LETTERS

Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*

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Spontaneous mutations are the source of genetic variation required for evolutionary change, and are therefore important for many aspects of evolutionary biology. For example, the divergence between taxa at neutrally evolving sites in the genome is proportional to the per nucleotide mutation rate, *u* (ref. 1), and this can be used to date speciation events by assuming a molecular clock. The overall rate of occurrence of deleterious mutations in the genome each generation (*U*) appears in theories of nucleotide divergence and polymorphism[2], the evolution of sex and recombination[3], and the evolutionary consequences of inbreeding[2]. However, estimates of *U* based on changes in allozymes[4] or DNA sequences[5] and fitness traits are discordant[6–8]. Here we directly estimate *u* in *Drosophila melanogaster* by scanning 20 million bases of DNA from three sets of mutation accumulation lines by using denaturing high-performance liquid chromatography[9]. From 37 mutation events that we detected, we obtained a mean estimate for *u* of 8.4×10^{-9} per generation. Moreover, we detected significant heterogeneity in *u* among the three mutation-accumulation-line genotypes. By multiplying *u* by an estimate of the fraction of mutations that are deleterious in natural populations of *Drosophila*[10], we estimate that *U* is 1.2 per diploid genome. This high rate suggests that selection against deleterious mutations may have a key role in explaining patterns of genetic variation in the genome, and help to maintain recombination and sexual reproduction.

Recurrent deleterious mutations have been implicated in several important evolutionary phenomena. For example, interference between deleterious mutations favours the spread of mutations that increase recombination or sex in finite populations[11]. Synergistic fitness effects of mutations may contribute to the maintenance of recombination and sex in large populations[3]. The positive correlation between recombination rate and nucleotide diversity in several species[12,13] may be caused by linked deleterious mutations reducing diversity in regions of low recombination[2]. Deleterious mutations are also thought to be a major contributor to inbreeding depression[2]. However, the role of deleterious mutations in these and other processes depends on the distribution of fitness effects and the number of deleterious mutations appearing in the genome in each generation (*U*).

Unfortunately, empirical estimates of *U* have been inconsistent and controversial. Two principal methods have been employed to infer *U*. The first is based on differences in fitness traits among mutation accumulation (MA) lines, which are initially genetically uniform and are subsequently maintained at a low population size in benign conditions, so that most new mutations behave neutrally and become fixed at random. However, this method will underestimate *U* because many deleterious mutations are unlikely to affect fitness detectably in the laboratory[6–8]. A second method[14] has no such a bias. *U* is estimated from the product of the mutation rate per nucleotide site per generation (*u*), the number of bases in the diploid genome (2*G*), and the fraction of sites in the genome that are subject to selective constraints (*C*):

$$U = 2uGC \quad (1)$$

C can be estimated from between-species genome comparisons[10,14]. In principle, *u* can be estimated from the nucleotide divergence in unselected genomic regions between a species pair[1] but is subject to uncertainty because the divergence date and generation interval are needed, and identifying neutrally evolving regions can be problematic. Alternatively, *u* can be estimated directly from the molecular divergence between MA lines. The first such estimate was based on electrophoretic mutations in *D. melanogaster*[4], but only three events were detected, and electrophoretic mutations can be related only indirectly to changes in the DNA. More recently, *u* has been estimated in *Caenorhabditis elegans* by sequencing MA-line DNA[5]. From this, an estimate of *U* for coding sequences was obtained, which is one to two orders of magnitude higher than an estimate from the phenotypic divergence of the MA lines, consistent with the expectation outlined above. Here we directly estimate *u* in *D. melanogaster* by scanning the genomes of MA lines, and infer *U* from equation (1).

We scanned 20 megabases (Mb) of DNA, comprising 277 segments (amplicons) of coding, intronic and intergenic DNA (Supplementary Tables S1 and S2, and Supplementary Fig. S1) from 133 MA lines of three genotypes (Florida-33, Florida-39 (ref. 15) and Madrid[16,17]), by denaturing high-performance liquid chromatography (DHPLC)[9]. The efficiency of DHPLC at detecting mutations was verified by analysing synthetic positive controls containing mutations. We successfully detected 45 out of 46 controls (Supplementary Table S3 and Supplementary Fig. S2), which is a similar rate to that in previous reports[18,19]. Putative mutations detected by DHPLC were verified and identified by sequencing. We found evidence for genetic variation in the inbred progenitor of the Florida-39 lines (see Methods and Supplementary Fig. S3 for more details). This manifested itself as fixed nucleotide differences between groups of MA lines for blocks of linked amplicons. Affected amplicons of these lines were excluded from the analysis.

Among 20,002,585 base pairs (bp) screened, we observed 37 mutations (Tables 1 and 2, and Supplementary Fig. S4), of which 3 segregated at a frequency of 0.5 in the line in which they occurred. The mutation detection rate was fairly uniform over the experiment (Supplementary Fig. S5). Our estimate of the single-nucleotide mutation rate per generation is 5.8×10^{-9} (95% confidence interval

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Table 1 | Mutation events detected by DHPLC and confirmed by sequencing

Amplicon	Line	Mutation type	Context
2L-CG8965-C	F33.49	complex cod	CCAAGGA**TG**TCTT→CCAAGGA**CCA**TCTT
3R-113648	M11	complex intron	CATAT**CGTT**CGCA**A**G→CATATCGCA**G**G
X-13003975	M62	complex intron	GATAT(A)$_4$T**T**T**G**CAACTATTTA→GATAT(A)$_4$T**A**T**AT**C**TTA(AT)$_8$T(AT)$_2$(A)$_4$TA**AACTATTT**C**A**
2L-20718966	M87	del.* interg.	GTAGTGTGT**TT...ATGT**AACC→TAAGAGTA(GT)$_3$AACC
2R-CG30377	F33.45	del.† intron	TCTAATG**CG...AG**TCA→TCTAATGTCA
2R-fus	M70	del. cod	AGG(CGG)$_2$**TGG**TTGTG→AGG(CGG)$_2$TTGTG
3R-7922936	F33.67	ins. intron	(T)$_5$AAGG(T)$_9$GTG→(T)$_5$AAGG(T)$_9$**T**GTG
3R-Fru-bis	F33.55	del. intron	AATGACT**CT**GATATT→AATGACTGATATT
3R-19561997	F33.42	del. intron	GGCGT**G**CCAAA→GGCGTCCAAA
X-3198685	M137	del. intron	AGAG(A)$_8$**A**GG→AGAG(A)$_8$GG
X-11335521	M148/M149	ins. intron	TT(A)$_9$CCTTG→TT(A)$_9$**A**CCTTG
3L-22018790	F33.5	TE interg.	CATATGGTAT→CATAT ***(Cr1a)***
2L-cul2-NC2	M78/ M79	ts interg.	AATG**T**ATG→AATG**C**ATG
2L-cul-2-C	F33.8	ts cod	CTT**A**AGCT →CTT**G**AGCT
2R-CG3136	F33.42	ts cod	GCAG**G**TC→GCAG**A**TC
2R-CG30377-up	F39.72	ts interg.	GTCT**T**GAT→GTCT**C**GAT
2R-3097863-down	F33.27	ts interg.	TAAA**C**GGT→TAAA**T**GGT
3L-Bab2-C	F33.8	ts cod	CTGT**G**GGG→CTGT**A**GGG
3L-22018790-down	F33.8	ts interg.	CTAG**G**AAG→CTAG**A**AAG
3L-BcDNAGHO3694	F33.6/ F33.71	ts cod	GGGT**C**ACT→GGGT**T**ACT
3R-113648	F33.49	ts intron	GTCGA**A**GGG→GTCGA**G**GGG
3R-19599719	F39.67	ts intron	ATG**G**GCG→ATG**A**GGCG
3R-19615776	F39.65	ts cod	ATTTCC**T**TTG→ATTTCC**C**TTG
3R-CG8968	F33.69	ts intron	CATC**G**CTT→CATC**A**CTT
3R-21787667-down	F33.49	ts interg.	CTT**G**CGCT→CTT**A**CGCT
X-11331631-down	M31	ts interg.	GTATA**T**ATGC→GTATA**C**ATGC
X-CG15745	F33.69	ts cod	TGCCC**G**GAG→TGCCC**A**GAG
X-CG15745	M75	ts cod	CGGAA**C**GAG→CGGAA**T**GAG
X-CG32495	F39.67	ts cod	CACC**G**AGG→CACC**A**AGG
2L-CG2955-NC	M73	tv interg.	CAA**T**(T)$_5$AAAG→CAA**A**(T)$_5$AAAG
2L-215156	F33.17/ F33.70	tv interg.	CCGAAA**G**TC→CCGAAA**C**TC
2R-3097863	M137	tv intron	CGAC**T**CAA→CGAC**G**CAA
2R-CG14748	M11	tv cod	GCGG**A**CG→GCGG**T**CG
3L-CG32050	F33.17 /F33.70	tv cod	CACA**A**GAT→CACA**C**GAT
3R-419892	F39.11	tv interg.	GCA**C**AAC→GCA**G**AAC
3R-21787667	M140	tv interg.	GCAT**T**TTGT→GCAT**G**TTGT
X-hiw	M100	tv cod	CAACT**T**GA→CAACT**G**GA

Abbreviations: cod, coding; interg., intergenic; del., deletion; ins., insertion; ts, transition, tv, transversion. *30-bp deletion. †65-bp deletion. Three mutations were segregating at a frequency of 0.5 within their respective MA lines: 2L-CG8965-C, 3L-22018790-down and X-CG32495. Mutations are indicated in bold.

(CI) 2.1×10^{-9} to 1.31×10^{-8}). This is about two-thirds of a direct estimate in *C. elegans*[5]. Our estimate of u for all mutation events is 8.4×10^{-9} (95% CI 3.6×10^{-9} to 1.6×10^{-8}). However, there is significant heterogeneity in u between the three genotypes (likelihood ratio test, $2\log L = 12.5$; $P = 0.002$). In pairwise tests, the mutation rate in Florida-33 is significantly higher than that in Madrid ($2\log L = 12.4$; $P < 0.001$) and nearly significantly higher than in Florida-39 ($2\log L = 3.6$; $P = 0.059$). Transitions were about twice as frequent as transversions (17 *versus* 8, Table 2); this is higher than the roughly 1:1 ratio observed in noncoding polymorphisms in *Drosophila*[20]. Insertion–deletion events (indels) were a minority of the mutations (eight, excluding transposable elements (TEs)). Among these, deletions (six) were more frequent than insertions (two), which is consistent with the high deletion/insertion ratio observed in *Drosophila* pseudogenes[21]. However, our findings are significantly different from the results of sequencing of *C. elegans* MA lines[5], in which indels substantially outnumbered point mutations (Fisher's exact test: $P = 0.05$) and insertions predominated among the indels ($P = 0.02$). Three events involved simultaneous indel and point mutations (Table 1); similar complex events also segregate within some *Drosophila* populations (P. Haddrill, personal communication). We detected only one TE insertion (of the family *Cr1a*), giving an insertion rate per base pair per generation of 2.7×10^{-10} (95% CI 6.8×10^{-12} to 1.5×10^{-9}), corresponding to an insertion rate per diploid of 0.06 per generation (95% CI 0.002 to 0.35). This is not significantly different from estimates obtained by extrapolating movement rates of active TE families in the Madrid MA lines[17]. Mutation rates were similar in coding, intronic and intergenic DNA (Supplementary Table S4; likelihood ratio test of heterogeneity of mutation rates $2\log L = 2.1$, $P = 0.35$), so an effect of transcription-coupled repair is not evident in our data. Two lines had two mutation events (none had more than two), and this is not significantly different from expectation under a Poisson distribution (randomization test: $P > 0.5$).

The euchromatic *Drosophila* genome size, G, is about 118 Mb, so our estimate of the mean diploid genomic mutation rate from all types of mutations is $2uG = 1.99$. From a comparison of the *D. melanogaster* and *D. simulans* genomes, the fraction of point mutations in *Drosophila* that are selectively eliminated, C, is estimated to be 0.58 (ref. 10). From equation (1), assuming that point mutations and indels are equally deleterious on average, the mean genomic deleterious mutation rate is $U = 1.15$ (95% CI 0.49–2.19). However, indels are more likely to be strongly deleterious than point mutations (Supplementary Fig. S6), and including this information gives a slightly higher estimate for U of 1.20 (95% CI 0.51–2.28; Table 2).

We may have underestimated the genomic mutation rate for three reasons. First, hypermutable, repetitive regions are probably underrepresented because amplicons containing them can be difficult to analyse by DHPLC. Second, we may have missed mutations because of the limitations of DHPLC, although our detection rate for positive controls was 98%. Third, C in equation (1) is likely to be an underestimate[10]. If, however, recessive modifiers that increased the mutation rate had become fixed in the MA line progenitors by inbreeding, we might have overestimated U for natural populations. This is a generic problem with experimental estimates of mutation rates that use inbred lines.

Table 2 | Results of scanning the *Drosophila* genome for new mutations

Mutation type	Mutation events detected			
	Madrid	Florida-33	Florida-39	Total
Complex events	2	0.5	0	2.5
Insertions	1	1	0	2
Deletions	3	3	0	6
TEs	0	1	0	1
Transitions	3	9.5	3.5	16
Transversions	5	2	1	8
Total events	14	17	4.5	35.5
Mutation rate parameter	**Mutation rate estimates**			
	Madrid	Florida-33	Florida-39	Overall
u (C + I) $\times 10^9$	2.0 (0.7–4.4)	5.6 (1.9–12.5)	0	2.6 (0.6–9.2)
u (SNM) $\times 10^9$	2.7 (1.2–5.4)	11.7 (5.9–20.6)	6.8 (2.1–16.6)	5.8 (2.1–13.1)
u (total) $\times 10^9$	4.8 (2.6–8.0)	17.2 (10.0–27.6)	6.8 (2.1–16.6)	8.4 (3.6–16.0)
U	0.66 (0.36–1.11)	2.56 (1.49–4.10)	0.94 (0.28–2.28)	1.20 (0.51–2.28)

Totals of 11,207,503 bp, 5,272,760 bp and 3,522,322 bp of Madrid, Florida-33 and Florida-39 DNA were scanned, respectively. u (C + I) is the mutation rate per site for complex and indel events, including TEs. u (SNM) is the mutation rate for single nucleotide mutation events (transitions and transversions). Ranges in parentheses are 95% confidence intervals. The overall estimates of mutation rates are averages, weighted by the average number of lines of each genotype successfully amplified per amplicon. We calculated confidence intervals for the overall mutation rates by maximum likelihood, under the assumption that each genotype's mutation rate is sampled from a log-normal distribution, with Poisson error on mutation numbers within genotypes. We calculated profile likelihoods as a function of the mean of the mutation rate distribution and obtained approximate confidence intervals on the basis of drops of 2 log likelihood units from the maximum likelihoods.

Our findings have several implications. First, we found significant genetic variation in the mutation rate between genotypes. Genetic variation in the mutation rate has been reported in *D. melanogaster*[22], and in the rate by which fitness declines due to MA in rhabditid nematodes[23]. Second, our estimate of the nucleotide site mutation rate is about 5-fold (95% confidence limits 2-fold and 12-fold) higher than a phylogenetic estimate from synonymous site divergence[24], assuming that wild flies undergo ten generations per year. This could be partly due to inaccurate estimates of species divergence times or to differences in generation times between laboratory flies and wild flies. Combined with the recent inference of pervasive selection against new mutations in *Drosophila*[10,25], our estimate for u indicates that U probably exceeds one event per diploid genome per generation in *Drosophila* and is unlikely to be less than 0.5. This is comparable with an estimate in *C. elegans* based on direct sequencing (0.96 for coding sequences only[5]).

However, genomic deleterious mutation rates estimated from the divergence of fitness traits in MA lines strongly disagree between these species; these are about 0.01 in *C. elegans*[23] and up to about 1.0 in *Drosophila*[6–8,26]. The distribution of fitness effects of deleterious mutations in *Drosophila* is likely to be highly leptokurtic[27], so it is unexpected that some *Drosophila* MA experiments should yield similar phenotypic[6] and DNA-based (our study) estimates of U. The reasons for this discrepancy remain obscure[2,7,8,23,26]. Last, our results have implications for the evolutionary maintenance of sex and recombination. Non-zero rates of recombination can be maintained by both Hill–Robertson interference[11] and synergistic epistasis[28], with genomic deleterious mutation rates as low as 0.5 (our lower confidence limit). However, our estimate of $U = 1.2$ seems too low for deterministic selection against deleterious mutations to allow the maintenance of sexual reproduction with a twofold cost, although the mechanism might work if U were as high as our upper confidence limit[28]. Additional factors that slow the spread of asexual mutants, such as population structure[29], might help to maintain sex in species with suitable population structure, even with U as low as 0.5.

METHODS

Mutation accumulation lines. We analysed *D. melanogaster* MA lines of three genotypes (Florida-33, Florida-39 and Madrid). Progenitors of Florida-33 and Florida-39 were derived independently from a common base population by brother–sister mating for 40 generations, then MA lines were maintained by full-sib mating until generation 90 (ref. 15), and by a mixture of full-sib and half-sib mating until generation 187, on average (D. Houle, personal communication). The Madrid progenitor was established by chromosome extraction[16]. MA lines were maintained by full-sib or double first-cousin mating until generation 47, then by full-sib mating until generation 262 (refs 16, 17). DNA was extracted from pools of 25 individuals per line.

Mutation detection by DHPLC. We randomly selected 77 nucleotide positions from the euchromatic genome sequence of *D. melanogaster* (Release 4.3 for chromosome 4, otherwise Release 3.1). A coding and a non-coding amplicon, each of 650–750 bp, were chosen close to each position. At an additional 56 random positions we selected either a coding or a non-coding amplicon. Finally, we selected 67 non-coding amplicons flanking suspected mutations (see below). For each amplicon, 5 ng of template was amplified by PCR with AmpliTaq Gold (Applied Biosystems), and the length and quality of products were verified on 1% agarose gels. Significantly weaker products than the others were excluded, because detection of variants at a frequency of less than 10% in a pooled sample is unreliable. The sequences of PCR products of the same MA-line genotype were compared by DHPLC[9]. Products were mixed in groups of four (labelled 'vials'), the mixtures were denatured and reannealed, and the fragments were separated on a Transgenomic Wave 3500A DHPLC instrument with a DNASep column at two to five temperatures with elution gradients chosen according to the sequence of the amplicon. In the absence of a mutation, vials gave similar elution profiles. If a line carried a mutation, the difference in retention time between heteroduplexes and homoduplexes resulted in its vial showing a wider profile or a double peak (Supplementary Fig. S4).

Positive controls. We assessed the detection rate of mutations by using positive controls for 46 amplicons, generated with the use of the relatively high misincorporation rate of traditional *Taq* polymerase. We amplified MA-line genomic DNA with a non-proofreading polymerase, and cloned and sequenced PCR products. Positive and negative controls were selected among the clones with one and zero mutations, respectively, compared with the wild-type sequence (Supplementary Table S3). Controls were then amplified by PCR along with the MA lines. Mixtures were produced between the positive control product and products from three MA lines of the same genotype, the negative and positive control products, and products of the negative control and the three MA lines of the same genotype. These were analysed by DHPLC along with the MA-line vials of that amplicon. Most positive controls are transitions (Supplementary Table S3), which are more difficult to detect by DHPLC than indels or transversions[30], making our positive control panel conservative.

Characterization of mutations. Whenever DHPLC elution profiles showed differences, the four lines of that vial were reamplified by PCR and directly sequenced in both directions. Some mutations were found to be segregating within a line; the strategy we used to investigate these is described in Supplementary Table S4.

Polymorphic sites not representing new mutations. Polymorphisms present at the start of the MA phase are expected to become fixed in different lines, and polymorphism is likely to affect a chromosomal region. We detected several regions having such characteristics in Florida-39, but not in Florida-33 or Madrid (Supplementary Fig. S3). Lines showing polymorphism in Florida-39 were excluded from the data on affected amplicons. Furthermore, to distinguish between genuine mutations and polymorphism blocks, noncoding amplicons closely linked to either side of putative mutations were analysed. This procedure makes it improbable that a polymorphism would be misclassified as a mutation

(Supplementary Fig. S3). In four cases, pairs of lines shared identical mutations (Table 1). In particular, the Madrid lines involved were consecutively numbered, and one of the Florida-33 events concerned lines sharing two mutations. These events presumably reflect breeding contamination between two MA lines[17]. We counted shared mutations only once, and reduced the total number of lines by 0.5 for each contaminant.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.M., C.H.-L. and M.D. performed the DHPLC analysis. M.D. cloned and sequenced putative variants. X.M. cloned and sequenced positive controls. D.L.H. analysed selective constraints on indel mutations. B.C. advised on *Drosophila* genetics and interpreting the data. P.D.K. conceived and designed the project. C.H.-L. and P.D.K. analysed the data and wrote the paper. All authors revised the draft manuscript.

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CORRIGENDUM

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In this Letter, David Houle was omitted from the author list. David Houle was responsible for producing the Florida mutation accumulation lines that were analysed in the experiment.