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Runaway evolution of the male-specific exon of the *doublesex* gene in Diptera

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Abstract

In Diptera (Insecta), alternatively spliced male-specific and female-specific products of the *doublesex* (*dsx*) gene play key role in regulating development of the adult genital structures from the genital disc. Analysis of the pattern of nucleotide substitution of different domains of the *dsx* gene in 29 dipteran species showed that, over short evolutionary times, purifying selection predominated on the domain common to both sexes, the female-specific exons, and the and male-specific exon. However, over longer the evolutionary time frames represented by between-family comparisons, the male-specific exon accumulated nonsynonymous substitutions at a much more rapid rate than either the common domain or the female-specific exon. Overall, the accumulation of nonsynonymous substitutions in the male-specific exon occurred at a significantly greater than linear rate relative to the common domain, whereas the accumulation of nonsynonymous substitutions in the female-specific exon occurred at less than linear rate relative to the common domain. The evolution of the male-specific exon of *dsx* thus shows a pattern reminiscent of that seen in the "runaway" evolution of male secondary sexual characters at the morphological level, consistent with the hypothesis that female choice is an important factor in the morphological diversification of insect male genitalia.

Keywords

Diptera; doublesex; genital development; sex-specific exon; sexual selection

1. Introduction

The great morphological variety of male genitalia in insects has been known for over a century and a half and has been exploited as a source of characters for species-level taxonomy of many insect groups. For many years, the most widely held explanation of this surprising diversity was the "lock-and-key" hypothesis, first advanced by the pre-Darwinian entomologist Dufour (1944), whereby different genital morphologies are hypothesized to pose a barrier to between-species copulation (Shapiro and Porter 1989). The lock-and-key hypothesis was subsequently "Darwinized" (in the terminology of Shapiro and Porter 1989) as an evolutionary hypothesis, whereby the evolution of distinctive male genital morphologies was seen as a form of pre-mating isolating mechanism, which arose by natural selection as a mechanism to prevent gametic wastage in non-viable interspecific copulation

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attempts (Jordan 1896). However, numerous objections to the lock-and-key hypothesis have been raised, including both (1) the observation that the male genitalia generally vary much more markedly among taxa than do female genitalia; and (2) data from several groups showing the lack of any obvious covariation between male and female genital morphology (Eberhard 1985; Eberhard and Ramirez 2004; Shapiro and Porter 1989).

More recently, hypotheses explaining the variety of male insect genital morphology as the result of sexual selection have become increasingly popular (Eberhard 1985; Arnqvist 1998; Hosken and Stockley 2004). As with many aspects of sexual selection, the exact mechanism that might have given rise to the diversity of insect male genitalia has so far proved elusive (Hosken and Stockley 2004). Eberhard (1985) favored a classic Fisherian (Fisher 1930) runaway process, while others have argued for a Zahavian mechanism (Zahavi 1975) whereby genital morphology provides a reliable indicator of male genetic quality (Arnqvist and Thornhill 1998).

An alternative to hypotheses proposing direct selection on genital structures themselves suggests that differences in genital morphology arose as pleiotropic effects of alleles selected for their effects on other characters (Mayr 1963). An objection to this hypothesis has been that it does not explain why such pleiotropic effects would impact the male genitalia specifically, rather than other morphological structures (Eberhard 1985). On the other hand, although some theoretical models have suggested a role of genetic drift in the evolution of traits used in mate choice (Lande 1981), the literature on insect genital morphology has largely ignored the possibility that interspecies differences in these structures have arisen through fixation of selectively neutral mutations by random genetic drift.

In *Drosophila melanogaster* (Diptera: Drosophilidae), the sex-determination gene *doublesex* (*dsx*) plays a key role in regulating development of the adult genital structures from the genital disc (Sánchez and Guerrero 2001; Vincent et al. 2001). By alternative splicing, the *dsx* gene encodes the female-specific transcription factor DsxF (exons 1, 2, and 3) and the male-specific transcription factor DsxM (exons 1, 2, and 4). In addition to their role in the development of the genitalia, these transcription factors play roles in the nervous system in regulating the development of sex-specific behaviors (Rideout et al. 2007; Siwicki et al. 2009; Waterbury et al. 1999). In transgenic *Drosophila melanogaster* with *dsx* genes from *Anastrepha obliqua* (Diptera: Tephiritidae), normal genital morphologies did not develop; this result demonstrates that amino acid changes in DsxF and DsxM can effect genital morphology (Alvarez et al. 2009).

Previous results have suggested that purifying selection acts on the amino acid sequences encoded by both the female-specific exon and the male-specific exon of dipteran *dsx* (Ruiz et al. 2007). Here I extend these analyses in order to compare the patterns of evolutionary diversification in functionally different regions of the *dsx* gene. Specifically, I test whether the pattern of nonsynonymous nucleotide substitutions in the male-specific exon of *dsx* is consistent with the hypothesis that such substitutions play a role to the diversification of male genitalia of Diptera. Moreover, I address functional constraints on *dsx* at different evolutionary time scales in order to gain insight into potential evolutionary mechanisms underlying male genital diversification. For example, if sexual selection has repeatedly favored novel male genital morphologies, rapid evolution of nonsynonymous sites in the male-specific exon might be predicted. Conversely, on the lock-and-key hypothesis, one might predict both covariation of male-specific and female-specific exons and rapid diversification between closely related species as a result of selection for isolating mechanisms.

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2. Methods

Coding sequences of *dsx* from 29 species of Diptera were analyzed (Table 1). *Anastrepha fraterculus* is a species complex including 4 recognized cryptic species, designated Sp1–Sp4 (Ruiz et al. 2007), for each of which sequence data were available (Table 1). The data included 11 species in the genus *Anastrepha* (family Tephritidae); 4 species in the genus *Bactrocera* (Tephiritidae) and 8 species in the genus *Drosophila* (Drosophilidae). Sequences for the common domain (exons 1–2), the female-specific domain (exon 3), and the male-specific domain were retrieved from the NCBI sequence database by homology search. In the case of *Drosophila* species, when predicted mRNAs in the Refseq database did not include all of these exons, they were located by homology search in genomic shotgun sequences (Table 1).

Using the MEGA 4.1 program (Tamura et al. 2007), sequences were aligned at the amino acid level, and the alignment imposed on the DNA sequence (Supplementary Figure S1). In pairwise comparisons of sequences within and between genera, any site at which the alignment postulated a gap in any sequence was excluded from all comparisons ("complete deletion"). However, when all available sequences were compared pairwise, partial deletion (i.e., deletion only of sites with gaps between the two sequences compared) was used; this was done to increase the number of sites compared in the case of the male-specific exon, for which the alignment postulated numerous gaps between distantly related sequences. However, in preliminary analyses, the results using complete and partial deletion showed the same trends (not shown).

A phylogenetic tree of the common domain was reconstructed using the neighbor-joining method (Saitou and Nei 1986), based on the maximum composite likelihood (MCL) distance (Tamura et al. 2007). The reliability of clustering patterns in the tree was assessed by bootstrapping (Felsenstein 1985); 100 boostrap samples were used. The number of synonymous substitutions per synonymous site (d_S) and the number of nonsynonymous substitutions per nonsynonymous site (d_N) were estimated by Nei and Gojobori's (1986) method. In preliminary analyses, other methods (Li 1993; Yang and Nielsen 2000) yielded similar estimates; therefore, only the results using the Nei and Gojobori (1986) method are presented, because that method is expected to have a lower variance than the others (Nei and Kumar 2000). The variance of mean d_S and d_N was estimated by the bootstrap method (Nei and Kumar 2000).

After computing d_N values for all pairwise comparisons separately for each domain (common, female-specific, and male-specific), I estimated the correlation coefficient between the d_N values for each domain and those for each other domain. Because the pairwise d_N values for a given domain are not independent of each other, randomization tests were used to test the significance of the correlation coefficients. This test involved creating 1000 random populations of paired d_N values by sampling with replacement from the observed d_N values.

In order to test for the linearity of the relationship of d_N in different domains, I used the technique of allometric regression, which involves regression of log-transformed values (Sokal and Rohlf 1971). In the present case, since d_N was often equal to zero in closely related comparisons, I used the natural logarithm of $d_N + 1$ (Sokal and Rohlf 1971). In significance tests regarding the slope of the allometric regression line, the standard error of the slope estimator was estimated from the randomization procedure described above.

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3. Results

3.1. Sister Pair Comparisons

The NJ tree of common domain sequences (Figure 1) showed a topology very similar to that obtained in previous analyses (Ruiz et al. 2007). On the basis of this tree, seven "sister" pairs of sequences were chosen; and d_S and d_N in the common domain, the female-specific exon, and the male-specific exon were estimated between these seven phylogenetically independent pairs of sequences (Table 2). In the common domain, mean d_S for the seven comparisons was significantly greater than mean d_N ($P < 0.05$; Table 2). Likewise, in the male-specific exon, mean d_S was significantly greater than mean d_N ($P < 0.05$; Table 2). In the female-specific exon, mean d_S and mean d_N did not differ significantly (Table 2). However, the latter result did not occur because of elevated d_N in the female-specific exon, but rather because there were no synonymous differences in the female specific exon between any of the seven pairs (Table 2). As a consequence, mean d_S in the female-specific exon was significantly lower than mean d_S in the common domain ($P < 0.05$; Table 2). However, there were no significant differences between mean d_N in the common domain and mean d_N in either of the two sex-specific exons (Table 2). Thus the sister-pair comparisons showed evidence of purifying selection on the common domain and both sex-specific exons, with no evident differences among these three regions with regard to the strength of purifying selection.

3.2. Comparisons within and between Genera

There were two genera of Tephritidae (*Anastrepha* and *Bactrocera*) and one of Drosophilidae (*Drosophila*) represented by at least 4 species in the data set. In order to examine the patterns of nucleotide substitution over a somewhat longer evolutionary time frame than that represented by the sister-pair comparisons, I estimated mean d_S and d_N in the different domains of *dsx* for all pairwise comparisons within and between these three genera (Table 3). Within each genus, the observed pattern was generally similar to that seen in the sister-pair comparisons. In the common domain and in the male-specific exon, mean d_S was significantly greater than mean d_N ($P < 0.001$ in every case; Table 3). On the other hand, in the female-specific exon, mean d_S and mean d_N did not differ significantly; but this pattern was due to greatly reduced d_S rather than elevated d_N (Table 3). In fact, not a single synonymous substitution was observed in the female-specific exon in any of the pairwise comparisons within the tephritid genera *Anastrepha* and *Bactrocera* (Table 3). In all three genera, mean d_S in the female-specific exon was significantly lower than that in the common domain ($P < 0.001$ in every case; Table 3).

In *Drosophila*, there were no nonsynonymous differences in all pairwise comparisons of the female-specific exon; and mean d_N was significantly lower in the latter exon than in the common domain ($P < 0.001$; Table 3). Mean d_N in the male-specific exon was significantly greater than that in the common domain in *Anastrepha* ($P < 0.001$) but not in the two other genera (Table 3). Thus, the within-genus comparisons showed purifying selection on all domains. In *Drosophila*, there was evidence that purifying selection on the female-specific exon was stronger than that on the common domain, while in *Anastrepha* there was evidence that purifying selection on the male-specific exon was more relaxed than that on the common domain.

In comparisons between *Drosophila* and the two tephritid genera, d_S in the common domain could not be estimated because of saturation of synonymous sites; and in the sex-specific exons, d_S estimates were very high and close to saturation (Table 3). However, in the comparison between *Anastrepha* and *Bactrocera*, mean d_S was significantly greater than mean d_N in the common domain ($P < 0.001$; Table 3). In all between-genus comparisons,

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mean d_S was significantly greater than mean d_N in the male-specific exon (Table 3). And in the comparison between *Bactrocera* and *Drosophila*, mean d_S was significantly greater than mean d_N in the female-specific exon (Table 3).

In all three between-genus comparisons, mean d_N in the male-specific exon was significantly greater than that in the common domain (Table 3). In the comparisons between both tephritid genera and *Drosophila*, mean d_N in the female-specific exon was significantly lower than that in the common domain; and in the comparison between *Anastrepha* and *Bactrocera*, mean d_S in the female-specific exon was significantly lower than that in the common domain (Table 3). Thus, the comparisons between genera again showed evidence of purifying selection on all domains, but there was a clear indication in these more distant comparisons that purifying selection on the male-specific exon was more relaxed than that on the common domain. By contrast, the female-specific exon showed evidence of stronger purifying selection and thus greater functional constraint than the common domain.

In order to examine possible causes of the reduced d_S in the female-specific exon of all three genera, I examined nucleotide usage in this exon. Mean % G+C at third codon positions in the female-specific exon was 48.1% in *Anastrepha*, 45.2% in *Bactrocera*, and 55.8% in *Drosophila*. These values did not show a consistent pattern of difference from those observed in the common domain (45.6%, 39.9%, and 70.5%, respectively) or the male-specific exon (46.0%, 41.9%, and 76.2% respectively). Thus, it did not appear that a biased nucleotide content was responsible for the reduced d_S. However, of 90 nucleotide sites in this exon, 69 were completely conserved in all species of the three genera in the data set. The conserved sites fell mainly in 7 blocks of 4 or more consecutive sites that were conserved in all three genera (Supplementary Figure S1). Most notable among these were two blocks each consisting of 11 consecutive nucleotides conserved in all three genera (sites 22–32 and 80–90; Supplementary Figure S1). These extensive conserved blocks included both synonymous and nonsynonymous sites, accounting for the the reduced d_S in the female-specific exon. On the other hand, these blocks were not conserved in comparisons with species outside Tephritidae and Drosophilidae.

3.2. All Pairwise Comparisons

In all pairwise comparisons among 29 dipteran species, mean d_N in the male-specific exon (0.7039 ± 0.0496) was significantly greater than that in the common domain (0.2096 ± 0.0143) or that in the female-specific domain (0.0515 ± 0.0174; Z-tests; $P < 0.001$ in each case). Likewise, mean d_N in the common domain was significantly greater than that in the female-specific domain (Z-test; $P < 0.001$). Thus, these comparisons showed that purifying selection was strongest on the female-specific exon and most relaxed on the male-specific exon. Overall, 18 of 30 (60%) of amino acid sites encoded in the female-specific exon were conserved in all 29 species. By contrast, there was only one amino acid residue, a proline (at position 69 of 152 encoded by the male-specific exon of *D. melanogaster*), that was conserved in all 29 species.

When the pairwise d_N values in the female-specific exon were plotted against those in the common domain, there was a strong positive correlation ($r = 0.935$; $P < 0.001$; randomization test; Figure 2A). Likewise d_N values in the male-specific exon were positively correlated with those in the common domain ($r = 0.948$; $P < 0.001$; randomization test; Figure 2B). However, inspection of the plots showed that d_N in the male-specific exon increased much more rapidly as a function of d_N in the common domain than did d_N in the female-specific exon (Figure 2). When the method of allometric regression was applied to the relationship between d_N in the male-specific exon and d_N in the common domain, a slope of 2.562 ± 0.132 was obtained. This slope was significantly greater than zero ($P < 0.001$; Z-test) and significantly greater than 1.0 ($P < 0.001$; Z-test), indicating that d_N in the male-

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specific exon increased as a function of d_N in the common domain at a greater than linear rate. By contrast, the slope of the allometric regression of d_N in the female-specific exon on d_N in the common domain 0.294 ± 0.016. The latter value was significantly greater than zero ($P < 0.001$; Z-test) but significant less than 1.0 ($P < 0.001$; Z-test). Thus, d_N in the female-specific exon increased as a function of d_N in the common domain at a less than linear rate.

Discussion

Analysis of the pattern of nucleotide substitution of different domains of the dipteran *dsx* gene showed that over short evolutionary times purifying selection predominated on the domain common to both sexes, the female-specific exons, and the and male-specific exon. These patterns were seen in comparisons between closely related species pairs and indeed all comparisons within the families Tephritidae and Drosophilidae. However, over longer evolutionary time frames represented by between-family comparisons, the male-specific exon accumulated nonsynonymous substitutions at a much more rapid rate than either the common domain or the female-specific exon. Overall, the accumulation of nonsynonymous substitutions in the male-specific exon occurred at a significantly greater than linear rate relative to the common domain, whereas the accumulation of nonsynonymous substitutions in the female-specific exon occurred at less than linear rate relative to the common domain. The evolution of the male-specific exon of *dsx* thus shows a pattern reminiscent of that seen in the “runaway” evolution of male secondary sexual characters at the morphological level (Eberhard 1985). Assuming that amino acid changes in the sex-specific exons of *dsx* play a role in the evolution of genital morphology, the present results are consistent with the hypothesis that female choice is an important factor in the evolution of insect male genitalia.

The fact that the male-specific exon and the female-specific exon of *dsx* evolve at very different rates is consistent with the fact that female genital morphology is evolutionarily more conservative than male genital morphology, an observation often cited as evidence against the lock-and-key hypothesis (Eberhard 1985). On the other hand, the fact that *dsx* plays a role not only in the development of the genitals but also a role in the development of sex-specific behaviors (Rideout et al. 2007; Siwicki et al. 2009; Waterbury et al. 1999) is intriguing in the light of Mayr’s (1983) pleiotropy hypothesis for male genital evolution. Because of the dual role of *dsx*, changes in male genital structures, but not other morphological traits, might occur as a pleiotropic consequence of selection for changes in male sexual behavior, answering a major objection to Mayr’s theory (Eberhard 1985). However, it is not clear what would be the basis for selection specifically focused on the behavioral role of *dsx* but not on its role in genital development. The observed evolutionary pattern is suggestive of a role for sexual selection in the evolution of the male-specific exon of *dsx*, whether as a consequence of its role in the genitalia, or its role in the nervous system, or both.

Most discussions of the evolution of male traits by sexual selection have assumed that positive, directional selection drives the process (Fisher 1930; Kirkpatrick and Ryan 1991; Pomiankowski and Isawa 1998). On the other hand, Lande (1981) presented a quantitative genetic model in which a runaway process could be started if female preferences were subject to genetic drift. Here I describe a simple model (Figure 3) to illustrate how changes in the focus of female choice might drive a runaway process in the absence of positive selection. This model is consistent with the hypothesis of Nei (2007) that phenotypic divergences can result from the random fixation of neutral or nearly neutral mutations, in combination with changes over time in the focus of functional constraints.

Assume that females choose mates on the basis of certain traits (A, B, and C) of a given male structure (such as the genitalia), and that these traits are not essential to the biological

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function of the structure (Figure 3). Female choice focusing on A, B, and C will result in stabilizing selection at the phenotypic level and purifying selection on the genetic basis of A, B, and C. Certain other features of the structure in question that are neither necessary for its function nor used by the female in mate choice will be free to vary at random. As a result, mutations causing changes to these traits will not be deleterious, and some of them may be fixed by drift. Suppose that one such mutation is fixed so that the structure in question now has the properties A, B, C, and D in nearly all males of the species (Figure 3). Under these circumstances, a mutation that causes the female to choose on the basis of B, C, and D rather than A, B, and C will confer no fitness disadvantage and may become fixed by drift. If the latter happens, females will choose on the basis of B, C, and D; and the genetic basis of A will no longer be subject to purifying selection. A mutation that eliminates A will no longer be selected against and may be fixed by genetic drift (Figure 3). Thus, by this process the properties of a male structure used by the female for mate choice will be subject to runaway evolution in the total absence of positive selection, simply due to changes in the focus of the purifying selection resulting from female choice.

Contrary to a widespread impression, there is no unambiguous "signature" of positive Darwinian selection at the molecular level that can detect cases where an individual nonsynonymous substitution was selectively favored (Hughes 2007). Thus it is possible that some of the nonsynonymous substitutions occurring in the male-specific exon of *dsx* were fixed by positive selection. Nonetheless, the overall pattern of nucleotide substitution in the *dsx* gene suggests that positive selection has not played an important role in the evolutionary diversification of this gene. The pattern of nonsynonymous substitution in the male-specific exon of *dsx* suggests strong purifying selection over a short to intermediate term. However, over longer periods, a relaxation of purifying selection is detectable, giving rise to the positively allometric relationship between d_N in the male-specific exon and d_N in the common domain. Such a pattern is most consistent with the hypothesis that the male-specific exon of *dsx* is subject to purifying selection, but that the set of residues subject to strong purifying selection changes over evolutionary time. On this hypothesis, it is the shift over time in the focus of purifying selection that gives rise to the runaway evolution of the male-specific exon, consistent with the model outlined above.

Although there is evidence that amino acid changes in dsxM can cause changes in male genital morphology (Alvarez et al. 2009), the mechanistic basis of this effect is not known. Thus, it remains to be determined whether the evolution of dipteran male genital structures is driven by the runaway evolution of the male-specific exon of *dsx*. However, the hypothesis of a non-Darwinian runaway process presents an attractive explanation for the evolution of male genitalia in insects and many other groups of animals. This hypothesis is consistent with evidence that selection on insect male genital morphology in contemporary populations of insects and spiders is largely stabilizing (Eberhard et al. 1998); with the observation that certain structures of male insect genitalia are not needed for successful mating (Scudder 1971); and with the apparent arbitrariness of male traits used in female choice (Eberhard 1985). Moreover, this hypothesis provides an attractive alternative model for the evolution of sexually selected traits in general that does not require the problematic assumption of a genetic correlation between female preference and male trait postulated by Fisher's (1930) model and its successors (Kirkpatrick and Ryan 1991). In the future, formal population genetics models as well as computer simulations may shed further light on the proposed mechanisms outlined in the above verbal model.

In contrast to the male-specific exon of *dsx*, the female-specific exon shows extraordinary conservation. Of the 30 amino acid positions in the female-specific exon, 60% were conserved in all 29 dipteran sequences analyzed here. Moreover, the conservation of this exon was not confined to the amino acid sequences encoded; rather, there were blocks of

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sequence, including both synonymous and nonsynonymous sites, there were conserved in all sequences from Tephritidae and Drosophilidae. This conservation suggests that the female-specific exon may have functions beyond protein-coding, perhaps a regulatory function in splicing of the *dsx pre*-mRNA. Indeed, it has been shown that insertion mutations in this exon can affect sex-specific splicing in *Drosophila melanogaster* (Nagoshi and Baker 1990). The strong conservation of the female-specific exons contrasts with the evolution of the male-specific exon, consistent with the distinct biological roles of DsxF and DsxM proteins (Sánchez and Guerrero 2001).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
NJ tree of the common domain of dsx from 29 species of Diptera, based on the maximum composite likelihood distance. Numbers on the branches represent the percentages of 1000 bootstrap samples supporting the branch; only values ≥ 50% are shown.

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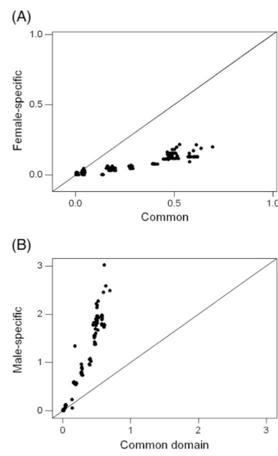

Figure 2.

The number of nonsynonymous substitutions per nonsynonymous site (d_N) in (A) the female-specific exon and (B) the male-specific exon (r = 0.948; P < 0.001; randomization test) plotted against d_N in the common domain for all pairwise comparisons among 29 dipteran species.

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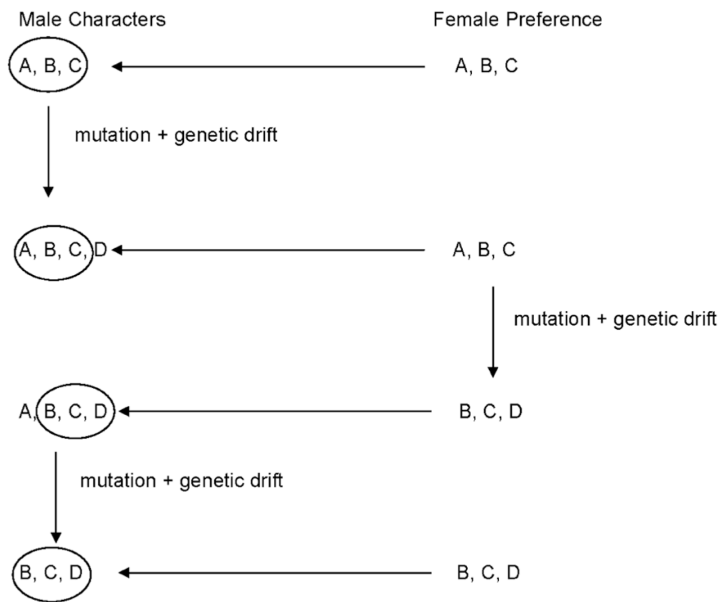

Figure 3.
Schematic illustration of a simple model whereby runaway evolution of traits used in mate choice by females can occur through a process of mutation and genetic drift, in the absence of positive Darwinian selection.

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Table 1

Sequences used in analyses.

Family	Species	Sequences
Tephritidae	*Anastrepha fraterculus* Sp1	DQ494334, DQ494344
	Anastrepha fraterculus Sp2	DQ494325, DQ494335
	Anastrepha fraterculus Sp3	DQ494326, DQ494336
	Anastrepha fraterculus Sp4	DQ494327, DQ494343
	Anastrepha amita	DQ494333, DQ494342
	Anastrepha serpentina	DQ494338, DQ494339
	Anastrepha obliqua	AY948420, DQ948421
	Anastrepha bistrigata	DQ494332, DQ494341
	Anastrepha striata	DQ494340, DQ494341
	Anastrepha sororcula	DQ494330, DQ494339
	Anastrepha grandis	DQ494328, DQ494337
	Bactrocera tryoni	AF029675, AF029676
	Bactrocera dorsalis	AY669317, FJ176944
	Bactrocera correcta	FJ185165, FJ185166
	Bactrocera oleae	AJ547621, AJ547622
	Ceratitis capitata	AF434087, AF434935
Drosophilidae	*Drosophila melanogaster*	NM_169202, NM_169203
	Drosophila erecta	XM_001979206, NW_001956552
	Drosophila sechellia	XM_002038714, NW_001999695
	Drosophila pseudoobscura	XM_001358983, NC_009005
	Drosophila persimilis	XM_002013310, NW_001985953
	Drosophila yakuba	XM_002086742, NT_167065
	Drosophila simulans	XM_002102506, NT_167061
	Drosophila ananassae	XM_001954765, NW_001939291
Muscidae	*Musca domestica*	AY461853, AY461854
Calliphoridae	*Lucilia cuprina*	GU784833, GU784834
Phoridae	*Megaselia scalaris*	AF283695, AF283696
Culicidae	*Anopheles gambiae*	XM_309601, XM560052
	Aedes aegypti	DQ440532, DQ440534

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Table 2

Numbers of synonymous (d_S) nucleotide substitutions per synonymous site and nonsynonymous (d_N) nucleotide substitutions per nonsynonymous site between sister pairs of *dsx* genes of Diptera.

Species comparison	Common		Female-specific		Male-specific	
	d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.
Anastrapha fraterculus Sp3 vs. *A. Serpentina*	0.0000 ± 0.0000	0.0031 ± 0.0031	0.0000 ± 0.0000	0.0147 ± 0.0148	0.0818 ± 0.0314	0.0085 ± 0.0060
Anastrepha fraterculus Sp2 vs. *A. Sororcula*	0.0256 ± 0.0068	0.0077 ± 0.0035	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0223 ± 0.0158	0.0042 ± 0.0043
Anastrepha striata vs. *A Bistrgiata.*	0.0144 ± 0.0083	0.0031 ± 0.0022	0.0000 ± 0.0000	0.0146 ± 0.0146	0.0225 ± 0.0160	0.0042 ± 0.0042
Batrocera tryoni vs. *B. Dorsalis*	0.1249 ± 0.0261	0.0070 ± 0.0034	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0458 ± 0.0231	0.0042 ± 0.0042
Drosophila pseudoobscura vs. *D. Persimilis*	0.0343 ± 0.0109	0.0011 ± 0.0011	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0256 ± 0.0149	0.0031 ± 0.0031
Drosophila yakuba vs. *D. erecta*	0.1330 ± 0.0229	0.0163 ± 0.0043	0.0000 ± 0.0000	0.0000 ± 0.0000	0.1482 ± 0.0511	0.0090 ± 0.0052
Drosophila simulans vs. *D. sechellia*	0.0351 ± 0.0112	0.0034 ± 0.0019	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0491 ± 0.0202	0.0030 ± 0.0030
Mean	0.0523 ± 0.0000	0.0060 ± 0.0020[a]	0.0000 ± 0.0000[b]	0.0042 ± 0.0027	0.0565 ± 0.0174	0.0052 ± 0.0010[a]

Paired t-tests of the hypothesis that mean d_S equals mean d_N:

[a] $P < 0.05$.

Paired t-tests of the hypothesis that mean d_S or d_N equals the corresponding value for the common domain:

[b] $P < 0.05$.

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Table 3

Mean numbers of synonymous (d_S) nucleotide substitutions per synonymous site and nonsynonymous (d_N) nucleotide substitutions per nonsynonymous site in *dsx* genes in comparisons within and between genera of Tephritidae and Drosophilidae.

Comparison	Common		Female-specific		Male-specific	
	d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.
Within genera						
Anastrepha	0.0428 ± 0.0083	0.0067 ± 0.0016[d]	0.0000 ± 0.0000[g]	0.0053 ± 0.0039	0.4361 ± 0.0797	0.0549 ± 0.0116[d]
Bactrocera	0.1622 ± 0.0210	0.0088 ± 0.0028[d]	0.0000 ± 0.0000[g]	0.0074 ± 0.0073	0.1276 ± 0.0314	0.0148 ± 0.0058[d]
Drosophila	0.5891 ± 0.0375	0.0375 ± 0.0069[d]	0.0786 ± 0.0433[g]	0.0000 ± 0.0000[g]	0.4914 ± 0.0812	0.0300 ± 0.0085[d]
Between genera						
Anastrepha vs. *Bactrocera*	0.9708 ± 0.1312	0.0351 ± 0.0208[d]	0.2817 ± 0.1713[f]	0.0055 ± 0.0042	0.7984 ± 0.1807	0.1052 ± 0.0237[d, e]
Anastrepha vs. *Drosophila*	--[a]	0.1692 ± 0.0208	0.8113 ± 0.4083	0.0481± 0.0253[g]	2.4335 ± 0.6452	0.5684 ± 0.0833[c, g]
Bactrocera vs. *Drosophila*	--[a]	0.1807 ± 0.0216	1.2927 ± 0.5395	0.0338 ± 0.0213[b,g]	2.4357 ± 0.5142	0.5582 ± 0.0795[d, g]

[a]The quantity could not be estimated because of saturation of synonymous sites.

Z-tests of the hypothesis that mean d_S equals mean d_N:

[b]P < 0.05;

[c]P < 0.01;

[d]P < 0.001.

Z-tests of the hypothesis that mean d_S or d_N equals the corresponding value for the common domain:

[e]P < 0.05.;

[f]P < 0.01;

[g]P < 0.001.