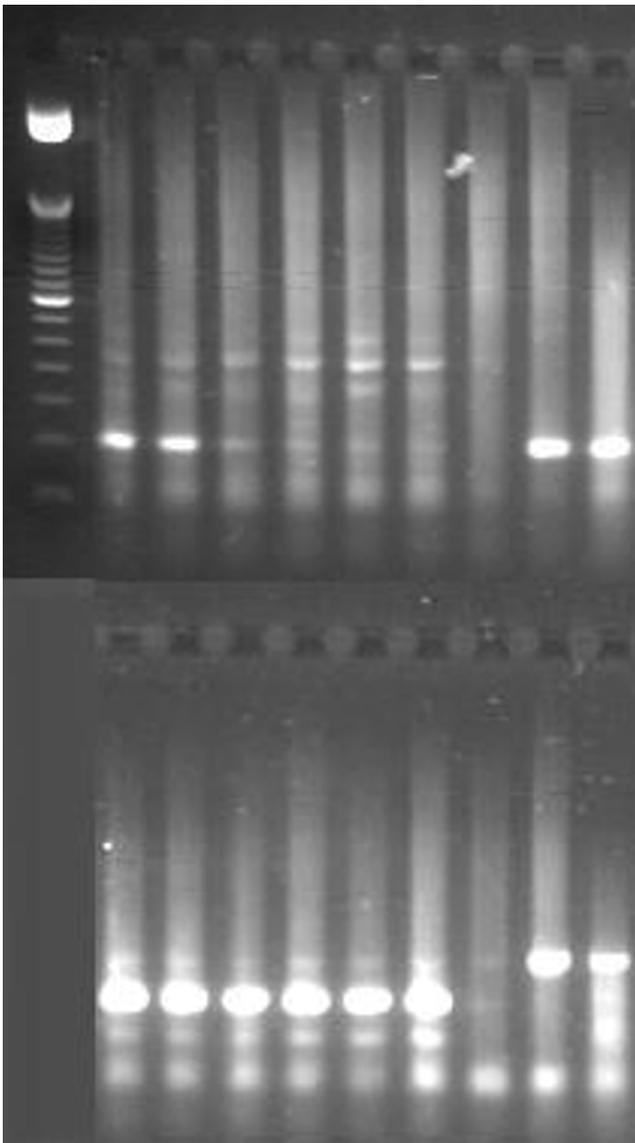
Figure 1
PCRs of transcript putatively misexpressed in hybrids (tmh) and a positive control (*Adh*). Upper lanes are, form the far left, 100-bp ladder, tmh from male *D. persimilis* cDNA, tmh from male *D. pseudoobscura* cDNA, tmh from male F$_1$ hybrid cDNA, tmh from female *D. persimilis* cDNA, tmh from female *D. pseudoobscura* cDNA, tmh from female F$_1$ hybrid cDNA, negative control of tmh PCR, tmh from *D. persimilis* genomic DNA, tmh from *D. pseudoobscura* genomic DNA. The lower nine lanes are as above but of *Adh* rather than tmh.

cations of all DD-PCRs within each strain or F$_1$ (r), no genetic variance within *D. pseudoobscura* or *D. persimilis* strains, and an inconsistency rate of 10% among replicates (q), we can calculate the probability of a transcript incorrectly appearing to be misexpressed as $p= [(1-q)^{2r}][q^r]$. This formula calculates a 0.053% probability of in-

correctly accepting a product as misexpressed in hybrids. With 5644 transcripts assayed, three should be false positives, and some variance in these estimates may partially explain our failure to confirm eight transcripts that appeared to be misexpressed via DD-PCR.

Our findings suggest that large-scale misexpression of transcripts is not a general feature of sterile adult male hybrids of these species. Three factors may confound our conclusion. First, "fast-evolving" genes may not have been surveyed adequately using our methodology. Genes highly divergent in sequence between *D. pseudoobscura* and *D. persimilis* may have been overlooked because amplification did not occur in both of the pure species. However, this complication is more improbable than it may appear. Screening for disruptions in gene expression in hybrids identifies factors that are not being transcribed properly, suggesting either that their promoter regions fail to interact with the proteins that regulate them or that their regulatory pathway was disrupted several steps earlier. Regulatory proteins that have diverged greatly in amino acid sequence [e.g., Odysseus: [24]] are not necessarily those that are misexpressed, and indeed, regulatory proteins that are highly divergent between species may control the expression patterns of several transcripts with no amino acid coding sequence divergence between the species.

A second possibility is that qualitative misexpression does occur in species hybrids, but the misexpression is confined to particular tissues. For example, actin may be transcribed normally throughout most of the body but completely absent from the brain. Such tissue-specific misexpressions can occur in hybrids [25]. We cannot exclude this possibility from our data, though transcripts expressed primarily or exclusively in a single tissue (e.g., testes) would still likely have been surveyed appropriately. Accordingly, 55% of ESTs from a large (3141) *D. melanogaster* testes EST collection were not found in both larger ovary (6034) and head (2086) EST collections [26].

Third, we have only assayed one-day post-eclosion adult males. Although transcripts in adult males comprise a relatively complete set of instructions for all stages of spermatogenesis [e.g., [27]], it is possible that failures in gene expression associated with hybrid sterility occur at the embryo, larval, or pupal stage.

Conclusions
We tentatively conclude from these findings that large-scale misexpression of transcripts is not a general feature across a large fraction of the bodies of sterile adult male hybrids of *Drosophila pseudoobscura* and *D. persimilis*. However, we have identified one male-specific transcript that is misexpressed in adult hybrids of these species. Our study

is a first attempt to elucidate why genes may fail to interact properly in hybrids resulting in sterility. We have shown hybrid male sterility can arise between diverging species independently of widespread, qualitative gene misexpression in adults. In addition to studies such as this one, genome-wide tests of other types of negative gene interactions in hybrids, such as failures in protein binding, are greatly needed. These types of approaches will ultimately lead us to understanding the molecular genetic nature of hybrid sterility, one of the contributors to the speciation process.

Methods

Differential display analyses
Total RNA was isolated from one-day post-eclosion adult *Drosophila pseudoobscura*, *D. persimilis*, and F_1 hybrid males (all bearing a *D. pseudoobscura* mother) using the QIAGEN RNeasy kit. One inbred isofemale line of each species was used, so genetic variation within each class should be minimal. One microgram of total RNA was reverse transcribed in a reaction bearing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 1 mM dNTPs, 20 U RNasin, 20 U reverse transcriptase (SuperScript II), and 2.5 µM of an 5'-$AAGCT_{11}M$-3' primer conjugated to IRDye (LI-COR, Inc.) where M is either A, G or C [28]. Differential display PCRs (DD-PCRs) were replicated three times with independent RNA and cDNA preparations from each species in 10 µl reactions including 2 µl cDNA, 1 µM arbitrary 13-mer primer (differs for different reactions), 1 µM IRDye-$AAGCT_{11}M$, 25 µM dNTPs, 1.5 mM $MgCl_2$, 5 mM KCl, 10 mM Tris-HCl and 1 U Taq polymerase [see [29]]. Thermal cycling was performed with 3 cycles of (94°C-60 s, 41°C-60 s, 72°C-30 s), 3 cycles of (94°C-60 s, 38°C-60 s, 72°C-30 s), 30 cycles of (94°C-60 s, 35°C-60 s, 72°C-30 s), and 1 cycle of 72°C-5 min. The products of these reactions were visualized on LiCor DNA sequencers. Each reaction produced approximately 50 bands between 100 bp and 350 bp. Band presence was scored manually and independently by two investigators, with very high consistency. At least three replicates of each sample were performed for each primer combination, and the consistency among replicates was >90%. Bands present in all samples of one species only or one species and the F_1 hybrid may indicate sequence differences between the strains being used at the primer binding sites. As such, we focus on bands present in the two species and not the F_1 hybrid or in the F_1 hybrid and not the two species as candidates for disruptions in gene expression.

Confirmation of differential display results
The DNAs amplified by DD-PCR were run on a low-melting point agarose gel, and several sections were extracted. DD-PCRs were then repeated to identify which of the sections contained the band of interest. The DD-PCR was repeated a second time with an unlabeled primer, and the product was run on a low-melting point agarose gel and gel-extracted using the QIAGEN Gel Extraction kit. The PCR product was then cloned using the Invitrogen TOPO-TA cloning kit, and the products were sequenced using the ABI BigDye kit. Product-specific primers were designed to confirm the misexpression via semiquantitative PCR [30].

For all of our semiquantitative PCR (sqPCR) assays, 1 µg of total RNA was reverse transcribed as above except with 2.5 µM of both a primer for a control transcript (*Adh*) and the primer of the transcripts being investigated. Two microliters of the cDNA from each sample were then PCR amplified in a 10 µl reaction volume following the conditions described previously [31], generally for about 25 cycles. Simultaneously, a 1/10 dilution of each RNA sample was also reverse transcribed and amplified in the same volumes. The ten-fold dilution serves as a positive control to estimate the extent of differences in expression [30]. Products were visualized on a 2% TBE agarose gel, and band intensity was analyzed using a gel documentation system and associated software (Intelligent Quantifier). This sqPCR assay consistently detected a four-fold misexpression in another study in our laboratory that was later confirmed by fluorescent real-time PCR [Noor, unpublished data]. This success confirms the sensitivity of the sqPCR assay to at least ten-fold and perhaps finer resolution. We use the "ten-fold" criterion to define qualitative differences in expression.

Authors' contributions
JR conducted all of the studies presented here, including developing and optimizing several of the protocols. MAFN conceived of the study, participated in its design and coordination, and prepared this manuscript.

Acknowledgements

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