Rapid Evolution of Sex Pheromone-Producing Enzyme Expression in *Drosophila*

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**Abstract**

A wide range of organisms use sex pheromones to communicate with each other and to identify appropriate mating partners. While the evolution of chemical communication has been suggested to cause sexual isolation and speciation, the mechanisms that govern evolutionary transitions in sex pheromone production are poorly understood. Here, we decipher the molecular mechanisms underlying the rapid evolution in the expression of a gene involved in sex pheromone production in Drosophilid flies. Long-chain cuticular hydrocarbons (e.g., dienes) are produced female-specifically, notably via the activity of the desaturase DESAT-F, and are potent pheromones for male courtship behavior in *Drosophila melanogaster*. We show that across the genus *Drosophila*, the expression of this enzyme is correlated with long-chain diene production and has undergone an extraordinary number of evolutionary transitions, including six independent gene inactivations, three losses of expression without gene loss, and two transitions in sex-specificity. Furthermore, we show that evolutionary transitions from monomorphism to dimorphism (and its reversion) in *desatF* expression involved the gain (and the inactivation) of a binding-site for the sex-determination transcription factor, DOUBLESEX. In addition, we documented a surprising example of the gain of particular cis-regulatory motifs of the *desatF* locus via a set of small deletions. Together, our results suggest that frequent changes in the expression of pheromone-producing enzymes underlie evolutionary transitions in chemical communication, and reflect changing regimes of sexual selection, which may have contributed to speciation among *Drosophila*.

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**Abbreviations:** CRE, cis-regulatory element; DSX-DBD, DOUBLESEX DNA binding domain; DSX-F, female specific DOUBLESEX isoform; DSX-M, male specific DOUBLESEX isoform; eGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; oe, oenocyte element.

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**Introduction**

Chemical communication is widespread in the animal world [1]. Pheromones can mediate aggregation, signal danger, attract mates, and elicit a variety of other behaviors. The species-specificity of pheromonal signals is of decisive importance in kin recognition and in sexual reproduction [2]. Evolutionary transitions in sexual communication have been suggested to govern the early stages of speciation [3]. Moreover, reproductively relevant traits such as sex pheromones are thought to evolve rapidly under sexual selection [4]. These observations raise the possibility that traits such as sex pheromones are thought to evolve rapidly under early stages of speciation [3]. Moreover, reproductively relevant transitions in sexual communication have been suggested to govern the early stages of speciation [3]. Additionally, reproductively relevant transitions in sexual communication have been suggested to govern the early stages of speciation [3].

*Drosophila* male courtship behavior is triggered in part by female pheromones that act either by direct contact and/or by transmission over short distances. Sex pheromones in *Drosophila* are largely fatty-acid derived hydrocarbons that are present on the fly’s cuticle [5]. Among Dipteran fly species, females exhibit considerable divergence in the number or position of double bonds in cuticular hydrocarbons [6]. In the *Sophophora* subgenus, females of some species, such as *D. melanogaster* [6–10], *D. sechellia* [6,9,11], and *D. erecta* [6] specifically produce long-chain dienes, which are hydrocarbons that contain two double bonds [6]. Alternatively, other species, such as *D. serrata*, *D. pseudoobscura*, and *D. persimilis* [12–14] produce these compounds in both sexes. Moreover, other species such as *D. simulans*, *D. mauritiana*, *D. yakuba*, *D. teissieri*, *D. aenea*, and *D. santomea* [6,7,9,13,16] do not produce dienes. Cuticular hydrocarbons are suggested to have multiple roles (e.g., protection against desiccation and cold resistance [17,18]), and evolutionary transitions in the production of these compounds have been linked to reproductive isolation [9,19]. Males from species that produce dienes dimorphically preferentially court heterospecific females that carry dienes over heterospecific females that do not harbor these compounds [9,20].
Author Summary

Mate selection is a complex process involving communication between potential partners on many levels, such as visual, aural, and olfactory cues. Many animals use chemical signals in the form of pheromones to communicate and correctly recognize individuals of the appropriate species and sex during reproduction. Evolutionary changes in the production of these chemicals have been suggested to contribute to speciation. Yet, the molecular mechanisms governing these transitions have seldom been addressed. Here, we show that expression of the gene desatF, which encodes an enzyme involved in the production of the Drosophila pheromones known as dienes, is highly variable and rapidly evolving across Drosophila species. Changes in desatF gene expression correlate with changes in sex- and species-specific production of dienes. Further, these changes in diene production can be explained by simple modifications in the regulatory regions of the desatF gene, providing a molecular level understanding of the evolution of pheromone production in Drosophila.

In D. melanogaster, the synthesis of dienes depends in part on the product of the desatF gene (also known as Fad2) [21], a desaturase that catalyzes the addition of a second double bond in cuticular hydrocarbons. This gene is transcribed female specifically in adults [21]. The loss of desatF activity causes both a decrease in the amount of dienes, and a decrease in the attractiveness of females to males during courtship [21], suggesting a crucial role in mate recognition. Others have hypothesized that differences at the desatF locus may contribute to the difference in diene production between D. simulans and D. sechellia [22] and other Drosophila species [23].

Here, we investigate the evolution and regulation of the desatF gene across the subgenus Sophophora. First, we show that the desatF locus and its expression are extremely rapidly evolving across the subgenus Drosophila. Second, we demonstrate that the female-specific isoform of the protein encoded by the sex differentiation gene, doublesex (dse), directly activates desatF expression in species that express desatF female specifically. Third, we reveal that one species evolved monomorphic expression of desatF by functional inactivation of an ancestral DSX-binding site in the desatF regulatory region. And finally, we uncover an apparent case of desatF inactivation of an ancestral DSX-binding site in the species evolved monomorphic expression of desatF gene, specifically, spatially restricted expression in oenocytes has not been demonstrated since all other analyses relied on reverse transcription (RT)-PCR on whole flies [21,23]. We therefore developed an in situ hybridization protocol to visualize mRNA transcripts in adult abdomens. Consistent with previous studies, desatF expression is female-specific in D. melanogaster. Our in situ analysis revealed desatF expression in a pattern that is entirely consistent with previous histological descriptions [26] of adult oenocyte cells (Figure 1A, purple stripes). Moreover, desatF expression in the adult abdomen is identical to the pattern revealed by a previously characterized GAL4 driver that is active in oenocyte tissue (see “desatF Expression in Female Oenocytes Is Directly Activated by the Female-Specific doublesex Isoform,” below; Figure 4A and 4B, [21]). desatF expression in oenocyte cells is consistent with its role in diene biosynthesis. Our in situ analysis was limited to oenocyte cells and we cannot rule out expression of desatF in other tissues.

Dynamic Evolutionary Changes in desatF Expression Correlate with Diene Production in the Sophophora Subgenus

The profile of cuticular diene production in the Sophophora subgenus exhibits several states, depending on the species (Figure 2, middle column) (unpublished data; [6–16]). Across this group, diene production displays an apparent transition from sexual monomorphism to dimorphism in an ancestor of the D. melanogaster species subgroup (Figure 2, left column, green arrowhead) and several transitions to a state of no production (Figure 2, left column, black arrow and not shown). Since desatF has been shown to be crucial for the production of dienes in D. melanogaster [21], we asked whether evolutionary changes at this locus could provide an explanation for these differences.

In order to do so, we tested whether desatF expression correlated with diene production. We cloned the desatF coding region and several kb of its upstream putative regulatory sequence from 24 species within the subgenus Sophophora. desatF expression was assessed by in situ hybridization in species where the gene lacked interruptedness in its reading frame. In 15 out of 24 species, desatF expression was assessed by in situ hybridization in species where the gene lacked interruptedness in its reading frame. In 15 of these species, desatF expression was assessed by in situ hybridization in species where the gene lacked interruptedness in its reading frame. In 15 of these species, desatF expression was assessed by in situ hybridization in species where the gene lacked interruptedness in its reading frame.

Results

desatF Is Expressed Female Specifically in Oenocytes

It has been suggested that the biosynthesis of cuticular hydrocarbons takes place in specialized cells called oenocytes [21,24,25], which are present underneath the dorsal and ventral abdominal cuticle. Histological, confocal, and electron microscopy studies have characterized adult oenocyte cells as being organized in metamer, transverse ribbon-like stripes, that do not cross the midline and are positioned just anterior to the intersegmental region of each segment in the dorsal abdomen [26]. Adult oenocyte cells are also present in each segment of the ventral abdomen [26]. While desatF is known to be transcribed female-specifically, spatially restricted expression in oenocytes has not been demonstrated since all other analyses relied on reverse transcription (RT)-PCR on whole flies [21,23]. We therefore developed an in situ hybridization protocol to visualize mRNA transcripts in adult abdomens. Consistent with previous studies, desatF expression is female-specific in D. melanogaster. Our in situ analysis revealed desatF expression in a pattern that is entirely consistent with previous histological descriptions [26] of adult oenocyte cells (Figure 1A, purple stripes). Moreover, desatF expression in the adult abdomen is identical to the pattern revealed by a previously characterized GAL4 driver that is active in oenocyte tissue (see “desatF Expression in Female Oenocytes Is Directly Activated by the Female-Specific doublesex Isoform,” below; Figure 4A and 4B, [21]). desatF expression in oenocyte cells is consistent with its role in diene biosynthesis. Our in situ analysis was limited to oenocyte cells and we cannot rule out expression of desatF in other tissues.
appeared to be intact (Figure 2, right column and summarized in Figure 7). desatF expression correlated with diene production (Figure 2, compare the middle and right columns). In species where diene production is monomorphic (e.g., *D. pseudoobscura*, *D. persimilis*, *D. serrata*), desatF is expressed in oenocytes of both sexes. In species that do not produce dienes, desatF was either not expressed (*D. simulans*, *D. mauritiana*, *D. santomea*, and *D. teissieri*) or the gene was not intact (*D. yakuba* and *D. orena*) (Figure 2, right column and summarized in Figure 7). Finally, in species where diene production is strongly female-biased, desatF was expressed only in female oenocytes (*D. melanogaster*, *D. sechellia*, and *D. erecta*). Our survey revealed that the expression of desatF has evolved with extraordinary rapidity (summarized in Figure 7). Of the 24

| Species          | desatF Males | desatF Females | Ref.          |
|------------------|--------------|----------------|---------------|
| *D. melanogaster*| +            | -              | [6-10, data not shown] |
| *D. simulans*    | -            | -              | [6, 7, 9, 16]  |
| *D. sechellia*   | +            | *              | [6, 9, 11]     |
| *D. erecta*      | +            | -              | [6]            |
| *D. takahashii*  | +            | +              | [data not shown] |
| *D. serrata*     | +            | +              | [12]           |
| *D. pseudoobscura*| +            | +              | [13, 14]       |

Figure 2. *desatF* expression correlates with evolutionary transitions in diene production from sexual monomorphism to dimorphism. Left column: phylogenetic relationships of the species used in this study. The *D. melanogaster* species subgroup is highlighted in red. Transitions in diene production from sexual monomorphism to dimorphism, and from dimorphism to amorphism, are indicated by a green and a black arrowhead, respectively. Middle column: diene production in males and females of these species. Diene production across the Sophophora subgenus shows several transitions: from monomorphism to dimorphism in the most recent common ancestor of the *D. melanogaster* species subgroup, and from dimorphism to amorphism in *D. simulans* (and other species not shown in the *D. melanogaster* species subgroup including *D. mauritiana*, *D. yakuba*, *D. santomea*, *D. teissieri*, and *D. orena*; summarized in Figure 7). We refer to dienes as any long-chain hydrocarbon (longer than 20 carbons) with two double bonds. Published reports of hydrocarbon profiles in these species are indicated in the column titled Ref. We have also independently validated the hydrocarbon profiles of *D. melanogaster* and *D. simulans* (unpublished data). Our analysis of *D. takahashii* males and females showed that they both produce a C23 diene (unpublished data). * Dienes are present on *D. sechellia* males, but account for less than 2% of the total amount of cuticular hydrocarbons present on the fly [6]. The putative expression of *desatF* in *D. sechellia* males is likely to be below the detection capabilities of in situ hybridization analyses. Right column: In situ hybridization of *desatF* expression performed on 4-d-old adults. In all species studied, we observed expression of *desatF* in abdominal oenocytes (purple stripes) in accordance with their status of diene production. doi:10.1371/journal.pbio.1000168.g002
species analyzed, spanning approximately 40 million years of evolution, we uncovered ten independent evolutionary transitions (not including an additional sex-specific transition discussed below) (summarized in Figure 7). On the basis of the phylogenetic tree (adapted from [27]), desatF was disrupted six times by deletions and insertions of repetitive DNA (summarized in Figure 7, red bars). The expression of an intact desatF was lost independently at least three times (summarized in Figure 7, black bars). Based on our expression data, and in agreement with the inferences of others [23], it appears that female-specific expression was gained once at the base of the D. melanogaster species subgroup (Figure 2, right column).

These ten evolutionary transitions in the state of desatF expression among such recently diverged species mark the fastest rate of regulatory evolution in the insect clade and are paralleled by changes in sex-specific gene expression [28]. The presence of a putative DSX-binding site in the desatF CREs raises the possibility that desatF is a direct target of DSX regulation [29–32]. In both cases, DSX activity completely lacks the expression of desatF in adult oenocyte cells (Figure S3). Furthermore, depletion of dxs transcripts in oenocytes using a UAS-dxs-RNAi transgene driven by an oenocyte-specific Gal4 transgene (OK72-Gal4; Figure 4A and 4B) caused a loss of desatF expression in adult oenocyte cells (compare controls in Figures 4C and 4E with Figure 4G).

We next tested whether the DSX protein bound to the putative DSX-binding sites in the desatF CREs from D. melanogaster and D. erecta. Electrophoretic mobility shift assays (EMSA) demonstrated that the DSX-DNA-binding domain (DSX-DDB) specifically and efficiently bound the wild-type site (Figure 4I, lanes 1–5), and this binding was abolished when the DSX-binding site was mutated (Figure 4I, lanes 6–10). Furthermore, mutations in the DSX binding site of an otherwise wild-type mel-oe1 (Figures 4J and 4K) and ere-oe (Figures 4N and 4O) caused a complete loss of reporter activity in vivo (Figure 4L and 4M and 4P and 4Q, respectively). Taken together, these data demonstrate that DSX-F directly activates desatF expression in adult oenocyte tissue. Note that while DSX-F is directly required for female-specific expression of desatF, it is not sufficient, and additional cis-regulatory inputs are also necessary for gene expression (see “cis-Regulatory Sites in desatF Were Gained by a Series of Small Deletions during D. melanogaster Evolution” below).

Our experiments did not indicate a repressive function for DSX-M in regulating desatF expression. The loss of DSX function in males did not lead to an upregulation of desatF in oenocyte cells (compare controls in Figure 4D and 4F with 4H), and mutations in the DSX binding site of mel-oe1 and ere-oe did not lead to a gain of reporter expression in males (Figure 4M and 4Q). Thus, while DSX-F is required directly to activate desatF expression in females (Figure 4), unlike other known targets of DSX proteins [30–32], DSX-M apparently does not regulate desatF in males. Our studies thus demonstrate an additional mode of DSX target gene regulation.

Our phylogenetic analysis of desatF expression in species within the Sophophora subgenus led us to infer that sexually dimorphic expression arose in the ancestor of the D. melanogaster species subgroup (Figure 2, left column, green arrowhead). Given that DSX-F directly activates desatF expression in female oenocytes of D. melanogaster and D. erecta, we posited that the origin of female-specific expression arose with the DSX-binding site. In order to test this hypothesis, we investigated the ancestry of this site.
The DSX-Binding Site at desatF Predates the *D. melanogaster* Species Subgroup

If the DSX-binding site at desatF evolved concomitantly with the origin of dimorphic expression, then all outgroup species to the *D. melanogaster* species subgroup analyzed in our study, which display monomorphic expression of desatF, should lack an orthologous DSX-binding site in the oenocyte CRE of desatF. While we did not find an orthologous DSX-binding site in the outgroup species *D. pseudoobscura, D. persimilis, or D. serrata,* which display monomorphic expression of desatF, we were surprised to find several outgroup species that contained an orthologous sequence similar to the DSX-binding site consensus [28] within the upstream regulatory region of desatF (Figure 5A). In *D. prostipennis, D. paralutea,* and *D. eugracilis,* all of which lack desatF expression in adult oenocytes, and in *D. takahashii,* which expresses desatF in both sexes, there is an orthologous sequence that matches the *D. melanogaster* DSX-binding site for at least ten out of 13 base pairs (Figure 5A, right panel). In *D. paralutea,* the orthologous site matches the *D. melanogaster* site perfectly. These data indicate that

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**Figure 3. cis-regulatory sequence evolution governs the gain of female-specific expression of desatF.** (A) Phylogenetic relationships of the species for which the activity of the desatF upstream regulatory region was assessed. Members of the *D. melanogaster* species subgroup are indicated in red. (B–M) Confocal images of the dorsal view of the abdomen from 4-d-old *D. melanogaster* females (B, D, F, H, J, and L) and males (C, E, G, I, K, and M) carrying two copies of the eGFP reporter transgene driven by the desatF CRE of each species indicated on the left. Note that, except for *D. sechellia,* all transgenes tested recapitulated the endogenous expression of the species they were derived from, indicating that functional differences in cis-regulatory sequences account for the transition from monomorphic to dimorphic expression of desatF.

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Figure 4. DSX-F is directly required to activate female-specific expression of desatF in adult oenocytes. The OK72-Gal4 driver specifically targets oenocyte tissue in females (A) and males (B). X-Gal staining performed on 4-d-old *D. melanogaster* of the genotype indicated. Blue staining indicates that the activity of the OK72-Gal4 driver is restricted to oenocytes (arrow) and two groups of cells (arrowhead) collinear to the dorsal vessel. These preparations retained fat body tissue, which appear to lack X-Gal activity, indicating that this driver does not target the fat body. (C–H) *In situ* hybridization for desatF performed on 4-d-old females (C, E, and G) and males (D, F, and H) of the *D. melanogaster* genotype indicated. Compared to the wild-type-like expression observed in control flies carrying just the GAL4 driver (C and D) and the dsx-RNAi (E and F) transgenes, desatF expression is lost in specimens expressing the dsx-RNAi driven by the OK72-GAL4 construct (G and H). (I) EMSAs were performed on annealed radiolabeled oligonucleotide probes containing the wild-type and mutant (mutated nucleotides in red) DSX-binding sites with increasing amounts of DSX-DBD protein. For probes containing the wild-type binding site, as the amount of DSX-DBD increased (lanes 1–5) a correlative increase in the amount of probe bound was observed. Protein binding was significantly reduced when the DSX-binding site was mutated (lanes 6–10). Arrow and arrowhead points to a single and pair of DSX-DBD monomers bound to the probe respectively. Asterisk identifies free probe. (J–Q) Confocal images of dorsal abdomens from 4-d-old *D. melanogaster* females (J, L, N, P) and males (K, M, O, Q) carrying two copies of an eGFP-reporter transgene. Reporter constructs are indicated at the top of the columns. The mel-oe (J and K) and ere-oe (N and O) transgenes recapitulate endogenous expression of desatF. However, when the DSX-binding is mutated in these constructs, they fail to drive eGFP-reporter expression in females (L and P, respectively). Reporter expression in males (M and Q, respectively) is not upregulated.

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the origin of the DSX-binding site most likely predated the origin of the D. melanogaster species subgroup (Figure 5A, red). If true, then the origin of the DSX-binding site would be deeper in the phylogeny (Figure 5A, green arrowhead) than the origin of dimorphic expression originally inferred from our phylogenetic expression analyses (Figure 5A, black arrowhead), and those from others [23].

**Monomorphic Expression of desatF in D. takahashii Evolved by Functional Inactivation of the DSX-Binding Site**

One interpretation of the observations above is that dimorphic expression of desatF actually arose with the acquisition of the DSX-binding site in the desatF CRE and was subsequently lost in the D. takahashii subgroup and in D. eugracilis. If this were true, then the state of desatF expression in D. takahashii, D. prostipennis, D. paraibana, and D. eugracilis evolved from an ancestor that expressed desatF female-specifically by direct DSX regulation. Moreover, monomorphic expression of desatF in D. takahashii would then be predicted to have evolved at least in part by loss of direct DSX regulation.

In order to test this possibility, we examined the D. takahashii desatF CRE and putative DSX-binding site in greater detail. We observed that the putative DSX-binding site present in the desatF CRE of D. takahashii diverges from the consensus sequence in two positions, one of which is located in the core of the site, where an A is found instead of the consensus C (Figure 5A). We note that this difference is likely to be derived in D. takahashii as all other species with the orthologous site contain a consensus C at this position (Figure 5A). Given that this change is located in the core of the sequence, it suggested to us that DSX proteins might not bind the D. takahashii site. EMSAs revealed that, in fact, the DSX-DBD failed to bind the orthologous site in D. takahashii desatF (Figure 5C, lanes 1–5). Furthermore, introducing the identical C-to-A mutation in the core of the D. melanogaster DSX-binding site greatly diminished binding of the DSX-DBD relative to wild type (Figure 5B, compare lanes 1–5 with lanes 6–10), and when introduced in an otherwise wild-type mel-oe1 (Figures 5D and 5E), this C-to-A mutation caused a complete loss of reporter activity (Figure 5F). These results show that the putative D. takahashii DSX-binding site is nonfunctional.

Our data are consistent with an evolutionary scenario in which monomorphic expression of desatF in D. takahashii evolved from a dimorphic ancestor by a loss-of-function mutation in the ancestral DSX-binding site. We tested this scenario by assessing whether the restoration of a functional DSX-site by an A-to-C transition in the D. takahashii oenocyte CRE would result in dimorphic reporter expression. A 296-bp CRE upstream of desatF from D. takahashii is fully sufficient to recapitulate monomorphic expression of desatF in transgenic reporter assays in D. melanogaster (Figure 5H and 5I). If this were true, then the state of desatF expression in D. takahashii as all other species with the orthologous site contain a consensus C at this position (Figure 5A). Given that this change is located in the core of the sequence, it suggested to us that DSX proteins might not bind the D. takahashii site. EMSAs revealed that, in fact, the DSX-DBD failed to bind the orthologous site in D. takahashii desatF (Figure 5C, lanes 1–5). Furthermore, introducing the identical C-to-A mutation in the core of the D. melanogaster DSX-binding site greatly diminished binding of the DSX-DBD relative to wild type (Figure 5B, compare lanes 1–5 with lanes 6–10), and when introduced in an otherwise wild-type mel-oe1 (Figures 5D and 5E), this C-to-A mutation caused a complete loss of reporter activity (Figure 5F). These results show that the putative D. takahashii DSX-binding site is nonfunctional.

One explanation for these results is that stabilizing selection has maintained phenotypic constancy for desatF expression while mutational turnover of functionally important sites has taken place. This phenomenon has been previously reported [33–38], but described for species that have diverged over relatively long periods of evolutionary time (40 millions y or more). It is surprising that drastic alterations in the cis-regulatory mechanisms at desatF occurred in a short period of time (D. melanogaster and D. sechellia diverged only 2–3 million y ago). More detailed investigation of the D. melanogaster CRE uncovered cis-regulatory sites specific to this species and important for desatF female-specific expression.

**Female-Specific Expression of desatF Is Consistent with Stabilizing Selection**

In the course of our studies of desatF regulation, we were surprised to discover that while D. melanogaster, D. sechellia, and D. erecta express desatF similarly in female oenocytes (Figure 2, right column), their respective oenocyte CREs were significantly different in structure. We found that sequences orthologous to the mel-oe2 CRE from D. erecta (i.e., ere-oe2; Figure S2) and D. sechellia (i.e., sec-oe2; Figure 3, compare 3B with 3D) failed to drive expression in transgenic reporter assays in D. melanogaster. These results suggested that female-specific desatF expression in D. erecta and D. sechellia rely at least in part on different cis-regulatory sites than those characterized in D. melanogaster.

Indeed, for D. erecta, additional sequences outside the ere-oe2 region are required for reporter activity. By extending the 5′-end of ere-oe2 by 190 bp (ere-oe3; Figure S2), we obtained full reporter activity in D. melanogaster female oenocytes. Importantly, this 190-bp region, by itself, is not sufficient for reporter function. Furthermore, the orthologous region from D. melanogaster is clearly not required for CRE function, as mel-oe2 is a fully functional CRE despite lacking the orthologous 190-bp region (Figure S1). Thus, the functional D. melanogaster and D. erecta CREs share common necessary features (e.g., the DSX-binding site), but also exhibit critical differences (e.g., the 190-bp region required for D. erecta CRE activity).

For D. sechellia, an exhaustive search of all intergenic sequences upstream and downstream of desatF failed to identify a region that drove reporter expression in the D. melanogaster genetic background (unpublished data). This may indicate that the D. sechellia CRE for oenocyte expression is located outside of the regions searched and/or that there are trans-acting regulatory differences between the species, which are key for desatF expression in D. sechellia females. We note there is a putative DSX-binding site in the species, which are key for desatF expression in D. sechellia females. We note there is a putative DSX-binding site in the species, which are key for desatF expression in D. sechellia females. We note there is a putative DSX-binding site in the species, which are key for desatF expression in D. sechellia females. We note there is a putative DSX-binding site in the species, which are key for desatF expression in D. sechellia females. We note there is a putative DSX-binding site in the species, which are key for desatF expression in D. sechellia females.
Figure 5. Monomorphic expression of desatF in D. takahashii evolved by functional inactivation of a DSX-binding site. (A) The DSX-binding site predates the D. melanogaster species subgroup (in red). Left panel: phylogenetic relationships of the species surveyed for the presence of a putative DSX-binding site (adapted from [27]). Others have positioned D. eugracilis and D. ficusphila differently in the phylogeny [63]. Our results are consistent in either case. Black arrowhead: inferred origin of desatF dimorphic expression, based on the phylogenetic distribution of desatF expression (see Figure 2). Green arrowhead: inferred origin of the DSX-binding site. Middle panel: sequences of the putative orthologous DSX-binding site of each species. Purple residues indicate positions that have diverged from the D. melanogaster site. Gray box identifies the critical residue within the core that has diverged in D. takahashii. Right panel: desatF expression summary. For D. trilutea, adult flies were not available and therefore desatF expression could not be assessed. desatF has been deleted in D. eugracilis and therefore assessing its expression was irrelevant. (B) EMSAs comparing
the ability of the DSX-DBD protein to bind annealed radiolabeled oligonucleotide probes containing the D. melanogaster DSX-binding site (lanes 1–5) and a mutated version of this site (lanes 6–10) containing a C to A point mutation (in red), as found in D. takahashii. This mutation greatly reduced binding of the DSX-DBD. (C) EMSAs comparing the ability of the DSX-DBD protein to bind annealed radiolabeled oligonucleotide probes containing the D. takahashii putative DSX-binding site and a mutated version of this site containing a C in the core of the putative site instead of an A, which is found in the consensus and in the D. melanogaster site. In contrast to the wild-type D. takahashii site (lanes 1–5), where no significant binding is observed, the DSX-DBD protein binds the mutant site relatively efficiently (lanes 6–10). The arrow and arrowhead point to a single and pair of DSX-DBD monomers bound to the probe. The asterisk marks the position of the free probe. (D–K) eGFP reporter expression in abdomens of 4-d-old D. melanogaster females (D, F, H, and J) and males (E, G, I, and K) and carrying two copies of the transgenes indicated at the top of the columns. Introducing an A to C point mutation in the putative DSX-binding site of D. takahashii abolishes eGFP reporter expression in females (compare D and F), while leaving the absence of expression in males unchanged (compare E and G). Introducing an A to C point mutation in the putative DSX-binding site of tak-oe produces sexually dimorphic eGFP expression (J, K), whereas a wild-type tak-oe drives monomorphic expression (H, I).

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Discussion

Pheromone differences between closely related species of Lepidoptera and Diptera suggest that their production is rapidly evolving [6,44,45]. While rapidly evolving traits have been characterized many times, especially regarding sexually related traits [46,47], few studies, to our knowledge, have identified the genes and mutations that give rise to these evolutionary changes [32]. We have investigated the evolution of the mechanisms that govern the production of pheromonal signals between males and females during Drosophila courtship. Our studies provide several insights into the molecular mechanisms of pheromone signal evolution.

We have shown that the desatF gene is rapidly evolving in the subgenus Sophophora. We found that changes in the desatF expression have evolved numerous independent times within 40 million y of Drosophila evolution, including six independent instances of gene loss, two modifications in sex-specific expression, and three independent losses of expression without gene loss. Altogether, among 24 species surveyed, we observed 11 transitions in the state of desatF expression (summarized in Figure 7). desatF has also been duplicated in some Drosophila species [23]. Our results reveal that the evolution of the desatF gene is extraordinarily dynamic, and displays, to our knowledge, the fastest evolving pattern of gene utilization observed to date.

We identified the CRE regulating desatF expression in D. melanogaster oenocytes and characterized DSX-F as a necessary and direct input for its female-specific expression. We found that transitions from sexual monomorphism to dimorphism, and the reverse, rely at least in part on the gain and loss of direct DSX regulation. Remarkably, simple evolutionary changes in cis-regulatory sequences were sufficient to explain the transition of desatF expression from dimorphism to monomorphism, as seen in D. takahashii.

Because desatF and dienes contribute strongly to D. melanogaster mating behaviors, it is likely that dimorphic expression of desatF is under sexual selection. Since the gain of female-specific expression, we count five losses of sexual dimorphism, which includes one transition to monomorphism and four transitions to amorphism (Figure 7, black dots). What does this pattern of evolutionary change suggest? The loss of sexually selected traits is widespread [48]. This pattern of frequent trait loss may be an indication of relaxed selection resulting from rapidly shifting regimes of sexual selection. For example, since fly courtship is regulated by multiple sensory cues (visual, chemosensory, auditory, etc), the modality that is under sexual selection may change, leading to trait loss.

Regulatory Evolution as a Mechanism to Evolve Sex-Specificity

A long-standing question in evolutionary biology is how sexually dimorphic traits evolve [49]. For example, monomorphic patterns can evolve from dimorphic patterns and vice versa, however, the molecular mechanisms that govern these transitions have seldom been addressed. In Lepidoptera and Diptera, duplication or structural changes of genes encoding desaturases have been suggested to [23] or shown to contribute to evolutionary alterations in pheromone signals [50–52], however, none of these phenomena alone could account for evolutionary transitions in sex-specificity of pheromone production. Here, we have provided evidence that cis-regulatory sequence evolution led to transitions from monomorphic to dimorphic expression of desatF, and its reversion, and concomitant changes in diene production.
Figure 6. Cis-regulatory information was gained by deletion during *D. melanogaster* evolution. (A) The AATTTG motif is statistically overrepresented in *mel-oe2*. Schematic representation of the AATTTG motifs (green dot over black bar) in *mel-oe2* and its orthologous sequence from *D. simulans* and *D. erecta*. (B and C) eGFP reporter expression in abdomens of 4-d-old *D. melanogaster* females carrying two copies of the transgenes indicated at the top of the images. The introduction of point-mutations in the clustered AATTTG motifs of *mel-oe1* (C) abolishes eGFP reporter expression driven in female oenocytes by a wild-type *mel-oe1* (B). The absence of reporter activity in males is not altered by these mutations (not shown). (D) Alignment of the desatF upstream region from *D. melanogaster*, *D. simulans*, and *D. erecta*. *mel-oe2* and its orthologous sequences from *D. simulans* and *D. erecta* are delineated by vertical black bars. The vertical red bar indicates the 3' end of *mel-oe1*. *mel-oe1* and *mel-oe2* begin at the same 5' position. AATTTG motifs are boxed in green (forward orientation) and blue (reverse orientation). Black stars (*) indicate conservation among the three species. The red plus sign (+) indicates conservation between *D. simulans* and *D. erecta*. The beginning of the coding region is in yellow. The *D. erecta* 190-bp sequence that is necessary (in addition to ere-oe2) to produce a construct capable of full reporter activity in *D. melanogaster* female oenocytes is represented in grey. Note the very well conserved indels in *D. simulans* and *D. erecta*, which disrupt each of the three AATTTG motifs in the cluster, indicating that those hexamer motifs evolved by a series of small deletions.

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![Image of Figure 6](https://www.plosbiology.org/)
By pinpointing one of these transitions at the level of individual base-pairs, we propose that monomorphic expression of desatF in D. takahashii evolved from a dimorphic ancestor through a derived mutation in a single critical residue inactivating the orthologous DSX-binding site (Figure 4). A simple model for the origin of monomorphic gene expression is that a mutation in the DSX-binding site abrogated repression by DSX-M, in turn, up-regulating desatF expression in males. Furthermore, the loss of regulation by DSX-F would lead to a decrease in desatF expression in females. These alterations, together, would produce monomorphic expression of desatF in D. takahashii. However, we note that this model is at odds with our finding that DSX-M appears to not

Figure 7. The desatF locus is rapidly evolving. (A) Phylogenetic relationships of the 24 species surveyed (adapted from [27]). (B) Schematic of the desatF locus in these species. The blue rectangles indicate the landmarks used in cloning. In D. ananassae, desatF was found in the genome, but not in synteny, which is indicated by the absence of the blue rectangles. The orange boxes indicate the coding region. A striped box indicates a mutation in the coding region leading to a loss of function of the protein (frameshift or nonsense mutation). Black and brown full circles represent regions with repetitive DNA. The six independent gene losses are indicated by red bars. Regulatory losses of expression without gene inactivation are marked by a black bar. Modifications in the sex-specificity of desatF expression are represented by a pink bar. Green “R” refers to regulatory transitions. Altogether, 11 independent evolutionary changes in desatF expression occurred in the approximate 40 millions y during which these species evolved. *, Note that the gene inactivations in D. yakuba and D. lutescens are not counted as such in our tally. In D. yakuba, the regulatory loss of desatF expression appears to have preceded the pseudogenization event. In D. lutescens, the ambiguous phylogenetic relationships in the clade prevents the accurate inference of transitions. Grey full circles indicate independent losses of dimorphism. (C) Status of desatF expression in oenocytes in 4-d-old adults aged. N/R: not relevant (because the gene was not functional based on sequence information).

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regulate desatF in *D. melanogaster*. This suggests that the ancestor of *D. melanogaster* and *D. takahashii* regulated desatF dimorphically by either a *D. melanogaster*-like mechanism, or by a mode that involved repression by DSX-M. While we currently cannot polarize these possibilities, both models implicate the inactivation of the ancestral DSX-binding site as a necessary step in the transition to monomorphic expression.

**desatF and Speciation**

In order to understand the mechanisms that drive speciation, the genetic changes that lead to reproductive isolation must be elucidated. It has been suggested, that “speciation genes are those that contribute to reproductive isolation, often in the form of hybrid inviability, sterility or behavioral aberration” [53]. While progress has been made in identifying genes that contribute to postzygotic isolation (such as *Xmnk2* [54,55], *Odh* [53], *Nap96* [56], see review [57]), little is known of genes that contribute to prezygotic isolation. We suggest that *desatF* could be one such gene.

There is evidence that diene production contributes to reproductive isolation. For example, it has been documented that dienes inhibit *D. simulans* male courtship behavior [58]. Moreover, *D. simulans/D. melanogaster* hybrid females lacking desatF expression elicit greater levels of courtship activity from *D. simulans* males, relative to hybrids expressing *desatF* female-specifically [59]. Taken together, these data indicate that expression of *desatF* and the production of dienes in *D. melanogaster* females contribute to the reproductive isolation between these sibling species.

*desatF* expression has evolved numerous times during *Drosophila* evolution. If, as others have suggested [12], transitions in dienes contribute to sexual behavior in species other than *D. melanogaster*, then the contribution of *desatF* to speciation may be widespread.

**Materials and Methods**

**Fly Stocks**

Wild-type stocks were obtained from the University of California, San Diego (UCSD) stock center (see Table S1). Gal4-UAS analyses were performed using the following lines: OK72-Gal4 was obtained from the Bloomington Stock Center; dsx+/p°, UAS-dsxRNAi, and UAS-lacZ were provided by M. McKeown (Brown University).

**Imaging of Fly Abdomens**

Images of in situ hybridizations and X-Gal stained adult abdomens were taken using an Olympus SZX16 Stereo Microscope equipped with an Olympus DP71 microscope digital camera. Adult transgenic eGFP-reporter line samples were imaged using an Olympus Fluoview FV 1000 confocal microscope and software. Wings and head were removed from 4-d-old adults, which were then mounted in Halocarbon 700 oil for confocal analysis.

**Sequence Analysis of Orthologous desatF Loci**

Sequences for *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, and *D. pseudoobscura* were obtained from their respective genome databases. All other sequences were obtained by cloning and sequencing of orthologous sequences using genomic DNA prepared from species stocks obtained from the UCSD Drosophila stock center (see Table S1). Sequences were PCR amplified using different sets of degenerate primers and then fused to give rise to the sequence of the whole locus. Details are available upon request to the authors. Novel sequences have been deposited in GenBank [http://www.ncbi.nlm.nih.gov/Genbank, submission numbers are listed in Table S1]. Orthologous sequences were aligned using ClustalW2 [60] with subsequent manual alignment in problematic regions. We used the GenePalette program to analyze our sequences (www.genepalette.org). We used Oligo-analysis to look for overrepresented motifs in our sequence [39]. This program calculates the probability that the analyzed sequence contains an oligonucleotide sequence at a frequency greater than that expected at random.

**In Situ Hybridization on Adult Abdomens**

In situ hybridization was performed as previously described [61] with minor modifications. The complete adult abdominal in situ protocol is available at http://www.molbio.wisc.edu/carroll/. Primers used to amplify probes are listed in Table S2.

**DNA-Binding Analyses**

EMSAs were performed as previously described [27,32]. PAGE-purified oligos used in EMSAs are listed in Table S3.

**Transgenic Fly Production**

All transgenic lines were produced by using the Phage φC31 Integrate system. Embryos from flies containing the X-chromosome attP docking site VK00046 [62] were injected as previously described [32]. Primers used to clone the constructs are listed in Table S4.

**Supporting Information**

**Figure S1** Screen of the *D. melanogaster* desatF locus for CREs. Numbers and adjacent bars indicate the *desatF* locus region surveyed in a given reporter construct. Green bars represent the regions that drove eGFP reporter activity in oenocytes in 4-d-old *D. melanogaster* flies, black bars represent regions that did not drive activity. Scale in base pairs is boxed. Additional information on the numbered eGFP-reporter gene constructs is in Table S4. Found at: doi:10.1371/journal.pbio.1000168.s001 (0.78 MB EPS)

**Figure S2** Screen of the *D. erecta* desatF locus for active CREs. Numbers and adjacent bars indicate the *desatF* locus region surveyed in a given reporter construct. Green bars represent the regions that drove eGFP reporter activity in oenocytes in 4-d-old *D. melanogaster* flies, black bars represent regions that did not show activity. Scale is boxed. Additional information on the numbered eGFP-reporter gene constructs can be found in Table S4. Found at: doi:10.1371/journal.pbio.1000168.s002 (0.75 MB EPS)

**Figure S3** DSX-F is required genetically to activate female-specific expression of desatF in adult oenocytes. In situ hybridization for *desatF* performed on 4-d-old *D. melanogaster*, dsx genotypes are indicated at the top of the columns, and sexual genotypes on the side. Compared to the heterozygous null controls (dsx+/TM6B, left panels) displaying a wild type pattern of *desatF* expression, *dsx* homozygous null mutants (dsx°, right panels) don’t show female specific expression (upper right panel), nor upregulation in males (bottom right panel). Found at: doi:10.1371/journal.pbio.1000168.s003 (3.76 MB EPS)

**Figure S4** The clustered AATTTG motifs contain regulatory information. eGFP reporter expression in abdomens of 4-d-old *D. melanogaster* flies carrying two copies of the transgenes indicated at the top of the images. The introduction of point-mutations that conserve AT content in the clustered AATTTG motifs of mel-oe1 (right panel) abolished eGFP reporter expression driven in female oenocytes by a wild-type mel-oe1 (left panel). This result indicate that rather than being important for structural
conformation of the enhancer, those hexamers are more likely binding sites for a transcription factor.

Found at: doi:10.1371/journal.pbio.1000168.s004 (2.44 MB TIF)

Table S1 List of the Drosophila species used in this study. The middle column refers to the UCSD stock center number. The right column refers to the GenBank accession number for the sequence orthologous to the D. melanogaster deslF locus. In addition, D. mimetica and D. tributa DNA was obtained from H. Malik. The sequences were referenced respectively as FJ869331 and FJ869337.

Found at: doi:10.1371/journal.pbio.1000168.s005 (0.04 MB DOC)

Table S2 Primers used to amplify probes for in situ hybridizations. The close proximity of some species allowed cross-hybridization. The D. melanogaster probe was hence also used on D. mauritiana, D. simulans, and D. sechellia. The D. santomea probe was also used on D. teissieri. The D. pseudosoba probe was also used on D. persimilis.

Found at: doi:10.1371/journal.pbio.1000168.s006 (0.05 MB DOC)

Table S3 Top strand EMSA oligonucleotides probes used in this study. The putative DSX-binding site is in bold.

References

1. Wyatt TD (2003) Pheromones and animal behaviour: communication by smell and taste. Cambridge: Cambridge University Press. xv.
2. Johansson BG, Jones TM (2007) The role of chemical communication in mate choice. Biol Rev Camb Philos Soc 82: 265–289.
3. Smadja C, Buxin RK (2009) On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity 102: 77–92.
4. Ellegren H, Parsch J (2007) The evolution of sex-biased genes and sex-biased gene expression. Nat Rev Genet 8: 689–698.
5. Ferveur JF (2005) Cuticular hydrocarbons: their evolution and roles in Drosophila pheromonal communication. Behav Genet 35: 279–295.
6. Jallon JM, David JR (1987) Variation in cuticular hydrocarbons among the eight species of the Drosophila melanogaster subgroup. Evolution 41: 294–302.
7. Jallon JM (1984) A few chemical words exchanged by Drosophila during courtship and mating. Behav Gen 14: 441–471.
8. Antony C, Davis TL, Carlson DA, Pechine JM, Jallon JM (1985) Compared behavioral responses of male Drosophila melanogaster (Canton S) to natural and synthetic aphrodisiacs. J Chem Ecol 11: 1617–1619.
9. Coyne JA, Crittenden AP, Mah K (1996) Genetics of a pheromonal difference contributing to reproductive isolation in Drosophila. Science 265: 1461–1464.
10. Pechine JM, Perez F, Antony C, Jallon JM (1985) A further characterization of Drosophila cuticular monoenes using a mass spectrometry method to localize double bonds in complex mixtures. Anal Biochem 145: 177–182.
11. Cobb M, Burnet B, Blizard R, Jallon JM (1989) Courtship in Drosophila sechellia: its structure, functional aspects, and relationships to those of other members of the Drosophila melanogaster species subgroup. J Insect Behav 2: 63–80.
12. Howard RW, Jackson LL, Banse H, Blores MW (2003) Cuticular hydrocarbons of Drosophila birchii and D. serrata: identification and role in mate choice in D. serrata. J Chem Ecol 29: 961–976.
13. Toolouc EG (1989) Laboratory evolution of epicuticular hydrocarbon composition and cuticular permeability in Drosophila pseudoobscura: effects on sexual dimorphism and thermal-acclimation ability. Evolution 43: 468–473.
14. Noor MA, Coyne JA (1996) Genetics of a difference in cuticular hydrocarbons between Drosophila pseudobscura and D. persimilis. Genet Res 68: 117–123.
15. Mas F, Jallon JM (2005) Sexual isolation and cuticular hydrocarbon differences between Drosophila santomea and Drosophila yakuba. J Chem Ecol 31: 2747–2752.
16. Coyne JA (1996) Genetics of differences in phenomeric hydrocarbons between Drosophila melanogaster and D. simulans. Genetics 143: 553–564.
17. Rouault JD, Marican C, Wicker-Thomas C, Jallon JM (2004) Relations between cuticular hydrocarbon (HC) polymorphism, resistance against desiccation and breeding temperature: a model for HC evolution in D. melanogaster and D. simulans. Genetica 120: 195–212.
18. Greenberg AJ, Moran JR, Coyne JA, Wu GJ (2003) Ecological adaptation during incipient speciation revealed by precise gene replacement. Science 302: 1754–1757.
19. Coyne JA, Oyama R (1995) Localization of phenomeric sexual dimorphism in Drosophila melanogaster and its effect on sexual isolation. Proc Natl Acad Sci U S A 92: 9505–9509.
20. Cobb M, Jallon JM (1990) Pheromones, mate recognition and courtship stimulation in the Drosophila melanogaster species sub-group. Anim Behav 39: 1058–1067.
21. Chernetps T, Duportes L, Labur C, Urayama M, Wicker-Thoma C (2006) A female-specific desaturase gene responsible for diene hydrocarbon biosynthesis and courtship behaviour in Drosophila melanogaster. Insect Mol Biol 15: 465–473.
22. Gleason JM, Jallon JM, Rouault JD, Ritchie MG (2005) Quantitative trait loci for cuticular hydrocarbons associated with sexual isolation between Drosophila simulans and D. sechellia. Genetics 171: 1789–1798.
23. Fang S, Ting CT, Lee CR, Chu KH, Wang CC, et al. (2009) Molecular evolution and functional diversification of fatty acid desaturases after recurrent gene duplication in Drosophila. Mol Biol Evol 26: 1447–1456.
24. Fan Y, Zurek L, Dykstra MJ, Schal C (2003) Hydrocarbon synthesis by enzymatically dissociated oenocytes of the abdominall segment of the German Cockroach, Blatta germanica. Naturwissenschaften 90: 121–126.
25. Ferveur JF, Saxavit F, O’Kane CJ, Surace G, Greenman RJ, et al. (1997) Genetic feminization of pheromones and its behavioral consequences in Drosophila males. Science 276: 1553–1558.
26. Krupp J, Kent C, Billette JC, Azancit R, So AK, et al. (2000) Social experience modifies pheromone expression and mating behavior in male Drosophila melanogaster. Curr Biol 10: 1373–1383.
27. Jeong S, Rokas A, Carroll SB (2006) Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in Drosophila evolution. Cell 125: 1387–1399.
28. Erdman SE, Chen HJ, Burns KC (1996) Functional and genetic characterization of the oligomerization and DNA binding properties of the Drosophila double-sex proteins. Genetics 144: 1639–1652.
29. Nagoshi RN, McKewon M, Burns KG, Belote JM, Baker BS (1988) The control of alternative splicing at genes regulating sexual differentiation in D. melanogaster. Cell 53: 229–236.
30. An W, Wensink PC (1995) Three protein binding sites form an enhancer that regulates sex- and fat-body-specific transcription of Drosophila yolk protein genes. Embryo 14: 1221–1230.
31. Coschigano KT, Wensink PC (1993) Sex-specific transcriptional regulation by the male and female doublesex proteins of Drosophila. Genes Dev 7: 42–54.
32. Williams TM, Selguge JE, Werner T, Gomperl N, Kopp A, et al. (2008) The regulation and evolution of a genetic switch controlling sexually dimorphic traits in Drosophila. Cell 134: 610–623.
33. Mitasia SA, Kafatos FC (1985) Regulatory elements controlling chorion gene expression are conserved between flies and moths. Nature 317: 453–456.
34. Xu PX, Zhang X, Hrnay S, Yoon A, Michelon AM, et al. (1999) Regulation of Pax6 expression is conserved between mice and flies. Development 126: 383–395.
35. Takahashi H, Mitani Y, Satoz G, Satoz N (1999) Evolutionary alterations of the minimal promoter for noctochord-specific Brachyury expression in ascidian embryos. Development 126: 3725–3734.
36. Ludvig MG, Bergman C, Patel NH, Kreitman M (2000) Evidence for stabilizing selection in a cukarotic enhancer element. Nature 403: 564–567.
37. Romano LA, Wray GA (2003) Conservation of Endo16 expression in sea urchins despite evolutionary divergence in both cis and trans-acting components of transcriptional regulation. Development 130: 4187–4199.

38. Tsong AE, Miller MG, Raisner RM, Johnson AD (2003) Evolution of a combinatorial transcriptional circuit: a case study in yeasts. Cell 115: 389–399.

39. van Helden J, Andre B, Collado-Vides J (1998) Extracting regulatory sites from the upstream region of yeast genes by computational analysis of oligonucleotide frequencies. J Mol Biol 281: 827–842.

40. Gasch AP, Moses AM, Chiang DV, Fraser HB, Berardini M, et al. (2004) Conservation and evolution of cis-regulatory systems in ascomycete fungi. PLoS Biol 2: e398. doi:10.1371/journal.pbio.0020398.

41. Wittkopp PJ (2006) Evolution of cis-regulatory sequence and function in Diptera. Heredity 97: 139–147.

42. Carroll SB (2005) Evolution at two levels: on genes and form. PLoS Biol 3: e245. doi:10.1371/journal.pbio.0030245.

43. Stone JR, Wray GA (2001) Rapid evolution of cis-regulatory sequences via local point mutations. Mol Biol Evol 18: 1764–1770.

44. Schulte S (2004) The chemistry of pheromones and other semiochemicals 1. Berlin; New York: Springer. ix.

45. Schulte S (2005) The chemistry of pheromones and other semiochemicals II. Berlin, New York: Springer. ix.

46. Eberhard WG (1985) Sexual selection and animal genitalia. Cambridge: Harvard University Press.

47. Civetta A, Singh RS (1998) Sex-related genes, directional sexual selection, and spermatogenesis. Mol Biol Evol 15: 903–909.

48. Wiena JJ (2003) Widespread loss of sexually selected traits: how the peacock lost its spots. Trends Ecol Evol 16: 317–321.

49. Darwin C (1871) The descent of man (and selection in relation to sex). London: John Murray.

50. Roelofs WL, Liu W, Hao G, Jiao H, Rooney AP, et al. (2002) Evolution of moth sex pheromones via ancestral genes. Proc Natl Acad Sci U S A 99: 13621–13626.

51. Roelofs WL, Rooney AP (2003) Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. Proc Natl Acad Sci U S A 100: 9179–9184.

52. Symonds MR, Elgar MA (2008) The evolution of pheromone diversity. Trends Ecol Evol 23: 220–229.

53. Ting CT, Tsaur SC, Wu ML, Wu CI (1998) A rapidly evolving homeobox at the site of a hybrid sterility gene. Science 282: 1501–1504.

54. Schartl M (1988) A sex chromosomal restriction-fragment-length marker linked to melanoma-determining Tu loci in Xiphophorus. Genetics 119: 679–685.

55. Wittbrodt J, Adams D, Maltschek B, Maule W, Rauf F, et al. (1989) Novel putative receptor tyrosine kinase encoded by the melanoma-inducing Tu locus in Xiphophorus. Nature 341: 415–421.

56. Presgraves DC, Balagopalan L, Abmayr SM, Orr HA (2003) Adaptive evolution drives divergence of a hybrid inviability gene between two species of Drosophila. Nature 423: 715–719.

57. Wu CI, Ting CT (2004) Genes and speciation. Nat Rev Genet 5: 114–122.

58. Marcillac F, Houot B, Ferveur JF (2005) Revised roles of Drosophila female pheromones. Chem Senses 30 Suppl 1: 273–274.

59. Legendre A, Miao XX, Da Lage JL, Wicker-Thomas C (2008) Evolution of a deaturase involved in female pheromonal cuticular hydrocarbon biosynthesis and courtship behavior in Drosophila. Insect Biochem Mol Biol 38: 244–255.

60. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

61. Jeong S, Rebeiz M, Andolfatto P, Werner T, True J, et al. (2008) The evolution of gene regulation underlies a morphological difference between two Drosophila sister species. Cell 132: 783–793.

62. Venken KJ, He Y, Hoskins RA, Bellen HJ (2006) P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. Science 314: 1747–1751.

63. Barmina O, Gonzalez M, McIntyre LM, Kopp A (2005) Sex- and segment-specific modulation of gene expression profiles in Drosophila. Dev Biol 288: 528–544.