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A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w^{1118}; *iso*-2; *iso*-3

Pablo Cingolani,[1-3] Adrian Platts,[4] Le Lily Wang,[1] Melissa Coon,[2] Tung Nguyen,[5] Luan Wang,[1,2] Susan J. Land,[2] Xiangyi Lu[1] and Douglas M. Ruden[1,2,*]

[1]Institute of Environmental Health Sciences; Wayne State University; Detroit, MI USA; [2]Department of Obstetrics and Gynecology; Wayne State University School of Medicine; C.S. Mott Center; Detroit, MI USA; [3]School of Computer Science & Genome Quebec Innovation Centre; McGill University; Quebec, Canada; [4]Department of Bioinformatics; McGill University; Quebec, Canada; [5]Department of Computer Sciences; Wayne State University; Detroit, MI USA

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We describe a new computer program, SnpEff, for rapidly categorizing the effects of variants in genome sequences. Once a genome is sequenced, SnpEff annotates variants based on their genomic locations and predicts coding effects. Annotated genomic locations include intronic, untranslated region, upstream, downstream, splice site, or intergenic regions. Coding effects such as synonymous or non-synonymous amino acid replacement, start codon gains or losses, stop codon gains or losses, or frame shifts can be predicted. Here the use of SnpEff is illustrated by annotating ~356,660 candidate SNPs in ~117 Mb unique sequences, representing a substitution rate of ~1/305 nucleotides, between the *Drosophila melanogaster* w^{1118}; *iso*-2; *iso*-3 strain and the reference y^1; cn^1 bw^1 sp^1 strain. We show that ~15,842 SNPs are synonymous and ~4,467 SNPs are non-synonymous (N/S ~0.28). The remaining SNPs are in other categories, such as stop codon gains (38 SNPs), stop codon losses (8 SNPs), and start codon gains (297 SNPs) in the 5'UTR. We found, as expected, that the SNP frequency is proportional to the recombination frequency (i.e., highest in the middle of chromosome arms). We also found that start-gain or stop-lost SNPs in Drosophila melanogaster often result in additions of N-terminal or C-terminal amino acids that are conserved in other Drosophila species. It appears that the 5' and 3'UTRs are reservoirs for genetic variations that changes the termini of proteins during evolution of the Drosophila genus. As genome sequencing is becoming inexpensive and routine, SnpEff enables rapid analyses of whole-genome sequencing data to be performed by an individual laboratory.

Introduction

When we re-sequenced the w^{1118}; *iso*-2; *iso*-3 genome in 2009,[1] bioinformatics tools were unable to rapidly categorize the ~356,660 SNPs as comparing to the y^1; cn^1 bw^1 sp^1 reference strain. The available tools at the time such as ENSEMBL's variant web application (ensembl.org) could only analyze a few hundred to a few thousand SNPs per batch. Therefore, over the past couple of years, we have been developing a new program called SnpEff (an abbreviation of "SNP effect") which is able to analyze and annotate thousands of variants per second and predict their possible genetic effects.

In this study, we analyze the output of SnpEff (version 1.9.6) analyses of the ~356,660 candidate SNPs that we identified in w^{1118}; *iso*-2; *iso*-3 with respect to the y^1; cn^1 bw^1 sp^1 reference strain as reported in our previous paper.[1] This is of great interest to the Drosophila community because thousands of transposon insertion stocks[5] and hundreds of deficiency stocks[6,7] were generated in the w^{1118}; *iso*-2; *iso*-3 genetic background. The presence of large number and potential severity of many SNPs in the two laboratory strains was a surprising finding and the possible evolutionary implications of this are discussed.

Program description. Genomic variants comprise single nucleotide polymorphism (SNPs), insertions and deletions (INDELs), and multiple nucleotide polymorphisms (MNPs). SnpEff annotates these variants based on their genomic locations, such as intronic, untranslated region (5' UTR or 3'UTR), upstream, downstream, splice site, or intergenic regions. It also predicts coding effects such as synonymous or non-synonymous amino acid replacement, start codon gains or losses, stop codon gains or losses, or frame shifts. Predicted effects are with respect to protein coding genes. Variants affecting non-coding genes are annotated and the corresponding bio-type is identified, whenever the information is available.

*Correspondence to: Douglas Ruden; Email: douglasr@wayne.edu
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Table 1. Output of SnpEff

# SNP	Gene_name	Effect	Old_AA/new_AA	Old_codon/New_codon	Codon_Num (CDS)	CDS_size
chr2L:10006682_C/T	CG31755	UPSTREAM: 541 bases				
chr2L:10006758_G/A	CG31755	UPSTREAM: 465 bases				
chr2L:10007289_G/A	CG4747	SYNONYMOUS_CODING	L/L	TTG/TTA	489	1809
chr2L:10007319_G/C	CG4747	SYNONYMOUS_CODING	G/G	GGG/GGC	499	1809
chr2L:10007356_A/T	CG4747	INTRON				1809
chr2L:10007363_T/A	CG4747	INTRON				1809

SNP, a description of the single nucleotide polymorphism (SNP) indicating chromosome arm (chr2L), coordinate in genome (10006682), and nucleotide change (e.g., C/T indicates that C is replaced by T in w^{1118}; *iso*-2; *iso*-3 at this position). Gene_name, official gene symbol of gene. Effect, description of SNP (e.g., upstream of transcription start site at position -541). Old_AA/new/AA, amino acid change, if any, in one letter code. Old_codon/New_codon, if a codon contains a SNP, the old (reference) and new (w^{1118}; *iso*-2; *iso*-3) codons are indicated. Codon_Num (CDS), the codon number of the coding sequence (CDS). CDS_size, the size of the protein in amino acids.

SnpEff (snpeff.sourceforge.net) is open source, platform independent and freely available for all users. The main features of SnpEff include: (1) speed - the ability to make thousands of predictions per second; (2) flexibility - the ability to add custom genomes and annotations; (3) web-based - the ability to integrate with Galaxy, an open access and web-based platform for computational bioinformatic research (gmod.org/wiki/Galaxy); (4) multi-organism - compatibility with multiple species and multiple codon usage tables (e.g., mitochondrial genomes); (5) integration with Broad's Genome Analysis Toolkit (GATK);[4] and (6) ability to perform non-coding annotations.

Before using SnpEff, a variants file must be generated using sequencing information. A variants file lists all SNPs, MNPs, and INDELs found by re-sequencing a genome. A simple walkthrough example on how to analyze sequencing data to calculate variants and their effects using SnpEff is shown in Listing SL1. In a nutshell, the analysis pipeline has three steps: (i) map the reads to the genome, (ii) call variants and (iii) use SnpEff to annotate variants. This example is intended for illustration purposes only since it omits many routine steps used in re-sequencing data analysis pipelines.

In addition to SnpEff, there are other recently developed programs for annotating genomic variants, most notably "Annotate Variation" (ANNOVAR)[2] and "Variant Annotation, and Analysis and Search Tool" (VAAST).[3] However, SnpEff differs from these programs in that it is an open source for all users, permits annotation of more genome versions, natively supports Variant Call Format (VCF)[9] files and it is marginally faster (although the speeds of SnpEff, ANNOVAR, and VAAST are comparable). **Table S1** shows a feature comparison of some currently available software packages.

At this time, SnpEff has been set up for annotating DNA polymorphisms of over 320 genome versions of multiple species including the human genome. Sources of information for creating these databases are ENSEMBL, UCSC as well as organism specific databases, such as FlyBase (*Drosophila melanogaster*), WormBase (*C. elegans*) and TAIR (*Arabidopsis thaliana*). Additional genomes can be added by the user or provided upon request.

Detailed information on how to download, install and run, as well as usage examples of the program can be found at the website (snpeff.sourceforge.net), also a frequently asked questions (FAQ) section that addresses most issues that a user might have in operating this program.

SnpEff has already been used by over 50 institutions and universities in the bioinformatics community. Rapid analyses of whole-genome sequencing data should now be feasible to perform by an individual laboratory.

Results

Table 1 shows the beginning portion of the output generated by SnpEff when the SNPs in w^{1118}; *iso*-2; *iso*-3 were compared with the reference genome, y^1; cn^1 bw^1 sp^1(*Drosophila melanogaster* release 5.3). A complete list of SnpEff output is shown in **Table 2**. We published a list of variant for w^{1118}; *iso*-2; *iso*-3 in our previous paper[1] and it was derived by comparing hundreds of millions of short sequence reads (~20-fold genome coverage).[8]

Heterozygosity is not considered in the w^{1118}; *iso*-2; *iso*-3 sequence because the stock was isogenized and only high quality (i.e., homozygous SNPs) were used for this analysis.[1] According to SnpEff (version 1.9.6), the largest number of SNPs in w^{1118}; *iso*-2; *iso*-3 are in introns (130,126) followed by those in upstream (76,155), downstream (71,645) and intergenic (51,783) regions (Fig. 1). "Upstream" is defined as 5 kilobase (kb) upstream of the most distal transcription start site and "downstream" is defined as 5 kb downstream of the most distal polyA addition site, but these default variables can be easily adjusted. SnpEff also found thousands of SNPs within the transcribed regions of genes. For example, there are 3,718 SNPs in the 3' untranslated regions (3' UTR), and 2,508 SNPs in the 5' UTR. The SNPs in the upstream, downstream, 5′ and 3′ UTR regions might affect transcription or translation, but the actual effects have to be confirmed case-by-case. In the next few sections, we present examples of several types of SNPs that might affect the protein function.

SNPs that generate new start codons. There are 297 SNPs that potentially generate a new translation initiation codon in the 5' UTR (referred to as start-gain SNPs). The most common translation initiation codon is AUG, which is coded by ATG in the genome. To be thorough, we also included CUG and UUG codons, which code for leucine, as these codons can also be used

Table 2. Detailed effect list from SnpEff

Effect	Notes
INTERGENIC	The variant is in an intergenic region
UPSTREAM	Upstream of a gene (default length: 5K bases)
UTR_5_PRIME	Variant hits 5'UTR region
UTR_5_DELETED	The variant deletes an exon which is in the 5'UTR of the transcript
START_GAINED	A variant in 5'UTR region produces a three base sequence that can be a START codon
SPLICE_SITE_ACCEPTOR	The variant hits a splice acceptor site (defined as two bases before exon start, except for the first exon)
SPLICE_SITE_DONOR	The variant hits a Splice donor site (defined as two bases after coding exon end, except for the last exon)
START_LOST	Variant causes start codon to be mutated into a non-start codon
SYNONYMOUS_START	Variant causes start codon to be mutated into another start codon
CDS	The variant hits a CDS
GENE	The variant hits a gene
TRANSCRIPT	The variant hits a transcript
EXON	The vairant hist an exon
EXON_DELETED	A deletion removes the whole exon
NON_SYNONYMOUS_CODING	Variant causes a codon that produces a different amino acid
SYNONYMOUS_CODING	Variant causes a codon that produces the same amino acid
FRAME_SHIFT	Insertion or deletion causes a frame shift
CODON_CHANGE	One or many codons are changed
CODON_INSERTION	One or many codons are inserted
CODON_CHANGE_PLUS_CODON_INSERTION	One codon is changed and one or many codons are inserted
CODON_DELETION	One or many codons are deleted
CODON_CHANGE_PLUS_CODON_DELETION	One codon is changed and one or more codons are deleted
STOP_GAINED	Variant causes a STOP codon
SYNONYMOUS_STOP	Variant causes stop codon to be mutated into another stop codon
STOP_LOST	Variant causes stop codon to be mutated into a non-stop codon
INTRON	Variant hist and intron. Technically, hits no exon in the transcript
UTR_3_PRIME	Variant hits 3'UTR region
UTR_3_DELETED	The variant deletes an exon which is in the 3'UTR of the transcript
DOWNSTREAM	Downstream of a gene (default length: 5K bases)
INTRON_CONSERVED	The variant is in a highly conserved intronic region
INTERGENIC_CONSERVED	The variant is in a highly conserved intergenic region

to initiate translation in rare genes in Drosophila and mammals.[10,11] There are 60 genes with ATG start-gain SNPs (**Table 5**), 99 genes with CTG start-gain SNPs, and 120 genes with TTG start-gain SNPs in *w*^{1118}; *iso*-2; *iso*-3, all by definition in 5′ UTR regions, compared with the reference genome (the reading frame is indicated on the SnpEff table). Most of the ATG start-gain SNPs are within 1 kb of the annotated translation start (**Table 5**), but this probably reflects the fact that most 5′ UTR sequences are less than 1 kb long. Less than expected by chance, only ~25% of the ATG start-gain SNPs are in the same open reading frame (ORF) as the annotated ORF (**Table 5**). Since 33% of the ATG start-gain SNPs are expected to be in-frame by chance, there might be a weak selection against this class of start-gain SNPs. Of the 60 genes with ATG start-gain SNPs, five genes have two ATG start-gain SNPs and one gene has 3 start-gain SNPs; the remaining 54 genes have a single start-gain SNP. Since SnpEff does not take into account of the Kozak consensus sequence flanking the AUG site, 5′ACC-AUG-G-3′, that is generally required for efficient translation,12 further assessment is required to determine whether a start-gain SNP is actually used.

Gene ontology (GO) pathway analysis of the genes affected by the 297 start-gain SNPs in *w*^{1118}; *iso*-2; *iso*-3 was done using DAVID (Database for Annotation, Visualization and Integrated Discovery).[13,14] We found that the GO categories "tissue morphogenesis," "immunoglobulin like," "developmental protein," and "alternative splicing" are significantly enriched after multiple-comparisons correction by false-discovery rate (FDR < 0.001; **Table 6**). These categories are interesting because they predominantly contain proteins that show a wide degree of intra- and interspecies variability. For example, the immunoglobulin loci,

Table 3. Information provided by SnpEff in tab separaOutput format (TXT)

Column	Notes
Chromosome	Chromosome name (usually without any leading 'chr' string)
Position	One based position
Reference	Reference
Change	Sequence change
Change type	Type of change (SNP, MNP, INS, DEL)
Homozygous	Is this homozygous or heterozygous (Hom, Het)
Quality	Quality score (from input file)
Coverage	Coverage (from input file)
Warnings	Any warnings or errors.
Gene_ID	Gene ID (usually ENSEMBL)
Gene_name	Gene name
Bio_type	BioType, as reported by ENSEMBL
Trancript_ID	Transcript ID (usually ENSEMBL)
Exon_ID	Exon ID (usually ENSEMBL)
Exon_Rank	Exon number on a transcript
Effect	Effect of this variant. See details below
old_AA/new_AA	Amino acid change
old_codon/new_codon	Codon change
Codon_Num(CDS)	Codon number in CDS
Codon_degenaracy	Codon degenaracy
CDS_size	CDS size in bases
Custom_interval_ID	If any custom interval was used, add the IDs here (may be more than one)

which are highly divergent among humans and other vertebrates, are used for antigen recognition.[15] Also, developmental proteins and proteins involved in tissue morphogenesis often have both conserved domains such as the Hox domain, and highly divergent domains such as the trans-activation domains.[16,17]

An example of a start-gain SNP is in the 5′UTR of *Ecdysone inducible protein 63E* (*Eip63E*) gene, which is predicted to be a cyclin J dependent kinase required for oogenesis and embryonic development (**Fig. 2**).[21] The potential start-gain SNP (A > G) in *Eip63E* changes 5′-ATA-3′ to 5′-ATG-3′ in the same reading frame with no intervening stop codons (**Fig. 2a**). If translation occurs at the new start-gain SNP, it would produce a protein with 57 additional N-terminal amino acids compared with the reference gene (**Fig. 2b**). However, the three bases prior to the new 5′-ATG-3′ sequence, 5′-AAT-3′, is a poor match to the Kozak consensus sequence, 5′-ACC-3′, discussed above.[12] Therefore, it is unclear whether the start-gain SNP in *Eip63E* is recognized by the translational machinery.

It is interesting that a BLASTp search of the protein database reveals that the N-terminal 57 amino acids in *Eip63E* are 63% identical (36/57) to the 58 N-terminal amino acids of the orthologous gene in Drosophila yakuba, but not to any other Drosophila species. This suggests that the 5′ UTR of *Eip63E* might be a source for genetic variations encoding novel N-terminal protein sequences that potentially modulates protein function (see **Discussion**).

SNPs that generate new stop codons or loss of stop codons. Another surprise in our SnpEff analysis was the identification of 28 stop-gain SNPs and 5 stop-lost SNPs in w^{1118}; *iso*-2; *iso*-3 (**Table 7**). A stop-gain SNP, classically called a nonsense SNP, has a coding codon changed to a stop codon, UAA, UAG, UGA.[22] Three genes, *oc/otd*, *LRP1*, and *trol9*, have two stop-gain SNPs. Surprisingly at least 8 of the stop-gain SNPs are in genes that encode essential proteins and these are *Dif*, *dp*, *ex*, *MESR4*, *mew*, *oc/otd*, *tai*, and *trol*. It is not known whether the other stop-gain SNPs also affect essential protein-coding genes because their functions have not yet been characterized (according to www.flybase.org). We note that a stop-gain SNP in w^{1118}; *iso*-2; *iso*-3 would be a stop-lost SNP in the reference strain and vice versa because the ancestral *Drosophila melanogaster* strain that gave rise to both of these strains is not known.

An important consideration with stop-gain SNPs is whether the expanded C-terminal amino acids in the longest version of the protein are conserved in other Drosophila species. If the additional C-terminal amino acids are not conserved, then these amino acids might not affect the essential function of the protein but they might exert modulatory effects. If the additional C-terminal amino acids are conserved in multiple Drosophila species, then their loss might adversely affect the function of the protein. Therefore, in **Table 7**, we further classify the stop-gain and stop-lost SNPs into four categories: Category 1, including 23 genes, with both the N-terminal and novel C-terminal tails conserved among Drosophila species and other organisms; Category 2, including only one gene, with the entire gene sequence not conserved even among other Drosophila species. This gene is therefore probably not a functional gene; Category 3, including two genes, with novel C-termini not conserved among other Drosophila species. In this category, the N-termini are conserved among Drosophila species, but this conservation is not maintained beyond the Drosophila genus. Genes in this class are likely novel genes that arose in the Drosophila genus); and Category 4, including seven genes, with novel C-termini conserved among other Drosophila species but not beyond the Drosophila genus. In this category, the N-terminus is conserved beyond the Drosophila genus. Genes in this class probably have C-terminal domains that exert modulatory roles in the Drosophila genus but not beyond the genus).

An example of an essential protein-coding gene in Category 4, where the novel C-terminus is not conserved outside the Drosophila genus, is *oceliless* (*oc*), also known as *orthodenticle* (*otd*) (**Fig. 3**). The *oc/otd* gene is a Hox-family transcription factor required for photoreceptor development in the compound eye and the light-sensing ocellus, embryonic development, and brain development segmentation.[23,24] The Hox domain contains 60 amino acids, 59 of which are identical with the human Otd protein. The Hox domains, which arose before invertebrates and vertebrates split several hundred million years ago, are among the most conserved protein domains in bilaterally-symmetric organisms in evolution.[25] The two stop-gain SNPs in w^{1118}; *iso*-2; *iso*-3

Table 4. Information provided by SnpEff in variant call format (VCF)

Sub-field	Notes
Effect	Effect of this variant. See details below
Codon_Change	Codon change: old_codon/new_codon
Amino_Acid_change	Amino acid change: old_AA/new_AA
Warnings	Any warnings or errors
Gene_name	Gene name
Gene_BioType	BioType, as reported by ENSEMBL
Coding	[CODING | NON_CODING]. If information reported by ENSEMBL (e.g., has 'protein_id' information in GTF file)
Trancript	Transcript ID (usually ENSEMBL)
Exon	Exon ID (usually ENSEMBL)
Warnings	Any warnings or errors (not shown if empty)

The information is added to the INFO fields using an tag 'EFF'. The format for each effect is "Effect (Effect_Impact | Codon_Change | Amino_Acid_change | Gene_Name | Gene_BioType | Coding | Transcript | Exon [| ERRORS | WARNINGS])".

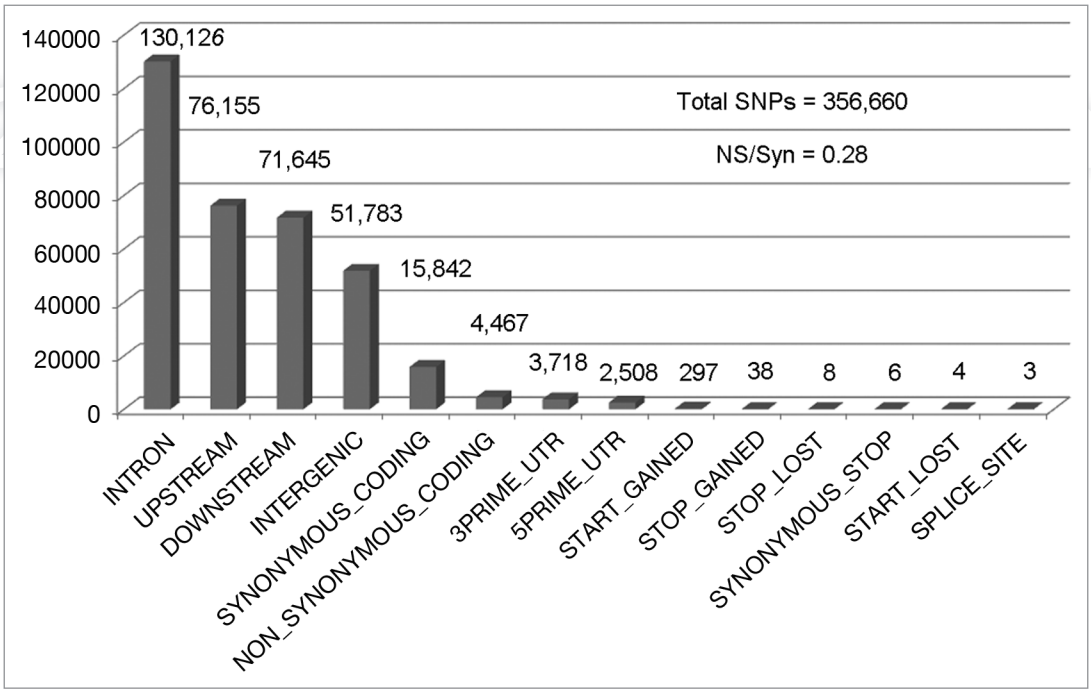

Figure 1. Classification of SNPs in w^{1118}; *iso*-2; *iso*-3. The number of NSPs in each class is shown above the bar. The quality score was arbitrarily set at 70 and above for this graph.

are in the non-conserved C-terminal region of Oc/Otd, which is thought to have a transcriptional-regulatory function. Since both strains are viable, both *oc/otd* genes are apparently functional although they encode a protein with 489 amino acids in w^{1118}; *iso*-2; *iso*-3 , and a protein with 543 amino acids in the reference genome (**Table 6**).

An example of a stop-lost gene in Category 3 c, where the C-terminus is not conserved among the Drosophila species, is *CG34326* that encodes a protein of unknown function (**Fig. 4**). In *w1118; iso-2; iso-3*, *CG34326* encodes a protein of 48 amino acids but in the reference genome it encodes a protein with 84 amino acids. When BLASTp was done with the non-redundant (nr) data set, there was not much homology beyond the 38th amino acid within the Drosophila genus. However, there was a near perfect (37/38) identity of the first 38 amino acids in four other Drosophila species: *Drosophila grimshawi, Drosophila yakuba, Drosophila erecta,* and *Drosophila virilus* (**Fig. 4**). This protein likely arose in the Drosophila genus since it has no known homologs outside of this genus.

There are also five stop-lost SNPs in w^{1118}; *iso*-2; *iso*-3 (**Table 6**). All of these SNPs are in predicted protein-coding genes, *metabotropic GABA-B receptor subtype 1* (*GABA-B-R1*), *CG13958, CG4975*, *brown* (*bw*), and *POU domain motif 3* (*pdm3*). It is not known whether any of these genes are essential in Drosophila besides bw, which is not required for viability. However, the GABA-B-R1 gene is required for normal behavior

Table 5. 60 Genes with start-gaineded SNPs with ATGs

Gene_name	Bases from TSS	Gene_name	Bases from TSS	Gene_name	Bases from TSS
a	386 (-)	CG4766	367 (-)	MESR3	454 (-)
Ace	652 (-)	CG4839	293 (-)	Mipp2	67 (-)
Axn	107 (-)	CG5103 (2)	104/17 (-/-)	osp	358 (-)
btsz	228 (+)	CG6024	269 (-)	p120ctn	119 (-)
Calx	582 (+)	CG7985	60 (+)	Pld	144 (+)
CAP	1224 (+)	CG8026	612 (+)	Pli	196 (-)
CG10186	402 (+)	CG8176	128 (-)	Pvr (2)	472/915 (-/+)
sesn	147 (+)	cpo	168 (+)	pxb (2)	50/76 (-/-)
CG12355	151 (-)	dac	103 (-)	rib	2 (-)
CG13802 (3)	490/575/635 (-/-/-)	dpr15	433 (-)	rn	142 (-)
haf	89 (-)	EcR	160 (-)	Samuel	517 (-)
CG15086	114	Eip63E	171 (+)	sli	307 (-)
CG15878	52 (-)	fdl (2)	307/437 (-/-)	so	5252 (-)
CG18522	40 (-)	frtz	196 (-)	Sobp	24 (+)
CG30419	253 (-)	GC	76 (-)	sprt	358 (-)
CG31163	998 (-)	Gug	70 (-)	Strn-Mlck (2)	210/228 (+/+)
Dscam3	269 (-)	inv	771 (+)	tai	203 (-)
CG31688	430 (-)	Ipk1	376 (-)	vn	1793 (-)
CG32048	63 (+)	klu	576 (+)	wg	231
CG32150	747 (+)	Mbs	10 (-)	Wnt4	680 (-)

Bases from TSS, bases from translation start site not including the ATG start-gained SNP. (+), in same reading frame as annotated ATG. (-), in different reading frame as annotated ATG.

Table 6. Genes with start-gained SNP GO categories in w^{1118}; *iso*-2; *iso*-3

Term	Count	%	p value	List total	Pop hits	Pop total	Fold enrichment	Bonferroni	Benjamini	FDR
tissue morphogenesis	21	8.898305	2.07E-08	147	247	7937	4.590515	2.37E-05	2.37E-05	3.33E-05
immunoglobulin-like	16	6.779661	3.40E-08	198	132	10196	6.241812	1.42E-05	7.08E-06	4.77E-05
developmental protein	29	12.28814	2.75E-07	229	540	12980	3.043992	3.99E-05	3.99E-05	3.27E-04
alternative splicing	31	13.13559	3.82E-07	229	616	12980	2.852464	5.53E-05	2.77E-05	4.53E-04

tissue morphogenesis	FRTZ, NRX-IV, ESG, WG, PBL, SFL, MBS, RIB, TOW, WNT4, FORM3, SLI, EIP63E, PHL, YRT, FAS, SRC64B, TWI, DLG1, BTSZ, HS6ST
immunoglublin-ilke	CG31814, DPR15, PVR, DPR16, CG14521, KLG, VN, CG12484, BEAT-IB, CG10186, DPR2, STRN-MLCK, CG34371, KEK5, FAS, CG15630
developmental protein	VN, ESG, DEI, INV, DAB, AWH, SCRIB, BICC, MST87F, WNT4, RIG, SLI, NUMB, PIP, INE, TWI, DLG1, FOXO, PTP10D, WG, AXN, EIP74EF, BUN, SO, FZ2, FDL, SCYL, SRC64B, POXN
alternative splicing	CPO, CPN, ECR, VN, CG11299, RN, DAB, AWH, SCRIB, INX7, SLI, PIP, NRV2, INE, DLG1, L(1)G0196, CG32048, FOXO, PTP10D, CYCT, WG, EIP74EF, BUN, CG13624, GLUT1, OSP, FDL, SSP4, PHL, SCYL, RDGC

Results of Gene ontology analysis for 297 start-gained SNPs in w^{1118}; *iso*-2; *iso*-3. Bottom, the genes in the indicated gene ontology category is listed.

in mice[26] and the ortholog is therefore likely also have a function in Drosophila, although no phenotypic data are available. The *bw* gene is a classic gene first described in 1921 by Waaler,[27] which causes the eyes to be brown rather than red and encodes an ATPase binding cassette (ABC) transporter.[28] The bw^{1} mutation in the reference strain is a spontaneous allele with a 412-transposon repeat insertion,[29] which would have been missed in our next-generation sequencing data because the input sequence

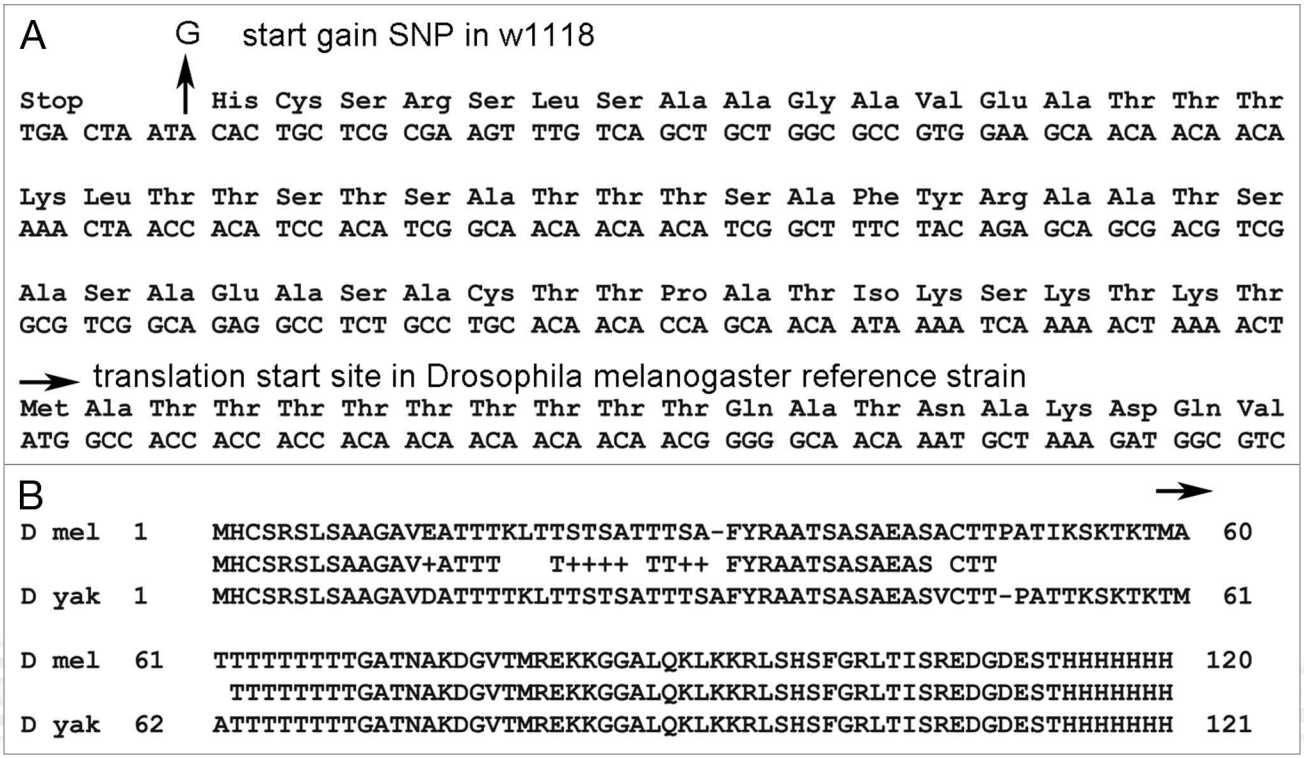

Figure 2. Analysis of Eip63E start-gained SNP in w^{1118}; *iso*-2; *iso*-3. (A), Location of the start-gained SNP at the Eip63E locus. Notice that the reading frame is the same as the normal translation start site (TSS). (B), Conservation of 60 amino acid N-terminal region of Eip63E in w^{1118}; *iso*-2; *iso*-3 with Drosophila yakuba orthologous gene. The other sequenced Drosophila species do not have this N-terminal sequence (not shown).

we analyzed contained only short-read sequences that mapped uniquely to the reference genome.

Not much is known about the functions of several genes with in-frame stop-gain SNPs. The *pdm3* gene is expressed in the larval and adult nervous system, and it encodes a highly-conserved Hox domain, but no phenotypic data are available (www.flybase.org). No phenotypic data are available for either CG13958 or CG4975. The protein encoding CG13958 has no known conserved domain, and its peak expression is observed within 6–24 h of embryogenes, during early larval stages, at stages throughout the pupal period, and in the adult male (www.flybase.org). The protein encoded by CG4975 has an Armadillo-like helical domain and an Ataxin-10 domain and has expression in the hind gut during the late larval and periods (www.flybase.org).[30]

Some of the stop-lost SNPs have interesting consequences. For example, a stop-lost SNP in w^{1118}; *iso*-2; *iso*-3 is in the CG13958 gene and causes an extension of 8 amino acids before the next stop codon in 3′UTR sequence is reached (**Fig. 5**). Since the C-termini of CG13958 vary in w^{1118}; *iso*-2; *iso*-3 and the reference strains of *Drosophila melanogaster*, it is conceivable that the C-terminus might also fluctuate in other Drosophila species. To test this idea, we investigated the C-terminal regions of CG13958 homologs in other Drosophila species.

We found that CG13958 homologs have variable C-terminal amino acids in different species of Drosophila. When the CG13958 protein is analyzed by protein Basic Local Alignment Search Tool (BLASTp) with the non-redundant (nr) protein database (www.ncbi.nlm.nih.gov), at least two Drosophila species have extended C-terminal amino acids and at least three Drosophila species have missing amino acids at the C-termini (**Fig. 5**). For example, *Drosophila pseudoobscura* has three of the extended amino acids found in w^{1118}; *iso*-2; *iso*-3 and *Drosophila mojavenais* has four of them. In contrast, *Drosophila simulans* is missing the last terminal amino acid, *Drosophila erecta* is missing the last two terminal amino acids, and *Drosophila yakuba* is missing the last three amino acids found in the reference strain (**Fig. 5**). The large number of stop-gain and stop-lost SNPs in Drosophila likely has important implications on the evolution of protein function (see **Discussion**).

Synonymous and non-synonymous SNPs in w^{1118}; iso-2; iso-3. There are 15,842 synonymous SNPs and 4,467 nonsynonymous SNPs in annotated ORFs in w^{1118}; *iso*-2; *iso*-3 (**Fig. 1**). A synonymous SNP (silent SNP) is defined as a SNP that does not change the amino acid in the protein, whereas a nonsynonymous SNP does. Evolutionary studies often compare the ratio (N/S) of nonsynonymous SNPs (N) to synonymous SNPs (S) in a gene to measure the evolutionary conservation of a particular protein or protein domain. For a highly conserved protein domain, such as the 60 amino acid Hox domain of Oc/Otd mentioned above (**Fig. 3b**), the number of synonymous SNPs is much higher than the number of non-synonymous SNPs. This is expected because nonsynonymous SNPs would probably disrupt the Hox protein function. The genome-wide normalized N/S ratio (*dN/dS*), also called ω (i.e., ω = *dN/dS*), is by definition normalized to 1 in most

Table 7. Stop gained and stop lost in w^{1118}; *iso-2*; *iso-3*

Stop gained	Location	Length	Phenotype	Stop gained	Location	Length	Phenotype
ade3	255K/*	435	ND[a]	ex	693Q/*	1428	Lethal[d]
CG10126	11W/*	228	ND[a]	lbk	1130Y/*	1174	ND[d]
CG15394	120Q/*	186	ND[a]	MESR4	1509E/*	2072	lethal[d]
CG31145	27L/*	764	ND[a]	mew	752Q/*	1050	Lethal[a]
CG31784	1049Q/*	1078	ND[a]	NFAT	12G/*	1420	ND[d]
CG32115	468W/*	476	ND[a]	oc/otd	389Y/*, 453Y/*	543	Lethal[d]
LRP1	2917Y/*, 2918E/*	4700	ND[a]	Pde9	255C/*	1527	ND[a]
CG34006	121R/*	202	ND[b]	rho-4	140W/*	418	ND[a]
CG34326	49Y/*	84	ND[c]	Synd	375S/*	495	ND[a]
CG3493	1419E/*	1490	ND[a]	tai	1420Q/*	2048	Lethal[d]
CG3964	509Y/*	983	ND[a]	trol	811Y/*, 808E/*	4180	Lethal[a]
CG4068	379Q/*	623	ND[d]	**stop lost**			
CG7236	70E/*	502	ND[a]	GABA-B-R1	*/L (+9 aa)	837	ND[a]
Cht6	4175L/*	4542	ND[a]	CG13958	*/G (+8 aa)	539	ND[a]
Cyp4s3	260W/*	496	ND[a]	CG4975	*/Q (+1aa)	353	ND[a]
Dif	263C/*	668	lethal[a]	bw	*/Q (+71 aa)	417	eye color[c]
Dp	17353L/*	22972	Lethal[a]	CG14755/pdm3	*/Q (+5 aa)	285	ND[a]

Stop gained, gene with stop gained SNP. Location, amino acid number changed to a stop codon (e.g., 255K/*, indicates lysine at amino acid changed to a stop codon). Length, the length of the protein in amino acids. Phenotype, not determined (ND), withdrawn (no longer considered a gene by FlyBase), and NPC (non-protein coding, such as a rRNA). For stop lost SNPs (bottom), */L (+9 aa) indicates that the next in frame stop is after nine additional amino acids are added. [a-d]refer to SNP categories 1–4 (see text).

evolutionary studies.[31] The non-normalized N/S ratio is ~0.28 in w^{1118}; *iso-2*; *iso-3* compared with the reference genome, y^1; cn^1 bw^1 sp^1 (i.e., N/S = 4,467/15,842; **Table 1**). For individual proteins, a dN/dS ratio < 1 (i.e., non-normalized N/S < 0.28) can indicate selection for a conserved protein that cannot withstand many amino acid substitutions. For genes with *dN/dS* ratio > 1, (i.e., non-normalized N/S > 0.28), the proteins or at least portions of the proteins, are able to withstand greater number of amino acid substitutions and are therefore probably lesser conserved.

We examined the genome-wide distribution of synonymous and nonsynonymous SNPs for w^{1118}; *iso-2*; *iso-3* and saw higher levels of both classes of SNPs in the middle of the chromosome arms and lower levels near the centromeres and telomeres (**Fig. 6**, left). This was expected because the number of SNPs is proportional to the recombination frequencies at different regions of the chromosomes.[32,33] Also, our previous analyses of the distribution of total SNPs revealed a similar pattern.[1] We observed higher N/S ratios near the telomeres and centromeres and lower N/S ratios in the middle of the chromosome arms (**Fig.6**, right). We speculate that this might reflect that a majority of conserved genes are located in highly recombinogenic regions near the middle of the chromosome arms. This finding requires confirmation in natural populations of *Drosophila melanogaster* since the origins of the two sequenced strains discussed in this paper are not known.

Discussion

To illustrate the use of SnpEff, we annotated ~356,660 SNPs in w^{1118}; *iso-2*; *iso-3* and place them into 14 different classes based on their predicted effects on protein function. In order of prevalence, these 14 classes are intron, upstream, downstream, intergenic, synonymous, non-synonymous, 3′UTR, 5′UTR, start-gain, stop-gain, stop-lost, synonymous-stop, start-lost, and splice-site SNPs (Fig. 1). The reason for cataloging these SNPs in w^{1118}; *iso-2*; *iso-3* is to get a better appreciation of evolution of genome sequences and genome organization in this common laboratory strain. We appreciate the fact that both w^{1118}; *iso-2*; *iso-3* and y^1; cn^1 bw^1 sp^1 are derived and highly manipulated laboratory strains and do not represent natural populations. Therefore, we do not mean to imply that the analyses in this paper are representative but rather just observational. To be representative, these observations need to be followed up with natural populations. Hundreds of Drosophila natural populations have already been or are in the process of being sequenced, so this type of analyses should be feasible in the near future with a program such as SnpEff.[34]

Our previous analyses suggest that most of these SNPs are probably genuine and can be validated by capillary sequencing.[1] A common worry about next-generation sequencing data are that SNPs are vastly over estimated. One might think that if a large fraction of the identified SNPs had the predicted "effects," the organism would not be viable. However, since short-read next-generation sequencing, such as the short-read sequences we obtained with the Illumina platform, has high error rates, further validation of specific SNPs is needed to be absolutely certain. Further validation of SNPs is best done with long-range DNA sequencing such as with traditional capillary sequencing or sequencing with the Roche,[18] Pacific Biosciences,[19] and many other third generation DNA sequencing instruments that are now available[20] (see ref.1 for validation examples with capillary sequencing). Many of the stop-gain and stop-lost SNPs in w^{1118};

iso-2; *iso-3* occur in essential genes that apparently still function after amino acid truncations caused by the stop-gain SNPs (**Table 6**). These non-critical effects of the stop codon SNPs are worth noting because nonsense SNPs generally result in nonfunctional protein products. For example, some genetic disorders, such as thalassemia and Duchenne muscular dystrophy (DMD), result from nonsense SNPs.[35-37] Also, nonsense SNP-mediated RNA decay exists in yeast, Drosophila, and humans, and usually ensures that mRNAs with premature stop codons are degraded.[38]

The stop-gain and stop-lost SNPs in essential genes, if they are validated, could have a profound evolutionary implication in that it might suggest the involvement of prions or mutations in translational termination factors that allow read-through of stop codons in the retention and selection of these SNPs. In 1965, Brian Cox, a geneticist working with the yeast *Saccharomyces cerevisiae*, isolated a yeast strain auxotrophic for adenine due to a nonsense mutation and found that it was able to survive in media lacking adenine when a [PSI+] mutation is present.[39] Reed Wickner showed in 1994 that the [PSI+] suppressor mutation resulted from a prion form of the translation termination factor Sup35[40] that allowed a read-though of the stop codon that caused the adenine auxotrophy. Lindquist and colleagues showed in 2008 that the [PSI+] prion provides survival advantages in several stressful environments, such as high salt conditions.[41] They speculate that Sup35 is an evolutionary capacitor that, when inactivated in the PSI+ form, allows translational read-through past stop codons and the expression of novel C-terminal amino acids in hundreds of proteins, some of which are beneficial in stressful environments.[41]

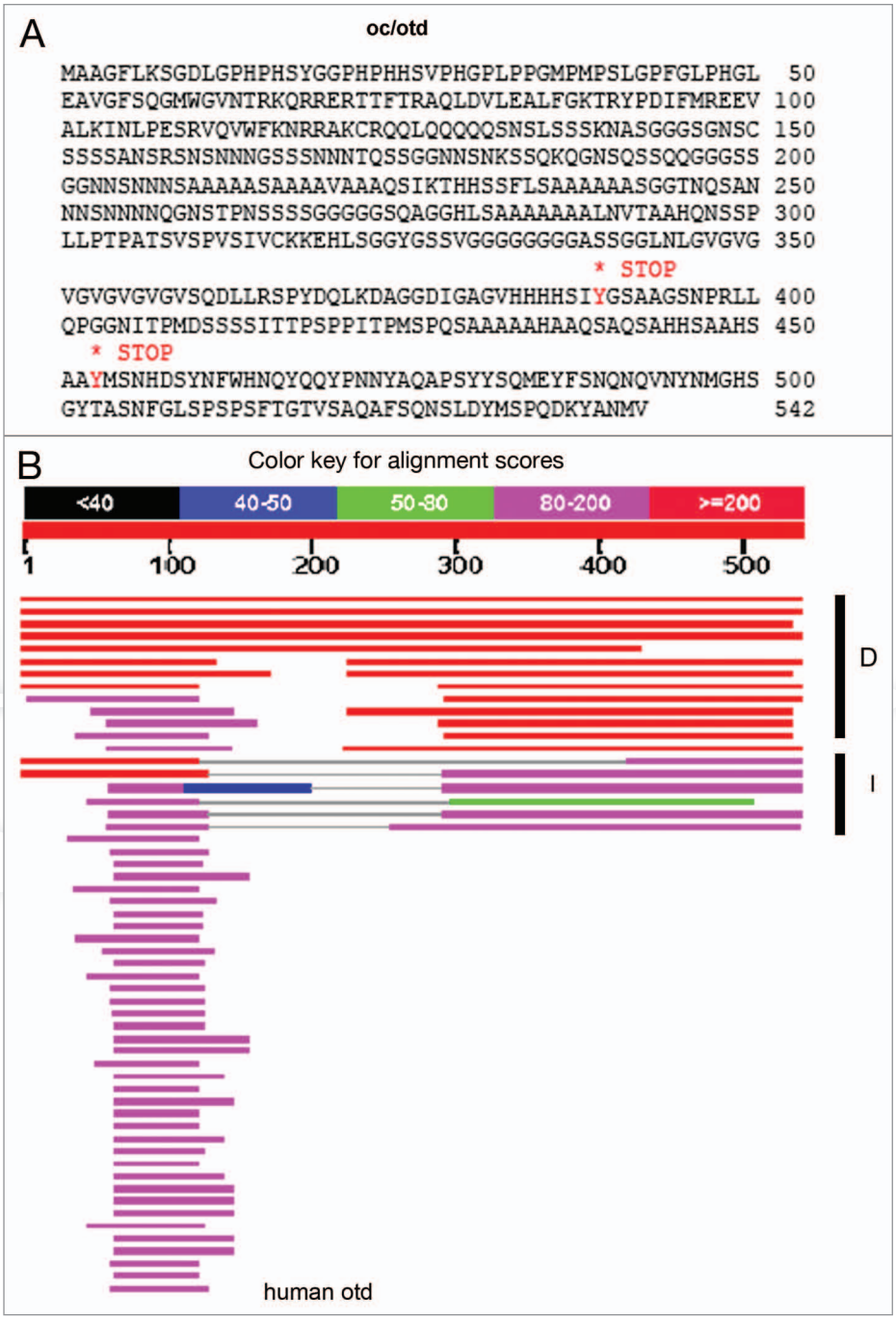

Figure 3. Oc/Otd has two stop-gained SNPs in w^{1118}; *iso*-2; *iso*-3. (A) Location of the two stop gained SNPs in oc/otd. (B) Protein BLAST of Oc/Otd against the non-redundant (nr) protein database shows that only the 60 amino Hox domain flanking amino acid 100 is conserved from Drosophila to humans. The color coding shows the alignment scores.

It is attractive to speculate that a similar prion-mediated evolutionary mechanism might occur in Drosophila, for both stop-loss and stop-gain SNPs, and that this might help explain the large number of SNPs that we see in these categories. We note that Drosophila has several Sup35 orthologs, some of which have N-terminal repeats known to be potentially prion-forming domains.[41] While most prions are thought to not directly mutate DNA sequences, they could provide an environment that would make the retention and selection of beneficial stop codon SNPs more likely. For example, a stop-lost SNP would allow a modified protein with the new C-terminal tail to be always expressed, even when the [PSI+] prion is lost.[41] Therefore, a stop-lost SNP would more likely occur in a gene with potential beneficial codons in the 3′ UTR because the cryptic C-terminal amino acids would provide a selective advantage in stressful environments when they are translated.

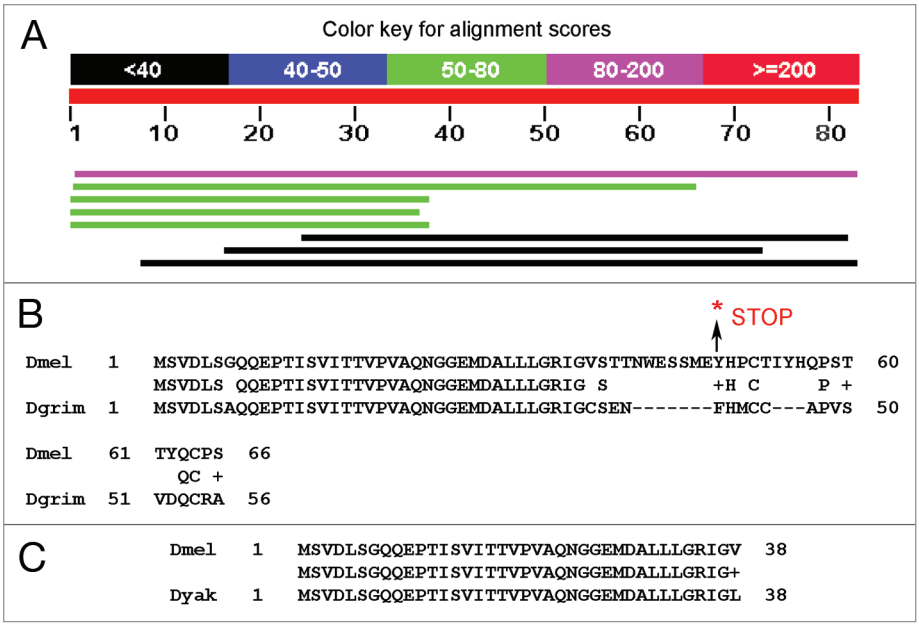

Figure 4. CG34326 has one stop-gained SNP in w^{1118}; *iso*-2; *iso*-3 in the non-conserved C-terminal region. (A) Protein BLAST of CG34326 against the non-redundant (nr) protein database shows that only the 38 N-terminal amino acids are conserved among Drosophila species and not beyond Drosophila. The colored lines represent the homologs from the following organisms: *Drosophila melanogaster*, *Drosophila grimshawi*, *Drosophila yakuba*, *Drosophila erecta*, *Drosophila virilus*, *Ixodes scapularis*, *Ixodes scapularis* and *Nycticebus coucang*. (B) Aligment of *Drosophila melanogaster* CG34326 with orthologous gene from *Drosophila grimshawi*. (C) Aligment of *Drosophila melanogaster* CG34326 with orthologous gene from *Drosophila yakuba*.

We acknowledge that this is a highly speculative explanation for the high numbers of start-gain and stop-lost SNPs, but we believe that it is worthy of further investigation.

The many potential start-gain SNPs in Drosophila might also have evolutionary implications. Similar to the cryptic genetic variation that is revealed by stop-lost mutations in the 3′ UTR, start-gain SNPs reveal cryptic genetic variation in the 5′ UTR. Uncovering the cryptic genetic variation in times of environmental stress, such as by inducing transcription initiation at start sites upstream of the normally-used transcription start sites, could be one mechanism to facilitate the use of potential start-gain SNPs. Further genetic drift and selection of potential start-gain SNPs, such as by introducing better Kozak consensus sequences or more commonly used 5′-AUG-3′ translation initiation codons, can stabilize the cryptic genetic variation further if these SNPs lead to improved survival or reproductive fitness in stressful environment.

Materials and Methods

SnpEff overview. The program is divided in two main parts (1) database build and (2) effect calculation.

(1) **Database build.** Since many databases containing genomic annotations are available with SnpEff distribution, this step is usually not run by the user. Databases are build using a reference genome and an annotation file. The reference genome must be in FASTA format. Genomic information can be parsed from four main annotation formats: GTF (version 2.2), GFF (versions 3 and 2), UCSC RefSeq tables, and tab separated text files (TXT). These annotation files are available at ENSEMBL, UCSC, or organism specific websites, such as FlyBase, WormBase and TAIR. SnpEff databases are compressed serialized objects that represent genomic annotations.

(2) **Effect calculations.** This can be performed once the user has downloaded or built the database. The program loads the binary database and builds a data structure called "interval forest" in order to perform an efficient interval search. Input files are parsed and each variant queries the data structures to find intersecting genomic annotations. All intersecting genomic regions are reported and whenever these regions include an exon, the coding effect of the variant is calculated. A list of the reported effects and annotations is shown in **Table 2**; additional information produced by the program is shown in **Tables 3 and 4** for different output formats.

SnpEff algorithms. In order to process thousands of variants per second, we implemented an efficient data structure that allows to query for arbitrary interval overlaps. We created an *interval forest*, which is a hash of i*nterval trees* indexed by chromosome. Each interval tree[42] is composed of nodes. Each node has five elements (i) a center point, (ii) a pointer to a node having all intervals to the left of the center, (iii) a pointer to a node having all intervals to the right of the center, (iv) all intervals overlapping the center point sorted by start position, and (v) all intervals overlapping the center point, sorted by end position. Querying an interval tree requires O(log n + m) time, where *n* is the number of intervals in the tree and *m* is the number of intervals in the result. Having a hash of trees optimizes the search by reducing the number of intervals per tree.

Input formats. Three input formats supported by SnpEff are variant call format (VCF), tab separated TXT format; and the SAMtools Pileup format.[8] VCF was created by the 1,000 Genomes project and it is currently the de facto standard for variants in sequencing applications. The TXT and Pileup formats are currently deprecated and being phased out.

Output formats. SnpEff also supports two output formats, TXT and VCF. The output information provided in both formats includes three main groups: (i) variant information (genomic position, the reference and variant sequences, change type, heterozygosity, quality and coverage); (ii) genetic information (gene Id, gene name, gene biotype, transcript ID, exon ID, exon rank); and (iii) effect information (effect type, amino acid changes, codon changes, codon number in CDS, codon degeneracy, etc.). Whenever multiple transcripts for a gene exist, the

effect and annotations on each transcript are reported, so one variant can have multiple output lines. **Table 3** shows the information provided in TXT format and **Table 4** shows the information provided in VCF format. When using the VCF format, the effect information is added to the information (INFO) fields using an effect (EFF) tag. As in the case of TXT output, if multiple alternative splicing products are annotated for a particular gene, SnpEff provides this information for each annotated version (see **Supplemental Data** for the complete SnpEff output for *w*^{1118}; *iso*-2; *iso*-3).

SnpEff accuracy. As part of our standard development cycle, we perform accuracy testing by comparing SnpEff to ENSEMBL "Variant effect predictor," which we consider to be the "gold standard." Current unity testing includes over a hundred test cases with thousands of variants, each to ensure that predictions are accurate.

SnpEff integration. SnpEff provides integration with third party tools, such as Galaxy,[43] which creates a web based interface for bioinformatic analysis pipelines. Integration with Genome analysis tool kit[4] (GATK) was provided by Broad's GATK team.

Data access. SnpEff Data can be accessed from the supplemental data file for *w*^{1118}; *iso*-2; *iso*-3 or by contacting D.M.R.

```
Drosophila melanogaster

Dm-ref    522  LVLQQCDSVQGYMEVSL*           538
               LVLQQCDSVQGYMEVSL*                    +8
Dm-w1118  522  LVLQQCDSVQGYMEVSLQIFNNINI*  546


Drosophila simulans

Dm-ref 522  LVLQQCDSVQGYMEVS  537
            LVLQQCDSVQGYMEVS                        -1
Sbjct  522  LVLQQCDSVQGYMEVS  537

Drosophila erecta

Dm-ref 522  LVLQQCDSVQGYMEV  536
            LVLQQCDSVQGYMEV                         -2
Sbjct  522  LVLQQCDSVQGYMEV  536

Drosophila yakuba

Dm-ref 481  LVLQQCDSVQGYME  535
            LVLQQCDSVQGYME                          -3
Sbjct  481  LVLQQCDSVQGYME  535

Drosophila mojavensis

Query  522  LVLQQCDSVQGYMEVS-LQIF  541
            LVLQQCDSVQGY+EV  L+IF                   +3
Sbjct  517  LVLQQCDSVQGYIEVRYLKIF  537


Drosophila pseudoobscura pseudoobscura

Query  522  LVLQQCDSVQGYMEVSLQIFN  542
            LVLQQCDSVQGY+EV   +F+                   +4
Sbjct  571  LVLQQCDSVQGYIEVFCALFH  591
```

Figure 5. CG13958 has a stop lost SNP in *w*^{1118}; *iso*-2; *iso*-3. The top comparison shows the alignment of the *Drosophila melanogaster* reference genome with *w*^{1118}; *iso*-2; *iso*-3. Notice that the stop lost causes an extension of 9 amino acids. The second through sixth comparisons shows the alignment of *Drosophila simulans*, *Drosophila erecta*, *Drosophila yakuba*, *Drosophila mojavensis* and *Drosophila pseudoobscura pseudoobscura* (Sbjct) with the *Drosophila melanogaster* reference genome (Dm-ref). The number of terminal amino acids missing or gained is shown (-1 to +3).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material may be found here:
http://www.landesbioscience.com/journals/fly/article/19695

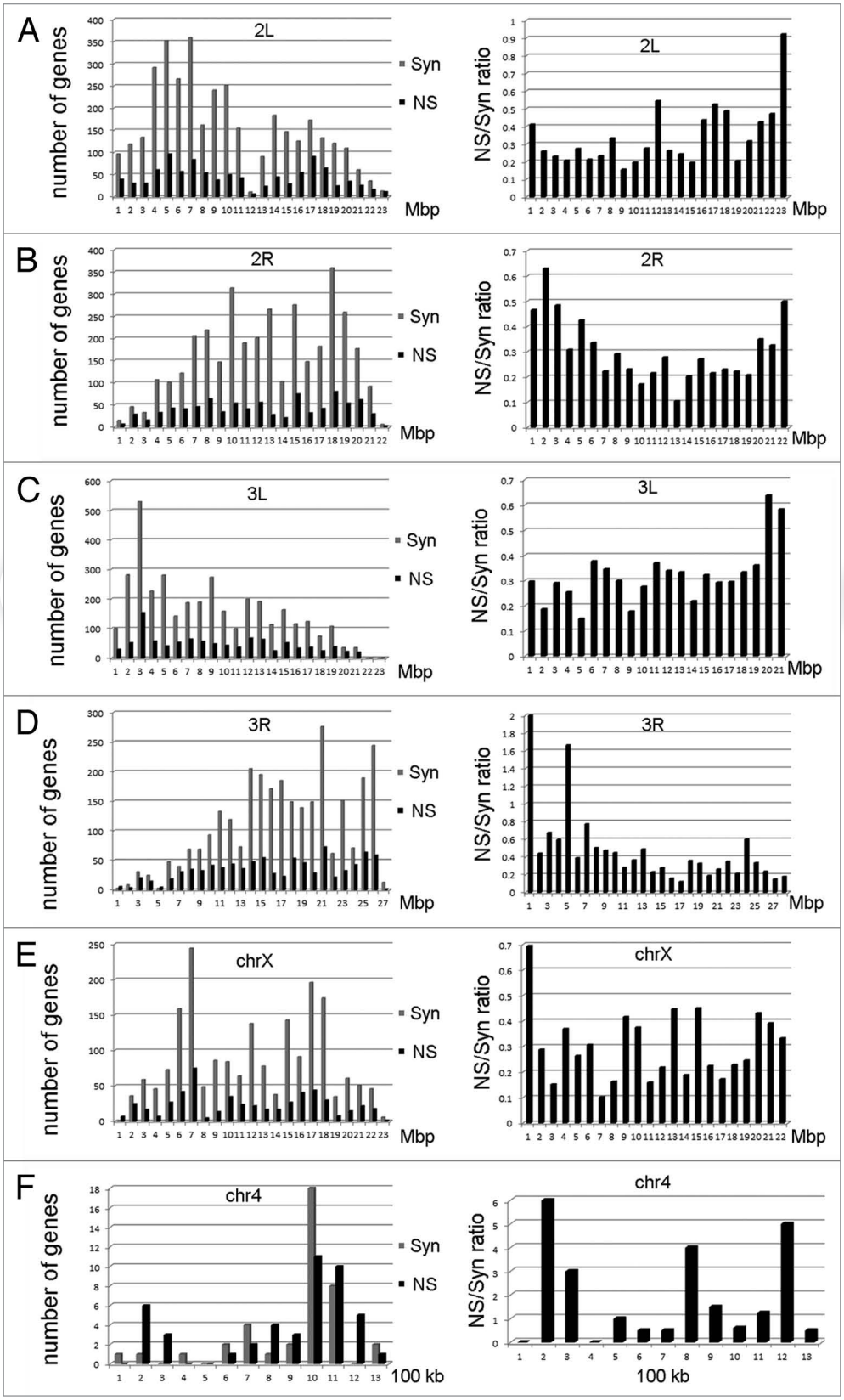

Figure 6. Nonsynonymous to synonymous ratios along the chromosome arms in w^{1118}; *iso*-2; *iso*-3. (A) Left, Nonsynonymous SNPs at 1 Mbp intervals along the 2L chromosome arm (black) and synonymous SNPs (gray). Right, N/S ratios (NS/Syn) along the chromosome arms. Notice that N/S ratios are higher near the centromere and telomere (see text). (B–F) as in (A), but for chromosome arms 2R, 3L, 3R, 4 and X.

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