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On the origin of new genes in *Drosophila*

Qi Zhou^{1,2,4}, Guojie Zhang^{1,2,3,4}, Yue Zhang^{1,4}, Shiyu Xu¹, Ruoping Zhao¹, Zubing Zhan^{1,2}, Xin Li^{1,2}, Yun Ding^{1,2}, Shuang Yang^{1,3}, Wen Wang^{1,#}

1. CAS-Max Planck Junior Research Group, State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
2. Graduate School of Chinese Academy Sciences, Beijing 100086, China
3. Beijing Genomics Institute-Shenzhen, Shenzhen 518083, China
4. These authors contributed equally to this work

Correspondence should be addressed to W.W. (wwang@mail.kiz.ac.cn)

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Abstract

Several mechanisms have been proposed to account for the origination of new genes. Despite extensive case studies, the general principles governing this fundamental process are still unclear at the whole genome level. Here we unveil genome-wide patterns for the mutational mechanisms leading to new genes, and their subsequent lineage-specific evolution at different time nodes in the *D. melanogaster* species subgroup. We find that, 1) tandem gene duplication has generated about 80% of the nascent duplicates that are limited to single species (*D. melanogaster* or *D. yakuba*); 2) the most abundant new genes shared by multiple species (44.1%) are dispersed duplicates, and are more likely to be retained and be functional; 3) *de novo* gene origination from non-coding sequences plays an unexpectedly important role during the origin of new genes, and is responsible for 11.9% of the new genes; 4) retroposition is also an important mechanism, and had generated approximately 10% new genes; 5) about 30% of the new genes in the *D. melanogaster* species complex recruited various genomic sequences and formed chimeric gene structures, suggesting structure innovation as an important way to help fixation of new genes; and 6) the rate of the origin of new functional genes is estimated to be 5 to 11 genes per million years in the *D. melanogaster* subgroup. Finally, we survey gene frequencies among 19 strains from all over the world for *D. melanogaster*-specific new genes, and reveal that 44.4% of them show copy number polymorphisms within population. In conclusion, we provide a panoramic picture for origin of new genes in *Drosophila* species.

[There are two Supplemental Data File, seven Supplemental Tables and two Supplemental Figures]

Introduction

The origin of new genes is of inherent interest to evolutionary biologists. But it was not until recently that the molecular details of the origin of new genes have been rigorously examined (Long et al. 2003). Multiple mechanisms including gene duplication, retroposition, horizontal gene transfer and *de novo* origination from non-coding sequences, have been proposed to be able to give birth to a new gene (Long et al. 2003). Nonetheless, a genome-wide comparison of all of these mechanisms remains desirable due to the light it could shed on their relative contributions to the origin of new genes.

Among the individual mechanisms, gene duplication has been considered as the singular most important one in creating new genes (Hughes 1994; Kimura and Ota 1974; Ohno 1970; Ohno 1973). Its contribution to the evolution of new functional genes have been widely demonstrated in various organisms (Arguello et al. 2006; Katju and Lynch 2003; Yang et al. 2008; Zhang et al. 2002), and gene duplication rate has been extensively studied in different species (Gao and Innan 2004; Gu et al. 2002; Hahn et al. 2007; Lynch and Conery 2000). Duplicate genes usually can be classified into tandem and dispersed duplicates, however the relative mechanistic contributions to their origins is largely unknown. In addition, the classic gene duplication model predicted that the most common fate for a newly duplicated copy is to become nonfunctional (Fisher 1935; Haldane 1933; Nei and Roychoudhury 1973; Ohno 1970). However, subsequent discoveries of numerous functional duplicated genes have inspired theoretical discussions on evolution of redundancy (Clark 1994; Nowak et al. 1997), subfunctionalization (Force et al. 1999; Lynch and Force 2000) and neofunctionalization (Walsh 2003; Walsh 1995). All of these theoretical

treatments overwhelmingly assume nascent duplicates are functionally and structurally redundant to their parental genes. Empirical evidence indicates however, pervasive structural heterogeneity can emerge between even young duplicates through the recruitment of new regulatory/coding sequences (Brosius and Gould 1992; Katju and Lynch 2003; Katju and Lynch 2006; Yang et al. 2008). Such chimeric structures, if non-deleterious, are expected to immediately confer novel functions that the parental genes do not have and thus may also well explain the existence of functional duplicates (Patthy 1999). Besides the reported cases of chimeric new genes found within different species (Arguello et al. 2006; Cordaux et al. 2006; Long et al. 2003; Patthy 1999; Xue et al. 2007), their generality, the mutational mechanisms, and selective forces responsible for their formation remains an intriguing question to be addressed.

Unlike regular gene duplication occurred at DNA level, retroposition events generates intronless new copies by reverse transcription of parental gene's mRNA (Brosius 1991). Numerous retrocopies have been identified in mammals (Marques et al. 2005; Vinckenbosch et al. 2006), plants (Wang et al. 2006; Zhang et al. 2005) and *Drosophila* (Bai et al. 2007; Betran and Long 2003). But the proportion of new genes derived from retroposition is still unknown except in *C. elegans* (Katju and Lynch 2006). Horizontal gene transfer has commonly occurred among prokaryotic species, and thus led to the introduction of new genes (Koonin et al. 2001). It is also possible that prokaryotic/eukaryotic parasites can occasionally transfer their genetic materials to their eukaryotic hosts (Bergthorsson et al. 2003; Hotopp et al. 2007). For example, Hotopp et al. recently reported the endosymbiont *Wolbachia* has transferred nearly its entire genome to its host *Drosophila ananassae* (Hotopp et al. 2007), but the functionality of these *Wolbachia* genes in the host is uncertain.

Each of the above three mechanisms are based on the Ohno's notion that "Each new gene must have arisen from an already existing gene" (Ohno 1970) and *de novo* gene origination from non-coding sequences should be rare, if not absent. In an influential essay, François Jacob also stated: "The probability that a functional protein would appear *de novo* by random association of amino acids is practically zero." (Jacob 1977) However, a pioneer study reported five *de novo* genes in *D. melanogaster* (Levine et al. 2006). This discovery urged a systematic evaluation regarding the extent that *de novo* formation has contributed to the evolution of protein-coding genes.

The most efficient approach to answer all the mentioned questions is to systematically study young genes which are in their early evolutionary stages (Long et al. 2003). Overall, we seek to tackle five central questions regarding both origination and evolution of new genes: what is the general picture for the relative contribution of each mutational mechanism creating new genes? How and how often would a new gene form a novel chimeric gene structure? What are the relative contributions of tandem and dispersed duplication to the creation of new duplicates? Is *de novo* origination an infrequent phenomenon during origin of new genes? And finally, beyond the apparent gene duplication rate, what is the rate of origin of functional new genes?

The *D. melanogaster* species subgroup is an excellent model for answering these questions due to their short divergence times (less than 12.8 million years) (Tamura et al. 2004) and the availability of abundant genetic data and techniques. Our previous studies characterizing complete origination process of several new genes within this clade have demonstrated that such a system allows scrutinization on the original process and the structural features after birth of new genes (Arguello et al. 2006; Long

et al. 2003; Wang et al. 2002; Wang et al. 2004; Wang et al. 2000; Yang et al. 2008). Now the availability of genome sequences for five species within this clade (Clark et al. 2007) offers an unprecedented opportunity to address the above questions at the whole genome level and thereby reveal the general principles governing origin of new genes.

Results

Identification and classification of new genes

To insure high quality results, we first focused on *D. melanogaster* and *D. yakuba* which have deep-coverage (more than 8×) genome sequences of high quality and a relatively short divergence time. We inferred the paralog number in *D. melanogaster* and *D. yakuba* after aligning 12,017 *D. melanogaster* cDNAs to their genome sequences. Taking both species as reciprocal outgroups, focal genes with extra copies in certain species were taken as candidates for new genes. New gene candidates in *D. melanogaster* were further divided into those specific to *D. melanogaster* and the *D. melanogaster* species complex by searching the genomes of *D. simulans* and *D. sechellia*. The latter group of new genes represents those originated in the common ancestors of *D. melanogaster*, *D. simulans* and *D. sechellia*. Most likely they should also exist in *D. mauritiana*, the single species whose genome sequence is not yet available in the *D. melanogaster* species complex. After the initial screen, we acquired 169 new gene candidates in *D. melanogaster*, 253 in *D. yakuba* and 191 shared by the *D. melanogaster* species complex.

We further performed extensive manual checks on UCSC Genome Browser (<http://genome.ucsc.edu/>) (Karolchik et al. 2003) and excluded spurious results caused by exon duplication/origination, repetitive elements or sequencing gaps in the

outgroup. Given the recently reported gene loss phenomenon in the 12 *Drosophila* genomes (Clark et al. 2007; Hahn et al. 2007), we also excluded candidates that resulted from loss events in the outgroup by comparison to genomes of *D. erecta* and *D. ananassae* as additional outgroups (see Materials and Methods). We only retained those well-annotated new genes with empirical support (i.e. expressed sequence tags or cDNAs) and intact open reading frames (ORF) in *D. melanogaster*, each of which has an annotation ID and is likely to be functional. For *D. yakuba*, we retained new gene copies with intact ORFs according to the *ab initio* prediction. With this stringent criteria, we finally acquired three new gene datasets: 72 new genes specific to *D. melanogaster*, 177 genes to *D. yakuba* and 59 new genes shared by the *D. melanogaster* species complex (Figure 1, Supplemental Table S1-3). We have compared our results in detail with other work characterizing orthology/paralogy in *Drosophila* species (Supplemental Data S1) (Bai et al. 2007; Bhutkar et al. 2007; Clark et al. 2007; Heger and Ponting 2007). The new gene numbers are very close to other two recent estimates of lineage-specific gene duplications in *D. melanogaster* (77 duplications) or *D. yakuba* (200 duplications) (Hahn et al. 2007; Heger and Ponting 2007). These identified new genes include the well-characterized new genes *sphinx* (Wang et al. 2002), *Sdic* (Nurminsky et al. 1998), *Dntf-2r* (Betran and Long 2003), *Acp29AB* (Aguade 1999; Clark et al. 1995) and five recently identified *de novo* genes in *D. melanogaster* (Levine et al. 2006). We didn't include recently reported new genes *Hun* (Arguello et al. 2006), *monkey king* (Wang et al. 2004), *hydra* (Chen et al. 2007) and several *de novo* new genes specific to *D. yakuba/D. erecta* (Begun et al. 2007), because they originated in lineages not focused by this study or their identifications depend on annotations other than those of *D. melanogaster*. Inclusion/exclusion of these experimentally verified new genes indicates the

robustness of our *in silico* screen. We subsequently calculated the ratio (Ka/Ks) of nonsynonymous substitution rate over synonymous substitution rate as an indicator of functionality between all the retained new genes and their parental genes. The average between-paralog Ka/Ks ratios are lower than 0.5 for *D. melanogaster* and *D. yakuba* specific new genes. And the average between-ortholog Ka/Ks ratio of the *D. melanogaster* species complex-specific new genes is lower than 1. In particular, 47.1% *D. melanogaster*-specific, 55.7% *D. yakuba*-specific and 71.4% the *D. melanogaster* species complex specific new genes show a Ka/Ks ratio significantly lower than 1 (Fisher's test, $P < 0.05$, Supplemental Table S1-4). These data likely indicate that a majority of the new genes are under functional constraint.

A majority of the constrained functional new genes are dispersed duplicates

We can estimate the ages of the identified new genes based on their phylogenetic distributions (Figure 1): The *D. melanogaster*-specific new genes are younger than 5.4 million years (myr), the *D. yakuba*-specific new genes younger than 12.8 myr, and the *D. melanogaster* species complex-specific new genes have originated within 5.4 to 12.8 myr (Tamura et al. 2004). To quantify the contribution of each mechanism creating new genes, we classified all the new genes into four types: tandem duplicates, dispersed duplicates, retrogenes and *de novo* originated genes (Table 1). Comparison among these different types of new genes should reveal their relative contributions not only at a genome-wide scale but also in a dynamic chronological order (Figure 1).

We found that the proportion of gene duplication (both tandem and dispersed) consistently outnumbers other mechanisms in generating new genes in all the three datasets (Figure 2). Specifically, tandem gene duplication predominantly accounts

for the emergence of nascent gene copies specific to *D. melanogaster* (59/72, 81.9%) and *D. yakuba* (138/177, 77.9%). Nevertheless, this proportion decreases to only 33.9% (20/59) for those constrained new genes shared by the whole *D. melanogaster* species complex. Accordingly, the proportion of new dispersed duplicates rises from only 8.3% (6/72) to 44.1% (26/59) (Figure 2). Since more new genes shared by the *D. melanogaster* species complex show selective constraints indicated by *Ka/Ks* ratios (see above), they are more likely to be functional than those younger ones only specific to *D. melanogaster* or *D. yakuba*. Thus we can conclude that new constrained functional genes are mainly dispersed duplicates in *Drosophila* species. Regarding the remaining mechanisms, retroposition generated about 10.2% (6/59) of the new genes and *de novo* genes, that have originated from non-coding sequences, unexpectedly comprise 11.9% (7/59) of the constrained new genes shared by the *D. melanogaster* species complex.

Tandem duplicates have younger ages but lower survivorships

We can also separately date each new gene using a molecular-clock-based method. Based on synonymous substitution rate (*Ks*) between ancestral and new genes (Table 2), and assuming the neutral substitution rate is about 5.8×10^{-9} per generation for *D. melanogaster* genome (Haag-Liautard et al. 2007), we estimated ages for *D. melanogaster*-specific, *D. yakuba*-specific and the *D. melanogaster* species complex-specific new genes. The average ages for the three groups of genes are 0.56 ± 0.12 , 1.27 ± 0.22 and 7.01 ± 1.35 myr respectively, indicating most of the *D. melanogaster* and *D. yakuba*-specific copies are much younger than the speciation time, but the ages of *D. melanogaster* species complex-specific genes are more or less consistent with the age of this lineage. In particular, the new tandem duplicates we identified have

an average K_s that is lower than dispersed duplicates (Table 2). The average ages of *D. melanogaster*-specific, *D. yakuba*-specific and the *D. melanogaster* species complex specific new tandem duplicates are estimated to be only 0.33-0.59 myr, 0.79-1.22 myr and 3.88-7.10 myr respectively, in contrast to 0.62-0.95, 1.59-2.89 myr and 7.50-12.38 myr for dispersed duplicates in these lineages. This result suggests that tandem duplicates are on average younger than dispersed ones, and that many new tandem duplicates identified in *D. yakuba* may have actually originated after the split of the ancestral lineage that led to *D. yakuba*, *D. santomea* and *D. teissirri* (6.8 myr, (Tamura et al. 2004)). We cannot completely exclude the possibility that gene conversion may homogenize some parental and new gene pairs (Gao and Innan 2004), but a recent study strongly suggests it might play a minor role in genome-wide scale in *Drosophila* species (Hahn et al. 2007).

We further investigated whether *D. melanogaster*-specific new genes have been fixed in the population. We conducted PCR experiments to test copy number variations (CNVs) according to the presence/absence of bands within different lines for 45 out of 72 (62.5%) *D. melanogaster*-specific genes. Most of the PCR products are designed to span the boundary sites of two duplicated segments where parental and new genes reside in, and we used multiple primer sets (3-4 sets) to minimize the effect of mispairing for most of PCR assays (see below, and Materials and Methods). In total, we inspected 10 South American iso-female strains as a local population sample, together with 9 other strains from different continents as a world wide sample. 44.4% (20 out of 45) of the nascent genes show CNVs among the 19 *D. melanogaster* strains (Table 3, Supplemental Table S5). A majority (75%) of these investigated nascent genes are tandem duplicates, and 58.8% of them have been fixed (Table 3). This is consistent with the proportion of tandem duplicates fixed in the *D.*

melanogaster complex, and suggests tandem new genes have a lower survivorship compared to other types of new genes.

Most new tandem duplicates reside in segmental duplications

All identified tandem duplicates except for one *D. melanogaster*-specific copy (CG18816-CG30160) are direct repeats. This differs from the pattern of *C. elegans* where most of the young duplicated genes (45%) are tandem duplicates with inverted directions (Katju and Lynch 2003). Further characterizations indicated that, 61.0% (36/59) of *D. melanogaster*-specific, 77.5% (107/138) of *D. yakuba*-specific and 65% (13/20) of the *D. melanogaster* species complex-specific tandem new genes reside in tandem segmental duplications (Supplemental Table S3 and S6). All of the *D. melanogaster*-specific, 84% *D. yakuba*-specific but only 20% *D. melanogaster* species complex-specific tandem segmental duplications are in immediate conjunction with each other with no or few intervening base pairs (less than 50 bp). A total of 22 such tandem segmental duplications whose lengths ranges from 580bp to more than 33kb generated 36 *D. melanogaster*-specific tandem duplicate genes (Supplemental Table S6). Among them, 8 transcribed new duplicates emerged with chimeric gene structures at the boundaries of the duplicated segments, through shuffling exons/introns of two genes located at the 5' and 3' end of the segments (Supplemental Figure S1).

Additionally, we found that 30.5% (18/59) *D. melanogaster*-specific, 11.8% (16/138) *D. yakuba*-specific and 25% (5/20) *D. melanogaster* species complex-specific tandem duplicates or their ancestral genes have at least one repetitive element at the duplication breakpoints. Interestingly, this proportion for dispersed duplicates rise up to 33.3% (2/6), 28.2% (11/39) and 46.2% (12/26) for each lineage respectively,

showing a higher correlation between dispersed new duplicates and repetitive elements, which conforms to our previous report (Yang et al. 2008).

Remarkable role of *de novo* new gene origination

We observed an unexpectedly high proportion of *de novo* new genes (11.9%, Figure 2) in the *D. melanogaster* species complex. Besides the previously reported cases (Levine et al. 2006), we identified a total of nine *de novo* genes comprised of seven in the *D. melanogaster* species complex and two specific to *D. melanogaster*. These genes are likely functional protein-coding genes based on two pieces of evidence: first, 7 of them are shared by the *D. melanogaster* species complex and have been retained in the genome for more than 5.4 million years. Second, all of them have transcriptional evidence provided by ESTs or full-length cDNAs. Because the *de novo* gene CG33666 was annotated without a start codon by FlyBase (www.flybase.org), we performed a 5' RACE (rapid amplification of cDNA ends, see Materials and Methods) experiment and re-annotated its intact ORF (Supplemental Table S1, Note). Thus all the *de novo* genes are predicted to have intact ORFs. Notably, the lengths of all the newly identified *de novo* genes' protein products are predicted to be longer than 100 amino acids. It is also noteworthy that both EST (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene>) and microarray (www.flyatlas.org) data has confirmed the gene CG33235 has evolved testis-specific expression pattern, which parallels previously reported *de novo* genes (Chen et al. 2007; Levine et al. 2006).

We searched the entire GenBank database and none of these *de novo* genes can retrieve any significant BLAST hits from organisms other than insects. All but one (CG31909) *de novo* genes can be mapped to their homologous non-coding

counterparts in *D. yakuba* (see Methods). Gene *CG31909* cannot find any homologous sequences in all the 12 *Drosophila* genomes. Interestingly, it is homologous to a region of non-coding sequence in honeybee, which raises the possibility that this gene may have originate through lateral gene transfer. All the other nine *de novo* genes appear to have evolved from ancestral non-coding sequences.

Based on the characterization of their homologous sequences, we found heterogeneity during the origin of these new genes (see Supplemental Data S2 for the alignments). More specifically, the flanking gene orders of four genes (*CG33666*, *CG32690*, *CG33235* and *CG2042*) are conserved between *D. melanogaster* and *D. yakuba* or *D. simulans*, suggesting they should have derived from orthologous ancestral sequences. Interestingly, *CG33235* originated from lineage-specific expansion of simple tandem repeats, forming a gene putatively encoding a protein with a length of 1584 amino acids. As mentioned above, it probably has evolved testis-specific expression pattern. It might be the first identified young gene with more than 75% coding regions comprising of repetitive sequences. *CG2042* is *D. melanogaster*-specific and emerged through recruiting a long interspersed element BS2 as its first exon. The formation process of four *de novo* genes (*CG40384*, *CG15323*, *CG32582* and *CG32824*) appears to have involved lineage-specific genomic rearrangements. Their flanking gene order is not conserved between species but they can be mapped to another homologous genomic region in *D. yakuba*. For example, the gene *CG32824* on chromosome X in *D. melanogaster* can be mapped to a region on chromosome 2 in *D. yakuba*. A DNA transposable element PROTO_B is adjacent to this region in *D. yakuba* and this gene's second exon in *D. melanogaster*. This suggests that ancient genomic rearrangements such as DNA transposition or chromosomal translocation events might lead to the emergence of

these genes. In summary, these data shed additional light on how non-coding sequences can be recruited in various ways to give rise to an entirely new gene.

Substantial proportion of new genes formed chimeric structures

The extensive annotations available for both parental and new genes in *D. melanogaster* gave us the unique opportunity to trace their gene structure changes. We focused on the comparison of the protein-coding (CDS) regions between ancestral and new genes in *D. melanogaster* and the *D. melanogaster* species complex due to the possible inadequate annotation in UTR regions (see Methods). Interestingly, a high proportion (29.2% and 32.2%, respectively) of new genes from these two levels of taxa formed chimeric gene structures by recruiting flanking sequences into their protein-coding regions (Table 4, Supplemental Table S7). The recruited regions come from a variety of genomic sources (Supplemental Figure S2) including coding regions of other genes (i.e. exon shuffling), intronic or intergenic sequences (i.e. exonization), and repetitive elements (long interspersed repeats or simple tandem repeats). The unique amino acids in the new genes are very likely to contribute to the process of neofunctionalization. We found that the proportion of new genes completely duplicating their ancestors' CDS decreases with increase of the new genes' ages (Figure 3). In fact, there are only about 16.3% of such new genes in the *D. melanogaster* species complex, in contrast to 40.9% in *D. melanogaster* (Figure 3). This indicates that functional redundancy resulting from complete duplication may have less of a chance to be fixed during new gene origination.

Chromosomal distribution of new genes

We found a significant (Fisher's exact test, $P < 0.01$) excess of new genes located on X

chromosome rather than chromosome 2 or 3 (Figure 4) in both lineages of *D. melanogaster* and the *D. melanogaster* species complex. *De novo* genes are overrepresented (66.6%, one gene *CG40384* doesn't have chromosome information) on X chromosome, consistent with the previously reported pattern (Levine et al. 2006). Also, retroposed new genes show a “gene traffic” pattern on X chromosome (Betran et al. 2002; Emerson et al. 2004): 4 out of 5 (80%) new retrogenes specific to *D. melanogaster* and 5 out of 6 (83.3%) retrogenes specific to the *D. melanogaster* species complex derived from an X-to-autosome or autosome-to-X retroposition event. In contrast, the new genes specific to *D. yakuba* is overrepresented on the third autosome but significantly underrepresented on X chromosome (Figure 4, Fisher's exact test, $P < 0.01$). The chromosomal distribution patterns of new genes in these species are in agreement with a recent analysis on lineage-specific duplications in *Drosophila* species (Heger and Ponting 2007), which found lineage-specific duplications have almost always been enriched within X chromosome in *D. melanogaster*/*D. simulans*. Further studies are required to answer whether such dramatically different patterns of chromosomal distribution of new genes in *D. melanogaster* and *D. yakuba* is due to a lack of reliable gene annotation in *D. yakuba* or different genomic evolution history of these species.

Discussion

A general picture for origin of new genes in *Drosophila*

The large disparities among organisms in gene numbers implies the fundamental process of new gene origination during evolution (Rubin et al. 2000; Tatusov et al. 1997). To date, various mechanisms have been demonstrated to be capable of generating new genes (Long et al. 2003). In this work, we selected the *D.*

melanogaster species subgroup with its high-quality genome data and its short divergence times to provide an integrative insight into these mechanisms. We compared the different mechanisms of the origin of new genes both within genomes and over evolutionary time. We uncovered that gene duplication (both tandem and dispersed) generated the most abundant new genes (Table 1). However, different types of gene duplication may be generated by different mechanisms and have different contributions to the emergence of new genes.

Regarding other mechanisms, we detected 8 putatively functional new retrogenes with transcriptional evidence, and shared by the *D. melanogaster* species complex (Table 1). These retrogenes account for about 10% of the new genes and translates to an origination rate of averagely 0.6-1.5 new retrogenes per myr. It supports previous reports that retroposition is an important mechanism creating new genes (Bai et al. 2007; Brosius 1991; Marques et al. 2005). However, given the compact genomes of *Drosophila* species (Clark et al. 2007; Petrov et al. 1996), their limited sources of retroposons may render the role of retroposition in origin of new genes not as significant as that in human and rice (more than 36% human and about 38% rice duplicates are generated from retroposition) (Marques et al. 2005; Pan and Zhang 2007; Wang et al. 2006). Most strikingly, we found new genes that have originated *de novo* from non-coding sequences have contributed as much as 11.9% of the putatively functional new genes. This further suggests its important yet underappreciated role during origin of new genes. By summarizing the contribution of each mechanism, we are able to, for the first time provide an integrative picture about all new genes on the genomic level.

We are also able to provide a rough estimate for origination rate of functional new genes, which is an important parameter for understanding the tempo of new gene

origination. Much attention has previously been paid to gene duplication rate (Gu et al. 2002; Hahn et al. 2007; Lynch and Conery 2000; Pan and Zhang 2007) and it has been estimated to range from 0.0010 (Hahn et al. 2007) to 0.0023 (Lynch and Conery 2000) per gene per myr in *Drosophila* species. Compared to the gene duplication rate, the rate of functional new gene origination makes more senses because only such new genes authentically contribute to evolution of organismal diversities. These new genes fixed in the *D. melanogaster* species complex provide a valuable dataset to estimate this parameter. The genes that are more likely to be functional are those that have been kept intact in the genomes for a long time (>5.4 myr). Therefore we estimate that the rate of new functional gene origination is 5 to 11 per myr in the *Drosophila* genome (60 new genes divided by 5.4 and 12.8 myr), which translates to 0.000391 to 0.000925 per gene per myr assuming 12,017 genes in *D. melanogaster* genome (the number used in this study). As expected, this rate is much lower than the estimated gene duplication rate. In fact, if we use all *D. melanogaster*-specific new genes including both fixed and un-fixed ones, the estimated new gene origination rate (0.0011 per gene per myr, 72 genes/5.4 million years, assuming 12,017 genes) is very close to the recent estimate of gene duplication rate in 12 *Drosophila* species (0.0010) (Hahn et al. 2007). However, our population data (Table 3) and a recent study on paralog of 12 *Drosophila* species (Heger and Ponting 2007) suggest that substantial gene duplicates used in previous studies perhaps are polymorphic within populations and thus many of them could disappear with time. In this sense, rate of new gene origination is a more realistic indicator than gene duplication rate to assess speed of generation of genetic novelties in evolutionary biology.

A dynamic view on new duplicates

From Table 1, we can see that tandem gene duplication comprises most of the nascent duplicates (about 80%, or more than 11 copies/myr) in both *D. melanogaster* and *D. yakuba*. However, only 58.8% of them are fixed throughout the populations of *D. melanogaster* (Table 3), and only 33.9% of the constrained new genes shared in the *D. melanogaster* species complex are tandem duplicates (Table 1, Figure 2). This is probably due to a deletion event or it can be explained by a rapid birth but infrequent fixation model (Heger and Ponting 2007). In contrast, although dispersed new duplicates seem to have a lower birth rate (Table 1, 1 copy/myr for *D. melanogaster*, 3 copies/myr for *D. yakuba*), they comprise the highest proportion (44.1%) among all the constrained new genes shared in the *D. melanogaster* species complex. These results suggest dispersed duplicates might have a higher survivorship than nascent tandem ones.

However, while many tandem duplicates may subsequently get lost, some may actually become dispersed. This expectation is based on the observation that there is a significantly (Table 1, Fisher's exact test, $P = 0.000129$) higher number of dispersed duplicates in the *D. melanogaster* species complex compared to that in *D. melanogaster*, and this cannot simply be explained from higher survivorship of original dispersed duplicates. Even if we assume all of the dispersed new duplicates are able to survive after their emergence, we would only expect 8% to 21% dispersed new genes to emerge in 5.4 to 12.8 myr shared by the *D. melanogaster* species complex (1 copy/myr for dispersed gene duplication in *D. melanogaster*, see above). Such a discrepancy from the observed 44.1% thus strongly suggests that some of the tandem duplicates might have become dispersed through subsequent mutation events (Clark et al. 2007; Heger and Ponting 2007). This scenario is consistent with our result showing that most nascent (all *D. melanogaster*-specific and 84% *D. yakuba*-

specific) tandem segmental duplications are in immediate conjunction with each other, while only 20% of them shared by the *D. melanogaster* species complex show this pattern. It is also supported by a recent study on gene families in human and mouse, which found that tandem duplicated members are of more recent origin than those interchromosomal ones (Friedman and Hughes 2003).

In addition to the evolutionary dynamics of tandem and dispersed duplicates, we can also infer that the underlying mechanisms of origin of these two types of duplicates might be different. Several genomic processes have been proposed to account for duplication events: non-allelic homologous recombination (NAHR) (Bailey et al. 2003; Fiston-Lavier et al. 2007; Stankiewicz and Lupski 2002), transposons-mediated transposition (Jiang et al. 2004; Lal et al. 2003; Yang et al. 2008) and illegitimate recombination (IR) (Roth and Wilson 1988; Roth et al. 1985; Slack et al. 2006). Duplicates generated by NAHR or transposons-mediated transposition usually have certain homologous sequences or repetitive elements at the breakpoint sites of the duplicated blocks. Alternatively, duplication could happen via IR (also called nonhomologous end joining, NHEJ) which depends on little or no sequence homology. Our recent experimental efforts characterizing new genes in 8 *D. melanogaster* subgroup species with comparative fluorescence *in situ* hybridization (cFISH) has found that a majority of new dispersed duplicate genes are associated with repetitive elements at the breakpoints (Yang et al. 2008). In this work, we interestingly found dispersed and tandem duplicated new genes show different degrees of associations with repetitive elements at their breakpoints. It suggests genomic processes depending on repetitive elements or homologous sequences such as NAHR or transposon-mediated DNA fragment transposition play an important role in creating both tandem and dispersed new genes. However, a lower association in

tandem duplicates (30.5% *D. melanogaster*-specific tandem new genes, 11.8% *D. yakuba*-specific tandem new genes) compared to dispersed duplicates (33.3% and 26.8%, respectively) suggests that IR is more important generating tandem duplicated genes.

A new perspective on *de novo* origination

Ohno stated: “In a strict sense, nothing in evolution is generated *de novo*” (Ohno 1970). New genes with novel functions were believed to be mainly derived from preexisting genes (Long 2007; Long et al. 2003; Ohno 1970). A lack of comparison of *de novo* origination with other mechanisms of new gene origination has hampered a proper evaluation for this mechanism. In this study, we unexpectedly found 11.9% of the constrained new genes emerge from non-coding sequences, suggesting that *de novo* origination is not rare and its role during the origin of new genes is important (Table 1, Figure 2). In fact, our current estimation for the contribution of this process is conservative for two reasons. First, we excluded several cases (e.g. *CG15930*) based on sequencing gaps located at their syntenic regions in the outgroup species. Second, our stringent criteria (see Methods) cannot find those *de novo* genes with alignable orthologous sequences which don’t have expression in the outgroup species. Given these two reasons, the proportion of *de novo* new genes could even be higher than 11.9%. It would be very intriguing to investigate whether *de novo* origination also play a prominent role in origin of new genes in species other than *Drosophila*.

In addition, for the first time, we have analyzed the orthologous non-coding regions of these *de novo* genes and reconstructed their origination processes to show how a region of non-coding sequences can give rise to an entirely new gene. We

revealed that, multiple non-coding genomic sources, including intergenic sequences, simple tandem repeats or long interspersed repetitive elements, have the potential to become a new gene. All of these *de novo* genes have intact ORFs and some of them might have acquired novel functions, which is supported by the fact that some of these genes (CG33235, and 5 previously reported cases) have evolved testis-specific expression pattern (Levine et al. 2006). It is also noteworthy that we found 13 *D. yakuba*-specific ESTs (data not shown) mapped to the *de novo* gene CG32690's orthologous region in *D. yakuba*. However, there is no evidence showing that this region contains an intact ORF which is capable of encoding a protein longer than 50 amino acids. Consistent with our finding on a *de novo* gene in budding yeast (Cai et al. 2008), this data suggests some *de novo* protein-coding genes may acquire the ability of transcription before being capable of encoding proteins (Casici 2008).

Chimeric structures significantly contribute to the evolution of new genes

Population genetic models have been developed to account for the fixation of new genes within a population (Clark 1994; Lynch and Force 2000; Lynch et al. 2001; Ohta 1988). Most of them assume gene duplication generates a new gene copy which is functionally and structurally redundant at birth. Under this assumption, the redundant new copy is most likely subject to nonfunctionalization, with a low probability of undergoing subfunctionalization or neofunctionalization. Consistent with such predictions, our structure analysis result for new genes show, structurally redundant copies (complete duplicates) are vulnerable to losses (Figure 3).

More importantly, we found 29.2% new genes specific to *D. melanogaster* formed chimeric structures. Given their extremely young ages, it suggests new genes are not necessarily structurally identical at birth. In particular, eight of them

(Supplemental Table S6, Supplemental Figure S1) arose from fusion events of partial coding regions/introns of two separate genes at the boundary position of two duplicated segments in less than 5.4 myr in *D. melanogaster*. This vividly demonstrates the process of exon-shuffling (Gilbert 1978). Since the involved duplicated segments are in immediate conjunction with each other, and 5 of such new genes exist without any synonymous substitutions (data not shown), it is likely that they originated as chimeric genes. We also found 32.2% of the new genes specific to the *D. melanogaster* species complex formed chimeric structures. These constrained new genes are more likely to be functional and thus it suggests chimeric structure formation is an important solution to the preservation of new copies within the population. Although previous theoretical models predict a low fixation probability of a new copy, they only consider structurally redundant cases (Lynch and Force 2000; Lynch et al. 2001). If a new copy has formed a chimeric structure, it has the potential to immediately confer a novel function which the parental gene does not bear (Patthy 1999).

The generality of chimeric new gene formation can be reflected by the fact that it is not restricted to certain species or certain type of new genes. Recent characterization of 37 young duplicates in *C. elegans* found 38% of them formed chimeric gene structures (Katju and Lynch 2003; Katju and Lynch 2006). And our parallel work in rice has found 42% transcribed retrogenes have formed chimeric structures (Wang et al. 2006). It is noteworthy that chimeric structures formed at the untranslated regions of the new genes could also contribute to the acquisition of novel functions by introducing new regulatory regions (Arguello et al. 2006; Begun 1997). Here, however, we only investigated protein-coding regions given their more reliable annotation. All these results indicate that forming chimeric gene structures is

probably a common path for new genes to acquire novel functions and thus being preserved within the population.

Methods

Identification of new genes

To identify the species-specific candidate new genes, we downloaded the genome sequences of *D. melanogaster* (dm2, Apr.2004), *D. simulans* (droSim1, Apr.2005) and *D. yakuba* (droYak2, Nov.2005) from UCSC (<http://hgdownload.cse.ucsc.edu/downloads.html>), as well as 19235 *D. melanogaster* cDNAs and their mapping information from FlyBase (<http://www.flybase.org/>). We removed redundancies resulting from alternative splicing and obtained 12570 cDNAs, each representing the longest transcript from a unique genome locus. We used both sequence similarity and syntenic gene order to infer the *bona fide* orthologous relationship (Gao and Innan 2004). We first aligned unique cDNAs to each other using BLASTN (Altschul et al. 1990) with the 10^{-5} as the *E*-value cutoff. To define paralogous genes, we grouped those cDNA pairs with an aligned length longer than 30% and sequence identity higher than 80% into 541 gene families. We picked up the longest cDNAs to represent each family. Together with 11476 single copy cDNAs, these 12017 genes comprise our query sequences. We aligned the non-redundant query cDNAs against genomes of *D. melanogaster*, *D. simulans* and *D. yakuba* using BLASTN (Altschul et al. 1990) with 10^{-5} as the *E*-value cutoff. We retained those hits with aligned lengths longer than 200bp or 30% of the query cDNA length, taking 80% as the sequence identity cutoff for aligned region. If a cDNA had

two or more hits in the *D. melanogaster* genome, we then investigated the conservation of syntenic gene order defined by two flanking genes of the focal cDNA/gene between *D. melanogaster* and its outgroup species to discriminate the parental gene (conserved) and daughter genes (not conserved). In a few cases, the corresponding region to the flanking gene contain sequence gaps on one side in the outgroup species. In this cases we used one available flanking gene to judge the synteny. For tandem gene duplications with the same flanking orthologous genes, we picked one at random as the new gene. It is reported lineage-specific chromosomal rearrangements could also break the conservation of synteny (Bhutkar et al. 2007). However, such events are rare for most investigated lineages (less than 5 events except for *D. ananassae*, also see Supplemental Data S1) (Bhutkar et al. 2007) in this work and the microsynteny, i.e., the order of three adjacent genes should be conserved (Heger and Ponting 2007). Based on the syntenic gene order and paralog number in three species, we acquired 169 candidate new genes in *D. melanogaster*, 253 genes in *D. yakuba* and 191 genes only shared by *D. melanogaster* and *D. simulans*.

Manual check and classification of new genes

First, we checked the homolog numbers with each candidate new gene's genomic sequences in *D. sechellia* (droSec1, Oct.2005), *D. erecta* (droEre2, Feb.2006) and *D. ananassae* (droAna3, Feb. 2006) with BLAT to exclude the possibility of gene loss in *D. simulans*, *D. yakuba* or *D. erecta* (Clark et al. 2007; Kent 2002). We counted homologous hits with BLAT scores higher than 100 in each species. If one of the outgroup species showed both same homolog number and conservation of synteny compared with *D. melanogaster* for a certain candidate gene, it was removed from the

dataset. Second, based on the annotation information, we excluded candidates that resulted from internal exon duplications or a sequencing gap in the outgroup. Only those new genes with annotation IDs (e.g. *CG10102*, Release 4.2 annotations from FlyBase) in *D. melanogaster* or intact ORF predicted by Genscan (Burge and Karlin 1997) in *D. yakuba* were retained as authentic new genes. We have also included 6 non-coding genes termed with *CR*-ids as new genes, because they have shown differential expression patterns during early developmental stages or in specific tissues (Chintapalli et al. 2007; Manak et al. 2006)(Supplemental Table S1-2, Notes). We initially defined *de novo* genes as those without significant sequence homology (aligned length longer than 200bp or 30% of the query sequence length) detected by BLASTN in all the outgroup species. We have 22 such candidates. To exclude possible double gene loss events in the two outgroup species (*D. erecta* and *D. ananassae*), genomic sequences of these genes were further subject to BLASTN using discontinuous megablast algorithm and then a TBLASTN search with their protein sequences against the whole GeneBank database. We excluded a candidate *CG15882*, which has homologous sequences detected by TBLASTN in *D. yakuba* and *D. erecta* and may be a *de novo* gene originated in the ancestor of *D. melanogaster* subgroup species. None of remaining candidates retrieved significant BLAST hits (score higher than 50) in organisms other than species within the *D. melanogaster* species complex. Using the more sensitive BLASTZ program and based on the “Alignment Net” information on UCSC Genome Browser, we further excluded 12 cases aligned to an annotated gene (mapped by BLASTZ) in *D. melanogaster* and reconstructed the origins of these genes (Schwartz et al. 2003). We also excluded a previously reported case, *CG32712* (Levine et al. 2006), which maintained its open reading frames in other insects’ genomes except *D. yakuba* and *D. erecta*. We

verified its expression with RT-PCR in *D. pseudoobscura* (data not shown). We performed a 5'RACE (FirstChoice RLM-RACE Kit, Ambion, Inc.) experiment using testis derived RNA of *D. melanogaster* and re-annotated ORF structure of CG33666 (Supplemental Table S2, Note).

We defined new genes without any introns compared to their intron-containing parents as retroposed new genes. Coding regions of these candidates were manually aligned back to the genome to assure that no hits in the introns of parental genes were detected. Finally we classified newly duplicated genes as tandem when they are adjacent to each other or reside in duplicated segments without any intervening genes. Those with intervening genes, or those located on different chromosomes, are classified as dispersed gene duplications. For all the duplicated new genes, we extended the flanking regions of gene pairs until they cannot align with each other by BLAT. Thus we determined the region of duplicated segment encompassing the focal genes in the genome. The segments with higher BLAT scores with outgroup species is considered as the segment containing the parental genes. Thereby, we defined the parental-daughter relationship for tandem gene duplications. To avoid assembly error, we further performed MEGABLAST against NCBI Trace Archive database (<http://www.ncbi.nlm.nih.gov/Traces/>) using sequence regions expanding the boundaries of the tandem duplicated segments as query and ensure there is at least one sequence read covering the boundary sites. We surveyed repetitive elements beyond the breakpoints (50bp around) of the duplicated segments or duplicated gene regions using RepeatMasker (Jurka 2000) and Tandem Repeat Finder (Benson 1999) information provided on the UCSC Genome Browser (Karolchik et al. 2003). The boundary sites were also surveyed for the orthologous regions in the outgroup species based on syntenic information.

Gene structure characterization and K_s calculation

We aligned the protein coding regions of both parental and daughter genes with *bl2seq*. We considered it as a chimeric gene if the new gene recruited a stretch of unalignable sequence longer than 50bp. The other new duplicates were assumed to generate from complete gene duplication if their length differences with parental genes are less than 50bp. If the differences are longer than 50bp and the new genes are shorter, they were characterized as partial duplications. We predicted gene regions and coding frames of parental and new genes in *D. yakuba* using GeneWise (Birney et al. 2004). Coding sequences were further aligned according to the protein sequences of *D. melanogaster* using Perl scripts. We calculated synonymous (K_s) and nonsynonymous substitution rate (K_a) with KaKs_Calculator_1.2 (<http://evolution.genomics.org.cn/software.htm>) using methods described by Yang and Nielsen (Yang and Nielsen 2000).

Population study

We extract genomic DNA of a single male fly from each of 19 iso-female *D. melanogaster* strains using the PUREGENE DNA Isolation Kit (Gentra). These strains included 10 Ecuador lines representing South American local population, 2 North American lines, 3 African lines, 1 European line and 3 Chinese lines. The Ecuador, North American and African lines were obtained from Prof. M Long's lab at University of Chicago. The European line was obtained from Prof. Aike Guo's lab at Institute of Neuroscience at Shanghai, and the Chinese lines were gifted by Dr. Wenxia Zhang of Peking University. Using these DNA samples, we used PCR to test if a new gene has been fixed in the population of *D. melanogaster* or not. We

designed PCR primers either surrounding the boundary sites of duplicated segments or at sites that were capable of discriminating parental and daughter genes. To exclude possible false negatives due to sequence mismatch in some strains, three to four non-redundant primer sets were used for each gene. We performed PCR amplification with rTaq(Takara Bio Inc.) for these 19 strains together with a negative control without adding templates. Presence/absence data were recorded for each line based on the PCR results.

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Figure Legends:

Figure 1. Phylogenetic distribution of new genes.

We designated numbers of new genes restricted to different lineages. Phylogenetic tree of investigated *Drosophila* species and their divergence times in each node are indicated (Tamura et al. 2004).

Figure 2. Quantification of contributions to new gene origination from different mechanisms.

‘*D. mel*’ stands for ‘*D. melanogaster*’, ‘*D. sch*’ for ‘*D. sechellia*’, ‘*D. sim*’ for ‘*D. simulans*’. These three species together stands for the *D. melanogaster* species complex. We didn’t identify new genes originate from retroposition or *de novo* process in *D. yakuba* lineage due to the lack of reliable annotations in this species.

Figure 3. Decrease of proportion of complete duplicated new genes with time.

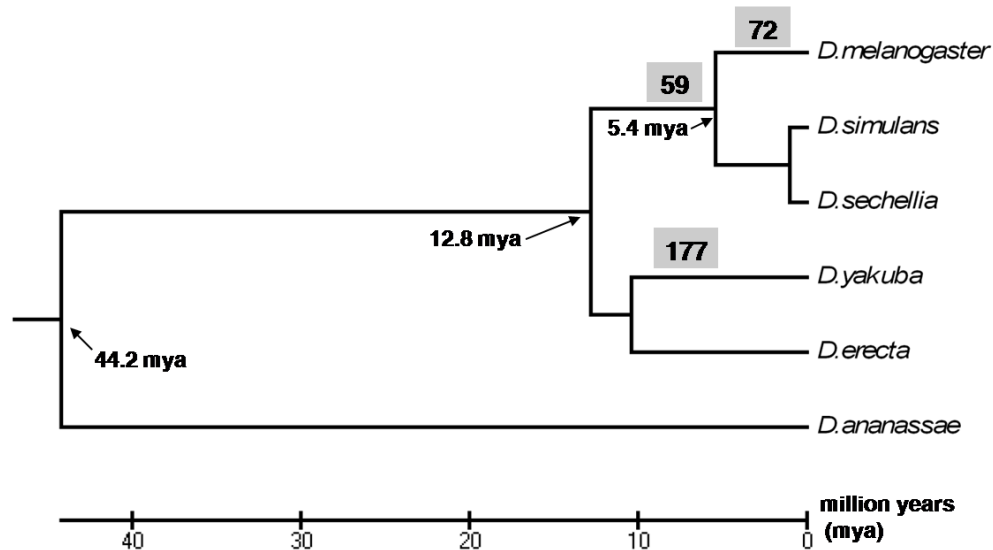
‘*D. mel*’ stands for ‘*D. melanogaster*’, ‘*D. sch*’ for ‘*D. sechellia*’, ‘*D. sim*’ for ‘*D. simulans*’. These three species together stands for the *D. melanogaster* species complex. We compared structures and lengths of parental and new genes in *D. melanogaster* and the *D. melanogaster* species complex. New genes completely duplicated its ancestors’ coding regions seems more vulnerable to subsequent loss or structure changes during evolution.

Figure 4. Chromosomal distribution of new genes in three datasets.

New gene numbers were divided by total gene numbers on the specific chromosome as a normalization. We marked significant overrepresentation ($P < 0.01$) of new

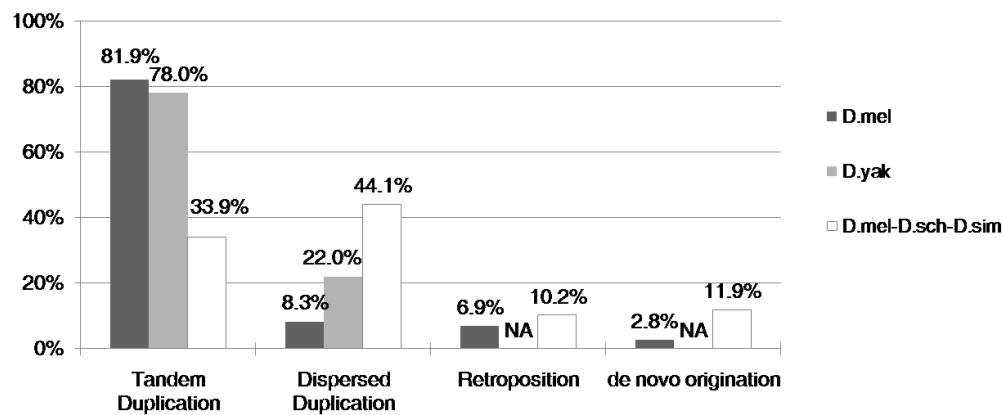
genes on certain chromosome with ‘*’, highly significant ($P < 0.001$) with ‘**’. We didn’t take chromosome 4 into account given the extremely low gene number on this chromosome.

Figure 1. Phylogenetic distribution of new gene datasets.



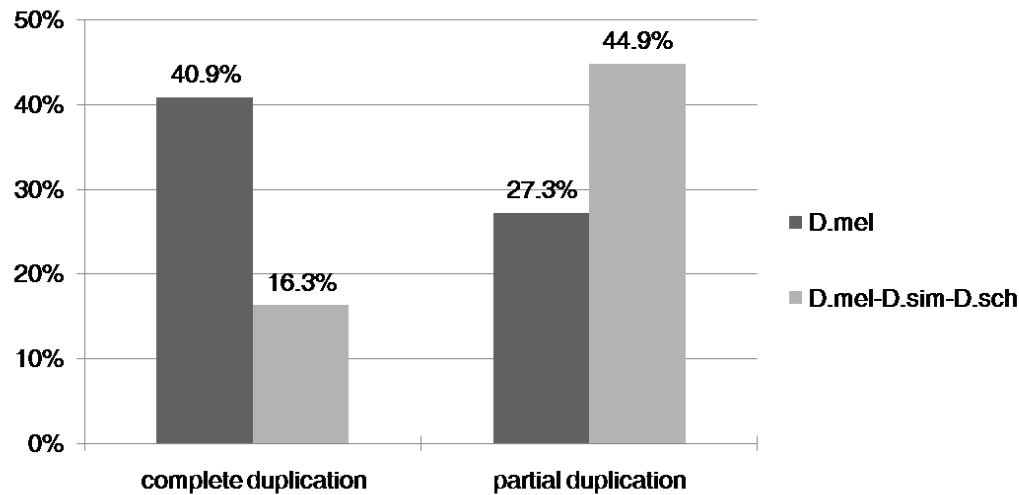
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Figure 2. Quantification of contributions to new gene origination from different mechanisms.



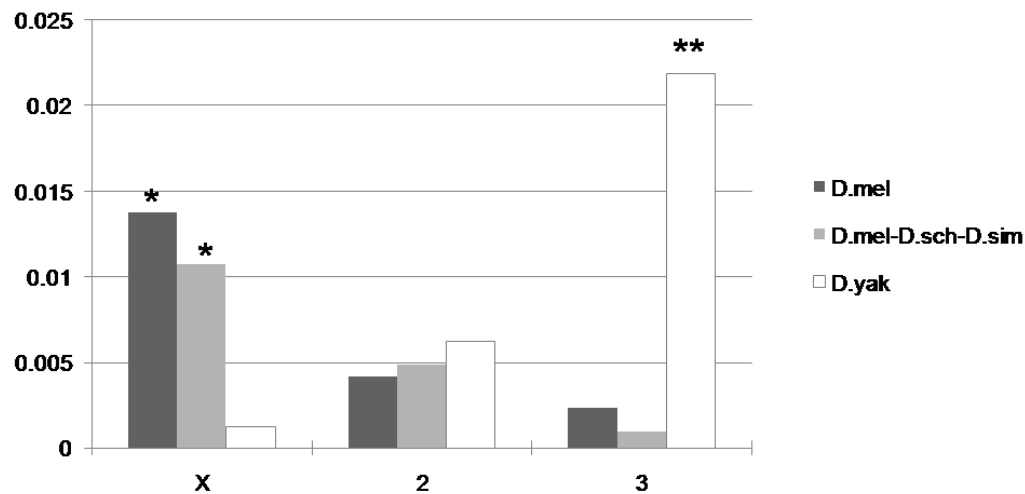
‘*D. mel*’ stands for ‘*D. melanogaster*’, ‘*D. sch*’ for ‘*D. sechellia*’, ‘*D. sim*’ for ‘*D. simulans*’. These three species together stands for the *D. melanogaster* species complex. We didn’t identify new genes originate from retroposition or *de novo* process in *D. yakuba* lineage due to the lack of reliable annotations in this species.

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New gene numbers were divided by total gene numbers on the specific chromosome as normalization. We marked significant overrepresentation ($P < 0.01$) of new genes on certain chromosome with ‘*’, highly significant ($P < 0.001$) with ‘**’. We didn’t take chromosome 4 into account given the extremely low gene number on this chromosome.

Table 1. New gene number categorized by different mechanisms

	Gene Duplication		Retroposition	<i>de novo</i>	Total
	Tandem	Dispersed			
<i>D. mel</i>	59	6	5	2	72
<i>D. mel-D. sch-D. sim</i>	20	26	6	7	59
<i>D. yak</i>	138	39	NA	NA	177

‘*D. mel*’ stands for ‘*D. melanogaster*’, ‘*D. sch*’ for ‘*D. sechellia*’, ‘*D. sim*’ for ‘*D. simulans*’. These three species together stands for the *D. melanogaster* species complex. Due to the lack of reliable annotation information of gene structure in *D. yakuba*, we didn’t identify new genes originated from retroposition and *de novo* origination.

Table 2. Synonymous substitution rate (K_s) calculated between paralogs in different categories of new genes

K_s	Tandem Duplication	Dispersed Duplication	Retroposition
<i>D. mel</i>	0.053 $\pm$ 0.015	0.091 $\pm$ 0.019	0.195 $\pm$ 0.054
<i>D. mel-D. sch-D. sim</i>	0.637 $\pm$ 0.187	1.153 $\pm$ 0.283	0.186 $\pm$ 0.032
<i>D. yak</i>	0.117 $\pm$ 0.025	0.260 $\pm$ 0.076	NA

‘*D. mel*’ stands for ‘*D. melanogaster*’, ‘*D. sch*’ for ‘*D. sechellia*’, ‘*D. sim*’ for ‘*D. simulans*’. These three species together stands for the *D. melanogaster* species complex. We calculated substitution rates between paralogs using the method described by Yang and Nielsen (Yang and Nielsen 2000). The standard error was shown. The detail ratio for each gene is also provided in Supplemental Table S1-3.

Table 3. Fixation pattern in different kinds of new genes specific to *D. melanogaster*

	Total Tested #	Fixed Gene #	Fixed Proportion
Tandem Duplication	34	20	58.8%
Dispersed Duplication	3	0	0
Retroposition	6	3	50%
<i>de novo</i>	2	2	100%
Total	45	25	55.5%

A gene is considered as ‘fixed’ if all the investigated strains can detect positive results with PCR assays.

Table 4. Proportion of chimeric new genes generated by different mechanisms

	<i>D. mel</i>	<i>D. mel-D. sch-D. sim</i>
Tandem Duplication	17/72 (23.6%)	8/59 (13.6%)
Dispersed Duplication	3/72 (4.2%)	8/59 (13.6%)
Retroposition	1/72 (1.4%)	3/59 (5%)
Total	29.2%	32.2%

‘*D. mel*’ stands for ‘*D. melanogaster*’, ‘*D. sch*’ for ‘*D. sechellia*’, ‘*D. sim*’ for ‘*D. simulans*’. These three species together stands for the *D. melanogaster* species complex.

Supplemental Data Legends

Supplemental Data S1. The detailed comparison between our identified new genes and other works' predictions (Bai et al. 2007; Bhutkar et al. 2007; Clark et al. 2007; Hahn et al. 2007; Heger and Ponting 2007)

Supplemental Data S2. BLASTZ alignments downloaded from UCSC Genome Browser for *de novo* genes (Karolchik et al. 2003; Schwartz et al. 2003). Numbers beside the alignments correspond to the genome positions in *D. melanogaster* (Apr. 2004 assembly) or other outgroup species.

Supplemental Figure Legends

Supplemental Figure S1. An example of chimeric gene formed at the boundary site of two tandem duplicated segments.

The figure was generated from USCS Genome Browser (Karolchik et al. 2003). The continuous black bars represent for the duplicated segments. The blue bars represent annotated genes. Segmental duplication concatenated exons of *esc1* and *CG12264* and formed a new gene *CG31864*.

Supplemental Figure S2. Diverse genomic sources of chimeric region in the new gene. We showed the proportion of different chimeric sources calculated from Supplemental Table S7 for new genes specific to *D. melanogaster* and the *D. melanogaster* species complex.

Supplemental Table Legends

Supplemental Table S1. *D. melanogaster*-specific new genes and their parental genes. We show *Ka/Ks* ratio calculated between paralogs for each new gene. For *de novo* genes, we show homologous BLASTZ hits information extracted from UCSC Genome Browser in 10 other insects' genome sequences (Schwartz et al. 2003). We also provided some expression information based on (Chintapalli et al. 2007; Manak et al. 2006) for some genes.

Supplemental Table S2. The *D. melanogaster* species complex specific new genes and their parental genes. We show *Ka/Ks* ratios calculated both between paralogs and between orthologs for each new gene. For *de novo* genes, we show homologous BLASTZ hits information extracted from UCSC Genome Browser in 10 other insects' genome sequences (Schwartz et al. 2003). We also provided some expression information based on (Chintapalli et al. 2007; Manak et al. 2006) for some genes.

Supplemental Table S3. *D. yakuba*-specific new genes and their parental genes. We show *Ka/Ks* ratio calculated between paralogs for each new gene. We also show duplicated gene/segment region for each parental-new gene pair. The position information is based on 'dro Yak2' version of *D. yakuba* genome sequences.

Supplemental Table S4. *Ka/Ks* ratio for three sets of new genes. We show average and median numbers of *Ka/Ks* ratios calculated for three new gene datasets. *Ka/Ks* ratios calculated between paralogs are shown for *D. melanogaster* and *D. yakuba* specific new genes. For the *D. melanogaster* species complex specific new genes, ratios calculated between orthologs are shown.

Supplemental Table S5. Copy number polymorphism pattern for *D. melanogaster*-specific new genes within 19 *D. melanogaster* strains.

‘T’ in the first column stands for tandem duplication, ‘D’ for disperse duplication, ‘retro’ for retroposition, ‘*de novo*’ for *de novo* origination. ‘1’ represents we detected the focal gene in the investigated strain, while ‘0’ represents the negative result. Genes marked in red have been detected within all the investigated strains.

Supplemental Table S6. *D. melanogaster*-specific tandem segmental duplications
The position and length of duplicated segments as well as involved new genes are provided. The genes marked in green represent those chimerical genes form at the boundaries of the duplicated segments.

Supplemental Table S7. Structure comparison of new genes with their parental genes
We divided gene structure changes of new genes into complete, partial or chimeric duplications (see Materials and Methods). For chimeric sources, we divided them into ‘E’ (from exons of another parental gene), ‘N’ (from intron or intergenic region), ‘R’ (simple tandem repeats or repetitive elements) and ‘U’ (unknown sources).

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