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Evolving *doublesex* expression correlates with the origin and diversification of male sexual ornaments in the *Drosophila immigrans* species group

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Abstract

Male ornaments and other sex-specific traits present some of the most dramatic examples of evolutionary innovations. Comparative studies of similar but independently evolved traits are particularly important for identifying repeated patterns in the evolution of these traits. Male-specific modifications of the front legs have evolved repeatedly in Drosophilidae and other Diptera. The best understood of these novel structures is the sex comb of *Drosophila melanogaster* and its close relatives. Here, we examine the evolution of another male foreleg modification, the sex brush, found in the distantly related *Drosophila immigrans* species group. Similar to the sex comb, we find that the origin of the sex brush correlates with novel, spatially restricted expression of the *doublesex* (*dsx*) transcription factor, the primary effector of the *Drosophila* sex determination pathway. The diversity of Dsx expression patterns in the *immigrans* species group closely reflects the differences in the presence, position, and size of the sex brush. Together with previous work on sex comb evolution, these observations suggest that tissue-specific activation of *dsx* expression may be a common mechanism responsible for the evolution of sexual dimorphism and particularly for the origin of novel male-specific ornaments.

INTRODUCTION

Some of the most striking examples of morphological variation can be seen in the differences between males and females of the same species. Even among closely related animals, sex-specific characters can differ dramatically. This simple observation implies that new sexual characters are gained, and old ones are lost, during the evolution of any animal lineage. Elucidating the genetic basis of these changes is essential to understand the origin of sexual dimorphism and biodiversity (Arnoult et al., 2013; Gompel, Prud'homme, Wittkopp, Kassner, & Carroll, 2005; Gotoh et al., 2014; Kijimoto, Moczek, & Andrews, 2012; Kopp, Duncan, & Carroll, 2000). Going beyond individual examples, we need to identify general patterns in the evolution of sexual dimorphism. How predictable are the genetic changes that give rise to new sex-specific traits? This question places a premium on research metamodels where similar traits have evolved independently in multiple lineages, each of which is amenable to experimental study (Kopp 2009).

The forelegs of true flies (Diptera) often show dramatic male-specific modifications (Hardy 1965; Sivinski 1979; Eberhard 2001; Ingram et al 2007; Stark & O'Grady, 2010; Daugeron, Plant, Winkler, Stark, & Baylac, 2011). Structures composed of modified bristles are especially common and have evolved independently in many Dipteran lineages. In the family *Drosophilidae* alone, examples include a variety of bristle brushes and bristle-filled spoon structures in Hawaiian *Drosophila* (Stark & O'Grady, 2010), pincushion-like outgrowths covered with fine bristles in *Zaprionus* (Tsakas and Chassagnard 1990; Chassagnard and Tsacas 1993), the thick, often massive "sex combs" in the *Drosophila melanogaster* and *obscura* species groups (Kopp, 2011; Hanna-Alava, 1958), and the densely packed bristle brush in *Drosophila immigrans* (Sturtevant, 1942) and its relatives (Figure 1). Repeated origin of male-specific bristle modifications in the same body region (most commonly, the tarsi of the front legs) suggests that across Diptera, the forelegs have been a target of sexual selection. It is theorized that ancestral Neoptera mated with females perched on top of males (Huber et al., 2007; McAlpine, 1981). This position places the male foreleg on the substrate, allowing it less opportunity to interact with the female. In many Diptera, however, there has been a shift to a mating posture with the male on top the female, allowing the male forelegs to interact with and potentially grasp the female. This shift in mating position may have contributed to the repeated evolution of sexually dimorphic structures in the Dipteran forelegs.

Independent evolution of morphologically distinct male-specific structures from homologous tissues presents an excellent opportunity to investigate the general rules that may govern the origin of novel sex-specific traits. The best studied male foreleg modification is the sex comb of the *Drosophila melanogaster* and *obscura* species groups: a regular array of enlarged bristles on the first and second tarsal segments (Figure 1). Sex combs play an important role in male courtship and mating success (Hurtado-Gonzales, Gallaher, Warner, & Polak, 2015; Ng & Kopp, 2008; Spieth, 1952). They develop as a male-specific modification of deeply conserved, sexually monomorphic transverse bristle rows (TBRs) that cover the ventral-anterior side of the foreleg (Tokunaga, 1962; Kopp, 2011). Like most other sexually dimorphic external structures in *Drosophila*, sex comb development requires the function of the gene *doublesex* (*dsx*), a transcription factor that acts downstream of the sex determination pathway to control sex-specific cell differentiation (Baker & Ridge, 1980; Hildreth, 1965). *dsx* expression in the foreleg is activated at the late larval and early pupal stage by *Sex combs reduced* (*Scr*), the HOX gene that controls the identity of the first thoracic segment (Tanaka, Barmina, Sanders, Arbeitman, & Kopp, 2011). In males, the male-specific isoform of Dsx upregulates *Scr*; the resulting feedback loop stabilizes the expression of both genes, which are jointly required to specify sex comb position and morphology. Females express a different Dsx isoform, which does not activate *Scr* and is not capable of inducing sex comb development (Tanaka et al., 2011).

In *Drosophila*, *dsx* is only expressed in cells that undergo sex-specific development (Camara, Whitworth, & Doren, 2008; Robinett, Vaughan, Knapp, & Baker, 2010). Expression in the male foreleg is restricted to the presumptive sex comb and sex-specific chemosensory organs (Tanaka et al., 2011; Mellert, Robinett, & Baker, 2012). In species that primitively lack sex combs, Dsx is not expressed in the homologous leg region, indicating that the origin of the sex comb coincides with the evolution of a novel *dsx* expression

domain (Tanaka et al., 2011). Given the central role of *dsx* in sexual development, this suggests a general model where changes in the spatial regulation of *dsx* are often a necessary first step in the evolution of sexual dimorphism (Kopp, 2012). For a new sex-specific structure to evolve in ancestrally monomorphic tissue, *dsx* expression must first be turned on in that tissue. Subsequent diversification in the size, position, and morphology of the new structure is likely to require further evolutionary changes in *dsx* regulation.

To test the generality of this mechanism, we decided to apply the metamodel approach (Kopp, 2009) by focusing on another male-specific foreleg modification that evolved independently in a distantly related group of *Drosophila*. The *immigrans* and *melanogaster* species groups diverged >20 million years ago (Izumitani, Kusaka, Koshikawa, Toda, & Katoh, 2016; Russo, Mello, Frazão, & Voloch, 2013). The “sex brush” of *D. immigrans* develops in the same leg region as the sex comb (ventral-anterior surface of the first and second tarsal segment of the male foreleg), but has a very different morphology (Markow & O’Grady Patrick M, 2006; Spieth, 1952; Wilson, Wheeler, Harget, & Kambysellis, 1969) (Figure 2). As expected of a sexually dimorphic structure, the cells that produce the sex brush also express Dsx (Tanaka et al., 2011). Here, we use phylogenetic analysis to reconstruct sex brush origin and diversification, and test for correlation between morphological evolution and the evolution of Dsx expression. We find that, similar to the sex comb, the origin of the sex brush coincides with the appearance of a new domain of Dsx expression, and that Dsx patterns in the developing forelegs correlate closely with the diversity of sex brush morphologies across species.

MATERIALS AND METHODS

Drosophila Strains:

To study sex brush evolution in the *immigrans* species group, we used the following subset of described species, representing all major subgroups: *D. immigrans* (Sturtevant, 1921), *D. formosana* (Duda, 1926; Sturtevant, 1927), *D. ruberrima* (De Meijere, 1911), *D. hypocausta* (Osten Sacken, 1882), *D. siamana* (Hihara & Lin, 1984; Ikeda, Hihara, Asada, Fujiwara, & Lin, 1983), *D. rubida* (Mather, 1960), *D. neohypocausta* (Lin & Tseng, 1973), *D. nasuta* (Lamb, 1914), *D. albomicans* (Duda, 1926), *D. sulfurigaster* (Duda, 1926), and *D. kepulauana* (Wilson et al., 1969). *D. quadrilineata* (De Meijere, 1911) was used as an outgroup. Stocks were obtained from both the Ehime University *Drosophila* Stock Center (*D. albomicans* KM55–5, *D. sulfrigaster* 5112-M, *D. formosana* 292.8 (E-13401), *D. ruberrima* OKNH8 (E-14601), *D. rubida* 23.01 CAR-316, *D. siamana* 1741.00-TC (E-22701), *D. neohypocausta* 1881.02-TC (E-22501), *D. quadrilineata* TMU (E14402) and the UC San Diego *Drosophila* Stock Center (*D. immigrans* 15111–1731.08, *D. hypocausta* 15115–1871.05, *D. kepulauana* 15112–1761.03, *D. nasuta* 15112–1781.00).

Phylogenetic Reconstruction:

We used partial genomic sequences of nine nuclear loci: *Alcohol dehydrogenase* (*Adh*), *Dopa decarboxylase* (*Ddc*), *extra sexcombs* (*esc*), *kinase suppressor of ras* (*ksr*), *Phospho-glucose isomerase* (*Pgi*), *Triose phosphate isomerase* (*Tpi*), *Xanthine dehydrogenase* (*Xdh*), *Amylase-related* (*Amyrel*), and *Glycerol 3 phosphate dehydrogenase* (*Gpdh*)

(Supplementary Table 1). Some of the sequences used in this study were generated by previous phylogenetic and population-genetic studies (Da Lage et al., 2007; Huang et al., 2002; Katoh, Nakaya, Tamura, & Aotsuka, 2007) and were obtained from GenBank. Sequences of *D. albomicans*, *D. nasuta*, *D. sulfurigaster*, and *D. immigrans* were extracted from the whole genome sequences of these species, kindly provided by Doris Bachtrog and Michael Eisen. The remaining sequences were amplified using the primers shown in Supplementary Table 2; these primers were modified from a previous study of the *melanogaster* species group phylogeny (Kopp, 2006).

DNA was extracted from 20 flies of each species using live strains maintained in our laboratory. Voucher specimens have been preserved in ethanol. Amplified DNA was either gel-purified and sequenced directly using the amplification primers (Supplementary Table 2) or ligated into pCRII (Invitrogen) and sequenced using the vector forward and reverse primers M13F and M13R. ABI chromatograms were examined by eye. Heterozygous nucleotide positions, if present, were represented by IUPAC ambiguity codes. Sequences were aligned and tiled using MUSCLE (Edgar 2004) through the program Geneious (Kearse et al., 2012). The lengths of available sequences varied from species to species, and the missing nucleotide positions at the ends of fragments were coded as gaps. To ensure correct alignments, all coding sequences were translated into proteins. Exons were determined by alignment to *D. melanogaster* cDNA. Due to difficulties in establishing homology, intronic sequences were removed from the dataset. The edited alignments were then partitioned by codon for analyses.

A published *Adh* sequence from *D. siamana* (GenBank accession AY044125) was found to closely resemble *D. albomicans*, which conflicted with the rest of our loci. We re-sequenced this locus from our stock *of D. siamana*. The resulting sequence is similar to the one found in Izumitani et al., 2016, GenBank accession AB261135. Our sequence was used as the representative sequence of *D. siamana* in further analysis.

In order to determine the best nucleotide substitution models, we used the program Partitionfinder (Lanfear, Calcott, Ho, & Guindon, 2012). Most of the codon partitions fit a GTR+G substitution model; while *Adh* codon position 2, *Amyrel* codon position 2, *Ddc* codon position 3, *Pgi* codon position 2, *Tpi* codon position 2, *Xdh* codon position 2 fit a TVM+I+G substitution model; *GpDH* codon position 2, *esc* codon position 2 fit a F81 substitution model; and *GpDH* codon position 3, *ksr* codon position 3 fit a HKY+G substitution model. *D. quadrilineata* has been repeatedly found to be basal, or closely related, to the *immigrans* lineage, making it a suitable outgroup (Da Lage et al., 2007; Huang et al., 2002; Izumitani et al., 2016; Katoh et al., 2007; Yan, Zeng, Yang, & Qian, 2006). Partitioned alignments were analyzed using BEASTv1.8.0 (Drummond, Suchard, Xie, & Rambaut, 2012) or MrBayes v3.2.1 (Ronquist et al., 2012), BEAST ran for 100,000,000 generations with the first 10,000,000 generations discarded. Trees were sampled every 1,000 generations (.xml file in Supplementary Text 1). MrBayes ran for 10,000,000 generations with 4 chains. Trees were sampled every 200 generations. The logs were analyzed in Tracerv1.6; for all parameters, the Effective Sample Sizes (ESS) were over 4000. Visual inspection of the traces indicated good mixing, and analysis of several independent chains showed convergence in tree topology and ancestral state reconstruction.

We used BayesTraits Version 3 (Pagel, Meade, & Barker, 2004), with the tree generated above, to test for correlation between the sex brush and Dsx expression. We performed three runs of both the independent and dependent models with a stepping stone model (250 stones each sampled 10,000 iterations). We averaged the log marginal likelihood from ten runs and calculated the Log Bayes Factors for these two models.

Immunohistochemistry and Microscopy:

Samples for immunohistochemistry were synchronized at pupariation, aged for 24 hours or longer depending on the species-specific rate of development, and processed and imaged as described in Mellert et al., 2012 and Tanaka et al., 2011. The primary antibodies used were rat anti-DsxCommon, 1:50 (Sanders and Arbeitman 2008), monoclonal mouse anti-Dsx[DBD], 1:10 (a gift from C. Robinett and B. Baker, Janelia Farm Research Campus, HHMI and available from the Developmental Studies Hybridoma Bank at the University of Iowa, DsxDBD), and mouse anti-Scr 6H4.1, 1:10 (Developmental Studies Hybridoma Bank, University of Iowa). The secondary antibodies were AlexaFluor 488 and 594, used at 1:200 (Invitrogen, Carlsbad, CA). Confocal images were collected on an Olympus FV1000 laser scanning confocal microscope with a 40X lens, with the gain adjusted for the dynamic range of each sample. In species where no Dsx expression was found, the gain was increased to detect even very weak signal, leading to higher background intensity in these samples.

For light microscopy, adult legs were dissected in water and mounted in PVA Mounting Medium (BioQuip) until fully cleared. Images were taken under bright field illumination with a 20X lens on a Leica DM500B microscope with a Leica DC500 camera. For scanning electron microscopy, adult flies were dehydrated in 100% ethanol, processed by critical point drying, coated with gold, and imaged on a Philips XL30 SEM microscope.

RESULTS

The Drosophila immigrans male-specific sex brush is composed of densely packed, hook-shaped bristles.

Although several studies have mentioned modified bristles on the foreleg of *D. immigrans* males (Spieth, 1952; Sturtevant, 1942), neither the morphology nor the interspecific diversity of the sex brush have been examined previously. In *D. immigrans* females, the chaetotaxy of the first tarsal segment of the foreleg is very similar to other *Drosophila* species. The ventral-anterior side of this segment is covered by regularly spaced transverse bristle rows (TBRs), which consist of bracted mechanosensory bristles (Figure 2A). In *D. immigrans* males, the sex brush replaces most of the TBR field, leaving only a few normal bristles in the most proximal TBRs. In addition, the sex brush occupies the ventral-anterior side of the second tarsal segment (Figure 2B). In contrast to females, the bristles comprising the male sex brush are not organized into linear rows, lack bracts, and are packed extremely densely: on the first tarsal segment, there are ~265 sex brush bristles in males, compared to ~55 TBR bristles in females. While the TBR bristles are straight with pointed tips, the much thinner sex brush bristles become curly and hooked near the tip (Figure 2C).

The sex brush of D. immigrans develops from an area of Dsx and Scr co-expression.

The sex comb of *D. melanogaster* and its relatives is established by coexpression of the HOX gene *Sex combs reduced* (*Scr*) and the sex determination gene *doublesex* (*dsx*) (Tanaka et al., 2011). Similarly, in the forelegs of *D. immigrans* male pupae, we find that Scr and Dsx expression patterns overlap on the ventral-anterior side of the first and second tarsal segments, where the sex brush will develop (Figure 3A–C). At the proximal end of the first tarsal segment, Scr expression is seen without Dsx, presumably corresponding to the remaining sexually monomorphic TBR bristles. Although Scr is expressed in a wider region than Dsx, Scr levels are highest in the Dsx expression domain (Figure 3A–C). In *D. albomicans*, an *immigrans* group species that lacks the sex brush and has unmodified TBRs in both sexes, we do not observe strong Dsx expression and, in contrast to *D. immigrans*, Scr expression is much weaker in the tarsus than in the distal tibia (Figure 3D–F). This observation suggests that, similar to the sex comb (Tanaka et al., 2011), Scr and Dsx co-expression in *D. immigrans* males may be maintained by a positive feedback loop and is required for the development of the male sex brush.

Revised phylogeny of the immigrans species group

The independent origin of the sex comb and sex brush in distantly related *Drosophila* lineages allows us to test whether novel Dsx expression is generally necessary for the gain and diversification of a new sex-specific trait. Our survey revealed extensive variation in the presence and morphology of the sex brush in the *immigrans* species group. Although previous studies have investigated phylogenetic relationships in this group, most of these studies used small non-overlapping datasets and produced conflicting results (Da Lage et al., 2007; Huang et al., 2002; Izumitani et al., 2016; Katoh et al., 2007; Yan et al., 2006). The branching order of the *immigrans, nasuta* and *hypocausta* subgroups, and the placement of *D. ruberrima* and *D. neohypocausta*, have remained controversial. Since resolving these relationships is essential for reconstructing sex brush evolution, we undertook a more robust phylogenetic analysis.

We generated an updated phylogeny using nine loci from twelve *immigrans* group species using Bayesian inference in BEAST (Drummond et al., 2012) and MrBayes (Ronquist et al., 2012) (Figure 4). Our analysis resulted in a fully resolved and well supported tree with posterior probabilities over 99% at all nodes. This tree identifies three lineages in the *immigrans* species group: the *nasuta* subgroup, including *D. nasuta, D. albomicans, D. kepulauana*, and *D. sulfurigaster*; the *immigrans* subgroup, including *D. immigrans* and *D. formosana*; and the *hypocausta* subgroup proper, including *D. hypocausta, D. siamana*, and *D. rubida*. Although *D. ruberrima* is traditionally assigned to the *immigrans* subgroup (Wilson et al., 1969), our analysis places it as sister to the *nasuta* subgroup (Node A, Figure 4). *D. neohypocausta*, described originally as part of the *hypocausta* subgroup (Osten Sacken, 1882), is more closely related to the *nasuta* subgroup and *D. ruberrima* than to the *hypocausta* subgroup (Node B, Figure 4). The *nasuta* and *immigrans* subgroups cluster together (Node C, Figure 4), followed by the branching of the *hypocausta* subgroup (Node D, Figure 4), and finally *D. quadrilineata* in the most basal position.

Sex brush diversity in the immigrans species group

We defined a species as having a sex brush if the foreleg TBRs contained at least a few male-specific bractless bristles. By this lenient definition, only the *nasuta* species subgroup and *D. quadrilineata* lack a sex brush (Figure 5); these species do not show overt sexual dimorphism in the morphology of TBRs (Supplementary Figure 1). However, all examined species show different degrees of sex-specificity in the number and size of chemosensory bristles, (Supplementary Figure 1), indicating that the front leg has the developmental capacity for sexual dimorphism. In the remaining *immigrans* group species, sex brushes vary widely in size and morphology (Figure 5). The largest and most densely packed sex brushes are found in the *immigrans* subgroup, *D. rubida* (*hypocausta* subgroup), and in *D. ruberrima*, which is sister to the brush-less *nasuta* subgroup. In these species, the sex brush replaces most of the TBRs in males. In *D. hypocausta*, *D. siamana*, and *D. neohypocausta*, sex brushes are smaller and look more similar to normal TBRs, but contain some supernumerary, irregularly arranged, bractless bristles. *D. neohypocausta* and *D. siamana* have male-specific bristles in only a subset of 3–5 TBRs (Supplementary Figure 1). In closely related species, the sex brush differs in both position and size. For example, the sex brush occupies first and second tarsal segments in *D. immigrans*, but is only found on the first segment of its sister species, *D. formosana*. In the *hypocausta* subgroup, *D. hypocausta* has small sex brushes on the first and second tarsal segments, while *D. rubida* has a very well developed sex brush but only on the first segment (Figure 5). Neither the species with the largest sex brushes, nor the ones with the smallest, cluster together. Rather, each clade within the *immigrans* species group shows a diversity of sex brush types.

Dsx expression correlates with sex brush origin and diversification

To test whether Dsx expression correlates with sex brush morphology, we stained the male pupal forelegs of *immigrans* group species with an antibody against the highly conserved DM domain of Dsx (Mellert et al., 2012). To confirm cross-reactivity, we stained the larval testes of several *immigrans* group species, and found Dsx expression similar to that in *D. melanogaster* (data not shown). Dsx expression in the leg corresponds well to the size and position of the sex brush (Figure 5). In species that lack a sex brush (the *nasuta* subgroup and *D. quadrilineata*), no Dsx expression is seen. In species with sex brushes on both first and second tarsal segments, Dsx is expressed in both segments (Figure 5). In species with a sex brush on only the first tarsal segment, Dsx expression is also confined to that segment (Figure 5). The size of the Dsx expression domain within the first tarsal segment also correlates with the size of the sex brush. For example, Dsx expression is most extensive in *D. immigrans, D. rubida*, and *D. ruberrima*, and much more restricted in *D. hypocausta* and *D. siamana* (Figure 5). The one exception is *D. neohypocausta*, where Dsx expression is present in both first and second tarsal segments, but only a few weakly differentiated male-specific bristles are seen in the first segment, and none in the second. This pattern may be related to an early specification of male-specific chemosensory bristles, which are especially large in this species (Supplementary Figure 1).

We used BEAST (Drummond et al., 2012) to estimate ancestral character states across the *immigrans* species group phylogeny, while accommodating for uncertainty in tree topology (Figure 6). Our analysis indicates strong but not overwhelming probability (87%) that the

last common ancestor of the *immigrans* species group had a male sex brush and associated Dsx expression (Node D, Figure 6). Although the phylogenetic distribution of the sex brush could in principle be explained either by multiple gains or by a single gain followed by reduction and loss, the latter model appears significantly more likely. In particular, the *nasuta* subgroup probably lost the sex brush secondarily.

We then used BayesTraits (Pagel et al., 2004) to test for correlation between the presence of the sex brush and Dsx expression. We found strong evidence that the sex brush and Dsx expression were correlated (Log Bayes Factor of 7.74). This suggests that Dsx expression is indeed associated with sex brush evolution – and, more broadly, supports our model that changes in Dsx expression are vital to the origin and loss of sexually dimorphic traits.

DISCUSSION:

To search for general patterns in the evolution of sexually dimorphic traits, we focused on the evolution of *doublesex* expression. *dsx* is the primary transcription factor responsible for inducing sex-specific differentiation of somatic cells in insects (Kopp, 2012). For example, *dsx* has been implicated in the development and evolution of sexually dimorphic traits such as male horns in the *Onthophagus* dung beetles (Kijimoto et al., 2012; Ledón-Rettig, E, Zattara, & Moczek, 2017) (Kijimoto et al., 2012; Ledón-Rettig et al., 2017) and *Trypoxylus dichotomus* (Ito et al., 2013), exaggerated mandibles in the stag beetle *Cyclommatus metallifer* (Gotoh et al., 2014), reduced male wings in the jewel wasp *Nasonia vitripennis* (Loehlin et al., 2010), the genital morphology of *Bombyx mori* (Xu et al., 2017), and sex-specific mimetic color patterns in *Papilio* swallowtail and *Agraulis vanilla* butterflies (Komata, Lin, Iijima, Fujiwara, & Sota, 2016; Kunte et al., 2014; Martin et al., 2014; Nishikawa et al., 2015).

Earlier work on the sex combs of *D. melanogaster* and its relatives suggested a model where the origin of a new sex-specific structure in a previously monomorphic tissue depends on evolutionary changes in the spatial regulation of *dsx* (Tanaka et al., 2011). Given the central role of *dsx* in sexually dimorphic cell differentiation, for any sex-specific structure to evolve, Dsx must be expressed in the cells that give rise to this structure. In tissues that already express Dsx, sexual dimorphism can evolve with relative ease if *dsx* acquires new downstream targets. In contrast, if a tissue is ancestrally monomorphic and does not express Dsx, changes in *dsx* regulation that result in its de novo expression in that tissue must be a necessary first step.

Here, we tested the generality of this model by focusing on a different sex-specific innovation – the sex brush that evolved in the *Drosophila immigrans* species group, which is distantly related to *D. melanogaster*. Similar to sex combs, we found abundant phenotypic diversity and a strong correlation between Dsx expression and sex brush morphology (Figure 4). In *D. quadrilineata*, the most basal species that lacks a sex brush, Dsx is not expressed in the foretarsal TBRs. In another similarity with sex combs, the foreleg-specific HOX gene *Scr* is coexpressed with Dsx and upregulated in the developing sex brush. Thus, it appears that independent evolution of male-specific foreleg modifications may have a

similar genetic basis in distantly related lineages. We predict that a similar pattern will be found in other Dipteran families.

One of the striking features of the *D. immigrans* sex brush is the hooked shape of the bristles. Although the selective forces that led to this bristle morphology are unknown, *D. immigrans* does not go through some of the stereotypical courtship steps found in most other *Drosophila*, such as licking. Instead, after circling and tapping the female's abdomen, the male lunges at the female, grabbing her abdomen with his forelegs, and attempts to inseminate the female (Spieth, 1952). Spieth conjectured that modified foreleg bristles in *D. immigrans*, as well as other foreleg modifications found throughout *Drosophila*, enable males to grasp the female more effectively during courtship.

This function may explain the frequent origin of new male-specific structures on the forelegs of *Drosophila* and other Diptera. For example, male flies in the genus *Zaprionus* have pincushion-like outgrowths on their front tarsi, covered with thin, densely packed bristles reminiscent of the sex brush bristles of *D. immigrans* and its relatives (Figure 1). These structures also express Dsx (Tanaka et al., 2011). Like *D. immigrans, Zaprionus* species have shortened courtship compared to other Drosophilidae (Spieth, 1952). The phylogenic position of *Zaprionus* within Drosophilidae is contentious, with some studies suggesting that *Zaprionus* and the *immigrans* species group are closely related (Yassin et al., 2010). If so, it is possible that the modified foreleg bristles of *Zaprionus* and the *immigrans* group, and the associated domains of Dsx expression, share a common origin.

The sex comb and sex brush share similar positions on the foreleg; this allows us to investigate the genetic constraints on establishing new sex-specific structures. However, the morphology of male-specific bristles differs greatly between the comb and the brush. Identifying the downstream targets of *dsx* in these two structures may illuminate the genetic and cellular pathways that transform a deeply conserved bristle type (TBRs) into two radically different shapes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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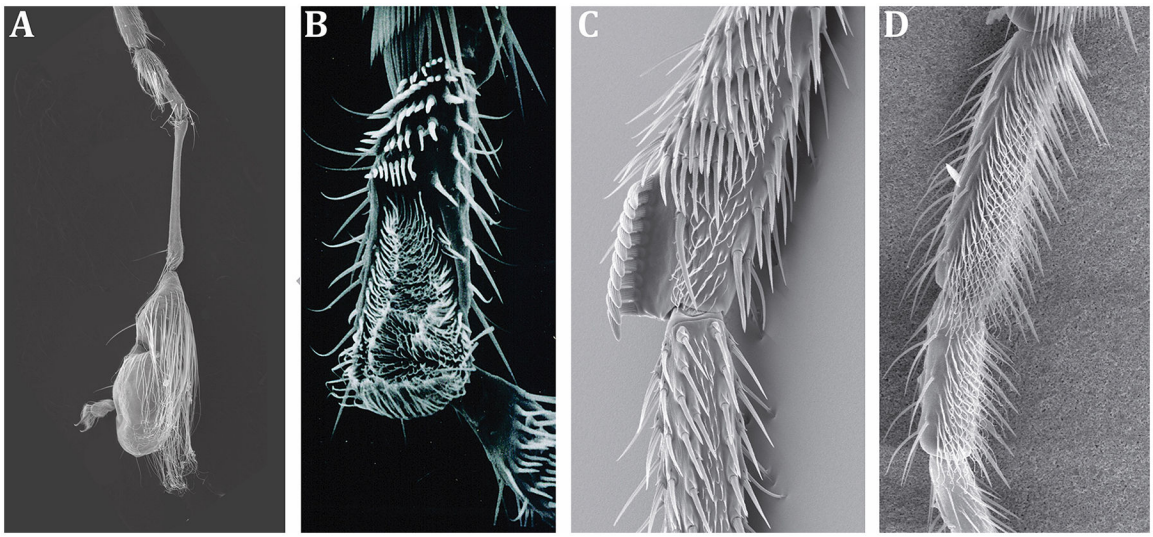

Figure 1. Many groups of *Diptera* have independently evolved diverse male-specific ornaments on front legs.

Scanning electron micrographs of adult male forelegs of **A)** *Empis (C.) jaschhoforum* (Daugeron et al., 2011), **B)** *Zaprionus inermis* (Museum of Paris), **C)** *Drosophila melanogaster* (Kopp & True, 2002), **D)** *Drosophila immigrans*. Reproduced with permission from Daugeron et al., 2011; Stark & O'Grady, 2010; and the Museum of Paris.

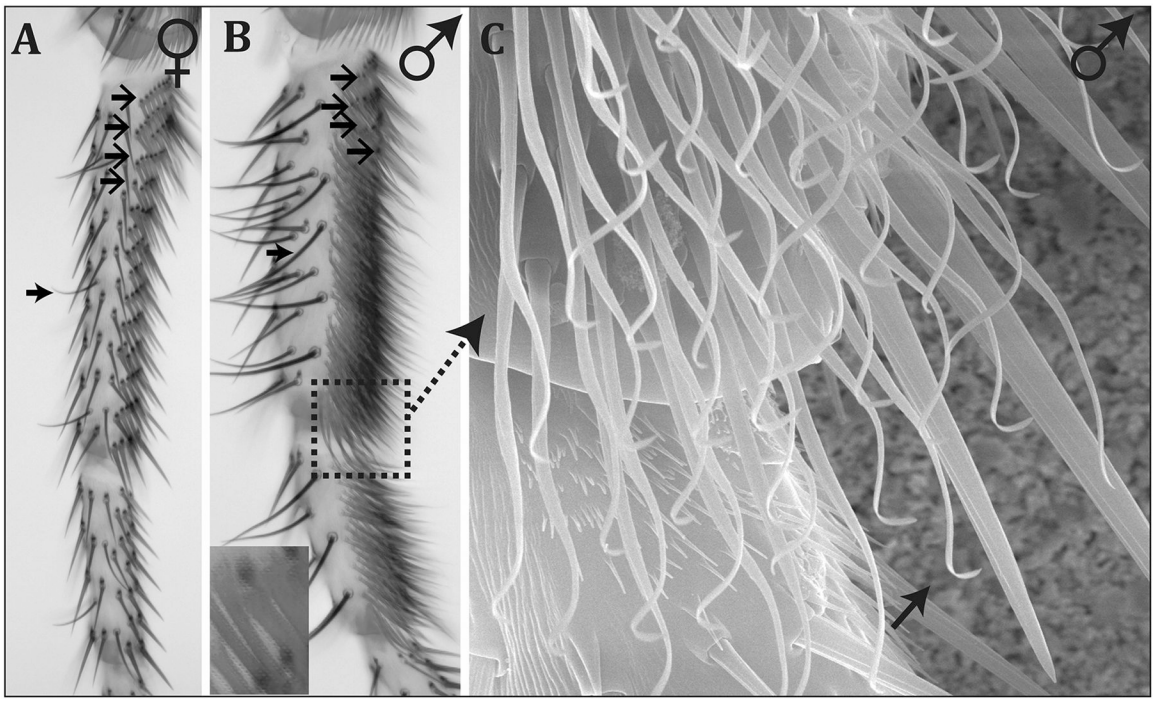

Figure 2. The male-specific sex brush of *D. immigrans* is composed of densely packed, hook-shaped bristles.

First and second tarsal segments of *D. immigrans* female (A) and male (B, C) forelegs. **A)** The first tarsal segment of *D. immigrans* females is covered by transverse bristle rows (TBRs, open face arrows). Anterior/dorsal (left) of the TBRs are chemosensory bristles (closed face arrow). Note that all TBR bristles have bracts. **B)** In males, the ventral/anterior surfaces of the first and second tarsal segments are mostly covered by the sex brush, which is composed of densely packed, bractless bristles with hooked tips. The most proximal (top) rows of bristles are composed of a mix of TBR (open faced arrows) and sex brush bristles (inset). Chemosensory bristles (closed face arrow) are larger and more numerous in males. The dashed box indicates the region shown in panel C. **C)** SEM image of the distal first tarsal segment showing the sex brush. The arrow highlights a single sex brush bristle, hooked at the distal end.

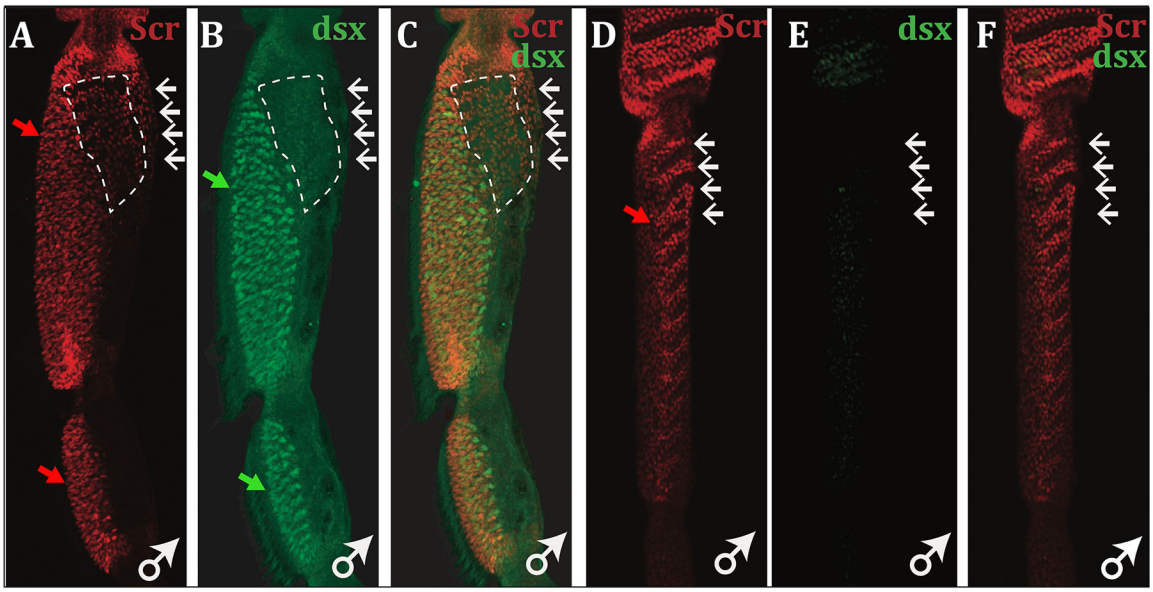

Figure 3. Overlapping Scr and Dsx expression in the sex brush primordium

The first and second tarsal segments of the pupal male forelegs are shown. *Scr* (red) is expressed in both TBR and sex brush regions, while Dsx expression (green), assayed with the anti-DsxCommon antibody (Sanders & Arbeitman, 2008), is limited to the sex brush. Expression of Dsx and Scr are highlighted by closed face green and red arrows respectively. As in the sex combs of *D. melanogaster* and its relatives (Tanaka et al., 2011), Dsx is expressed predominantly in bristle precursor cells, whereas Scr is present mainly in the surrounding epithelial cells. A-C) *D. immigrans* male pupal forelegs at 48 hrs after pupariation. Scr expression that does not overlap Dsx likely represents the proximal TBR bristles (open faced arrow). Scr expression is lower in regions where Dsx is absent (dashed white box). D-F) *D. albomicans* male pupal forelegs at 30 hrs after pupariation. Scr expression can still be seen in the TBRs, but Dsx is not expressed in the corresponding region.

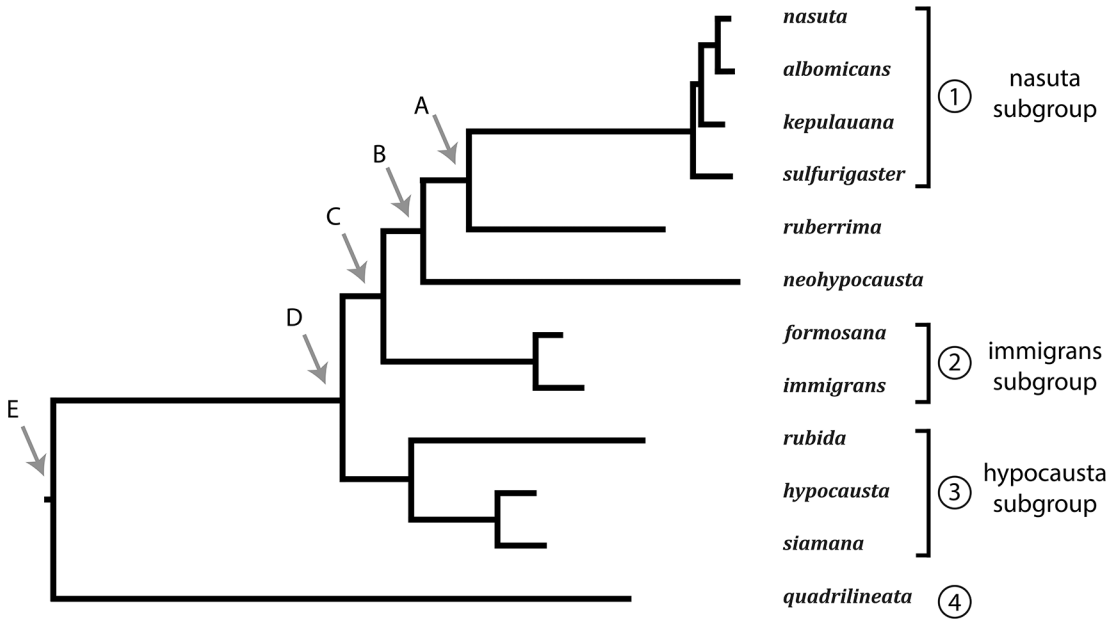

Figure 4. Phylogeny of the *Drosophila immigrans* species group.

Phylogenetic tree produced by Bayesian analysis in MrBayes. Posterior probabilities at all nodes are >99%. This tree identifies three monophyletic clades: *nasuta* subgroup (1), *immigrans* subgroup (2), and *hypocausta* subgroup proper (3). Although *D. ruberrima* is traditionally assigned to the *immigrans* subgroup (De Meijere, 1911), our analysis places it as sister group to the *nasuta* subgroup (Node A). *D. neohypocausta* was originally described as part of the *hypocausta* subgroup (Osten Sacken, 1882), but here it is also placed closer to the *nasuta* subgroup (node B). The *immigrans* subgroup forms a monophyletic clade with the *nasuta* subgroup + *D. ruberrima* + *D. neohypocausta* (node C), and the *hypocausta* subgroup occupies the most basal position in the *immigrans* species group (node D). *D. quadrilineata* was used as an outgroup to root the tree.

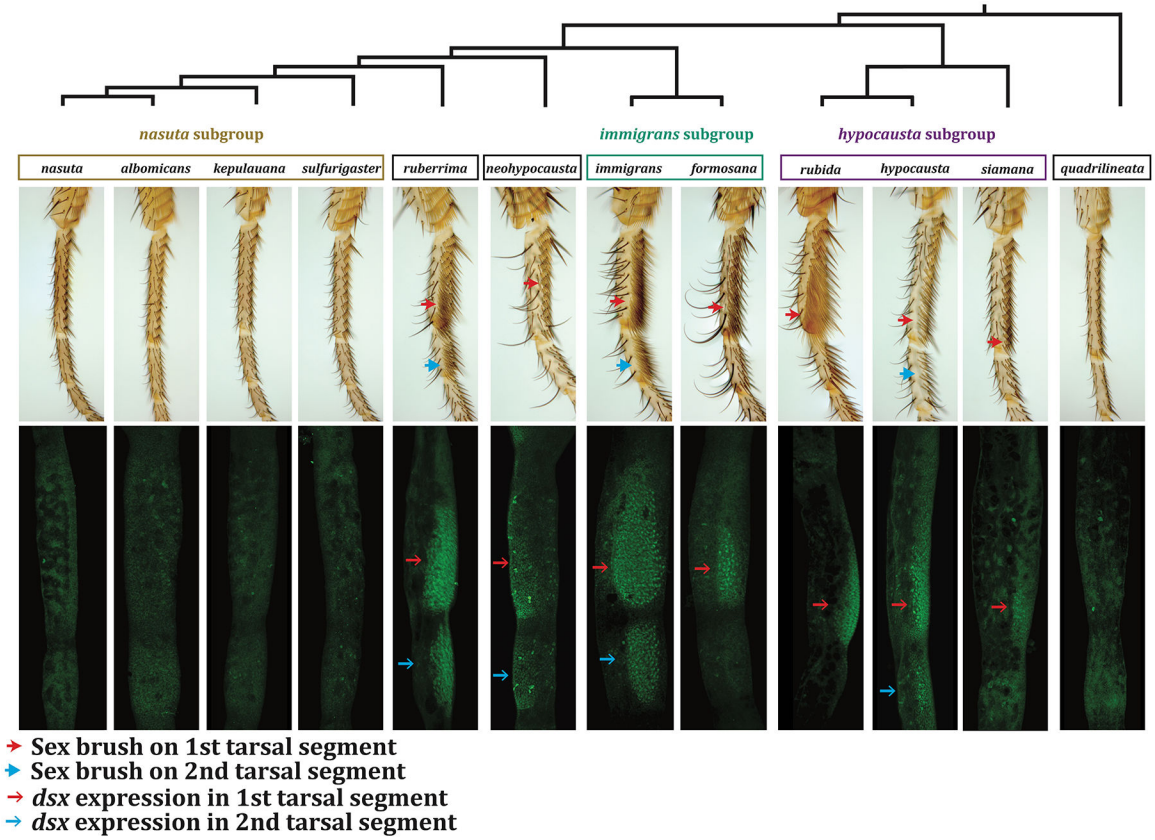

Figure 5. Dsx expression in the *D. immigrans* species group correlates with sex brush morphology.

Adult male phenotypes and phylogenetic relationships are shown in the top row. The bottom row shows Dsx expression, assayed by the Dsx[DBD] antibody (Mellert et al., 2012), in male pupal legs at 24 hours after pupariation. Dsx immunostaining (green) is seen in sex brush bristles (arrows). In most species the position and size of Dsx expression corresponds with the sex brush. In *D. neohypocausta*, Dsx expression is seen throughout the first and second tarsal segments, yet only two bractless bristles, suggestive of a rudimentary sex brush, are found on the first tarsal segment (red arrow in the top row). More intense Dsx staining correlates with the positions of chemosensory bristles (see also Supplementary Figure 1).

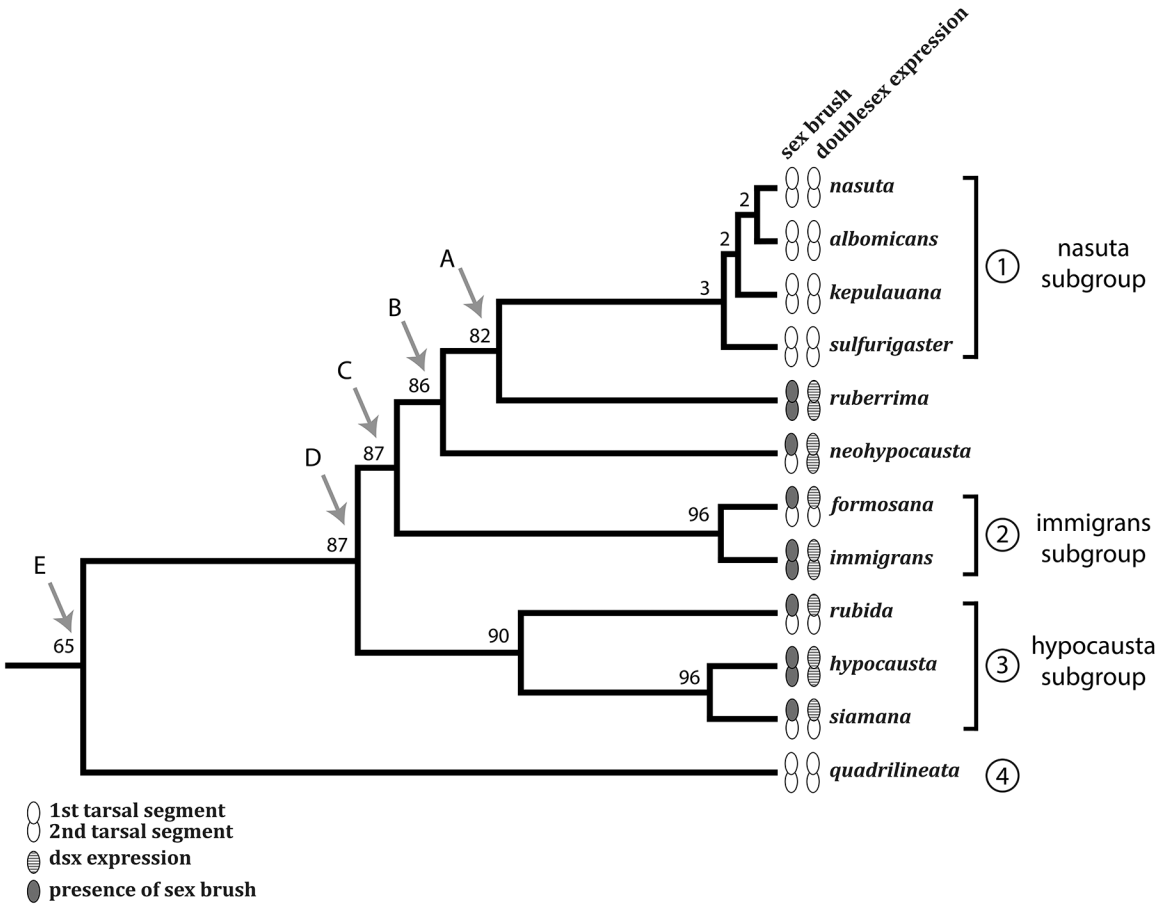

Figure 6. Phylogenetic reconstruction of sex brush evolution.

BEAST was used to estimate the ancestral state of the sex brush and Dsx expression at each node of the phylogeny. Grey ovals show the presence of a sex brush, and striped ovals show the presence of Dsx expression, in extant species. Open ovals represent the lack of sex brush or Dsx expression. The numbers at each internal node indicate posterior probabilities that the last common ancestor of the corresponding clade had a sex brush, which are identical to the probabilities of expressing Dsx. The posterior probabilities were estimated in BEAST using a Speciation: Birth-death model with incomplete sampling.