THESIS

GENETIC DRIFT AND MUTATIONAL HAZARD IN THE EVOLUTION OF SALAMANDER GENOMIC GIGANTISM

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Erik Mohlhenrich

Department of Biology

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Advisor: Rachel Mueller

Daniel Sloan
William Black IV

ABSTRACT

GENETIC DRIFT AND MUTATIONAL HAZARD IN THE EVOLUTION OF SALAMANDER GENOMIC GIGANTISM

Salamanders have the largest nuclear genome sizes among tetrapods and, with the exception of lungfishes, among vertebrates as a whole. Lynch and Conery (2003) have proposed the mutational hazard hypothesis to explain variation in genome size and complexity. Under this hypothesis, non-coding DNA imposes a selective cost by increasing the target for degenerative mutations, i.e. the mutational hazard. Expansion of non-coding DNA, and thus genome size, is expected to be driven by increased levels of genetic drift and/or decreased mutation rates; the former determines the efficiency with which excess non-coding DNA can be selected against, while the latter determines the level of mutational hazard. Here, we test the hypothesis that salamanders have experienced stronger long-term, persistent genetic drift than frogs, a clade with more typically sized vertebrate genomes. To test this hypothesis, we compared *dN/dS* and *Kr/Kc* values between these clades. Our results reject this hypothesis; we find that salamanders have not experienced stronger genetic drift than frogs. Additionally, we find evidence consistent with a lower nucleotide substitution rate in salamanders. This result, along with previous work showing lower rates of small deletions and ectopic recombination in salamanders, suggests that a lower mutational hazard may contribute to genome expansion in this clade. Taken together, these results further underscore the importance of studying large genomes and indicate that salamanders provide an important model system for the study of how non-drift processes (i.e. mutation, natural selection) shape the evolution of genome size.

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INTRODUCTION

Nuclear genome sizes span eight orders of magnitude across the Tree of Life and vary 6,650-fold (0.02 to 130 Gb) within animals alone (Gregory, 2014). Nuclear genome size has many correlates (e.g. nucleus and cell sizes, cell cycle duration, invasiveness, rates of development, metabolism, and extinction (Olmo and Morecalchi 1975; Sessions and Larson 1987; Jokusch 1997; Gregory 2003; Gregory 2005), suggesting that genome size impacts both the phenotypes of organisms as well as the evolutionary trajectories of lineages. Explaining genome size diversity across the Tree of Life is a central goal of genome biology (Vinogradov 2004; Oliver et al. 2007).

In 2003, Lynch and Conery proposed the body of theory known as the mutational-hazard hypothesis to explain variation in genome size and complexity (Lynch and Conery 2003). The central premise of the mutational-hazard hypothesis is that non-coding DNA is a mutational liability because it increases the risk of a harmful mutation occurring, whether that be a disruption of an essential genomic function (e.g. disruption of sites required for intron splicing) or a deleterious gain-of-function mutation (e.g. a premature start codon in a 5' UTR) (Lynch 2002; Lynch 2007; Lynch and Walsh 2007; Lynch et al 2011). Therefore, insertion mutations with no immediate fitness effect will still behave in a non-neutral (i.e. deleterious) manner because they increase the mutational hazard.

Under the mutational-hazard hypothesis, the accumulation of non-coding DNA is driven by two factors – effective population size and the mutation rate; the mutation rate (encompassing all forms of mutation in this usage) defines the selective cost of excess DNA, while the effective population size determines the ability of selection to oppose its fixation (Lynch and Walsh

2007). Effective population size (N_e), approximately the number of individuals contributing genes to the next generation, is proportional to the strength of genetic drift. At low N_e, the power of drift can overwhelm selection, and mutations with small fitness effects (specifically, those with selection coefficients $<|1/N_e|$) will behave in a neutral manner (Ohta and Kimura 1971). Thus, variation in effective population size and mutation rate is expected to drive the evolution of genome size. In lineages with low N_e and/or low mutation rates, increased proportions of insertions will be effectively neutral and therefore more likely to drift to fixation, leading to genomic expansion over time. Conversely, in lineages with high N_e and/or high mutation rates, insertions are less likely to be effectively neutral and therefore more likely to be exposed to selection, thus opposing genomic expansion.

Across tetrapod vertebrates, genomic expansion has been most pronounced in salamanders, an amphibian clade composed of 665 extant lineages with genomes ranging from 14 Gb to 120 Gb in size (mean = 35 Gb) (Gregory 2015; AmphibiaWeb 2015). The other two amphibian clades — frogs (Order: Anura) and caecilians (Order: Gymnophiona) — have smaller genomes more comparable in size to the majority of vertebrate genomes; frog genome sizes range from 0.9 Gb to 13 Gb (mean = 4.6 Gb), and caecilian genome sizes range from 3.6 Gb to 14 Gb, although data are limited (Gregory, 2015). Salamanders split from frogs ~300 mya (Zhang et al 2005; Hedges et al 2006; Roelants et al 2007; San Mauro et al 2010). Fossil evidence, along with the nested phylogenetic position of salamanders within the vertebrate clade, indicates that large genome size is a derived trait in salamanders (Organ et al 2011, Laurin et al 2015). The potential causes of this genomic expansion in salamanders can be broadly categorized as (1) changes in the mutational process that lower mutational hazard (e.g. through lower nucleotide substitution rates, lower indel rates, or lower rates of ectopic recombination between

repetitive sequences) and/or change the balance between insertions and deletions (e.g. increased transposable element proliferation, decreased rates of DNA deletion); (2) changes in the selective regime targeting one of the correlates of genome size (e.g. selection on metabolic rate or developmental rate, assuming that genome size is causative in this relationship); or (3) changes in the strength of genetic drift. These potential causes are not mutually exclusive. A growing number of studies have addressed the roles of mutation and selection in shaping genomic expansion in salamanders (Sessions et al 1987; Licht et al 1991; Roth et al 1997; Gregory 2002; Sun et al 2012a,b; Sun and Mueller 2014; Frahry et al 2015). However, to date, no one has tested the hypothesis that unusually strong genetic drift, as a result of low N_e throughout the history of the salamander clade, has contributed to genomic gigantism.

Here, we test the hypothesis that salamanders have experienced stronger persistent, long-term genetic drift than frogs by comparing *dN/dS* and *Kr/Kc* values between these clades (Daubin and Moran 2004; Kuo et al 2009; Whitney and Garland 2010). Our results reject this hypothesis; we find that salamanders have not experienced stronger drift than frogs throughout their evolutionary history. Additionally, we find evidence consistent with a lower nucleotide substitution rate in salamanders. This finding and others (Sun and Mueller 2014; Frahry et al 2015) suggest that a lower mutational hazard may contribute to genome expansion in this clade. This suggests that salamanders provide an important model for the study of how non-drift processes (i.e. mutation, natural selection) shape the evolution of genome size.

METHODS

Dataset

Our dataset comprises transcriptome data from six salamander species and six frog species (Table 1). These species were chosen in order to maximize the sampling of higher-level phylogenetic diversity within Anura and Caudata; in total, five frog families (out of 55) and four salamander families (out of ten) are represented (Amphibiaweb, 2015). The basal phylogenetic split is represented within each clade by inclusion of the most basal lineage (Salamanders – Hynobiidae; Frogs – Bombinatoridae) (Pyron and Wiens 2011). With the exception of *Ambystoma tigrinum,* all sequences were obtained from a single transcriptome sequencing experiment per species. In order to maximize the number of *A. tigrinum* sequences for downstream analysis, sequence data from multiple transcriptomes were pooled with EST sequences downloaded from Genbank.

Contig sequences were downloaded for each species from the sources listed in Table 1 with the exception of *Bombina maxima* and *Ensatina eschscholtzii picta.* For *Bombina maxima,* the reads were downloaded from the NCBI sequence read archive and assembled *de novo* using Trinity (Haas et al 2013) with default parameters. For *Ensatina eschscholtzii picta,* Two adults individuals — one female, one male — were collected in June, 2012 from Crescent City, California (male) and Six Rivers National Park, California (female) and euthanized by immersion in chlorotone following protocols approved by Colorado State University's Institutional Animal Care and Use Committee. RNA was extracted from skin, eyes, brain, liver, tail, testes, and ovaries using TRIzol Reagent (Life Technologies) according to the manufacturer's protocols. RNA quality was assessed using a BioAnalyzer. Samples were DNase

treated (Ambion), and ribosomal RNA was depleted using RiboMinus (InVitrogen). ds-cDNA synthesis was performed using random primers, and TruSeq libraries were constructed using the Apollo library preparation machine and Apollo reagents (IntegenX). Libraries were sequenced on an Illumina MiSeq, 2 x 150 bp reads. Library construction and Illumina sequencing were performed by the Institute for Bioinformatics and Evolutionary Studies (IBEST) at the University of Idaho.)

Data Filtering and Coding Sequence Prediction

The total number of contigs for each species is listed in Table 2. The following steps were performed on the contigs from each species in order to remove redundancy and predict the longest unique protein-coding sequences: 1) CD-HIT-EST (Fu et al 2012) was run with default parameters to remove initial redundancy in the data and increase computational tractability; 2) Transdecoder (Haas et al 2013) with default settings and the PFAM search option for identification of possible protein domains was used to identify protein-coding sequences *de novo*; 3) CD-HIT-EST was run a second time with more stringent parameters in order to eliminate splicing isoforms and other semi-redundant coding sequences. The results of each step for every taxa are presented in Table 2.

Gene Annotation and Construction of Initial Six-taxon and 12-taxon Datasets

To identify orthologs across all 12 species, all remaining contigs were searched against the Uniprot database using BLASTX (e-value < 1×10^{-5}). In total, 22,371 genes received at least one hit. From these genes, two datasets were constructed. The "taxon-rich" dataset included all 12 species (known hereafter as the 12-taxon dataset), while the "gene-rich" dataset included six species – three frogs (*B. maxima, O. margaretae, X. tropicalis*) and three salamanders (*H.*

chinensis, C. pyrrhogaster, A. mexicanum) (known hereafter as the six-taxon dataset). In addition to maximizing the sampling of phylogenetic diversity, the taxa in the six-taxon dataset were chosen based on their relatively high numbers of contigs and average contig length compared to the other taxa. 794 genes received a hit from all taxa in the 12-taxon dataset, and 6,494 genes received a hit from all taxa in the six-taxon dataset. Custom Bash and perl scripts were used to select the longest sequence per taxa for all genes in both datasets.

Overview of Gene Annotation and Alignment Methodology

The deep divergences present among our taxa (Hedges et al 2006), along with the obvious need to analyze high quality alignments of orthologous sequences, informed our annotation and alignment methodology. Initial steps in our pipeline were less stringent (low e-value, default Gblocks parameters) in order to maximize the number of sequences retained. The final steps (alignment filtering steps) in our pipeline were more stringent in order to produce confidence in the quality of the resulting dataset.

Alignment

TranslatorX (Abascal et al 2010) was used to translate sequences into proteins and align the resulting amino acid sequences using MAFFT (Katoh and Standley 2013). Alignments were then cleaned with Gblocks using default parameters (Castresana 2000). Only alignments of at least 210 nucleotides (70 amino acids) were considered.

Branch Length Filtering

Phylogenetic trees for all further analyses were adapted from Pyron and Wiens (2011). To detect improperly aligned sequences and sequences of questionable orthology, branch lengths (nuc. substitutions per codon) were estimated in the CODEML program in PAML (v. 4.7) (Yang

2007) for each alignment. Because misaligned regions and alignments containing paralogous sequences would likely produce excessively long branch/tree lengths, alignments producing outlier branch lengths were removed from further analysis. The following criteria were used to identify and filter out alignments with outlier branch lengths in the 12-taxon dataset: 1) Trees with a sum total of branch lengths longer than 10 were removed, and 2) alignments with a single branch length greater than three were removed. The following criteria were used to identify and filter out alignments with outlier branch lengths in the 6-taxon dataset: 1) Trees with a sum total of branch lengths longer than 15 were removed, and 2) alignments with a single branch length greater than six were removed. These branch/tree length cutoffs were determined empirically for each dataset, taking into account differences in taxon sampling and rates of molecular evolution among taxa.

T-COFFEE Alignment Filtering

To further detect improperly aligned sequences and sequences of questionable orthology, individual sequences in each alignment were given conservation scores using T-COFFEE (Notredame et al 2000). If one sequence in an alignment received a conservation score below 95, the whole alignment was removed from further analysis.

dN/dS Analysis

The ratio of *dN* (nonsynonymous substitution rate) to *dS* (synonymous substitution rate) (*dN/dS*, ω hereafter) can be used to quantify the strength of genetic drift (Eyre-walker et al 2002; Daubin and Moran 2004; Woolfit and Bromham 2005; Popadin et al 2007; Kuo et al 2009). Large ω values (closer to 1) indicate that the efficiency of purifying selection has been limited by strong genetic drift, whereas small ω values (closer to 0) indicate that weak genetic drift is

allowing purifying selection to act efficiently. The hypothesis that salamanders have experienced stronger persistent, long-term genetic drift than frogs would be supported by a pattern of consistently higher ω in salamanders.

For the 12-taxon (212 filtered genes) and six-taxon (3302 filtered genes) datasets, PAML (v. 4.7) (Yang, 2007) was used to estimate the likelihood and ω values of two different models. The null model (the single-ω) model hereafter) estimated one ω value for all salamander and frog branches (8 branches total). The second model (the two- ω model hereafter) estimated two ω values – one for all salamander branches (4 branches) and one for all frog branches (4 branches). Branch lengths and the transition/transversion ratio (κ) were treated as free parameters and estimated separately in each model. Given that these models are nested within each other, a likelihood ratio test (LRT) was used to determine significance. Significant single-ω models support the hypothesis that there are no differences in the strength of genetic drift between frogs and salamanders; a single ω value for both clades best fits the data. Significant two- ω models that estimate a higher ω value for salamanders support the hypothesis that salamanders have experienced stronger genetic drift than frogs throughout their evolutionary histories. ω estimates from extremely short and long branches can be unreliable (Wolf et al 2009); given that the ω estimate for each clade was derived from the sequences of three taxa, it could be problematic for ω estimation if any terminal branch had an extremely high or low amount of synonymous substitutions. Thus, any gene that was estimated to have a *dS* value > 2 or < 0.1 for any individual terminal branch was removed from further analysis in the six-taxon dataset (197 genes removed, 3105 genes remaining). The greater number of taxa (and therefore branches) in the 12-taxon dataset created shorter branches overall and also might be expected to make any one branch less problematic for estimation of a clade-wide ω value; accordingly, many genes had at

least one terminal branch with a *dS* value <0.1 but did not have an unusually high ω. Thus, only genes that were estimated to have a dS value >2 were removed (6 genes removed, 206 genes remaining). Because PAML takes unrooted trees as input for ω analyses, the basal frog branch and the basal salamander branch are jointly estimated as a single branch; because we are testing for differences between frogs and salamanders, this frog + salamander branch was excluded from further analysis.

The two-ω model estimates a single ω value for each of the two focal clades; thus, ω values could be disproportionately affected by one branch with a dramatically higher or lower ω value. To ensure that any detected differences in ω values between salamanders and frogs are clade-wide, rather than due to single outlier branches, we conducted branch model. Genes that were estimated to have any terminal branch with a $dS > 2$ value or < 0.1 were removed from further analysis. Additionally, genes that estimated any terminal branch to have a $\omega > 1$ were also removed as these values are likely an artifact of low *dS* and not indicative of relaxed levels of purifying selection and/or positive selection (Wolf et al 2009) (908 genes removed in total, 2394 genes remaining). The high level of parameterization and shorter branches of the 12-taxon dataset made a significant proportion of the per-branch ω estimates unreliable ($\omega > 1$ or < 0.001); therefore, the branch model analysis was conducted only on the six-taxon dataset.

Functional Enrichment Analyses of ω Estimates

The goal of this study is to test for differences in the efficiency of purifying selection genome-wide due to differences in effective population size (N_e). However, differences in the strength of purifying selection (i.e. selective constraint) between salamanders and frogs would also produce differences in ω between the two clades, independent of any differences in demography. Thus, we tested whether any detected differences in ω were driven by a set of

genes with a particular biological function, as this would suggest differences in selective constraints. The FatiGO tool (Al-Sharour et al 2004) was used with a one-sided Fisher's exact test in the Babelomics platform version 5.0 (Alonso et al 2015) to test whether genes with significant M2 models were enriched in a particular function. Two comparisons were made for the six-taxon dataset: genes indicating significantly higher ω values in salamanders vs. all other genes in the dataset (654 genes vs. 2451 genes) and genes indicating significantly higher ω values in frogs vs. all other genes in the dataset (289 genes vs. 2816 genes). The low number of genes with significantly higher ω for either clade precluded analysis in the 12-taxon dataset. Human GOslim and GO cellular components classifications were used.

Examination of Factors Affecting Reliability of ω Estimates

Much recent work has focused on the dependence of ω estimates on synonymous branch length (*dS*) (Rocha et al 2006; Li et al 2009; Wolf et al 2009; dos Reis and Yang 2013; Mugal 2014), suggesting that comparing taxa with different substitution rates can be problematic. The causes of the relationship between *dS* and ω are unclear; however, evidence suggests that methodology and real biological effects can both play a role, depending on the dataset (Li et al 2009; Wolf et al 2009). Some evidence shows that salamanders and frogs have different substitution rates (Pyron and Wiens 2011; Evans et al 2014), raising the possibility that our ω comparison might be affected. Thus, for each gene in the six-taxon dataset (3105 genes), synonymous branch lengths were summed for frog and salamander branches separately (four branches in each clade). These summed *dS* values were used to 1) test for correlations with the ω values estimated for a given clade from the two-ω model, and 2) filter the dataset to genes in which *dS* values were most similar between the two clades. For the 12-taxon dataset, Summed *dS*

values were tested for correlations with ω values, but the lower number of genes precluded a meaningful filtering of the dataset by *dS* similarity.

Previous work has also indicated that GC content and synonymous codon bias can affect estimates of ω (Bielawski et al 2000; Bierne and Eyre-Walker 2003; Bullaughey et al 2008; Li et al 2009; Weber and Hurst 2009; Bay and Bielawksi 2013). As with the relationship between dS and ω, the causes of the relationships among GC content, codon bias, and ω are unclear and may reflect both real biological effects and methodology (Piganeu et al 2002; Hellman et al 2003; Meunier and Duret 2004; Betancourt et al 2009; Galtier et al 2009; Weber and Hurst 2009; Clement and Ardnt 2013; Gossman et al 2014). GC content and the extent of codon bias in frogs and salamanders are largely unknown, but a slightly higher GC content in salamanders has been suggested by a previous study (Vinogradov 1998). Thus, the codon deviation coefficient (CDC) (Zhang 2012) and GC3 content were calculated for all genes in both datasets using the Codon Analysis Toolkit (Zhang 2012). The codon deviation coefficient is a measure of codon bias that improves upon previous methods by accounting for background nucleotide compositions tailored to specific codon positions (Zhang 2012). For each gene in both datasets, average frog and salamander CDC and GC3 values were calculated by averaging the values for the sequences representing each clade. The average CDC and GC3 values for each clade were used to 1) test for correlations with the ω values estimated for a given clade from the two-ω model, and 2) filter results to genes in which these values were most similar between the clades (only in six-taxon dataset). Again, for the 12-taxon dataset, Average GC3 and CDC values were tested for correlations with ω values, but the lower number of genes precluded a meaningful filtering of the dataset by GC3 and CDC similarity.

Kr/Kc Analysis

The strength of genetic drift can also be quantified by calculating the ratio of radical (*Kr*) to conservative (*Kc*) amino acid substitution rates (Zhang 2000). Amino acids can be classified into different groups based on a physiochemical property (e.g. charge, volume, polarity) or some combination of these properties. A radical substitution is a substitution that results in the replacement of one amino acid by another in a different group (e.g. a non-polar amino acid replacing a polar amino acid), whereas a conservative substitution replaces one amino acid with another in the same group (e.g. a non-polar amino acid replacing another non-polar amino acid). Although it is difficult to predict the effect that any one amino acid substitution may have on protein function, radical substitutions are more likely to be deleterious than conservative ones (Smith 2003). Therefore, *Kr/Kc* can be used in a similar manner to ω because an excess of radical substitutions relative to conservative ones indicates that strong genetic drift has limited the efficiency of purifying selection. In accordance with this notion, *Kr/Kc* is positively correlated with ω (the level of correlation varying based on amino acid classification), and numerous studies have found *Kr/Kc* to be higher in lineages expected to have experienced stronger genetic drift (Zhang 2000; Eyre-Walker et al 2002; Smith 2003; Hanada et al 2007; Wernegreen 2011). In addition, *Kr/Kc* may offer an advantage over ω for some comparisons because it avoids issues associated with the synonymous branch length dependence of ω. The hypothesis that salamanders have experienced stronger persistent, long-term genetic drift than frogs would be supported by a pattern of consistently higher *Kr/Kc* in salamanders.

The low numbers of amino acid substitutions generally present in each alignment yield noisy per-gene estimates of *Kr/Kc* (Weber et al 2014); thus, sets of fifty genes were randomly concatenated 1000 times prior to calculating *Kr/Kc*. Ancestral sequence reconstructions and the

transition/transversion ratio (kappa) were inferred from these concatenated gene sets using the maximum likelihood method implemented in PAML (Yang 2007). *Kr* and *Kc* were estimated for all branches in both datasets using the methodology of Zhang (2000) in the HON-NEW program. Custom Perl scripts (available upon request) were used to parse output files from HON-NEW. All *Kr* and *Kc* values were adjusted with a Jukes-Cantor correction (Jukes and Cantor 1969) using the formula $K_{(corr)} = -0.75* \ln(1-(4/3*K_{(orig)}))$, where $K_{(orig)}$ is the uncorrected value and $K_{(corr)}$ is the corrected value. The shorter branch lengths (lower numbers of amino acid substitutions) and much smaller number of genes (206 genes vs. 3302 genes) in the 12-taxon dataset make it less informative for a *Kr/Kc* analysis than the six-taxon dataset; therefore, the *Kr/Kc* analysis was only conducted with the six-taxon dataset.

Three classification schemes were used to categorize radical and conservative amino acid substitutions: 1) classification by polarity and volume (Miyata et al 1979); 2) classification by charge and aromaticity (Hanada et al 2007); and 3) the classification that maximizes correlation with ω, referred to as the Hanada classification hereafter (Hanada et al 2007). The three classification schemes are presented in Table 3.

Examination of Factors Affecting Reliability of *Kr/Kc* Estimates

Previous work has indicated that levels of amino acid divergence and GC content can affect *Kr/Kc* estimates (Smith 2003; Wernegreen 2011). Because salamanders and frogs may differ in substitution rates (Evans et al 2014), and GC content (Vinogradov 1998), correlations were calculated between *Kr/Kc* estimates based on the polarity and volume classifications and both the conservative substitution rate (*Kc*) and GC content.

RESULTS

Annotation and Alignment

For the six-taxon dataset, average alignment length was 902 base pairs (bp) (median = 744 bp). For the 12-taxon dataset, average alignment length was 417 bp (median = 372 bp). The number of genes removed from the dataset at each step in the annotation and alignment process are summarized in Table 4.

*dN/dS (*ω) Analysis

The results of the single-ω /two-ω model LRT for both datasets are summarized in Table 5. In the 12-taxon dataset, 66.99% (138/206) of the genes indicate that the single-ω model is the best-fitting model. The remaining genes are nearly evenly split between frogs and salamanders having a significantly higher ω value. For 15.05% (31/206) of the genes, the two-ω model is the best fit with salamanders having the higher ω. For 17.96% (37/206) of the genes, the two-ω model is the best fit with frogs having the higher ω. The ω values estimated from the 12-taxon dataset are largely overlapping for salamanders and frogs; the median ω for salamanders is 0.048 ± 0.047 (range (0.0001-0.219) and the median ω value for frogs is 0.046 ± 0.046 (range (0.0001-0.235).

In contrast to the results from the 12-taxon dataset, the results from the six-taxon dataset reveal a pattern of slightly higher ω values for salamanders. The percentage of genes indicating that the single-ω model is the best-fitting model is 69.63% (2162/3105), consistent with the results from the 12-taxon dataset. However, for 21.06% (654/3105) of the genes, the two-ω model is the best fit with salamanders having the higher ω value, whereas only 9.31% (289/3105) of genes show a significantly higher ω value for frogs. The median ω values for the

6-taxon dataset also reveal this slight pattern; the median ω value for salamanders is 0.060 ± 0.050 (range (0.0001-0.338)) and the median ω value for frogs is 0.054 ± 0.040 (range (0.0001-0.330).

Synonymous Branch Lengths and *dN/dS (*ω) Estimates

Synonymous branch lengths for each taxon in the six-taxon dataset are summarized in Table 6. Correlations between summed *dS* and ω for both datasets are presented in Fig. 1. The correlation between *dS* and ω in the six-taxon dataset is significantly negative (Pearson's r = -0.154, $p < 2.2 \times 10^{-16}$), but is non-significant in the 12-taxon dataset (Pearson's r = -0.062, p = 0.213), suggesting that synonymous branch lengths may be affecting our comparison between the two clades in the six-taxon dataset. In accordance with this notion, restricting analysis to only genes that have similar *dS* values between frogs and salamanders (Table 7) reduces the proportion of genes for which salamanders have a significantly higher ω value (salamanders - 11.928%; frogs – 14.911%) and reduces the difference in median ω values between frogs and salamanders (salamanders = 0.051; frogs = 0.054) to the point that frogs actually have slightly higher ω values. Thus, differences in *dS* between frogs and salamanders do appear to strongly impact the results from the six-taxon dataset, contributing to the differences in ω estimates between the two clades in the six-taxon dataset.

GC3 Content, Codon Bias, and ω Estimates

GC3 content and codon deviation coefficients (CDC) for each taxon in both datasets are summarized in Table 8 and Table 9. Correlations between GC3, CDC, and ω for both datasets are presented in Fig. 2 (6-taxon) and Fig. 3 (12-taxon). The relationship between CDC and ω in our dataset is significantly positive in the six-taxon dataset (Pearson's r = 0.111,

2.2 x 10^{-16}), but is non-significant in the 12-taxon dataset (Pearson's r = -0.062, p = 0.212). Considering only genes in which CDC values were most similar between the clades (Table 10) shows a negligible effect on the results relative to those of the total six-taxon dataset, likely because no considerable differences exist between salamanders and frogs in CDC estimates. Thus, codon bias, as measured by CDC, is unlikely to be affecting our comparison in any significant way in either dataset.

In contrast to codon bias, differences in GC content between frogs and salamanders do seem to be having an effect on our comparison. The correlation between GC3 content and ω is significantly negative in the six-taxon dataset (Pearson's r = -0.136, $p < 2.2 \times 10^{-16}$), but is non-significant in the 12-taxon dataset (Pearson's r = -0.0760, p = 0.124). Because salamanders possess higher GC3 than frogs (Table 8 and Table 9), GC content affects our comparison in the opposite direction of *dS*. Restricting our analysis to only genes with similar GC3 content between frogs and salamanders (Table 11) increases the proportion of genes for which salamanders have a higher ω value (salamanders - 26.0397%; frogs – 7.7757%) and increases the difference in median ω values between the two clades (salamanders - 0.061, frogs - 0.050). Thus, differences in GC3 content between frogs and salamanders do appear to impact the results from the six-taxon dataset, masking differences in ω between the two clades.

Branch Model Analyses

The results of the branch model analysis for the six-taxon dataset are presented in Table 12. Overall, these results indicate that the two-ω model estimates are not disproportionately affected by an outlier branch with a drastically higher or lower ω value. Rather, the difference in ω between frogs and salamander in the two-ω models appears to reflect a consistent difference between all branches in the two clades. These results are also consistent with *dS* strongly

affecting estimates of ω. The internal branches within both clades are by far the shortest branches in the phylogeny, and they also have the highest estimates of ω by a large margin – consistent with the negative correlation between *dS* and ω described above.

Functional Enrichment Analyses

The results of the functional enrichment analyses are presented in Table 13. Two GO categories are over-represented in the subset of genes for which salamanders have higher ω values than frogs. Six categories are over-represented in the subset of genes for which frogs have higher ω than salamanders. Analysis of a subset of the total dataset in which the genes annotated to these functionally enriched categories are removed (1015 genes removed, 2090 remaining) shows a slight decrease in the proportion of genes for which frogs have a higher ω value (salamanders – 21.43%, frogs – 7.65%) and an slight increase in the difference in median ω values between the two clades (salamanders - 0.064, frogs - 0.055). However, it does not appear that any differences in selective constraint between frogs and salamanders are having a large effect on the results of the six-taxon dataset.

Kr/Kc Analysis

The results of the *Kr/Kc* analysis are presented in Table 14. Correlations between Kc, GC content, and *Kr/Kc* are presented in Fig. 4. Overall, the *Kr/Kc* results do not suggest that there are persistent, long-term differences in the strength of genetic drift between frogs and salamanders. For all three classifications, the *Kr/Kc* values for the terminal branches of frogs and salamanders are very similar to one another, with frog *Kr/Kc* estimates being approximately ~ 0.03 - 0.05 higher. The two internal branches for each clade produce lower *Kr/Kc* values than the terminal branches, with the frog internal branch in particular being approximately ~0.1 lower

than all other *Kr/Kc* values. Fig. 4 shows that GC content is significantly negatively correlated with *Kr/Kc* (Pearson's $r = -0.238$, $p < 2.2 \times 10^{-16}$), while the conservative branch length (*Kc*) is significantly positively correlated with *Kr/Kc* (Pearson's $r = 0.528$, $p < 2.2 \times 10^{-16}$). The negative correlation between GC content and *Kr/Kc* mirrors that seen with GC3 content and ω in the six-taxon ω comparison; in contrast, the positive correlation between Kc and *Kr/Kc* is opposite to the correlation found between ω and its denominator, *dS* (negative correlation). These opposing correlations are mirrored by the opposing patterns in the results of the two comparisons; salamanders had slightly higher ω values while frogs have slightly higher *Kr/Kc* values. This observation suggests that the higher *Kr/Kc* values of frogs are a reflection of their higher *Kc* values. Further supporting this notion are the lower *Kr/Kc* values of the internal branches in both clades as these branches are both substantially shorter (lower Kc) than the terminal branches. As with the relationships between *dS*, GC3, and ω, the causes of the relationships among *Kc*, GC content, and *Kr/Kc* are unclear and may reflect both real biological effects and methodological bias.

DISCUSSION

Our results represent the first in-depth comparative analysis of the efficiency of purifying selection — a function of the strength of genetic drift — between salamanders and other vertebrates with more typical (i.e. smaller) genome sizes. The three primary results of this study are 1) a comparison of ω estimates from six frog and six salamander taxa for 206 genes (12-taxon dataset), 2) a comparison of ω estimates from three frog and three salamander taxa for 3105 genes (six-taxon dataset), and 3) a comparison of *Kr/Kc* estimates originating from the 3105 genes in the six-taxon dataset. Stronger genetic drift in salamanders relative to frogs would be reflected in less efficient purifying selection across genes, which, in turn, would be reflected in consistently higher ω and *Kr/Kc* values. Our results do not show this pattern. Our results do show differences in ω and *Kr/Kc* between salamanders and frogs, but these differences are in opposite directions and slight in magnitude and may be best explained by other factors aside from differing strengths of genetic drift. Taking these results in conjunction, no clear pattern emerges that would allow us to conclude any difference exists in the strength of genetic drift between frogs and salamanders.

The three primary results of this study reveal contrasting patterns – 1) The ω values for frogs and salamanders (i.e. proportions of genes for which each clade had the significantly higher ω value in the two-ω models, median ω) are nearly identical in the 12-taxon dataset, 2) The ω values for salamanders are higher in the 6-taxon dataset, and 3) The *Kr/Kc* values for frogs are higher in the concatenated gene sets derived from the 6-taxon dataset. The pattern of higher ω values for salamanders in the 6-taxon dataset is the only result of the three that supports the hypothesis of stronger genetic drift in salamanders. However, this pattern is very slight

(difference in median ω between frogs and salamanders is ~ 0.06), and when considered with the conflicting results of the 12-taxon ω comparison and the *Kr/Kc* comparison, it seems likely that methodological bias and/or other biological factors aside from genetic drift are responsible for this pattern. Accordingly, several lines of evidence support this interpretation.

Synonymous branch lengths were found to be significantly negatively correlated with ω in the six-taxon dataset (Pearson's r = -0.154, $p < 2.2 \times 10^{-16}$). Given that *dS* was shorter in salamanders than in frogs, controlling for this difference removed the pattern of slightly higher ω in salamanders and rendered ω nearly identical between the two clades. This parallels the results of the 12-taxon dataset which demonstrated highly similar ω values between frogs and salamanders and a non-significant correlation between *dS* and ω. The origin of the relationship between *dS* and ω is unclear, but as noted above, it is likely some combination of methodology and real biological phenomenon (Rocha et al 2006, Li et al 2009, Wolf et al 2009, dos Reis 2013, Mugal 2014). For very closely related lineages, inclusion of segregating non-synonymous polymorphisms as fixed differences between lineages may cause higher estimates of ω; this likely has a negligible effect on our dataset, given the deep divergences present among our taxa (Zhang et al 2005; Hedges et al 2006; Roelants et al 2007; San Mauro et al 2010 ; Irisarri et al 2012). Other proposed biological explanations center around epistatic and mutation rate effects (e.g. higher mutation rates increase the potential for adaptation) (Wyckoff et al 2005, Wolf et al 2009, Weber et al 2014). Methodological explanations also clearly play a role as different algorithms and substitutions models can affect the direction and strength of the relationship (Li et al 2009, Dos Reis and Yang 2013). Finally, this relationship may also be inherent to taking the ratio of two randomly distributed variables that are nonlinearly correlated (Wolf et al 2009).

GC content, as measured by GC3 content, also appears to have affected the ω comparison in the six-taxon dataset. A significant negative correlation between GC3 content and ω was found (Pearson's r = -0.136, $p < 2.2 \times 10^{-16}$); however, the higher GC3 content of salamanders meant that controlling for GC3 content actually had the opposite effect of controlling for dS and resulted in an increased difference in ω between salamanders and frogs. The correlation between GC3 and ω was non-significant in the 12-taxon dataset, suggesting a negligible effect of GC content on the ω comparison in this dataset. The interpretation and relative importance of this effect in the six-taxon dataset is unclear. It is possible that in attempting to control for GC3 content we are selecting a subset of genes that are under different evolutionary forces in the two clades; GC content and the strength of purifying selection are related in complex ways, mediated through the effects of recombination, GC-biased gene conversion, non-equilibrium GC dynamics, CpG sites, and double stranded-breaks (Piganeu et al 2002, Hellman et al 2003, Meunier & Duret 2004, Betancourt et al 2009, Galtier et al 2009, Weber & Hurst 2009, Clement & Ardnt 2013, Gossman et al 2014). It has also been shown that the relationship between ω, dS, and GC content can vary by method of ω estimation, substitution model, and evolutionary lineage (warm or cold-blooded) (Li et al 2009). Finally, it should be noted that two of the taxa included in the six-taxon dataset are outliers in terms of average GC content for their respective clades; *H. chinensis (* 0.551) has the highest average GC3 by a significant margin, while *B. maxima* (0.441) has the lowest average GC3 by a similar margin. Thus, the difference in GC3 between frogs and salamanders as measured in the six-taxon dataset may not be truly representative of the two clades in general.

The results of the *Kr/Kc* analysis further support the notion that the pattern of slightly higher ω in salamanders in the six-taxon dataset is caused by factors other than differing

strengths of genetic drift. *Kr/Kc* values are highly similar between frogs and salamanders, but a slight pattern of higher *Kr/Kc* values for frogs is apparent – opposite to the pattern in the six-taxon ω comparison. This opposing pattern is mirrored by an opposite effect of the denominator, Kc (conservative branch length), on *Kr/Kc* estimation; Kc shows a weak positive correlation with *Kr/Kc* (Pearson's $r = 0.528$, $p < 2.2 \times 10^{-16}$) (*dS* has a negative relationship with ω), indicating that the slightly higher *Kr/Kc* of frogs is caused by their higher *Kc*. The opposing results and biases seen in the six-taxon ω comparison and the *Kr/Kc* comparison favor the interpretation that neither is reflective of a true difference in the strength of genetic drift, particularly in light of the results and interpretation of the 12-taxon ω comparison. It is interesting to note that there is precedence for a discordance between ω and *Kr/Kc*. Weber et al (2014) found that, in accordance with theoretical expectations, larger birds, expected to have smaller effective population sizes, were found to have higher *Kr/Kc* values than smaller birds; however, contrary to theoretical expectations, larger birds had lower ω values. The interpretation of their results is not entirely clear, but it may indicate that *Kr/Kc* (due to the sensitivity of ω to *dS*, GC content, and other factors) is actually a better indicator of the long-term strength of genetic drift in a lineage.

The mutational-hazard hypothesis proposes that two variables drive the evolution of genome size – effective population size and mutation rate. Our results demonstrate that there are no persistent, long-term differences in the strength of genetic drift between frogs and salamanders; it is therefore unlikely that an unusually low effective population size throughout the evolutionary history of salamanders is causing their genomic gigantism. However, our results are consistent with lower nucleotide substitution (point mutation) rates in salamanders. This suggests that mutational hazard may be lower in salamanders; non-coding sequence will experience fewer nucleotide substitutions and thus be less likely to incur a substitution that has a

deleterious effect. A lower mutational hazard indicates that insertions of non-coding DNA are less likely to be opposed by selection in salamanders, and thus more likely to accumulate and lead to genomic expansion.

Synonymous branch lengths (nucleotide substitutions per synonymous site) are, averaged across all genes in our dataset, shorter in salamanders compared to frogs. The large-scale comparison of frog and salamander substitution rates in Evans et al (2014) and the amphibian phylogeny of Pyron and Wiens (2011) also corroborate this result using different methodologies. Given that divergences times among the frog taxa and the salamander taxa in our study are very similar (Zhang et al 2005; Roelants et al 2007; San Mauro et al 2010; Hedges et al 2011, Irisarri et al 2012), this result implies a slower nucleotide substitution rate in salamanders. Inferring nucleotide substitution rate from the accumulation of substitutions at synonymous sites can be problematic because the assumption that synonymous sites evolve neutrally is often violated; however, the equal levels of codon bias between frogs and salamanders in our study suggest that any non-neutral evolution at synonymous sites will likely have a negligible effect on this inference. Salamanders have lower metabolic rates than frogs, and the lowest among tetrapods overall (Gatten et al 1992; Chong and Mueller 2013). The precise mechanism of the relationship is unknown, but metabolic rate is correlated with nucleotide substitution rates in a wide variety of taxa, including amphibians (Martin and Palumbi 1993; Gilooly et al 2005; Baer et al 2007; Santos 2012). Salamanders also have lower rates of small deletions (<30 bp) and smaller indels on average relative to other vertebrates (Sun et al 2012b). The size and abundance of indels is correlated with increased nucleotide substitution in adjacent sequence (Tian et al 2008, De and Babu 2009, Hollister et al 2009, Zhu et al 2009), suggesting that lower indel rates could either directly or indirectly lower the nucleotide substitution rate in salamanders. Additionally, the

lower rate of small deletions and smaller size of indels in salamanders further lowers the mutational hazard of non-coding sequence aside from any specific effect on nucleotide substitution rates.

Transposable elements (TEs) may also have a lower mutational hazard in salamanders relative to other vertebrates. Transposable elem0ents, and other types of repetitive DNA, make up the bulk of many eukaryotic genomes, including salamanders (Pritham 2009; Venner et al 2009, Sun et al. 2012a); consequently, insertion and deletion of TEs is a primary factor in genome size evolution (Gregory 2005; Vitte and Panaud 2005; Sun et al 2012a; Agren and Wright 2011). The propensity to undergo ectopic recombination, and the concomitant increase in large deletions and chromosomal rearrangements, is one of the primary causes for the negative fitness effects of repetitive sequences (Petrov et al 2003; Furano et al 2004; Song and Boissinot 2007; Delprat et al 2009; Petrov et al 2011). Frahry et al (2015) recently demonstrated that LTR retrotransposons in salamanders undergo lower rates of ectopic recombination relative to other vertebrates, thereby suggesting that a major class of insertion mutations in salamanders may be less deleterious. A related, but untested possibility, is that LTR retrotransposons may be less deleterious in some lineages because of differences in their propensity to target "safe havens" (i.e. non-functional sequence) for insertion (Gao et al 2008).

The exceptionally large genomes of salamanders have long attracted interest as a system for studying the causes and consequences of genome size evolution (Sessions and Larson 1987, Wiggers and Roth 1991, Hanken and Wake 1993, Roth et al 1997, Jockusch 1997; Mueller et al 2008, Wake 2009). Our results indicate that the evolution of genomic gigantism in salamanders is not driven by long-term differences in the strength of genetic drift which would accompany long-term reductions in Ne relative to other taxa with smaller genomes. Our results also suggest

that salamanders may have lower rates of nucleotide substitution than other taxa with smaller genomes (i.e. frogs), consistent with (1) previous studies, as well as (2) predictions based on the relationship between metabolic rate and mutation rate. Combined with previous work showing that rates of small deletions and large deletions (as caused by ectopic recombination) are relatively low in salamanders, we hypothesize that lower mutational hazard may contribute to genome expansion in this clade. More generally, this study further underscores the importance of studying large genomes and suggests that salamanders provide an important model for studying how variation in the other forces that shape genome size — mutation and selection — yield changes in overall genome content.

TABLES

Table 1. The taxa and sources for the sequences used in this study. For Xenopus laevis and Xenopus tropicalis, coding sequences were download from Xenbase; the version refers to the genome release from which the sequences were derived.

Order	Family	Species	Data Source
Anura	Bombinatoridae	*Bombina maxima**	Zhou et al 2014
Anura	Hylidae	*Pseudacris regilla*	Robertson and Cornman 2014
Anura	Pipidae	*Xenopus laevis*	Xernbase(v8.0)
Anura	Pipidae	*Xenopus tropicalis**	Xenbase (v7.1)
Anura	Ranidae	*Lithobates clamitans*	Robertson and Cornman 2014
Anura	Ranidae	*Odorrana margaretae**	Qiao et al 2013
Caudata	Ambystomatidae	*Ambystoma mexicanum**	Reads – Stewart et al 2013, Assembly – Li et al 2014
Caudata	Ambystomatidae	*Ambystoma tigrinum*	Doyle et al 2013, Smith et al 2005, Putta et al 2004
Caudata	Hynobiidae	*Hynobius chinensis**	Che et al 2014
Caudata	Plethondontidae	*Ensatina eschscholtzii picta*	Personal Data
Caudata	Salamandridae	*Cynops pyrrhogaster**	Nakamura et al 2014
Caudata	Salamandridae	*Notophthalmus viridescens*	Looso et al 2013

Table 2. The number of sequences remaining after each step of redundancy filtering (CD-HIT) and coding sequence prediction (Transdecoder). The final step was the second and more stringent CD-HIT filter; these sequences (numbers in bold) were used for the BLASTX search against Uniprot and are the sequences for which the average length is reported. † The *Ambystoma tigrinum* sequence contained ESTs and contigs from transcriptomes (42,333 contigs, 20,495 ESTs). ‡ CD-HIT previously run on downloaded Lithobates and Pseudacris sequences so these sequences did not decrease in number after the initial CD-HIT filter.

Taxon	Sequences	CD-HIT (default)	Transdecoder	CD-HIT (stringent)	Average Length (bp)
Salamanders					
H. chinensis†	146142	139731	38586	**36819**	783
A. mexicanum	173130	140606	32485	**28098**	690
A. tigrinum	†62828	42183	10744	**8330**	530
C. pyrrhogaster	237120	202087	52016	**41382**	979
N. viridescens	56401	53790	32856	**27954**	715
E. picta	17958	17659	4702	**4492**	556
Frogs					
B. maxima	155212	148470	30495	**27931**	930
X. tropicalis	22718	20012	20246	**17915**	1690
X. laevis	62823	51289	40671	**30019**	1434
P. regilla	48213	‡48213	18822	**18399**	574
L. clamitans	50238	‡50238	17673	**17915**	575
O. margaretae†	37906	35903	19896	**18543**	1062

Table 3. The three radical/conservative classifications utilized in the *Kr/Kc* analysis.

Radical/Conservative Classifications	
Polarity and Volume Classification	
Special	C
Neutral and small	A G P S T
Polar and relatively small	N D Q E
Polar and relatively large	R H K
Nonpolar and relatively small	I L M V
Nonpolar and relatively large	F W Y
Charge and Aromatic Classification	
Acidic	D E
Neutral and no aromaticity	Q A V L I C S T N G P M
Neutral and aromaticity	F W Y
Basic	K R H
Hanada Classification (maximization of *dN/dS* correlation)	
Neutral and small	A N C G P S T
Neutral and large	I L M V
Basic acid, aromaticity, and relatively small	R Q H K F W Y
Acidic charge and relatively large	D E

Table 4. The number of genes remaining after each annotation and alignment step. Sequences were annotated by a BLASTX (e-value < 1 x 10-5) search against the Uniprot sequences. A gene is considered annotated if it was found to have at least one sequence for each taxa in the given dataset. The longest sequence per taxa was chosen and aligned with MAFFT; only alignments longer than 210bp (70AA) were considered. Alignments that produced excessively long branch lengths (derived from PAML) were then removed as this would likely indicate paralagous/misaligned sequences. T-COFFEE conservation scores were then used to score alignments and find smaller regions of misaligned sequences; an alignment was removed from further analysis if a sequence contain therein had a conservation score <95. Lastly, an alignment was removed from further analysis if it produced a synonymous branch length (*dS*) >2 or less <0.1 in the six-taxon dataset and a *dS* >2 in the 12-taxon dataset.

Dataset	Annotated Genes	Alignments (>210bp)	Branch length filtering	T-COFFEE filter	*dS* filter
Six-taxon	6493	5137	4498	3302	3105
12-taxon	794	340	304	212	206

Table 5. The results of the ω analysis for both datasets. Likelihood ratio tests were carried out to determine significance between a single-ω model (one ω for all taxa) and a two-ω model which estimated one ω value for frogs and one for salamanders. Significant two-ω models could either indicate frogs or salamanders as having the higher ω value. Median ω values were calculated from all genes in a given dataset (six-taxon – 3105 genes, 12-taxon – 206 genes)

***dN/dS* (ω) analysis**	**6-taxon dataset**	**12-taxon dataset**
Alignments	3105	206
Single-*ω*	69.63% (2162/3105)	66.99% (68/206)
Two-ω – Salamanders	21.06% (654/3105)	15.05% (31/206)
Two-*ω* – Frogs	9.31% (289/3105)	17.96% (37/206)
Median Salamander *ω* (SD)	0.06 (0.050)	0.048 (0.047)
Median Frog *ω* (SD)	0.054 (0.040)	0.046 (0.046)

Table 6. The median *dS* values (substitutions per synonymous site) and standard deviations for each branch in the six-taxon dataset.

Taxa	Median *dS* (SD)
Salamanders	
A. mexicanum	0.351 (0.132)
C. pyrrhogaster	0.358 (0.129)
H. chinensis	0.279 (0.148)
Internal branch	0.109 (0.113)
Frogs	
B. maxima	0.555 (0.242)
O. margaretae	0.727 (0.294)
X. tropicalis	0.642 (0.273)
Internal branch	0.116 (0.136)

Table 7. The results of the ω analysis filtered by Σ*dS* similarity between frogs and salamanders. The results of the full six-taxon dataset are presented for comparison. The average difference in Σ*dS* between salamanders and frogs in the full dataset is 1.02 (frogs >). Alignments were filtered by similarity in Σ*dS* between salamanders and frogs; only genes with a difference in Σ*d*S < 0.5 were considered (503 genes). The average difference in Σ*dS* between salamanders and frogs in the filtered subset dataset is 0.260 (frogs >).

***dN/dS* (ω) analysis**	**6-taxon dataset (1.02)**	**Σ*dS* similarity filter (0.260)**
Alignments	3105	503
Single-*ω*	69.63% (2162/3105)	73.16% (368/503)
Two-ω – Salamanders	21.06% (654/3105)	11.93% (60/503)
Two-*ω* – Frogs	9.31% (289/3105)	14.91% (75/503)
Median Salamander *ω*	0.060	0.051
Median Frog *ω*	0.054	0.054

Table 8. The average GC3 and CDC (codon deviation coefficient) for each taxon across the 206 genes in the 12-taxon dataset.

Taxa	GC3 Average	CDC Average
Salamanders		
A. mexicanum	0.490	0.186
A. tigrinum	0.489	0.187
C. pyrrhogaster	0.489	0.187
H. chinensis	0.521	0.186
N. viridescens	0.483	0.185
E. eschscholtzii picta	0.479	0.185
Frogs		
B. maxima	0.444	0.192
L. clamitans	0.478	0.194
O. margaretae	0.484	0.193
P. regilla	0.502	0.193
X. laevis	0.460	0.192
X. tropicalis	0.475	0.189

Table 9. The average GC3 and CDC (codon deviation coefficient) for each taxon across the 3105 genes in the six-taxon dataset.

Taxa	GC3 Average	CDC Average
Salamanders		
A. mexicanum	0.521	0.141
C. pyrrhogaster	0.512	0.141
H. chinensis	0.552	0.140
Frogs		
B. maxima	0.441	0.141
O. margaretae	0.492	0.143
X. tropicalis	0.478	0.142

Table 10. The results of the ω analysis filtered by average CDC (codon deviation coefficient) similarity between frogs and salamanders with the results of the full six-taxon dataset presented for comparison. Average CDC represents the average CDC of the three sequences in the given clade. The difference (for a given alignment) in average CDC between salamanders and frogs averaged across all alignments in the full dataset is 0.002 (frogs >). Genes were filtered by similarity in average CDC between salamanders and frogs; only genes with a difference in average CDC of <0.01 were considered (1106 alignments). The difference (for a given alignment) in average CDC between salamanders and frogs averaged across all alignments in the full dataset is 0.0004 (frogs >).

***dN/dS* (ω) analysis**	**6-taxon dataset (0.002)**	**CDC similarity filter (0.0004)**
Alignments	3105	1493
Single-*ω*	69.63% (2162/3105)	67.92% (1014/1493)
Two-ω – Salamanders	21.06% (654/3105)	22.23% (332/1493)
Two-*ω* – Frogs	9.31% (289/3105)	9.85% (147/1493)
Median Salamander *ω*	0.060	0.058
Median Frog *ω*	0.054	0.052

Table 11. The results of the ω analysis filtered by average GC3 content similarity between frogs and salamanders with the results of the full six-taxon dataset presented for comparison. Average GC3 represents the average GC3 of the three sequences in the given clade. The difference (for a given alignment) in average GC3 between salamanders and frogs averaged across all alignments in the full dataset is 0.058 (salamanders >). Genes were filtered by similarity in average GC3 between salamanders and frogs; only genes with a difference in average GC3 of <0.05 were considered (1106 alignments). The difference (for a given alignment) in average GC3 between salamanders and frogs averaged across all alignments in the full dataset is 0.006 (salamanders >).

***dN/dS* (ω) analysis**	**6-taxon dataset (0.058)**	**GC3 similarity filter (0.006)**
Alignments	3105	1106
Single-*ω*	69.63% (2162/3105)	66.18% (732/1106)
Two-ω – Salamanders	21.06% (654/3105)	26.04% (288/1106)
Two-*ω* – Frogs	9.31% (289/3105)	7.78% (86/1106)
Median Salamander *ω*	0.06	0.061
Median Frog *ω*	0.054	0.050

Table 12. The results of the branch model analysis. ω was estimated separately for each branch in the phylogeny.

Taxa	Median ω	Median *dS*
Salamanders		
A. mexicanum	0.061	0.365
C. pyrrhogaster	0.061	0.369
H. chinensis	0.071	0.291
Internal branch	0.015	0.071
Frogs		
B. maxima	0.055	0.569
O. margaretae	0.054	0.752
X. tropicalis	0.062	0.636
Internal branch	0.018	0.044

Table 13. The results of the ω analysis with over-represented GO categories removed presented along the results of the full six-taxon dataset for comparison. Eight GO categories were found to be over-represented in genes with significant two-omega models for salamanders or frogs; all genes in these GO categories were removed (1015 genes removed, 2090 remaining).

***dN/dS* (ω) analysis**	**6-taxon dataset**	**Enriched GO categories removed**
Alignments	3105	2090
Single-*ω*	69.63% (2162/3105)	70.91% (1482/2090)
Two-ω – Salamanders	21.06% (654/3105)	21.40% (448/2090)
Two-*ω* – Frogs	9.31% (289/3105)	7.66% (160/2090)
Median Salamander *ω*	0.06	0.065
Median Frog *ω*	0.054	0.055

Table 14. The results of the *Kr/Kc* analysis for all three classifications. All values represent the average of the *Kr/Kc* values from the 1000 concatenated gene sets. The polarity and volume classification was used to test for correlations between *Kr/Kc* and *Kc*; the average polarity and volume *Kc* value for each taxa is presented in the parentheses.

Taxa	Polarity and Volume (*Kc*)	Charge and Aromaticity	Hanada (*dN/dS* maximization)
Salamanders			
A. mexicanum	0.413 (0.041)	0.457	0.371
C. pyrrhogaster	0.395 (0.042)	0.428	0.352
H. chinensis	0.408 (0.035)	0.456	0.367
Internal branch	0.389 (0.015)	0.420	0.334
Frogs			
B. maxima	0.455 (0.055)	0.521	0.411
O. margaretae	0.425 (0.075)	0.496	0.383
X. tropicalis	0.419 (0.069)	0.474	0.379
Internal branch	0.335 (0.014)	0.354	0.289

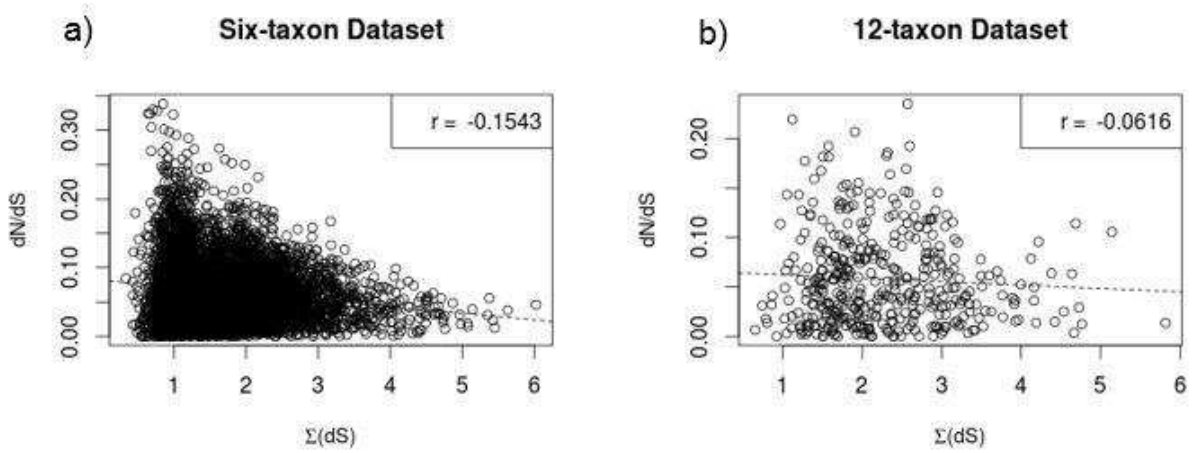

Figure 1. The relationship between Σ*dS* and ω (*dN/dS*) for the two-ω six-taxon dataset (a) and the 12-taxon dataset (b). For each two-ω model in both datasets, synonymous branch lengths (*dS*) were summed for frog and salamander branches separately and these values were correlated with the ω values estimated from the two-ω model for that given clade (six-taxon dataset – 6210 points, 12-taxon dataset - 412 points). The relationship between Σ*dS* and ω in the six-taxon dataset is negative and significant (Pearson's r = -0.1543, $p < 2.2 \times 10^{-16}$). The relationship between Σ*dS* and ω in the 12-taxon dataset is negative, but non-significant (Pearson's *r* = -0.062, p = 0.213).

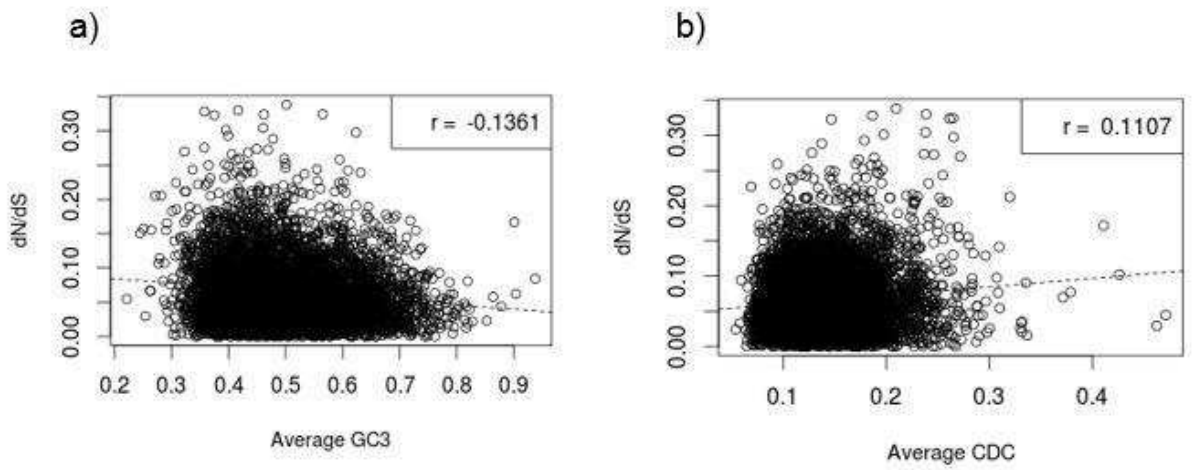

Figure 2. The relationship between average GC3 content and ω (*dN/dS*) (a) and average CDC (codon deviation coefficient) and ω (*dN/dS*) (b) for the six-taxon dataset. Average GC3 and CDC values were calculated by averaging the values from all the sequences representing a clade (three sequences each for frogs and salamanders). These values were correlated with the ω values estimated from the two-ω model for that given clade (6210 points). (a) The relationship between average GC3 and ω is dataset is negative and significant (Pearson's $r = -0.1361$, $p < 2.2 \times 10^{-16}$). (b) The relationship between average CDC and ω is dataset is positive and significant (Pearson's $r = 0.1107$, $p < 2.2 \times 10^{-16}$).

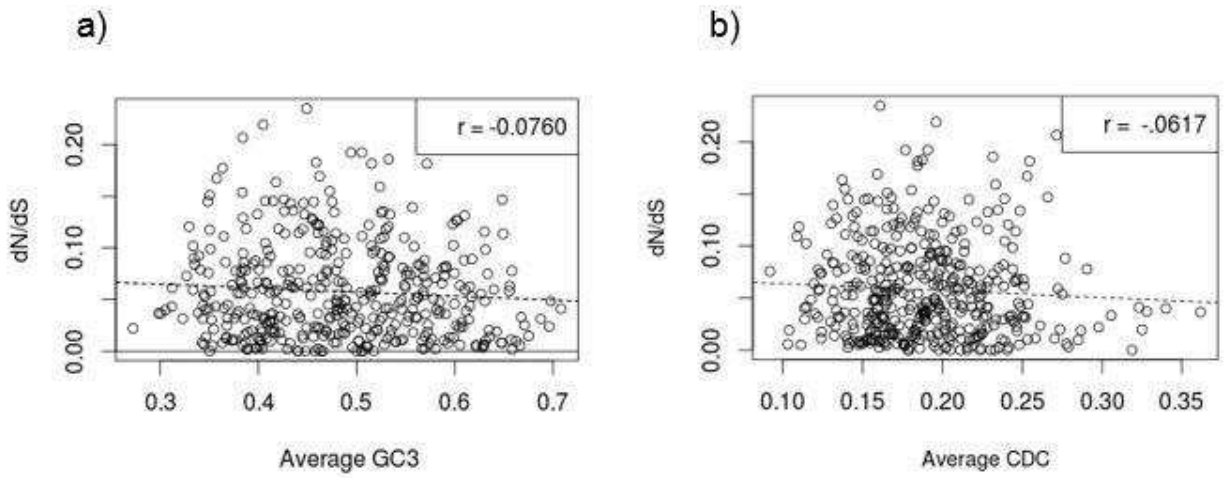

Figure 3. The relationship between average GC3 content and ω (*dN/dS*) (a) and average CDC (codon deviation coefficient) and ω (*dN/dS*) (b) for the 12-taxon dataset. Average GC3 and CDC values were calculated by averaging the values from all the sequences representing a clade (six sequences each for frogs and salamanders). These values were correlated with the ω values estimated from the two-ω model for that given clade (412 points). (a) The relationship between average GC3 and ω is dataset is negative and non-significant (Pearson's r = -0.0760, p = 0.124). (b) The relationship between average CDC and ω is dataset is negative and non-significant (Pearson's r = -0.0617, p = 0.212).

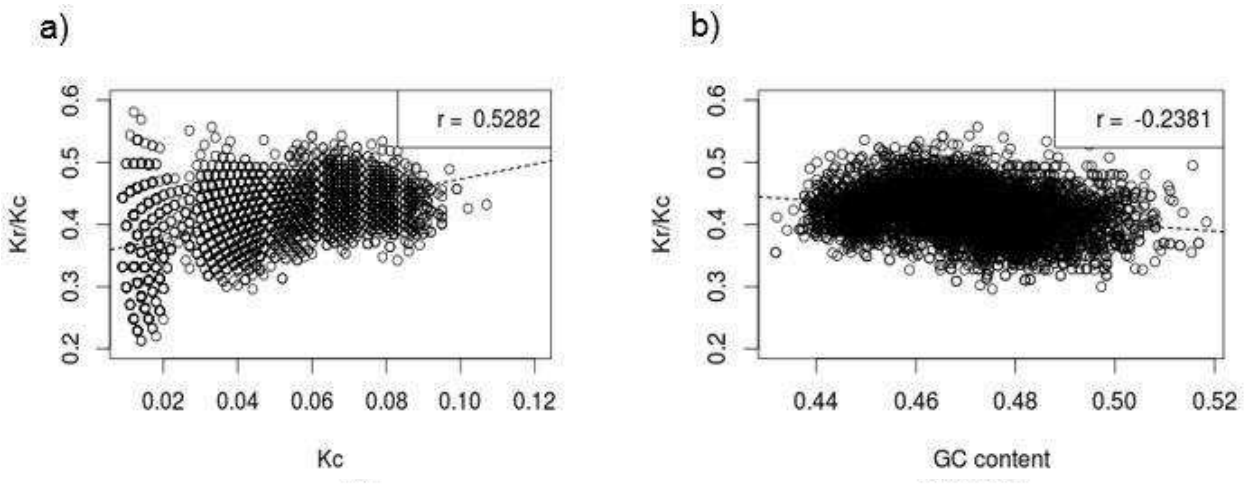

Figure 4. The relationship between average *Kc* and *Kr/Kc* (a) and GC content and *Kr/Kc* (b) for the 1000 concatenated gene sets derived from the six-taxon dataset. *Kc* values and GC content was correlated with *Kr/Kc* values for every branch in the phylogeny (eight branches total – four branches from both clades, 8000 total points). The relationship between *Kc* and *Kr/Kc* is positive and significant (Pearson's $r = 0.5282$, $p < 2.2 \times 10^{-16}$). The relationship between GC content and *Kr/Kc* in the 12-taxon dataset is negative and significant (Pearson's $r = -0.2381$, $p < 2.2 \times 10^{-16}$).

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