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Diversification of *takeout*, a male-biased gene family in *Drosophila*

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ABSTRACT

The display of courtship behavior has evolved in response to sexual selection driven by competition to obtain mates. Sexually dimorphic mate selection rituals are likely controlled at least in part by genes with sex-biased patterns of expression. In *Drosophila melanogaster,* male courtship behavior has been well described and consists of a series of stereotyped behaviors. The *takeout* gene is predominantly expressed in males and affects male courtship behavior. In this study, we examine the patterns of expression and evolution in *takeout* and the family of related proteins. We show that a number of genes in the *takeout* gene family show male-biased expression in *D. melanogaster*, largely in non-reproductive tissues. Phylogenetic analysis reveals that this gene family is conserved across insects. As expected for genes with male-biased expression, we also find evidence of positive selection in some lineages. Our results suggest that the genes in this family may have evolutionarily conserved sex specific roles in male mating behavior across insects.

1. Introduction

Many animal species have evolved elaborate courtship displays in response to sexual selection driven by competition to gain access to mates (Williams and Carroll, 2009). Sexually dimorphic courtship rituals are generally controlled by genes that have sex-biased expression. Courtship rituals in *Drosophila melanogaster* consist of a series of stereotyped behaviors displayed by the male in order to gain access to and mate with females (Hall, 1994; Greenspan and Ferveur, 2000). This male-specific behavior is regulated by the general sex determination pathway that controls sex-specific expression of the two master regulators *doublesex* (*dsx*) and *fruitless* (*fru*) (McRobert and Tompkins, 1985; Taylor et al., 1994; Billeter et al., 2006; Yamamoto, 2007). Very little is known about their downstream target genes that control mating behavior, but one of them, *takeout*, is male-specifically expressed and mutations in *takeout* result in reduced male courtship behavior (Dauwalder et al., 2002).

takeout was isolated in screens for circadian-regulated genes and in a screen for male-specifically expressed genes in *D. melanogaster* (Sarov-Blat et al., 2000; Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Dauwalder et al., 2002; Lin et al., 2002). Experimental evidence has demonstrated that *takeout* expression depends on the male-specific isoforms of both Doublesex and Fruitless (Dauwalder et al., 2002). Male-specific expression of *takeout* in *D. melanogaster* has been observed in the head fat body, a secretory tissue that surrounds the brain. In addition, *takeout* is expressed in the antennae in both sexes (Dauwalder et al., 2002). The *takeout* protein has been shown to be secreted and function as a secreted protein (Lazareva et al., 2007). In addition to its role in courtship, the gene has a function in the larval response to starvation (Sarov-Blat et al., 2000). *takeout* proteins have characteristics of small soluble proteins and are most similar to juvenile hormone binding proteins (Sarov-Blat et al., 2000; So et al., 2000; Dauwalder et al., 2002).

21 homologs of *takeout* have been identified in *D. melanogaster* (Sarov-Blat et al., 2000; So et al., 2000; Dauwalder et al., 2002). Their specific functions are unknown, however several exhibit circadian regulated expression, and all encode proteins with predicted signal sequences indicative of secreted proteins. In addition to *takeout*, we have previously shown that two additional homologs also exhibit male-biased expression (Dauwalder et al., 2002). *takeout* homologs have since been identified in several other insect species in a variety of tissues, including olfactory organs (Du et al., 2003; Fujikawa et al., 2006; Ghanim et al., 2006; Saito et al., 2006; Hagai et al., 2007; Jordan et al., 2008; Hamiaux et al., 2009; Schwinghammer et al., 2010). Together, current data suggest that *takeout* is part of a large gene family found throughout insects with potential roles in metabolism, circadian behavior, aging, and male courtship behavior.

A large fraction of the *D. melanogaster* genome displays sex-biased gene expression (Jin et al., 2001; Arbeitman et al., 2002; Ranz et al., 2003; Chang et al., 2011). Most of these sex-biased genes are expressed in reproductive organs (Parisi et al., 2003). Evidence is mounting linking sex-biased expression to accelerated rates of evolution in a wide array of genes, with genes having male-biased expression evolving the most rapidly (Ellegren and Parsch, 2007; Haerty et al., 2007). Here, we examine the evolution of a gene family with many genes having male-biased expression largely in non-reproductive tissues. First, we show that additional *D. melanogaster*

Abbreviations: BLAST, Basic Local Alignment Search Tool; *dsx*, *doublesex*; *fru*, *fruitless*; *to*, *takeout*; ω, ratio of nonsynonymous to synonymous substitution rates.

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takeout family members are preferentially expressed in male heads. Then we investigate the evolutionary history of the *takeout* gene family across insects, with particular emphasis on *Drosophila*. We find that this gene family is broadly conserved across insects, but with many instances of gene duplication and loss and evidence of positive selection in some lineages, consistent with the action of sexual selection on male-specifically expressed genes. These results will help guide future research into the functions of *takeout* family genes, and further elucidate the genetic determinants and evolutionary history of courtship behavior in *Drosophila*.

2. Materials and methods

2.1. Northern blot analysis of gene expression

PolyA$^+$ RNA from isolated heads and bodies of males and females was prepared as described by Chandler et al.(2001). Northern blots were performed as described by Mattox and Baker(1991). 10 μg of polyA$^+$ RNA was loaded per lane. Gene specific probes were amplified from sequences in the predicted 3′ untranslated region of each gene to avoid potential cross-hybridization among family members. The sequence of primers used to generate the probes was: CG14259 s-1: 5′ CACTCATCGTTGAGACGGTGGA, CG14259 as-2: 5′GGGGATGGCGTACTACAAGTT, CG13618 s-1: 5′ACGAACTAAAGGTGGGATATGC, CG13618 as-2: 5′CTATGTAGTAACATAAATTAAGCA, CG7079 s-1: 5′ACGTTTAAGTCATCAGTACTG, CG7079 as-2: 5′CTGGATTATGTGGCTAGACAA, CG10407 s-1: 5′TGGGCTTCACTAGATATTTTACA, CG10407 as-2: 5′ ACATCACCATGCGAACCGAGC. Probes were generated by PCR labeling (Mertz and Rashtchian, 1994).

2.2. Identification of takeout homologs

We searched for homologs of the *Drosophila melanogaster takeout* gene in the 12 sequenced species of *Drosophila* (Clark et al., 2007) and 9 additional insect species with fully sequenced genomes: *Culex pipiens*, *Aedes aegypti*, *Anopheles gambiae*, *Bombyx mori*, *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, *Acyrthosiphon pisum*, and *Pediculus humanus corporis*. Annotated or predicted protein databases (Supplementary file 1) were searched using BLAST. 22 *Drosophila melanogaster takeout* family protein sequences had been previously identified (Dauwalder et al., 2002; this study identified 21 homologs, but one, CG7096, was identified as a tandem duplication and was split into two genes in release 3 of the genome annotation (http://flybase.org/reports/FBrf0148886.html)). These *D. melanogaster* sequences were used as the initial queries in blastP searches, with an *E*-value cutoff of 1. For each species, iterative BLAST searches were performed using the proteins recovered from that species as the search queries. Proteins were excluded if less than 1/3 of the iterated searches recovered that protein. Similar searches of *C. elegans* and mammals were performed in GenBank, but no homologs were identified outside of insects.

2.3. Phylogenetic and evolutionary analysis of the takeout gene family

Protein sequences were aligned using ClustalW (Larkin et al., 2007) (implemented in Geneious (Drummond et al., 2009)), using the BLOSUM cost matrix. Sequences that were not unambiguously alignable were removed. We also removed sequences that were shorter than 109 amino acids, which we chose as a cut off to allow for inclusion of all *Drosophila* genes and the majority of other genes; genes shorter than this cut off appeared to be partial sequences or misannotated (see Supplementary file 2). Sequences identical at the nucleotide level were not included. Sequences from *P. humanis* were excluded because of ambiguity created in alignment due to the large genetic distance between these and other sequences (Supplementary file 2). The filtered alignment consisted of 408 genes from 20 species. A neighbor-joining phylogeny, with 100 bootstrap replicates, was constructed based on the filtered alignment using MEGA (Tamura et al., 2007).

Using the resultant phylogeny, we defined orthologous clusters as clades with >65% bootstrap support that contained a homolog from the majority of the *Drosophila* species. The *Drosophila* sequences in each of these clusters were further analyzed independently. For each cluster, nucleotide sequences were aligned by Translation Align (in Geneious) using ClustalW with a BLOSUM matrix, and adjusted by eye. Maximum likelihood genealogies were built from these alignments using PAUP* (Swofford et al., 2003) (as implemented in Geneious).

Within the melanogaster group of *Drosophila* species, we tested for evidence of positive selection in each cluster using codeml in PAML (Anisimova and Yang, 2007). First, we assumed a homogenous ratio of nonsynonymous to synonymous substitution rates (ω) across lineages within a cluster, and compared model M7 to M8. Model M7 fits codon variation with a beta distribution and M8 includes an additional parameter that allows for a class of codons with $\omega > 1$ (Yang et al., 2000). The two models were compared using likelihood ratio tests.

We then tested for lineage specific effects using a branch-site test of positive selection (Yang et al., 2005). For each cluster, every branch was designated in turn as the foreground branch, on which sites with $\omega > 1$ are allowed. This model was compared, by likelihood ratio test, to a model where positive selection is not allowed ($\omega = 1$). To make the test conservative, we used χ^2 as the null distribution (Zhang et al., 2005) and controlled for multiple tests within a cluster using Bonferroni's correction (Anisimova and Yang, 2007). One node was collapsed (*D. simulans* and *D. sechellia* with *D. melanogaster*) because genealogies of more than half of the clusters failed to consistently recover those relationships with sufficient support.

3. Results

3.1. Male-biased gene expression

We have previously shown that, in addition to *takeout*, two other family members (CG5867 and CG7096) are male-specifically expressed (Dauwalder et al., 2002). To identify additional *D. melanogaster* family members that might have sex-specific roles, we performed additional Northern blot analyses. In order to avoid potential cross-hybridization, we prepared probes from the predicted 3′ untranslated regions of the RNAs. Transcription in non-adult stages, or in a very few number of cells, would not have been detected using our protocol. Transcripts were observed for four additional genes (CG14259, CG13618, CG7079 and CG10407 (Fig. 1)). Transcript levels for these genes were very low, indicating that the genes are expressed at low levels or only in a small number of cells. This may also explain why no transcripts were observed on Northern blots for a number of the genes examined (as indicated in Table 1). All genes for which transcripts have been detected are preferentially expressed in males, suggesting a family of genes with largely male-biased expression. Transcripts vary in their relative abundance in heads versus bodies. One of the genes with strong expression in male bodies (CG10407) has previously been reported to be enriched in testes based on microarray studies (Parisi et al., 2004). In addition to the seven *takeout* family members that show male-biased expression by Northern blot analysis ((Dauwalder et al., 2002) and this study), expression data from microarray and RNA-seq data indicate that at least 14 of the 23 genes are preferentially expressed in males (Gnad and Parsch, 2006) (Table 1).

3.2. Takeout gene family in insects

Our genome database search and filtering algorithms identified 408 *takeout* homologs from 20 insect species, including a previously unidentified homolog in *D. melanogaster* (CG15497). Phylogenetic analysis revealed several well-supported clades containing at least

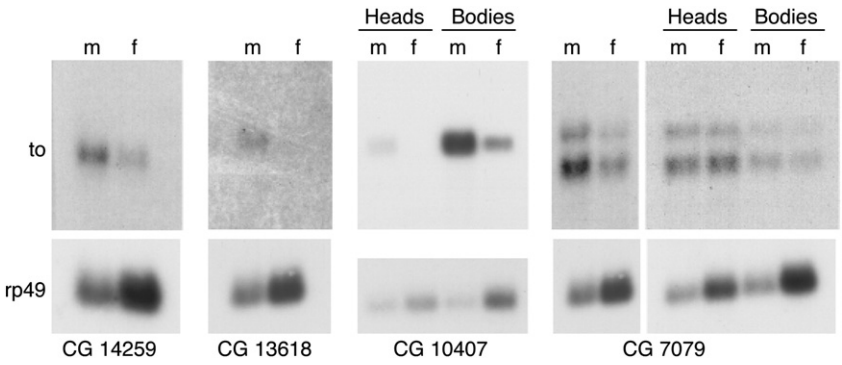

Fig. 1. Northern analysis of *takeout* family members. 10 μg of poly A^+ RNA prepared from either whole males (m) or females (f), or from isolated heads and bodies from male or female flies was loaded per lane as indicated. Gene specific probes were generated from sequences in the predicted 3' untranslated region of each gene to avoid potential cross-hybridization among family members. Hybridization of the same blot with rp49, a probe recognizing ribosomal protein 49 RNA, which is commonly used as a reference, is shown in the lower panel for quantitation. Note that most lanes containing female RNA contain substantially more RNA than male lanes.

one member of the previously defined *takeout* gene family in *D. melanogaster*. We defined a clade as a "cluster" when bootstrap support for that clade was >65% and it contained homologs from at least 9 of the 12 *Drosophila* species studied. Twenty such clusters were identified (Fig. 2). Only one *Drosophila* sequence did not fall into one of the clusters (Fig. 2, Supplementary file 2). For 7 clusters, there was sufficient phylogenetic support (>65% bootstrap support) to include genes from other insect species in addition to *Drosophila*. If we lower our bootstrap threshold to 50%, two additional clusters also contain genes from other insect genera (Fig. 2).

While we are unable to completely resolve deep relationships between clusters, we do recover support for several clades containing multiple clusters (Figs. 2, 3). Mapping the relationships between clusters onto their chromosomal locations in *D. melanogaster* reveals a close association between genomic location and evolutionary relationship between clusters (Fig. 3). This suggests that much of the expansion of the *takeout* gene family occurred via tandem duplication.

One clade, designated cluster 4, contains several paralogs from all of the *Drosophila* species, including four from *D. melanogaster*, but could not be adequately resolved into separate clusters based on a neighbor joining analysis of protein sequences. Thus, we did not include cluster 4 when assessing gene loss and gain (see below). However, by aligning and building phylogenies from the nucleotide sequences of genes in this cluster, we identified four clades that each contained a *D. melanogaster* homolog and genes from 6 to 10 other *Drosophila* species (with bootstrap support of >75% from maximum parsimony analysis and Bayesian posterior probability >0.90). These clades, designated as clusters 4.1–4.4, contain 37 out of the 46 total genes in cluster 4 (Supplementary file 4).

The remaining 19 clusters were also analyzed independently. Maximum likelihood phylogenies were constructed based on aligned nucleotide sequences from *Drosophila* spp. and gene duplications and losses within clusters were inferred using parsimony (Fig. 4). We infer multiple gene duplications and gene losses, 10 gains and 5 losses, distributed across the history of this gene family. Five of the duplications are tandemly arranged. Among the five duplicates that are not tandem, there is no evidence that any of these arose by transposition, i.e. introns remain intact and no poly-A tract is found. We confirmed the inferred losses by querying the genome via blastN and TblastX with the homolog of that cluster from the sister taxa; no genes were recovered that met our criteria for inclusion. This finding of multiple gene gains and losses is consistent with previous evidence demonstrating that genes with male-biased expression have high effective birth and extinction rates (Zhang et al., 2007).

Table 1
Sex-biased expression and test of selection in *Drosophila* clusters.

Cluster	*D. melanogaster* ortholog	M/F ratio[a]	Northerns[b]	ω[c]
1	CG15497	1.57[d]	NE	0.096
2	CG14259	1.87[d]	this study	0.073
3	*takeout*	1.62[d]	Dauwalder et al., 2002	0.073
4.1	CG31189	1.35[d]	NE	0.217
4.2	CG7079	1.85[d]	this study	0.105
4.3	CG31207	1.92[d]	Dauwalder et al., 2002	0.149
4.4	CG17279	1.07	ND	0.181
5	CG16820	3.75[d]	ND	0.184
6	CG10264	1.00	ND	0.061
7	CG14661	1.26	ND	0.056
8	CG2016	1.43	ND	0.049
9	CG1124	1.38[d]	ND	0.057
10	CG33680	n/a	NE	0.312[e]
11	CG10407	6.56[d]	this study	0.095
12	CG13618	3.42[d]	this study	0.047
13	CG11852	1.25	ND	0.048
14	CG11854	2.71[d]	ND	0.117
15	CG2650	1.02	NE	0.210
16	CG17189	1.43[d]	ND	0.113
17	CG14457	0.96	ND	0.095
18	CG14258	1.08	ND	0.147
19	CG5867	2.02[d]	Dauwalder et al., 2002	0.079
20	CG5945	2.62[d]	ND	0.132

[a] Ratio of male to female expression in *D. melanogaster* from SEBIDA meta-analysis (Gnad and Parsch, 2006).
[b] Northern blot expression data demonstrating evidence of male-biased expression in *D. melanogaster* from this or previous studies; ND no transcripts detected in Northern blot analysis; NE not examined.
[c] Ratio of synonymous to nonsynonymous rates of substitution (ω) in a cluster (using model M7 in PAML).
[d] Significantly male-biased expression ($p<0.05$) (Gnad and Parsch, 2006).
[e] Significant result of log-LRT comparing M7 to M8 ($p<0.05$).

3.3. Selection on takeout family members in Drosophila

All clusters were assessed for evidence of selection in the melanogaster group of species. First, we estimated the ratio of nonsynonymous to synonymous substitution rates (ω) with a single beta-distributed rate across a gene. ω<1 is evidence of purifying selection and ω>1 is evidence of positive selection. All clusters have ω substantially less than one (Table 1) suggesting broad purifying selection across this gene family.

We additionally tested for evidence of positive selection using codon-based models, first assuming a homogenous ω across lineages but allowing for a class of codons with ω>1, then by additionally allowing individual lineages to have ω>1. Assuming a homogenous ω across lineages, one cluster displays evidence of positive selection (Table 1). Allowing lineages to vary in ω, we identify 5 lineages, out

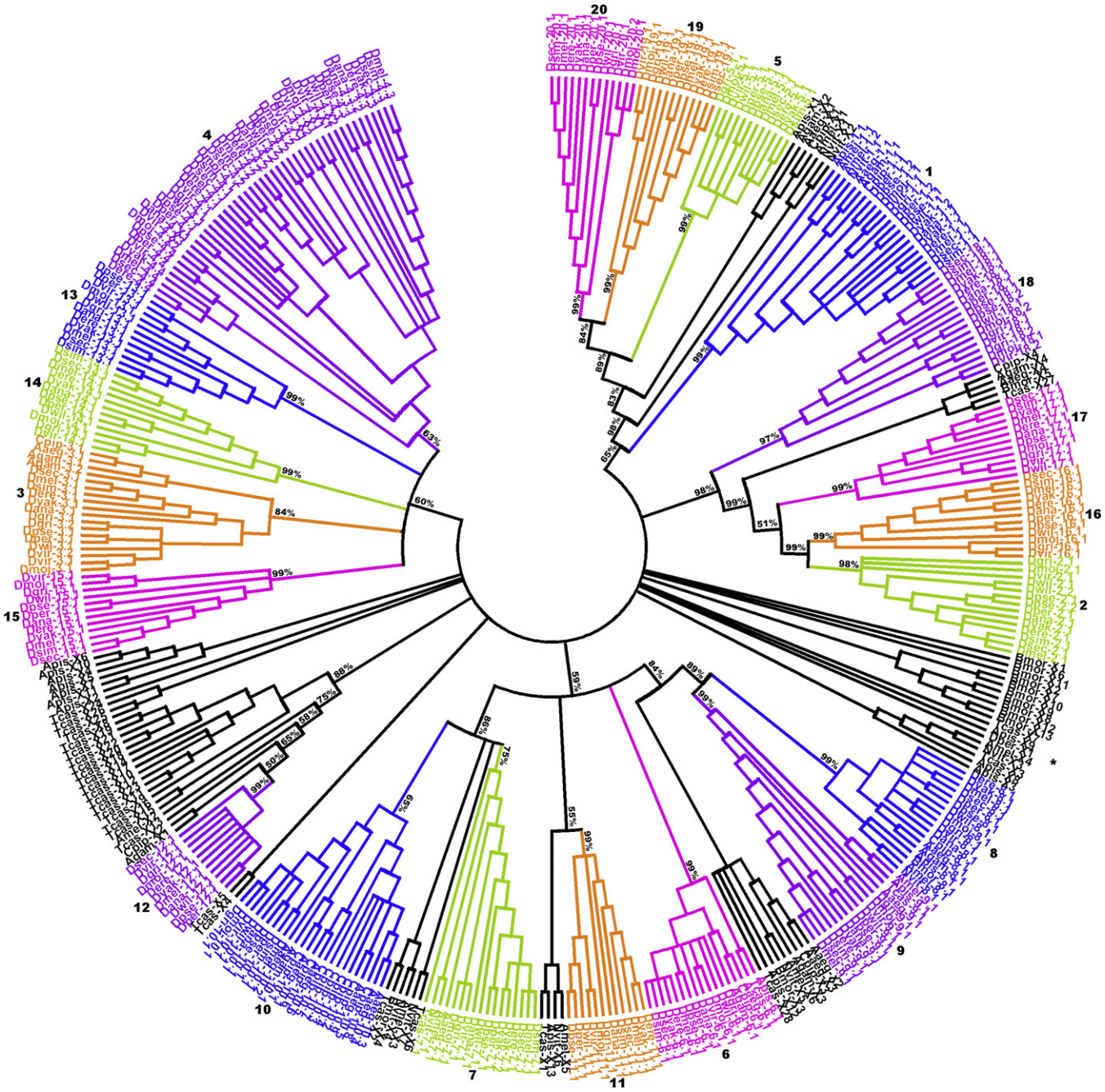

Fig. 2. Relationships between the 408 *takeout* family proteins in 20 insect species. The phylogeny shows the consensus of 100 bootstrap replicates using neighbor-joining analysis. Different colors illustrate each of the 20 *takeout* clusters (defined as described in Materials and methods); cluster numbers correspond to those in Table 1. Bootstrap support is shown at the nodes defining each cluster and relationships between clusters. Lineages in black are those that were not part of a cluster; most of these proteins were in species other than *Drosophila*. The *Drosophila* gene that was not part of a cluster is indicated by *.

of 9 examined, that have experienced positive selection in at least one cluster, with a total of 8 gene lineages that have experienced positive selection in this gene family over the evolutionary history of the melanogaster group of *Drosophila* (Fig. 4, Supplementary file 3).

4. Discussion

Previous studies based on large-scale expression data and comparative genomics have demonstrated a trend of rapid diversification in genes with male-biased expression (Ellegren and Parsch, 2007). One such study found evidence of positive selection in *takeout* (Haerty et al., 2007), which had previously been shown to be male-specifically expressed in the head of *D. melanogaster* (Dauwalder et al., 2002). A recent study suggests that sex-biased genes expressed in non-reproductive tissue evolve rapidly not due to sex-bias but rather due to a narrow expression profile (Meisel, 2011). Because *takeout* is part of a larger gene family whose members may share functions involved in courtship and other sex-specific behaviors, we examined this gene family more closely for patterns of expression and diversification.

In addition to *takeout*, we previously identified two *D. melanogaster takeout* family members that are preferentially expressed in

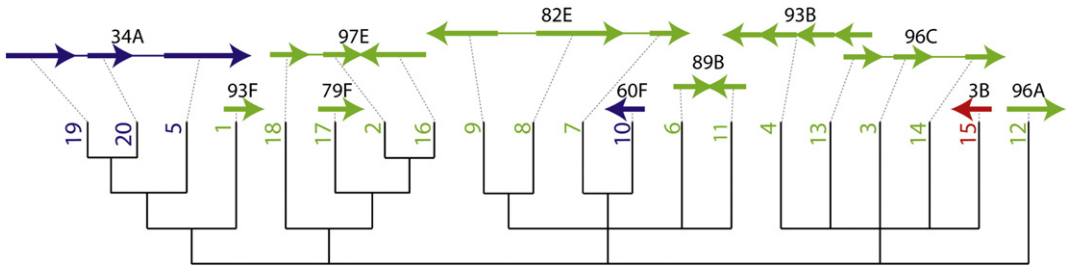

Fig. 3. Mapping of genomic location on phylogeny of clusters. Phylogenetic relationships between clades are derived from the phylogeny in Fig. 2 and numbers on branches correspond to the clusters as defined in Fig. 2. Chromosomal locations of the *D. melanogaster* homologs from each cluster are shown above the phylogeny. Arrows indicate gene size and orientation. Colors indicate the *D. melanogaster* chromosome where genes are located: red, chromosome 1; blue, chromosome 2; green, chromosome 3.

males (Dauwalder et al., 2002). We extended these studies and performed Northern analysis on the other genes. We detected rare transcripts in *D. melanogaster* for four additional genes. This is consistent with a narrow expression profile, however further work is needed to determine the localization and timing of expression of these genes. In addition, these genes show male-biased patterns of expression. This result, in combination with previous data (Table 1), suggests that this gene family has a historical pattern of male-biased expression, i.e. the ancestral expression state of the *takeout* family prior to gene duplication and diversification was likely male-biased. However, our expression data are only from one species, thus we present this conclusion with caution until *takeout* expression is studied in other species. At present, few *takeout* family members have been well described in other species (Du et al., 2003; Fujikawa et al., 2006; Ghanim et al., 2006; Saito et al., 2006; Hagai et al., 2007; Jordan et al., 2008; Hamiaux et al., 2009; Schwinghammer et al., 2010). Some *takeout* family members in other *Drosophila* species have been identified in isolated antennae, a tissue in which *D. melanogaster takeout* expression is not sex-biased (Dauwalder et al., 2002), it remains to be seen whether their expression in other tissues is sex-biased. The observation of extensive categorical changes in sex-bias class, especially for genes with male-biased expression (Ranz et al., 2003; Nuzhdin et al., 2004), suggests that it will be of interest to more closely examine the expression patterns of these genes in other species.

In *D. melanogaster*, *takeout* expression is determined by *dsx* and *fru*, downstream regulators in the sex determination pathway. *dsx* appears to play a role in sex determination across insects (Zarkower, 2001), with conservation in sequence and splicing pattern in lineages as distant as *Drosophila* and *Apis* (Cho et al., 2007). Male-specific expression of *fru* has been observed in the majority of *Drosophila* species examined (Yamamoto et al., 2004; Usui-Aoki et al., 2005) and it has been shown that *Fru* displays functional conservation among lineages at least as divergent as *Drosophila* and *Anopheles* (Gailey et al., 2006). Likewise, we find that members of the *takeout* gene family are found in all of the insect species we examined. Despite *Pediculus* sequences being too divergent to be included in the phylogenetic analyses, the

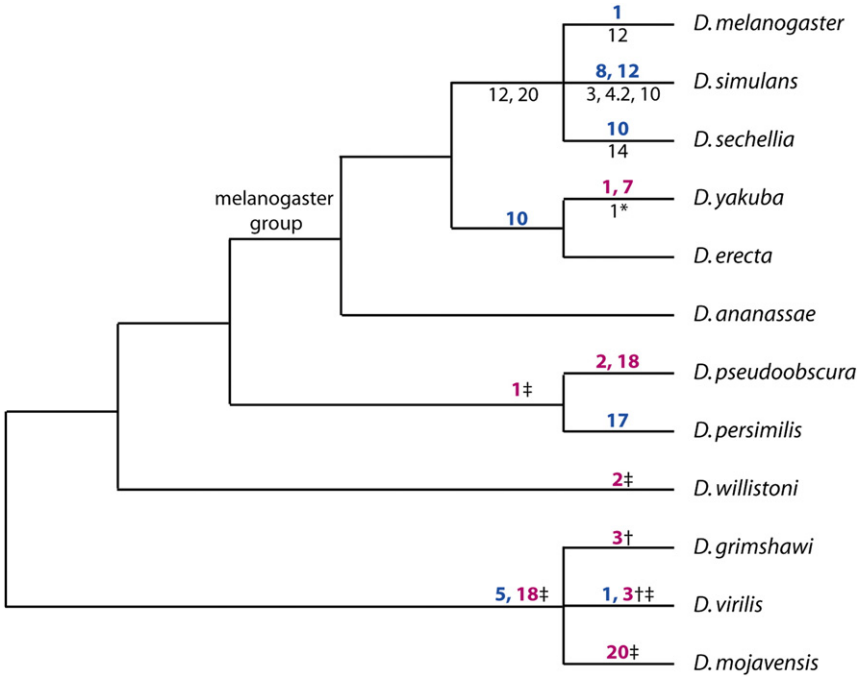

Fig. 4. *Drosophila* lineages with evidence of positive selection and losses or gains of *takeout* genes. Above each branch, numbers in blue show clusters that have lost a gene in that lineage; numbers in pink are clusters with a gene gain. † indicates that an equally parsimonious reconstruction would infer a gene gain in cluster 3 prior to the divergence of *D. grimshawi*, *D. virilis*, and *D. mojavensis* and a subsequent gene loss in *D. mojavensis*. ‡ indicates that duplicates are arranged tandemly in the genome. Below branches of species in the melanogaster group are shown clusters with evidence of positive selection in that lineage. * indicates positive selection that is associated with a gene duplication (see Supplementary file 3).

fact that we were able to identify *takeout* homologs in all insect species examined indicates that the *takeout* gene family is >350 million years old (Gaunt and Miles, 2002). Despite a long divergence time between the insect lineages studied, greater than one third of the clusters contain genes from genera besides *Drosophila*, indicating that this gene family arose early in insect evolution and has been maintained throughout the diversification of insects. This finding of ancient paralogs across species in conjunction with evidence of broad purifying selection within clusters leads to the hypothesis that the *takeout* gene family is a group of conserved proteins that may have maintained similar functional roles across species.

Our results suggest that the *takeout* gene family is old and conserved across insects, but it has experienced intermittent bouts of diversification. In contrast to the pattern of overall protein sequence conservation, some genes have experienced positive selection in specific lineages. However, there is no obvious pattern found among the positively selected genes; they are found in several lineages and many gene clusters (Fig. 4). If these instances of adaptive evolution are in fact a result of selection on courtship behavior, this may suggest that the paralogs function in courtship differently in different species. Alternatively, given that *takeout* in *D. melanogaster* affects not only courtship, but also aging, metabolism, and other behaviors, it remains possible that selection on these genes is not only in response to a role in courtship. Previous evidence has suggested that positive selection plays an important role in the evolution of proteins in the sex determination pathway (e.g. *fru* (Sobrinho and de Brito, 2010)). It is unclear whether diversification in these sex determination genes should result in positive selection on the downstream targets, such as *takeout*.

5. Conclusions

The results presented here will be critical in further elucidating the role of *takeout* in the evolution of courtship behavior in insects. Our results predict a high degree of functional conservation across this gene family, but experimental tests are necessary to confirm these predictions. Likewise, experimental tests will allow us to assess the functional consequences of adaptive evolution.

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2011.10.003.

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