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ORIGINAL ARTICLE

Hybrid sterility and evolution in Hawaiian *Drosophila*: differential gene and allele-specific expression analysis of backcross males

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The Hawaiian *Drosophila* are an iconic example of sequential colonization, adaptive radiation and speciation on islands. Genetic and phenotypic analysis of closely related species pairs that exhibit incomplete reproductive isolation can provide insights into the mechanisms of speciation. *Drosophila silvestris* from Hawai'i Island and *Drosophila planitibia* from Maui are two closely related allopatric Hawaiian picture-winged *Drosophila* that produce sterile F_1 males but fertile F_1 females, a pattern consistent with Haldane's rule. Backcrossing F_1 hybrid females between these two species to parental species gives rise to recombinant males with three distinct sperm phenotypes despite a similar genomic background: motile sperm, no sperm (sterile), and immotile sperm. We found that these three reproductive morphologies of backcross hybrid males produce divergent gene expression profiles in testes, as measured with RNA sequencing. There were a total of 71 genes significantly differentially expressed between backcross males with no sperm compared with those backcross males with motile sperm and immotile sperm, but no significant differential gene expression between backcross males with motile sperm and backcross males with immotile sperm. All of these genes were underexpressed in males with no sperm, including a number of genes with previously known activities in adult testis. An allele-specific expression analysis showed overwhelmingly more *cis*-divergent than *trans*-divergent genes, with no significant difference in the ratio of *cis*- and *trans*-divergent genes among the sperm phenotypes. Overall, the results indicate that the regulation of gene expression involved in sperm production likely diverged relatively rapidly between these two closely related species.
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INTRODUCTION

Reduced hybrid fitness in the form of hybrid sterility can play an important role in speciation by acting as a post-zygotic isolating barrier (Coyne and Orr, 2004). Haldane (1922) first documented that in crosses between species sterility is more likely to occur in hybrid individuals of the heterogametic sex. Since then, this observation has been shown to occur in almost all animals; especially in *Drosophila* spp., and is called 'Haldane's rule' (Coyne and Orr, 2004). In *Drosophila* species, hybrid male sterility (HMS) has been shown to function as an evolutionarily early limiting factor to introgression between species and consequently maintain species integrity (Noor and Feder, 2006). Hybrid incompatibility and sterility are thought to occur when epistatic interactions of alleles from different species are dysfunctional (Johnson, 2000), creating incompatible developmental pathways, ecological detriments or altered (and therefore, unsuccessful) mating behavior (Coyne and Orr, 2004). Genetic models have been developed that indicate that this type of fitness reduction may require at least two genetic changes—one from each species—but can be much more complex and be the result of multiple gene interactions (Johnson, 2000; Coyne and Orr, 2004). Multiple studies of HMS in the well-studied allopatric species pair *Drosophila mauritiana* and *Drosophila simulans* showed interactions of at least three genes or more (Johnson, 2000), and the identification of over 100 genes contributing to HMS on the X chromosome (Wu *et al.*, 1996), as well as many genes on the autosomes (Tao *et al.*, 2003; Araripe *et al.*, 2010; Dickman and Moehring, 2013).

In addition to extensive X and autosomal HMS loci studies, gene expression studies in *Drosophila* have shown a number of spermatogenesis genes differentially expressed between hybrids and parental species (Landry *et al.*, 2007a). Theoretical models suggest that the effects of accumulation of regulatory incompatibilities in the architecture of transcriptional networks can be a part of hybrid incompatibilities directly influencing the process of speciation (Porter and Johnson, 2002; Johnson and Porter, 2007). Techniques such as microarrays have been used to characterize gene expression, and have uncovered several genes that are deregulated in hybrids between species, especially in *D. mauritiana* and *D. simulans* (Michalak and Noor, 2003; Moehring *et al.*, 2007). *D. mauritiana/D. simulans* sterile male F_1 hybrids are more likely to succumb to downregulation of genes associated specifically with male reproduction, implying a

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possible genetic cause to their sterility (Michalak and Noor, 2003, 2004). Closely related species pairs can provide further insights into the genetic mechanisms of speciation because of incomplete reproductive isolation in the form of sterile F_1 males but fertile F_1 females that can be backcrossed to parental species. Analyzing the differences in gene expression among backcrossed (BC) individuals will provide insights into the variation in expression of testis-specific genes and also to potential candidate genes that lead to reproductive isolation by comparing transcriptomes between sterile and fertile BC siblings. Such a comparison between backcross hybrids having similar genomic backgrounds but distinct fertility phenotypes provides a higher resolution of the association between genes and phenotypes than that between F_1 hybrids and parental species, as every subsequent generation of backcrossing on average halves the amount of HMS-unrelated heterozygosity (Michalak and Noor, 2004).

HMS is generally observed to occur earlier in the divergence of species (that is, more closely related species) than hybrid inviability, suggesting stronger selection on sterility-causative genes (Orr *et al.*, 2004). DNA sequence divergence and expression levels of sex-related genes in many studies support the idea that genes involved in male fertility diverge faster between species than other types of genes (Orr *et al.*, 2004). Hybrid sterility-causative genes, such as *Odysseus site homeobox* (*OdsH*) in *Drosophila* (Ting *et al.*, 1998), *Meisetz* (*Prdm9*) in mice (Oliver *et al.*, 2009) and *AEP2/OLI1* in yeast (Lee *et al.*, 2008), are often characterized by rapid sequence evolution and distinct expression patterns. Candidate genes for speciation, therefore, include genes responsible for spermatogenesis and sperm motility and other genes that cause reproductive incompatibilities in hybrids.

HMS is an important post-zygotic reproductive isolating mechanism in many eukaryotic organisms, but only recently has genome-wide gene expression analysis been used to investigate the full suite of genes involved in the expression of this complex trait (Gomes and Civetta, 2015). Here we analyze the differential gene expression and allele-specific expression (ASE) of testis-level fertility in BC males between two closely related endemic Hawaiian *Drosophila* species in the picture-wing clade under *planitibia* group and IVβ subgroup, *Drosophila planitibia* and *Drosophila silvestris* (Spieth, 1986). *D. silvestris* is endemic to Hawai'i Island and *D. planitibia* is endemic to the island of Maui, and diverged ~0.7 Mya (O'Grady *et al.*, 2011; Magnacca and Price, 2015). Both species are bark breeders, whereby females oviposit eggs on and larvae develop in the decaying bark of the endemic Hawaiian flowering plant, *Clermontia* spp. ('oha wai) (Magnacca *et al.*, 2008). The subgroup is known for selecting leks in more open spaces as opposed to other subgroups that prefer a hidden location. Current phylogenetic analyses show that *D. planitibia* shares a close ancestor with both *D. silvestris* and *D. heteroneura* as the latter two species were established on Hawai'i Island (Magnacca and Price, 2015).

Previous studies have shown that successful mating can occur between the two species, creating sterile F_1 hybrid males, but fertile F_1 hybrid females (Craddock, 1974). Fertile F_1 females can be used to create a backcross generation whereby males exhibit different sperm phenotypes. Using the RNA sequencing platform and a *de novo* transcriptome assembly, we identified 71 differentially expressed (DE) genes across three BC phenotypes, showing a clear underexpression of key functional genes in BC individuals that lack sperm compared with those individuals who possess large numbers of motile sperm. These results demonstrate a directional gene expression change correlated with HMS, providing important insight into the mechanisms of reproductive isolation.

MATERIALS AND METHODS

Drosophila stocks

The *D. planitibia* and *D. silvestris* populations used in this study as parental population were initiated from individuals recently captured from wild populations: ~20 *D. planitibia* individuals captured in Waikamoi Preserve, east Maui (GPS coordinates 20.811286, −156.241901), in December 2012 and ~20 *D. silvestris* individuals collected in South Kona Forest Reserve, Kukuiopae unit (GPS coordinates 19.297281, −155.811710), Hawai'i Island in June 2013. All parental, F_1 and BC populations were maintained in a controlled-environment room maintained at a constant 18 °C, 70% relative humidity with a 12:12 light/dark cycle. This is a standard environment for rearing Hawaiian *Drosophila* (Uy *et al.*, 2015) with a generation time of ~3–4 months. Adults were housed in 4-l glass jars with a damp fine sand floor and containing 3 to 5 vials of adult food. The 25×95 mm adult food vials contained a yeastless Wheeler–Clayton medium and a small tissue moistened with a tea made with the leaves of *Clermontia* spp. on which females oviposited as described by Carson (1987). Adult food vials in which females deposited eggs on tissue were replaced weekly and placed in larvae-rearing trays. Larvae food, a yeast–cornmeal–molasses medium, was provisioned to each vial with active larvae several times per week. At 4 weeks, maturing larvae were transferred to emergence jars containing moistened large-grain sand for burial pupation. Emerging adult flies were aspirated from emergence jars twice a week and separated into male or female jars to ensure flies were virgins for future experiments.

Adult *D. silvestris* and *D. planitibia* used to produce F_1 and BC offspring were ~14–21 day post eclosion when the flies reach reproductive maturity and placed in mating vials (28.5×95 mm) that contained adult food and a tissue moistened with *Clermontia* spp. tea. Each cross was labeled, dated and numbered to ensure identification. Adults were transferred to new mating vials each week to ensure ideal mating and egg-laying conditions. The old mating vials were placed in larvae trays labeled with type of cross, date and identification number and fed larvae food. As larvae reached the third instar, the larval vials were placed in 2-l pupation/emergence jars, and emerging adult male and female flies were removed weekly from the pupation/emergence jars, before becoming sexually mature, and segregated into jars by each species or hybrid and sex. Hybrids and BCs were attempted during parental generations F_4–F_7. The production of BC individuals was accomplished by mating F_1 hybrid females (from the *D. silvestris* females×*D. planitibia* males cross) to emerged *D. silvestris* males of the parental species after flies reached sexual maturity (Figure 1). Because of the difficult nature of obtaining hybrids and backcrosses, all BC males used in this study were full siblings from one successful F_1 hybrid female/*D. silvestris* parental male pair.

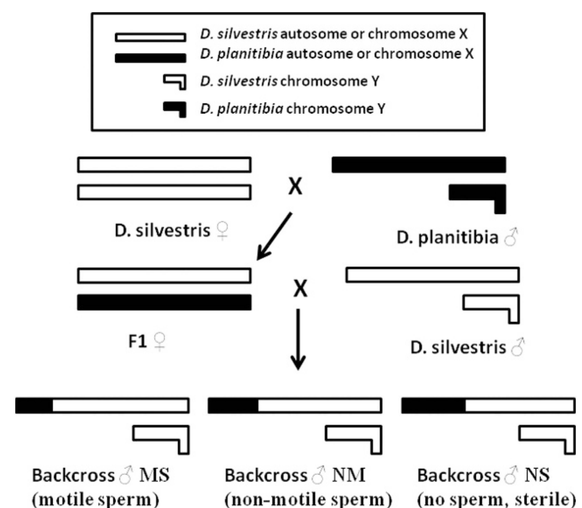

Figure 1 Schematic diagram of backcrossing of F_1 *D. silvestris*× *D. planitibia* female to *D. silvestris* with three distinct sperm phenotypes among male offspring.

Testes dissection and RNA collection

D. planitibia, *D. silvestris*, F_1 hybrid males and BC adult male flies that had reached sexual maturity (4 weeks post eclosion) were dissected at room temperature under similar housing and stabilization conditions using sterilized Dumont No. 5 fine forceps (Dumont, Montignez, Switzerland), tungsten needles and a 100×20 mm glass dissection dish that were sprayed with RNaseZAP (Sigma-Aldrich, St Louis, MO, USA) before each dissection to remove RNase contamination. Males were housed in their own individual glass vials with food and dissected after a 24-h stabilization period in the laboratory. Testes were dissected under a compound light microscope. One testis and its accessory glands were bifurcated and placed into a RNase-free 2 ml round-bottomed tube (Eppendorf, Hamburg, Germany) filled with RNALater (Life Technologies, Carlsbad, CA, USA). The other testis was prepared for a live testis squash by placing the tissue on a 20×20 mm cover slip with 10 μl drop of testis buffer and covered with a clean glass slide. The prepared slide was inverted and placed under a compound light microscope at ×10 and ×40 magnification for observation.

BC males were categorized into three phenotype groups based on testis and sperm observations and cross-referenced with Craddock's classifications (Craddock, 1974): males that exhibited complete absence of mature sperm (BC-NS) and were thus sterile, males that had sperm that was nonmotile and often clumped inside the testes (BC-NM) and males that had motile sperm (BC-MS). Because of the low numbers of mature BC males in the laboratory, we did not have extra BC males to conduct fertility experiments to determine the ability of BC-MS and BC-NM males to father progeny.

Sample preparation and sequencing

Twenty-four testis samples were prepared for RNA extraction: 3 *D. planitibia*, 3 *D. silvestris*, 2 *D. silvestris*×*D. planitibia* F_1 hybrids, 7 BC-MS, 4 BC-NM and 5 BC-NS. RNA extraction and sequencing were conducted at the Virginia Bioinformatics Institute, Virginia Tech (Blacksburg, VA, USA) and University of Hawai'i at Hilo (Hilo, HI, USA). Total RNA was extracted using Trizol Reagent (Life Technologies) following the manufacturer's instructions. Using TruSeq RNA sample preparation kit (Illumina, FC-122-1001/1002), mRNA from 1 μg of total RNA with RNA integrity number ⩾8.0 was converted into a library of template molecules suitable for subsequent cluster generation and sequencing with Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). The libraries generated were validated using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and quantitated using Quant-iT dsDNA HS Kit (Invitrogen, Waltham, MA, USA) and quantitative PCR. Individually indexed complementary DNA libraries were pooled, clustered onto a flow cell using Illumina's TruSeq SR Cluster Kit v3 (GD-401-3001) and sequenced for 101 cycles using two TruSeq SBS Kit HS (FC-401-1002) on HiSeq 2500.

de novo transcriptome assembly

Adapter sequences were removed from the raw sequencing reads. Duplicated and low-quality reads were discarded using FastqMcf (Aronesty, 2013) with default parameters. To exclude possible contamination, all reads were aligned to a bacterial database downloaded from National Center for Biotechnology Information (NCBI), and only unmapped reads were used to assemble the transcriptome. The processed reads from all samples were merged together and assembled with Trinity (Grabherr *et al.*, 2011) (with parameter –trimmomatic). TransDecoder (Broad Institute, Cambridge, MA, USA) was used to identify candidate coding regions within assembled transcripts, and transcripts with open reading frame lengths <300 (100 amino acids) were filtered out. The final transcriptome assembly was used as a reference for gene annotation and expression calculation.

Gene annotation

Transcripts/genes were mapped to NR database (nonredundant protein database from NCBI) using BLAST (v. 2.2.28, NCBI). Alignments with threshold *e*-values >1e-20 or identity <50% were discarded. The *e*-values determine significant matches to the database by describing the number of hits by chance. The top hit from *Drosophila* species was used to annotate the query genes (giving priority to *Drosophila melanogaster*, if present), and genes without *Drosophila* hits were discarded to further exclude possible contamination.

Gene expression

The clean reads were mapped to the reference assembly using Bowtie (Langmead *et al.*, 2009) (v. 1.0.0) with parameters set to '−l 25 -I 1 -X 1000 -a -m 200' (seed length 25, the minimum and maximum insert sizes for paired-end alignment of 1 and 1000, respectively, and report alignments <200). RSEM (Li and Dewey, 2011) was used to calculate the gene expression with default parameters. The differential expression of genes was calculated using the DEseq (Anders and Huber, 2010) package in R software (http://www.r-project.org/), with Benjamini–Hochberg adjusted *P*-values <0.05 considered to be significant.

Allele-specific expression

RNA sequencing reads were mapped to the reference transcriptome using BWA (v. 0.7.6; Li and Durbin, 2010) with default parameters. Genotypes were identified by UnifiedGenotyper from GATK package (McKenna *et al.*, 2010) with default parameters except setting heterozygosity to 0.01 and indel heterozygosity to 0.00125. Genotypes with genotyping quality <40 or mapping quality <30, or covered depth <10 were discarded. Only homozygous species-specific loci from parental *D. planitibia* and *D. silvestris* samples were chosen as ASE candidate sites. To reduce the bias of reference preference during the mapping process, a masked reference was generated by changing the reference to a third genotype different from the homozygous genotypes in *D. planitibia* and *D. silvestris* in all ASE candidate positions. Genotypes for other samples were generated as above, using the masked sequence as reference. Loci of heterozygous genotypes in F_1 and BC samples with a minimal genotyping score of 40, mapping score of 30 and coverage depth of 10 were tested with a binomial test, and candidates with Benjamini–Hochberg adjusted *P*-values of <0.05 were kept. Simulated reads around the candidate ASE sites were generated with half of the reads carrying the *D. silvestris* genotype and the other half carrying the *D. planitibia* genotype. Simulated reads that mapped against the masked reference and the simulated genotypes generated with binomial test *P*-value of <0.05 were excluded from the ASE candidate pool as false positives. As the analysis was confined to heterozygous loci, we were unable to detect extreme cases of ASE in which only one allele was expressed. This bias against extreme ASE leads to an underestimate of the total ASE levels but at the same time makes our analysis more conservative.

Using a general framework of *cis*/*trans* divergence with respect to allelic ratios (Wittkopp *et al.*, 2004; Landry *et al.*, 2005), we formulated *cis*-score (S_{cis}) and *trans*-score (S_{trans}) as follows:

$$S_{cis} = 1 - \frac{1}{4}\cdot|(|p-f| + |p-b|)$$

$$S_{trans} = 1 - \left(\frac{1}{4}\cdot|f-b| + \frac{1}{4}\cdot(|f| + |b|)\right)$$

where *p*, *f*, and *b* are allelic ratios for parental, F_1 and BC samples, respectively. For each gene, $P=(E_{pla}-E_{sil})/(E_{pla}+E_{sil})$ was calculated as the ratio based on the gene expressions of *D. planitibia* (E_{pla}) and *D. silvestris* (E_{sil}); $f=(C_{pla}-C_{sil})/(C_{pla}+C_{sil})$ was calculated as a ratio based on the number of reads corresponding to the *D. planitibia* allele (C_{pla}) and the number of reads corresponding to the *D. silvestris* (C_{sil}) allele in F_1 samples; *b* was calculated in a similar way as *f* but in BC samples. The gene was called either *cis*- or *trans*-divergent in each BC group when one score was larger than the other. If the difference of the two scores was <0.05, the gene was classified as *cis/trans* synergy. This formalization is consistent with the idea that *cis* divergence pattern is characterized by equal parental (*p*) and hybrid (*f*) ratios, whereas *trans* divergence pattern is characterized by the *f* ratio (but not necessarily *p* ratio) approaching zero. We expanded the framework to include heterozygous BC hybrids, assuming that sequence divergence exerts the same ASE effects in F_1 and backcross hybrids, and hence *f* and *b* should be convergent. In addition to each phenotypic BC group classified separately, all BC individuals (combined BC) were analyzed as one group for a combined BC *cis/trans* divergence measure. A subset of 800 genes containing informative single-nucleotide polymorphism between *D. planitibia* and *D. silvestris* were used for this analysis.

Gene Ontology (GO) enrichment

All functional enrichment analyses were carried out on the DAVID (Database for Annotation, Visualization and Integrated Discovery) site (v. 6.7) (http://

david.abcc.ncifcrf.gov/). The list of DE genes was uploaded to the DAVID website that cross-referenced the gene IDs among many databases to provide gene ontology annotations as well as categorizing the genes into broader functional groups.

Interaction network

Interaction network for *cis*- and *trans*-divergent genes was generated using GeneMania (Mostafavi *et al*., 2008), whereas DroID (Yu *et al*., 2008) data were incorporated as an additional data source.

RESULTS

Testes and sperm morphologies

Parental species males ($N=42$ *D. silvestris*, $N=44$ *D. planitibia*) consistently exhibited fully formed testes with dense, coiled sperm that was motile after the testis squash (Figures 2a and b). F_1 hybrid males ($N=23$) were sterile, containing no sperm inside an intact testis (Figure 2c). Full-sib BC males showed a range of sperm morphologies (Figures 2d–f) consistent with the results reported in Craddock (1974). BC-MS males ($N=19$) exhibited a large number ($>80\%$) of highly motile sperm inside an intact testis (Figure 2d); BC-NM males ($N=15$) exhibited nonmotile sperm in intact testes (Figure 2e); and BC-NS males ($N=15$) exhibited a completely empty testis where no sperm was present similar to F_1 hybrid males (Figure 2f). Of the 16 BC individuals dissected in this study, the distribution of the three sperm phenotype categories were as follows: 7 were BC-MS (motile sperm), 4 were BC-NM (nonmotile sperm present) and 5 were BC-NS (completely sterile, absence of sperm).

Transcriptome assembly and annotation

After quality control for raw sequencing reads, an average of 2.84 Gb clean data per sample was generated. Clean reads from all samples were used in the *de novo* transcriptome assembly. Trinity output yielded 102 981 genes (170 887 transcripts) with an average contig/transcript length of 554 base pairs (bp). To assure enrichment of mRNAs, contigs were post-processed by filtering out open reading frames <300 bp. A remaining total of 24 320 genes (55 038 transcripts) with average length of 991 bp and total length of 54.5 Mb were used for mapping. Out of the 24 320 genes, 21 307 (87.6%) mapped to the NR database under our criteria, with 16 761 (68.9%) of them with hits to *Drosophila*. Gene IDs were assigned according to the hits, with the majority coming from the well-annotated *D. melanogaster* (13 655) and *D. grimshawi* (2933), the only Hawaiian *Drosophila* species sequenced to date.

Analysis of differential gene expression and candidate genes

Gene expression was compared among BC groups. There were 65 genes significantly DE between BC-MS and BC-NS and 33 between BC-NM and BC-NS, but no significant differential gene expression was found between BC-MS and BC-NM (Figure 3, Supplementary Figure 1 and Supplementary Table 1). Interestingly, all significantly DE genes found between BC-MS and BC-NS, as well as BC-NM and BC-NS, were downregulated in BC-NS. For comparison, 41 genes showed higher expression in BC-MS and 30 genes had higher expression in BC-NM for the comparison between BC-MS and BC-NM, although differential expression was not statistically significant for any of these genes (Supplementary Table 1 and Supplementary Figure 1).

Among the 33 DE genes between BC-NM and BC-NS, 27 were shared in DE genes between BC-MS and BC-NS groups. The most significant shared DE gene was *CG31467*, known to be expressed at moderate levels in *D. melanogaster* adult testis according to FlyAtlas (http://flyatlas.org/). No significant GO enrichment was found for DE genes, presumably because of the relatively low level of functional annotation. Cellular components, biological processes and molecular functions for each gene hit were recorded if the data were available in Flybase (Table 1). Nevertheless, we have analyzed GO term enrichments for the top 5% misexpressed genes in the three comparisons between BC groups (Supplementary Table 2). The analysis showed high similarities between the comparisons, especially between BC-MS/BC-NS and BC-NM/BC-NS, in which GO terms related to microtubule cytoskeleton were among the most overrepresented.

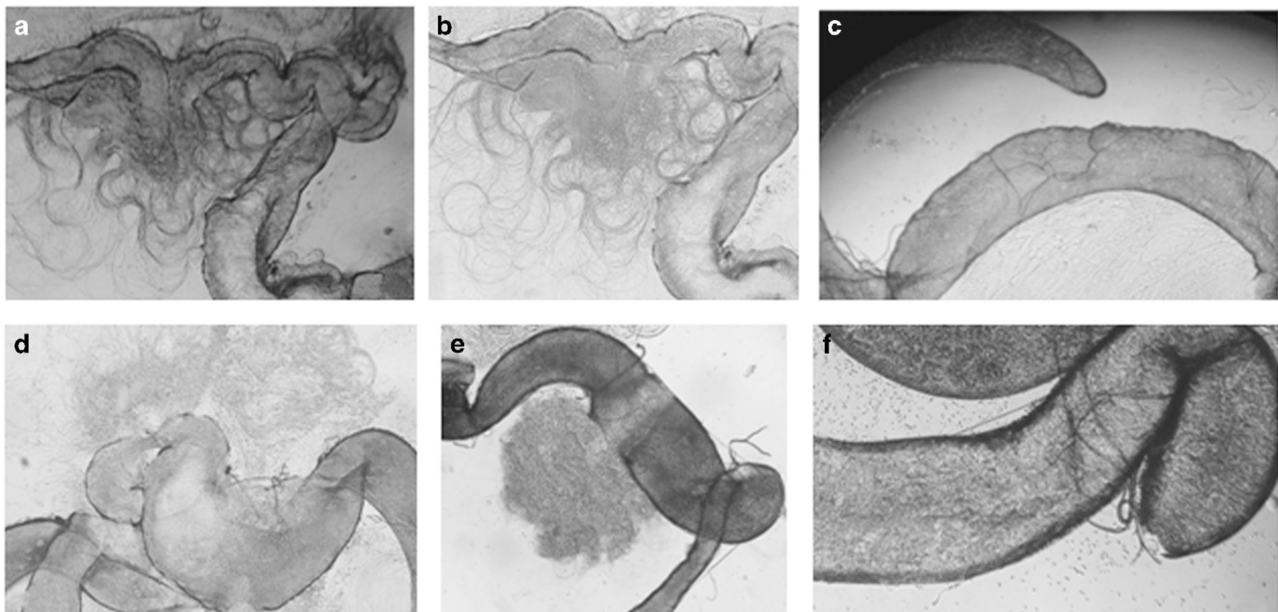

Figure 2 Photographs of representative testis phenotypes post testis squash exhibited by (**a**) *D. silvestris*, (**b**) *D. planitibia*, (**c**) F_1 hybrid, (**d**) BC-MS (motile sperm), (**e**) BC-NM (sperm present but non-motile) and (**f**) BC-NS (no sperm present). All dissections and photographs were taken under a light compound microscope at either $\times 10$ or $\times 40$ magnification.

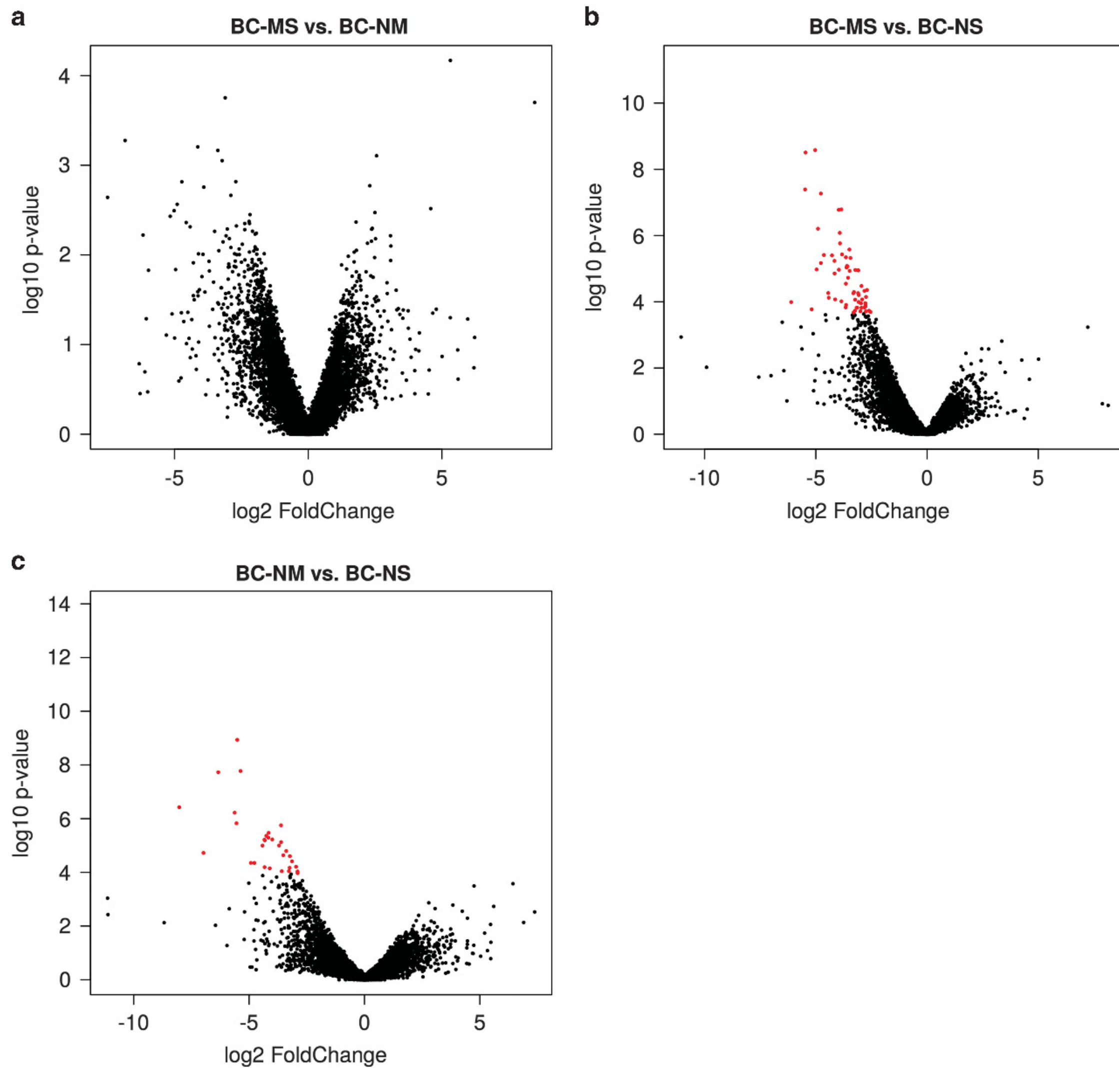

Figure 3 Volcano plot for differential expression analysis among the testes of backcross male groups. The x-axis is the log2 fold change of the differences in gene expression (**a**): BC-MS > BC-NM, (**b**) BC-MS > BC-NS and (**c**) BC-NM > BC-NS. The y-axis is the negative of the log10 false discovery rate (FDR) values of the comparison between the two BC male groups, with red dots indicating significant DE genes.

Of the 65 DE genes between BC-MS and BC-NS, 4 genes have known spermatogenesis-related function(s) in *D. melanogaster* (Table 1). Of the 33 DE genes between BC-NM and BC-NS, at least three genes have spermatogenesis-related function: *kl-5*, *pendulin* and *CG15161* (Table 1). Other important functions represented by the remaining DE genes include microtubule activities (*LP11180p*, *kl-2*, *Dgri\GH10382*, *gb:AAN71272.1*), phosphatase binding (*CG31467*, *Dgri\GH13925*), oxidation-reduction processes (*Dgri\GH19779*, *Dgri\GH22053*, *Dgri\GH17535*) and hydrolase activities (*Dgri\GH11455*, *PpV*). Of the 71 DEs found among BC groups, all of them also showed differential expression between parental species and BC-NS, and 42 of them showed differential expression between parental species and both BC-MS and BC-NM.

ASE analysis

There were more *cis*-divergent than *trans*-divergent genes with no significant difference in the ratio of *cis*- and *trans*-regulated genes among three BC male groups ($\chi^2=1.29$, d.f.$=2$, $P=0.52$, Table 2). BC-MS had 335 *cis*-regulated, 97 *trans*-regulated and 169 synergistic genes; BC-NM had 283 *cis*-regulated, 67 *trans*-regulated and 125 synergistic genes; BC-NS had 333 *cis*-regulated, 90 *trans*-regulated and 143 synergistic genes; and the combined BC groups had 333 *cis*-regulated, 108 *trans*-regulated and 180 synergistic genes (Table 2). Out of the 71 significantly misexpressed genes (Table 1 and Supplementary Table 1), only four had polymorphism (species-specific single-nucleotide polymorphisms in transcribed sequences and heterozygosity in backcrosses) permitting computations of *cis/trans* scores and *cis/trans* divergence classification. Two out of the four genes were *cis/trans* synergistic (*Octbeta3R* and CG5196) and two were *cis*/regulated (Dgri\GH10450 and Dgri\GH13801). To test for a possible contribution of compensatory *cis/trans* evolution (manifested as misregulation of *cis/trans* synergistic genes in hybrids; Landry *et al.*, 2005) to backcross hybrid sterility, we estimated Spearman's rank correlations between *cis/trans* score differences (being low for *cis/trans* synergy) and expression fold changes between the BC groups. The correlation was positive but not significant ($r=0.04$, $P=0.34$), inconsistent with the scenario that compensatory *cis/trans* evolution significantly contributed to differences between the BC groups. In addition, an interactome network of *cis*- and *trans*-divergent genes was generated (Supplementary Figure 2).

Table 1 Differentially expressed genes among the testes of BC-MS, BC-NM and BC-NS and their known biological processes and molecular functions

Gene ID[a]	*F/S*[b]	*NM/S*[b]	*Biological processes*	*Molecular function*
[1,2]*CG31467*	S	S	Negative regulation of phosphatase activity	Phosphatase binding
[1,2]*kl-5*	S	NS	Microtubule-based movement	ATPase activity, coupled; motor activity
[2]*pendulin*	S	NS	Centrosome duplication; lymph gland development; sperm individualization	Protein transmembrane transporter activity
[1,2]*Octbeta3R*	S	NS	G protein-coupled receptor signaling pathway	G protein-coupled amine receptor activity
[1,2]*LP11180p*	S	NS	Microtubule-based movement	Motor activity; ATPase activity, coupled
[2]*Dgri\GH10450*	S	NS	Metabolic processes; transmembrane transport	Catalytic activity
[1,2]*pen*	S	S	Apposition of dorsal and ventral imaginal disc-derived wing surfaces	RNA binding
[2]*Dgri\GH13925*	S	S	Negative regulation of phosphatase activity	Phosphatase binding
[1,2]*Dgri\GH19779*	S	S	Oxidation-reduction process	Oxidoreductase activity
[1,2]*Dgri\GH20554*	S	S	rRNA transcription	Endoribunuclease activity
[1,2]*Dgri\GH11455*	S	S	Metabolic process	Hydrolase activity
[2]*CG5196*	S	S	Golgi organization; protein palmitoylation	Protein-cysteine S-palmitoyltransferase activity; zinc ion binding
[1,2]*CG5718*	S	S	Electron transport chain; tricarboxylic acid cycle	Succinate dehydrogenase (ubiquinone) activity
[1,2]*lobo (lost boy)*	S	NS	Sperm motility; sperm storage	NA
[1,2]*Dgri\GH10382*	S	NS	Microtubule nucleation	NA
[2]*PpV*	S	S	Mitotic cell cycle; protein dephosphorylation	Hydrolase activity
[2]*Prosbeta7*	S	S	Cell proliferation; cellular response to DNA Damage stimulus; mitotic spindle	Endopeptidase activity
[2]*Dgri\GH11042*	S	NS	Protein phosphorylation	ATP binding; protein kinase activity
[2]*CG15161*	S	S	Cilium assembly; intraciliary transport	NA
[1,2]*kl-2*	S	NS	Microtubule-based movement	ATP binding; ATPase activity, coupled; motor activity
[2]*ZnT33D*	S	S	Cellular zinc ion homeostasis	Zinc ion transmembrane transporter activity
[2]*Dgri\GH23359*	S	S	Protein glycosylation	Fucosyltransferase activity
[1,2]*CG9173*	S	NS	Regulation of cell cycle	NA
[1,2]*Dgri\GH22053*	S	NS	NA	Cytochrome-*c* oxidase activity
[1,2]*Dgri\GH10111*	S	NS	Lipid metabolic process	Phosphoric diester hydrolase activity; starch binding
[2]*Dgri\GH15386*	S	NS	NA	Lysozyme activity
[2]*Tpc2*	S	NS	Transmembrane transport	Transmembrane transporter activity
[1,2]*Dgri\GH17535*	S	NS	Oxidoreductase activity	Oxidation-reduction process
[2]*Vha16-1*	S	NS	dsRNA transport; imagine disc-derived wing morphogenesis	Hydrogen ion transmembrane transporter activity
[2]*Dgri\GH25085*	S	NS	Metabolic process	Catalytic activity
[2]*brv3*	NS	S	Calcium ion transport	Calcium channel activity; calcium ion binding
[2]*Dgri\GH16238*	NS	S	Protein glycosylation	Fucosyltransferase activity
[2]*gb:AAN71272.1*	S	S	Microtubule-based movement	Motor activity; ATPase activity, coupled
[1,2]*unc80*	NS	S	Locomotor rhythm	Cation channel activity

Abbreviations: BC, backcrosses; BC-MS; backcrosses with motile sperm; BC-NM, backcrosses with sperm present but nonmotile; BC-NS, backcrosses with no sperm present; dsRNA; double-stranded RNA; NA, not available; NS, nonsignificant differential expression; S, significant differential expression.
Only genes with known functions are included in this table.
[a]Superscript 1 indicates differential expression between parental species and both BC-MS and BC-NM; superscript 2 indicates differential expression between parental species and BC-NS.
[b]F/S and NM/S indicate comparison between BC-MS and BC-NS, and between BC-NM and BC-NS, respectively.

Table 2 Total number of *cis*-divergent, *trans*-divergent and *cis/trans* synergistic genes expressed in the testes of each BC group and all BC groups combined

BC group	*cis*	*trans*	*cis/trans*
BC-MS	335	97	169
BC-NM	283	67	125
BC-NS	333	90	143
All BC	333	108	180

Abbreviations: BC, backcrosses; BC-MS; backcrosses with motile sperm; BC-NM, backcrosses with sperm present but nonmotile; BC-NS, backcrosses with no sperm present.

DISCUSSION

Sperm morphologies

Our analysis of sperm production and motility in this study is consistent with the study of Craddock (1974) showing similar proportions of the three fertility phenotypic groups in the BC males. The results show that the underlying physiological processes in the BC males of these two species results in three distinct phenotypic classes with one group lacking sperm in the testis (BC-NS), a second group with nonmotile sperm (BC-NM) and a third group with testis filled with motile sperm (BC-MS). The F_1 hybrid males are similar to the sterile BC males (BC-NS) and both parental species are similar to the fertile BC males (BC-MS). These results also suggest that HMS may be caused by two processes with one disrupting sperm production and the other disrupting sperm motility.

Differential gene expression

Our RNA sequencing analyses on BC adult male testes also showed highly significant differential gene expression between two of the three phenotypic groups of BC males. There were 65 DE genes observed between BC-MS and BC-NS and 33 DE genes between BC-NM and

BC-NS. Three potential candidate genes of interest are identified as *D. melanogaster* genes *lost boys, male fertility factor kl-2* and *male fertility factor kl-5*. The *lost boys* is a gene involved in ciliar motility and encodes a conserved flagellar protein CG34110 that is localized along fly sperm flagella and is highly expressed in ciliated respiratory epithelia and sperm (Yang *et al*., 2011). Phenotypic analysis in *D. melanogaster* showed that *lost boys* specifically affected sperm movement into the female storage receptacle (Yang *et al*., 2011). Therefore, it is a gene that is involved directly in sperm motility and sperm storage.

Male fertility factor kl-2 and *male fertility factor kl-5* are two genes that reside in the long arm of the Y chromosome in *D. melanogaster*, and are two of seven fertility factors identified in *D. melanogaster* (Carvalho *et al*., 2000). Deletion studies showed that the lack of *kl-5* results in the loss of the outer arm of the sperm tail axoneme; sperm in males lacking *kl-2* and *kl-5* were missing important heavy chain proteins, and therefore the individuals produced immotile sperm (Carvalho *et al*., 2000). *kl-5* is known to code for an axonemal β-dynein heavy chain expressed in the testis; these heavy chains are known to be responsible for the motility of flagella and cilia (Carvalho *et al*., 2000). We note that all of the BC males in this study have the same *D. silvestris* Y chromosome. If *kl-2* and *kl-5* genes are on the Y chromosome of *D. silvestris* and *D. planitibia*, it would suggest that in order for there to be DE between the backcross males, given that all have the same Y chromosome, *kl-2* and *kl-5* genes would have to interact with genes or regulatory factors located elsewhere in the genome on either an autosome or X chromosome. The construction of chromosomal maps for these species would be valuable in determining whether these genes are located on the Y chromosome.

Other functionally annotated genes of interest in this set include *CG31467* and *Octbeta3R*. *CG31467* was the top DE gene and plays a role in phosphatase binding and is expressed moderately in the adult testis of *D. melanogaster* (FlyBase). Protein phosphatases have been known to modulate sperm motility in mammals (Fardilha *et al*., 2011), and a gene encoding acylphosphatase (*Acyp*) has been found to be associated with HMS in F_1 and backcross hybrids between *D. simulans* and *D. mauritiana* (Michalak and Ma, 2008; Michalak and Noor, 2004). *Octbeta3R* is homologous to β-adrenergic receptors in vertebrates that play multifunctional roles in insects, and is an octopamine (Farooqui, 2012). In *D. melanogaster*, octopamines have been detected in pathways relating to different behaviors such as olfactory learning and memory, aggression, locomotion and grooming and conditional courtship (Farooqui, 2012). *Octbeta3R* specifically has been known to partially restore ovulation and fecundity in sterile females.

DE genes between BC-MS and BC-NS and between BC-NM and BC-NS were all downregulated in the sterile phenotype that lacked sperm (BC-NS) compared with the fertile phenotype (BC-MS) and the nonmotile sperm phenotype (BC-NM). This result suggests that the sterile phenotype does not express testis function genes compared with the purported fertile phenotype, as expected. It should be noted that many of the downregulated genes in BC-NS do not necessarily need to be responsible for, or even associated with, HMS. Indeed, many of these genes are also downregulated in BC-MS and BC-NM relative to the parental species, suggesting that this misexpression may be a more general feature of backcross hybrids regardless of their fertility phenotype. On the other hand, it would also be premature to claim that such genes cannot be related to fertility for this reason, as we cannot rule out a possibility that even fertile backcross males are subfertile relative to parental species. Another complication in this and other studies of gene expression in HMS is due to tissue structural alterations, including gonadal atrophies and sperm deficiencies, leading to spurious expression changes among genes with tissue-specific activity, difficult (if possible at all) to distinguish from true gene silencing. As BC-NS males lack sperm, genes with sperm-specific expression will necessarily be underrepresented in the analysis. To minimize the effect of sperm absence on the expression profile, we excluded genes with zero-level expression.

Interestingly, we did not find significant differential expression between BC-MS and BC-NM. Both BC-MS and BC-NM contain sperm, but BC-MS contains many motile sperm, and BC-NM contains less dense, nonmotile sperm. Although such sperm motility-related genes as *lost boys*, *male fertility factor kl-2* and *male fertility factor kl-5* were significantly underexpressed in BC-NS, their expression alterations were subtler in BC-NM. Indeed, there were a number of genes that exhibited differential expression between the BC-MS and BC-NM phenotypic groups that did not reach statistical significance with the sample sizes in this study (Figure 3). For both BC-MS/BC-NS and BC-NM/BC-NS, the top DE gene was *CG31467* that is moderately expressed in the testis. Alternatively, BC-NM phenotype could be conveyed through regulatory changes at post-transcriptional, translational or post-translational levels, including protein–protein interactions. Lastly, we did not capture interfamily variation, as all BC males used in this study were offspring from a single F_1 hybrid female/*D. silvestris* parental male pair, and this has its advantages (genetically related brothers with distinct phenotypes) and also disadvantages (difficulty with extrapolations to the population levels).

Allele-specific expression

The interaction of *cis*- and *trans*-regulatory factors during transcription can affect gene expression (reviewed in Bell *et al*., 2013), and both factors are subject to mutational changes that may result in transcriptional alterations. Sensitivity of gene expression to mutations increases with both increasing trans-mutational target size and the presence of a TATA box (Landry *et al*., 2007b). These regulatory networks are primarily composed of regulatory and structural genes (Wittkopp *et al*., 2004). We found overwhelmingly more *cis*-divergent genes than *trans*-divergent genes in all BC groups. This result is consistent with those from other *Drosophila*, including closely related *Drosophila p. pseudoobscura* and *D. p. bogotana* (Gomes and Civetta, 2015), as well as more distantly related *D. melanogaster* and *D. simulans* (Wittkopp *et al*., 2004, 2008). A review of regulatory experiments by Bell *et al*. (2013) concludes that generally *cis*-regulatory changes account for more divergent expression between more genetically divergent parents (for example, interspecific) than *trans*-effects that account for a higher proportion of variation in gene expression between less divergent parents (for example, intraspecific).

Wittkopp *et al*. (2004) models of regulatory divergence suggest structural genes tend to be more *cis*-regulatory than *trans* regulatory because of their proximity to terminal nodes of the network where expression of genes is not regulated within the network. In contrast, some of the *cis* genes of interest had regulatory functions. However, we did not observe an excess of DNA-binding gene activities (or other significant GO term enrichments) among *trans*-divergent genes (Supplementary Tables 3). The most abundant groups of genes among *cis*-divergent genes were functionally related to cytoskeleton (12 genes) and reproductive cellular processes (7 genes). Unlike Landry *et al*. (2005), we did not observe increased misexpression of *cis* × *trans* synergistic genes that is likely related to the fact that comparisons between backcross hybrids display less extensive misexpressions than comparisons between F_1 hybrids and parental species (Michalak and Noor, 2004), whereas *cis* × *trans* synergy does not disproportionally contribute to backcross sterility.

Evolutionary implications of regulatory divergence

The combination of *cis*- and *trans*-regulatory elements in these BC groups allows further insight into the gene expression within and between groups, and the potential genetic architecture contributing to their divergence. Gene regulation has been shown to be heritable (Pavey *et al*., 2010; Yang *et al*., 2014) and an important part of divergence among species and variation within populations, but *cis*- and *trans*-acting elements differ in their evolutionary influence (Meiklejohn *et al*., 2014). For example, if *cis* elements are stronger than *trans* elements, then changes in a species pair may have evolved one gene at a time, instead of through a broad sweep as would be inferred by a *trans*-dominant regulation that affects many genes (Wittkopp *et al*., 2004). Understanding regulatory networks can lead to further insight into the potential associations among gene expression, adaptive genetic divergence and reproductive isolation, as this link is not conclusive in many experiments and needs further testing (Pavey *et al*., 2010). The combination of molecular techniques and fitness assays will provide a more robust analysis of the relationship between isolation and divergence relative to gene expression (Pavey *et al*., 2010). These studies could also be used as a proxy for comparing and contrasting the ecological divergence of other pairs of closely related Hawaiian *Drosophila* species, such as the sympatric, strongly reproductively isolated *D. heteroneura* and *D. silvestris*. Potential differences in *cis*- and *trans*-acting factors may be important in providing a more robust analysis and unraveling the evolutionary histories among species.

Overall, the HMS between the closely related *D. silvestris* and *D. planitibia* species highlights the relatively rapid divergence of gene regulation for testis development and sperm production in Hawaiian *Drosophila*. The physiological and developmental processes that are involved in testis formation and sperm production appear to have diverged to such an extent that the two systems have become incompatible within ~ 0.7 million years. The evolution of these species on separate islands also suggests that selection operating separately on male gamete production within each species is an important contributor to divergence between these two species. Furthermore, the potential for interactions between genes on the Y chromosome with genes or genetic factors located either on the X chromosome or autosomes is consistent with the proposition that this sterility occurs, at least in part, through epistatic interactions of alleles from the two species, which leads to incompatible systems (Johnson, 2000). As gonad formation begins in the larval stage and is maintained into the adult stage (Williamson and Lehmann, 1996), it will be important to examine gene expression patterns throughout the developmental process to determine where the compatibility of the genomes breaks down in the formation of testes and the production of sperm. Further research is also necessary to examine the potential for a smaller number of genes to create the initial incompatible developmental pathways that then lead to other changes in developmental and gene expression patterns to create hybrid male sterility (Coyne and Orr, 2004; Johnson, 2000). The comparison of gamete formation between more species in a phylogenetic context could shed light into how gene expression patterns and the gamete physiological and developmental systems evolve over time within and between species (Porter and Johnson, 2002; Johnson and Porter, 2007).

DATA ARCHIVING

Data were deposited at NCBI SRA under accession number SRP068366.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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