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Patterns of evolution of genes disrupted in expression in *Drosophila* species hybrids

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Summary

Divergence between species in regulatory pathways may contribute to hybrid incompatibilities such as sterility. Consistent with this idea, genes involved in male fertility often evolve faster than most other genes both in amino acid sequence and in expression. Previously, we identified a panel of male-specific genes underexpressed in sterile male hybrids of *Drosophila simulans* and *D. mauritiana* relative to pure species, and we showed that this underexpression is associated with infertility. In a preliminary effort to assess the generalities in the patterns of evolution of these genes, I examined patterns of mRNA expression in three of these genes in sterile F_1 hybrid males of *D. pseudoobscura* and *D. persimilis*. F_1 hybrid males bearing *D. persimilis* X chromosomes underexpressed all these genes relative to the parental species, while hybrids bearing *D. pseudoobscura* X chromosomes underexpressed two of these three genes. Interestingly, the third gene, *CG5762*, has undergone extensive amino acid evolution within the *D. pseudoobscura* species group, possibly driven by positive natural selection. We conclude that some of the same genes exhibit disruptions in expression within each of the two species groups, which could suggest commonalities in the regulatory architecture of sterility in these groups. Alternative explanations are also considered.

1. Introduction

The search for common patterns in the evolution of reproductive isolation has been one of the most fruitful avenues for understanding speciation. For example, the observation that, when one hybrid sex is sterile or inviable, it is nearly always the heterogametic sex (Haldane's Rule: Haldane, 1922) stimulated decades of research that substantially enhanced our understanding of the genetic basis of hybrid dysfunctions (e.g. Orr, 1997). In the so-called genomics era, we are now accumulating a wealth of information that can also be used for comparative studies to understand speciation and other evolutionary processes. Several studies have shown that genes involved in spermatogenesis or other male-specific functions often evolve very quickly in amino acid sequence (e.g. Swanson & Vacquier, 2002*a*, *b*; Kulathinal & Singh, 2004) or expression (Meiklejohn *et al*., 2003; Parisi *et al*., 2003; Ranz *et al*., 2003; Nuzhdin *et al*., 2004) between species. Complementary work, largely in *Drosophila*, has shown that male-specific genes are disproportionately prone to hybrid disruptions (Michalak & Noor, 2003; Noor *et al*., 2003; Ranz *et al*., 2004). However, no one has investigated whether the same genes or regulatory pathways are disrupted in hybrids of multiple species groups.

Our laboratory previously identified a panel of genes significantly underexpressed in sterile hybrids of *Drosophila simulans* and *D. mauritiana* (Michalak & Noor, 2003). We also showed that the underexpression of five of these genes is strongly associated with hybrid male sterility (Michalak & Noor, 2004) and that their expression was influenced by loci at or near the *Odysseus* gene, which has been implicated in hybrid male sterility (Ting *et al*., 1998; Sun *et al*., 2004). This observation could suggest that these genes are downstream targets of the genetic changes that cause hybrid male sterility in these species.

In this preliminary note, I assess whether some of the same genes are disrupted in sterile male hybrids of

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two distantly related species groups and study the pattern of evolution of one of these genes. This information can identify genes or pathways that may be intrinsically prone to disruption in sterile hybrids. Some pathways may be particularly prone to disruption because of a high complexity of interacting factors, large interacting regions, or the rapid evolution of genes within these pathways. Studying such pathways may also ultimately help to characterize the nature of the genetic interactions resulting in hybrid dysfunction.

We have surveyed expression in sterile hybrid males of *D. pseudoobscura* and *D. persimilis* of three genes underexpressed in sterile hybrid males of *D. simulans* and *D. mauritiana*. These two species pairs share several features making them suitable for comparisons of misexpression patterns that may contribute to hybrid male sterility. For example, the two species within each pair diverged approximately 500 000 years ago, and F_1 hybrid males are sterile. The sterility of hybrid males is meiotic or postmeiotic, as spermatogenesis proceeds to meiosis normally in both pairings. F_1 hybrid males of *D. simulans* and *D. mauritiana* bear morphologically normal testes despite their sterility (Wu *et al.*, 1992). This is also true in hybrid male offspring of *D. pseudoobscura* females × *D. persimilis* males (hereafter, F1Xps), but hybrid male offspring with *D. persimilis* mothers (hereafter, F1Xper) have anomalously small and misshapen testes, suggesting a more severe developmental disruption (Dobzhansky & Boche, 1933; Dobzhansky, 1934; Dobzhansky & Powell, 1975).

Transcription of genes associated with spermatogenesis in *Drosophila* is almost exclusively premeiotic (Fuller, 1998). Because F1Xps males lack documented premeiotic developmental defects in spermatogenesis or testis formation, we infer there was opportunity for transcription of spermatogenesis-related genes in these hybrids, as in hybrids of *D. simulans* and *D. mauritiana*. Misexpression of these genes, or genes genetically upstream of them in the regulatory pathway, may contribute to hybrid dysfunctions such as hybrid male sterility. In contrast, because F1Xper males have atrophied testes, we anticipate underexpression of most spermatogenesis-related transcripts. Therefore, surveying F1Xper effectively serves as a positive control for our tests.

2. Methods

(i) *Genes and fly strains surveyed*

We surveyed expression of *Acylphosphatase* (*Acyp*), *always early* (*aly*) and *CG5762* (equivalent to *GA19111*) in *Drosophila pseudoobscura* (ps) males, *D. persimilis* (per) males and reciprocal F_1 hybrid males of these two species (F1Xps and F1Xper). For comparison, we also surveyed expression of these genes in *D. simulans*, *D. mauritiana* and sterile hybrid males bearing *D. simulans* mothers (F1Xsim). *Acyp* and *CG5762* were among the five genes studied by Michalak & Noor (2004). *always early* (*aly*) resides in a known spermatogenic regulatory pathway upstream of two of the other genes used by Michalak & Noor (2004): *Mst84Dc* and *Mst98Cb*. We attempted to survey the fifth gene used by Michalak & Noor (2004), *CG14718*, but were unable to obtain amplification from *D. persimilis* after many attempts.

Strains assayed in the expression portion of this study were *D. pseudoobscura* Flagstaff 1993, *D. persimilis* Mount St Helena 1993, *D. simulans* Florida City and *D. mauritiana* SYN. We also report expression assays on F_1 males from a cross between *D. persimilis* Mount St Helena 1993 and *D. persimilis* Mather 39. DNA sequences for *CG5762* were obtained from the above strains as well as *D. miranda* Mather 28 and the following strains of *D. pseudoobscura*: Goldendale 17, Goldendale 20, Goldendale 26, Goldendale 35, Mather 32, Mather 48, Mather 52, Mesa Verde 17, James Reserve 032 and Zapotitlan Mexico.

(ii) *DNA sequence analyses*

To design TaqMan primers and probes for real-time reverse transcription polymerase chain reaction (RT-PCR), we obtained DNA sequences of the strains being assayed to ensure perfect match. Primers and probes for *Acyp* and *CG5762* in *D. simulans* and *D. mauritiana* were used previously (Michalak & Noor, 2004). We designed PCR and sequencing primers for *aly* in *D. simulans* and *D. mauritiana* using the *D. melanogaster* sequence in GenBank (Accession No. AJ277307). Sequences were obtained using standard protocols and have been deposited in GenBank (Accession Nos. AY857738–AY857750). *CG5762* sequences were analysed using DnaSP (Rozas & Rozas, 1999), SITES (Hey & Wakeley, 1997) and PAML (Yang, 1997).

(iii) *Expression assays*

Expression assays followed the protocol of Michalak & Noor (2004) except that *actin5C* was used as the control. In all cases, we observed virtually no variance among samples in amplification of *actin5C*, and analyses were identical with or without normalization for *actin5C*. We present the normalized results here for simplicity. Transcript relative abundances were estimated using the threshold cycle number (C_T) in the dilution series and experimental samples, and in each case we further normalized each set of RT-PCRs by assigning either the *D. simulans* or *D. pseudoobscura* concentration to 1·000. Detailed protocols, quantitative RT-PCR primer sequences

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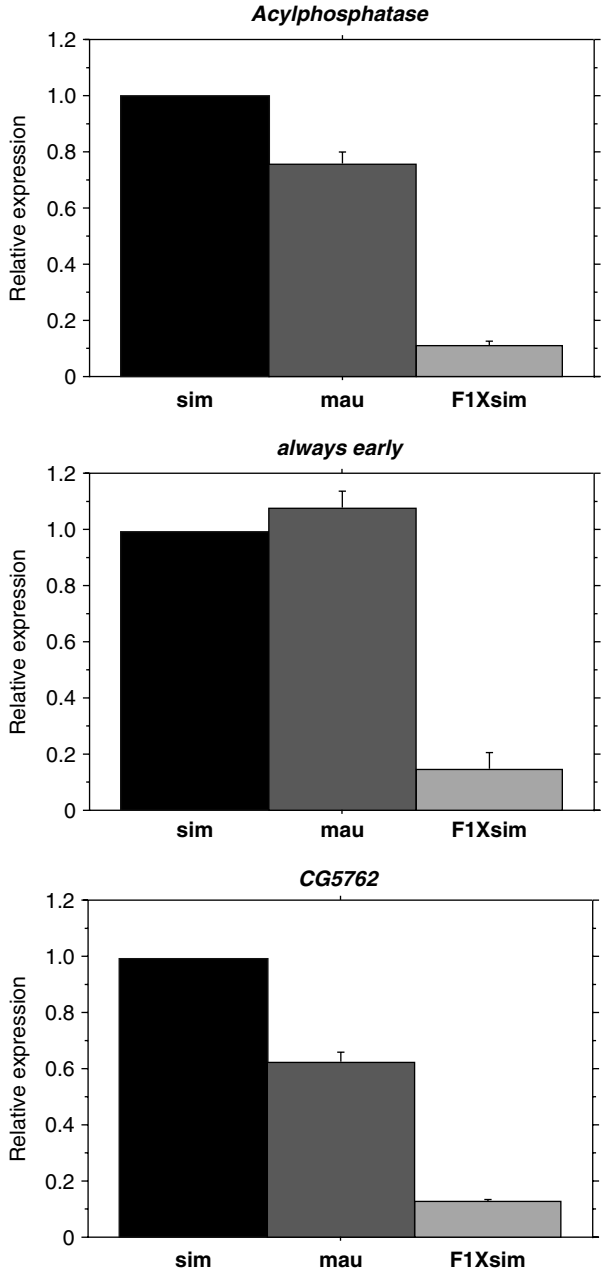

Fig. 1. Relative expression of *Acylphosphatase* (*Acyp*), *always early* (*aly*) and *CG5762* in adult male *D. simulans* (sim), *D. mauritiana* (mau) and F_1 hybrids (F1Xsim). Error bars denote 1 standard error.

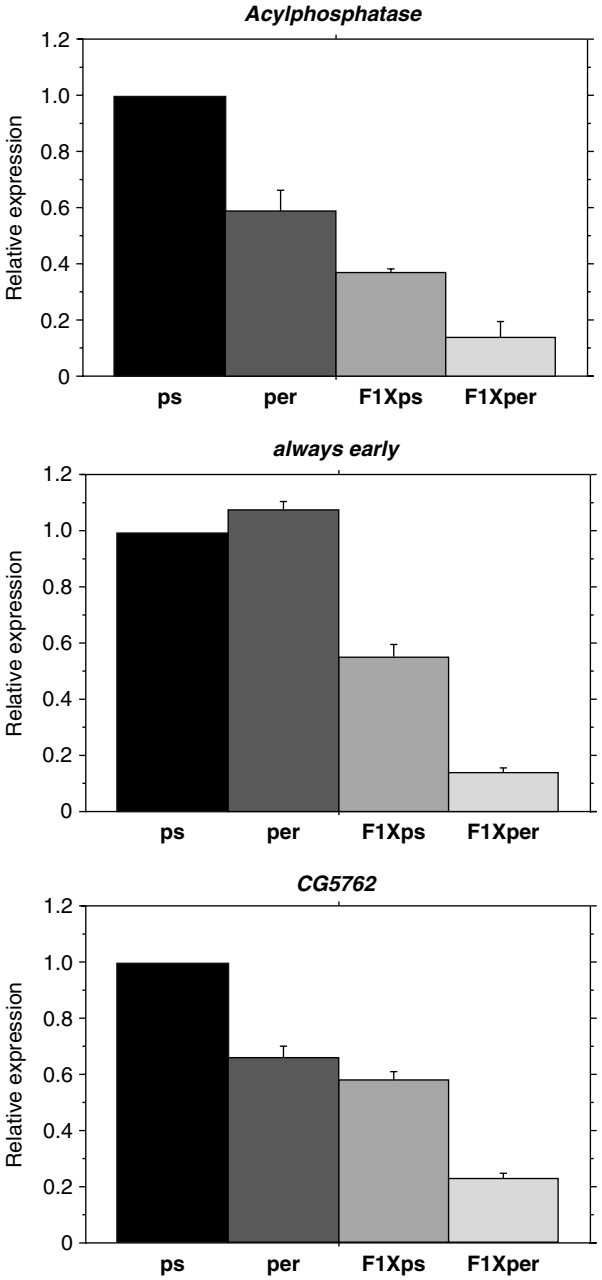

Fig. 2. Relative expression of Acylphosphatase (*Acyp*), *always early* (*aly*) and *CG5762* in adult male *D. pseudoobscura* (ps) *D. persimilis* (per) and F_1 hybrids (F1Xps, F1Xper). Error bars denote 1 standard error.

and fluorescent probe sequences are available upon request.

3. Results

(i) *Hybrid underexpression*

We confirmed significant underexpression of all three genes in F_1 hybrids of *D. simulans* and *D. mauritiana* relative to the parental strains (Fig. 1). In each case, F1Xsim expressed the transcript at a significantly lower level than either pure species parent, and at a level between 10% and 20% of *D. simulans* males.

We assayed transcription of these three genes in hybrids of *D. pseudoobscura* and *D. persimilis* relative to the parental strains (Fig. 2). Consistent with our *a priori* expectations given their atrophied testes, hybrid males bearing a *D. persimilis* X chromosome (F1Xper) always greatly and significantly underexpressed the transcripts relative to males of both parental species, at a level between 15% and 25% of *D. pseudoobscura* males. Both *aly* and *Acyp* were also

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significantly underexpressed in F1Xps males relative to males of both parental species. This underexpression was subtler than in F1Xper or F1Xsim hybrids: F1Xps hybrids expressed the transcripts at between 35% and 55% the level in *D. pseudoobscura* males. *CG5762* was also expressed significantly lower in F1Xps than in *D. pseudoobscura*, but there was no significant difference between its expression in F1Xps and *D. persimilis*. The low expression of *CG5762* in F1Xps may therefore not constitute or contribute to a hybrid dysfunction.

The parental *D. pseudoobscura* and *D. persimilis* strains differed significantly in expression at both *CG5762* and *Acyp*. We were concerned that these differences in expression may reflect laboratory inbreeding of *D. persimilis*, which had the lower level of these transcripts. To test this possibility, we also assayed expression of these transcripts in male offspring of a cross between two *D. persimilis* strains. We found no difference in expression between the outcrossed and inbred *D. persimilis* in any of the three transcripts, suggesting inbreeding did not cause this difference.

(ii) *Further analyses of* CG5762 *expression and sequence*

Unlike the other two genes, the sequence of *CG5762* in *D. pseudoobscura* is dramatically different from those of *D. melanogaster* and *D. simulans*. Indeed, BLAST (Altschul *et al*., 1997) analyses of either the *D. melanogaster* or *D. simulans* sequences to the genome sequence of *D. pseudoobscura* produced no significant matches. *CG5762* in *D. melanogaster* encodes a putative 200 amino acid protein and bears a single intron. In *D. pseudoobscura*, we observed an open reading frame (ORF) flanked by the same two genes as in *D. melanogaster* that appears to encode a 119 amino acid protein not bearing an intron. Only 54 of the amino acids can be aligned with confidence between the *D. melanogaster* and *D. pseudoobscura* sequences. We used RT-PCR and sequencing to confirm the absence of an intron anywhere within the putative ORF of *CG5762* in *D. pseudoobscura*. Through further RT-PCRs in *D. pseudoobscura*, we also observed that this gene is not transcribed in adult females or in male heads, but it is transcribed in the male body, consistent with its isolation from a testis library in *D. melanogaster* (Andrews *et al*., 2000).

We searched the Baylor College of Medicine *Drosophila pseudoobscura* sequence for other sequences resembling the *D. pseudoobscura CG5762*, and we identified two duplicates contained within transposon-bearing regions similar to ISY3 (Steinemann & Steinemann, 1992, 1993). These duplicates bore frameshift mutations or premature stop codons in the sequence resembling the ORF. Our attempts to isolate a transcript from one duplicate by RT-PCR from adult male RNA were unsuccessful. Nonetheless, for all assays above and below, we selected primer sequences that rested within indels differentiating the copies to ensure that we were working with the original gene and not a duplicate.

To study the recent evolutionary history of this gene in the *Drosophila pseudoobscura* species group, we surveyed sequences of this gene in 11 strains of *D. pseudoobscura* and one strain each of the related species *D. persimilis* (estimated divergence 500 000 years) and *D. miranda* (estimated divergence 2 million years). Within *D. pseudoobscura*, we identified five synonymous polymorphisms and six non-synonymous polymorphisms. We also observed two deletions in one strain, of which one was shared with the published genome sequence. The weighted average value of Watterson's (1975) estimator, θ, of the population mutation rate parameter $3N\mu$ (where N is the effective population size and μ is the neutral mutation rate) was 0·010 in *D. pseudoobscura*, which is comparable to other loci surveyed in this species (0·0099 on average: Machado *et al*., 2002). Tajima's D (Tajima, 1989) was $-0{\cdot}303$, suggesting a non-significant excess of rare alleles. The *D. persimilis* sequence differed from all the *D. pseudoobscura* sequences by only a single synonymous difference.

In contrast, the sequence of this gene from *D. miranda* was strikingly divergent from the *D. pseudoobscura* sequences. In *D. miranda*, the putative ORF encoded 133 amino acids, differing from all the *D. pseudoobscura* sequences by two or three indels, depending on the alignment used. Only 86 amino acids matched between these two species. The three-indel alignment of DNA sequences differed at five synonymous sites and 26 non-synonymous sites. The contrast of divergence between species to the polymorphism data within *D. pseudoobscura* yielded a marginally significant McDonald–Kreitman (1991) test (chi-square = 3·85, $P = 0{\cdot}049$), suggesting the potential action of positive natural selection. The two-indel alignment differed at six synonymous and 33 non-synonymous sites, yielding a significant McDonald–Kreitman test (chi-square = 4·52, $P = 0{\cdot}034$). The estimated ratio of non-synonymous to synonymous substitutions (dN/dS) calculated using Yang *et al*.'s (2000) approach was 1·35, again indicating an excess of non-synonymous differences between these species and the action of positive natural selection.

We confirmed transcription of this sequence in *D. miranda* by RT-PCR, and we also amplified via PCR a large fragment containing *CG5762* and the known flanking genes (and not the insertion sequences associated with the duplicates) to confirm that we isolated the same gene in this species as in *D. pseudoobscura*. The longer sequence in *D. miranda* appears to be ancestral because the indel regions align

in length with regions of this gene in *D. melanogaster* and because the regions deleted in the *D. pseudoobscura* ORF are present in the duplicate copies in *D. pseudoobscura*. Hence, two regions were deleted in the active copy of *CG5762* in *D. pseudoobscura*.

4. Discussion

Previously, we identified a panel of genes underexpressed in sterile adult male hybrids of *D. simulans* and *D. mauritiana* relative to pure species males (Michalak & Noor, 2003). In this study, we assayed three of these genes in sterile adult male hybrids of *Drosophila pseudoobscura* and *D. persimilis* to evaluate whether expression of some of the same genes may be disrupted in these different species groups, possibly associated with their hybrid sterility. In sterile hybrids bearing *D. persimilis* X chromosomes, all three genes assayed were significantly underexpressed, consistent with their severe gonadal atrophy and the likely transcription of these genes in the testes or germline. However, sterile hybrids bearing *D. pseudoobscura* X chromosomes, which do not suffer from any documented premeiotic disruptions in gametogenesis and which bear normal-sized testes, also underexpress two of these three transcripts relative to pure-species males. This latter finding could suggest regulatory similarities in the genetic control of hybrid sterility between these disparate species pairs, but it could also result from undocumented premeiotic disruptions in spermatogenesis or gonadogenesis in these hybrids preventing expression of multiple downstream genes in both species pairs.

Distinguishing between these explanations requires knowledge of the disruptions in sterile F1Xps and F1Xsim hybrids. In both cases, no premeiotic defects have been documented in spermatogenesis, and the testes appear morphologically normal, suggesting that the opportunity for transcription existed for all genes involved in spermatogenesis. In F1Xsim, other genes in the *always early* regulatory pathway are known to be disrupted, such as *Mst98Cb* and *Mst84Dc* (Michalak & Noor, 2003). Recently, Carlos Machado (pers. comm.) used microarrays to survey expression differences between F1Xps and its parent species, and one of the transcripts he identified as underexpressed in hybrids had strong sequence similarity to *Mst84Dc*. Combining our data with his, we thus observe that multiple genes in this pathway are underexpressed in hybrids of both species pairs, perhaps as a cause or consequence of their sterility.

We identified the potential action of positive natural selection in *CG5762* in the *D. pseudoobscura* species group. The coding sequence of *CG5762* bears an excess of non-synonymous divergence between *D. pseudoobscura* and *D. miranda* relative to synonymous divergence, and the amino acid sequences are barely alignable with those of *D. melanogaster* or *D. simulans*. Curiously, although male reproductive genes often exhibit accelerated amino acid substitution rates (Swanson & Vacquier, 2002*a*, *b*), *CG5762* bears no sign of rapid evolution in the *D. simulans* species group (Michalak & Noor, 2004). Therefore, if selection has acted upon this gene, its action is episodic across the history of the genus. It is also of interest, that the sequence of *CG5762* appears to have been captured recently by a transposable element within *D. pseudoobscura*, although the duplicate sequences do not appear to be expressed.

We have varying levels of information regarding the normal functions of these genes or their effects when disrupted or misexpressed. The *always early* gene is known to be essential for spermatogenesis in *D. melanogaster* (White-Cooper *et al.*, 1998, 2000), where it may regulate chromatin conformation in primary spermatocytes. Knocking out this gene prevents the expression of a host of other transcripts (including *Mst84Dc*) and causes sterility. However, it is unknown whether a 2-fold or 10-fold reduction in its expression would necessarily cause underexpression of its downstream targets or sterility. The *Acylphosphatase* gene produces a protein that may contribute to the control of ion transport across membranes in vertebrates, but the function of the *Drosophila* homologue may be completely different (Pieri *et al.*, 1998). Finally, *CG5762* was essentially unknown prior to our studies except in that it was expressed in testes (Andrews *et al.*, 2000). We previously showed that both *Acylphosphatase* and *CG5762* underexpression were associated with sterility in *D. simulans*/*D. mauritiana* in fifth-generation backcross hybrid males (Michalak & Noor, 2004).

We propose the hypothesis that some of the same regulatory pathways may be disrupted in the hybrid male sterility of multiple species groups. We must now identify these regulatory pathways and then determine where and at how many points in those pathways the disruptions occur. Johnson & Porter (2000; Porter & Johnson, 2002) modelled the evolution of hybrid sterility via regulatory divergence as a cumulative effect where multiple individual interactions were slightly disrupted. We can potentially test this model by identifying the points in the regulatory pathways where regulatory disruptions occur and assessing the magnitude of these disruptions. The observation that *always early* is underexpressed in these groups is fortuitous in this regard because this gene lies in a pathway that has been well characterized through mutagenesis studies in *D. melanogaster* (Fuller, 1998; Perezgasga *et al.*, 2004). The next steps will therefore be to assay expression in hybrids across this pathway in a stepwise fashion, determine whether underexpression of these genes in *D. pseudoobscura* hybrids is also associated with

sterility, and compare the results in multiple species groups.

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