The organization and evolution of the Dipteran and Hymenopteran *Down syndrome cell adhesion molecule* (*Dscam*) genes

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ABSTRACT

The *Drosophila melanogaster Down syndrome cell adhesion molecule* (*Dscam*) gene encodes an axon guidance receptor and can generate 38,016 different isoforms via the alternative splicing of 95 variable exons. Dscam contains 10 immunoglobulin (Ig), six Fibronectin type III, a transmembrane (TM), and cytoplasmic domains. The different Dscam isoforms vary in the amino acid sequence of three of the Ig domains and the TM domain. Here, we have compared the organization of the *Dscam* gene from three members of the *Drosophila* subgenus (*D. melanogaster*, *D. pseudoobscura*, and *D. virilis*), the mosquito *Anopheles gambiae*, and the honeybee *Apis mellifera*. Each of these organisms contains numerous alternative exons and can potentially synthesize tens of thousands of isoforms. Interestingly, most of the alternative exons in one species are more similar to one another than to the corresponding alternative exons in the other species. These observations provide strong evidence that many of the alternative exons have arisen by reiterative exon duplication and deletion events. In addition, these findings suggest that the expression of a large Dscam repertoire is more important for the development and function of the insect nervous system than the actual sequence of each isoform.

Keywords: *Down syndrome cell adhesion molecule* (*Dscam*); exon duplication; alternative splicing; *Drosophila*

INTRODUCTION

The *Drosophila melanogaster Down syndrome cell adhesion molecule* (*Dscam*) gene encodes a trans-membrane receptor required for axon guidance and target selection (Schmucker et al. 2000; Wang et al. 2002; Hummel et al. 2003; Zhan et al. 2004). The extracellular portion of the Dscam protein contains 10 immunoglobulin (Ig) domains and 4 fibronectin type III domains whereas the C terminus contains a single trans-membrane domain and a cytoplasmic tail that interacts with Dock (Schmucker et al. 2000). *Dscam* encodes an extraordinarily diverse collection of isoforms by virtue of extensive alternative splicing. *Dscam* contains 95 alternative exons that are organized into four clusters. The exon 4, 6, 9, and 17 clusters contain 12, 48, 33, and 2 variable exons, respectively, and the exons within each cluster are alternatively spliced in a mutually exclusive manner (Schmucker et al. 2000). As a result, *Dscam* potentially encodes 38,016 different isoforms. Alternative splicing of the exon 4, 6, and 9 clusters alters three of the Ig domains whereas alternative splicing of the exon 17 cluster modifies the trans-membrane domain. Different Dscam isoforms are expressed in specific spatial and temporal patterns (Celotto and Graveley 2001; Neves et al. 2004; Zhan et al. 2004) and individual cells appear to express on the order of 50 different isoforms (Neves et al. 2004). It is thought that the collection of isoforms expressed by each neuron plays an important role in determining the specificity of neural wiring in *Drosophila*.

Here, we compare the organization of the *Dscam* gene from five insects spanning ~250 million years of evolution—the fruit flies *D. melanogaster*, *Drosophila pseudoobscura*, and *Drosophila virilis*, the mosquito *Anopheles gambiae*, and the honeybee *Apis mellifera*. These sequence comparisons provide insight into the evolution of the *Dscam* gene and provide strong evidence that many of the alter-

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native exons have arisen by multiple exon duplication and deletion events. These findings also suggest that expressing a diverse Dscam repertoire is more important than the actual sequence of each isoform.

RESULTS AND DISCUSSION

To obtain insight into the importance of Dscam diversity in neural function, the mechanisms involved in regulating *Dscam* expression, and the evolution of *Dscam* we have compared the sequence of the *Dscam* genes from a variety of insects. The sequence of the *Dscam* genes from the fruit fly *D. pseudoobscura* (http://www.hgsc.bcm.tmc.edu/projects/drosophila/) and the mosquito *A. gambiae* (Holt et al. 2002) were obtained from public databases. We also cloned and sequenced the portion of *D. virilis Dscam* encoding the extracellular domain, as well as the entire *Dscam* gene from the honeybee *A. mellifera,* a member of the order Hymenoptera. The sequences from these five species allowed us to analyze the evolution of the *Dscam* gene over at least 250 million years and across phylogenetic orders.

Organization of the *Drosophila Dscam* genes

A graphical overview of the exon–intron organization of the five genes is shown in Figure 1 and a nucleotide alignment of all five genes is shown in Figure 2. The overall organization of the *Dscam* genes of the three *Drosophila* species is quite similar, but there are a few subtle differences. Each species contains 12 exon 4 variants and two exon 17 variants (note that the clone obtained from *D. virilis* did not contain sequence downstream of exon 16), but differ in the number of exon 6 and exon 9 variants. Whereas *D. melanogaster* contains 48 exon 6 variants, *D. pseudoobscura* and *D. virilis* contain 49 and 52, respectively. Likewise, both *D. pseudoobscura* and *D. virilis* contain 32 exon 9 variants rather than 33 as in *D. melanogaster*. Consequently, *D. pseudoobscura* and *D. virilis* encode 36,864 and 39,936 potential isoforms, respectively, compared to *D. melanogaster*, which can potentially generate 38,016 different isoforms.

We next analyzed the evolutionary relationship of the alternative exons within the three *Drosophila* species. As shown in Figure 3A, all 12 exon 4 variants have orthologs in each species. In the exon 6 and 9 clusters, there are 36 and 27 examples, respectively, in which orthologous exons are clearly present in all three species (Fig. 3B,C). There are also many cases in the exon 6 and 9 clusters of exons that are common to only two of the three organisms. For example, *D. pseudoobscura* exon 6.48 is orthologous to *D. virilis* exon 6.52, but *D. melanogaster* does not contain an exon orthologous to either of these two exons. Finally, there are also several species-specific exons in the exon 6 and 9 clusters. The most divergent, species-specific exons are *D. melanogaster* exon 6.11 and *D. virilis* exons 6.19 and 9.15. However, there are several other species-specific exons that are highly related to other exons in the cluster. For example, *D. virilis* exon 6.16 appears to be species specific, but is highly related to *D. virilis* exon 6.17, which is orthologous to *D. pseudoobscura* exon 6.15 and *D. melanogaster* exon 6.14 (Fig. 3B).

Perhaps the most interesting observations are two instances of exon rearrangements. The first case involves ex-

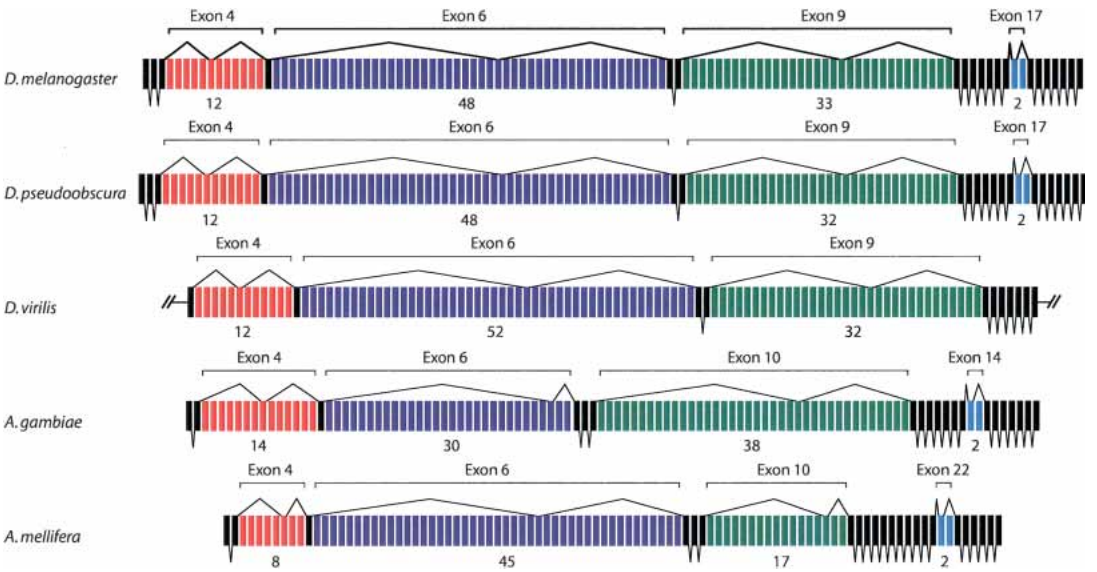

FIGURE 1. Overview of *Dscam* genes from *D. melanogaster*, *D. pseudoobscura*, *D. virilis*, *A. gambiae*, and *A. mellifera*. The exon–intron organization of the *Dscam* gene from each organism is shown. The black exons are constitutively spliced. The alternative exons in the exon 4, 6, 9, and 17 clusters are shaded in red, purple, green, and blue, respectively. The number of variable exons within each cluster in each organism is indicated *below* each cluster. The exon 10 clusters in *A. gambiae* and *A. mellifera* correspond to the exon 9 cluster in the *Drosophila* species. The exon 14 and 22 clusters in *A. gambiae* and *A. mellifera*, respectively, correspond to the exon 17 clusters in the *Drosophila* species.

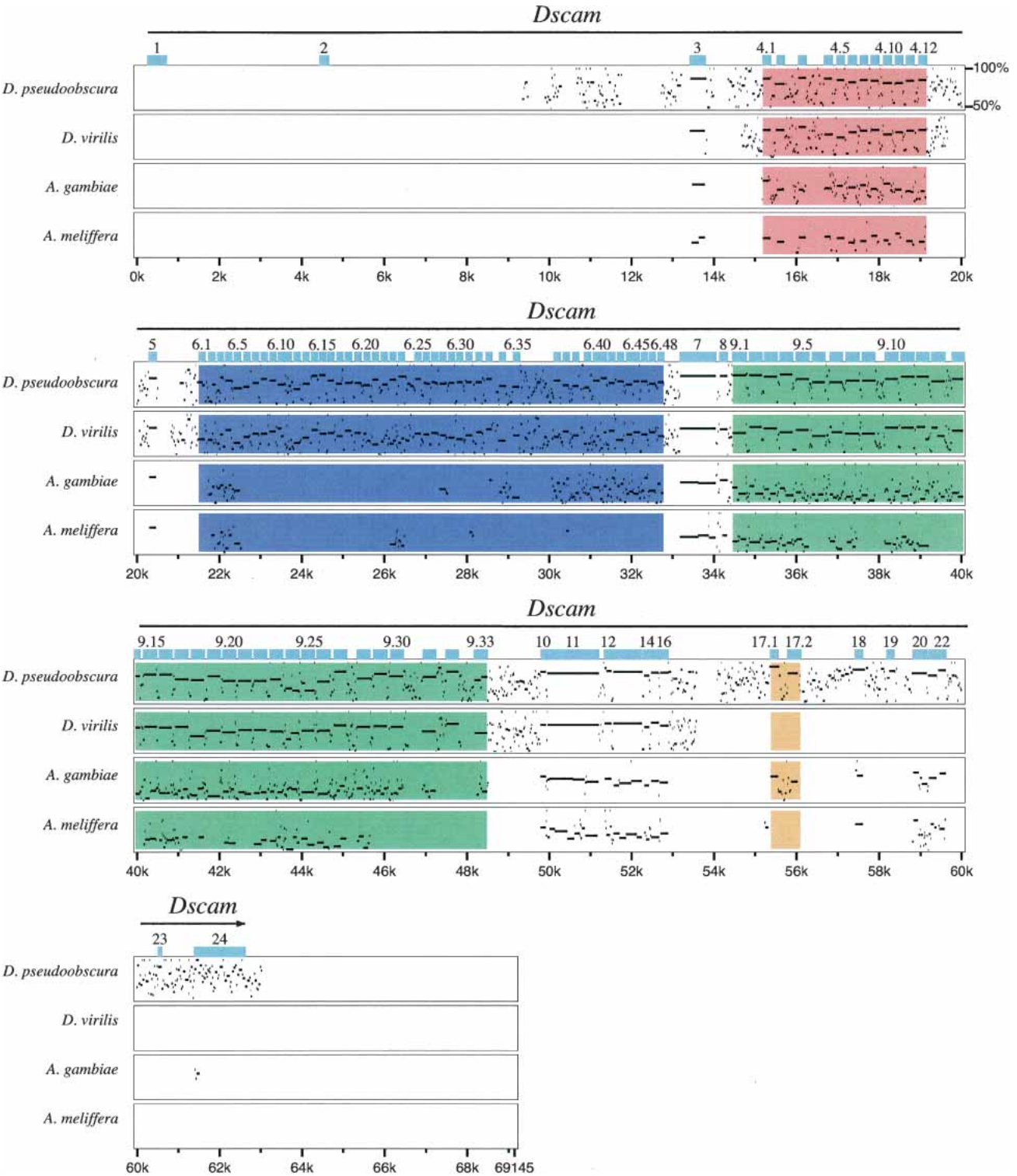

FIGURE 2. Percent identity plot of the nucleotide sequence of the insect *Dscam* genes. The nucleotide sequences of the *Dscam* genes from *D. melanogaster*, *D. pseudoobscura*, *D. virilis*, *A. gambiae*, and *A. mellifera* were aligned using MultiPipmaker (Schwartz et al. 2000; http://bio.cse.psu.edu/pipmaker/). The locations of the exons in the *D. melanogaster Dscam* gene are shown on the *top* of the alignment. Each box depicts regions in the *Dscam* gene of the indicated organism that are 50%–100% identical to the *D. melanogaster* gene. The exon 4, 6, 9, and 17 clusters are colored in red, blue, green, and orange, respectively.

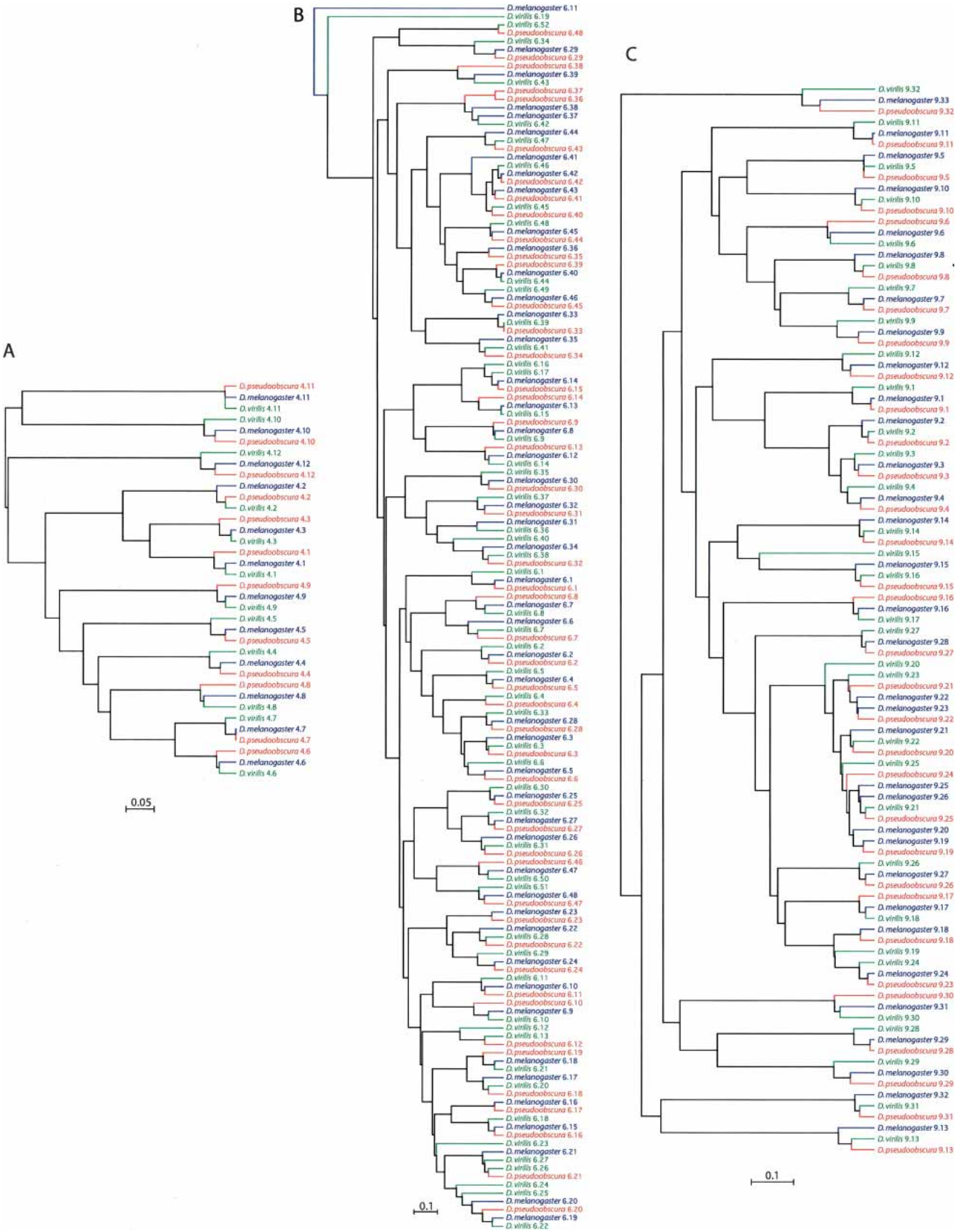

FIGURE 3. Evolutionary relationships of the variable exons between *D. melanogaster*, *D. pseudoobscura*, and *D. virilis*. Each variable exon was conceptually translated. For each cluster, the amino acid sequences of each variable exon from each species were aligned using ClustalW and phylogenetic trees generated using MacVector with the UPGMA method. Branches containing *D. melanogaster*, *D. pseudoobscura*, and *D. virilis* sequences are colored in blue, red, and green, respectively. The branches are colored until two different species intersect. Separate trees were generated for the exon 4 cluster (*A*), the exon 6 cluster (*B*), and the exon 9 cluster (*C*).

ons 6.33 and 6.34 in *D. melanogaster*, which are orthologous to exons 6.32 and 6.33 in *D. pseudoobscura* and exons 6.38 and 6.39 in *D. virilis*. These pairs of exons are in the same linear order in *D. pseudoobscura* and *D. virilis*, but are reversed in *D. melanogaster*. A similar situation is observed for *D. melanogaster* exons 6.42 and 6.43, which are most similar to *D. pseudoobscura* exons 6.42 and 6.41, respectively (Fig. 3B). Interestingly, *D. virilis* lacks an exon orthologous to *D. melanogaster* exon 6.43. This strongly indicates that a rearrangement has occurred that reversed the relative order of these exons since *D. melanogaster* and *D. pseudoobscura* last shared a common ancestor.

Another intriguing observation is that in many cases in each cluster, proximal exons are more similar to one another than distal exons. For instance, in the exon 4 cluster, exons 4.1, 4.2, and 4.3 cluster together, as do exons 4.10, 4.11, and 4.12 (Fig. 3A). In the exon 6 cluster, exons 6.25, 6.26, and 6.27 cluster together (Fig. 3B) and in the exon 9 cluster, exons 9.5 though 9.11 cluster together as do exons 9.1–9.4 (Fig. 3C).

Divergence of the *Drosophila, Anopheles,* and *Apis Dscam* genes

We next analyzed the organization of the fruit fly, mosquito, and honeybee *Dscam* genes (Figs. 1, 2, 4). For these analyses we used *D. melanogaster* as the representative *Drosophila* species. In contrast to the situation in the closely related members of the *Drosophila* subgroup, the organization of the *Drosophila* gene differ significantly from those of *A. gambiae* and *A. mellifera*. First, although each organism contains exons orthologous to *D. melanogaster* exons 17.1 and 17.2, the numbers of variable exons contained within the exon 4, 6, and 9 clusters are dramatically different (Fig. 1). For example, the *A. gambiae Dscam* gene contains 14 exon 4 variants, 30 exon 6 variants, and 38 exon 10 variants (which correspond to the *Drosophila* exon 9 variants) and can potentially synthesize 31,920 different isoforms. Likewise, the *A. mellifera* gene contains eight exon 4 variants, 45 exon 6 variants, and 17 exon 10 variants (which correspond to the *Drosophila* exon 9 variants) and can therefore generate only 12,240 potential isoforms.

Remarkably, there are only three instances in which clear orthologs of a particular exon 4 variant are present in each species (Fig. 4A). These involve *D. melanogaster* exons 4.4, 4.5, and 4.11 and the homologous exons in the other species. There are, however, several instances in which exons appear to be common to only two of the three organisms. For instance, *D. melanogaster* exon 4.12 and *A. mellifera* exon 4.8 appear to be orthologous, but a clear ortholog is not present in *A. gambiae*. Likewise, *D. melanogaster* exon 4.9 and *A. gambiae* exon 4.10 appear to be orthologous, but are not significantly similar to any exon in *A. mellifera*. Finally, both *D. melanogaster* and *A. gambiae* contain species-specific exons. For example, both *D. melanogaster* and *A. mellifera* lack exons that are clearly orthologous to *A. gambiae* exons 4.7, 4.8, and 4.9.

It is more difficult to establish the evolutionary relationships of exons in the exon 6 and exon 9 clusters between *D. melanogaster*, *A. gambiae*, and *A. mellifera* because the sequences of these exons are quite different in each organism (Fig. 2). However, the sequences of the constitutive exons are quite conserved (Fig. 2), suggesting that the constitutive and alternative exons are under different functional constraints. Interestingly, for both the exon 6 and exon 9 clusters, there is significantly more similarity between the variable exons within each species than there is across species. For example, the exon 9 variants from one species are more similar to one another than they are to any of the exon 9 variants in the other two species (Fig. 4C). Moreover, as was observed to a limited extent when comparing the three *Drosophila* species, in many instances, neighboring exons cluster together, indicating that proximal exons are more similar to one another than distal exons. For example, *A. gambiae* exons 6.13–6.24 are more similar to one another than they are to any of the exon 6 variants in either *D. melanogaster* or *A. mellifera* (Fig. 4B). A striking example of this species-specific evolution involves 24 contiguous exons in the *A. gambiae* exon 10 cluster (exons 10.5–10.28) (Fig. 4C). Within this group, there are three cases of particularly similar neighboring exons. The most telling example of this involves *A. gambiae* exons 10.6, 10.7, and 10.8, which are all ~90% identical to one another at the nucleotide level and 95% identical at the protein level. Likewise, *A. gambiae* exons 10.23, 10.24, and 10.25 are 80%–97% identical at the nucleotide level and 87%–97% identical at the protein level. Interestingly, the introns separating these highly conserved neighboring exons are also very highly conserved. For instance, the intron separating exons 10.23 and 10.24 is 94% identical to the intron separating exons 10.24 and 10.25. Likewise, the intron separating exons 10.6 and 10.7 is 77% identical to the intron separating exons 10.7 and 10.8. Similar trends are observed for the introns separating the other highly similar adjacent exons (data not shown).

The implications of DNA recombination and Dscam function on *Dscam* evolution

The organizational differences we have observed provide insight into the evolution of the alternative exons in the insect *Dscam* genes. The differences in the number of exons in the variable exon clusters between each species indicate that the compositions of these regions are extraordinarily fluid. This is most likely a consequence of frequent recombination events between nearly identical exons or introns. Recombination between adjacent exons would result in the loss of an exon on one sister chromosome and generate a region containing three nearly identical exons separated by

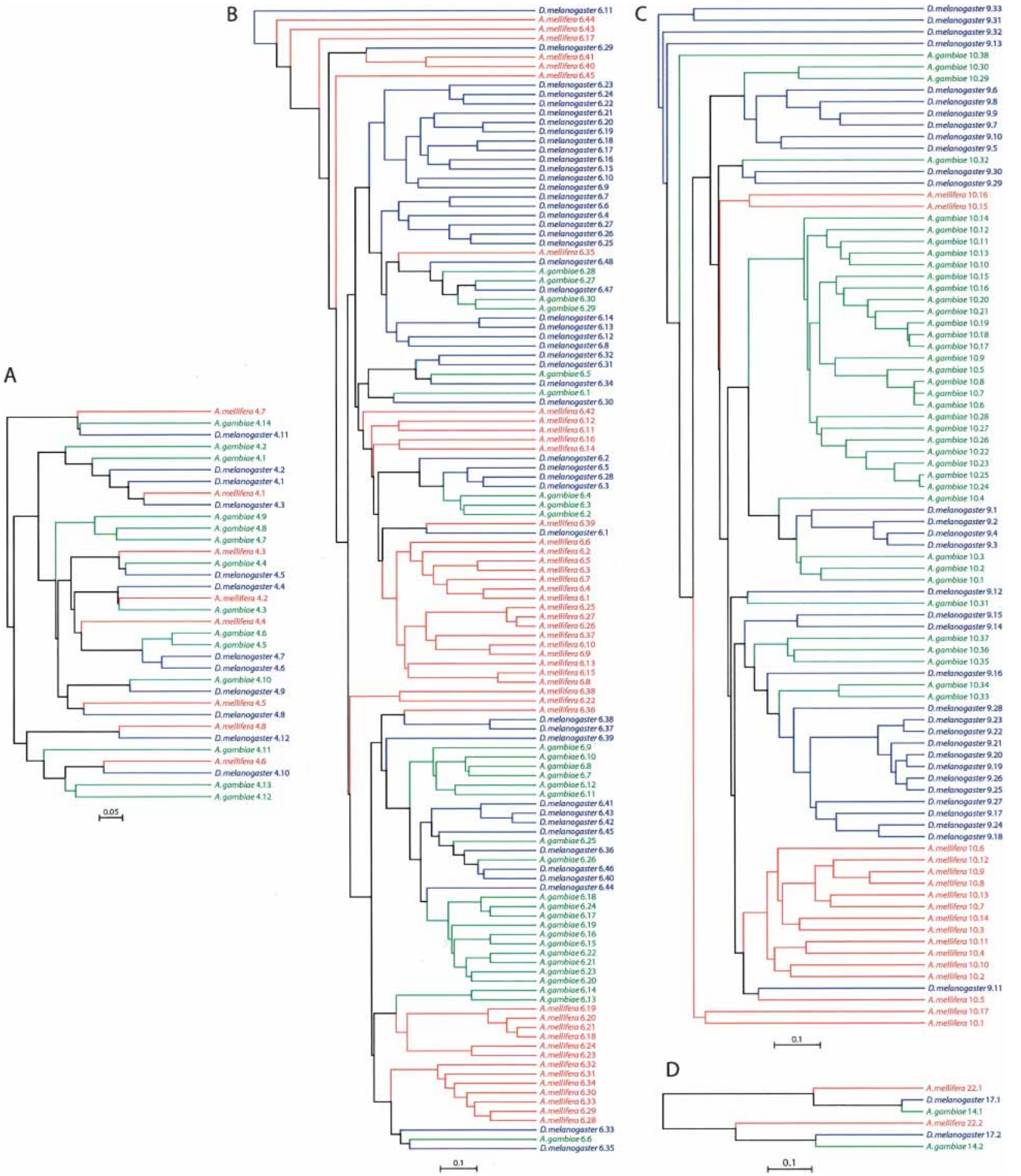

FIGURE 4. Evolutionary relationships of the variable exons between *D. melanogaster*, *A. gambiae*, and *A. mellifera*. Phylogenetic trees were generated as described in Figure 3 and Materials and Methods. Branches containing *D. melanogaster*, *A. gambiae*, and *A. mellifera* sequences are colored in blue, green, and red, respectively. The branches are colored until two different species intersect. Separate trees were generated for the exon 4 cluster (*A*), the exon 6 cluster (*B*), the exon 9 cluster (*C*), and the exon 17 cluster (*D*).

nearly identical introns on the other sister chromosome. The observation that *A. gambiae* exons 10.6–10.8, 10.17–10.19, and 10.23–10.25 and their flanking introns are nearly identical suggests that these regions recently underwent such a recombination event. Multiple reiterations of such deletions and duplications after the divergence of two species would eventually result in the homogenization of the variable exons such that the exons within each species would be more similar to one another than they would be to the corresponding exons in the other species. This is precisely what we observed for the exon 6 and 9 clusters.

The extracellular portion of Dscam has recently been shown to engage in isoform-specific, homophilic interactions and the Ig domains encoded by the exon 4, 6, and 9 clusters all contribute to the specificity of these interactions (Wojtowicz et al. 2004). Each Dscam isoform that has been tested strongly interacts with itself, but not with isoforms that are closely related (Wojtowicz et al. 2004). We propose that the exons within one species are more similar to one another than to the exons in the other species because in addition to residing in recombination hot spots, the primary functional constraint on each exon is that they maintain the ability to engage in homotypic interactions. As a result, exons created by the recombination mechanism described above would be immediately functional. Any subsequent divergence of an exon would be allowed, provided that the encoded isoform could still interact with itself.

Whereas each insect *Dscam* gene we analyzed can potentially generate tens of thousands of isoforms, the mammalian *DSCAM* gene (Yamakawa et al. 1998) and its paralog *DSCAML1* (Barlow et al. 2002) each appear to encode only three different mRNA isoforms, and their roles in neural wiring is unclear. However, mammals have evolved other genes that play important roles in axon guidance. For example, the odorant receptor gene family, which contains thousands of members, is required for proper wiring of mammalian olfactory receptor neurons (Vassalli et al. 2002). Thus, although different genes and mechanisms are involved, it appears as though mammals and insects both utilize a large repertoire of proteins to solve the problem of how to specify neural wiring—mammals have invoked gene duplication of the odorant receptor genes and insects utilize alternative splicing of *Dscam*.

It is striking, given the complexity of the mammalian nervous system, that mammalian genes that encode as many alternatively spliced mRNAs as the insect *Dscam* genes have not been discovered. Although there are several genes in the *Drosophila* genome that contain clusters of more than two mutually exclusive alternative exons, despite much effort, we have been unable to find a single mammalian genes sharing this organization (B.R. Graveley, unpubl. data). There are, however, numerous mammalian genes containing only two mutually exclusive exons. Thus, despite the fact that exon duplication events are quite common in mammalian genomes (Kondrashov and Koonin 2001; Letunic et al. 2002), the generation of extraordinary protein diversification by alternative splicing of multiple cassette exons has not been exploited in mammals as it has in insects.

Unfortunately, this comparative sequence analysis has not provided much insight into the mechanisms involved in either regulating *Dscam* alternative splicing or ensuring that the alternative splicing of the variable clusters is mutually exclusive. There are a few sequences in the introns that flank the variable exon clusters (i.e., the intron between exons 3 and 4.1 or the intron between exons 4.12 and 5) that are conserved to different degrees in each species analyzed. However, it is by no means obvious what, if any, functional role these conserved elements play in the regulation of *Dscam* alternative splicing. Additionally, although the sequences of the splice sites flanking orthologous sets of variable exons are conserved in many cases, this is by no means universally true. Nonetheless, these analyses have highlighted regions to focus on to experimentally elucidate the mechanisms involved in *Dscam* splicing regulation.

Although the sequences of the *Dscam* alternative exons are quite diverse between distantly related species, the ability to generate tremendous Dscam diversity by alternative splicing is highly conserved. Thus, Dscam diversity is clearly extraordinarily important for the development of the insect nervous system. Moreover, our results strongly suggest that the expression of a large Dscam repertoire is more important for the development and function of the insect nervous system than the actual sequence of each isoform.

MATERIALS AND METHODS

Cloning and sequencing of *D. virilis Dscam*

A full-length *D. melanogaster Dscam* cDNA served as a template in a random primer labeling reaction. The resulting probe was used to screen a *D. virilis* genomic library (kindly provided by John Tamkun, Univ. of California, Santa Cruz) at low stringency (2× SSC/0.2% SDS, room temperature washes). Two robustly positive clones were selected for further characterization and were found to contain partially overlapping genomic sequences that encode *D. virilis Dscam*. The inserts of both clones were fully sequenced, resulting in 96 contiguous sequences that were assembled into a single sequence spanning 43,944 nt using AssemblyLIGN (Oxford Molecular). The sequence has been deposited in GenBank (accession number AY686597).

Cloning and sequencing of *A. mellifera Dscam*

An *A. mellifera* EST clone (GenBank accession number BI514254) homologous to a portion of the *D. melanogaster Dscam* cDNA was identified by a BLAST search. The EST clone was obtained and sequenced and found to contain exons 21, 22.1, 23, and 25–28. A PCR product was generated from the EST clone and used as a template in a random primer labeling reaction. The probe was hybridized to an arrayed *A. mellifera* genomic DNA BAC library

(obtained from Clemson University Genomics Institute). Thirty positive BAC clones were identified and ordered. DNA was purified from *Escherichia coli* containing each BAC and slot-blotted onto a nylon membrane. To identify BACs likely to contain the full-length *Dscam* gene, the membrane was hybridized with a probe synthesized from PCR products amplified from a pool of 12 *D. melanogaster* cDNA clones, each containing a different exon 4 variant. Ten BAC clones that hybridized to this probe were grown in LB media with chloramphenicol for 14 h at 37°C and DNA was prepared on the Autogen 740 DNA Isolation System in accordance with the manufacturers' instructions. Restriction enzyme digests of BAC DNA were done using EcoRI and HindIII, run on a 1% agarose gel for 20 h at 250 V, stained with vista green (Amersham Biosciences), and imaged by using a Molecular Dynamics 595 fluorimager. Two BACs were selected for sequencing and DNA from each clone was fragmented by sonication, end repaired, and electrophoresed to select insert sizes of 2–5 kb. Insert was ligated into pUC18 vector, transformed, and plasmid DNA was made by using the Eppendorf-5 prime PERFECTprep robot. DNA templates were sequenced at both ends with a one-eighth dilution of the Applied Biosystems Big dye terminator kit reagents and electrophoresed on ABI 3700 capillary DNA sequencers. Raw chromatographic data were base called with Phred and assembled with Phrap (Ewing and Green 1998). Finishing was done using oligonucleotide-direct sequencing as needed. The sequence has been deposited in GenBank (accession number AY686596).

Sequence analysis

The sequence analysis was performed using MacVector 7.2 software (Accelery's). In addition, cross-species nucleotide sequence analysis was performed using MultiPipmaker (Schwartz et al. 2000). To generate the phylogenetic trees, each variable exon was first conceptually translated. The amino acid sequences of the variable exons from each cluster from each organism were aligned using ClustalW. From this alignment a phylogenetic tree was generated using the unweighted pair group method with arithmetic mean (UPGMA).

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