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Phylogenetic and Genomewide Analyses Suggest a Functional Relationship Between *kayak*, the Drosophila Fos Homolog, and *fig*, a Predicted Protein Phosphatase 2C Nested Within a *kayak* Intron

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ABSTRACT

A gene located within the intron of a larger gene is an uncommon arrangement in any species. Few of these nested gene arrangements have been explored from an evolutionary perspective. Here we report a phylogenetic analysis of *kayak* (*kay*) and *fos intron gene* (*fig*), a divergently transcribed gene located in a *kay* intron, utilizing 12 Drosophila species. The evolutionary relationship between these genes is of interest because *kay* is the homolog of the proto-oncogene *c-fos* whose function is modulated by serine/threonine phosphorylation and *fig* is a predicted PP2C phosphatase specific for serine/threonine residues. We found that, despite an extraordinary level of diversification in the intron–exon structure of *kay* (11 inversions and six independent exon losses), the nested arrangement of *kay* and *fig* is conserved in all species. A genomewide analysis of protein-coding nested gene pairs revealed that ~20% of nested pairs in *D. melanogaster* are also nested in *D. pseudoobscura* and *D. virilis*. A phylogenetic examination of *fig* revealed that there are three subfamilies of PP2C phosphatases in all 12 species of Drosophila. Overall, our phylogenetic and genomewide analyses suggest that the nested arrangement of *kay* and *fig* may be due to a functional relationship between them.

THE vast majority of genes are not nested in the introns of other genes. The first nested gene to be described in *Drosophila melanogaster* was located within the *Gart* locus (Henikoff *et al.* 1986). Subsequently, a set of three nested genes was identified in the *dunce* locus (Furia *et al.* 1990). In both cases, no functional relationship was identified between the nested genes. Neufeld *et al.* (1991) conducted the first phylogenetic analysis of a *D. melanogaster* nested gene pair and determined that *sina* and its intronic gene *Rh4* were not nested in *D. virilis*. However, 7% of *D. melanogaster* genes are predicted to contain a nested gene (Adams *et al.* 2000), and 85% of these have a protein-coding intronic gene (15% have a noncoding RNA; Misra *et al.* 2002). To date, little evidence is available upon which to determine if nesting indicates a functional relationship between the genes.

Here we report a phylogenetic analysis of *kayak* (*kay*) and *fos intron gene* (*fig*), a divergently transcribed gene located in a *kay* intron, utilizing 12 Drosophila species. The structure and transcriptional activity of the *D. melanogaster kay* gene, the homolog of the human proto-oncogene *c-fos*, is complex and has not been fully determined. In humans, *c-fos* encodes part of the AP-1 transcription factor and is known to be misregulated in a number of tumors (Perkins *et al.* 1988). Utilizing genome annotations for *D. melanogaster* and confirmation with a variety of mRNA-based techniques, we generated a new model for the structure of *kay* (Hudson 2006). That study showed that *kay* is a substantial gene (27.5 kb) with three distinct promoters. In addition, nested within a large (17.5 kb) intron of *kay* there is a predicted, divergently transcribed gene (CG7615) that we have named *fos intron gene* (*fig*).

Our new model determined that *kay* has three transcription initiation sites that create alternative 5′ exons, each containing their own predicted initiator methionine (Figure 1A). Each of these 5′ exons splices to a common 3′ exon (*kay-mainbody*) that encodes the Basic domain (DNA binding) and the leucine-zipper domain (dimerization) essential for Kay activity. The centromere proximal promoter (most distant from *kay-mainbody*) gives rise to the *kay*-α transcript. The middle promoter generates the *kay*-β transcript. The closest promoter leads to the *kay*-γ transcript. Analysis of the divergently transcribed, nested locus *fig* showed that it

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ858474 (*kayak*-α), DQ858476 (*kayak*-γ), and DQ858472 (*fig*).

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generates an intronless transcript and encodes a protein phosphatase 2C (PP2C).

The complexity of this region surprised us, and we wondered if each of the alternative first exons for *kay* and *fig* were conserved in distant Drosophila species. Further, we wondered if the nested arrangement of *kay* and *fig* in *D. melanogaster* was due to a functional relationship or was just a random recent occurrence. Numerous studies have shown that, when comparing *D. virilis* (subgenus Drosophila) and *D. melanogaster* (subgenus Sophophora), sequence conservation strongly indicates functional importance (*e.g.*, Newfeld *et al.* 1993). For comparison, the 63-MY divergence between these species (Tamura *et al.* 2004) is roughly two-thirds of the divergence between human and mouse (93 MY; Kumar and Hedges 1998).

Evidence of a functional relationship between these genes is of interest because constitutive *c-fos* activity can lead to tumors and *c-fos* activity is stimulated by serine/threonine phosphorylation (Deng and Karin 1994). Upon activation by serine/threonine kinases, *kay* functions in Drosophila in the same manner as *c-fos* (*e.g.*, Xia and Goldstein 1999; Ciapponi *et al.* 2001). How *kay* serine/threonine phosporylation is regulated is not thoroughly known, but the *puckered* serine/threonine phosphatase regulates *kay* activity in embryos and adults (Dobens *et al.* 2001). Since *fig* is a predicted PP2C phosphatase (specific for serine/threonine), it would not be surprising if *fig* plays a role in regulating *kay* function.

To address these questions, we examined the *kay–fig* genomic region in 12 species of Drosophila. We found a wide variety of gene structures for *kay*, as shown by the presence of multiple inversions and the repeated loss of individual *kay* 5′ exons. Nevertheless *fig* is divergently transcribed and nested in a *kay* intron in all species—a level of conservation that may indicate a functional relationship between them. This hypothesis is supported by our genomewide analysis of nested gene pairs that revealed that ~20% of nested pairs in *D. melanogaster* are also nested in *D. pseudoobscura* and *D. virilis*. Overall, our study illustrates the power of phylogenetics to suggest experimentally testable hypotheses for the function of poorly characterized genes.

MATERIALS AND METHODS

DNA sequence retrieval: DNA sequences were obtained at http://rana.lbl.gov/drosophila, http://evoprinter.ninds.nih.gov, and http://www.flybase.org and are attributed to the following labs: Agencourt (*D. erecta*, *D. ananassae*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*), Washington University (*D. simulans* and *D. persimilis*), The Broad Institute (*D. sechellia* and *D. persimilis*), and Baylor University (*D. pseudoobscura*). Sequences corresponding to *D. melanogaster* accession numbers for *kayak*-α (DQ858474), *kayak*-β (AF332657, AF332658, AF332659, and AF332660; Rousseau and Goldstein 2001), *kayak*-γ (DQ858476), and *fig* (DQ858472) were utilized in BLAST to acquire homologous sequences from each of the 12 species. The FEX gene-finding program was utilized to complete predicted coding sequences as necessary (http://www.softberry.com; Solvyev and Salamov 1997). Individual sequence identifiers are listed in supplemental Tables 1–7 at http://www.genetics.org/supplemental/. *Anopheles gambiae* and *Apis mellifera* were accessed via http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=insects.

DNA sequence analysis: Alignments of predicted amino acid sequences were generated in ClustalX (Thompson *et al.* 1997) and amino acid conservation highlighted with Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were generated using the neighbor-joining method with bootstrap resampling in MEGA version 3.1 (Kumar *et al.* 2004). Protein domains were identified via the EMBL-EBI database at http://www.ebi.ac.uk/interpro.

Genomewide studies: For the annotation analysis, the complete set of nested protein-coding genes in *D. melanogaster* (Release 5.1) and the same set in *D. pseudoobscura* (Release 2.0) were obtained using GFF files from http://www.flybase.org. Only nested gene pairs that mimic the *kay–fig* structure were retrieved: two protein-coding genes with one gene completely contained within the limits of the other gene. We excluded partially overlapping genes (only an exon of one gene is within the limits of the other gene), but did include gene pairs nested on the same strand (unlike *kay–fig*) and on the opposite strand (like *kay–fig*) for completeness. We then compared these sets to identify loci where gene 1 is nested in gene 2 in *D. melanogaster* and the homolog of gene1 is nested in the homolog of gene 2 in *D. pseudoobscura*. Attributions of homology between genes in *D. melanogaster* and *D. pseudoobscura* were derived from FlyBase annotations. For the tBLASTn analysis, we began with the complete set of nested protein-coding genes in *D. melanogaster* (Release 5.1) obtained above. Then all exons of each nested gene pair were identified and their translated in-frame amino acid sequences were extracted. These amino acid sequences were then aligned against the *D. pseudoobscura* and *D. virilis* genomes using the tblastn algorithm (Altschul *et al.* 1997). tblastn results were filtered to ensure that *D. pseudoobscura* and *D. virilis* sequences matching exons of a *D. melanogaster* gene were located nearby on the same scaffold. Subsequently, for each nested pair in *D. melanogaster*, the *D. pseudoobscura* and *D. virilis* exon matches were examined to determine if matching sequence for the nested gene was located fully within the bounds of the matching sequence for the containing gene.

RESULTS

Nested arrangement of *kay* and *fig* is maintained in all 12 Drosophila species: Our first task was to visualize the entire 27-kb *kay–fig* region, located on chromosome 3R at polytene band 99B10-C1 in *D. melanogaster*, in each of the 12 species of Drosophila that have been fully sequenced. To accomplish this, we utilized BLAST to identify and retrieve sequences corresponding to the protein-coding domain of each *D. melanogaster kay* exon and of *fig*. We found that the *kay*-γ, *fig*, and *kay-mainbody* coding regions are present in all species and that their location in each species fits with the chromosomal synteny identified by Muller. Each *kay–fig* region is located in the E group of the Muller synteny table (FlyBase 2006). This suggests that these genes were present in the common ancestor of the 12 Drosophila species.

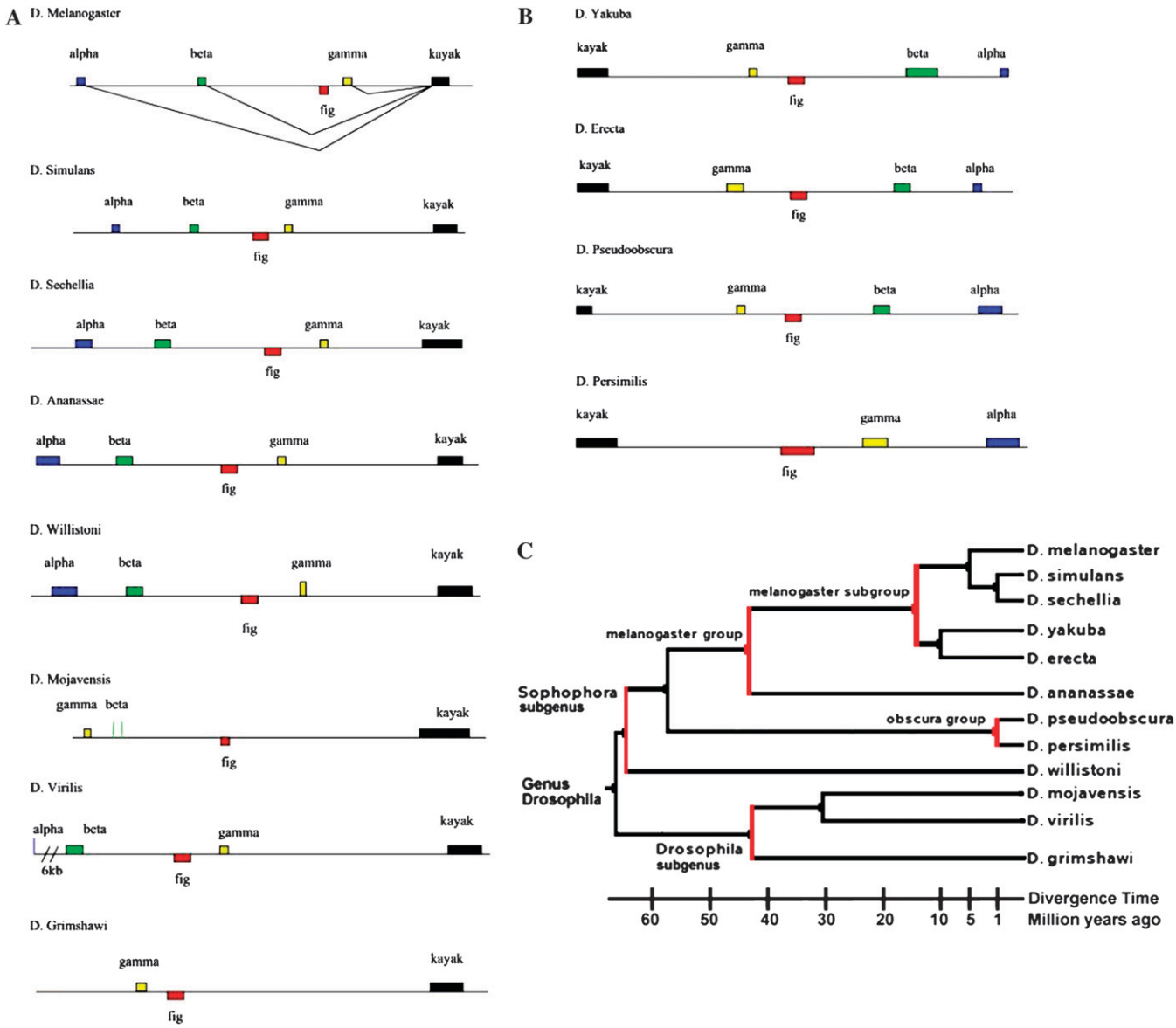


FIGURE 1.—Gene structure of the *kay–fig* region in 12 Drosophila species. The region is shown to scale in all 12 species. The coding portions of each exon are shown in color: *kay*-α (blue), *kay*-β (green), *fig* (red, divergently transcribed), *kay*-γ (yellow), and *kay-mainbody* (*kayak*, black). (A) Eight species have the 5′-end of the *kay* transcription unit at the proximal end of this 27.5-kb region (closest to the centromere and depicted with 5′ to the left). The vertical blue line in *D. virilis* represents a segment displaying a low level of DNA sequence similarity to *kay*-α but no similarity at the protein level. The pair of vertical green lines in *D. mojavensis* represents a segment displaying a low level of DNA sequence similarity to *kay*-β but no similarity at the protein level. (B) Four species have a large inversion that includes this region and thus the 5′-end of the *kay* transcription unit is at the distal end (depicted with 5′ to the right). (C) Phylogenetic tree for the 12 Drosophila species utilized in this analysis, modified from FLYBASE (2006) to match the timeline of TAMURA *et al.* (2004).

We then determined that scaffolds surrounding each exon-specific sequence were contiguous and that *fig* was divergently transcribed and nested in a *kay* intron in all species (Figure 1). This highly conserved relationship stands out in stark contrast to the extensive diversity of gene structures for *kay* present in the 12 species. From our analysis, it is clear that there have been multiple chromosomal inversions and repeated loss of individual *kay* 5′ exons. The largest and most obvious difference among these species is a reversal in proximal–distal orientation affecting the entire *kay–fig* region. Eight of the species, including *D. melanogaster*, have the 5′-end of *kay* at the proximal end of the region (closest to the centromere; Figure 1A). Alternatively, four species have an inversion that includes this region and places the 5′-end of *kay* at the distal end of the region (closest to the telomere; Figure 1B). However, the four species with the 5′ distal arrangement are not monophyletic (Figure 1C). Therefore, the most parsimonious explanation of this distribution requires two independent inversions of

the ancestral 5′ proximal orientation within the subgenus Sophophora. One inversion occurred in the branch leading to the *obscura* group and a second in the branch leading to *D. yakuba* and *D. erecta* in the *melanogaster* subgroup. As both inversions affect the entire *kay–fig* region, the relationship of the two sets of inversion breakpoints to each other is unknown.

In addition, multiple inversions are evident within the *kay–fig* region. In *D. melanogaster*, the relative order of the coding regions from 5′ to 3′ for *kay* (regardless of orientation to the centromere) is *kay*-α, *kay*-β, *fig* (transcribed from the opposite strand), *kay*-γ, and *kay-mainbody*. This organization is present in 9 of the 12 species. It is not present in three species where the exon order is inverted: *D. persimilis*, *D. mojavensis*, and *D. grimshawi*. In these three species, *fig* is closer to *kay-mainbody* than to *kay*-γ. However, the three species with an inversion affecting *fig* and *kay*-γ are not monophyletic (Figure 1C). As above, the most parsimonious explanation of this distribution requires three independent inversions.

There are two scenarios in which these three events could have occurred. Both scenarios require an inversion in the recent past in the *D. persimilis* lineage after its divergence from *D. pseudoobscura*. One scenario has two additional independent inversions: one in the *D. grimshawi* lineage and one in the *D. mojavensis* lineage after separation from *D. virilis*. Alternatively, there could have been an inversion in the subgenus Drosophila lineage leading to *D. mojavensis*, *D. grimshawi*, and *D. virilis* and a reversion (likely not identical to the initial inversion but one moving *kay*-γ and *fig* back to their original orientation) in *D. virilis*.

One consequence of the inversions that move *fig* closer to *kay-mainbody* than *kay*-γ is the reorientation of the *fig* and *kay*-γ open reading frames. The inversions would reverse the direction of the reading frames for *fig* and *kay*-γ in comparison to the other nine species and to their present orientation in these species. To reorient the reading frames, two small independent inversions (one each for *fig* and *kay*-γ) must be invoked in each species (six inversions total).

Evidence for these reorienting small inversions is found in *D. mojavensis*. Here an additional inversion involving *kay*-β and *kay*-γ reversed their order (*kay*-β is now closer to *kay-mainbody* than *kay*-γ; Figure 1A). This inversion rectifies the orientation of *kay*-γ but reverses the orientation of *kay*-β. The reversal of *kay*-β orientation suggests why the *kay*-β open reading frame was lost in this species. The *kay*-β reading frame is also absent in *D. persimilis* and *D. grimshawi*, the other two species with the inversion that moves *fig* closer to *kay-mainbody* than *kay*-γ, perhaps for the same reason.

If we employ chromosomal inversions as the sole mechanism for generating the diversity of gene structures seen in all species for the *kay–fig* genomic region, then 11 independent intragenic inversions are required. Utilizing the formula of Bartolomé and Charlesworth (2006), this equals a rate of 0.899 inversions/Mb/MY [(11 inversions/0.0275-Mb region)/445 MY total distance between all 12 species]. Other chromosomal mechanisms (*e.g.*, reading frame maintaining transpositions) may have been involved reducing the number of events, but incorporating them would be pure speculation.

We then determined how the intragenic inversion frequency of the *kay–fig* region compares to published intergenic inversion frequencies. Bartolomé and Charlesworth (2006) report an intergenic inversion frequency for a two-species comparison (*D. melanogaster* and *D. pseudoobscura*) of the E group of the Muller synteny table (this includes the *kay–fig* region) of 0.013/Mb/MY. The rate at which we detected intragenic inversions in the *kay–fig* genomic region was 69-fold greater than this intergenic rate. Thus, either the *kay–fig* region has an anomalously high rate of inversions or the intergenic inversion frequency significantly underestimates the actual rate of inversion. Analysis of additional genes across the 12 Drosophila genomes will be needed to distinguish between these alternatives.

Genomewide analysis of nested gene pairs reveals that ∼20% maintain this arrangement in distant Drosophila species: The absolute conservation of the nested arrangement of *kay* and *fig* contrasted sharply with the multiple inversions that we noted in the region. This led us to wonder if the conservation of nesting across distant Drosophila species for pairs of protein-coding genes was common. If it is common, then this suggests that the nested arrangement is not maintained by natural selection but rather the frequency of mutation is simply insufficient to displace what is actually a serendipitous structure. In this case, a finding of conserved nesting would indicate that the probability that the two genes are functionally related is low—on par with the likelihood that two adjacent genes have a meaningful connection.

Alternatively the conservation of nesting across distant Drosophila species for pairs of protein-coding genes could be uncommon. If it is uncommon, then this suggests that the arrangement was maintained by selection, perhaps to facilitate a functional relationship between the genes. As we could find no genomewide information on the extent of conservation for nested protein-coding gene pairs in Drosophila, we conducted this analysis by two different methods, utilizing three distantly related Drosophila species.

First, we employed a method that relied on genome annotations. Utilizing the annotations, we identified a set of 1261 nested protein-coding gene pairs in the newest release (5.1) of the *D. melanogaster* genome (supplemental Table 9 at http://www.genetics.org/supplemental/). Given the *D. melanogaster* total gene count of 14,124 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=drosoph), these nested protein-coding genes account for ∼8.95%, a slight increase from the assessment of Adams *et al.* (2000).

We utilized annotations to identify a set of 1363 nested protein-coding gene pairs in the latest release (2.0) of the *D. pseudoobscura* genome (supplemental Table 10 at http://www.genetics.org/supplemental/). A comparison of the two sets determined that 215 pairs (17.1% of those found in *D. melanogaster*) are nested in both species (supplemental Table 11 at http://www.genetics.org/supplemental/).

Second, we employed a method that exploited genome sequences. We extracted and translated the exons of each nested gene pair in *D. melanogaster* (derived from annotations as described above). These amino acid sequences were utilized to identify counterpart exons in the *D. pseudoobscura* and *D. virilis* genome sequences using tblastn. Finally, the *D. pseudoobscura* and *D. virilis* exon matches were examined to determine if a nested arrangement that corresponded to the arrangement in *D. melanogaster* was present. This method revealed that 391 nested pairs are conserved in *D. pseudoobscura* (31.0%), 376 are conserved in *D. virilis* (29.8%), and 318 are nested in all three species (25.2%; supplemental Table 12 at http://www.genetics.org/supplemental/).

The high degree of conservation of nesting observed in a relatively small set of genes (~20% if one averages the results of the two analyses) supports the hypothesis that conservation may be linked to a functional relationship between the nested genes, although this is not by itself significant enough to make that argument without further evidence. Given the additional information that serine/threonine phosphatase activity is known to regulate *kay* and that *fig* is a predicted PP2C phosphatase specific for serine/threonine, we believe that the conservation of the nested relationship between these two genes in 12 Drosophila species supports the possibility that there is a functional relationship between them.

Phylogenetic analysis of *kay* protein-coding regions: The coding region of the *kay*-α exon is present only in the nine members of the subgenus Sophophora (Figure 2A). However, in the subgenus Drosophila, *D. virilis* has a segment with low-level DNA similarity to the *kay*-α exon, but it does not encode a recognizable protein. This suggests that this species originally had the *kay*-α coding region, but that it has become degraded by mutation over time. Given the complete absence of the *kay*-α exon in the other two species in the subgenus Drosophila and that *D. virilis* and *D. mojavensis* are more closely related than *D. mojavensis* and *D. grimshawi*, we can construct two possible evolutionary histories for this exon in the subgenus Drosophila. One possibility is that the *kay*-α exon was lost independently and at different times in each lineage. For example, a point mutation resulting in loss of the initiator methionine would eliminate the possibility of selection maintaining the encoded protein. Alternatively, one could postulate a single loss in the lineage leading to all three subgenus Drosophila species with the caveat that the exon degraded at different rates in each lineage. We prefer the first possibility because there is no evidence of differential mutation rates in these lineages.

Bioinformatic analyses of this coding region show that 16% (44/286) of the positions contain an identical/similar amino acid in all species. The *kay*-α tree has one difference from the species tree: *D. ananassae* is shown as an outlier to the *melanogaster* group (Figure 2B). However, bootstrap support for this arrangement is weak and thus this discrepancy is not likely meaningful. Overall, our analysis suggests that the *kay*-α exon predates the divergence of these species, is moderately conserved, and was lost independently in three species.

The coding region of the *kay*-β exon is present in 9 of the 12 species (Figure 3A) and in members of both subgenera. It is not present in the three species, *D. persimilis*, *D. mojavensis*, and *D. grimshawi*, with an inversion affecting *fig* and *kay*-γ. In *D. mojavensis*, there is a segment with low-level DNA similarity to the *kay*-β exon, but it does not encode a recognizable protein. As discussed above, this suggests to us that *D. mojavensis* originally had the *kay*-β coding region but that it has been rendered unusable by the small inversion that rectified the orientation of the *kay*-γ exon. *D. grimshawi* and *D. persimilis* have no trace of the *kay*-β exon.

Bioinformatic analyses of this coding region show that 11% (8/74) of the positions contain an identical/similar amino acid in all species. The *kay*-β tree has two strongly supported differences from the species tree (Figure 3B). First is the clustering of *D. pseudoobscura* with *D. willistoni*. Most likely this reflects the loss of this exon in *D. persimilis*. Second is the movement of *D. virilis* (subgenus Drosophila) between two subgenus Sophophora groups: *D. ananassae* (*melanogaster* group) and *D. pseudoobscura* (*obscura* group). Most likely this reflects the loss of this exon in the other subgenus Drosophila species. Overall, our analysis suggests that the *kay*-β exon predates the divergence of these species, is moderately conserved, and was lost independently in three species.

The *kay*-γ exon is the only one of the alternative 5′ exons that is present in all species (Figure 4A). Bioinformatic analyses of this coding region show that 74% (20/27) of the positions contain an identical/similar amino acid in all species. The *kay*-γ tree shows considerable differences from the species tree. However, the very short length of the alignment leads to a lack of statistical significance (Figure 4B). Overall, our analysis indicates that the *kay*-γ exon predates the divergence of these species and is very highly conserved.

The *kay-mainbody* coding region is present in all 12 species (Figure 5A). We noted a gap in the middle of this sequence in *D. simulans*, so this species was not used in the analysis. Bioinformatic analyses found that the Basic region and leucine-zipper regions are highly conserved. Thirty-six percent (193/541) of the positions contain an identical/similar amino acid in all species.

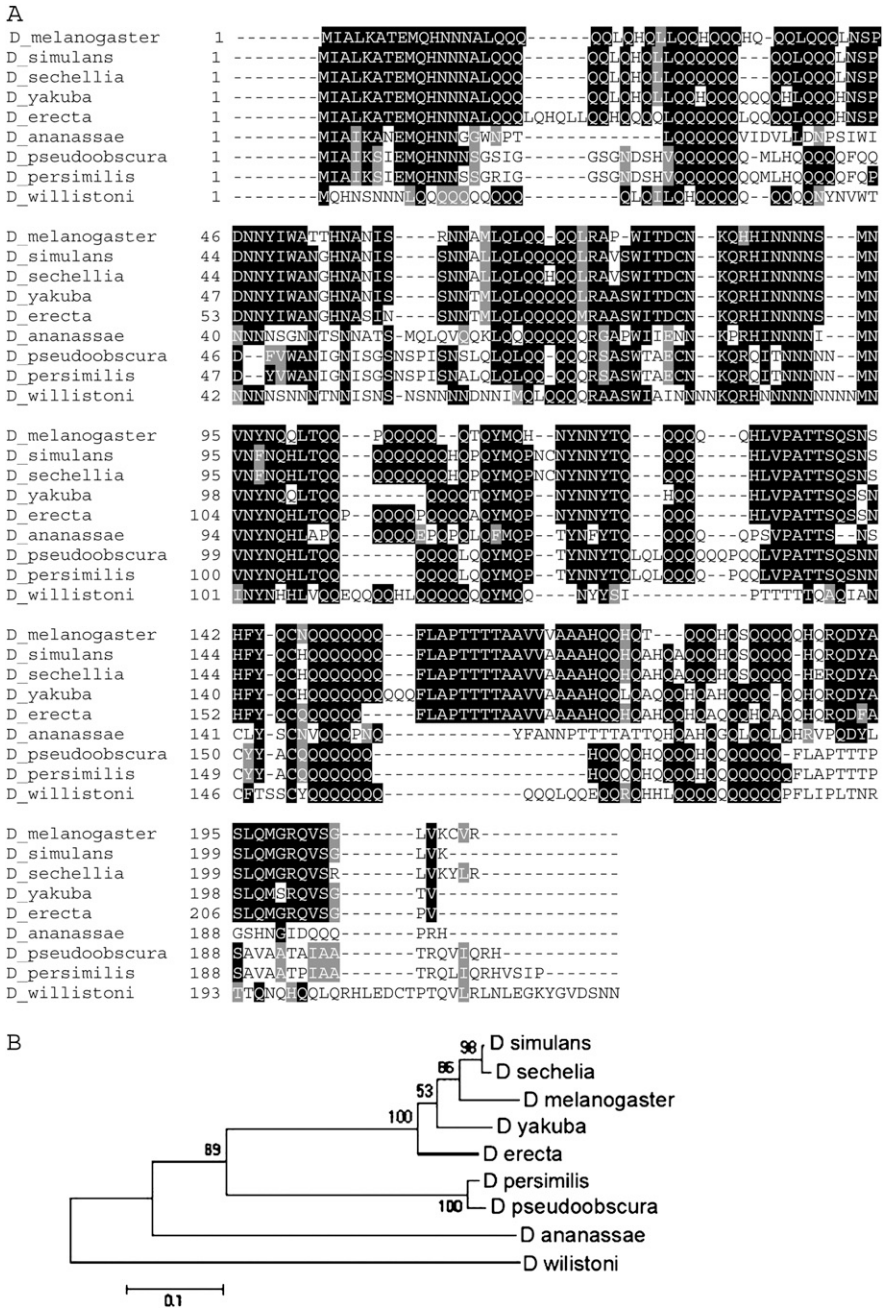


FIGURE 2.—Alignment and phylogenetic tree of the *kay*-α exon. (A) Alignment of the coding region from nine species that have an α exon, utilizing the one-letter amino acid code. The total length of the alignment is 286 amino acids. Amino acid numbers are shown to the left. Amino acids are noted if, at a particular position, more than half of the species have the same amino acid (solid background) or a similar amino acid (shaded background). Dashes represent gaps inserted to maximize amino acid similarity between the sequences. The coordinates for each sequence are shown in supplemental Table 1 at http://www.genetics.org/supplemental/. (B) Phylogenetic tree of the coding region from nine species that have the *kay*-α exon. The tree is unrooted and branch lengths are drawn to scale. The scale bar shows the number of amino acid substitutions per site between two sequences. Branches with the highest bootstrap values are indicated and those >70 are considered statistically significant (SITNIKOVA 1996). Here, five of the eight branches are significant.

This fits well with the demonstrated functions for these two regions: the leucine zipper holds the functional *c-fos* dimer together and the Basic region immediately preceding the zipper binds DNA to stimulate transcription of target genes (CHEN *et al.* 1996). The *kay-mainbody* tree, even without *D. simulans*, is identical to the species tree (Figure 5B). Overall, our analysis indicates that the *kay-mainbody* exon predates the divergence of these species and is well conserved.

In summary, our analysis of *kay* protein-coding exons indicates that all four exons predate the divergence of these species. However, there is variation in their conservation level. The *kay*-α and *kay*-β exons are moderately conserved and have been lost independently six times. The *kay*-γ and *kay-mainbody* exons are present in all species and are highly conserved.

Phylogenetic analysis of *fig*: Initially, we wondered if there were any well-characterized proteins similar to *fig* in other species that would suggest a function for this gene. To identify such sequences, we conducted BLASTp and protein domain searches. The domain analysis identified *fig* as a PP2C enzyme (InterPro database accession no. IPR000222) and, of the top 14 BLAST matches, 12 were PP2C Phosphatases (supplemental Table 8 at http://www.genetics.org/supplemental/). The other two matches (the top matches) were to other predicted *D. melanogaster* genes (CG15035 and CG12091). None of the BLAST matches were insubstantial as their

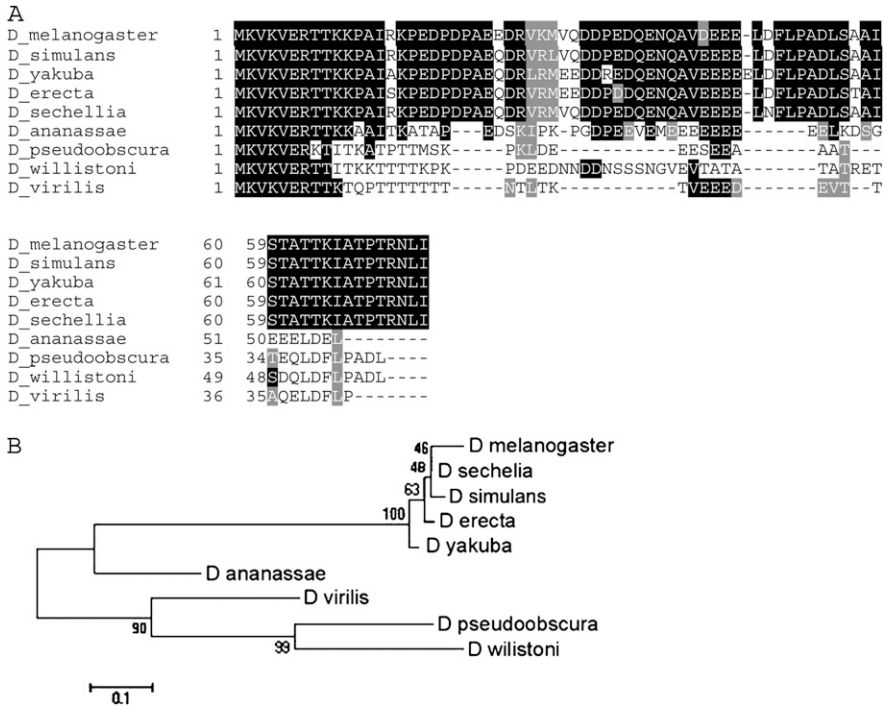


FIGURE 3.—Alignment and phylogenetic tree of the *kay*-β exon. (A) Alignment of the coding region from nine species that have a *kay*-β exon. The total length of the alignment is 74 amino acids. Amino acid numbering and shading are as in Figure 2. The coordinates for each sequence are shown in supplemental Table 2 at http://www.genetics.org/supplemental/. (B) Phylogenetic tree of the coding region from nine species that have a *kay*-β exon. The tree is drawn as in Figure 2. Bootstrap values indicate that only three of the eight branches are significant.

scores ranged from 5*e*-61 to 5*e*-39 and they represented organisms from flies to humans. Thus, *fig* is very likely a member of a large family of PP2C phosphatases. These enzymes are serine/threonine-specific protein phosphatases that are active on a wide variety of substrates. They are found in plants, animals, and bacteria (*e.g.*, Paramecium). Interestingly, this nearly universal species distribution is similar to that of *fos* (if one considers the *c*- and *v*- forms).

The *fig* coding region is present in all 12 Drosophila species (Figure 6A). Bioinformatic analyses show that *fig* is highly conserved. Thirty-three percent (126/382) of the positions contain an identical/similar amino acid in all species, a level of conservation nearly identical to that seen for *kay-mainbody*. The *fig* tree agrees with the species tree with two minor exceptions (Figure 6B). First, in the *fig* tree, *D. willistoni* falls within the subgenus Drosophila (with a 100% bootstrap value) instead being the most distinct species in the subgenus Sophophora. However, this is likely due to an unreadable stretch of nucleotides in the coding region that result in a gap of 19 amino acids (Figure 6A). Second, the *obscura* group including *D. willistoni* is considered more distant from the *melanogaster* group than the subgenus Drosophila. However, without a bootstrap value, this is likely not meaningful. Overall, our analysis indicates that *fig* predates the divergence of these species and is well conserved and that its evolutionary history is the same as that of *kay-mainbody*.

We could find only a single published study of a PP2C family member in Drosophila (DICK *et al.* 1997). Therefore, to gather additional information on Drosophila PP2C family members, we decided to examine the relationship between *fig* and the two most similar genes in the database (supplemental Table 8 at http://www.genetics.org/supplemental/)—the predicted Drosophila genes CG15035 and CG12091. Given the identity of the other BLAST hits (all with less convincing matches to *fig*), it seems safe to assume that these are also PP2C phosphatases.

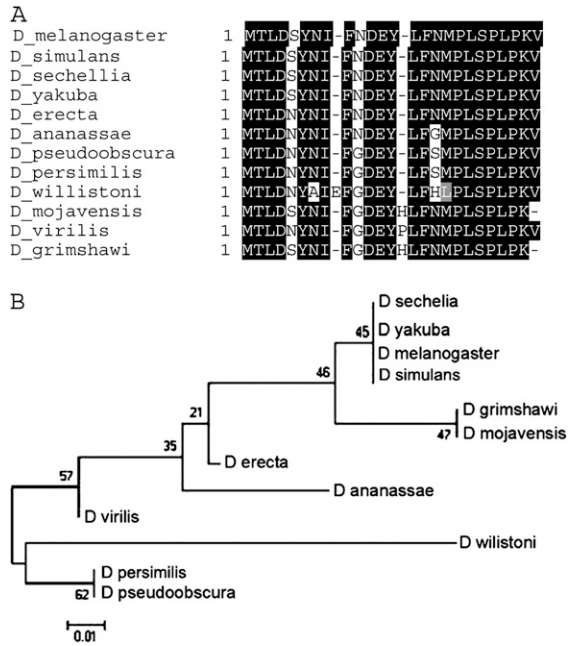


FIGURE 4.—Alignment and phylogenetic tree of the *kay*-γ exon. (A) Alignment of the coding region from the *kay*-γ exon in all 12 species. The total length of the alignment is 27 amino acids. Amino acid numbering and shading are as in Figure 2. The coordinates for each sequence are shown supplemental Table 6 at http://www.genetics.org/supplemental/. (B) Phylogenetic tree of the coding region from the *kay*-γ exon in all 12 species. The tree is drawn as in Figure 2. Due to the short length of the alignment, bootstrap values are not significant.

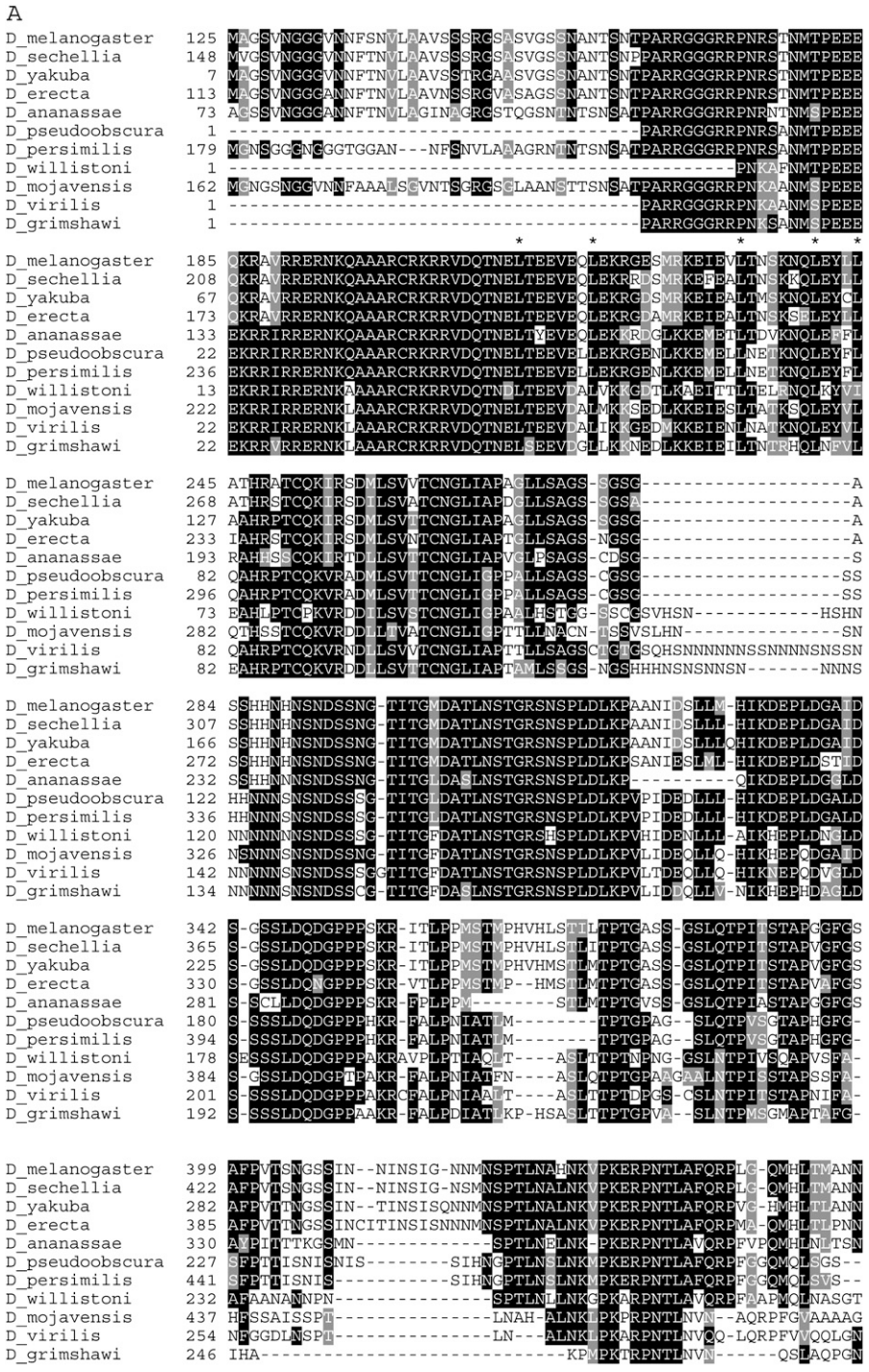


FIGURE 5.—Alignment and phylogenetic tree of the *kay-mainbody* exon. (A) Alignment of the coding region from the *kay-mainbody* exon in 11 species. What is shown begins at the equivalent of the *D. melanogaster* amino acid 125 because the location of the splice donor for this exon is highly variable. *D. simulans* was not used due to incomplete sequence in the database. The Basic region and leucine zipper begin at positions 186 and 212, respectively, in *D. melanogaster.* The five invariant leucines are indicated by asterisks above the *D. melanogaster* sequence. The total length of the alignment is 541 amino acids. Amino acid numbering and shading are as in Figure 2. The coordinates for each sequence are shown in supplemental Table 7 at http://www.genetics.org/supplemental/. (B) Phylogenetic tree of the coding region from the *kay-mainbody* exon in 11 species. The tree is drawn as in Figure 2. Bootstrap values indicate that 7 of the 10 branches are significant.

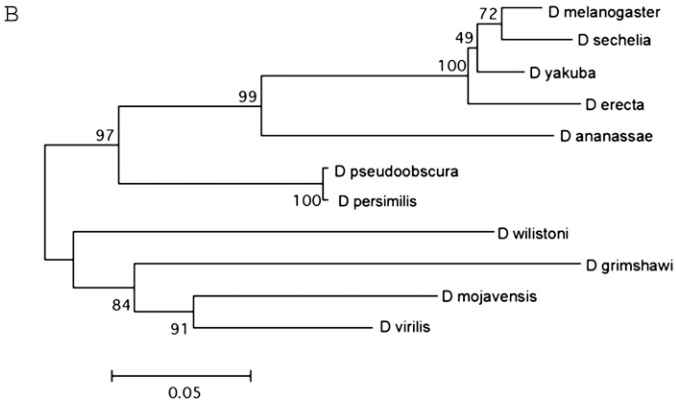
First we determined that each of these genes is present in all species. This was unsurprising as most species have multiple PP2C genes. For example, there are five PP2C genes in mammals (JIN *et al.* 2004). However, due to technical issues (gaps and N's) in the genome sequences, we could retrieve only 35 sequences, and three of these are not full length. *D. simulans* CG15035 could not be retrieved, as it is barely visible alongside a gap that takes out most of the coding region. *D. ananassae* CG15035 is truncated at its amino terminus, and *D. persimilis* CG12091 is truncated at its carboxy terminus by gaps. Also, as noted above, *D. willistoni fig* has an unreadable stretch of 19 amino acids. Sequence identifiers for each gene are found in supplemental Tables 3–5 (http://www.genetics.org/supplemental/).

We then generated an alignment of the 35 sequences (supplemental Figure 1 at http://www.genetics.org/supplemental/). The alignment revealed that the *obscura* group species CG15035 genes have 5′ extensions of 130 amino acids and that their *fig* sequences have 3′ extensions of 19 amino acids not found in any other species. Excluding these extensions, a core stretch of

```
D_melanogaster   455 -----KAGGPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLVPNSSSTNKHPLELP
D_sechellia      478 -----KAGGPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLVPNSSSTNKHPLELP
D_yakuba         339 N----KPGGPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLVPNSSSANKHPLELP
D_erecta         444 -----KAGGPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLVPNSSSTNKHPLELP
D_ananassae      376 HNKM-PGSGPTHIQGVPIQTPTTG-FNFDSLMDGGTGLTPVSGPLVP---SQNKHPLELP
D_pseudoobscura  277 ------GRAPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLIPNCTSQNKHPLELP
D_persimilis     488 ------GRAPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLIPNCTSQNKHPLELP
D_willistoni     277 GGVVDGKGAPIQIQGVPIQTPSTGVFNFDSLMDGGTGLTPVSGPLIPNCSSQNKHPLELP
D_mojavensis     479 DG----KAPPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLMPTCSSQNKHPLELP
D_virilis        295 DGN---KAPPTQIQGVPIQTPSTGTFNFDSLMDGGTGLTPVSGPLVPTCSSQNKHPLELP
D_grimshawi      271 IAI---GKTPMQIEGVPIQTPSAGNFNFDSLMAGGTGLTPVSGPLVPTCSSQNKHPLELP

D_melanogaster   510 TPTAEPSKLVSL
D_sechellia      533 TPTAEPSKLVSL
D_yakuba         395 TPTAEPSKLVSL
D_erecta         499 TPTAEPSKLVSL
D_ananassae      431 TPTAEPSKLVSL
D_pseudoobscura  331 TPTTEPSKLVSL
D_persimilis     542 TPTTEPSKLVSL
D_willistoni     337 TPTTEPSKLCPL
D_mojavensis     535 TPTSEPSKLVSL
D_virilis        352 TPTSEPSKLVSL
D_grimshawi      328 TPTSEPSKLVSL
```

FIGURE 5.—*Continued.*



~250 amino acids is well conserved in all sequences. Four discrete regions that encompass over half of the core sequence (~150 amino acids) and are very highly conserved were identified. Within these regions, between 39 and 71% of the positions have an identical/similar amino acid in all species (here we ignore gaps due to the incomplete nature of several sequences).

The tree of the 35 Drosophila PP2C coding regions (Figure 7) identifies three different distinct subfamilies with statistically significant support: a *fig* subfamily (top), a CG12091 subfamily (middle), and a CG15035 subfamily (bottom). The CG15035 sequences do not actually form a cluster like the other two in which all sequences connect to a single originating branch. The CG15035 sequences form a loose group of smaller clusters based on exclusion from the other subfamilies. Nevertheless, each subfamily contains one member from each species.

In the PP2C tree, the *fig* subfamily tree is identical to the tree obtained when only *fig* sequences were analyzed (Figure 6) and is strongly supported as 8 of its 11 branches have statistically significant bootstraps. Given the exception noted above for *D. willistoni*, the *fig* subfamily tree is different from the species tree in that it has the subgenus Drosophila between the *melanogaster* group and the *obscura* group. However, unlike the *fig* tree, in the PP2C tree this change has significant support.

The CG12091 subfamily tree is unaffected by the truncation of *D. persimilis*, and this species remains in the *obscura* group. The CG12091 subfamily tree is different from the species tree in two ways, one major and one minor. Like the *fig* subfamily tree, the CG12091 subfamily tree gives statistically significant support for placing the subgenus Drosophila between the *melanogaster* group and the *obscura* group. In a minor difference, the CG12091 subfamily tree groups *D. ananassae* with *D. willistoni*. In the CG15035 subfamily tree, there are two differences from the species tree but neither is strongly supported. *D. ananassae* is clustered with the *obscura* group most likely due to its truncation and *D. willistoni* is again more distant from the *melanogaster* group than the subgenus Drosophila.

Clearly, all of the PP2C subfamilies predate the divergence of the species and each subfamily tree strongly resembles the species tree. In each subfamily tree, the subgenus Drosophila and the *melanogaster* subgroup (8 of the 12 species) appear as they do in the species tree. Differences between the species tree and the subfamily trees that are not explained by sequence gaps are limited to the intervening species, *D. ananassae*, *D. willistoni*, and the *obscura* group. One possible explanation is that when a single species is utilized to represent a large number of species (*e.g.*, in Figure 1C, *D. ananassae* representing the 148 species of the *melanogaster* group that do not belong to

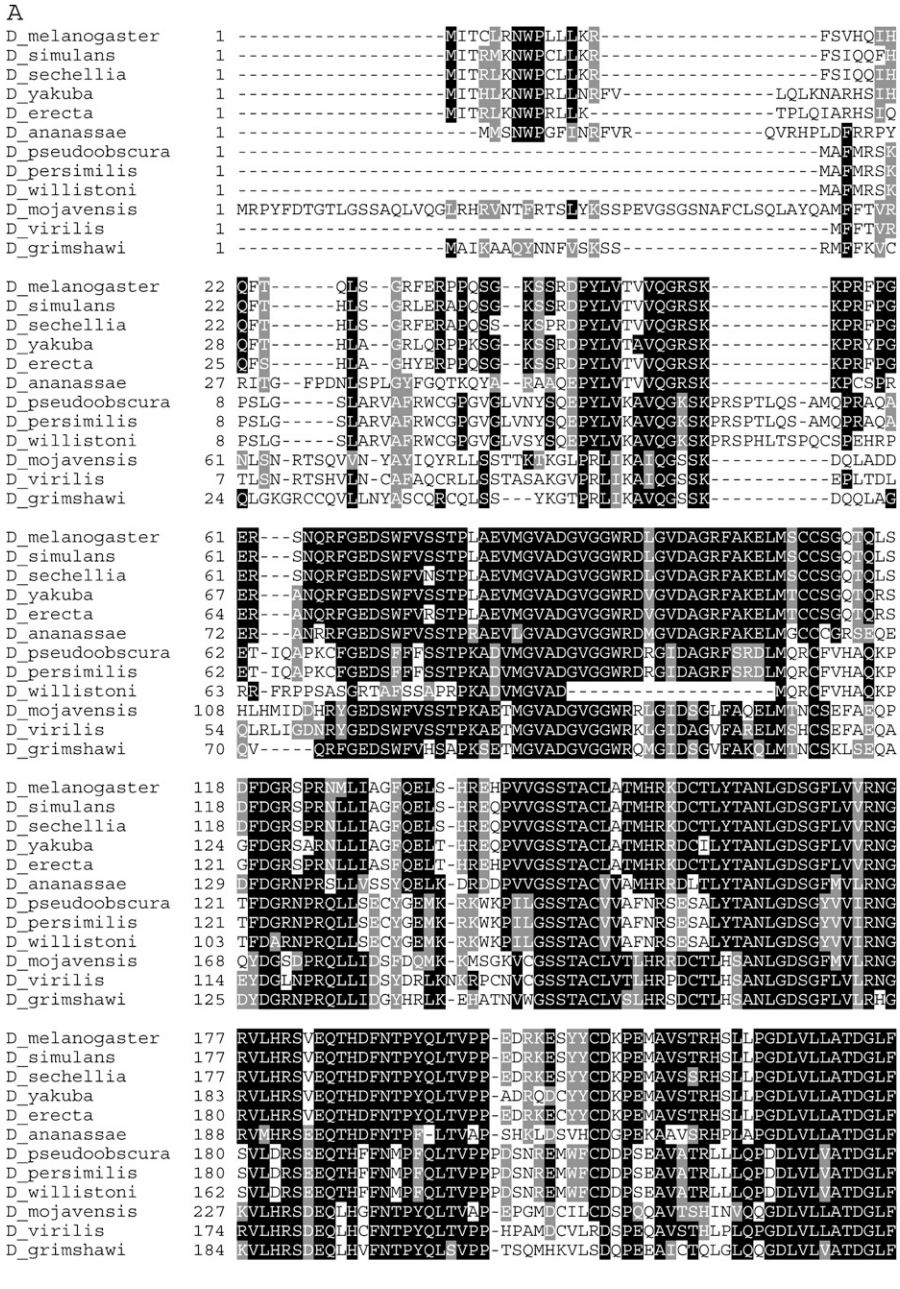


FIGURE 6.—Alignment and phylogenetic tree of *fig*. (A) Alignment of the coding region of *fig* from all species. The total length of the alignment is 382 amino acids. Amino acid numbering and shading are as in Figure 2. The coordinates for each sequence are shown in supplemental Table 3 at http://www.genetics.org/supplemental/. (B) Phylogenetic tree of the coding region of *fig* from all species. The tree is drawn as in Figure 2. Bootstrap values indicate that 9 of the 11 branches are significant. The placement of *D. willistoni* in the *obscura* group is likely due to a gap of 19 amino acids.

the *melanogaster* subgroup; ASHBURNER 1989), stochastic changes that occurred in that lineage can have an excessive impact on that species' placement in the tree.

DISCUSSION

Our analysis provides a new illustration of the power of phylogenetic analysis to suggest experimentally testable hypotheses for the function of poorly characterized genes. We, and others, have typically employed phylogenetic analysis to address large-scale questions (*e.g.*, NEWFELD *et al.* 1999; NEWFELD and WISOTZKEY 2006), utilizing sequences from widely disparate organisms such as humans and flies that have an estimated divergence of 950 MY (WANG *et al.* 1999). In these studies, the goal is to determine the evolutionary relationship among members of a multigene family in different species. Information on these relationships (homology, orthology, paralogy) is then employed by experimental biologists as a framework in which to meaningfully interpret experiments outside their own model system.

The near-complete sequencing of the genomes of 12 species of Drosophila allows us to expand the application of phylogenetic analysis to address small-scale questions utilizing sequences from closely related organisms (divergence of the Sophophora and Drosophila subgenera is estimated at 63 MY; TAMURA *et al.* 2004). Here, phylogenetics can be employed to test hypotheses about the structure of a single gene and the functional relationships among genes in the same species.

Our analysis of *kay* structure reveals an intriguing juxtaposition of conservation (the nested arrangement

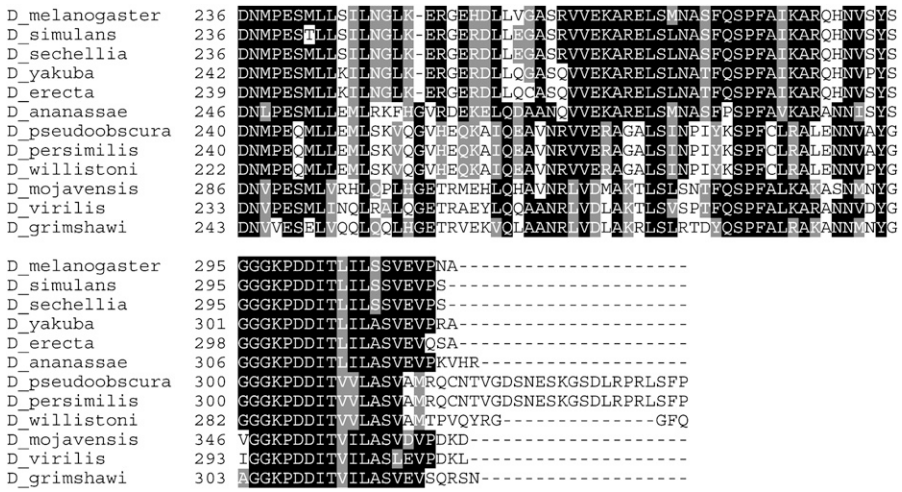
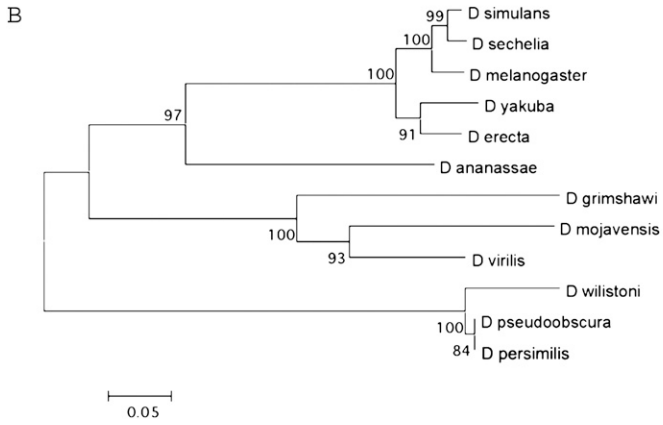


FIGURE 6.—*Continued.*



and divergent orientation of *kay* and *fig* transcription) and diversification (a 69-fold greater rate of intragenic inversion than previously reported rates of intergenic inversion). This shows, at the molecular level, the ability of natural selection to maintain the functionality of an essential gene in spite of the mutations and chromosome rearrangements that are an inevitable feature of DNA replication and mitosis/meiosis. Regarding a potential *kay–fig* functional relationship, our analysis reveals that *fig* is divergently transcribed and nested in a *kay* intron in all 12 species. Since our genomewide analysis determined that only 20% of the nested gene pairs in *D. melanogaster* are conserved in *D. pseudoobscura* and *D. virilis*, we believe that the absolute conservation of the nested relationship strongly supports the possibility that there is a functional relationship between *kay* and *fig*.

Taken together, our data also lead us to propose a model for the genetic mechanism underlying the origin and maintenance of the nested arrangement of *kay* and *fig*. For the origin, we propose a genetic event (an inversion seems likely, given their frequency in the region) predating the divergence of the 12 Drosophila species that placed *fig* upstream of *kay* in a head-to-head orientation. At that time, *kay* consisted of its most highly conserved exons (*kay*-γ and *kay-mainbody*) with a sole promoter region adjacent to the 5′-end of *kay*-γ. A head-to-head orientation of two genes requires that they be divergently transcribed, and the inversion placed the 5′-end of *fig* near the *kay*-γ promoter. This orientation still exists for the 9 species in the subgenus Sophophora. Given the proximity of the two 5′-ends, perhaps the *kay*-γ promoter began to influence *fig* transcription and it became a bidirectional promoter. Bidirectional promoters have been reported for other gene pairs with head-to-head orientations (*e.g.*, DHFR and Rep-3 in mice; LINTON *et al.* 1989).

In a subsequent step (or steps), also prior to the divergence of the 12 species, the *kay*-α and *kay*-β exons and their respective promoter regions were created downstream of *fig* with an orientation that allowed them to splice to the *kay-mainbody* exon. The creation of these new exons (perhaps by transposable element or illegitimate recombination mechanisms; LONG 2001; PAVLICEK *et al.* 2002) would then place *fig* in a *kay* intron. According to this model, the nested arrangement of *kay* and *fig* has been maintained because over time the *kay*-γ promoter became an irreplaceable component of *fig* transcription.

As such, any event that moved *fig* away from the 5′-end of *kay*-γ or reversed its direction of transcription would be strongly selected against or compensated for by a subsequent event. In fact, a reversal in *fig* transcription was not observed in any species even though *fig* was moved to the 3′ side of *kay*-γ by an inversion in three

Figure 7.—Phylogenetic tree of Drosophila PP2C phosphatases. The tree was derived from an alignment of the coding regions of 35 PP2C phosphatase sequences. See supplemental Figure 1 at http://www.genetics.org/supplemental/ for the alignment in which amino acid numbering and shading are as in Figure 2. What is shown begins at the equivalent of the *D. pseudoobscura* and *D. persimilis* CG15035 amino acid 121 as these species have unique 5′ extensions. Each of the 12 species has three PP2C phosphatases except *D. simulans* CG15035 could not be retrieved. *D. ananassae* CG15035 is truncated at its amino terminus and *D. persimilis* CG12091 at its carboxy terminus. *D. willistoni fig* has a gap of 19 amino acids. The total length of the alignment is 518 amino acids. The coordinates for each sequence are shown supplemental Tables 3–5. Numbers in parentheses identify individual sequences in supplemental Tables 4–5 and in supplemental Figure 1. Bootstrap values indicate that the presence of three subfamilies containing one sequence from each species is significant: *fig* (top), CG12091 (middle), and CG15035 (bottom). Within these subfamilies, CG15035 (6 of 10) and *fig* (8 of 11) have a majority of significant branches while CG12091 (4 of 11) has less than half of its branches as significant.

species. Evidence of a subsequent reorienting inversion affecting *kay*-γ is visible in one of these species and is therefore inferred in the other two. Finally, as our model has *kay*-α and *kay*-β as relatively new exons, it is not surprising that they have been lost numerous times.

We sought to gain support for this model by examining the arrangement of *kay* and *fig* in an insect outside the genus Drosophila. First, we analyzed the mosquito *An. gambiae*, the closest relative to Drosophila with a genome-sequencing project. Unfortunately, the *kay–fig* region is incomplete at this time. We determined that the *kay-mainbody* exon is present as two exons in mosquitoes. The first contains amino acids 173–252 and the second (located 22.8 kb downstream) contains amino acids 434–521 according to the *D. melanogaster* sequence (Figure 5). Upstream of the first of these exons is a gap of ~17.7 kb. When we BLAST the mosquito genome with *kay*-α, *kay*-β, or *kay*-γ, we retrieve nothing meaningful possibly because they are located in the gap. A BLAST with *fig* retrieves only a CG12095-like sequence. Thus, at this time we obtain only negative evidence to support our model: either there is no *fig* in mosquitoes or perhaps it lies in the gap upstream of *kay-mainbody* with *kay*-γ. The genome of the honeybee *A. mellifera* is even more incomplete: no evidence of *kay-mainbody* (which should be present as the *kay* homolog *c-fos* is present in humans) could be obtained by BLAST.

Our model presents a number of experimentally testable hypotheses. For example, to determine if *kay* and *fig* are connected by common regulation, reporter genes can be constructed carrying the region between *kay*-γ and *fig* to learn if it contains a promoter. Further, these reporters can be designed to test our idea that this promoter is bidirectional. Alternatively, to determine if these genes have a biochemical connection, one can examine the ability of *fig* to dephosphorylate Kay constructs that have phosphates attached at one or more of its Jun-amino terminal kinase or ERK phosphorylation sites in cell culture assays. In addition, a comparison of gene expression patterns may indicate that *fig* or another of the PP2C phosphatases substantially overlaps with *kay*. New information gained from these experiments will immediately suggest new avenues of investigation into the human *c-fos* proto-oncogene.

In summary, our analysis demonstrates that phylogenetics can be profitably employed to generate testable hypotheses regarding gene structure or the relationship between two genes in the same species, an extension of current practice. The application of this approach to understanding the *kay–fig* nested gene pair suggested that the arrangement was functional. With the availability of 12 sequenced Drosophila genomes, studies such as this will become an increasingly important tool for researchers.

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