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Evolutionary cytogenetics in salamanders

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Abstract

Salamanders (Amphibia: Caudata/Urodela) have been the subject of numerous cytogenetic studies, and data on karyotypes and genome sizes are available for most groups. Salamanders show a more-or-less distinct dichotomy between families with large chromosome numbers and interspecific variation in chromosome number, relative size, and shape (i.e. position of the centromere), and those that exhibit very little variation in these karyological features. This dichotomy is the basis of a major model of karyotype evolution in salamanders involving a kind of 'karyotypic orthoselection'. Salamanders are also characterized by extremely large genomes (in terms of absolute mass of nuclear DNA) and extensive variation in genome size (and overall size of the chromosomes), which transcends variation in chromosome number and shape. The biological significance and evolution of chromosome number and shape within the karyotype is not yet understood, but genome size variation has been found to have strong phenotypic, biogeographic, and phylogenetic correlates that reveal information about the biological significance of this cytogenetic variable. Urodeles also present the advantage of only 10 families and less than 600 species, which facilitates the analysis of patterns within the entire order. The purpose of this review is to present a summary of what is currently known about overall patterns of variation in karyology and genome size in salamanders. These patterns are discussed within an evolutionary context.

Karyology

Chromosome number and shape

Recent morphological and molecular phylogenetic analyses identify 10 families of salamanders (Order Caudata; Larson & Dimmick 1993, Weins *et al*. 2005). Karyological analyses split these salamander families into two groups on the basis of chromosome numbers and shape (Figures 1 and 2). The majority (60%) of salamander families are karyologically uniform with diploid chromosome numbers between 22 and 28 and (with a few exceptions) completely bi-armed chromosomes, while four families have much larger chromosome numbers and also show much more extensive variation in chromosome number and shape between species within and/or between families and even within genera (Figure 2; Morescalchi 1973, 1975, Green & Sessions 1991, 2007).

The large-chromosome-number group, with diploid numbers ranging from 38 to 78 chromosomes of diverse size and shape (Figures 1 and 2), includes the mainly east Asian Hynobiidae (2n=40–78), the east Asian/eastern North American Cryptobranchidae (2n=60), the south-eastern N. American Sirenidae (2n=46–64), and the family Proteidae (eastern N. American and eastern Europe) (2n=38). The hynobiids are not only the most speciose of these groups but are chromosomally by far the most diverse family in the order Caudata, representing the entire range of chromosome numbers in the high-number groups. Within the Hynobiidae the largest

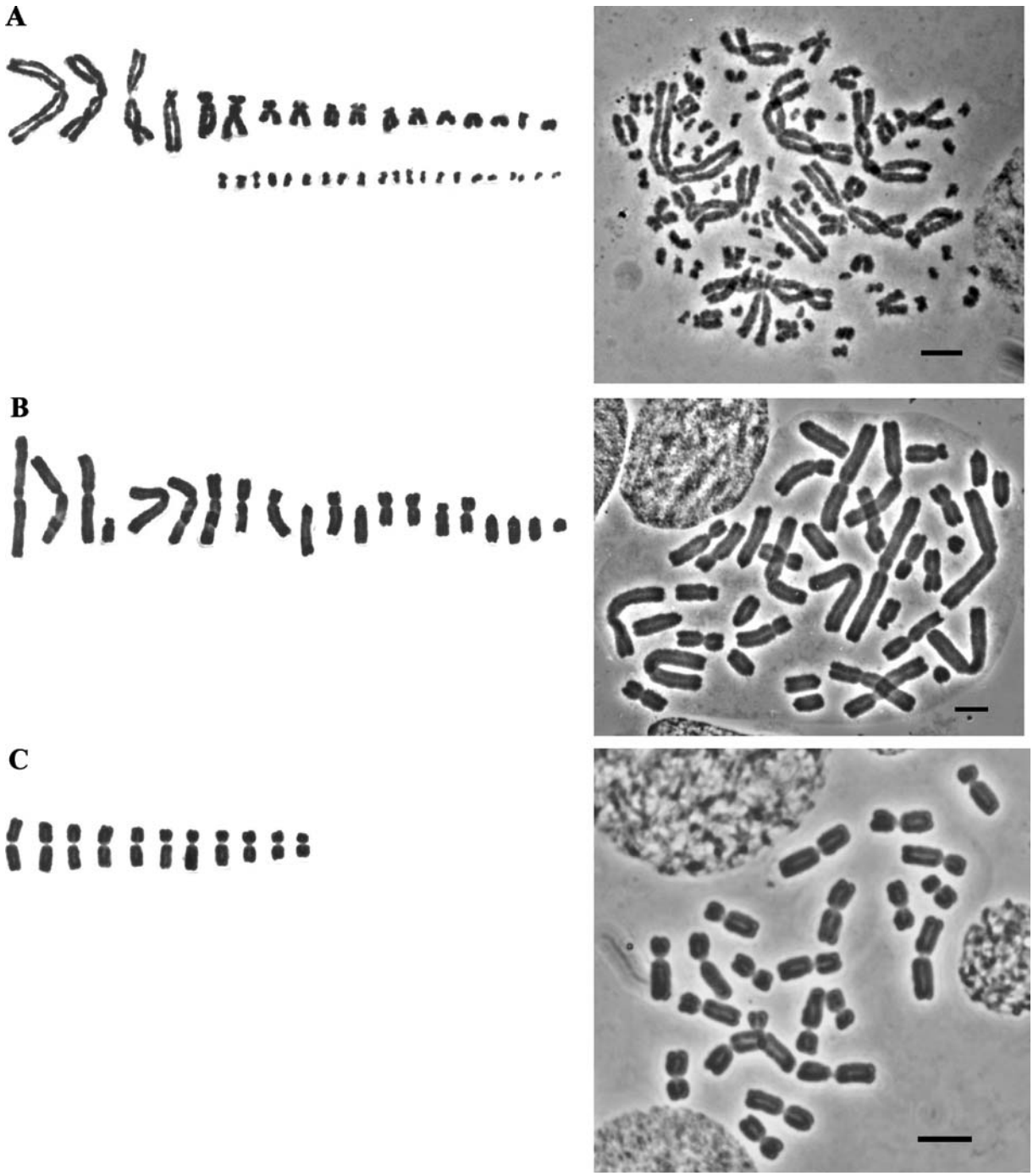

Figure 1. Chromosome spreads and haploid karyotypes representing three different levels of karyological organization in terms of chromosome number and morphology. **(A)** *Onychodactylus fischeri* (Hynobiidae); **(B)** *Necturus maculosus* (Proteidae); **(C)** *Notophthalmus viridescens* (Salamandridae). Scale=10 μm.

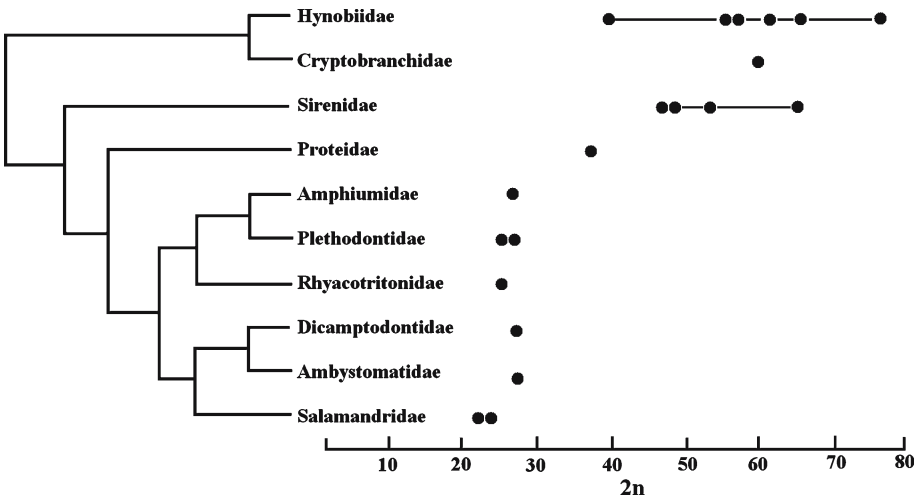

Figure 2. Phylogenetic tree and diploid chromosome numbers in Caudate amphibians based on the phylogenetic analysis of Weins *et al*. (2005); the Proteidae are here presented as a basal group.

chromosome number is found in *Onychodactylus* (2n=78; Figure 1; Iizuka & Yazawa 1994, Green & Sessions 2007), which is also the largest chromosome number of any urodele. Somewhat lower chromosome numbers are found in diverse hynobiid genera, e.g. *Ranodon* (2n=66), *Batrachuperus* (2n=62 and 66), and *Salamandrella keyserlingii* (2n=62). The lowest hynobiid chromosome numbers are found in the most speciose genus *Hynobius*, in which species with 'stream-adapted' larvae have 2n=58 and those with 'pond-adapted' larvae have 2n=56. An exceptional case is *H. retardatus* with an apparently reduced karyotype of 2n=40 and pond-type larvae. The karyotypes of most hynobiids (and cryptobranchids) show a more-or-less smooth transition within the karyotype from the largest chromosomes to substantially smaller elements, with a mixture of bi-armed and telocentric chromosomes. Despite the relatively smooth transition in relative sizes, the chromosomes are often sorted into a group of 13–14 pairs of large to medium-sized chromosomes ('macrochromosomes') plus 15 or more microchromosomes, even though it is often difficult to distinguish between the largest microchromosome and the smallest macrochromosome (Sessions *et al*. 1982, Kohno *et al*. 1991). *Hynobius retardatus* has apparently reduced its chromosome number by eliminating all but six pairs of microchromosomes (Kohno *et al*. 1991).

The next most variable salamander family, in terms of chromosome number and shape, is the Sirenidae, comprising only two genera: *Siren* (2n=46 and 52) and *Pseudobranchus* (2n=48 and 64). Karyotypes in both genera may be either symmetrical (all bi-armed) or asymmetrical (combination of bi-armed and telocentric chromosomes) with no microchromosomes (León & Kezer 1974, Morescalchi & Olmo 1974, Moler & Kezer 1993, Green & Sessions 2007). There is some evidence that polyploidy may have been involved in the karyological evolution of sirenids (see below).

In rather stark contrast to these three karyologically variable groups, the majority of salamander families have karyotypes with mostly bi-armed chromosomes that are relatively uniform in both relative size and shape, with no microchromosomes (Figure 1). Three exclusively North American families, the western N. American Dicamptodontidae (with the single genus *Dicamptodon*), the widespread Ambystomatidae (two genera, *Ambystoma* and *Rhyacosiredon*) and the south-east N. American Amphiumidiae (with the single genus *Amphiuma*), have 2n=28 chromosomes and a fourth family, the Plethodontidae (primarily North American but with

a few species in southern Europe and one newly-discovered species in Asia; Sessions *et al*. 2008), includes species with either 2n=26 or 2n=28 chromosomes (Morescalchi 1973, Sessions & Kezer 1991, Green & Sessions 2007). The western North American family Rhyacotritonidae, with the single genus *Rhyacotriton*, is characterized by 2n=26 chromosomes (Humphrey 1958, Morescalchi 1973, Sessions 1984). Finally, the primarily Eurasian family Salamandridae has the smallest chromosome numbers recorded for salamanders, 2n=22 (the two N. American genera) or 24 (18 Eurasian genera) all bi-armed chromosomes.

In some ways, the family Proteidae (*Necturus* of eastern North America, and *Proteus* of eastern Europe) occupies an 'intermediate' position in which all species have 2n=38 chromosomes (Figures 1 and 2) but show interspecific differences in the number of telocentric chromosomes (Sessions & Wiley 1985, Green & Sessions 2007). Microchromosomes are absent except for possibly the smallest chromosome pair in *Necturus* (Morescalchi 1979, Sessions 1980, Sessions & Wiley 1985).

Chromosomal variation

There are a few outstanding exceptions to the karyological uniformity seen among the low-chromosome-number families, including telocentrics, heteromorphic sex chromosomes, somatic polymorphisms, B chromosomes and polyploidy. Chromosome variation due to polyploidy and supernumerary (or B) chromosomes in salamanders and other amphibians has recently been reviewed by Green & Sessions (2007) and will not be dealt with in any great detail here. Suffice it to say that polyploidy is not uncommon among salamanders, can be experimentally induced, and shows up sporadically in almost all groups. The best example of relatively stable and widespread polyploidy is the so-called *Ambystoma jeffersonianum* complex (Uzzell 1964, Macgregor & Uzzell 1964, Bogart 1980, 1982, 2003, Sessions 1982, Bogart & Licht 1986, Bogart *et al*. 1987, Spolsky *et al*. 1992, Bi & Bogart 2006), possibly the most ancient unisexual polyploidy complex known in vertebrates (Gregory & Mable 2005). It is possible that polyploidy has played a major role in the cytogenetic evolution of salamanders and this is discussed within the context of karyological evolution (see below).

According to the model of chromosome evolution in salamanders presented by Morescalchi (1973, 1975), telocentric chromosome structure is considered to be plesiomorphic (ancestral) and bi-armed chromosomes are derived. Telocentrics are conspicuous in the karyotypes of certain cryptobranchoid salamanders, such as *Onychodactylus*, *Batrachuperus*, *Salamandrella*, *Andrias* and *Cryptobranchus*, and these species are also the most plesiomorphic salamanders in terms of morphology and reproduction. Among other hynobiids (e.g. species of the genus *Hynobius*), telocentrics are mainly found among the microchromosomes. But telocentric chromosomes also show up in nearly every other salamander group and are sometimes involved in sex chromosome heteromorphisms or fixed and floating somatic chromosomal polymorphisms. All species of the western North American genus *Dicamptodon* (in the monotypic Dicamptodontidae) for example, have a pair of telocentric chromosomes at position 12 of the karyotype (Sessions 1984, Green 1991). Among plethodontids, the N. American genera *Aneides* and *Eurycea* and the Neotropical genera *Chiropterotriton* and *Oedipina* are characterized by one or more pairs of telocentric chromosomes, always among the smallest members of the karyotype (Figure 3). The telocentrics found in species of *Aneides* and in *Eurycea wilderae* are involved in intraspecific polymorphisms (Macgregor & Jones 1977, Sessions & Kezer 1987, Sessions &

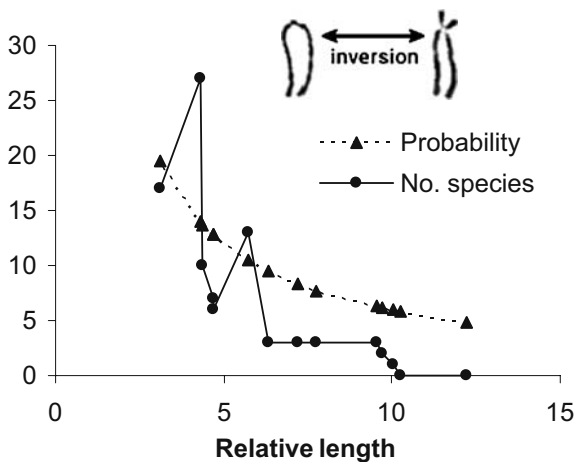

Figure 3. The observed incidence of uni-armed (telocentric) chromosomes among the largest 14 pairs of chromosomes in urodeles and the probability of telocentric morphology (via pericentric inversions) as a function of relative length.

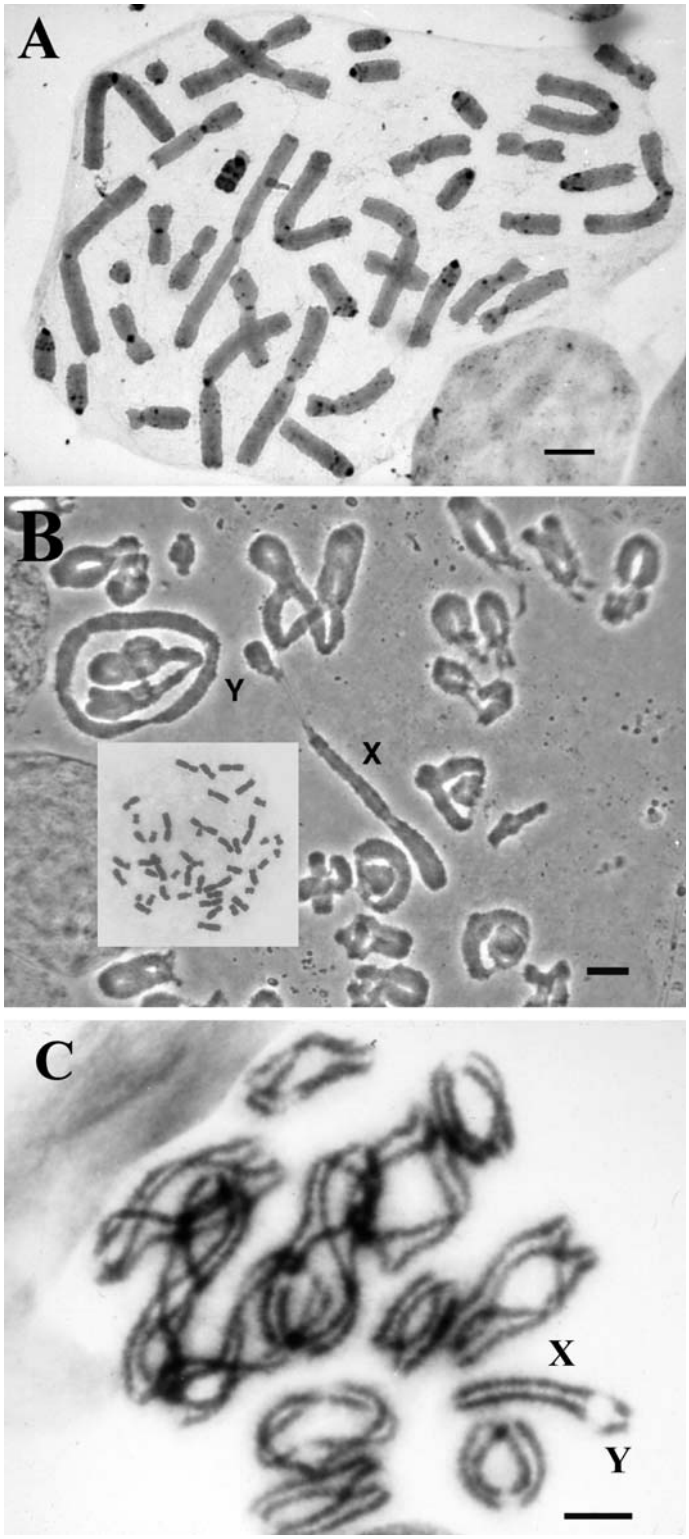

Figure 4. Heteromorphic sex chromosomes. **(A)** The proteid *Necturus maculosus* (C-banded to show the heterochromatic Y). **(B)** X and Y bivalent of *N. maculosus* at prometaphase of the first meiotic division (insert shows the relative size of human chromosomes). **(C)** X and Y bivalent of the Neotropical plethodontid *Oedipina uniformis* (hypotonic treated late diplotene spread). (Fig. 4C is reproduced from Kezer *et al*. (1989) with permission from Springer-Verlag, Berlin/Heidelberg.) Bar=10 μm.

Wiktorowski 2000). Similar inversion polymorphisms have been observed in hynobiid salamanders (Ikebe *et al*. 1990). Species of the plethodontid genus *Oedipina* have the most 'highly derived' karyotypes in the family, with two pairs of telocentrics (nos. 9 and 13) and a highly differentiated sex chromosome heteromorphism (Figure 4; Kezer *et al*. 1989, Sessions & Kezer 1991).

One interesting question concerning the incidence of telocentrics and other asymmetrical chromosomes is why they usually involve only the smallest chromosomes in the karyotype. This pattern is easily understood if telocentric chromosomes result from pericentric inversions that shift the position of the centromere. If these rearrangements occur at random with respect to chromosome size and involve similar-sized bits of chromatin, then the probability of a centromere shift generating a telocentric chromosome increases with decreasing chromosome size. The observed incidence of telocentrics among salamanders fits this pattern (Figure 3). Thus, the presence of telocentrics can be interpreted, at least in part, as the result of high rates of chromosome rearrangements (Sessions & Kezer 1987, Sessions & Wiktorowski 2000), especially if they show up among the larger elements of the karyotype. Thus, an alternative interpretation of the prevalence of telocentrics and other asymmetrical chromosomes in some cryptobranchoids is unstable karyotypes that are undergoing rapid rates of rearrangement.

Perhaps the strangest example of a somatic chromosome heteromorphism in any organism represents a cytogenetic and developmental synapomorphy defining a distinct lineage of European newts of the *Triturus cristatus* complex (*Neotriton*) family Salamandridae (Callan & Lloyd 1960, Macgregor & Horner 1980, Sims *et al*. 1984, Sessions *et al*. 1988, Macgregor *et al*. 1990, Macgregor 1991). This chromosome heteromorphism involves massive amounts of constitutive heterochromatin in the long arm of the largest chromosome of the karyotype, and represents a system of balanced, recessive lethals, resulting in developmental arrest and death of homozygotes accounting for 50% of the embryos in every clutch among five species of *Triturus* (Macgregor & Horner 1980, Sessions *et al*. 1988). It is puzzling how such a cytogenetic catastrophe could have withstood multiple speciation events over millions of years. Are these newts showing us, in slow motion, how an organism can rid itself of a problematic chromosome (cf. Morgan 1978, Muller 1964)? Is it just happenstance that the two Nearctic genera (*Notophthalmus* and *Taricha*), known to be derived from a Palearctic ancestor, have reduced their chromosome number by one pair?

Heteromorphic sex chromosomes

Heteromorphic sex chromosomes, including both XY and ZW systems, have been observed in nearly 50 species of salamanders (although there are probably at least twice that number) representing at least 15 genera and six families (Table 1) (Schmid *et al*. 1991, Green & Sessions 2007). Although both XY (male heterogametic) and ZW (female heterogametic) heteromorphisms have been reported among salamanders, XY systems are far more common (~90%) and range from small differences in C-band heterochromatin to substantial differences in chromosome size and shape. The N. American proteid genus *Necturus* stands out in having one of the most highly differentiated XY sex chromosome heteromorphisms among amphibians (Figure 4; Sessions 1980, Sessions & Wiley 1985, Green & Sessions 2007). Strongly heteromorphic XY sex chromosomes

Table 1. Heteromorphic sex chromosomes in salamanders. The number of examined species is indicated and, in parentheses, the total number in that taxon that probably have sex chromosomes

Taxon	No. of species	X/Y	Z/W
Hynobiidae			
Hynobius	3	X	
Cryptobranchidae			
Andrias	1		X
Proteidae			
Necturus	5	X	
Ambystomatidae			
Ambystoma	1		X
Salamandridae			
Lissotriton	4	X	
Pleurodeles	2		X
Triturus	6	X	
Plethodontidae			
Aneides	1		X
Chiropterotriton	1		X
Cryptotriton	3 (6)	X	
Dendrotriton	5 (6)	X	
Hydromantes (*Speleomantes*)	5	X	
Nototriton	4 (13)	X	
Oedipina	7 (23)	X	
Thorius	6 (23)	X	

have been found in all five recognized species of *Necturus*, but are apparently absent in the only other member of the family, the European genus *Proteus* (Kezer *et al*. 1965). Species of the Neotropical plethodontid genera *Cryptotriton*, *Dendrotriton*, *Nototriton*, *Oedipina*, and *Thorius* also stand out in having strongly heteromorphic XY sex chromosomes (Figure 4) (Sessions & Kezer 1991). A single species of *Chiropterotriton*, *C. dimidiatus*, has ZW sex chromosomes involving a simple pericentric inversion. It is clear that heteromorphic sex chromosomes have played a major role in the cytogenetic evolution of Neotropical plethodontid salamanders (Sessions & Kezer 1991). It seems likely that additional examples of heteromorphic sex chromosomes in salamanders will be found as more groups are examined using modern cytogenetic techniques.

Chromosome mapping: chromosome repatterning or homosequentiality?

Very little is known about the underlying arrangement of genes and other kinds of sequences on the chromosomes of salamanders. Observed variation in interstitial C-bands and AgNOR loci in various groups of salamanders suggests that conservation of chromosome number and shape is accompanied by rearrangements of genes and other sequences on the chromosomes, as expected for highly diverged species (Sessions *et al*. 2008). Variation in NOR positions for species of salamandrids, for example, was interpreted as 'chromosome repatterning' via translocations (Mancino *et al*. 1977). On the other hand, Macgregor & Sherwood (1979) interpreted variation in NOR position in plethodontid salamanders to be consistent with 'homosequentiality' in which apparent repatterning is achieved without translocation through growth or decline in the numbers of repeats in clusters of gene sequences that were already more or less widely scattered throughout the ancestral chromosome. According to the latter hypothesis, variation in *cytologically visible* ribosomal gene loci reflects differences in the size of the gene clusters generated by unequal crossing over within the clusters (Macgregor & Sherwood 1979).

Variation in C-band heterochromatin (known to contain large amounts of highly repetitive (hr) DNA) has also long been a topic of interest in salamander cytogenetics (Macgregor & Sessions 1986a,b, King 1991, Green & Sessions 2007). Most salamanders have few C-bands, with most of the darkly staining heterochromatin localized to the centromeres and to pericentromeric bands, and interstitial or telomeric bands are much less common (Figure 4). From an analysis of the distribution and molecular structure of hrDNA in the salamandrid genus *Triturus*, Macgregor & Sessions (1986a,b) presented a model of the evolution of chromosome structure in which centromeric DNA first accumulates *de novo* around centromeres, and later disperses outward into the chromosome arms. This model predicts that pericentric satellite sequences would be older, and show more interspecific homologies, than centromeric sequences. This model is supported by a recent mapping analysis using high-resolution FISH to study the chromosomal distribution and nucleotide sequences of satellite DNA sequences in salamandrids, showing that pericentric sequences are well conserved between species while centromere-specific satellite DNAs are not (Murakami *et al*. 2007).

High-resolution banding necessary for the identification of homoeologous chromosomes between species has generally not been available for most salamander groups. In most vertebrates this is done with G-banding and/or physical mapping of DNA sequences but, for some reason, G-banding has not been accomplished in salamanders. High-resolution R-banding (revealing underlying differences in AT- vs GC-rich sequences), however, has been successfully used to identify homoeologous chromosomes in hynobiid salamanders including 13 species of *Hynobius* (2n=40, 56 and 58) and one species of *Salamandrella* (2n=62) (Kohno *et al*. 1991). R-banding revealed approximately 90% homology among the chromosomes of 12 species of *Hynobius*. Approximately 65% homology was found between these species and *H. retardatus* (with a reduced chromosome number of 2n=40), and only 13% homology was seen between these species and *Salamandrella*. In every case, most of the homologies were seen among the large to medium-sized chromosomes since few of the microchromosomes had discernible banding patterns. These data reveal that R-bands are strongly conserved among species of hynobiid salamanders and that observed differences appear to be phylogenetically informative. A Southern blot analysis of highly repetitive DNA in these same species showed differences in hybridization patterns indicating that conserved chromosome banding patterns belie deeper molecular repatterning. Attempts to

R-band *Onychodactylus japonicus*, representing the largest chromosome number (2n=78) among hynobiid salamanders, failed to produce usable bands, indicating that *Onychodactylus* is phylogenetically remote from other hynobiids. *Onychodactylus* also has the largest genome size among hynobiids (see below), so it is also possible that evolutionary changes in genome size may alter the molecular structure of chromosomes in ways that affect visible banding patterns.

More recently, genetic linkage analysis has been used to construct linkage maps and to identify homologous chromosome segments between ambystomatid salamanders and other vertebrate species (Voss *et al*. 2001, Putta *et al*. 2004). Another important breakthrough is the use of genomic *in situ* hybridization (GISH) to identify intergenomic recombination among homoeologous chromosomes in unisexual allopolyploid salamanders of the *Ambystoma jeffersonianum* complex (Bi & Bogart 2006). This kind of information about the molecular organization of salamander chromosomes along with physical mapping of defined and especially homologue-specific sequences to chromosomes may soon make it possible to resolve these old issues of chromosome structure as well as to use chromosomes to construct and test phylogenetic hypotheses in salamanders.

Phylogenetics and cytotaxonomy of salamanders

The use of chromosomal data to understand phylogenetic relationships among salamanders depends on an analysis of information from at least four hierarchical levels of genomic organization: chromosome number, chromosome morphology (size and shape), banding patterns, and molecular fine-structure. A 'big picture' model of karyological evolution in amphibians based on chromosome number and shape was presented by Morescalchi (1973, 1975). The impetus for this model is the observation that karyotypes with relatively large chromosome number, asymmetrical chromosome shape (including telocentrics), and 'microchromosomes', are found only in the more primitive (i.e. plesiomorphic) members of each of the three amphibian orders. Morescalchi called such karyotypes asymmetrical (A), because they contain a relatively large number of telocentrics and subtelocentrics, and bimodal (b) because they include both 'microchromosomes' and 'macrochromosomes' (Figure 1). According to this model, such asymmetrical/bimodal (Ab) karyotypes are plesiomorphic in amphibians and have been transformed, independently in different lineages, into symmetrical/unimodal (Su) karyotypes with reduced chromosome number (Figure 1). These transformations occurred through loss of microchromosomes and loss of telocentrics, presumably through chromosome fusion/translocation/inversion events. Microchromosomes are lost first in this process, so that intermediate karyotypes are asymmetrical/unimodal (Au) karyotypes (Figure 1). Since the Ab→Su transition is thought to have happened independently in all lineages of amphibians, this model can be considered a kind of karyotypic orthoselection (White 1973, 1978) resulting in parallel evolution in the karyotypes of the three amphibian orders. Although the observed karyological variation in amphibians as a whole is clearly more complicated than that (Green & Sessions 2007), the pattern of karyological variation in urodeles does seem to support Morescalchi's model in some respects (Figures 1 and 2).

Mapping chromosome numbers on a phylogenetic tree for salamanders based on molecular data (Weins *et al*. 2005, Vieites *et al*. submitted) yields different results depending on assumptions about the ancestral state. Using a primitive anuran (such as the Tailed Frog, *Ascaphus*) as an outgroup supports the idea that asymmetrical, bimodal karyotypes are ancestral in urodeles and that cryptobranchoids retain the primitive condition (e.g. 2n=60–62). Evolutionary changes featured stepwise reductions in chromosome numbers, with no increases, occurring independently in different lineages (i.e. the Morescalchi scenario). On the other hand, if low chromosome number (e.g. 2n=28) is presumed ancestral, then an approximately equal number of increases and decreases must have occurred. However, if no assumptions are made about ancestral chromosome number, and the diploid numbers are partitioned on the tree to minimize the amount of change along each branch (Sessions & Larson 1987), then the number of inferred reductions in chromosome number exceeds the number of increases by approximately 6:1. Thus two of these scenarios support Morescalchi's model of karyological evolution, suggesting that cytogenetic evolution in salamanders has indeed featured persistent decreases of chromosome number in independent lineages.

Hynobiid salamanders appear to provide further evidence of persistent evolutionary decreases in chromosome number (Figure 5). The highest chromosome numbers are found in a diverse array of

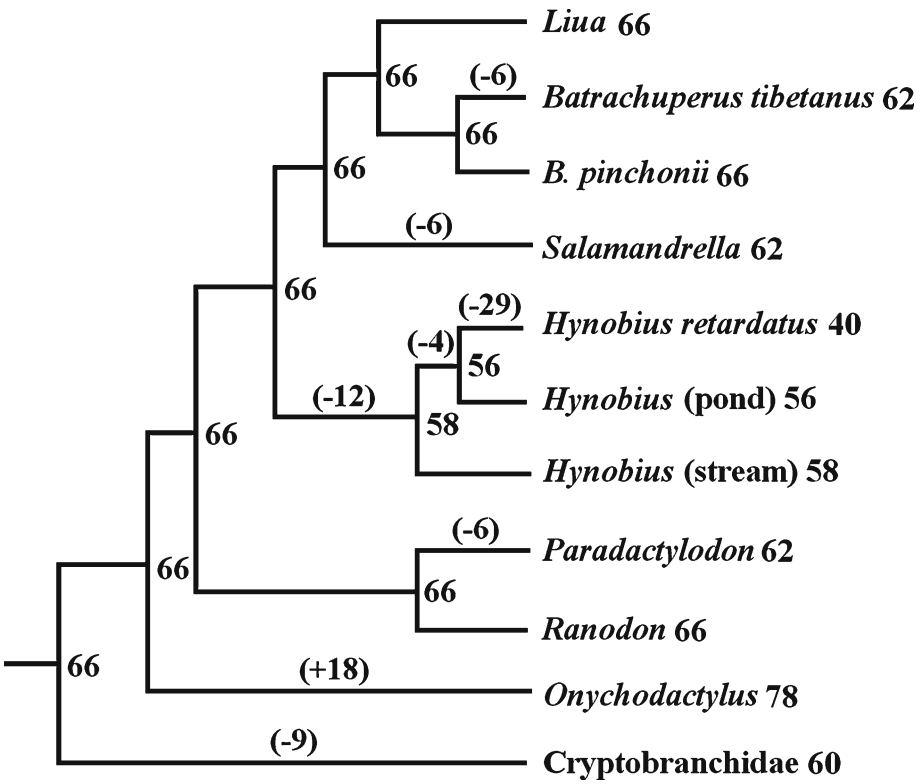

Figure 5. Inferred changes (% of ancestral values) in diploid chromosome number in hynobiid salamanders partitioned on a phylogenetic tree based on mitochondrial genome sequences (Zhang *et al*. 2006).

plesiomorphic genera, including *Onychodactylus*, *Paradactylotriton*, *Ranodon*, *Salamandrella*, *Liua*, and *Batrachuperus*, and are reduced in the more derived and successful genus *Hynobius*. Within the genus *Hynobius*, the more derived species with pond-type larvae have a reduced karyotype compared to the more plesiomorphic species with stream-type larvae (Iizuka *et al*. 1989). Finally, within the pond-type group, *H. retardatus* has clearly reduced its diploid chromosome number from 56 to 40. Mapping chromosome number onto a phylogenetic tree based on mitochondrial DNA sequences (Zhang *et al*. 2006) shows that decreases in chromosome number have prevailed over increases in the evolutionary history of the group (Figure 5). Another clear example of reduction in chromosome number is provided by the N. American salamandrid genera *Notophthalmus* and *Taricha*, which have reduced their diploid chromosome number to 22 from a 24-chromosome Eurasian ancestor. The highly successful and diverse 26-chromosome bolitoglossine plethodontids probably represent another case of reduction in chromosome number, from a 28-chromosome ancestor.

An alternative approach to understanding the pattern of variation in chromosome number among salamanders is to examine a histogram of chromosome number (Figure 6). This graph emphasizes the fact that the vast majority of salamander groups have low chromosome numbers (n=11–14). The remaining species (representing the cryptobranchoids, sirenids, and proteids) form what looks almost like a normal, bell-shaped distribution of chromosome numbers from n=19 to n=39 (Figure 6). Seen this way, six salamander families, including the most successful (speciose) salamander groups (ambystomatids, plethodontids, and salamandrids) happen to have chromosome numbers representing one tail of this distribution. On the other hand, this pattern might be telling us that low chromosome number is ancestral, since it is shared among most families and species, and that the karyotypes of the remaining groups represent an ancient polyploidy series. The way this would work is that an ancestral

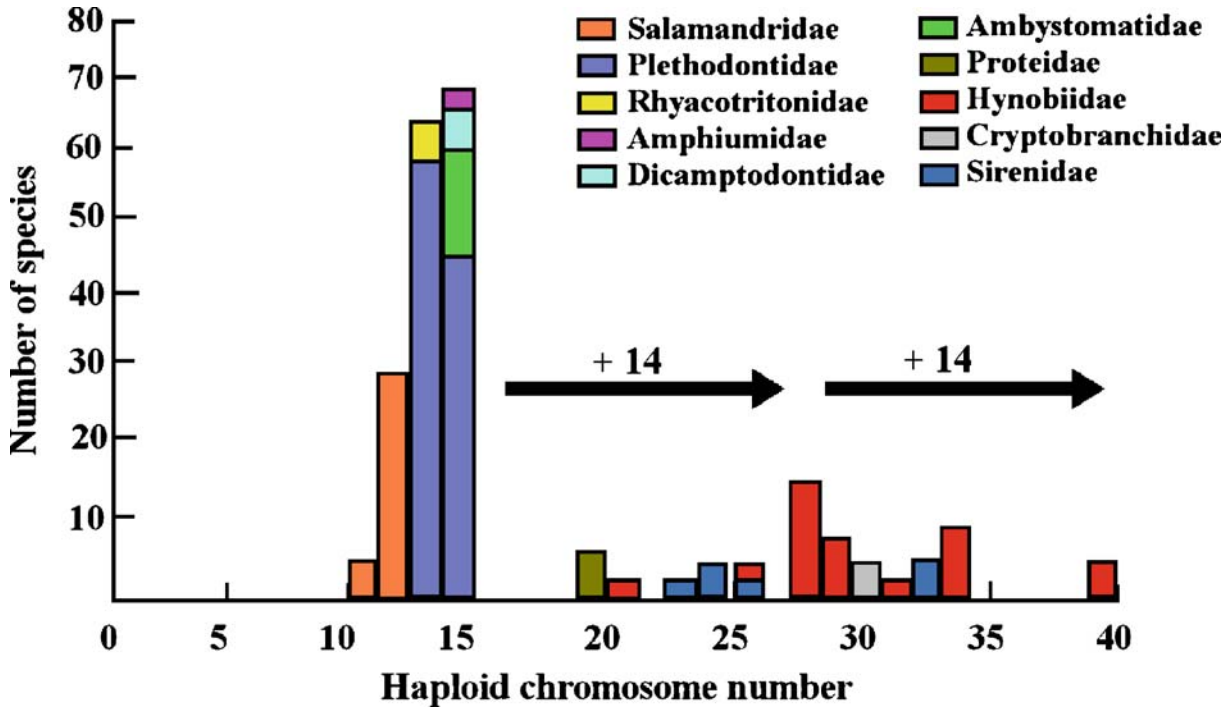

Figure 6. Haploid chromosome numbers in salamander families. Six out of the 10 families have 11–14 pairs of chromosomes. The higher numbers seen in hynobiids *et al*. could have been generated by two rounds of polyploidy followed by different degrees of 'diploidization'.

n=14 karyotype underwent polyploidization to generate a n=28±karyotype (hynobiids and sirenids), and a subsequent polyploidization event (adding 14 to 28) generated n=42±karyotypes (similar to what is seen in *Onychodactylus*). Subsequent diploidization and other minor evolutionary changes (e.g. fusions and fissions) would generate the scattering of chromosome numbers observed in living species, cytogenetic 'noise' around these ancestral numbers.

In support of the polyploidy hypothesis, the karyotypes of some extant salamanders do show similarities in size and shape between non-homologous chromosomes that may reflect remnants of ancient polyploidy in their karyotypes. The Sirenidae have long been thought to show possible signs of ancestral polyploidy because many of the chromosomes within a karyotype can be arranged in groups of three or four (Morescalchi & Olmo 1974, Morescalchi 1975). A more recent karyological study of *Pseudobranchus* revealed two chromosome numbers (n=24 and 32) but failed to find evidence of polyploidy (Moler & Kezer 1993). Instead, since the 32-chromosome karyotype contains many asymmetrical chromosomes (telocentrics and subtelocentrics) while the 24-chromosome karyotype was more symmetrical (bi-armed), it seemed more likely that the differences were due to translocations and inversions (Moler & Kezer 1993), although the direction of change (e.g. 24→32 via fissions, or 32→24 via fusions) is not clear. Distinguishing between the Morescalchi model and a polyploidy hypothesis would require the kind of molecular cytogenetic analyses that have not yet been performed in urodele amphibians, mostly because of their large genome sizes and the lack of genetic markers (although progress is being made with species of *Ambystoma*; Voss *et al*. 2001).

Genome size

The large amount of variation in genome size in eukaryotes, and especially among amphibians, has long attracted the attention of biologists. Genome size, or C-value, is usually defined as the mass of DNA per *haploid* set of chromosomes, expressed as picograms (1 pg=approximately 1000 Mb), and that is the convention that will be used here. Salamanders show greater variation in genome size, in terms of

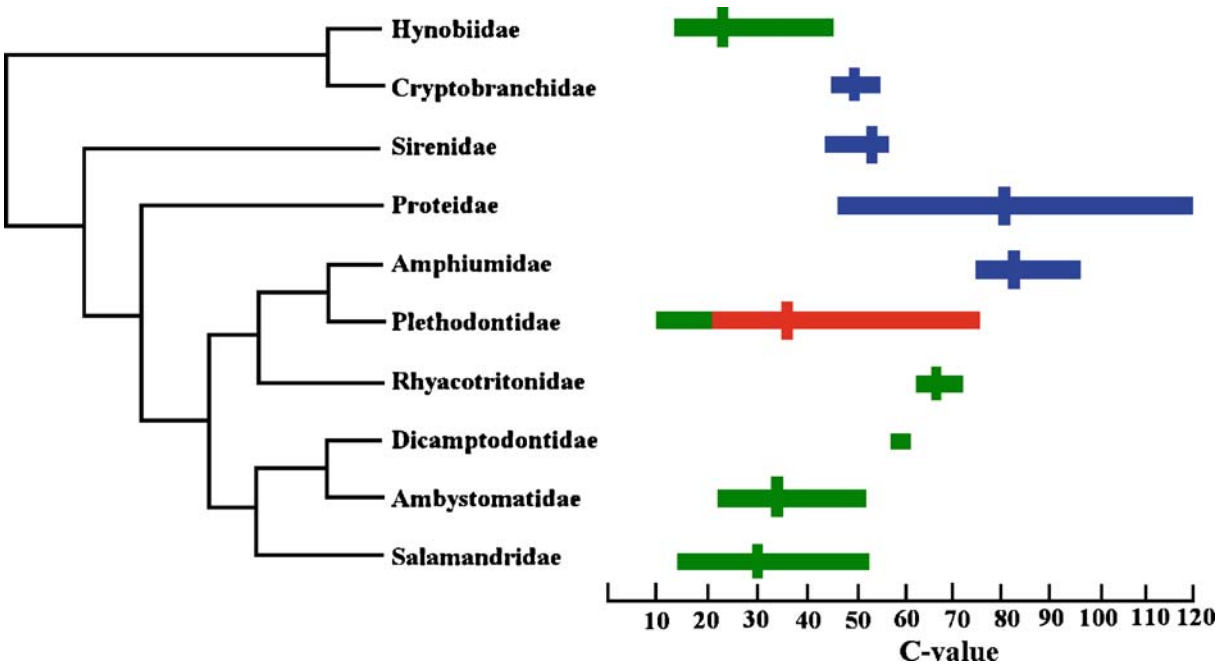

Figure 7. Distribution of genome sizes among families of salamanders. Green: species with aquatic larvae and metamorphosis. Red: direct development. Blue: strongly paedomorphic with partial or no metamorphosis.

absolute mass of DNA, than any other animal taxon (Figure 7). As in most other organisms, genome size in urodele amphibians is related to chromosome size but not to chromosome number, and the observed variation in genome size has nothing to do with polyploidy, which is a separate issue (Green & Sessions 2007). All species of Neotropical bolitoglossine salamanders, for example, have 13 pairs of similar-shaped chromosomes, and yet their genome sizes vary over 3-fold (Sessions & Kezer 1991). Small differences in genome size may be generated by heteromorphic sex chromosomes and by the presence of B-chromosomes, but the total range of variation seen among salamanders involves massive amounts of DNA that have been added to or deleted from karyotypes independent of changes in chromosome number and shape (Mizuno & Macgregor 1974, Horner & Macgregor 1983). Thus, evolutionary changes in genome size must have involved additions or deletions of DNA sequences distributed more-or-less uniformly among all chromosomes of the karyotype in such a way that chromosome shape was not substantially altered (Figure 8) (Mizuno & Macgregor 1974, Macgregor & Mizuno 1976). Evidence is accumulating that this genome size variation has significant phenotypic effects at the organismal level, particularly in urodele amphibians (Sessions 1984, Sessions & Larson 1987, Gregory 2005a) and is correlated not only with phylogeny but also with life history, morphogenesis, and biogeography.

Patterns of genome size variation in salamanders

The so-called 'C-value paradox' is that genome size is not correlated with organismal complexity. As we shall see, salamanders turn this paradox on its head and show that organismal complexity in salamanders is *negatively* correlated with genome size! A more accurate and useful restatement of the C-value paradox is that genome size is largely unrelated to the number of protein-coding genes in the eukaryotic genome. Urodele amphibians illustrate this well. Genome size (i.e. the haploid amount of DNA) ranges from approximately 14.0 pg in *Desmognathus wrighti* to approximately 120 pg in *Necturus lewisi*, an approximately 9-fold range (Figure 7) (Gregory 2005b). The mean genome size for urodeles (~37 pg) is more than 10 times that of *Homo sapiens* (3.5 pg, about the average for mammals) (see Figure 4b).

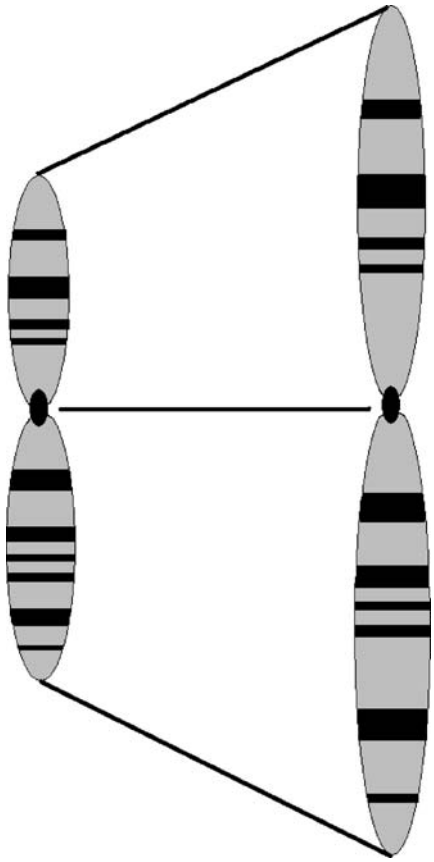

Figure 8. Genome size variation in salamanders involves change in chromosome size but not shape.

The C-value paradox was resolved by the discovery that the vast majority of the DNA sequences constituting the eukaryotic genome are non-genic (i.e. non-coding) sequences. In the human genome, for example (which is tiny from an amphibian perspective; Figure 4b), protein-coding sequences account for only about 1.5% of the genome (Gregory 2005a). The remaining sequences include transposable elements of various types (45%), introns (26%), simple sequence repeats (3%), sequential duplications (5%), miscellaneous heterochromatin (8%), and miscellaneous unique sequences (12%; Gregory 2005a). Much less information is available for the molecular structure of salamander genomes, but there is no evidence that salamanders have a larger number of protein-coding genes than mammals. We can therefore conclude that protein-coding sequences in the larger salamander genomes represent a vanishingly small component of the genome, most of which is composed of non-coding repetitive sequences (Mizuno & Macgregor 1974, Mizuno *et al*. 1976, Morescalchi 1990). Since it is unlikely that the number of protein-coding genes differs among (nonpolyploid) salamanders, variation in genome size is due to variation in the amount of these non-coding sequences. Although genome size variation is thus no longer a paradox, we still do not fully understand its origin and biological significance.

Several different workers have measured genome sizes in different groups of salamanders over the years, and most of the published values are remarkably consistent within and between species, regardless of the way they were measured (Gregory 2005b). Most studies have used microdensitometry of Feulgen-stained RBC nuclei (a possible source of confusion since these are diploid nuclei and genome size is conventionally reported as haploid C-value). A limitation to this approach is the availability of good densitometers and the expense of scanning spectrophotometers. A recent breakthrough is the use of digital photomicroscopy and computer-assisted image analysis software in which pixels are used as the units of optical density measurements (Hardie *et al*. 2002, Gregory 2005a). Genome size data are now available for approximately 370 species of salamanders (Gregory 2005b) and this list will continue to grow.

Salamanders show more variation in absolute mass of DNA (107 pg) than any other animal taxon. Perhaps not surprisingly, the groups with the largest range of genome sizes are also the most species-rich. Hence, the salamander family with the largest amount of genome size variation is the Plethodontidae (Figure 7; Sessions & Larson 1987, Sessions & Kezer 1991, Green & Sessions 2007) representing approximately 2/3 of all living species of salamanders. The genome size range for the Plethodontidae (14–76 pg DNA/haploid nucleus) encompasses the ranges of nearly all other urodele families combined (Sessions & Kezer 1991, Green & Sessions 2007), with genomes at the high end that are exceeded only by species of the genera *Amphiuma* and *Necturus* (which include the largest genomes of any tetrapod). Within the Plethodontidae, the largest range of genome sizes is seen, again, in the most speciose group, the Neotropical bolitoglossines, ranging from 21 pg in *Parvimolge townsendi* to 69 pg in *Bolitoglossa subpalmata* (Sessions & Kezer 1991).

Phenotypic correlates of genome size

Many decades of research have indicated that genome size has some important phenotypic correlates at the cellular level, especially nucleus and cell size and cell cycle time, that express themselves as effects on rates of organismal growth, development, and regeneration (Sessions & Larson 1987, Bennett & Leitch 2005, Gregory 2005a). In general, the larger the genome, the larger the nucleus and cell (Figure 9), the longer the cell cycle (Cavalier-Smith 1985, Gregory 2005a) and the slower the rates of growth, development, and regeneration (Sessions & Larson 1987, Bennett & Leitch 2005, Gregory 2005a). The most widely accepted explanation for these correlates is that they are the mechanical effects of the mass of nuclear DNA, effects that Bennett & Smith (1971) referred to as 'nucleotypic' to distinguish them from genotypic. Thus the nucleotype has effects on the phenotype that are independent of the affects of the genotype. At the molecular level, however, intron size also shows a weak positive correlation with genome size (Vinogradov 1999), suggesting that genome size, through its effects on intron length, could also have an impact on the regulation of gene expression. Perhaps this helps explain the correspondence between large genomes and paedomorphosis in salamanders (Fig. 7).

It would not be surprising if these cellular and molecular correlates of genome size had effects at the organismal level. Indeed, among Neotropical bolitoglossine salamanders, a weak positive correlation is seen between adult body size and genome size (Sessions 1984) and the smallest genomes are found in lineages that are characterized by morphological miniaturization, suggesting that genome size, through its effects on cell size, may be a morphogenetic constraint at lower extremes of body size (Sessions 1984, Roth *et al*. 1994). Cell size is so large relative to body size in some direct developing plethodontids that the femur in hatchlings may be only three or four cells in diameter, and portions of the tongue skeleton in adults are only a single cell in diameter (Sessions, unpublished)! Additional evidence that genome size is a morphogenetic constraint in salamanders is the fact that the largest genomes are found in four lineages (Amphiumidae, Cryptobranchidae, Proteidae, and Sirenidae) that are all permanently aquatic 'salamonsters', gigantic paedomorphic salamanders that retain larval features in an adult morphology that is characterized by morphological oddities. *Amphiuma*, for example, can be over 1 meter long,

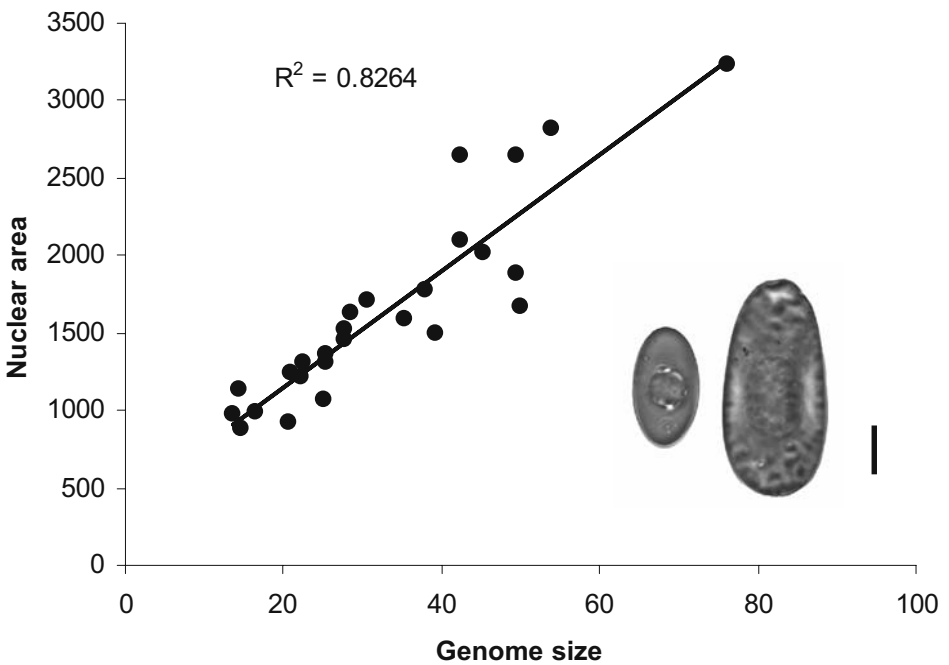

Figure 9. Nuclear area versus genome size in 27 species of salamanders. Insert: RBCs of *Gyrinophilus porphyriticus* (21 pg) on the left, and *Necturus maculosus* (86 pg) on the right. Scale bar is 20 μm.

cylindrical (eel shaped) with astonishingly tiny limbs only a few centimeters long bearing reduced numbers of digits.

Not surprisingly, cell size (positively correlated with genome size) was found to predict morphological complexity in the brains of frogs and salamanders, with reduced complexity correlated with large genomes (Roth *et al*. 1994).

An analysis of limb regeneration and genome size variation within the Plethodontidae revealed a pronounced negative impact of large genomes on rates of limb regeneration (Sessions & Larson 1987), a characteristic that is likely to be exposed to selection. Salamanders with the smallest measured genomes were able to almost complete regeneration within about 60 days whereas those with the largest genomes took over nine months, again suggesting that genome size (through its effects on cell size, cell division, and rates of growth and development) may be a morphogenetic constraint in salamanders.

Genome size also appears to be related to life history in amphibians, again probably reflecting morphogenetic constraints (Morescalchi 1990, Gregory 2005a). Among urodeles, species with aquatic larvae and metamorphosis tend to have the smallest genomes and show the smallest ranges in genome size (Fig. 7), suggesting that genome size may be a constraint on rates of growth and development. Within the family Plethodontidae, the groups which show the largest range of genome sizes, including some of the largest genomes known for any vertebrate, are characterized by direct development in which aquatic larvae and metamorphosis have been eliminated (Fig. 7). Different degrees of paedomorphosis are pronounced in these species. Most of these species also live in the Neotropics with extended or nonseasonal breeding activity, again suggesting that variation in genome size may reflect selection on rates of growth and development. Genome size thus may represent compromises between large cell size (imposed by large genomes) and rapid developmental rates (allowable with small genomes). These results are easiest to explain by interaction between selection at the molecular level (intragenomic selection) favouring the accumulation of selfish/junk DNA elements in the genome, and selection acting on nucleotypic effects of this DNA (via cell size and cell cycle time) at the cellular and whole organismal levels. In other words (as pointed out by Gould 1983, cited in Gregory 2005a), understanding the evolution of genome size requires a hierarchical view of natural selection operating at multiple levels of organismal complexity.

Genome size and biogeography

Genome size variation also shows strong correlations with biogeography, at least in some groups (Figure 10) (Sessions 1984, Litvinchuk *et al*. 2007, Sessions *et al*. 2008). Within the Plethodontidae, species found in eastern North America, regardless of clade, have smaller genomes than those found elsewhere, including western N. America, Middle America, South America, Asia, and southern Europe. Among Neotropical bolitoglossines, genome size increases

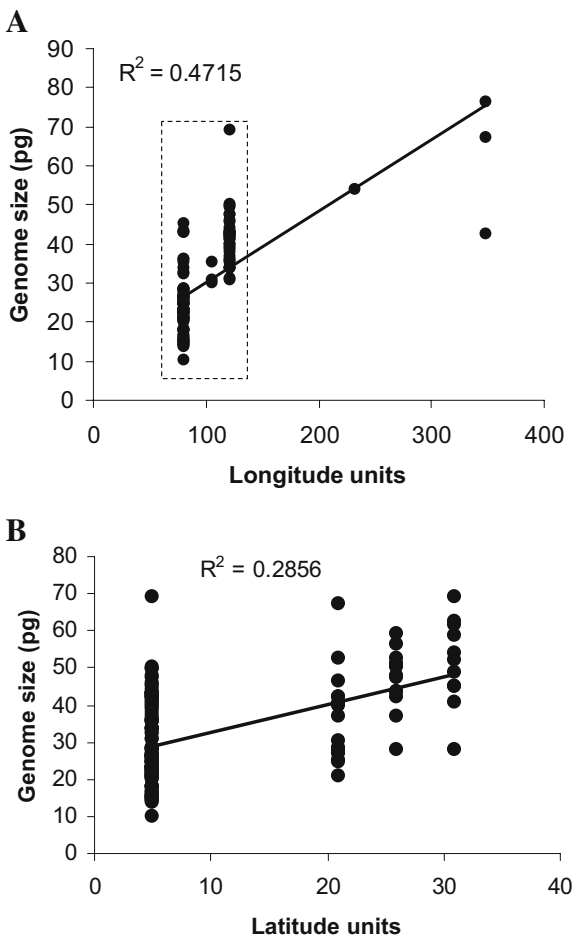

Figure 10. Regression analysis showing the relationship of genome size with longitude **(A)** and with latitude **(B)** in plethodontids. In both cases, longitude and latitude are expressed as a distance measurement. If only North American species are considered (box in graph **A**), the R^2 value increases to 0.58.

with latitude from north to south (Figure 10B). Sessions (1984) interpreted this pattern as the result of persistent independent evolutionary increases in genome size with distance from a presumed centre of origin for the family in the Appalachian Mountains of eastern N. America. Recent phylogenetic analyses, however, suggest that plethodontids may have originated elsewhere (Vieites *et al*. submitted), in which case it seems likely that the small genomes of species in eastern N. America were derived from larger genomes that characterize the family (Sessions *et al*. 2008). Most of the groups with the smallest genomes in the family have aquatic larvae with metamorphosis, which fits the relationship between genome size and life history already noted in the order as a whole. But this does not explain the fact that eastern species of *Plethodon*, all direct developers, have smaller genomes than western species of *Plethodon*.

Genome sizes are also correlated with biogeography in salamandrids (Litvinchuk *et al*. 2007), a salamander family that is thought to have its origins in the Palearctic. In salamandrids, the smallest genomes are found among western Palearctic species, the largest genomes in east Asiatic species, and intermediate-sized genomes in the Nearctic species. Since the Nearctic species (representing two genera) have a reduced chromosome number relative to Palearctic salamandrids, it seems likely that their genome sizes are derived as well. Litvinchuk *et al*. (2007) also found very strong correlations between genome size and embryonic development time that seemed to be related to climate and breeding season, where the smallest genomes were found in winter-breeding species, and the most between-species variation was seen in spring breeders. Also, viviparous species have larger genomes than oviparous species.

In both salamandrids and plethodontids, therefore, geographic patterns of genome size variation transcend phylogenetic relationships. These correlates of genome size may be easier to understand in salamandrids, most of which have aquatic larvae and metamorphosis, than in terrestrial plethodontids, which have eliminated aquatic larvae and metamorphosis in favour of direct development. Indeed, direct development (in plethodontids), may have relaxed selection on genome size, since the entire embryonic plus larval period occurs within the protection of the egg capsule, attended by a parent. A similar argument applies to salamandrid species that experience environmentally imposed slow rates of development, and those that give birth to live young, both of which have the larger genomes (Litvinchuk *et al*. 2007).

Evolution of genome size

Several models of genome size evolution have been proposed (for a recent review see Gregory 2005a). According to one of these, extra, non-coding DNA may be 'junk', i.e. completely non-functional DNA derived from the accidental duplication of various kinds of DNA sequences including protein-coding genes (Ohno 1970). As 'so much junk', this DNA would not be subject to natural selection in the same way as functional DNA sequences are. Most known mechanisms for random, quantitative change in DNA, such as gene duplication and unequal crossing-over within non-coding regions, will lead to DNA accumulation if unchecked by natural selection (Horner & Macgregor 1983). This 'junk DNA' hypothesis predicts that the majority of non-coding sequences in the genome will be pseudogenes and/or tandemly duplicated repetitive sequences. Some kinds of DNA sequences, though, may be 'selfish' (Doolittle & Sapienza 1980, Orgel & Crick 1980), such as transposable elements that are able to independently replicate and spread within genomes as a kind of molecular 'parasite'. Inactive remnants of transposable elements constitute nearly 50% of the human genome (Kidwell 2005). Both junk and selfish DNA would generate a kind of mutation pressure in which there would be a tendency for genomes to increase in size (Gregory 2005a). The molecular composition of giant salamander genomes is not nearly as well understood, but available data indicate that they consist almost entirely of repetitive sequences of various kinds (Morescalchi 1990). The relative contributions of 'junk' versus 'selfish' DNA to genome size variation in amphibians is yet to be worked out.

Looking at the ranges of genome sizes in the context of recent phylogenetic analyses of salamander families shows a rather complex pattern that is not clearly useful in understanding how genome size evolved (Figure 7). As pointed out by Green & Sessions (2007), we do not understand enough about the mechanisms of genome size change to make the kinds of assumptions we would need to use genome size as a character for constructing phylogenetic

hypotheses. But we can use phylogenetic hypotheses based on other characters (e.g. molecular data) to generate hypotheses about genome size evolution. One way to do this is to partition genome sizes on a phylogenetic tree based on other kinds of data such as nuclear markers (Figure 11) (Sessions & Larson 1987, Weins *et al*. 2005, Vieites *et al*. submitted). The picture that emerges from this kind of analysis depends on initial assumptions. Working backwards along the tree to infer ancestral genome sizes using maximum parsimony to minimize the number of evolutionary changes required on each branch of the tree results in an inferred ancestral genome size for most lineages that is approximated by the average genome size of the four largest families (Ambystomatidae, Hynobiidae, Plethodontidae, and Hynobiidae), approximately 30 pg (Figure 11). This tree requires a total of seven changes, six of which are massive increases of approximately 67–170% of inferred ancestral genome sizes (Figure 11). These huge increases in genome size occurred in lineages that are characterized by pronounced paedomorphosis: Cryptobranchidae, Sirenidae, Proteidae, Amphiumidae, Rhyacotritonidae, and Dicamptodontidae. The only large (i.e. >10%) inferred decrease in genome size was in the Hynobiidae, a lineage characterized by aquatic larvae and metamorphosis. As an alternative, taking the smallest known salamander genome size (14 pg) as ancestral requires eight changes, all increases and no decreases. Another alternative, of course, is that giant genomes are ancestral, in which case the largest decreases are on lineages (ambystomatids, hynobiids, and salamandrids) that have aquatic larvae and complete metamorphosis.

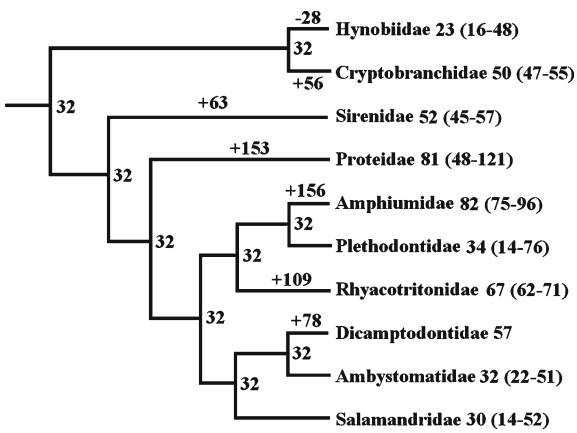

Figure 11. Genome sizes partitioned on a phylogenetic tree (modified from Weins *et al*. 2005) using maximum parsimony to minimize number of changes (expressed as percentage increase or decrease relative to inferred ancestral values).

Choosing the most likely scenario for genome size evolution may be helped by the realization that increases in genome size appear to be more likely than decreases, based on known molecular cytogenetic mechanisms that could generate changes in genome size. As pointed out by Mizuno & Macgregor (1974), changes in genome size in salamanders appears to involve sequences that are finely dispersed along the lengths of chromosomes, making it difficult to remove sequences once they have been added. Indeed, a phylogenetic analysis of genome size variation in plethodontid salamanders also concluded that increases in genome size predominated over decreases and that these changes occurred independently in several different lineages (Sessions & Larson 1987). The only inferred evolutionary decreases in genome size were seen in miniaturized species, underlining the significance of genome size as a morphogenetic constraint.

Treating genome size as a purely continuous character, Martin & Gordon (1995) postulated the existence of a 'junk DNA' molecular clock to infer times of divergence among lineages. By comparing the ages (from fossil records) of several neotenic lineages of salamanders with their genome size values, Martin & Gordon (1995) postulated a constant, gradual rate of junk DNA expansion among these species and suggested that this junk DNA molecular clock might be useful in the inference of phylogenetic relationships among the species studied. The ages of the lineages, as given by Martin & Gordon (1995), though, are dubious, as is the junk DNA molecular clock idea itself, due in part to its neglect of rare but important events such as genome size doublings (Green & Sessions 2007). As is the case with chromosomes, phylogenetic relationships constructed from other kinds of data are more likely to reveal information about genome size evolution in salamanders than vice versa, at least with our present understanding.

The picture presented here is of genome size, and 'extra' DNA, that is neither purely selfish nor of precise adaptive significance. Evolutionary pressure to increase in size is balanced and constrained by considerable phenotypic ramifications. Hopefully,

these ideas have opened up possible avenues for future research concerning the biological significance of genome size and, in general, the interaction between evolutionary events at the molecular and organismal levels.

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