Chromosome Research (2008)
DOI: 10.1007/s10577-008-1197-7

Cytogenetic analysis of the Asian Plethodontid salamander, *Karsenia koreana*: Evidence for karyotypic conservation, chromosome repatterning, and genome size evolution

Stanley K. Sessions[1]*, Matthias Stöck[2], David R. Vieites[3], Ryan Quarles[1], Mi-Sook Min[4] & David B. Wake[3]
[1]*Department of Biology, Hartwick College, Oneonta, NY, 13820, USA; Tel: +1-607-431-4764; E-mail: sessions@hartwick.edu;* [2]*Department of Ecology and Evolution (DEE), University of Lausanne, UNIL, Biophore, CH-1015, Lausanne, Switzerland;* [3]*Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, CA, 94720-3160, USA;* [4]*Conservation Genome Resource Bank for Korean Wildlife, BK21 Program for Veterinary Science, Laboratory of Wildlife Conservation Genetics, College of Veterinary Medicine, Seoul National University, 151–742, Seoul, South Korea*
* *Correspondence*

Received 15 October 2007. Received in revised form and accepted for publication by Herbert Macgregor 14 December 2007

Key words: cytogenetics, evolution, genome size, *Karsenia*, Plethodontidae, salamander

Abstract

A cytogenetic analysis, including the karyotype, C-bands, silver-stained nucleolus organizer regions and genome size, was performed on the recently discovered species, *Karsenia koreana*, the first plethodontid salamander from Asia. The karyotype consists of 14 pairs of bi-armed chromosomes, with no evidence of heteromorphic sex chromosomes. C-banding reveals a concentration of heterochromatin at the centromeres as well as at interstitial locations. The smallest chromosome (pair number 14) has symmetrical interstitial C-bands in each arm, resembling chromosome no. 14 of North American species of its sister group taxon, supergenus *Hydromantes*. A comparative analysis of C-band heterochromatin and silver-stained nucleolus organizer regions of *Karsenia* and other plethodontid genera reveals that chromosomal evolution may have featured chromosome 'repatterning' within the context of conserved chromosome number and shape in this clade. Genome size is correlated with geographic distribution in plethodontids and appears to have important phenotypic correlates as well. The genome size of *Karsenia* is relatively large, and resembles that of the geographically closest plethodontids from western North America, especially species of the genus *Hydromantes*. The biological significance of these cytogenetic characteristics of plethodontid salamanders is discussed within an evolutionary context.

Introduction

The Plethodontidae is the most speciose family of salamanders, with a centre of biodiversity in the Americas. A single group (supergenus *Hydromantes*) includes species in western North America and in a small Mediterranean region in Europe. The absence of plethodontids from elsewhere in Europe or anywhere in Asia has been a major biogeographic puzzle for decades. The recent discovery of an Asian plethodontid from South Korea, *Karsenia koreana* (Min *et al*. 2005), has helped to clarify the phylogenetic relationships and historical biogeography of Holarctic plethodontids. Molecular phylogenetic studies of these species have led to a reassessment of their taxonomy and phylogenetic relationships (Mueller *et al*. 2004, Wiens *et al*. 2005; Vieites *et al*. 2007). In conflict with traditional taxonomy (Wake

1966), molecular evidence from complete mitochondrial genomes and multiple nuclear loci identifies two distinct subfamilies: Plethodontinae and Hemidactyliinae (Figure 1) (Vieites *et al*. 2007). This study found that *Karsenia* is most closely related to members of the subfamily Plethodontinae. Within this subfamily, *Karsenia* superficially resembles a western species of *Plethodon* in overall morphology and habitat, but molecular evidence and divergence time estimates suggest that it is more closely related to the biogeographically widely distributed Sg. *Hydromantes*. The common ancestor of *Karsenia* and *Hydromantes* is estimated to have diverged from other plethodontids in the Late Cretaceous, and these two diverged from each other ~69 million years ago (Mya) (Vieites *et al*. 2007).

Nothing is known about the cytogenetics of *Karsenia*. All plethodontids show strong karyological similarities to each other in chromosome number and shape (Mizuno & Macgregor 1974, Sessions & Kezer 1991, Sessions & Wiktorowski 2000, Green & Sessions 2007, Sessions 2008). An extensive review of karyological diversity across the taxonomic diversity of plethodontids (~115 species; Green & Sessions 2007, Sessions 2008) showed that plethodontids are characterized by karyological uniformity with either 13 or 14 pairs of chromosomes, bi-armed in over 85% of these species. Very little is known, however, about the underlying arrangement of genes and other kinds of sequences on the chromosomes of salamanders, although this is an old, controversial problem (Macgregor & Sherwood 1979).

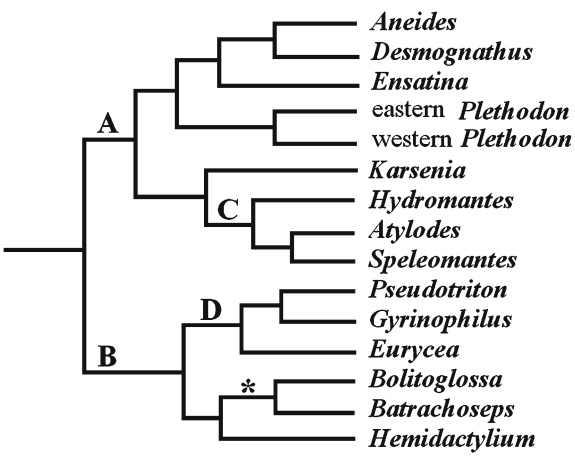


Figure 1. Phylogenetic tree showing evolutionary relationships among major groups of plethodontid salamanders based on the molecular phylogenetic analysis of Vieites *et al*. (2007). **A**: subfamily Plethodontinae; **B**: subfamily Hemidactyliinae; **C**: supergenus *Hydromantes*; **D**: the Spelerpines. Asterisk indicates the bolitoglossines with reduced chromosome number from n=14 to n=13.

In contrast to chromosome number and shape, genome size (defined as the mass of DNA in a *haploid* nucleus) shows a wide range of variation in plethodontids. Plethodontids, like salamanders in general, are characterized by astonishingly large genomes including genomes with 20 times the nuclear DNA of a human genome (Gregory 2001). Genome sizes within the family ranges from ~14 pg (the smallest among urodeles) in *Desmognathus wrighti* (Halley *et al*. 1986), to ~72 pg (one of the largest among urodeles and tetrapods) in *Speleomantes italicus*, Sg. *Hydromantes* (Sessions & Larson 1987, Gregory 2001). Not only do species of Sg. *Hydromantes* have the largest genome sizes of any terrestrial salamanders (Green & Sessions 2007), but the clade also experienced the fastest rates of molecular evolution among Holarctic plethodontids (Vieites *et al*. 2007). These unusual features of salamanders of the Sg. *Hydromantes*, together with their long-term biogeographic isolation (two clades in southern Europe and one in California) and deep divergence from other plethodontid genera, make its sister taxon *Karsenia* an ideal candidate for investigating chromosome and genome size evolution. In this study we examine the karyotype and genome size of *Karsenia*, together with taxa belonging to its closest relatives, including C-banding (to visualize patterns of constitutive, C-band heterochromatin, CBH) and AgNOR banding (to localize ribosomal gene loci on mitotic chromosomes), in order to compare these cytogenetic characteristics with those of related taxa in an evolutionary context.

Materials and methods

Specimens of *Karsenia koreana* were collected in 2005 and 2006 from the type locality (Min *et al*. 2005), by DV and MSM. Chromosome preparations were done in the field for two female specimens in 2005, with poor results. Three specimens (one female and two males) collected in 2006, were kept alive and transported to the laboratory to be analysed in this study. Chromosomes were also prepared from specimens of *Aneides lugubris* and *Ensatina eschscholtzii* (from Berkeley, California), *Plethodon*

dunni (from Portland, Oregon), *Hydromantes platycephalus* (from Lyons Lake, California) and *Desmognathus ochrophaeus* (from Oneonta, New York). C-banding and AgNOR banding for these species have not been reported previously. Mitotic chromosomes were prepared from the intestinal epithelial cells of colchicine-treated animals using the technique of Sessions and Kezer (Sessions & Kezer 1987, Sessions 1996). Unstained preparations were then examined and photographed using phase-contrast optics on an Olympus BH-2 compound microscope with a digital camera attached to a computer. Slide preparations were made permanent using the technique of Conger and Fairchild (Conger & Fairchild 1953, Sessions 1996). For C-banding we followed the procedure of Schmid *et al*. (Schmid *et al*. 1979, Sessions 1996), and for silver nitrate staining of the nucleolus organizer regions (AgNOR) banding, we used the procedure of Hsu (Hsu 1981, Sessions 1996).

Genome sizes were estimated using optical density measurements and nuclear areas (in arbitrary units) from digitized images of Feulgen-stained RBC nuclei (Mizuno & Macgregor 1974, Sessions & Larson 1987, Hardie *et al*. 2002) using imaging software. Genome size is conventionally reported as 'C-value', which is the mass of DNA in picograms (1 pg $\approx$ 1000 Mb) in a haploid nucleus (Macgregor & Varley 1983), and this convention is followed here even though our measurements were made on *diploid* nuclei. Genome size and C-value will be used interchangeably with the same meaning: the haploid mass of nuclear DNA (Gregory 2005) RBC nuclei were photographed with a digital camera attached to a compound microscope and computer. For optical density measurements, blood was obtained directly from the heart using a heparinized glass pipette. A drop of the blood was placed at one end of a glass slide and another clean glass slide was used to pull the blood across the slide, creating a thin smear (Humason 1972). The slides were then air-dried and fixed in fresh ethanol–acetic acid (3:1) for 5 min. They were stained with Schiff's reagent (SR) using the following procedure (Humason 1972, Sessions & Larson 1987): slides were rinsed briefly in distilled water, hydrolysed in 5 M HCl for 20 min at room temperature, rinsed three times in distilled water, and stained in SR for 90 min at room temperature. Slides were then soaked in three changes of 'bleaching solution' (0.5% potassium metabisulfite in 0.05 M HCl), 5 min each, rinsed with distilled water, and dehydrated in 70%, 95% and 100% ethanol for 1 min each, followed by two changes of xylene. Slides were then mounted in a xylene-based mounting medium and covered with a glass coverslip for microscopy. Digital images were taken at 100$\times$ oil, and optical density measurements were averaged from a total of 100 nuclei for each specimen. The C-value (genome size, expressed as picograms) of *Karsenia* was estimated using simultaneously stained RBC nuclei of a single specimen of *Aneides lugubris* (C-value=45.1 pg, averaged from three published values ranging from 42.8 to 49.6 pg; Gregory 2001) as a standard. An estimate was also made using nuclear areas computed by the software, again using *A. lugubris* as a standard. The final estimated C-value was checked against a regression analysis of genome size versus nuclear area using measurements of RBC nuclei and published genome size data for eight urodele species representing a range of genome sizes (Gregory 2001). For the genome size versus nuclear area regression analysis, fresh blood smears were prepared, fixed in 3:1 fixative, and stained with 8% Giemsa for 5 min. Nuclear areas (expressed as μm^2) were calculated using measurements made from digitized images of the stained RBC nuclei and converting to micrometres.

Results

We examined mitotic chromosome spreads from one female and two male specimens of *Karsenia,* as well as meiotic diplotene spreads from the testes of the male. A total of 57 mitotic spreads (32 female, 25 male) and 5 diplotene spreads were examined. Karyotypes are shown in Figure 2. We counted a total of 28 chromosomes in mitotic spreads and 14 bivalents in meiotic spreads. Therefore, *Karsenia* has a haploid chromosome number of 14 pairs of chromosomes (2n=28), and all of the chromosomes are bi-armed (metacentric or submetacentric) with no indication of heteromorphic sex chromosomes or any other obvious heteromorphism.

A total of 21 C-banded mitotic spreads were examined (10 female, 11 male). C-banding showed a fairly 'typical' (for plethodontids) distribution of C-band heterochromatin (CBH) concentrated at the centromeres with a few interstitial bands (Figure 3). Chromosome no. 14, the smallest in the set, showed a symmetrical pattern of interstitial CBH in both arms (Figure 3A). Clear AgNOR bands were obtained only

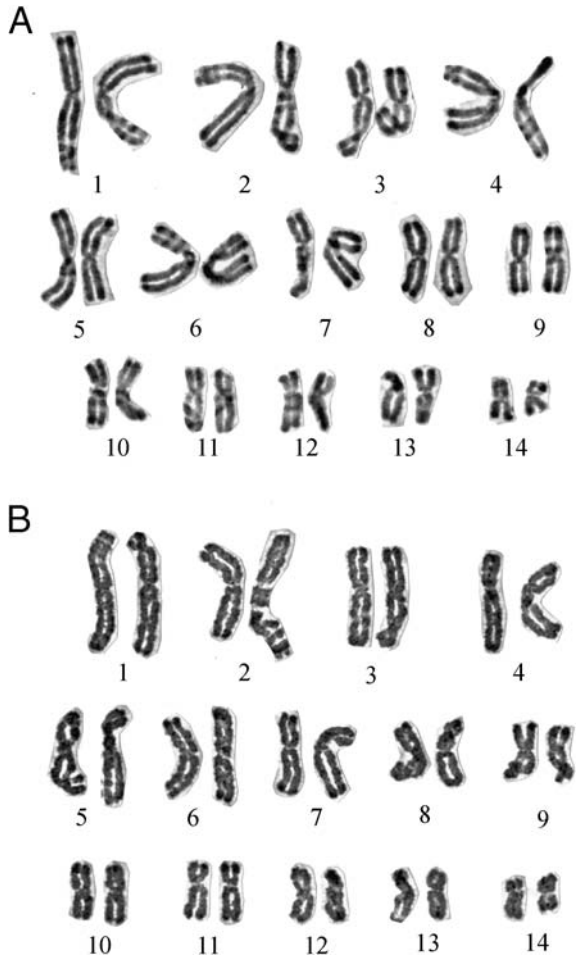


Figure 2. Karyotypes of *Karsenia koreana*: (**A**) female and (**B**) male.

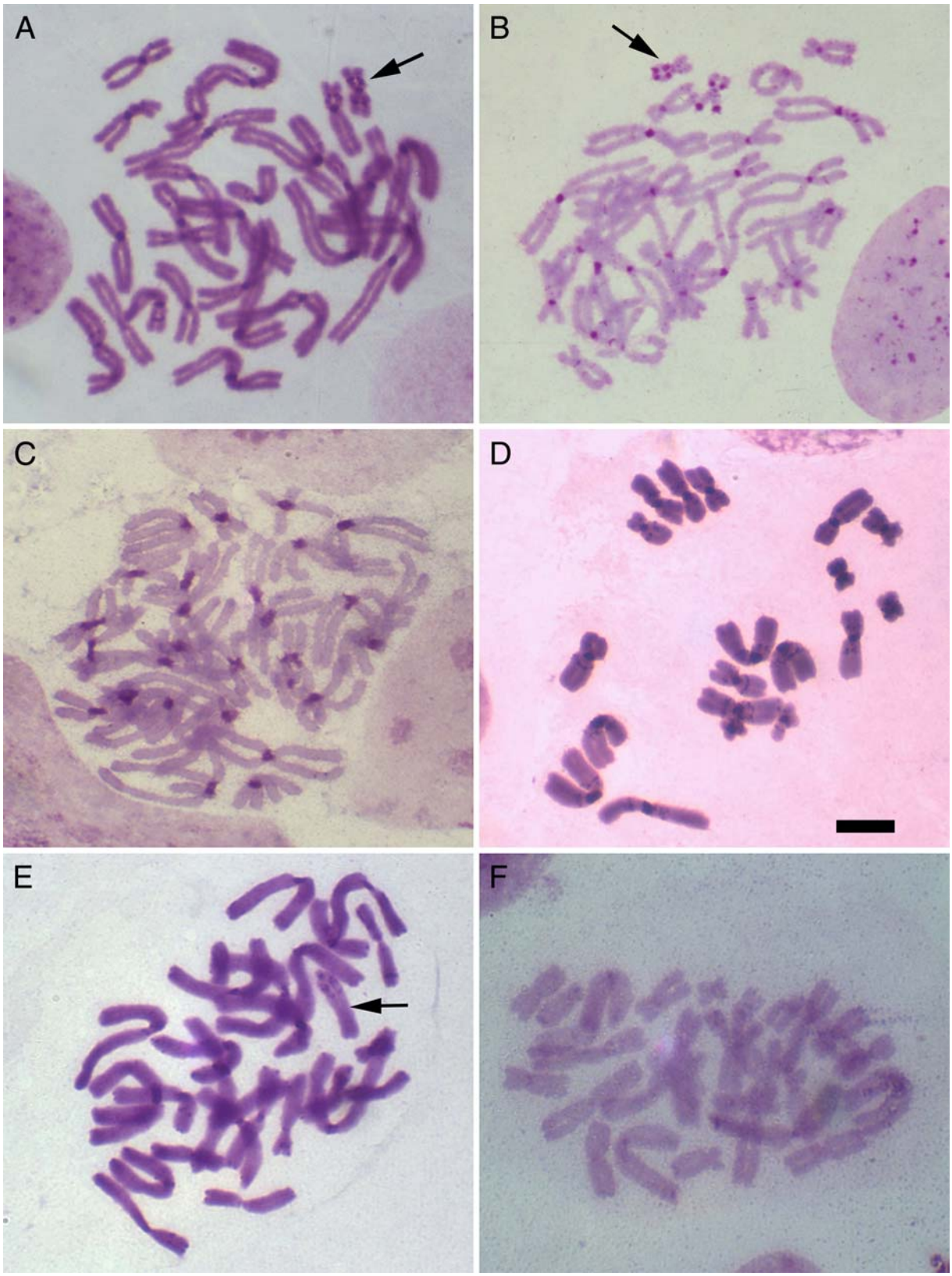
for a single male specimen. Interphase nuclei show four active (silver-stained) nucleoli corresponding to two main AgNOR loci found in the arms of two of the largest chromosomes (Figures 4A, 5A), one near the telomere of the long arm of chromosome no. 4, and one in the middle of the long arm of chromosome no. 1 (Figure 5A). Additional small sliver spots were sometimes seen in interphase nuclei, indicating the possible existence of one or two additional NOR loci not visible in the mitotic chromosomes. No obvious similarities in AgNOR loci were seen between *K. koreana* and other plethodontids (Figure 5). An idiogram summarizing these chromosomal data is presented in Figure 5A.

Representative specimens of the major clades within the subfamily Plethodontinae were also examined for this study (Figures 3, 4, 5), including *Hydromantes* (*H. platycephalus*), *Aneides* (*A. lugubris*), *Ensatina* (*E. eschscholtzii*), western *Plethodon* (*P. dunni*), and *Desmognathus* (*D. ochrophaeus*). Data were obtained for fresh specimens and also compared with available data from the literature (Sessions & Kezer 1991). *Hydromantes platycephalus* has a karyotype consisting of 14 pairs of bi-armed chromosomes and CBH that is mostly concentrated in the centromeres with a few interstitial bands (Figures 2B, 5B) (Sessions & Kezer 1991). Chromosome no. 14 of *H. platycephalus* is distinguished by polymorphic interstitial C-bands in both the short arms and long arms (as also seen in other North American species of *Hydromantes*; Sessions & Kezer 1991) but not in European members of the supergenus, probably indicating a high rate of chromosomal rearrangement in that chromosome (Sessions & Kezer 1991). In *H. platycephalus*, a single AgNOR locus is found near the telomere of the long arm of one of the largest chromosome pairs (Figure 4B).

Ensatina also has a karyotype consisting of 14 pairs of bi-armed chromosomes, relatively large amounts of CBH that is more concentrated in the centromeric regions and shows fewer interstitial C-bands, and an AgNOR located near the telomere of the long arm of the smallest chromosome, no. 14, in close proximity to an interstitial C-band (Figures 3C, 4C, 5C). *Plethodon dunni* has a karyotype also consisting of 14 pairs of bi-armed chromosomes with C-band heterochromatin concentrated at the centromeres but with symmetrical pericentric C-bands on each chromosome (Figures 3D, 5D). *Plethodon dunni* has a single AgNOR locus at an interstitial locus in the short arm of a medium-sized submetacentric chromosome near an interstitial C-band (Figures 4D, 5D).

Aneides lugubris has 14 pairs of chromosomes including two pairs of telocentric chromosomes (nos. 12 and 13; Figure 5E). As reported elsewhere (Sessions & Kezer 1987), chromosome nos. 12 and 13 may be either bi-armed (subtelocentric, st) or

Figure 3. C-banded chromosome preparations. (**A**) *Karsenia koreana*, arrow points to chromosome no. 14; (**B**) *Hydromantes platycephalus*, arrow points to chromosome no. 14; (**C**) *Ensatina eschscholtzii*; (**D**) *Plethodon dunni*; (**E**) *Aneides lugubris*, arrow points to a telocentric; (**F**) *Desmognathus ochrophaeus*. Scale=10 μm.


A
B
C
D
E
F

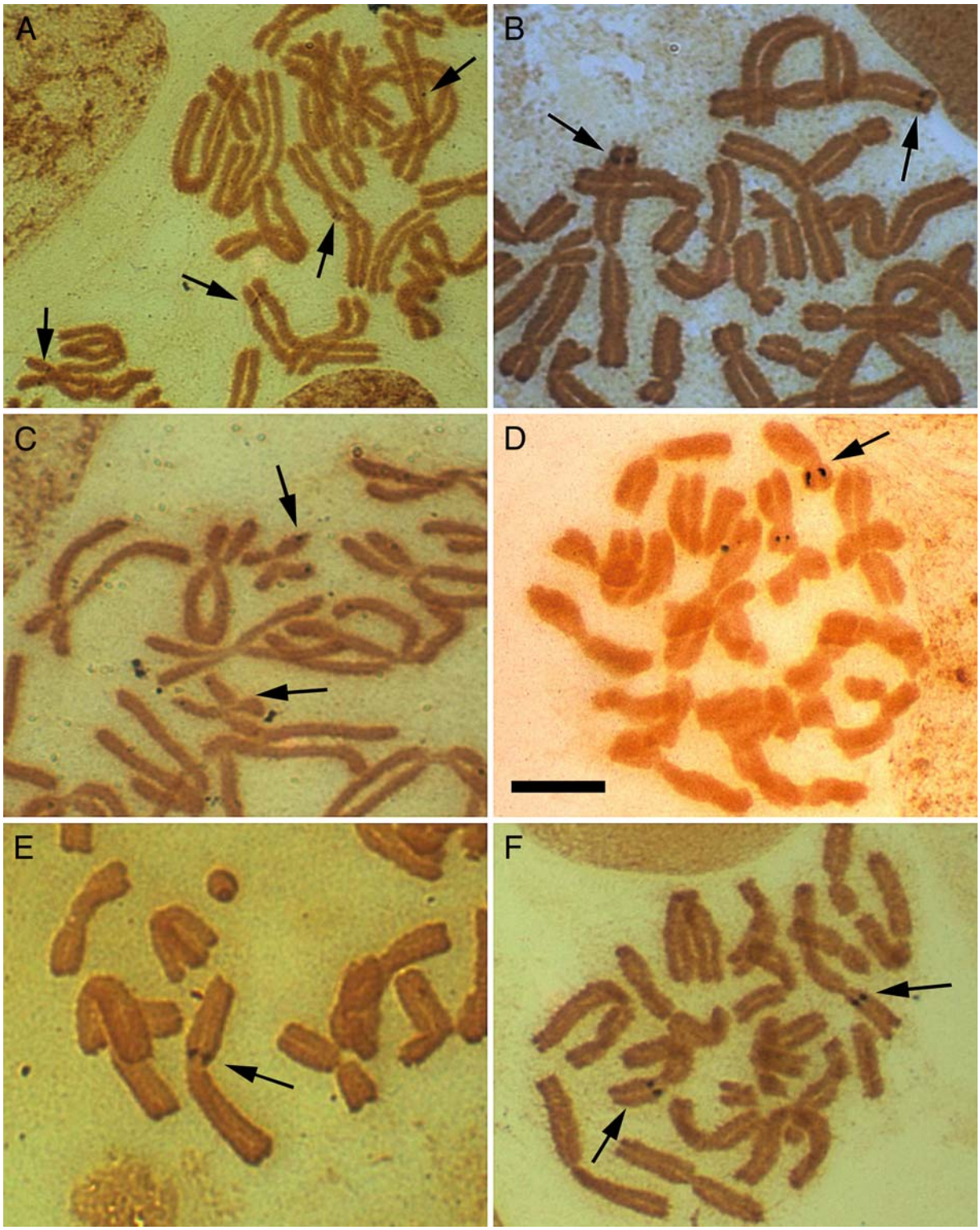


Figure 4. AgNOR banding. (**A**) *Karsenia koreana*; (**B**) *Hydromantes platycephalus*; (**C**) *Ensatina eschscholtzii*; (**D**) *Plethodon dunni*; (**E**) *Aneides lugubris*; (**F**) *Desmognathus ochrophaeus*. Arrows point to AgNOR bands. Scale=10 μm.

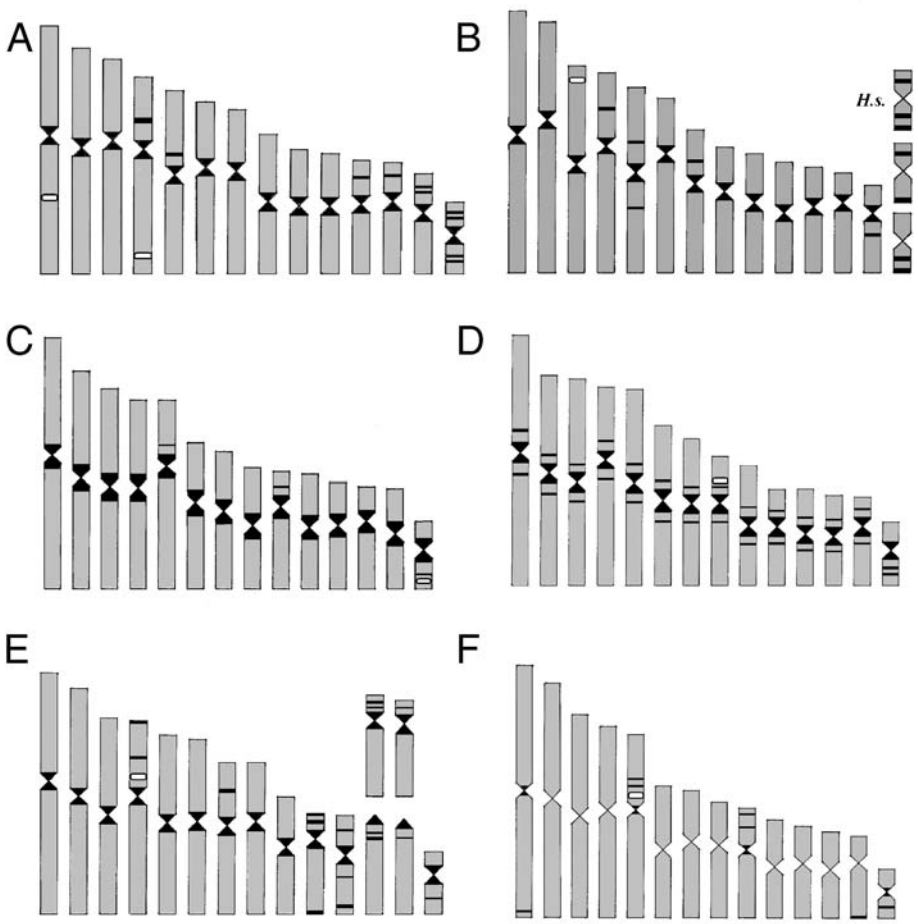


Figure 5. Idiograms showing relative lengths of chromosomes, chromosome morphologies, C-bands (dark areas) and AgNOR loci (open ovals): (**A**) *Karsenia koreana*; (**B**) *Hydromantes platycephalus* (*H.s.*=chromosome no. 14 in *H. shastae*; Sessions & Kezer 1991); (**C**) *Ensatina eschscholtzii*; (**D**) *Plethodon dunni*; (**E**) *Aneides lugubris* (heteromorphic chromosome nos. 12 and 13; Sessions & Kezer 1987); (**F**) *Desmognathus ochropheaus*.

telocentric (t) depending on the population, with one reported zone of hybridization (Figure 5E; Sessions & Kezer 1987). C-bands in *A. lugubris* are mostly concentrated at the centromeres but with a few interstitial and telomeric loci (Figures 3E, 5E), and a single AgNOR is located in the pericentric region of one of the largest chromosomes (Figures 4E, 5E). *Desmognathus ochrophaeus* also has 14 pairs of all-bi-armed chromosomes, a single AgNOR located in the short arm of a medium-sized submetacentric chromosome, and strikingly little detectable C-band heterochromatin (Figures 3F, 4F, 5F). Detectable C-bands in *D. ochrophaeus* include telomeric bands (Figure 5F). Finally, all species examined, including *Karsenia*, have a large asymmetrical chromosome (usually in position 4 or 5 of the karyotype) with an interstitial C-band in the short arm (Figure 5). An AgNOR is found in the short arm of this chromosome in *Aneides* and *Desmognathus*, but not in the other species examined. *Karsenia* has an AgNOR on this chromosome, located near the telomere of the long arm (Figure 5A).

Optical density measurements of *Karsenia* RBC nuclei, using *Aneides lugubris* (45.1 pg) as the standard, generated a C-value of 52.1 pg (range 38.1–71.4; SD 6.37), whereas nuclear area comparison generated an estimated C-value of 55.6 pg (range 45.4–63.5; SD 3.65). Using the line equation from a regression analysis of genome size on nuclear area for eight different salamander species, we

estimated a C-value of 52.9 pg for *Karsenia*. The mean of these three values for *Karsenia* is 53.5 pg.

Discussion

The results of our cytogenetic investigation provide critical information concerning the only known Asian plethodontid and its closest known relatives. The family Plethodontidae is by far the largest family of urodele amphibians, containing nearly 68% of the 560 described species of salamanders. The centre of diversity for this clade is North and Middle America, where the vast majority (98%) of species are found. A few (six or seven) representatives of the Sg. *Hydromantes* (*Hydromantes*, *Atylodes* and *Speleomantes*) are found in Europe and Western North America. The discovery of *Karsenia* in Asia, and the presence of its long-separated sister taxon Sg *Hydromantes* in both western North America and the eastern Mediterranean region suggest a wider distribution of the family in Eurasia in the past (Vieites *et al*. 2007). Despite this high species diversity and the long evolutionary history of plethodontids, the karyotypes are almost indistinguishable and only two chromosome numbers are known (Sessions & Kezer 1991, Green & Sessions 2007, Sessions 2008).

Most Holarctic genera of plethodontid salamanders, with the exception of the hemidactyliine *Batrachoseps*, have 14 pairs of chromosomes, while *Batrachoseps* and all neotropical plethodontids (Sg *Bolitoglossa*) have 13 pairs (Figure 1) (Sessions & Kezer 1991). In the vast majority of cases, the chromosomes are all bi-armed (metacentric or submetacentric) and remarkably uniform in size and shape (Mizuno & Macgregor 1974). Sex chromosomes at different degrees of differentiation characterize several neotropical genera and have also been found in one North American species, and in *Speleomantes* (Sg *Hydromantes*) of S. Europe (Sessions & Kezer 1991, Green & Sessions 2007, Sessions 2008). Otherwise the Plethodontidae is characterized by great karyological uniformity. Species included in the subfamily Plethodontinae also show general karyological uniformity in terms of chromosome number, although most species of *Aneides* show fixed and floating polymorphisms involving pericentric inversions among the smallest chromosome pairs (Macgregor & Jones 1977, Sessions & Kezer 1987). *Hydromantes*, long considered to be a relative of *Batrachoseps* and Sg *Bolitoglossa* (bolitoglossines of Wake 1966), stood out as the only lineage with 14 pairs of chromosomes; all other bolitoglossines have 13 pairs (Sessions & Kezer 1991). We now know that Sg *Hydromantes* is a plethondontine and not a bolitoglossine (Mueller *et al*. 2004; Vieites *et al*. 2007). In this respect *Karsenia* and Sg *Hydromantes* are typical members of the Plethodontinae, of which *Plethodon* is also typical. *Ensatina* also has 14 pairs of chromosomes in a *Plethodon*-like, all-bi-armed karyotype. While all species of desmognathine salamanders have 14 pairs of bi-armed chromosomes, they are distinguished in having the smallest genomes in the family in terms of the amount of nuclear DNA (Sessions & Larson 1987, Green & Sessions 2007, Sessions 2008), and correspondingly small chromosomes.

Neither C-banding nor AgNOR banding shows clade-specific cytogenetic affinities among these plethodontid groups. The differences observed among species in the location of heterochromatin and AgNOR loci raise the possibility that, unlike chromosome number and shape, which have remained 'frozen' throughout extremely long periods of evolution, underlying chromosomal structure has undergone substantial change. C-banding reveals relatively large amounts of heterochromatin concentrated mainly at the centromeres in the chromosomes of *Karsenia*, a pattern seen in other species with similar-sized genomes, including North American *Hydromantes* (Sessions & Kezer 1991). However, each species shows an essentially unique pattern of interstitial C-bands (Figure 5). North American species of *Hydromantes* are unusual in showing variation in the C-banding pattern in the smallest chromosome pair, no. 14 (Sessions & Kezer 1991), and in European *Speleomantes* this chromosome pair is involved in an XY sex chromosome heteromorphism (Nardi 1991, Sessions & Kezer 1991). *Karsenia* has a conspicuously large amount of C-band heterochromatin organized as two sets of bands in both arms of chromosome no. 14, superficially resembling *Hydromantes* chromosome no. 14, but we found no evidence of variation in this pattern between specimens (although our sample size is very small). Species of *Aneides* show inversion polymorphisms in one or more pairs of the three smallest chromosomes, including chromosome no. 14 (Sessions & Kezer 1987), suggesting that increased rates of chromosomal rearrangements occurred during their evolutionary history (Sessions & Kezer 1987,

Sessions & Wiktorowski 2000). The functional significance of these cytogenetic features remains unclear. *Desmognathus ochrophaeus* stands out among the species examined in exhibiting little detectable C-band heterochromatin, probably reflecting its exceptionally small genome; other desmognathines have similar karyotypes and genome sizes (Hally *et al*. 1986, S.K. Sessions, unpublished).

No apparent patterns are seen in the variation of active ribosomal gene loci, as represented by AgNOR banding, each species having essentially unique AgNOR loci. The similarity in AgNOR bands between *A. lugubris* and *D. ochrophaeus* is likely homoplastic in that each species is deeply nested within the respective genera. The hemidactyliine *Eurycea wilderae* also exhibits a similar locus (Sessions & Wiktorowski 2000). However, variation in AgNOR loci is also seen in these genera (Sessions & Kezer 1987, S.K. Sessions, unpublished). AgNORs appear to be located at random relative to other chromosomal 'landmarks' such as centromeres, telomeres and C-bands (Figure 5). Multiple AgNORs as seen in *Karsenia* (Figure 5) have also been reported in some bolitoglossine salamanders with large genomes (Sessions & Kezer 1991). The AgNOR locus in North American *Hydromantes* (*H. platycephalus*) is totally different from that seen in European species of *Atylodes* and *Speleomantes*, which have an AgNOR locus in the pericentric region of a single small chromosome, no. 10 or 12 (Nardi 1991). Whether these differences represent chromosome repatterning or homosequentiality remains to be determined.

Within the context of conserved chromosome number and shape, plethodontid salamanders show a very wide range of extraordinarily large genome sizes, from approximately 14 to 72 pg per haploid nucleus, a more than 5-fold difference (Figure 6; Sessions & Larson 1987, Gregory 2001, Sessions 2008). Very large genomes are found both in the Plethodontinae (e.g., *Hydromantes*, *Speleomantes*, *Karsenia*) and the Hemidactyliinae (e.g., *Bolitoglossa*). Genome size in the Plethodontidae has a curious geographic distribution that appears to transcend phylogenetic relationships (Figure 6; Sessions 1984, 2008). The smallest genomes are found in groups restricted to eastern North America, including *Desmognathus*, the spelerpines (*Eurycea*, *Gyrinophilus*, *Pseudotriton* and *Stereochilus*), *Hemidactylium*, and eastern species of *Plethodon*. Genomes are substantially larger among western North American plethodontid species, including *Hydromantes*, *Ensatina*, *Aneides*, *Batrachoseps* and *Plethodon*, representing the two major plethodontid lineages. The only eastern species of plethodontid salamander with a large genome comparable to those of western North American plethodontids (~44 pg) is *Aneides aeneus* (Gregory 2001), an eastern member of an otherwise western clade. Among western species of *Plethodon*, *P. larselli* (~49 pg) and especially *P. vandykei* (69 pg) stand out in having exceptionally large genomes (Mizuno & Macgregor 1974, Sessions & Larson 1987, Gregory 2001, Sessions 2008).

Genome sizes show the most variation in the most speciose taxon, the neotropical plethodontids (bolitoglossines), including some of the smallest and largest genomes reported for the family (Figure 6; Sessions & Kezer 1991, Sessions 2008). The largest genome of any plethodontid (~72 pg), representing the largest genome of any terrestrial vertebrate, is found in the European *Speleomantes italicus* (Gregory 2001, Sessions & Kezer 1991). The genome size of *Karsenia* (~54 pg per haploid nucleus) is relatively large by almost any standards, similar to the genome sizes of North American *Hydromantes* and some tropical bolitoglossine species (Figure 6; Sessions & Larson 1987, Sessions & Kezer 1987, 1991, Gregory 2001). Thus, western plethodontids that are geographically and phylogenetically closest to *Karsenia*, including *Ensatina*, *Aneides*, *Hydromantes* and western *Plethodon*, also have relatively large genomes, and of these the C-value of *Karsenia* is closest to that of *H. platycephalus* (50 pg). Within the Plethodontinae, the desmognathines stand out in having extraordinarily small genomes (Figure 6), and the most parsimonious explanation from current phylogenies (Vieites *et al*. 2007) is that genome size must have undergone extensive decreases in this group.

Variation in genome size among plethodontids also appears to have important phenotypic correlates in terms of morphological complexity and rates of development (Sessions & Larson 1987, Roth *et al*. 1994, Gregory 2002, Sessions 2008). Positive correlations between genome size, cell size and cell cycle time have been documented in a variety of organisms, including amphibians (Olmo & Morescalchi 1975, Horner & Macgregor 1983, Gregory 2005, Litvinchuk *et al*. 2007). Sessions & Larson (1987) found that species of plethodontid salamanders with larger genomes regenerated their limbs more slowly than those with smaller genomes, suggesting that

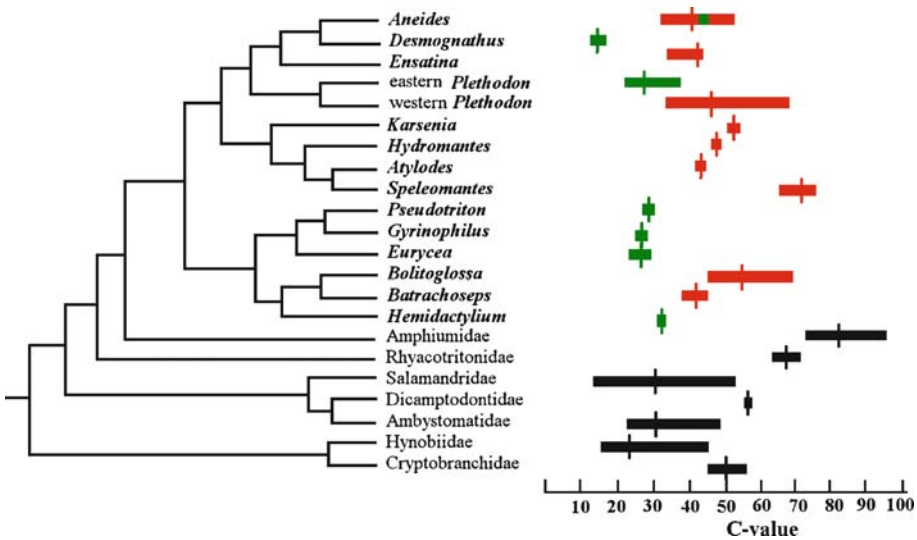


Figure 6. Diagram showing the distribution of C-values among plethodontids and other salamanders based on a molecular phylogenetic analysis of Vieites *et al*. (2007). Eastern species of plethodontids are indicated by green bars and species found elsewhere (western North America, Asia, Europe) by red bars. Bars show ranges and means.

genome size may affect development rate. The most extensive variation in genome size, including the largest genomes, are found in salamanders with direct development and no aquatic larval stage, possibly reflecting the importance that genome size has for rates and modes of development. Salamanders with large genomes that have larval stages are often perennibranchiate (e.g., *Necturus*) or fail to complete metamorphosis (e.g., *Amphiuma*), suggesting that metamorphosis may be difficult to attain with large genome size. The proximal cause for this phenomenon is likely related to the presence of large, slowly dividing cells and reduced metabolic rates in species with very large genomes, which retard the rapid developmental changes demanded during amphibian metamorphosis. From this perspective, the relatively small genomes of *Desmognathus* may have enabled the homoplasic evolution of larvae in this genus (Chippindale *et al*. 2004, Mueller *et al*. 2004). The spelerpines and *Hemidactylium*, all of which have a larval stage, also have relatively small genomes (Figure 6). Similar correlations between genome size, geographic distribution and embryonic development have recently been reported for species of the salamander family Salamandridae (Litvinchuk *et al*. 2007). These observations underline the importance of future research in understanding the relationship of genome size, biogeography and evolutionary changes in modes of development among plethodontids as well as other groups of salamanders.

Our results have important implications for cytogenetic evolution of plethodontid salamanders. The variation in interstitial C-bands and AgNOR loci suggests that the conservation of chromosome number and shape may have been accompanied by underlying changes in the positions of genes and other sequences on the chromosomes, as expected for highly diverged species. Similar variation in NOR positions for species of salamandrids was interpreted as 'chromosome repatterning' via translocations by Mancino *et al*. (1977). On the other hand, Macgregor & Sherwood (1979) interpreted variation in NOR position in plethodontid salamanders as 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). Limitations of C-bands and AgNOR bands notwithstanding, our results appear to point towards chromosome repatterning, but resolution of this old controversy will require additional molecular cytogenetic studies, namely *in situ* hybridization, which we encourage.

The geographically nearest relatives of *Karsenia* are species of western plethodontids, and *Karsenia* appears to show at least some cytogenetic similarities. Paleogeographic and paleoclimatic evidence indicates that physical connections and environmental conditions would have allowed many opportunities for migration between North America and Asia over the last 90 million years up to at least the Late Miocene (reviewed in Vieites *et al*. 2007). Molecular-based time estimates suggest that *Karsenia* diverged from its closest relatives, Sg. *Hydromantes*, in the late Cretaceous, experiencing a long period of independent evolution (Vieites *et al*. 2007). The fact that *Karsenia* has the same chromosome number and similar genome size as groups from which it has been evolving independently for tens of millions of years is a testament to how extraordinarily stable chromosome number and genome size can be in this clade.

Acknowledgements

We thank S. Karsen, R. Bonett and S. Yang for help in obtaining specimens, and the Korean authorities for collecting permits. This work was supported in part by NSF grants EF-0334939 to D.B.W., and DEB-0515536 to S.K.S.. This paper is dedicated to the memory of Dr. James Kezer, pioneer in salamander cytogenetics.

References

Chippindale PT, Bonett RM, Baldwin AS, Wiens JJ (2004) Phylogenetic evidence for a major reversal of life history evolution in plethodontid salamanders. *Evolution* **58**: 2809–2822.

Conger AD, Fairchild LM (1953) A quick freeze method for making smear slides permanent. *Stain Technol* **28**: 281–283.

Green DM, Sessions SK (2007) Karyology and cytogenetics. In: Heatwole H, ed. *Amphibian Biology*, Vol. 7. Chipping Norton, Australia: Surrey Beatty & Sons, pp. 2757–2842.

Gregory TR (2001) *Animal Genome Size Database*. www.genomesize.com.

Gregory TR (2002) Genome size and developmental complexity. *Genetica* **115**: 131–146.

Gregory TR (2005) Genome size evolution in animals. In: Gregory TR, ed. *The Evolution of the Genome*. Burlington, MA: Elsevier Academic Press, pp. 3–87.

Hally MK, Rasch EM, Mainwaring HR, Bruce RC (1986) Cytophotometric evidence of variation in genome size of desmognathine salamanders. *Histochemistry* **85**: 185–192.

Hardie DC, Gregory TR, Hebert PDN (2002) From pixels to picograms: a beginner's guide to genome quantification by Feulgen image analysis densitometry. *J Histochem Cytochem* **50**: 735–749.

Horner HA, Macgregor HC (1983) C value and cell voume: their significance in the evolution and development of amphibians. *J Cell Sci* **63**: 135–146.

Hsu TC (1981) Polymorphism in human acrocentric chromosomes and the sliver staining method for nucleolus organizer regions. *Karyogram* **7**: 45.

Humason GL (1972) *Animal Tissue Techniques*. San Francisco, CA: WH Freeman.

Litvinchuk S, Rosanov JM, Borkin LJ (2007) Correlations of geographic distribution and temperature of embryonic development with the nuclear DNA content in the Salamandridae (Urodela, Amphibia). *Genome* **50**: 333–342.

Macgregor HC, Jones C (1977) Chromosomes, DNA sequences, and evolution in salamanders of the genus *Aneides*. *Chromosoma* **63**: 1–9.

Macgregor HC, Sherwood S (1979) The nucleolus organizers of *Plethodon* and *Aneides* located by in situ nucleic acid hybridization with *Xenopus* ^{3}H-ribosomal RNA. *Chromosoma* **72**: 271–280.

Macgregor H, Varley J (1983) *Working with Animal Chromosomes*. New York: Wiley.

Mancino G, Ragghianti M, Bucci-Innocenti S (1977) Cytotaxonomy and cytogenetics in European newt species. In: Taylor DH, Guttman SI, eds. *The Reproductive Biology of Amphibians*. New York: Plenum, pp. 411–447.

Min MS, Yang SY, Bonett RM, Vieites DR, Brandon RA, Wake DB (2005) Discovery of the first Asian plethodontid salamander. *Nature* **435**: 87–90.

Mizuno S, Macgregor HC (1974) Chromosomes, DNA sequences and evolution in salamanders of the genus *Plethodon*. *Chromosoma* **48**: 239–296.

Mueller RL, Macey JR, Jaekel M, Wake DB, Boore JL (2004) Morphological homoplasy, life history evolution, and historical biogeography of plethodontid salamanders from complete mitochondrial genomes. *Proc Natl Acad Sci USA*. **101**: 13820–13825.

Nardi I (1991) Cytogenetics of the European Plethodontid Salamanders, Genus *Hydromantes*. In: Green DM, Sessions SK, eds. *Amphibian Cytogenetics and Evolution*. New York: Academic Press, pp. 131–156.

Olmo E, Morescalchi A (1975) Evolution of the genome and cell sizes in salamanders. *Experientia* **31**: 804–806.

Roth G, Blanke J, Wake DB (1994) Cell size predicts morphological complexity in the brain of frogs of salamanders. *Proc Natl Acad Sci USA* **91**: 4796–4800.

Schmid M, Olert J, Klett C (1979) Chromosome banding in amphibian III. Sex chromosomes in *Triturus*. *Chromosoma (Berlin)* **71**: 29–55.

Sessions SK (1984) *Cytogenetics and evolution in salamanders*. PhD dissertation, University of California, Berkeley.

Sessions SK (1996) Chromosomes: molecular cytogenetics. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular Systematics*, 2nd edn. Sunderland, MA: Sinauer Associates, pp. 121–168.

Sessions SK (2008) Evolutionary cytogenetics in salamanders. *Chromosome Res* **16**: 183–201.

Sessions SK, Kezer J (1987) Cytogenetic evolution in the plethodontid salamander genus *Aneides*. *Chromosoma (Berlin)* **95**: 17–30.

Sessions SK, Kezer J (1991) Evolutionary cytogenetics of Bolitoglossine Salamanders. In: Green DM, Sessions SK, eds. *Amphibian Cytogenetics and Evolution*. New York: Academic Press, pp. 89–130.

Sessions SK, Larson A (1987) Developmental correlates of genome size in plethodontid salamanders and their implications for genome evolution. *Evolution* **41**: 1239–1251.

Sessions SK, Wiktorowski JL (2000) Population cytogenetics of the Plethodontid salamander *Eurycea wilderae*. In: Bruce RC, Jaeger RG, Houck LD, eds. *The Biology of Plethodontid Salamanders*. New York: Kluwer Academic/Plenum, pp. 327–343.

Vieites DR, Min MS, Wake DB (2007) Rapid diversification and dispersal during global warming periods by plethodontid salamanders. *Proc Natl Acad Sci USA* **104**: 19903–19907.

Wake DB (1966) Comparative osteology and evolution of the lungless salamanders, family Plethodontidae. *Mem S Calif Acad Sci* **4**: 1–111.

Wiens JJ, Bonett RM, Chippindale PT (2005) Ontogeny discombobulates phylogeny: paedomorphosis and higher-level salamander relationships. *Syst Biol* **54**: 91–110.