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Genome size, cell size, and the evolution of enucleated erythrocytes in attenuate salamanders

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Abstract

Within the salamander family Plethodontidae, five different clades have evolved high levels of enucleated red blood cells, which are extremely unusual among non-mammalian vertebrates. In each of these five clades, the salamanders have large genomes and miniaturized or attenuated body forms. Such a correlation suggests that the loss of nuclei in red blood cells may be related, in part, to the interaction between large genome size and small body size, which has been shown to have profound morphological consequences for the nervous and visual systems in plethodontids. Previous work has demonstrated that variation in both the level of enucleated cells and the size of the nuclear genome exists among species of the monophyletic plethodontid genus *Batrachoseps*. Here, we report extensive intraspecific variation in levels of enucleated red blood cells in 15 species and provide measurements of red blood cell size, nucleus size, and genome size for 13 species of *Batrachoseps*. We present a new phylogenetic hypothesis for the genus based on 6,150 bp of mitochondrial DNA sequence data from nine exemplar taxa and use it to examine the relationship between genome size and enucleated red blood cell morphology in a phylogenetic framework. Our analyses demonstrate positive direct correlations between genome size, nucleus size, and both nucleated and enucleated cell sizes within *Batrachoseps*, although only the relationship between genome size and nucleus size is significant when phylogenetically independent contrasts are used. In light of our results and broader studies of comparative hematology, we propose that high levels of enucleated, variably sized red blood cells in *Batrachoseps* may have evolved in response to rheological problems associated with the circulation of large red blood cells containing large, bulky nuclei in an attenuate organism.

Keywords

Batrachoseps; Plethodontidae; Nucleus; Miniaturization; Red blood cells

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Introduction

Plethodontidae is the largest family of salamanders, with over 375 named species, all of which are lungless (AmphibiaWeb, 2008). The genus *Batrachoseps* is a clade of plethodontids comprising 20 named species with distributions in the western United States and Baja California, Mexico. Despite high levels of genetic diversity (Yanev, 1980; Jockusch and Wake, 2002), most species of *Batrachoseps* exhibit extreme overall morphological stasis. Body form within the genus evolved from a fairly robust ancestral morphology to an attenuated body form with miniaturized limbs and a slender trunk, head, and tail. Three members of the genus, which together form the sister group to all other *Batrachoseps*, retain the ancestral robust morphology; these three species together form the subgenus *Plethopsis*. Most other members of the genus *Batrachoseps* have the attenuated morphology and together form the subgenus *Batrachoseps*, hereafter called “the attenuate clade.” However, one member of the attenuate clade, *Batrachoseps stebbinsi*, has secondarily evolved a more robust body form. Previous analyses based on the mitochondrial *cytochrome-b* gene have demonstrated five well-supported, monophyletic species groups within the attenuate clade (the *nigriventris* group, the *pacificus* group, the *attenuatus* group, the *gabrieli* group, and the *relictus* group), but the relationships among them have remained unresolved (Jockusch and Wake, 2002).

An unusual red blood cell (RBC) morphology has been reported in three species within the attenuate clade (Emmel, 1924; Villolobos et al., 1988). Instead of the nucleated RBCs characteristic of almost all non-mammalian vertebrates, these salamanders possess high levels (> 80 %) of circulating enucleated RBCs. The non-attenuate species of the subgenus *Plethopsis* also possess such cells, but at much lower levels (1–10 %) (Stebbins and Lowe, 1949). Villolobos et al. (1988) surveyed 85 out of the 378 currently recognized species of plethodontids, representing 21 out of 27 recognized genera (www.amphibiaweb.org), and found that high levels (> 80 %) of enucleated RBCs have also evolved independently within four other genera: *Bolitoglossa*, *Nototriton*, *Oedipina*, and *Thorius*. These four genera, as well as *Batrachoseps*, are members of the subfamily Bolitoglossinae (Chippindale et al., 2004). Low levels of enucleated RBCs are present in other bolitoglossine species and in species within the remaining plethodontid subfamilies (Emmel, 1924; Villolobos et al., 1988). Enucleated salamander RBCs differ from the analogous cells in mammals in their retention of an ellipsoid shape, a marginal band of microtubules, and other cytoskeletal structures that are absent from mature mammalian RBCs (Cohen, 1982).

All plethodontid species with high levels of enucleated RBCs share two characteristics: (1) miniaturized or attenuated body form, and (2) large genome size (haploid nuclear DNA content) relative to other vertebrates (Villolobos et al., 1988). Salamanders exhibit extreme variation in genome size among species, from ~15 pg (e.g. genus *Desmognathus*) to ~120 pg (genus *Necturus*). Within plethodontids alone, genome sizes range from ~15 pg (genus *Desmognathus*) to ~76 pg (*Hydromantes italicus*) (Gregory, 2006). Evolutionary changes in genome size within salamanders have been linked to several traits, including: (1) rate of embryonic development (Jockusch, 1997; Gregory, 2002); (2) rate of limb regeneration following injury (Sessions and Larson, 1987); and (3) complexity of the developmental program, in particular the presence/absence and intensity of metamorphosis (Gregory, 2002). In addition, genome sizes are correlated with both nuclear and cell sizes across a variety of tissue types and taxonomic groups, including salamanders (Olmo and Morescalchi, 1975; Olmo, 1983; Gregory, 2001a, 2003). The presence of both a miniaturized/attenuated body form and a large genome in clades with enucleated RBCs suggests that the evolution of enucleation five times within Plethodontidae may be related to the “biological size” of these organisms, which takes into account both the size of the organism and the size of the cells from which it is constructed (Hanken and Wake, 1993). The interaction between large cell size and small body size has been shown to have profound morphological consequences for both the nervous

and visual systems of plethodontid salamanders. Such morphological consequences include simplification and/or loss of structures resulting from spatial constraints on the total number of large cells that can fit into any region of a miniaturized animal at a given point in its ontogeny. These spatial constraints are compounded by the relatively slow rates of cell division and differentiation in organisms with large genomes (Sessions and Larson, 1987; Roth et al., 1994, 1997), which also contribute to structural simplification. Enucleated RBCs may result from an interaction between large genome size and small body size in one of at least two ways: (1) such cells may result from random breakage of large, nucleated RBCs during circulation, causing nuclear loss (Villolobos et al., 1988); or (2) such cells may have evolved in response to a physical constraint against the circulation of large, fully nucleated cells through the circulatory system of a miniaturized/attenuated animal (Mueller, 2000; Gregory, 2003).

In this study, we report genome size, nucleus size, and nucleated and enucleated cell sizes for 13 species of *Batrachoseps*. We also report extensive intraspecific variation in the levels of enucleated RBCs in 15 species. In addition, we provide a phylogenetic hypothesis for the clade based on 6,150 bp of mitochondrial DNA sequence data from nine exemplar taxa. We use this new phylogenetic hypothesis to examine the relationships between *Batrachoseps* genomic and cytological parameters in a phylogenetic context. Finally, we present a hypothetical evolutionary scenario explaining the appearance of enucleated RBCs in salamanders in light of our results and broader studies of comparative hematology.

Materials and methods

Specimens

Individuals from different mitochondrially-diagnosed population-level lineages (called species for simplicity, although not all meet criteria for species status) were selected to represent each of the major clades of *Batrachoseps* (Jockusch and Wake, 2002) for measurements of genome size, cell size, RBC enucleation, and phylogenetic analyses. Attempts were made to use the same exemplar taxa for all analyses, but this was not always possible. Measurements of cell, nucleus, and genome sizes required fresh specimens; measurement of RBC enucleation required a series of individuals collected together; and estimates of phylogenetic relationships required taxa that spanned the basal split within each major *Batrachoseps* clade to decrease the chance of long branch attraction artifacts. Fresh specimens were collected between 2001 and 2002, and all specimens have been deposited in the Museum of Vertebrate Zoology, Berkeley. Specimen information is summarized in Appendix A (online supplementary table).

Phylogenetic analysis

Whole genomic DNA was extracted from frozen tissue in the Museum of Vertebrate Zoology collection. A > 6,150 bp fragment including *trnI*, *trnQ*, *trnM*, *nad2*, *trnW*, *trnA*, *trnN*, the origin of light strand replication (*O_L*), *trnC*, *trnY*, *cox1*, *trnS2*, *trnD*, *cox2*, *trnK*, *atp8*, *atp6*, *cox3*, *trnG*, *nad3*, and *trnR* was PCR amplified from each specimen using 5'–3':TACGACCTCGATGTTG(C/G)ATCAGG and 5'–3':TCTACGTGGGCTTTTGGTAGTCA (all taxa except *B. attenuatus* and *B. nigriventris*) or 5'–3':AAACT(G/A)GGATTAGATACCC(C/T)ACTA and 5'–3':TCTACGTGGGCTTTTGGTAGTCA (*B. attenuatus* and *B. nigriventris*). Sequences are deposited in GenBank (Accession Nos. EU117187–EU117195). PCR products were sheared to ~1.5 kb with a HydroShear device (GeneMachines) and enzymatically repaired to blunt their ends. Products were gel-extracted, ligated into pUC18 vector, and electroporated into competent cells (InVitrogen) using a Gene Pulser II (BioRad). Plated cells were grown overnight. Colonies were picked using a Qbot robotic colony picker (Genetix) and processed robotically through the following steps: (1) rolling circle amplification of plasmids, (2)

sequencing reactions using fluorescent dideoxynucleotide terminators, (3) reaction clean-up, and (4) loading onto either ABI 3730XL or Megabace 4000 DNA sequencing machines.

Sequences from each half-genome were assembled into contigs using PHRAP and confirmed visually using CONSED v.13 (Gordon et al., 1998). Half-genomes were annotated manually or using DOGMA (Wyman et al., 2004). Sequences of each gene were aligned using CLUSTAL W (Thompson et al., 1994) (gap creation and extension costs set to the defaults = 10 and 5, respectively), adjusted to preserve reading frame and tRNA secondary structure, and concatenated for phylogenetic analysis, producing a final alignment of 6,150 bp. For one species, *B. nigriventris*, 2,310 bp were not sequenced and were coded as missing data. The alignment is available from TreeBASE (S1866).

Bayesian phylogenetic analysis was implemented using MRBAYES v. 3.04b (Huelsenbeck and Ronquist, 2001). Analyses were performed using four data partitions to improve the fit of the substitution model to the data in light of heterogeneous nucleotide substitution processes (Mueller et al., 2004; Nylander et al., 2004; Brandley et al., 2005). These four partitions comprised the following subsets of the dataset: (1) the concatenated tRNA genes and the presumed O_L , (2) all first codon positions, (3) all second codon positions, and (4) all third codon positions. For each of the four partitions, the best-fitting nucleotide substitution model was selected using the Akaike Information Criterion implemented in MRMODELTEST (v. 1.1b), modified from MODELTEST (Posada and Crandall, 1998) by J.A.A. Nylander. Flat Dirichlet distributions were used for substitution rates and base frequencies, and default flat prior distributions were used for all other parameters. Metropolis-coupled Markov chain Monte Carlo analyses were run with one cold and three heated chains (temperature set to the default = 0.2) for 15 million generations and sampled every 1,000 generations. Stationarity was confirmed by examining plots of $-\ln L$ scores and parameter values; 10 million generations were discarded as burn-in. The tree was rooted with *Batrachoseps wrightorum* + *Batrachoseps campi*. Equally-weighted maximum parsimony analysis was performed using PAUP*4.0b10 (Swofford, 1998). A heuristic search was performed with ten random addition replicates and TBR branch-swapping. Non-parametric bootstrap proportions for clades were assessed using 1,000 pseudo-replicates.

Genome, nucleus, and cell sizes

Air-dried blood smears were prepared on standard microscope slides for use in measurements of genome size, cell and nucleus sizes, and percentage of enucleated cells. Estimates of nuclear genome size were made using Feulgen image analysis densitometry as described previously (Hardie et al., 2002). Blood smears were post-fixed overnight in MFA solution (85 methanol : 10 formalin : 5 glacial acetic acid), rinsed in tepid tapwater, and hydrolyzed for 120 min in 5N HCl at room temperature before being stained for 120 min in freshly prepared Schiff reagent and passed through a series of bisulfite and distilled water rinses. Stained slides were stored in the dark until analysis with the BIOQUANT image analysis package described in Hardie et al. (2002). The sums of all nuclear pixel point densities (integrated optical densities, or IODs) of 50 nuclei were analyzed per individual under 630 X magnification, with 1–4 individuals assessed per species according to availability. Due to the highly compact nature of DNA in salamander nuclei, it is important to use an appropriate standard for converting IODs into absolute nuclear DNA contents in picograms (Sessions and Larson, 1987; Hardie et al., 2002). The mean IOD of two individuals of the salamander species *Ambystoma jeffersonianum* (1 C = 28.8 pg) (Vinogradov, 1998) was used to calculate absolute genome size, and nuclei from as similar a compaction level as possible were measured for all species. Blood smears from chicken (*Gallus domesticus*) and rainbow trout (*Oncorhynchus mykiss*) were also included to confirm staining efficacy, and several species of non-attenuate

salamanders with nucleated blood were included for comparison with the attenuate species (Table 1).

Cell and nucleus sizes were assessed using the same blood smears as for genome size (and/or smears from the same individuals) following treatment with Wright-Giemsa stain. Dry cell and nucleus areas were measured in pixels using the BIOQUANT True Color Windows 98 v. 3.50.6 image analysis software package (R&M Biometrics; Nashville, TN) under 400 X magnification and converted to absolute areas in μm^2 using a spatial calibration slide. Although both genome and nucleus sizes are determined by measurements of nuclear parameters, the two measurements are distinct; genome size is measured using IOD, whereas nucleus size is measured using number of pixels. Different enucleated RBC morphologies were observed in *Batrachoseps* species, and these were measured separately: (1) nucleated RBCs; (2) RBCs following primary enucleation, which retain the elliptical shape of nucleated RBCs; and (3) RBCs following secondary enucleation (Fig. 1), which result either from subdivision of primarily enucleated RBCs or through a pinching off of a portion of the cytoplasm from nucleated cells (Emmel, 1924; Fig. 2).

Relationships among genome, nucleus, and cell sizes

Pearson correlations and least-squares linear regression analyses were performed using $\log_{10}$ -transformed data to test for relationships between genome size and nucleus size, nucleated cell size, primarily enucleated cell size, and secondarily enucleated cell size without correcting for phylogeny. In addition, analyses were performed correcting for statistical non-independence resulting from underlying phylogenetic relationships (Felsenstein, 1985). A topology including all species with genome, nucleus, and cell size data was constructed based on combined results from the present study and Jockusch and Wake (2002). Branch length estimates were obtained using *cytochrome-b* sequences published by Jockusch and Wake (2002) or collected since and generously provided by those authors (online supplementary table at Appendix A). A full likelihood evaluation of these 13 sequences was performed in PAUP*4.0b10 using the best-fitting model of nucleotide substitution (TVM + I + Γ) and associated model parameters selected using the Akaike Information Criterion implemented in MODELTEST (Posada and Crandall, 1998). The topology and branch length estimates were used to calculate phylogenetically independent contrast values for genome, cell, and nucleus sizes using CAIC v. 2.6.9 (Purvis and Rambaut, 1995). Because of substantial intra-specific variation, median values for each species were used for calculation of independent contrasts to reduce the effects of outliers. Analyses were repeated using species means and all branch lengths = 1 in the PDAP module (Midford et al., 2002) in Mesquite v. 1.11 (Maddison and Maddison, 2006); the two methods gave very similar results. Resulting contrast values were tested for normality across species using the Shapiro-Wilk W test and were $\log_{10}$ -transformed to approximate normal distributions. Contrast regression analyses were forced through the origin (Garland et al., 1992).

RBC enucleation

Measurements of the percentage of enucleated cells within and among *Batrachoseps* species were performed using both fresh and preserved material. For fresh material, the same air-dried slides used for the measurements of genome, nucleus, and cell sizes were used. Five microscope fields were assessed by image analysis at 100 X magnification using a manual counting feature (900–2,500 cells total per individual, 3–5 individuals collected together/species) (Fig. 3). In addition, slides were prepared from museum specimens previously fixed in 10 % magnesium carbonate-buffered formalin and stored in 70 % ethanol from 15 species (5–23 individuals collected together/species). Whole preserved salamander specimens were submerged in 70 % ethanol under a dissecting microscope, an incision through the pectoral girdle was made, and a 27G1/2 needle was inserted into the heart. Blood cells were collected in suspension and placed

on glass microscope slides to dry. Slides were stained according to the following protocol: (1) ddH₂O for three minutes; (2) thionine-methyl green for nucleic acids for ten minutes (0.1 g chloroform-extracted methyl green; 0.0165 g thionine; 42 ml 0.01M HCl, 58 ml 0.01 M sodium citrate); (3) three rinses in ddH₂O; (4) three changes of 4:1 1-butanol:ethanol for three minutes each; and (5) one rinse in 100 % ethanol (Presnell and Schreibman, 1997). Air-dried slides were examined under 400 X magnification using a Zeiss MC 63 microscope, and total numbers of nucleated and enucleated cells were recorded (14–976 cells per individual, median = 419). The use of preserved museum specimens allowed larger sample sizes necessary to capture variation within *Batrachoseps* species. Where possible, both fresh and preserved samples of the same species were measured for verification of methods.

Results

Phylogenetic relationships

Partitioned Bayesian and maximum parsimony analyses of 6,150 bp of mitochondrial genome sequence yielded congruent topologies (Fig. 4A). All nodes are supported by Bayesian posterior probabilities of 100 %. All but two nodes are supported by parsimony non-parametric bootstrap proportions of 1.00; exceptions are *nigriventris* group + *relictus* group (0.98) and *pacificus* group + *gabrieli* group (0.66). These results are congruent with those of Jockusch and Wake (2002), who reported high support for the following nodes: (1) the basal split between *Plethopsis* and the attenuate clade; (2) the split within the attenuate clade between *B. attenuatus* and all remaining lineages; and (3) the sister group relationship between *B. gabrieli* and the *pacificus* group. The relationships between the *B. gabrieli* + *pacificus* group, *nigriventris* group, and *relictus* group were resolved differently, and with low support, by different analyses in Jockusch and Wake (2002).

Genome, nucleus, and cell sizes

Genome sizes reported for *Batrachoseps* species range from 26 pg to 37 pg and are listed in Table 1. Previous estimates for members of this genus are available for two species: *B. attenuatus* at 39.8 pg (Sessions and Larson, 1987) or 42.0 pg (Olmo and Morescalchi, 1975) and *B. campi* at 35.9 pg (Sessions and Kezer, 1991). The present data are ~8 % lower than previous estimates. This inconsistency likely reflects the values assumed for the standard species rather than errors in staining or density measurements, as the ratio between *B. attenuatus* and *B. campi* obtained here is identical to that based on the earlier measurements of Sessions and Larson (1987) and Sessions and Kezer (1991). Although the non-attenuate species *B. campi* and *B. robustus* exhibit genome sizes at the high end of the observed range, they are not exceptional among *Batrachoseps* (Table 1).

Erythrocyte areas vary substantially among salamander species (Table 1), with notable variation also occurring within species and individual organisms. In the attenuate clade, species mean values of nucleated RBC areas range from 439 μm^2 (*B. gavilanensis*) to 676 μm^2 (*B. attenuatus*), whereas the values are considerably higher among members of the non-attenuate subgenus *Plethopsis* (*B. campi* = 811 μm^2 , *B. robustus* = 833 μm^2). Mean areas of cells following primary enucleation are smaller than those of nucleated cells. In attenuate species, these range from 324 μm^2 (*B. gavilanensis*) to 470 μm^2 (*B. attenuatus*). Primarily enucleated cells are rare in the non-attenuate subgenus *Plethopsis*, but are larger than those of the attenuate species (*B. campi* = 700 μm^2 , *B. robustus* = 787 μm^2). Species means of cells following secondary enucleation in attenuate species range from 164 μm^2 (*B. nigriventris*) to 301 μm^2 (*B. gregarius*) and are extremely rare or absent among non-attenuate species. Differences in living cell sizes between enucleated and nucleated cells exceed differences in dry area because nucleated cells are biconvex rather than flat (Chien et al., 1971). Mean nucleus sizes within the attenuate clade range from 84 μm^2 (*B. gavilanensis*) to 117 μm^2 (*B. attenuatus*); again,

values are generally higher in the non-attenuate species (*B. robustus* = 117 μm^2 , *B. campi* = 125 μm^2). *B. campi* has the largest nuclei and exhibits the lowest DNA compaction level in the genus, indicated by comparisons of mean pixel density of Feulgen stained nuclei (IOD divided by nuclear area in pixels).

Relationships among genome, nucleus, and cell sizes

Without controlling for phylogeny, genome size is positively correlated with nucleus size in the genus *Batrachoseps* ($r^2 = 0.77$, $p = 0.0001$, $n = 13$). This correlation persists when the non-attenuate members of the subgenus *Plethopsis* are excluded from the analysis to test for this relationship solely among attenuate species with enucleated RBCs ($r^2 = 0.83$, $p = 0.0001$, $n = 11$). Genome size is also positively correlated with nucleated RBC size across the entire genus ($r^2 = 0.45$, $p < 0.02$, $n = 13$). Removal of the non-attenuate species renders the relationship non-significant ($p = 0.067$); however, this result is contingent on the inclusion of data from the Fairview mitochondrially-diagnosed lineage (Jockusch and Wake, 2002). Within the attenuate clade, but excluding Fairview because it appeared to disproportionately affect the analysis, the relationship between genome size and nucleated RBC size is positive ($r^2 = 0.63$, $p < 0.01$, $n = 10$). Overall, nucleated erythrocytes in the genus *Batrachoseps* are smaller per unit DNA content than those of salamanders with fully nucleated erythrocytes and similar genome sizes (~25–40 pg; Fig. 5). Size of cells following primary enucleation also correlates positively with genome size across the genus as a whole ($r^2 = 0.33$, $p < 0.04$, $n = 13$) as well as within the attenuate clade when Fairview is excluded ($r^2 = 0.43$, $p < 0.04$, $n = 10$; Fig. 5); however, the intercept of the regression is lower for primarily enucleated cells than for nucleated cells (Fig. 5). Size of cells following secondary enucleation, by contrast, is not correlated with genome size in *Batrachoseps* with or without non-attenuate species (all $p > 0.34$).

Linear regression of phylogenetically independent contrasts for nucleus size versus genome size demonstrates a significant, positive relationship ($r^2 = 0.65$, $p < 0.001$, $n = 12$) (Fig. 6). Analyses performed excluding the non-attenuate members of the subgenus *Plethopsis* yield similar results ($r^2 = 0.79$, $p < 0.001$, $n = 10$). All other phylogenetically controlled regression analyses involving genome size (i.e. genome size versus nucleated cell size and cell sizes following primary and secondary enucleation) yield non-significant results (all $p > 0.22$).

RBC enucleation

Measurements of the degree of enucleation are generally consistent between fresh and preserved samples for *B. major*, *B. gavilanensis*, *B. nigriventris*, and *B. attenuatus*. However, for *B. gregarius* and *B. luciae*, measurements based on fresh specimens are lower (*B. gregarius*: fresh $N = 6$, 39–65 %; preserved $N = 16$, 90–99 %. *B. luciae*: fresh $N = 3$, 58–74 %; preserved $N = 9$, 79–97 %). Regardless of methodology, percentages of enucleated cells (primary and secondary combined) vary substantially both within and among species of *Batrachoseps* (Fig. 7). Differences in RBC morphology exist between the members of the non-attenuate subgenus *Plethopsis* and the attenuate clade. Specifically, non-attenuate species have much lower levels of enucleated cells and larger, more elliptical RBCs (Fig. 7). Overall, median species values range from 1.3 % enucleated cells in *B. campi* to 98.5 % enucleated cells in *B. attenuatus*, consistent with the results of earlier studies examining several species within the attenuate clade (Emmel, 1924) and a different species of the subgenus *Plethopsis* (*B. wrightorum*, 6 % enucleated cells) (Stebbins and Lowe, 1949). A single individual of the *Plethopsis* species *B. robustus*, examined in the current study, had ~1 % enucleated cells. Varying numbers of free nuclei, with little to no associated cytoplasm, were also detected in some species (Fig. 1). Free nuclei in non-attenuate species were very rare and were less compacted than those in the attenuate species.

Discussion

Phylogenetic correction and the relationships among genome, nucleus, and cell sizes

Differences exist between the results of our non-phylogenetically corrected and phylogenetically corrected analyses. Without correcting for phylogeny, genome size is positively correlated with nucleus size and nucleated cell size in the genus *Batrachoseps*, as it is in all other vertebrates (Gregory, 2001b). This positive correlation remains when the non-attenuate members of the subgenus *Plethopsis* are excluded from the analysis (nucleus size) and when *Plethopsis* and the attenuate outlier Fairview are excluded from the analysis (nucleated cell size). Genome size is also positively correlated with cell size following primary enucleation across the genus as a whole and within the attenuate clade alone when Fairview is excluded (Fig. 5). Cell size following secondary enucleation is not correlated with genome size, with or without phylogenetic correction. In contrast, phylogenetically independent contrast analyses demonstrate a positive relationship between genome size and nucleus size in both the attenuate clade and the genus as a whole, but not between genome size and nucleated or enucleated cell sizes. This discrepancy in results between non-phylogenetically corrected and phylogenetically corrected analyses indicates one of two things: (1) the correlations between genome size and nucleus and cell sizes shown by the non-phylogenetically corrected analyses are false, resulting from an inflation of the degrees of freedom in direct correlation analyses because of statistical non-independence of related species (Felsenstein, 1985); or (2) the correlations between genome size and nucleus and cell sizes are real, but are not demonstrated by our phylogenetically corrected analyses. Our relatively small sample size (13 taxa) produces broad confidence intervals in independent contrast analysis (66–72 % based on simulations, depending on the underlying mode of character evolution) (Martins et al., 2002), which may explain the non-significant phylogenetically corrected result.

RBC size and the evolution of enucleation

Enucleated RBCs in mammals scale with genome size (Gregory, 2000). Mammalian aerobic metabolic demands are high, and the evolution of mammalian enucleated RBCs was likely related to a requirement for efficient gas exchange based on surface area to volume ratio. Such efficiency requires a high concentration of hemoglobin, which in turn requires either large or numerous RBCs. An increase in cell size can cause a decreased rate of gas exchange, whereas an increase in cell number can increase blood viscosity and strain the heart by decreasing cellular aggregation (Chien et al., 1971; Cavalier-Smith, 1978). A necessary balance between these effects may underlie the narrow range of RBC sizes in mammals. Enucleation of mammalian RBCs decreases cell volume per unit genome size. As a result, the upper limit on mammalian genome size may be less constrained by energetic demands on RBC morphology than is the upper limit on genome size in similarly highly aerobic birds, which retain tightly compacted nuclei (Cavalier-Smith, 1978; Olmo, 1983; Hughes, 1999; Gregory, 2002; Waltari and Edwards, 2002). RBC enucleation may enable mammals to have the smallest RBCs among vertebrates, despite having genome sizes that average more than twice as large as birds'. Loss of the nucleus in a large percentage of *Batrachoseps* RBCs also permits smaller cells per unit genome size (Fig. 5), both by reducing the length of the elliptical axes and by changing the geometry of the cell from ellipsoid to flat. Similar to the situation in mammals, the size of primarily enucleated RBCs is correlated with genome size (based on non-phylogenetically corrected analyses). In both groups, this correlation implies that genome size, possibly via nucleus size, irreversibly influences cell growth and/or division during the cell cycle prior to the ejection of nuclei (Gregory, 2000, 2001a). However, unlike mammals, salamanders have extremely low aerobic metabolic requirements and possess a “frugal” metabolic strategy (Monnickendam and Balls, 1973; Szarski, 1983; Gatten et al., 1992). Cell and genome sizes are at best only very weakly related to metabolic rate in salamanders (Gregory, 2003), and the red-spotted newt *Notophthalmus viridescens* is capable of surviving a near-total experimental

ablation of erythrocytes (Grasso and Shephard, 1968). Therefore, the evolutionary cause of enucleated RBCs in salamanders is likely to differ from that in mammals.

Based on our data, we present a hypothetical scenario for the evolution of enucleated RBCs in *Batrachoseps* and other plethodontids in which a rheological constraint selected for high levels of enucleated RBCs. We hypothesize that this constraint resulted from problems associated with circulation of large RBCs with large nuclei, both resulting from large genomes, through the circulatory system of a miniaturized/attenuated animal. Because nucleus size scales with genome size in *Batrachoseps*, large genome sizes may magnify negative effects of the nucleus on blood flow. Possession of a nucleus decreases RBC deformability, which in turn increases resistance to flow, especially at the entrance to capillaries (Chien et al., 1971; Nash and Egginton, 1993). In addition, possession of a nucleus decreases the ability of RBCs to aggregate (Ohta et al., 1992), and high levels of aggregation lower overall blood viscosity (Chien et al., 1971). RBC enucleation would reduce the effects of a large nucleus on blood flow, in addition to reducing overall RBC size. The effects of organism-level miniaturization that may contribute to a rheological constraint have not been examined in miniaturized/attenuated salamanders. Possibilities include decreased capillary diameters and decreased numbers of capillaries per unit of tissue.

In addition to the smaller RBCs resulting from nuclear loss (with or without further subdivision), members of the attenuate clade also have relatively small nucleated RBCs (Fig. 5). Smaller nucleated RBC size per unit genome size may demonstrate an additional cell-level evolutionary response to possible physical constraints on circulation resulting from large genome and cell sizes and small body size. One such response may be increased DNA compaction in the RBC nucleus of attenuate species, which may reduce cell size via reduced nucleus size. Consistent with this possibility, *B. campi*, a non-attenuate *Plethopsis* species, has the lowest DNA compaction level.

The presence of extensive intraspecific variation in levels of enucleated RBCs is also consistent with a rheological constraint. In the presence of such a constraint, some minimal level of enucleated RBCs would be required for circulation to proceed. All levels in excess of this minimum would be functionally equivalent, producing the range of levels of enucleated cells seen in *Batrachoseps* species. This may also explain the discrepancy between fresh and preserved specimen-based measures of enucleation in *B. gregarius* and *B. luciae* because such disagreement may reflect real variation across space and time.

The evolution of extensive enucleated RBCs in the genus *Batrachoseps* occurred in the common ancestor of the attenuate clade. Within the attenuate clade, *B. attenuatus*, the sister taxon to the remaining lineages, (Fig. 4) has the highest percentage of circulating enucleated cells, suggesting two possible transformation series: (1) lower/more variable levels of enucleated RBCs evolved first, which were maintained in all other attenuate species, and higher levels evolved in the *B. attenuatus* lineage; or (2) higher levels of enucleated RBCs evolved first, which were maintained in *B. attenuatus*, and lower/more variable levels evolved at the base of (*nigriventris* group + *relictus* group) + (*pacificus* group + *gabrielii* group). In either case, genome sizes have not changed dramatically along with shifts in body form or degree of enucleation and instead remain typical of plethodontid salamanders. Thus, enucleation may have allowed maintenance of an ancestrally large genome in the face of changing physical constraints within the organism. Villolobos et al. (1988) suggested that high levels of enucleated RBCs in plethodontids may result from random breakage of cells as they circulate through narrow blood vessels of miniaturized/attenuated animals. However, the appearance of a more robust body form in *B. stebbinsi* within the attenuate clade did not coincide with a reversal to predominantly nucleated RBCs, as would be expected if enucleated cells resulted from random breakage (Fig. 7). *Batrachoseps stebbinsi* retains both blood morphology and

osteology characteristic of its immediate ancestral, attenuate form (Marlow et al., 1979; Wake, 1989). Similarly, no negative relationship exists between body size and enucleation within four species of plethodontids with high levels of enucleated RBCs (*Oedipina poelzi*, *O. uniformis*, *Nototriton picadoi*, and *Bolitoglossa subpalmata*) (Villolobos et al., 1988). Such a negative relationship would be expected to result from random breakage because larger animals would have fewer enucleated cells. Further investigation of the developmental mechanisms underlying enucleated RBC evolution will require broader comparative analyses with specific emphasis on the effects of miniaturization on the circulatory system. Such analyses should include: (1) other miniaturized salamander species with large genomes and similar RBC morphology, (2) non-miniaturized plethodontids with low (< 10 %) levels of enucleated RBCs, and (3) members of the plethodontid genus *Lineatriton*, which have attenuate body forms and genome sizes comparable to *Batrachoseps*, but have nucleated RBCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Photomicrographs of erythrocytes from the attenuate salamander *Batrachoseps luciae* showing various cell types found in circulation. N = nucleated, PE = primarily enucleated, SE = secondarily enucleated (following subdivision of primarily enucleated cells), FN = free nucleus. 400 X magnification. Scale bar = 20 μ m.

Fig. 2.

Photomicrographs of erythrocytes from different species of *Batrachoseps* showing varying degrees of nuclear loss and cell subdivision. (A) Enucleation in progress in the non-attenuate species *B. campi*. Members of *B. campi* have only a few enucleated cells. (B) Enucleation in progress in the attenuate species *B. attenuatus*. Members of *B. attenuatus* have ~99 % enucleated cells. (C) Secondary enucleation in progress via formation of a “figure 8” (Cohen, 1982) in an enucleated *B. campi* cell. (D) Secondary enucleation in progress in an enucleated *B. major* cell. 400 X magnification. Scale bar = 20 μ m.

Fig. 3. Photomicrographs of erythrocytes from three species of *Batrachoseps* showing varying degrees of enucleation. (A) *B. campi* (< 1 % enucleated). (B) *B. kawia* (~50 % enucleated). (C) *B. attenuatus* (~99 % enucleated). 100 X magnification. Scale bar = 40 μm .

Fig. 4.

Batrachoseps phylogeny. Boxes and circles indicate taxa that belong to larger groups indicated in the upper left. *Plethopsis* is a subgenus; other clades do not have Linnaean taxonomic ranks. (A) Phylogenetic relationships among nine species of *Batrachoseps* inferred from partitioned Bayesian analysis of 6,150 bp of mitochondrial genome sequence. Numbers above internal branches are Bayesian posterior probabilities, and numbers below internal branches are parsimony non-parametric bootstrap proportions. (B) *Cytochrome-b* maximum likelihood branch length estimates (TVM + I + Γ) for 13 species (italics) or distinct mitochondrial lineages (Fairview, Southern *B. nigriventris*) of *Batrachoseps*. Topology is compiled from Bayesian analysis of 6,150 bp of mitochondrial genome sequence (Fig. 4a) and the results of Jockusch and Wake (2002). This topology and these branch lengths were used to perform independent contrast analysis on genome size (pg) and RBC and nucleus size (μm^2).

Fig. 5.

Relationships between erythrocyte sizes (dry cell areas measured in μm^2) and genome size (pg DNA/haploid nucleus) in salamanders with nucleated and enucleated cells. Circles represent nucleated cells; solid line = 15 species of non-*Batrachoseps* salamanders with genome sizes of ~25–40 pg (data from present study and literature; see Gregory, 2003); o, long dashes = 13 species of *Batrachoseps*, both attenuate and non-attenuate. Triangles represent enucleated cells for 13 species of *Batrachoseps*; medium dashes = primarily enucleated cells; Δ , short dashes = secondarily enucleated cells.

Fig. 6. Relationship between nucleus size (dry area measured in μm^2) and genome size (pg DNA/haploid nucleus) in 13 species of *Batrachoseps*, both attenuate and non-attenuate. Plot shows linear regression of nucleus-size independent-contrast values onto genome-size independent-contrast values.

Fig. 7.

Box plot depicting the percentages of RBCs that are enucleated for 15 named species (in italics) or distinct mitochondrial lineages. Each box plot contains the information for 5 to 23 individuals collected at the same time and place, (14–976 cells/individual, median = 419). Box plots show a line at the median percentage, a surrounding box containing the middle 50 % of the data, and whiskers extending to encompass the middle 80 % of the data. Dots indicate outlier points. Species groups are indicated at the top of the figure. *B. attenuatus* is not a member of a larger species group. *B. campi* is a member of the non-attenuate subgenus *Plethopsis*. Asterisks indicate species for which measurements were corroborated with fresh specimens. For *B. gregarius* and *B. luciae*, percentages based on fresh specimens were lower.

Means and standard errors of genome size, nucleus size, and red blood cell (RBC) size for 13 species of *Batrachoseps*. Genome size and nucleated RBC size for seven species of non-attenuate salamanders with fully nucleated RBCs.

Species	Genome size (mean pg DNA/ haploid nucleus)	SE	N	Nucleus Size (mean μm^2)	SE	N	Nucleated RBC Size (mean μm^2)	SE	N	Primarily Enucleated RBC Size (mean μm^2)	SE	N	Secondarily Enucleated RBC Size (mean μm^2)	SE	N
<i>B. attenuatus</i>	36.69	0.93	2	116.50	4.37	5	676.01	33.44	5	469.95	43.35	5	192.80	10.47	5
<i>B. campyl</i> ^a	33.1	0.64	2	125.47	8.04	2	810.98	17.34	2	699.69	—	1	—	—	—
Fairview	26.2	—	1	90.83	—	1	583.61	—	1	447.62	—	1	244.01	—	1
<i>B. gabrieli</i>	34.93	0.77	4	112.03	5.26	4	546.10	13.26	4	460.16	11.72	4	231.27	14.97	4
<i>B. gavilarensis</i>	26.82	—	1	83.72	—	1	438.67	20.06	2	324.26	14.14	2	208.99	16.07	2
<i>B. gregarius</i>	28.08	0.72	4	89.78	1.75	6	503.06	12.18	6	439.27	7.23	6	301.48	6.60	6
<i>B. incognitus</i>	30.62	—	1	101.26	—	1	567.20	—	1	435.97	—	1	242.34	—	1
<i>B. kavita</i>	33.2	—	1	113.12	—	1	482.95	—	1	370.04	—	1	222.43	—	1
<i>B. luciae</i>	29.68	0.17	3	94.77	2.89	3	459.59	16.20	3	363.96	12.89	3	224.71	33.96	3
<i>B. major</i>	31.52	0.8	4	100.77	3.60	4	557.50	33.66	5	378.36	13.85	5	221.05	8.70	5
<i>B. nigriventris</i>	26.06	0.71	3	94.60	4.16	3	444.37	13.24	3	341.47	20.03	3	164.17	19.91	3
<i>B. robustus</i> ^a	37.1	—	1	116.55	—	1	832.73	—	1	787.12	—	1	—	—	—
southern <i>B. nigriventris</i>	30.17	0.31	4	95.70	2.63	5	488.26	13.00	5	334.09	7.33	5	204.37	4.52	5
<i>Ambystoma jeffersonianum</i>	28.80 ^b	—	1	—	—	—	629.78	—	1	—	—	—	—	—	—
<i>Aneides lugubris</i>	35.68	—	1	—	—	—	698.34	—	1	—	—	—	—	—	—
<i>Ensatina eschscholtzii croceator</i>	40.50	—	1	—	—	—	824.08	—	1	—	—	—	—	—	—
<i>Neurergus strauchi</i>	25.67	—	1	—	—	—	536.64	—	1	—	—	—	—	—	—
<i>Notophthalmus viridescens</i>	30.03	0.39	5	—	—	—	534.29	—	1	—	—	—	—	—	—
<i>Plethodon cinereus</i>	22.64	0.09	3	—	—	—	430.77	—	1	—	—	—	—	—	—
<i>Taricha torosa</i>	32.12	—	1	—	—	—	704.27	—	1	—	—	—	—	—	—

N is the number of individuals measured.

SE is standard error.

^aNon-attenuate member of sub-genus *Plethopsis*. All other *Batrachoseps* species are attenuate.

^bUsed as standard, from Vinogradov (1998).