Genetica (2009) 136:359–369
DOI 10.1007/s10709-008-9304-4

Analysis and implications of mutational variation

Peter D. Keightley · Daniel L. Halligan

Received: 16 July 2008 / Accepted: 16 July 2008 / Published online: 29 July 2008

Abstract Variation from new mutations is important for several questions in quantitative genetics. Key parameters are the genomic mutation rate and the distribution of effects of mutations (DEM), which determine the amount of new quantitative variation that arises per generation from mutation (V_M). Here, we review methods and empirical results concerning mutation accumulation (MA) experiments that have shed light on properties of mutations affecting quantitative traits. Surprisingly, most data on fitness traits from laboratory assays of MA lines indicate that the DEM is platykurtic in form (i.e., substantially less leptokurtic than an exponential distribution), and imply that most variation is produced by mutations of moderate to large effect. This finding contrasts with results from MA or mutagenesis experiments in which mutational changes to the DNA can be assayed directly, which imply that the vast majority of mutations have very small phenotypic effects, and that the distribution has a leptokurtic form. We compare these findings with recent approaches that attempt to infer the DEM for fitness based on comparing the frequency spectra of segregating nucleotide polymorphisms at putatively neutral and selected sites in population samples. When applied to data for humans and *Drosophila*, these analyses also indicate that the DEM is strongly leptokurtic. However, by combining the resultant estimates of parameters of the DEM with estimates of the mutation rate per nucleotide, the predicted V_M for fitness is only a tiny fraction of V_M observed in MA experiments. This discrepancy can be explained if we postulate that a few deleterious mutations of large effect contribute most of the mutational variation observed in MA experiments and that such mutations segregate at very low frequencies in natural populations, and effectively are never seen in population samples.

Keywords Mutation · Quantitative traits · Fitness · Distribution of effects

P. D. Keightley (✉) · D. L. Halligan
Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, UK
e-mail: pk.genetica2008@gmail.com

Introduction

Variation from new mutations, the ultimate source of all genetic variation, impacts on several areas of quantitative genetics. For example, it is important for understanding the levels of quantitative genetic variation that are observed in natural and artificial populations (Bürger 2000; Zhang and Hill 2005), the long term response to selection (Keightley 2004a), and the causes of inbreeding depression and heterosis (Charlesworth and Charlesworth 1999). In addition, the rate and distribution of effects of new mutations (DEM) for fitness impacts on several important theoretical questions in evolutionary and population genetics, including the evolution of recombination and sex (Otto and Lenormand 2002).

Early experiments by Mukai and co-workers (Mukai 1964; Mukai et al. 1972; Ohnishi 1977) suggested that recurrent spontaneous mutations can cause rapid losses of fitness if their fixation is unopposed by natural selection. This result encouraged a great deal of work on the impact of new mutations on the fitness of small populations or populations in which selection has been relaxed (Lande 1994; Lynch et al. 1995; Gilligan et al. 1997). In the 20 years since the 2nd International Conference on Quantitative Genetics (ICQG) in Raleigh in 1987, a great deal of

empirical and theoretical research on the nature and implications of new mutational variation for quantitative traits in general has been carried out, and particularly the nature of the DEM for fitness (Eyre-Walker and Keightley 2007). In this paper, we will attempt to review the major analytical and experimental advances that have been made since the 2nd ICQG concerning the elucidation of the nature of new mutational variation for quantitative traits. We shall describe statistical methods that have been developed to jointly infer the mutation rate and the DEM for quantitative traits, and describe the results of the application of these methods to experimental data. We shall also describe how high-throughput molecular methods, which make it possible to scan the whole genome or large samples of the genome for new mutations, are beginning to alter our perception of the nature of mutational variation. Finally, we shall describe how data on the frequencies of nucleotide polymorphisms in natural populations can be used to shed light on the DEM for fitness.

New mutational variation for quantitative traits

Two parameters that quantify the influence of new mutational variation on a quantitative trait are D_M and V_M, the per generation changes in the mean and variance of a trait from new mutations. In order to allow comparisons between different traits or different species, D_M is usually expressed scaled by the population mean (μ) as D_M/μ, and V_M by the environmental variance (V_E) or μ to yield the mutational heritability, $h_M{}^2 = V_M/V_E$, or the mutational coefficient of variation, $CV_M = (V_M)^{1/2}/\mu$. One way of estimating mutational variation (or heritability) is to artificially select in an initially inbred line (Clayton and Robertson 1955). Assuming that the line is initially homozygous at all loci, under an infinitesimal model of many additive mutations of very small effect, the selection response is a function of V_M and other parameters that can be inferred from the experiment (Hill 1982a, b). It is also possible to estimate V_M under a model of mutations that have large additive effects, brought rapidly to fixation by selection (Hill 1982b; López and López-Fanjul 1993). A drawback with inferring mutational heritability from a selection experiment is that it is limited to the trait under selection, and potentially quite sensitive to the details of the underlying genetics, such as the per locus strength of selection (Hill and Rasbash 1986). A more general approach, that can be used to obtain information on multiple traits, is the mutation accumulation (MA) experimental design, in which spontaneous mutations are allowed to accumulate in conditions of minimal selection in initially homozygous lines of very small effective size (e.g., chromosomes protected from selection by balancer chromosomes, selfed lines, full-sib mated lines). Most work has been on the homozygous effects of mutations on quantitative traits, which are assayed after several tens of generations of MA.

Two extensive reviews of data from selection and MA experiments suggest that spontaneous h_M^2 usually falls in the range 10^{-4}–10^{-2} (Lynch 1988; Houle et al. 1996), although mutagenesis can increase this by one or two orders of magnitude (Ohnishi 1977; Mackay 1988). There is an indication that long-generation species tend to have higher values for h_M^2, perhaps reflecting higher per locus, per generation spontaneous mutation rates (Lynch et al. 1999). Although the contribution to variation from new mutations is therefore generally small to modest relative to the existing genetic and environmental variation for the trait, theoretical results suggest that a balance between mutation and selection may lead to the maintenance of substantial genetic variation. However, predictions depend heavily on the details of the strength and mode of selection on individual mutations (Bulmer 1989; Falconer and Mackay 1996; Bürger 2000). The contribution of new mutations to the long-term response to selection can also be substantial, particularly if there are mutations of large dominant or semi-dominant effect that become quickly selected to high frequencies (Hill 1982a, b). The impact of new mutations on long-term selection response has recently been reviewed (Keightley 2004a), and there have also been reviews of the magnitude of D_M/μ (García-Dorado et al. 1999; Lynch et al. 1999).

Analysis of MA experiments to infer the DEM and the genomic mutation rate

In most MA experiments that have been carried out, significant variance between MA lines (V_b) is detected for quantitative or life history traits. In a diploid organism, this can be equated to the mutational variance by $V_b = 2tV_M$, where t is the number of generations of MA (Lynch and Hill 1986). Significant decreases in mean values for traits such as viability and fertility (and other traits related to fitness) are also generally observed (Bataillon 2000). For traits related to fitness, advantageous mutations seem to be uncommon, although cases have been reported where a substantial proportion of MA lines have evolved increased values for life history traits (Shaw et al. 2000, 2002; Joseph and Hall 2004) and one experiment has demonstrated rapid fitness recovery of mutationally degraded lines of *C. elegans* when maintained in large population sizes under competitive conditions (Estes and Lynch 2003). It is possible, however, that the net effects of most of these mutations on fitness in natural conditions are negative, although this point has been debated (Keightley and Lynch

2003; Shaw et al. 2003; Bataillon 2003; Shaw and Chang 2006).

The simplest way to go beyond changes of means and variances to obtain information about the genetic basis of new mutational variation is to employ the approach suggested by Bateman (1959) and Mukai (1964) in which the genomic mutation rate (U) and the mean mutational effect $E(a)$ are estimated from simple functions of D_M and V_M (see Lynch and Walsh 1998 for details). However, the Bateman-Mukai method makes the unrealistic assumption that mutations have equivalent, unidirectional effects. This is expected to lead to downwardly and upwardly biased estimates of U and $E(a)$, respectively, if there is variation in the magnitude of mutational effects or if there are both positive and negative mutational effects, as is expected for many quantitative traits. It has long been argued that the distribution of allelic effects is likely to be leptokurtic (Robertson 1967; Hill 1982b; Kimura 1983; Crow and Simmons 1983), and this has encouraged several attempts to estimate parameters of a fitted distribution of mutational effects, $f(a)$, along with the genomic mutation rate, from MA data.

Four approaches have been developed that use MA data to make joint inferences about $f(a)$ and the genomic mutation rate. One simple method has been suggested that relies on an extension of the Bateman-Mukai approach (Elena and Moya 1999), and incorporates the variance of mutation effects, $Var(a)$, as well as U and $E(a)$ into the functions for D_M and V_M. Different distributions of mutation effects (e.g. uniform or gamma) can then be compared by obtaining expressions for $Var(a)$ under each model and estimating the parameters of these expressions by fitting the model to the data using least-squares nonlinear regression. Thus, different distributions of mutational effects can be compared, and the variation in mutation effects can be quantified.

Keightley (1994) developed a maximum likelihood (ML) method that analyses data from an MA experiment consisting of phenotypic measurements from a set of mutation-free control lines and a set of MA lines from generation t. The control line data are assumed to be normally distributed with mean M and variance V_E, and the MA lines values are assumed to be the sum of an environmental deviate drawn from the same distribution as the control lines plus a mutational deviate. The normality assumption was relaxed subsequently by Halligan et al. (2003). The mutational deviate is the sum of n mutational effects, where n is Poisson distributed with parameter Ut. Each mutational effect is assumed to be an independent draw from $f(a)$. In most cases $f(a)$ has been modelled as a gamma distribution or a gamma distribution reflected about zero with a proportion P of mutations with effects greater than zero. By allowing a reflected distribution, positive and negative mutational effects are allowed, which overcomes one of the key weaknesses of the Bateman-Mukai approach. The gamma distribution has two parameters, α specifying its scale, and β its shape. Varying β allows the distribution to take a wide variety of shapes, ranging from a spike at the distribution's mean (β/α) if $\beta \to \infty$, to an increasingly leptokurtic form with most of the density located close to zero if $\beta \to 0$. The parameters of the model (M, V_E, U, α, β, and P) are estimated by maximizing the likelihood of the control and MA line data, fitting the distribution by numerical integration, and summing over the distribution of mutation numbers per line. A weakness of the approach is that it does not allow more than one generation of MA line data to be analysed simultaneously (i.e., generations in addition to the controls plus generation t), whereas MA experimental data often consist of measurements of the same MA lines at several time points. This limitation was partially overcome by Keightley and Bataillon (2000), but only for the case of equal mutational effects, which makes the modified procedure only marginally more useful than the Bateman-Mukai approach.

García-Dorado (1997) and García-Dorado and Marin (1998) developed a "minimum distance" (MD) method to estimate U and $f(a)$ that makes similar assumptions to the ML method. The statistical procedure used to find the best-fitting value of U and distribution parameters was somewhat different, however. Parameter values were found that minimized a statistic, the Cramer-Von Mises distance, between the observed distributions of control and MA line phenotypic data and their expected distributions, given the model parameters. In principle this allows any family of distribution of effects to be fitted, although most work has been carried out with reflected gamma distributions. Variances of the parameter estimates have been obtained by bootstrapping over MA lines. In common with the standard ML method, covariances between MA line genotypic values are not incorporated in the analysis, so the method is limited to using a control line and a single generation of MA line data. However, as above, assuming reflected distributions overcomes the weaknesses of the Bateman-Mukai approach of equivalent, unidirectional mutational effects. The relative merits of the MD and ML approaches have been investigated (García-Dorado and Gallego 2003; Keightley 2004b).

The fourth, and most sophisticated, method for analysing MA line data was developed by Shaw et al. (2002). The main assumptions and parameters are the same as for the ML and MD approaches, but the procedure incorporates genetic covariances caused by different MA lines accumulating different numbers of mutations with different effects between the same MA lines assayed at different generations. This method, which estimates parameters by Markov Chain Monte Carlo ML, is therefore expected to

extract a greater amount of information from the data than the previous two methods. In principle, any DEM can be modelled, but Shaw et al. (2002) have focussed on "shifted" gamma distributions, in which a constant ρ is added to each gamma deviate and the mean of the distribution is therefore $\beta/\alpha + \rho$, although there has been debate about whether this or the reflected gamma distribution is more biologically plausible (Keightley and Lynch 2003; Shaw et al. 2003).

Whatever the relative efficiencies of the four approaches, it is clear that two fundamental problems severely limit their usefulness. First, the true DEM is likely to differ substantially from that assumed in the analysis. In particular, the true DEM may be more complex than a reflected gamma distribution, and may have multiple modes. Second, even if the true DEM is similar to that assumed in the analysis (e.g., a gamma distribution), the amount of data required to obtain accurate estimates is large, and parameter estimates therefore tend to have large confidence intervals (Keightley 1998; Shaw et al. 2002; García-Dorado and Gallego 2003). Related to this point, the parameters of the model are highly confounded with one another, such that U and β are negatively correlated (Keightley 1998). If the true DEM is leptokurtic, simulations show that the best-fitting model is often a distribution with $\beta \to 0$ and $U \to \infty$ (Keightley 1998; García-Dorado and Gallego 2003).

In spite of their limitations to estimate accurately the DEM, these methods have been applied to numerous MA experiments. Estimates of the shape parameter (β) and mean mutational effect $E(a)$ from published experiments are summarised in Table 1. Estimates of β below one imply that the distribution of effects is more leptokurtic than an exponential distribution, whereas estimates above one imply that there is evidence for a mode of mutational effects away from zero. Although it is clear from those estimates that are reported with confidence limits that the estimates of β are noisy, 20 of the 29 estimates are larger than one (seven significantly so), whereas only nine are less than one (two significantly so). Thus, data from MA experiments tends to support the conclusion that spontaneous mutation effects have a distribution with a mode away from zero, with the caveat that the gamma distribution assumed in the analyses may not adequately model the true DEM.

Incorporating information on nucleotide mutation rates

Because the genomic mutation rate and $f(a)$ are difficult to jointly estimate in the same analysis, independent information on the number of mutations accumulated could be useful in inferring the DEM. Two MA and mutagenesis experiments using the nematode worm *C. elegans* have utilised such information. Both experiments, which are described below, report large reductions in mean and large increases in variance for traits related to fitness as a consequence of spontaneous MA or mutagenesis.

Davies et al. (1999) measured the effect of ethylmethane sulphonate (EMS) mutagenesis on life history traits in *C. elegans*. By assuming a gamma distribution of effects in an ML analysis of EMS-treated lines and control lines, a minimum estimate for the mean mutational effect on fitness was 15%, and a minimum estimate for β was 1.6. This distribution is therefore quite platykurtic (i.e., it is more platykurtic than an exponential distribution for which $\beta = 1$), and mutations with small fitness effects (e.g., $< \sim 1\%$) contribute less than half of the density of this distribution. The ML estimate for U, which in this case is the mean number of mutations affecting fitness per haploid MA line genome, was less than 1.4. However, the mutagen dose was calibrated so that an estimated average of ~ 200 mutation events per haploid genome were generated (primarily G/C $\to$ A/T transitions). Since at least 50% of the *C. elegans* genome appears to be subject to selective constraints (Webb et al. 2002), at least 100 of these mutations would be expected to deleterious in natural conditions. This discrepancy can be explained if we postulate that the DEM contains a large class of deleterious mutations that are not detected in the laboratory environment. The ML analysis picks up mutations of large effect (averaging about 15% in this case) that generate most of the mutational variance and decline in mean fitness observed in the laboratory.

Similar conclusions concerning the distribution of spontaneous mutational effects can be drawn from the experiment of Denver et al. (2004), who analysed the longest running spontaneous MA experiment in *C. elegans* (~ 350 generations; Vassilieva et al. 2000). An ML analysis of the fitnesses of MA and control lines gave an estimate for the mean mutational effect on fitness of $\sim 20\%$, and suggested that the DEM for fitness is fairly platykurtic (Vassilieva et al. 2000; Table 1). U was estimated to be only about 0.005 per haploid genome per generation, consistent with other spontaneous MA experiments in *C. elegans* (Keightley and Caballero 1997; Baer et al. 2005). However, by direct DNA sequencing of samples of the genome from the MA lines, Denver et al. (2004) estimated that about two mutation events per haploid genome per generation had occurred. Assuming again that one-half of all mutations in the *C. elegans* genome are deleterious in natural conditions, this implies that the "true" deleterious mutation rate could be more than two orders of magnitude higher than the estimate based on the fitnesses of the MA lines, which is similar to what was inferred more indirectly by Davies et al. (1999). The

Table 1 Estimates of parameters of the DEM obtained from analysis of MA experiments

Species	Reference	Method	Model	Trait	β [95% CI]	$E(a)$ [95% CI]
VSV	1	BM	Standard	Fitness[a]	19.4 [5.02/33.8][b]	–
		BM	Standard	Fitness[a]	31.2 [12.7/49.8][b]	–
		BM	Standard	Fitness[a]	3.97 [1.82/6.11][b]	–
ϕ6 Virus	2	ML[c]	Standard	Fitness	$\rightarrow\infty$[d]	−0.021
S. cerevisiae	3	ML[c]	Reflected	Fitness	2[0/$\rightarrow\infty$]	−0.061 [−0.077/0.00]
A. gloriosa	4	ML[c]	Standard	No. flowers	2[<0.5/$\rightarrow\infty$]	−0.050 [−0.18/−0.019]
A. douglasiana	4	ML[c]	Standard	No. flowers	$\rightarrow\infty$ [>1/$\rightarrow\infty$]	−0.18 [−0.25/−0.14]
		ML[c]	Standard	Dry weight	$\rightarrow\infty$[<1/$\rightarrow\infty$]	−0.030 [−0.050/−0.017]
A. thaliana	5	MCML	Displaced	Fruits × 0.1	3.53 [0.00/7.75]	−0.010 [−0.087/0.059][e]
		MCML	Displaced	Seeds per fruit	1.91 [1.09/2.71]	0.00061 [−0.0046/0.0049][f]
C. elegans	6	ML[c]	Standard	Productivity	$\rightarrow\infty$ [>1/$\rightarrow\infty$]	−0.21 [−0.29/−0.06]
	7	ML[g]	Standard	*r*	1.2 [1.0/2.0][d]	−0.058 [−0.12/−0.0007]
	8	ML[g]	Standard	Body Size[h]	1.2 [<0.063/$\rightarrow\infty$]	−0.12 [−0.24/0.00][b]
		ML[g]	Standard	Body Size[i]	5 [0.063/$\rightarrow\infty$]	−0.25 [−0.32/−0.18][b]
	9	ML[g]	Standard	Directness	0[d]	−0.08 [−0.14/−0.04]
		ML[g]	Standard	Velocity	0.17 [0.16/0.59][d]	−0.07 [−0.31/−0.05]
		ML[g]	Standard	Turn Rate	0.73 [0.69/2.69][d]	0.25 [0.18/0.63]
D. melanogaster	10	MD	Reflected	Wing length	1.25[j]	–
		MD	Reflected	Abdom bristles	0.63[j]	–
		MD	Reflected	Stern bristles	0.13[j]	–
	11	MD	Reflected	Viability[k]	3.12[j]	−0.19
		MD	Reflected	Viability[l]	0.90[j]	−0.23
		MD	Reflected	Viability[m]	3.35 [2.02/11.9][b,j]	−0.10 [0.041/0.17][b]
		MD	Reflected	Fitness[n]	1.59[j]	−0.27
	12	ML[c]	Standard	Viability[i]	$\rightarrow$0 [$\rightarrow$0/1.69][j]	$\rightarrow$0 [−0.034/$\rightarrow$0]
		ML[c]	Standard	Viability[j]	$\rightarrow$0 [$\rightarrow$0/0.25][j]	$\rightarrow$0 [−0.020/$\rightarrow$0]
	13	MD	Reflected	Viability	2.2	−0.034
		ML[c]	Reflected	Viability	$\rightarrow\infty$	−0.092
	14	ML[c]	Standard	Viability	$\rightarrow$0 [$\rightarrow$0/<∞]	−0.024

The mean effect of a new mutation $E(a)$ is expressed relative to the population mean

[a] Data from three different monoclonal antibody-resistant mutant (MARM) clones, MARM X, MARM C, MARM R

[b] Approximate 95% confidence interval calculated from 2 × standard error

[c] As implemented in Keightley and Ohnishi (1998)

[d] β calculated from reported estimate of coefficient of variation of mutational effects

[e] Scaled relative to the estimated mean number of fruits per plant ×0.1 at generation zero reported in Shaw et al. (2000)

[f] Scaled relative to the estimated mean seeds per fruit at generation zero reported in Shaw et al. (2000)

[g] As implemented in Vassilieva et al. (2000)

[h] Using lines produced by Keightley and Caballero 1997

[i] Using lines produced by Vassilieva and Lynch 1999

[j] β calculated from reported estimate of kurtosis

[k] Data from Mukai et al. 1972

[l] Data from Ohnishi (1977)

[m] Data from Fernández and López-Fanjul (1996)

[n] Data from Houle et al. (1992)

References: (1) Elena and Moya (1999), (2) Burch et al. (2007), (3) Joseph and Hall (2004), (4) Schoen (2005), (5) Shaw et al. (2002), (6) Keightley and Caballero (1997), (7) Vassilieva et al. (2000), (8) Azevedo et al. (2002), (9) Ajie et al. (2005), (10) García-Dorado and Marin (1998), (11) García-Dorado et al. (1999), (12) Keightley (1994), (13) Ávila et al. (2006), (14) Fry et al. (1999)

discrepancy between the phenotypic and molecular-based estimates of the genomic deleterious mutation rate also suggests that the DEM was inadequately modelled in the ML analysis of the phenotypic data. This conclusion is consistent with the finding that in spontaneous MA experiments with *C. elegans* populations of moderate size (i.e., >10), the decline in fitness seen in smaller populations is largely arrested, presumably because selection against deleterious mutations of large to moderate effect is effective in populations of even moderate size (Estes et al. 2004). A comparable under-estimation of the "true" mutation rate inferred on the basis of phenotypic assays has also been observed in mismatch-repair deficient *C. elegans* (Estes et al. 2004; Denver et al. 2005). In the RNA virus ϕ 6, however, a far higher proportion of mutations have fitness effects that are detectable in laboratory assays (Burch et al. 2007).

Nucleotide polymorphism frequencies and the DEM for fitness

The analysis of MA line data has therefore provided only limited information concerning the DEM for small effect mutations, so alternative approaches to tackle this problem have been sought. Recently, large data sets of DNA polymorphism data have started to become available in a number of species, and methods have been developed to use these to make inferences about the DEM for new spontaneous mutations. The methods are limited to inferring the DEM for new single nucleotide mutations for fitness, $f(s)$, based on comparing the diversities or frequency spectra of alleles at sites under selection with sites thought to be evolving neutrally. They therefore focus on the part of the distribution corresponding to deleterious mutations of small effect that stand a chance of being observed segregating in a population sample. They make use of the fact that selection against deleterious mutations increases the proportion of low frequency polymorphisms while decreasing the proportion of intermediate frequency polymorphisms, relative to neutral sites, and the stronger the selection the stronger this effect. They make the assumptions that adaptive mutations are essentially absent from polymorphism data, which is reasonable if selection on them is relatively strong (Smith and Eyre-Walker 2002), and that variation in the DNA is not maintained by balancing selection. Recent evidence in humans suggests that this is uncommon (Bubb et al. 2006). The most recent developments have aimed to infer $f(s)$ for amino acid-changing mutations, assuming independent sites, in the nuclear genome. Other methods have been applied to mitochondrial DNA diversity (Nielsen and Yang 2003; Piganeau and Eyre-Walker 2003; Sawyer et al. 2003). In all these analyses, without information about the mutation rate, estimates of the strength of selection affecting amino acid sites are obtained as the product of the effective population size and selection coefficient (Ns).

Loewe et al. (2006) developed an approach to estimate $f(s)$ that contrasts estimates of nucleotide diversity at putatively neutral and selected sites from two species that have different effective population sizes, and applied their method to polymorphism data from *D. miranda* and *D. pseudoobscura*. Loewe et al. (2006) method uses summary statistics derived from the polymorphism data sets, such as nucleotide diversity, π. Other methods have attempted to more fully use the information available in polymorphism data by analysing the frequency distributions of alleles at selected and neutral sites in a sample; these frequency distributions are usually referred to as site frequency spectra (SFS). In interpreting the SFS, a crucial problem is that changes in population demography can change the SFS in ways that resemble selection. For example, a recent population expansion leads to a SFS containing an excess of rare alleles compared to the neutral expectation, which is also the pattern seen under purifying selection. Methods have therefore attempted to co-estimate parameters of an explicit demographic model along with the DEM, or have applied corrections to account for demographic changes. This is possible because population demography affects both neutral and selected sites.

The first approach that fully utilises the SFS was developed by Eyre-Walker et al. (2006), and applied to a large human polymorphism data set obtained by resequencing candidate loci in samples of individuals from the American population as part of the "Environmental Genome Project" (EGP; Livingston et al. 2004). Under the assumption that $f(s)$ is a gamma distribution, Eyre-Walker et al. (2006) used ML to estimate the distribution parameters that best explain the SFS for nonsynonymous sites (assumed to be under purifying selection), using intronic sites as a neutrally evolving standard. They used population genetics theory to infer the expected number of alleles at a particular frequency in a sample of alleles, given a strength of selection Ns, while applying a correction for demography by estimating additional parameters to account for departures of the intronic SFS from its neutral expectation. Subsequently, methods have been developed by Keightley and Eyre-Walker (2007) and Boyko et al. (2008), which are similar to Eyre-Walker et al. (2006) in that their inferences are based on the SFS. However, the modelling of demographic changes is more explicit, since they apply population genetics formulae (Boyko et al. 2008) or transition matrix methods (Keightley and Eyre-Walker 2007) to infer the expected allele frequency distribution after population expansion or contraction, to calculate the likelihood of the SFS. Keightley and Eyre-Walker (2007)

applied their method to the EGP data set, another large human polymorphism data set (PGA) also obtained by resequencing, and a large data set of *D. melanogaster* polymorphism data (Shapiro et al. 2007).

The results of these analyses are presented in Table 2. In general, there is a reasonable degree of consistency across the different methods and data sets within species, and a number of general conclusions have emerged. First, the DEM for new amino acid mutations is leptokurtic in both humans and *Drosophila*. Second, there is a tendency for the shape of the distribution of effects to be more leptokurtic in humans than *Drosophila*, the distribution for humans having a substantially more leptokurtic form. The biological mechanism(s) underpinning this apparent difference are unknown. In general, the confidence limits on estimates of the shape parameter are reasonably narrow, whereas those for $E(s)$ are quite wide (see the original papers for details). The wide confidence limits on $E(s)$ may reflect the inability to estimate the effects of strongly deleterious mutations, which are expected almost never to appear in the modest samples of alleles that have been analysed, but these can have a large influence on the mean of the distribution. The differences in the DEM parameters between humans and *Drosophila* leads to striking differences in the proportion of mutations with effects in different Ns ranges (Table 3), such that effectively neutral mutations (those with $Ns < 1$) are much more common in humans than *Drosophila*, whereas strongly deleterious mutations (e.g., $Ns > 10$) are much more frequent in *Drosophila*. Third, there is an indication that selection is more effective against deleterious mutations in African than European human populations, presumably reflecting differences in recent effective population size.

Conclusions

At the time of the last International Conference on Quantitative Genetics, there was essentially no empirical information about the distribution of effects of new mutations. This situation has changed during the last 20 years, and there has been a good deal of progress in understanding the nature of the DEM, particularly for fitness traits. This has come from many new long term MA experiments, from new statistical techniques developed to analyse these experiments, from a rapidly expanding database of high quality nucleotide polymorphism data, particularly in humans and *Drosophila*, and from the development of methodology to make inferences based on these data.

MA and polymorphism data allow inferences to be made about the DEM at opposite ends of the spectrum. MA lines can only provide substantial information about the DEM for mutations that have relatively large effects, i.e., those whose effects can be measured on the basis of a change of phenotype. At the opposite end of the distribution, the analysis of polymorphism data can provide information only about the fitness effects of those mutations that have an appreciable chance of segregating in a population sample. Since effective population sizes in nature are typically in the tens of thousands and greater, these mutations are therefore expected to have selection coefficients less than a fraction of a percent. An interesting feature that emerges from the analysis of spontaneous MA experiments is that estimates of the shape parameter β are often quite high, so the inferred DEM is platykurtic (Table 1). We argue that this implies that the DEM may be bimodal, and that analysis methods for MA experiments are highly sensitive to the presence of large-effect

Table 2 Estimates of shape parameter and mean selective effect of new amino acid-changing mutations based on analyses that make use of allele frequencies in polymorphism surveys

Species	Population, ethnicity	Parameter estimates		Reference
		β	NE(s)	
H. sapiens	USA, mixed	0.23	850	1
	African (a)	0.10	5,300	2
	African (b)	0.15	2,500	
	European (a)	0.19	51	
	European (b)	0.29	61	
D. miranda/pseudoobscura	N. American	0.30	1,200	3
D. melanogaster	African	0.38	1,800	2
	Non-African	0.27	14,000	

(1) Eyre-Walker et al. (2006)

(2) Keightley and Eyre-Walker (2007)

(3) Loewe et al. (2006)

(a) PGA data set

(b) EGP data set (Livingston et al. 2004)

Table 3 Proportions of amino acid mutations with effects in different *Ns* ranges

Species	Population	Reference	Ns range			
			0–1	1–10	10–100	>100
H. sapiens	USA, mixed	1	0.17	0.12	0.19	0.52
	Africa (a)	2	0.34	0.09	0.12	0.45
	Europe (a)		0.37	0.20	0.27	0.15
	Africa (b)		0.24	0.10	0.15	0.51
	Europe (b)		0.23	0.22	0.36	0.19
D. melanogaster	N. American	3	0.09	0.09	0.18	0.63
	Africa	2	0.05	0.06	0.15	0.74
	Non-African		0.06	0.05	0.09	0.79

(1) Eyre-Walker et al. (2006)

(2) Keightley and Eyre-Walker (2007)

(3) Loewe et al. (2006)

(a) PGA data set

(b) EGP data set (Livingston et al. 2004)

mutations. On the other hand, the polymorphism data imply that the DEM for fitness is strongly leptokurtic, with a peak at, or close to, zero (Table 2). In these cases, strongly deleterious mutations that contribute to the phenotypic changes that are observed in MA experiments segregate at very low frequencies, and scarcely contribute to the outcome of the analysis.

The fact that the two approaches make inferences pertaining to opposite ends of the DEM is illuminated by considering the magnitude of V_M, the new genetic variance produced by one generation of spontaneous mutation. In *D. melanogaster*, an empirical estimate for V_M for fitness from an MA experiment is 10^{-3} (Houle et al. 1992). There are also estimates for other life history traits, such as viability, which are about ten times lower (García-Dorado et al. 1999). What V_M is predicted by the DEM parameter estimates derived from the analysis of nucleotide polymorphism? Under the assumption of unlinked mutations with additive effects, this can be obtained from

$$V_M = \sum uE(s^2)/2 \qquad (1)$$

(Hill 1982a), where u is the mutation rate per site and the summation is over sites in the genome. Focussing solely on amino-changing mutations in *D. melanogaster*, the number of nonsynonymous sites in the genome is about 1.3×10^7, obtained from the product of the number genes in the genome (about 13,000), the average length of a gene in bases (about 1,500), and the fraction of coding nucleotides that are nonsynonymous (about two-thirds). A recent direct estimate of u in *D. melanogaster* is 8.4×10^{-9} (Haag-Liautard et al. 2007), so our estimate for $\Sigma u = 0.109$. We can infer $E(s^2)$ from the gamma distribution shape (β) and scale (α) parameters estimated from the polymorphism data, i.e., $E(s^2) = \beta(\beta + 1)/\alpha^2$. For the African *Drosophila* population, we have an estimate for $\beta = 0.46$ (Table 2), but the scale of the distribution is only known as a function of the product $N_e s$. We therefore need to divide our estimate of $N_e E(s)$ by an estimate of N_e in *D. melanogaster* (1.5×10^6; Eyre-Walker et al. 2002), yielding $E(s)$ of a nonsynonymous mutation in the African population of 0.00047. Equating this to the mean of the gamma distribution (β/α), we obtain $\alpha = 986$, and $E(s^2) = 6.91 \times 10^{-7}$. From Eq. 1 V_M is then 3.8×10^{-8}, which is more than four orders of magnitude smaller than the value estimated by Houle et al. (1992) from their MA experiment (10^{-3}). Assuming the parameter estimates for non-African flies (Table 2), the discrepancy is a factor of about 900.

There are at least four possible explanations for the discrepancy between V_M for fitness estimated in MA experiments and values inferred from DNA polymorphisms. The first possibility is that N_e in *D. melanogaster* has been grossly over-estimated, but an over-estimation of the required magnitude seems unlikely. Second, point mutations in noncoding DNA could make a major contribution to V_M. However, noncoding polymorphisms segregate at higher frequencies than nonsynonymous polymorphisms (Andolfatto 2005), implying lower selection coefficients at such sites and substantially lower variance contributed per mutation. Further research is needed to investigate the relative contribution of noncoding mutations to mutational variation, since there are many more noncoding than coding sites under selection in *Drosophila* (Halligan and Keightley 2006). Third, a substantial fraction of the mutational variance for fitness detected in *Drosophila* MA experiments may be generated by transposable element insertions, whose average effects seem to be quite high (Lyman et al. 1996; Houle and Nuzhdin 2004). Consistent with this, transposable elements

segregate at very low frequencies within populations (Charlesworth and Langley 1989). The final explanation is that the DEM is inadequately described by a gamma distribution, and may be multimodal: a few large-effect mutations effectively do not contribute to DNA polymorphisms in natural populations, but are the major contributors to V_M in MA experiments. This is consistent with evidence from MA experiments in which the number of mutations in the DNA has been estimated. Many more experiments to estimate the mutation rate at the DNA level involving high throughput genome sequencing technologies are likely to follow in the future, and may shed light on these issues.

Acknowledgements We thank Penny Haddrill, Bill Hill and Mark Kirkpatrick for helpful comments.

References

Ajie BC, Estes S, Lynch M, Phillips PC (2005) Behavioral degradation under mutation accumulation in *Caenorhabditis elegans*. Genetics 170:655–660. doi:10.1534/genetics.104.040014

Andolfatto P (2005) Adaptive evolution of non-coding DNA in *Drosophila*. Nature 437:1149–1152. doi:10.1038/nature04107

Ávila V, Chavarrías D, Sánchez E, Manrique A, López-Fanjul C, García-Dorado A (2006) Increase of the spontaneous mutation rate in a long-term experiment with *Drosophila melanogaster*. Genetics 173:267–277. doi:10.1534/genetics.106.056200

Azevedo RB, Keightley PD, Laurén-Määttä C, Vassilieva LL, Lynch M, Leroi AM (2002) Spontaneous mutational variation for body size in *Caenorhabditis elegans*. Genetics 162:755–765

Baer CF, Shaw F, Steding C, Baurngartner M, Hawkins A, Houppert A et al (2005) Comparative evolutionary genetics of spontaneous mutations affecting fitness in rhabditid nematodes. Proc Natl Acad Sci USA 102:5785–5790. doi:10.1073/pnas.0406056102

Bataillon T (2000) Estimation of spontaneous genome-wide mutation rate parameters: whither beneficial mutations? Heredity 84:497–501. doi:10.1046/j.1365-2540.2000.00727.x

Bataillon T (2003) Shaking the 'deleterious mutations' dogma? Trends Ecol Evol 18:315–317. doi:10.1016/S0169-5347(03)00128-9

Bateman AJ (1959) The viability of near-normal irradiated chromosomes. Int J Radiat Biol 1:170–180. doi:10.1080/09553005914550241

Boyko AR, Williamson SH, Indap AR, Degenhardt JD, Hernandez RD, Lohmueller KE et al (2008) Assessing the evolutionary impact of amino acid mutations in the human genome. PLoS Genet 4:e1000083. doi:10.1371/journal.pgen.1000083

Bubb KL, Bovee D, Buckley D, Haugen E, Kibukawa M, Paddock M et al (2006) Scan of human genome reveals no new loci under ancient balancing selection. Genetics 173:2165–2177. doi:10.1534/genetics.106.055715

Bulmer MG (1989) Maintenance of genetic variability by mutation-selection balance: a child's guide through the jungle. Genome 31:761–767

Burch CL, Guyader S, Samarov D, Shen H (2007) Experimental estimate of the abundance and effects of nearly neutral mutations in the RNA virus ϕ6. Genetics 176:467–476. doi:10.1534/genetics.106.067199

Bürger R (2000) The mathematical theory of selection, recombination and mutation. Wiley, Chichester

Charlesworth B, Langley CH (1989) The population genetics of *Drosophila* transposable elements. Annu Rev Genet 23:251–287. doi:10.1146/annurev.ge.23.120189.001343

Charlesworth B, Charlesworth D (1999) The genetic basis of inbreeding depression. Genet Res 74:329–340. doi:10.1017/S0016672399004152

Clayton G, Robertson A (1955) Mutation and quantitative variation. Am Nat 89:151–158. doi:10.1086/281874

Crow JF, Simmons MJ (1983). The mutation load in *Drosophila*. pp 1–35. In: Ashburner M, Carson HL, Thompson JN (eds) The genetics and biology of *Drosophila*, vol 3C. Academic Press, London

Davies EK, Peters AD, Keightley PD (1999) High frequency of cryptic deleterious mutations in *Caenorhabditis elegans*. Science 285:1745–1747. doi:10.1126/science.285.5434.1748

Denver DR, Morris K, Lynch M, Thomas WK (2004) High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome. Nature 430:679–682. doi:10.1038/nature02697

Denver DR, Feinberg S, Estes S, Thomas WK, Lynch M (2005) Mutation rates, spectra, and hotspots in mismatch repair-deficient *Caenorhabditis elegans*. Genetics 170:107–113. doi:10.1534/genetics.104.038521

Elena SF, Moya A (1999) Rate of deleterious mutation and the distribution of its effects on fitness in vesicular stomatitis virus. J Evol Biol 12:1078–1088. doi:10.1046/j.1420-9101.1999.00110.x

Estes S, Lynch M (2003) Rapid fitness recovery in mutationally degraded lines of *Caenorhabditis elegans*. Evolution 57:1022–1030

Estes S, Phillips PC, Denver DR, Thomas KW, Lynch M (2004) Mutation accumulation in populations of varying sizes: the distribution of mutational effects for fitness correlates in *Caenorhabditis elegans*. Genetics 166:1269–1279. doi:10.1534/genetics.166.3.1269

Eyre-Walker A, Keightley PD (2007) The distribution of fitness effects of new mutations. Nat Rev Genet 8:610–618. doi:10.1038/nrg2146

Eyre-Walker A, Keightley PD, Smith NGC, Gaffney D (2002) Quantifying the slightly deleterious model of molecular evolution. Mol Biol Evol 19:2142–2149

Eyre-Walker A, Woolfit M, Phlelps T (2006) The distribution of fitness of new deleterious amino acid mutations in humans. Genetics 173:891–900. doi:10.1534/genetics.106.057570

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman, London

Fernández J, López-Fanjul C (1996) Spontaneous mutational variances and covariances for fitness-related traits in *Drosophila melanogaster*. Genetics 143:829–837

Fry JD, Keightley PD, Heinsohn SL, Nuzhdin SV (1999) New estimates of rates and effects of mildly deleterious mutation in *Drosophila melanogaster*. Proc Natl Acad Sci USA 96:574–579. doi:10.1073/pnas.96.2.574

García-Dorado A (1997) The rate and effects distribution of viable mutation in *Drosophila*: minimum distance estimation. Evolution 51:1130–1139. doi:10.2307/2411042

García-Dorado A, Marin JM (1998) Minimum distance estimation of mutational parameters for quantitative traits. Biometrics 54:1097–1114. doi:10.2307/2533860

García-Dorado A, Gallego A (2003) Comparing analysis methods for mutation-accumulation data: A simulation study. Genetics 164:807–819

García-Dorado A, López-Fanjul C, Caballero A (1999) Properties of spontaneous mutations affecting quantitative traits. Genet Res 74:341–350. doi:10.1017/S0016672399004206

Gilligan DM, Woodworth LM, Montgomery ME, Briscoe DA, Frankham R (1997) Is mutation accumulation a threat to the

survival of endangered populations? Conserv Biol 11:1235–1241. doi:10.1046/j.1523-1739.1997.96215.x
Haag-Liautard C, Dorris M, Maside X, Macaskill S, Halligan DL, Charlesworth B et al (2007) Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. Nature 445:82–85. doi:10.1038/nature05388
Halligan DL, Keightley PD (2006) Ubiquitous selective constraints in the *Drosophila* genome revealed by a genome-wide interspecies comparison. Genome Res 16:875–884. doi:10.1101/gr.5022906
Halligan DL, Peters AD, Keightley PD (2003) Estimating numbers of EMS-induced mutations affecting life history traits in *Caenorhabditis elegans* in crosses between inbred sublines. Genet Res 82:191–205. doi:10.1017/S0016672303006499
Hill WG (1982a) Rates of change in quantitative traits from fixation of new mutations. Proc Natl Acad Sci USA 79:142–145. doi:10.1073/pnas.79.1.142
Hill WG (1982b) Predictions of response to artificial selection from new mutations. Genet Res 40:255–278
Hill WG, Rasbash J (1986) Models of long term artificial selection in finite population with recurrent mutation. Genet Res 48:125–131
Houle D, Nuzhdin SV (2004) Mutation accumulation and the effect of *copia* insertions in *Drosophila melanogaster*. Genet Res 83:7–18. doi:10.1017/S0016672303006505
Houle D, Hoffmaster D, Assimacopolous S, Charlesworth B (1992) The genomic mutation rate for fitness in *Drosophila*. Nature 359:58–60. doi:10.1038/359058a0
Houle D, Morikawa B, Lynch M (1996) Comparing mutational variabilities. Genetics 143:1467–1483
Joseph SB, Hall DW (2004) Spontaneous mutations in diploid *Saccharomyces cerevisiae*: more beneficial than expected. Genetics 168:1817–1825. doi:10.1534/genetics.104.033761
Keightley PD (1994) The distribution of mutation effects on viability in *Drosophila melanogaster*. Genetics 138:1315–1322
Keightley PD (1998) Inference of genome wide mutation rates and distributions of mutation effects for fitness traits: a simulation study. Genetics 150:1283–1293
Keightley PD (2004a) Mutational variation and long-term selection response. Plant Breed Rev 24(part 1):227–247
Keightley PD (2004b) Comparing analysis methods for mutation-accumulation data. Genetics 167:551–553. doi:10.1534/genetics.167.1.551
Keightley PD, Caballero A (1997) Genomic mutation rates for lifetime reproductive output and lifespan in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 94:3823–3827. doi:10.1073/pnas.94.8.3823
Keightley PD, Ohnishi O (1998) EMS-induced polygenic mutation rates for nine quantitative characters in *Drosophila melanogaster*. Genetics 148:753–766
Keightley PD, Bataillon TA (2000) Multi-generation maximum likelihood analysis applied to mutation accumulation experiments in *Caenorhabditis elegans*. Genetics 154:1193–1201
Keightley PD, Lynch M (2003) Towards a realistic model of mutations affecting fitness. Evolution Int J Org Evolution 57:683–685
Keightley PD, Eyre-Walker A (2007) Joint inference of the distribution of fitness effects of deleterious mutations and population demography based on nucleotide polymorphism frequencies. Genetics 177:2251–2261
Kimura M (1983). The neutral theory of molecular evolution. Cambridge University Press, Cambridge
Lande R (1994) Risk of population extinction from fixation of new deleterious mutations. Evolution 48:1460–1469. doi:10.2307/2410240
Livingston RJ, von Niederhausern A, Jegga AG, Crawford DC, Carlson CS, Rieder MJ et al (2004) Pattern of sequence variation across 213 environmental response genes. Genome Res 14:1821–1831. doi:10.1101/gr.2730004
Loewe L, Charlesworth B, Bartolomé C, Nöel V (2006) Estimating selection on non-synonymous mutations. Genetics 172:1079–1092. doi:10.1534/genetics.105.047217
López MA, López-Fanjul C (1993) Spontaneous mutation for a quantitative trait in *Drosophila melanogaster* I. Response to artificial selection. Genet Res 61:107–116
Lyman RF, Lawrence F, Nuzhdin SV, Mackay TFC (1996) Effects of single P-element insertions on bristle number and viability in *Drosophila melanogaster*. Genetics 143:277–292
Lynch M (1988) The rate of polygenic mutation. Genet Res 51:137–148
Lynch M, Hill WG (1986) Phenotypic evolution by neutral mutation. Evolution 40:915–935. doi:10.2307/2408753
Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer, Sunderland, MA, USA
Lynch M, Conery J, Burger R (1995) Mutation accumulation and the extinction of small populations. Am Nat 146:489–518. doi:10.1086/285812
Lynch M, Blanchard J, Houle D, Kibota T, Schultz S, Vassilieva L et al (1999) Perspective: spontaneous deleterious mutation. Evolution Int J Org Evolution 53:645–663. doi:10.2307/2640707
Mackay TFC (1988) Transposable element-induced quantitative genetic variation in *Drosophila*. In: Weir BS, Eisen EJ, Goodman MM, Namkoong G (eds) Proceedings of the second international conference on quantitative genetics. Sinauer, Sunderland, Massachusetts
Mukai T (1964) The genetic structure of natural populations of *Drosophila melanogaster* I. Spontaneous mutation rate of polygenes controlling viability. Genetics 50:1–19
Mukai T, Chigusa SI, Mettler LE, Crow JF (1972) Mutation rate and dominance of genes affecting viability in *Drosophila melanogaster*. Genetics 72:333–355
Nielsen R, Yang Z (2003) Estimating the distribution of selection coefficients from phylogenetic data with applications to mitochondrial and viral DNA. Mol Biol Evol 20:1231–1239. doi:10.1093/molbev/msg147
Ohnishi O (1977) Spontaneous and ethyl methanesulfonate-induced mutations controlling viability in *Drosophila melanogaster* II. Homozygous effect of polygenic mutations. Genetics 87:529–545
Otto SP, Lenormand T (2002) Resolving the paradox of sex and recombination. Nat Rev Genet 3:252–261. doi:10.1038/nrg761
Piganeau GV, Eyre-Walker A (2003) Estimating the distribution of fitness effects from DNA sequence data: implications for the molecular clock. Proc Natl Acad Sci USA 100:10335–10340. doi:10.1073/pnas.1833064100
Robertson A (1967) The nature of quantitative genetic variation. In: Brink RB (ed) Heritage from Mendel. University of Wisconsin Press, Madison, Milwaukee and London, pp 265–280
Sawyer SA, Kulathinal RJ, Bustamante CD, Hartl DL (2003) Bayesian analysis suggests that most amino acid replacements in *Drosophila* are driven by positive selection. J Mol Evol 57:S154–S164. doi:10.1007/s00239-003-0022-3
Schoen DJ (2005) Deleterious mutation in related species of the plant genus *Amsinckia* with contrasting mating systems. Evolution 59:2370–2377
Shapiro JA, Huang W, Zhang C, Hubisz MJ, Lu J, Turissini DA et al (2007) Adaptive genic evolution in the *Drosophila* genomes. Proc Natl Acad Sci USA 104:2271–2276. doi:10.1073/pnas.0610385104
Shaw RG, Chang SM (2006) Gene action of new mutations in *Arabidopsis thaliana*. Genetics 172:1855–1865. doi:10.1534/genetics.105.050971

Shaw RG, Byers DL, Darmo E (2000) Spontaneous mutational effects on reproductive traits of *Arabidopsis thaliana*. Genetics 155:369–378

Shaw FH, Geyer CJ, Shaw RG (2002) A comprehensive model of mutations affecting fitness and inferences for *Arabidopsis thaliana*. Evolution Int J Org Evolution 56:453–463

Shaw RG, Shaw FH, Geyer C (2003) What fraction of mutations reduces fitness? A reply to Keightley and Lynch. Evolution 57:686–689

Smith NGC, Eyre-Walker A (2002) Adaptive protein evolution in *Drosophila*. Nature 415:1022–1024. doi:10.1038/4151022a

Vassilieva LL, Lynch M (1999) The rate of spontaneous mutation for life-history traits in *Caenorhabditis elegans*. Genetics 151:119–129

Vassilieva LL, Hook AM, Lynch M (2000) The fitness effects of spontaneous mutations in *Caenorhabditis elegans*. Evolution 54:1234–1246

Webb CT, Shabalina SA, Ogurtsov AY, Kondrashov AS (2002) Analysis of similarity within 142 pairs of orthologous intergenic regions of *Caenorhabditis elegans* and *Caenorhabditis briggsae*. Nucleic Acids Res 30:1233–1239. doi:10.1093/nar/30.5.1233

Zhang XS, Hill WG (2005) Genetic variability under mutation selection balance. Trends Ecol Evol 20:468–470. doi:10.1016/j.tree.2005.06.010