A Generation Time Effect on the Rate of Molecular Evolution in Invertebrates

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Abstract

The rate of genome evolution varies significantly between species. Evidence is growing that at least some of this variation is associated with species characteristics, such as body size, diversification rate, or population size. One of the strongest correlates of the rate of molecular evolution in vertebrates is generation time (GT): Species with faster generation turnover tend to have higher rates of molecular evolution, presumably because their genomes are copied more frequently and therefore collect more DNA replication errors per unit time. But the GT effect has never been tested for nonvertebrate animals. Here, we present the first general test of the GT effect in invertebrates, using 15 genes from 143 species spread across the major eumetazoan superphyla (including arthropods, nematodes, molluscs, annelids, platyhelminthes, cnidarians, echinoderms, and urochordates). We find significant evidence that rates of molecular evolution are correlated with GT in invertebrates and that this effect applies consistently across genes and taxonomic groups. Furthermore, the GT effect is evident in nonsynonymous substitutions, whereas theory predicts (and most previous evidence has supported) a relationship only in synonymous changes. We discuss both the practical and theoretical implications of these findings.

Key words: generation time, nearly neutral theory, phylogenetic comparative methods, substitution rate, genetic draft, effective population size.

Introduction

Variability in rates of molecular evolution is well documented, and there is now much evidence to suggest that a strict global molecular clock does not hold (Bousquet et al. 1992; Thomas et al. 2006; Welch et al. 2008). Furthermore, it has been demonstrated that substitution rates can be affected systematically by certain species characteristics; these include ecological factors such as environmental energy (Davies et al. 2004), aspects of evolutionary history such as population size (Woolfit and Bromham 2005), or life-history traits such as body size (Bromham et al.1996). One of the most prominent explanations for variation in substitution rates between lineages is differences in species generation times (GTs). The GT hypothesis states that species with shorter GTs should have a greater rate of mutation per year, as they will copy their genomes more frequently and therefore may accrue more DNA replication errors per unit time.

The GT hypothesis stems from observations in early DNA hybridization studies, where differences in GT were proposed to explain the finding that rates of molecular evolution for DNA sequences in rodents appeared to be much faster than those found in primates (Laird et al. 1969; Kohne 1970). This was in contrast to the initial and somewhat surprising observation of a "molecular clock" in amino acid sequence evolution (Zuckerkandl and Pauling 1965). In protein coding sequences, the GT effect should be observed in synonymous substitutions because these are influenced predominantly by the underlying mutation rate (Kimura and Ohta 1971; Ohta and Kimura 1971; Ohta 1972). Nonsynonymous substitutions, however, are more likely to be under selection, and theory suggests that the rate of nonsynonymous substitution should be influenced not only by the underlying mutation rate but also by the effective population size (Kimura and Ohta 1971; Ohta and Kimura 1971; Ohta 1972). This is because mutations of small selective effect are expected to go to fixation more frequently in small populations due to genetic drift. Because species with longer GTs (and thus a slower accrual of copy-error mutations) also tend to have smaller N_e (and thus higher fixation rates), it has been suggested that in many cases these 2 effects will cancel each other out, making the relationship between GT and substitution rate less predictable for nonsynonymous substitutions (Ohta and Kimura 1971; Ohta 1972, 1993; Gillespie 1995; Ohta and Gillespie 1996).

These predictions have been borne out by a number of studies of molecular evolution in vertebrates. A GT effect has been observed for synonymous substitutions in mammals, birds, and reptiles (Li and Tanimura 1987; Ohta 1993; Mooers and Harvey 1994; Bromham et al. 1996;

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Bromham 2002; Nabholz et al. 2008; Welch et al. 2008), whereas nonsynonymous substitutions and ribosomal RNAs (rRNAs) show rate variation (Ohta and Gillespie 1996; Smith and Eyre-Walker 2003) but far less dependency on life-history traits such as GT (Bromham et al. 1996; Gissi et al. 2000; Spradling et al. 2001; Welch et al. 2008; but see Nabholz et al. 2008; Gillooly et al. 2007). Furthermore, similar results have been observed in plants, where in both monocots and dicots, DNA substitution rates are found to be faster in annual species than in related woody perennials (Laroche et al. 1997; Ainouche and Bayer 1999; Laroche and Bousquet 1999; Andreasen and Baldwin 2001; Smith and Donoghue 2008) and in monocots, minimum GT (time to flowering) appears also to be related to rates of molecular evolution (Gaut et al. 1996, 1997).

However, despite evidence that provides support for a GT effect, a number of other life-history traits have also been put forward to explain the observed differences in substitution rates. This is because, particularly in vertebrates, many life-history traits covary strongly with each other (e.g., a mammal with a short GT is also likely to have a smaller body size, faster metabolism, higher fecundity, and larger population size). Other prominent explanatory variables include metabolic rate (Martin and Palumbi 1993), DNA repair efficiency (Britten 1986), fecundity and longevity (Nabholz et al. 2008; Welch et al. 2008), and the number of developmental germline cell divisions (Goetting-Minesky and Makova 2006). Teasing apart the causal effects of different life-history traits on rates of molecular evolution in vertebrates is a difficult task, and partial correlations between life-history traits in mammals have provided support not only for GT but also for body size, fecundity, and longevity (Bromham et al. 1996; Nabholz et al. 2008; Welch et al. 2008).

As yet, there have been no comprehensive studies examining the GT effect in nonvertebrate metazoans. Invertebrates, though a paraphyletic assemblage, are a useful group in which to investigate the GT effect. The relationships between the different life-history traits, such as size, GT, metabolic rate, and longevity, are thought to be more variable in invertebrates than in most vertebrate taxa (see Discussion), and this may help to tease out the possible confounding factors that could cause an association between GT and substitution rates. In this study, we set out to investigate whether a GT effect on substitution rates might be operating in invertebrate metazoans, using a phylogenetic comparative approach. We collected DNA sequences and GT data for over 300 invertebrate species across 8 different phyla and 15 different mitochondrial and nuclear genes. We then tested for an association between rates of molecular evolution and GT using phylogenetically independent comparisons and both nonparametric and parametric statistics. We found evidence that invertebrate eumetazoan species with shorter GTs have faster rates of molecular evolution in ribosomal RNA genes in mitochondrial and nuclear sequences and in nonsynonymous substitutions and 4-fold degenerate transversions in protein-coding mitochondrial genes.

Methods

Data Collection

GT measurements for invertebrate Eumetazoa (including arthropods, nematodes, molluscs, annelids, platyhelminthes, cnidarians, echinoderms, and urochordates) were gathered from the literature (see supplementary tables 1 and 2, Supplementary Material online). Our preferred index of GT was the average generation time in days. Where this was unavailable, estimates of developmental time were used, for example, age at sexual maturity or first reproduction. However, such measurements were not used for groups known to undergo phenomena such as diapause, for example, some terrestrial arthropods.

We aimed to maximize the statistical power of our analysis by balancing the number of comparisons we could use with the amount of DNA sequence data available for each comparison. Consequently, we compiled GT data for all invertebrate species for which the whole mitochondrial genome had been sequenced (available from Genbank [ncbi.nlm.nih.gov]). This data set consisted of 214 species with both GT measurements and sequence data (including rRNAs, but excluding transfer RNAs, ATP6 and ATP8, which could not be aligned between phyla). In addition, to investigate the GT effect in the nuclear genome, we collected a separate data set comprising 113 species with 2 nuclear rRNA sequences (28S and 18S). We were unable to obtain sufficient data to test for a GT effect in nuclear protein coding genes.

Selection of Phylogenetically Independent Pairs

The comparative analyses in this study used phylogenetically independent sister pairs (see Felsenstein [1985]; Harvey and Pagel [1991]; Bromham et al. [1996]; and Welch and Waxman [2008]). Because species may share similar trait and rate values as a result of shared ancestry, rather than separate instances of correlated evolutionary change, a failure to account for phylogeny can lead to the same evolutionary changes being included in more than one data point. Such pseudoreplication can yield spurious evidence of an association between variables.

The number of independent pairs we were able to choose was limited by continued uncertainty about the taxonomy of many large groups of Eumetazoa. In addition, we also needed pairs to be both sufficiently deep, for differences in substitution rates to be reliably detectable (Welch and Waxman 2008), and sufficiently shallow, to avoid saturation. Nevertheless, we were able to choose 54 comparisons for the mitochondrial data set (comprising 27 arthropods, 7 molluscs, 6 platyhelminthes, 5 nematodes, 4 cnidarians, 2 annelids, 2 echinoderms, and 1 urochordate) and 20 for the nuclear genes (comprising 6 molluscs, 5 arthropods, 5 annelids, 2 platyhelminthes, and 2 nematodes). These pairs encompassed 8 different phyla, including representatives of the major bilaterian superorders (Ecdysozoa, Lophotrochozoa, and invertebrate

Deuterostomia), and a small number of pairs from the cnidaria. The supplementary tables 1 and 2 (Supplementary Material online) contains full details of all pairs, including the taxonomic hypotheses on which the assumption of independence was based.

Substitution Rate Estimation

All sequences were aligned by eye in Se-Al v2.0 (Rambaut 1996), excluding any highly variable regions that could not be aligned with confidence. Species pairs were then grouped with another pair or with a single outgroup species of suitable distance, for branch length estimation. For the primary results, all genes of each sequence type (mitochondrial rRNA, mitochondrial protein coding, and nuclear rRNA) were concatenated for rate estimation as follows: 12S + 16S; COI + COII + COIII + CYTB + ND1 + ND2 + ND3 + ND4 + ND4L + ND5 + ND6 and 18S + 28S. However, to test the consistency of any effect across the genome, we also split the protein alignments into separate genes or, for very short genes, gene pairs (ND2 + ND3; ND4 + ND4L; ND5 + ND6).

All molecular branch lengths were estimated using maximum likelihood in PAML v 4.0 (Yang 2007). For rRNA genes, the appropriate substitution model for each pair was determined in Modeltest v3.6 (Posada and Crandall 1998), using the Akaike information criterion, which is generally superior to standard hierarchical likelihood ratio tests (Posada and Buckley 2004). For protein-coding genes, we used the recently developed LG amino acid replacement matrix of Le and Gascuel (2008), with gamma distributed rates across sites. To translate the sequences, the source code for PAML was altered to implement the variant mitochondrial codes for platyhelminthes (Garey and Wolstenholme 1989; Ohama et al. 1990; Telford et al. 2000).

In addition to the amino acid substitution rates, we also set out to estimate synonymous substitution rates in protein-coding genes using the codon-based model of Goldman and Yang (1994). However, synonymous sites were highly saturated for almost every pair in our data set (53/54), making estimates of synonymous rate highly unreliable (codon-based estimates of amino acid–changing rate remained very close to those obtained from the translated sequences). Furthermore, data to construct a sufficient number of shallower pairs, which are less likely to be saturated, were not available. Therefore, in order to investigate the effects of GT on synonymous substitution rates, given the problems of saturation at synonymous sites, we considered only transversions (which occur at a slower rate) at 4-fold degenerate sites at codons that were conserved across the comparison pair and outgroups. This removes the effects of transition substitutions from the estimate of substitution rate, which are likely to reach saturation significantly more quickly than transversions. Parametric statistics could not be performed for this data set, because sequence lengths differed substantially between pairs, with rapidly evolving pairs having fewer conserved codons. This clearly violates the assumptions of homogeneous variance for the linear regression and is difficult to correct for in a principled way. Nevertheless, nonparametric tests could still be used (see below). Details of all data are given in supplementary tables 1 and 2 (Supplementary Material online).

Statistical Analysis

To test for significant variation in substitution rates between the members of each comparison pair, we used likelihood ratio tests, comparing the fit of a 2-rate model (in which the pair had a common substitution rate and the outgroup species another rate) with a 3-rate model (in which both pair members and the outgroup had separate rates). Twice the difference in log likelihood was then compared with a χ^2 distribution, with 1 degree of freedom. Z-tests were employed to test whether this rate variation applied across the data set as a whole (Whitlock 2005).

To test for a relationship between GT and substitution rate, for each pair we calculated the contrast in GT as the log ratio of the GT measurements for the 2 species [i.e., $\ln(GT_1/GT_2)$] and the contrast in substitution rate as the log ratio of their molecular branch lengths [i.e., $\ln(BL_1/BL_2)$]. We first used a nonparametric 2-tailed sign test to test for a relationship across the data set in the product of the 2 variables: $\ln(GT_1/GT_2) \times \ln(BL_1/BL_2)$. For this test, a significant excess of negative signs indicates that increases in GT tend to be associated with decreases in species substitution rate.

As a more powerful test of the GT effect, we also used parametric linear regression forced through the origin (see Felsenstein [1985]; Garland et al. [1992]). To check that the assumptions of this test were met (e.g., standardized variance across contrasts), we used the methods of Freckleton (2000), Garland et al. (1992), and Welch and Waxman (2008) and the suite of regression diagnostics implemented in R (R Development Core Team 2008). To standardize the variance across contrasts, it is usually necessary to weight each contrast by some measure of the pair's divergence time, which serves to correct for the fact that more distantly related pairs are likely to be associated with more evolutionary change and so generate contrasts of larger magnitude (Felsenstein 1985; Garland et al. [1992]). For this purpose, we used the summed branch lengths for the pair and found that dividing each contrast by $(BL_1 + BL_2)^{1/4}$ gave a good fit for both GT and rate contrasts.

To assess whether our results applied consistently across taxonomic groups, we formally compared the strength of the GT effect in the 2 superphyla with sufficient comparisons, namely the Ecdysozoa and Lophotrochozoa. For this purpose, we used the common slope test of Warton and Weber (2002), as implemented in the smatr package in R (Warton and Ormerod 2007). This test uses standardized major axis regression (more appropriate for the purpose of comparing slopes) and compares the fit of a single regression line applied to both Lophotrochozoan and Ecdysozoan comparisons with a model in which each group is allowed its own slope.

Table 1. The Invertebrate GT Effect.

		Sign Test		Regression			Slope Comparison		
Data Set Sequences	*n*	Negative	*P*	Slope	r^2	*P*	Ecdy	Loph	*P*
Mitochondrial synon. tv.	54	35	0.0175*	—	—	—	—	—	—
Mitochondrial proteins	54	39	0.0004**	−0.143	0.128	0.0073**	−0.358	−0.501	0.269
Mitochondrial rRNAs	53	34	0.0241*	−0.124	0.064	0.0658	−0.522	0.540	—
	52[a]	34	0.0124*	−0.145	0.117	0.0124*	−0.521	−0.284	0.070
Nuclear rRNAs	20	17	0.0026**	−0.162	0.304	0.0096**	−0.465	−0.228	0.083

NOTE.—*$P < 0.05$; **$P < 0.01$; Ecdy: Ecdysozoan pairs; Loph: Lophotrochozoan pairs; synon. tv.: transversions at 4-fold degenerate sites in conserved codons.
[a] Outlier removed.

Results

Evidence of Rate Variation

Using triplet likelihood ratio tests, we found evidence of significant substitution rate variation across our data set within all sequence types. For the nuclear rRNAs, significant variation at the 5% level was observed in a fifth (4/20) of all comparisons, whereas for the mitochondrial sequences, significant variation was observed in 43% (23/54) of comparisons for protein coding genes and in 18% (10/54) for rRNAs. *Z*-tests (Whitlock 2005), used to combine multiple *P* values, yielded highly significant results for the data set as a whole, in all cases (mitochondrial proteins: $P < 1 \times 10^{-5}$; mitochondrial rRNAs: $P < 1 \times 10^{-5}$; nuclear rRNAs: $P < 1 \times 10^{-5}$).

Evidence for a GT Effect

After accounting for saturation at synonymous sites, we found evidence for a significant negative relationship between GT and mitochondrial synonymous transversions in invertebrates. A sign test indicated that in more comparison pairs than would be expected by chance, the species with the shorter GT had the faster molecular rate ($P = 0.0175$; table 1). We found even stronger evidence of a significant negative relationship for amino acid–changing substitutions in mitochondrial genes ($P = 0.0004$; table 1). For these nonsynonymous substitutions, we were also able to carry out a regression analysis, which too was highly significant ($P = 0.0073$; fig. 1*a*; table 1). Furthermore, this analysis was robust to the removal of any or all of the mild outliers evident in figure 1*a*. The observed GT effect appeared to be consistent across taxonomic groups, with the slope comparison test showing no significant difference between the Ecdysozoa and Lophotrochozoa ($P = 0.269$; see table 1). The effect also appeared to be consistent across the mitochondrial genome. All 8 single-gene or gene pair analyses (not shown) yielded negative slopes, and half of these were individually significant (COI, COIII, CYTB, ND2 + ND3).

For the mitochondrial rRNAs, the amount of sequence divergence was much smaller, and we had to exclude a single comparison (the cnidaria pair *Briareum-Pseudopterogorgia*) due to a zero-valued branch length. Nevertheless, there was also evidence for a significant influence of GT on substitution rates in these sequences (fig. 1*b*). This was evident from the sign test ($P = 0.024$; table 1), but the regression was nonsignificant, and the best-fit slope for the Lophotrochozoa was actually positive (table 1). However, examination of the regression diagnostics indicated that the assumptions of the regression were poorly met (e.g., Shapiro–Wilks test $P = 0.004$), making this result unreliable. This was found to be due to a single outlier (a shallow comparison pair between the congeners *Octopus ocellatus* and *O. vulgaris*), as identified by a number of diagnostics and evident in figure 1*b*. (The sensitivity of parametric tests to shallow pairs, with poorly estimated rate changes, is also expected on theoretical grounds, e.g., Welch and Waxman 2008). The removal of this outlier improved the diagnostics (e.g., Shapiro–Wilks $P = 0.880$) and gave a significant regression analysis ($P = 0.0124$). Both Lophotrochozoan and Ecdysozan slopes were now negative and not significantly different from each other ($P = 0.070$).

Finally, although there were far fewer comparisons, there was also evidence of a GT effect operating in invertebrate nuclear rRNA sequences (fig. 1*c*). Both the sign test and regression test were highly significant ($P < 0.01$ in both cases; table 1), and again, the slope comparison test indicated no significant differences in the GT effect between the 2 major superphyla ($P = 0.083$).

Discussion

Using a large data set of 143 species and 15 genes, across 8 different phyla, we have found evidence that differences in GT can affect rates of molecular evolution in invertebrates. This effect is observed in both nuclear and mitochondrial ribosomal RNAs and in nonsynonymous and synonymous substitutions in mitochondrial protein-coding sequences and is evident despite heterogeneity in quality in our estimates of GT and inevitable errors in the estimation of molecular branch lengths. This result is important for 2 reasons. Firstly, the demonstration of a GT effect in animals has thus far been restricted to vertebrates, whereas we show that it is a general phenomenon observable across a wide range of animal taxa. Secondly, in much of the previous literature, the GT effect was assumed to be a feature of synonymous changes only (e.g., Gillespie 2001), but we show that it can also be detected for nonsynonymous substitution rates.

The GT effect is generally thought to arise from the effect of GT on the accrual of copy-error mutations. Mechanistic explanations assume that substitution rate depends

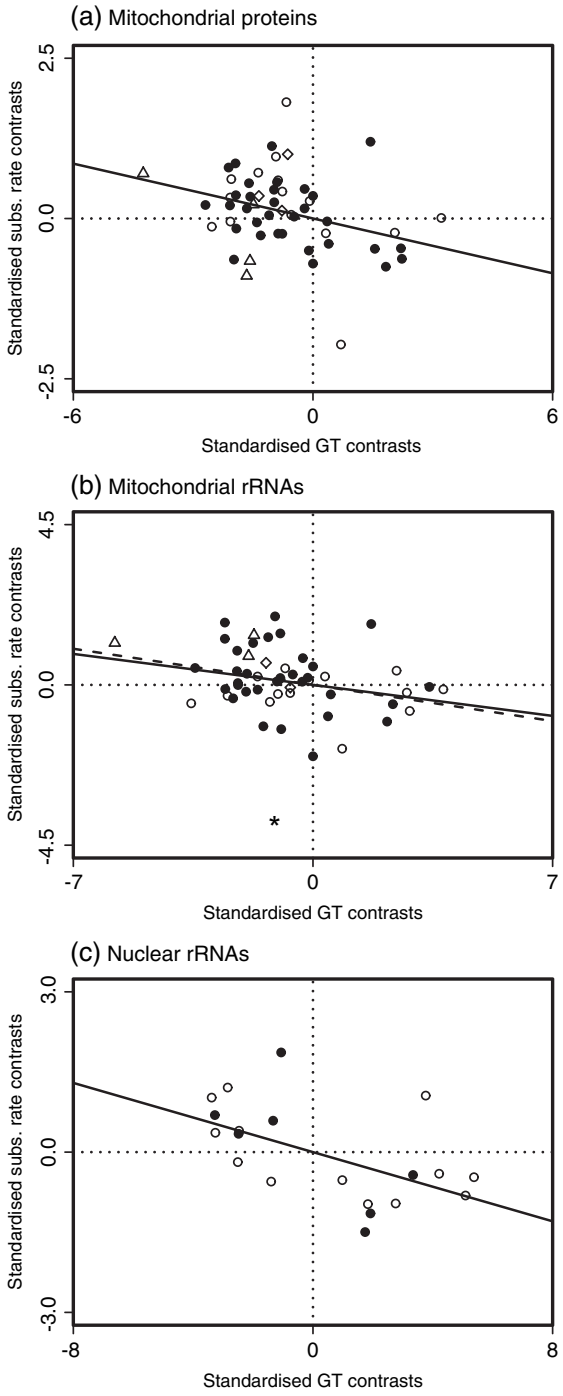

Fig. 1. Plots of phylogenetically independent sister-pair comparisons of substitution rate, against GT, with regression lines forced through the origin (table 1; dotted line shows the regression with the Lophotrochozoan outlier, indicated as "asterisk," removed). The different symbols indicate major taxonomic groups: Ecdysozoa (filled circles), Lophotrochozoa (empty circles), invertebrate Deuterostomia (diamonds), and cnidaria (triangles). Substitution rate estimated from (*a*) 11 mitochondrial protein-coding genes (the complete complement excluding ATP6 and ATP8), (*b*) the 2 mitochondrial rRNA-coding genes (12S and 16S), and (*c*) 2 nuclear rRNA-coding genes (18S and 28S).

strongly on the mutation rate, and the mutation rate per year decreases with increasing GT. If every time the genome is copied, there is a chance of replication error, then if species with shorter generations undergo more replications per unit time, they should have higher mutation rates. Several factors might weaken the resulting correlation between GT and the mutation rate per year. These include mutations due to unrepaired DNA damage (Cooke et al. 2003), between-lineage variation in replication fidelity (Promislow 1994), the number of germline replications per generation (Bauer and Aquadro 1997; Drake et al. 1998), natural selection acting on the mutation rate (Sniegowski et al. 2000), and the phenomenon of germline mosaicism, where a large fraction of mutations in the nuclear genome appear in just 1 or 2 meiotic divisions per organismal generation, thereby obscuring between-lineage variation in the rate of mutation accrued in the preceding mitotic divisions (Drost and Lee 1998). Nevertheless, despite all these factors, the importance of replication error in the overall mutation rate is attested both by direct estimates (Iyer et al. 2006) and phenomena such as "male-driven evolution" (Li et al. 2002).

All other factors being equal, an increase in the mutation rate provides an explanation for the observation of greater rates of synonymous substitutions in species with short GTs. Synonymous changes are generally considered to be effectively neutral (Kimura and Ohta 1971; Ohta and Kimura 1971; Ohta 1972) and therefore expected to reflect the underlying mutation rate. Previous failures to observe a GT effect in nonsynonymous substitution rates in vertebrates have been explained as the result of a correlation between life-history traits because species with shorter GTs tend to also have larger population sizes. Thus, an increase in the rate of nearly neutral mutations may be balanced by an increased effectiveness of selection preventing these mutations going to fixation, so these 2 effects on substitution rates may cancel each other out (Ohta and Kimura 1971; Ohta 1972, 1993; Gillespie 1995; Ohta and Gillespie 1996). In contrast to most of the vertebrate results, however, we do find evidence of a GT effect in nonsynonymous substitutions, and this appears to be no less strong than the effect in synonymous sites. This suggests that a "canceling out" of GT and N_e is not occurring for these sequences in these taxa. Why not?

One possibility is that Ohta's predicted canceling out of N_e and GT for nonsynonymous substitutions relies on both the proportion of effectively neutral mutations and the GT being inversely proportional to population size to exactly the same extent. If, however, this is not the case, and GT declines rapidly with population size, but the fraction of effectively neutral mutations declines more slowly, then we would still expect to observe a GT effect, even if the nearly neutral theory holds. Although a direct correlation between N_e and GT in vertebrates may be a reasonable assumption (Nei and Graur 1984; Chao and Carr 1993), the extent to which these traits correlate within invertebrates is not well documented, as the published studies contain only a few measurements for the genus *Drosophila*.

If GT does scale widely with N_e across metazoan species, there are still a number of ways that a GT effect may be observed in nonsynonymous sites. For one, substitutions

at nonsynonymous sites may not all be weakly deleterious. If a significant proportion of nonsynonymous substitutions are strictly neutral, then their rate of fixation should be independent of the population size and instead reflect the underlying mutation rate (Kimura and Ohta 1971; Ohta 1972), thus generating a relationship between GT and the nonsynonymous substitution rate. This might appear unlikely for our data, as we are analyzing highly conserved housekeeping genes, the sites in which are likely to be under strong selection. However, a GT effect might still be observed so long as all the substitutions that do occur are strictly neutral (however few there may be). A second possibility is that most of the substitutions we are observing are adaptive rather than weakly deleterious. There is indeed evidence of high rates of adaptive substitution in some species of *Drosophila* (Eyre-Walker 2006). In this case, the nonsynonymous substitution rate would be expected to increase with N_e, as the fixation of adaptive substitutions is expected to be more efficient in larger populations. Consequently, if GT was inversely proportional to N_e, we might observe a "generation time" effect for nonsynonymous sites as a result of differences in N_e rather than GT. A GT effect that is stronger in selected than nonselected sites might suggest a role for adaptive molecular evolution proceeding more rapidly in lineages with shorter generations. However, the lack of power in the synonymous transversion analysis prevents us from testing this rigorously, and in any case, an explanation based on mutation rates is more parsimonious.

Alternatively, it could be that the assumptions underlying Ohta and Kimura's explanation are violated in quite a different way. One key supposition of the nearly neutral theory is that substitutions take place independently of one another. However, for species with large N_e and tight linkage between sites, linked selection (genetic draft or hitchhiking) may be a stronger stochastic force in molecular evolution than genetic drift (Maynard Smith and Haigh 1974; Gillespie 2001). If this is so, then neutral and nearly neutral mutations may be dragged to fixation because they are linked to adaptive substitutions. Under these conditions, although a GT effect is still expected to operate for neutral mutation rates, genetic draft is predicted to remove the effects of population size on the fixation of nearly neutral mutations (their fixation would instead depend on the rate of strongly selected adaptive substitutions per generation) (Gillespie 2001). Consequently, the effects of N_e would no longer cancel out the effects of GT on substitution rates, and a GT effect would be expected in rates of nonsynonymous substitution (Gillespie 2001). Although Gillespie (2001) hypothesized that the rate of adaptive substitution per generation might be inversely proportional to GT (removing any GT effect under draft) in order to account for the widely assumed lack of a GT effect in proteins, our results would suggest that this cancelation may not apply.

Support for the genetic draft hypothesis is particularly strong in invertebrate taxa. Many invertebrate species are likely to have relatively high effective population sizes (Lynch and Conery 2003), and substitutions in mitochondrial sequences in particular are likely to be tightly linked (due to low or absent recombination) (Ballard and Whitlock 2004). In addition, draft relies on adaptive substitution occurring at a nonnegligible rate (Gillespie 2001), and comparisons of polymorphism and divergence do suggest that rates of adaptive substitution are substantially higher in invertebrates than in vertebrates (Bazin et al. 2006; Eyre-Walker 2006; Meiklejohn et al. 2007). Furthermore, recent studies have shown that levels of mitochondrial polymorphism across invertebrates do not appear to change with N_e, as they would be expected to under the nearly neutral theory (Bazin et al. 2006; Meiklejohn et al. 2007). This is in contrast to results in vertebrates (Mulligan et al. 2006; Piganeau and Eyre-Walker 2009), which have much smaller population sizes, and where the effects of genetic drift would be expected to have a greater influence than draft on substitution rates (Gillespie 2001).

There are consequently several possible explanations for the observed GT effect in invertebrate nonsynonymous sites. Although the case for GT as a true causal factor is strong, it is important to acknowledge that many other factors have been proposed as predictors of the mutation rate (e.g., body mass, longevity, fecundity, and metabolic rate) (Britten 1986; Martin and Palumbi 1993; Bromham et al. 1996; Gillooly et al. 2005; Nabholz et al. 2008; Welch et al. 2008; Bromham 2009). Recent analyses suggest that GT might not be the strongest of these predictors in mammals (Nabholz et al. 2008; Welch et al. 2008), but strong life-history correlations between GT and other proposed factors make all such results difficult to interpret. The extent to which these life-history traits covary in invertebrates is less well known, but it is likely to be less tightly constrained than it is in vertebrates (Thomas et al. 2006). For example, the relationship between body size and GT in ectotherms is also likely to be significantly affected by temperature (environmental and developmental) as well as other related factors such as season length, resource availability, and latitude (Olive 1995; Huntley and Lopez 1992; Chown and Gaston 1999; Chown and Klok, 2003). If life-history correlations are indeed less strong in invertebrates, then this is consistent with the failure of other life-history traits, such as body size and metabolic rate, to predict invertebrate substitution rate (Thomas et al. 2006; Lanfear et al. 2007). However, the failure to observe a correlation can always be attributed to lack of power, a particularly problematic issue in the study of molecular rates (Fontanillas et al. 2007; Welch et al. 2008; Welch and Waxman 2008). Indeed, yet another possible explanation for the differences in results between vertebrates and invertebrates could simply be that invertebrate comparison pairs in published studies tend to be deeper and therefore more divergent.

The results reported here have important implications not only for understanding the drivers of molecular evolution in metazoans but also for the growing use of molecular data in biology. Regardless of the possible causes, our results imply that the "protein molecular clock" of Zuckerkandl and Pauling (1965) may be a taxonomically restricted

phenomenon. Although assumptions of rate constancy in molecular dating are no longer prevalent, the observations of a GT effect in both mitochondrial nonsynonymous changes and in mitochondrial and nuclear rRNA genes demonstrate that variation in rates of substitution may not only be widespread but also be systematic. This is relevant to molecular phylogenetic and dating methods that allow rates of molecular evolution to vary between lineages, as it has the potential to cause bias in molecular dates rather than simply adding noise to date estimates. However, although such systematic correlations of substitution rate may complicate models of rate variation in molecular evolution, they may also offer a potential future solution: If we can identify strong correlates of rate variation, then it may be possible to use biological characteristics such as GT to inform model choice in phylogenetic reconstruction and molecular dating analyses. Consequently, the practical benefits of the molecular clock need not be lost because a widely applicable GT effect could be exploited as a priori information in molecular dating studies.

Supplementary Material

Supplementary tables 1 and 2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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