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The dynamic evolutionary history of genome size in North American woodland salamanders

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Abstract

The genus *Plethodon* is the most species rich salamander genus in North America, and nearly half of its species face an uncertain future. It is also one of the most diverse families in terms of genome sizes, which range from 1C = 18.2 pg to 69.3 pg, or 5-20 times larger than the human genome. Large genome size in salamanders results in part from accumulation of transposable elements and is associated with various developmental and physiological traits. However, genome sizes have been reported for only 25% of *Plethodon* species (14 of 55). We collected genome size data for *P. serratus* to supplement an ongoing phylogeographic study, reconstructed the evolutionary history of genome size in Plethodontidae, and inferred probable genome sizes for the 41 species missing empirical data. Results revealed multiple genome size changes in *Plethodon*; genomes of western *Plethodon* increased, whereas genomes of eastern *Plethodon* decreased, followed by additional decreases or subsequent increases. The estimated genome size of *P. serratus* was 21 pg. New understanding of variation in genome size evolution, along with genome size inferences for previously unstudied taxa, provide a foundation for future studies on the biology of plethodontid salamanders.

Keywords: C-value, Feulgen densitometry, *Plethodon serratus*, Plethodontidae

Introduction

In most non-amphibian tetrapod families, genome sizes (haploid nuclear DNA content) tend to be fairly stable, varying up to 5 pg [1 pg = 0.978 gigabases (Gb)] among species (Gregory 2016). However, amphibians show much larger variation in genome size within families: up to a 12 pg difference within frog families and up to 96 pg within families within salamanders (Gregory 2016). The exceptionally wide variation within salamander families suggests that major genome size changes have occurred independently among lineages (Jockusch 1997).

Plethodontidae is the most species rich salamander family, with 451 species in 28 genera (AmphibiaWeb 2016). Among salamander families, Plethodontidae also has the widest range of genome sizes (Herrick and Sclavi 2014). Genome size in salamanders is related to chromosome size, not chromosome number (Sessions 2008). In Plethodontidae, *Batrachoseps* and the neotropical genera have 2n=26 chromosomes; all other genera have 2n=28 chromosomes (Leon and Kezer 1978). The genomes of plethodontid salamanders contain much larger quantities of transposable elements (TEs) than are found in most other vertebrate clades, but within Plethodontidae, TE content does not appear to be correlated with genome size (Sun et al. 2012). This suggests that factors in addition to TE proliferation more strongly drive genome size evolution within the family Plethodontidae.

Genome size data play a critical role in designing effective laboratory methods of modern DNA sequence data collection methods for phylogenetic and phylogeographic studies. Large genome sizes limit the efficiency of genomic preparation techniques for next-generation sequencing data sets (Hodges et al. 2009; McCartney-Melstad et al. 2016), as well as subsequent assembly of highly repetitive sequences. Established laboratory protocols have been successfully

used with salamanders with genome sizes below average for the order (though still large relative to non-salamander tetrapods) (Newman and Austin, *in press*). However, even those species yielded sequence data with lower read depth and percentage of on-target reads than comparable studies with other vertebrates, suggesting that species with even larger genomes may present additional challenges. Study design therefore depends on the genome size(s) of the species of interest. Unfortunately, of the 686 salamander species (AmphibiaWeb 2016), only 179 species (26%) have published empirical genome size data (Gregory 2016).

Because of the wide variation in genome size, plethodontid salamanders in particular would benefit from a thorough phylogenetic and genome range assessment. The genus *Plethodon* is the most species rich and ecologically diverse salamander genus in North America, with 55 species. The backbone phylogeny of the major lineages of *Plethodon* is well-established (Highton and Larson 1979; Highton 1995), but some relationships among closely related species remain unresolved (Fisher-Reid and Wiens 2011; Highton et al. 2012). In addition, extreme morphological stasis within *Plethodon* likely masks identification of cryptic species (Highton 1995, Mueller et al. 2004; Wake 2009, Highton et al. 2012; Pelletier et al. 2015). *Plethodon* is thus an interesting and species rich lineage for phylogeographic and species delimitation research. Nonetheless, mitochondrial genes have been shown to be sometimes misleading in *Plethodon* (Fisher-Reid and Wiens 2011), and low numbers of nuclear loci are sometimes not sufficient to fully resolve relationships (e.g., Newman & Austin 2015). Therefore, multilocus genetic data sets with hundreds to thousands of loci are often required, necessitating knowledge of genome size to choose appropriate techniques. However, only 14 of 55 *Plethodon* species have estimated genome sizes (Gregory 2016).

Here, we estimate the genome size for *P. serratus* as part of an ongoing phylogeographic study of the species (Newman and Austin 2015). We incorporate our data with available genome size and genetic sequence data to reconstruct the evolutionary history of genome size in plethodontid salamanders under a maximum-likelihood (ML) framework and infer likely genome sizes for extant species that currently lack empirical estimates.

Materials and Methods

Genome size

Haploid genome size (1C-value) data for 14 *Plethodon* species and outgroup species *E. bislineata*, *A. lugubris*, *E. eschscholtzii*, *D. quadramaculatus*, and *D. fuscus* were downloaded from the Animal Genome Size Database (Gregory 2016) (Table S1). For species with multiple genome size estimates in the database, we used the median in downstream analyses rather than the mean to minimize bias due to potentially erroneously high genome size values reported in one study. In particular, the genome sizes for *E. bislineata*, *D. quadramaculatus*, *P. glutinosus*, and *P. jordani* reported in Bachmann (1970) are substantially larger than values reported for the same species in more recent studies (Olmo 1973, 1974; Mizuno and Macgregor 1974; Hally et al. 1986; Sessions and Larson 1987; Licht and Lowcock 1991). This is potentially due to the use of a higher than usual genome size for *Rana pipiens* as a reference to convert relative values to absolute (Olmo 1974). However, because estimates of genome size do vary within species, we chose to incorporate but minimize the bias, rather than exclude the unusually high data points. Results from the same analyses using mean instead of median genome sizes were not qualitatively different (data not shown).

In addition, we generated the first estimate of genome size for *P. serratus* as part of a phylogeographic and phylogenomic study of the species. Blood smears were made for five individuals of *P. serratus* collected at Sicily Island Hills Wildlife Management Area in Louisiana. Blood was collected on site from the caudal vein of live salamanders after removing the tail tip for a future genetic resource. All blood and tissue samples were deposited in the Louisiana State University Museum of Natural Science Herpetology Collection. Field work was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 2011), under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University (permit number 13-060).

Genome size was estimated for *P. serratus* using the Feulgen Image Analysis Densitometry (FIAD) method described in detail by Hardie et al. (2002). Air-dried blood smears were post-fixed overnight in 85:10:5 methanol : formalin : glacial acetic acid, rinsed in running tap water, and then hydrolyzed in 5N HCl for 2 hours, followed by staining in freshly-prepared Schiff reagent for 2 hours. The slides were passed through a series of metabisulfite and distilled water rinses before being dried and stored in the dark until analysis. Genome size was estimated by using the Bioquant Life Science image analysis package along with a Leica DM2500 microscope using a 63x oil-immersion lens connected to a Retiga EXi digital camera. Integrated optical densities (IODs) were measured for at least 50 nuclei per specimen and converted to absolute genome size by comparison with nuclei of the salamander *Ambystoma jeffersonianum* (1C = 28.8 pg; Licht and Lowcock 1991). Chicken and rainbow trout blood were also included as internal checks of the Feulgen staining.

Phylogenetic analysis

Genetic sequence data from several previously published studies (Wiens et al. 2006; Vieites et al. 2007; Bonett et al. 2009; Fisher-Reid and Wiens 2011; Martin et al. 2015) were downloaded from GenBank (Benson *et al.* 2013) http://www.ncbi.nlm.nih.gov/genbank, retrieved 19 Feb. 2016 for 55 species in the family Plethodontidae, including 50 *Plethodon* species (Table S2). The concatenated alignment contained a total of 4,057 bp and included the following nuclear loci: BDNF (707 bp), GAPD (644 bp), ILF3 (281 bp), Mlc2a (253 bp), POMC (481 bp), RAG-1 (1,467 bp) and RHO (224 bp). For each locus, sequences were aligned in Geneious v.6.0.5 using the ClustalW algorithm. We generated two data sets for downstream analyses: one including all species, and another including only species with empirical genome size data.

Intentionally, our genetic data set overlapped almost entirely with the nuclear-only data set in Fisher-Reid & Wiens (2011), the most recent multilocus plethodontid phylogenetic study with extensive taxon sampling within *Plethodon*. We thus implemented the same partitioning scheme, models of nucleotide evolution, and software settings described in the previous paper to generate a phylogeny under a Bayesian framework in MrBayes v.3.2.6 (Ronquist et al. 2012). We conducted two MCMC runs of 6 million generations, sampling every 1000 generations. Convergence was assessed in Tracer v.1.6 (Rambaut and Drummond 2007) by ensuring effective sample sizes (ESSs) > 200. The first 10% of samples were discarded as burn-in.

Ancestral character reconstruction

Genome sizes of ancestral nodes (most recent common ancestor, MRCA) were estimated in a likelihood framework under a Brownian motion model using the phytools package v.0.5.20 (Revell 2012) in R v.3.2.1 (R Core Team 2015). This method of ancestral character

reconstruction (ACR) allows for missing character data for some tips on the phylogeny and, in addition to reconstructing ancestral characters, also estimates values for the tips missing empirical data. To assess whether or not the large amount of missing character data (73%) influenced reconstruction of ancestral states, we performed the same analysis using a phylogeny comprised only of species with empirical genome size data. Lastly, we ran the analysis on the full phylogeny but excluding the empirical genome size data for *P. serratus* to test the accuracy of the method's character state estimation for extant taxa.

Results

The estimated haploid genome size for *P. serratus* was 21.01 pg ± 0.77 SE (range: 19.23 – 24.21 pg) (Table 1, Fig. 1), within the range of previously available data for the *P. cinereus* clade (18.20 – 26.20 pg). Partitioned Bayesian analysis of both sets of taxa yielded strong support (posterior probability [*P*] ≥ 0.9) for all major clades: Plethodontinae, genus *Plethodon*, western *Plethodon*, eastern *Plethodon*, and, within eastern *Plethodon*, the *P. cinereus*, *P. wehrlei* + *P. welleri*, and *P. glutinosus* groups (Fig. S1). The sister relationship of the *P. cinereus* group to the remainder of eastern *Plethodon* is also strongly supported. This topology is congruent with the previous study that used the same genetic data and analyses (Fisher-Reid and Wiens 2011).

The genome size of the MRCA of all *Plethodon* was estimated to be 33.82 pg (Table 2, Fig. 2), which falls slightly above the median empirical genome size of all salamanders (30.07 pg). Within *Plethodon*, the estimated genome size was smaller for the MRCA of the eastern *Plethodon* (25.71 pg) but larger for the MRCA of the western *Plethodon* (38.71 pg). It is thus likely that, relative to salamanders as a group, modest genome size is the ancestral state for *Plethodon*, with a subsequent increase in genome size among the western species and decrease

among the eastern species. Within the eastern *Plethodon*, estimated MRCA genome sizes ranged from 22.22 pg for the *P. cinereus* group to 29.07 for the *P. glutinosus* group. This suggests that the *P. glutinosus* group has undergone expansion of genome size since diverging from the remainder of the eastern *Plethodon*. Genome size appears to have been relatively stable along the lineage leading to *Plethodon* before diversification, as the genome size of the MRCA of all Plethodontinae was estimated to be 32.63.

There were no qualitative differences between estimated genome sizes of ancestral nodes when the ACR analysis was run on the phylogeny including only species with empirical genome size data and analysis with the full phylogeny (Table S3; Figs. S3, S4). When the ACR analysis was run without the empirical *P. serratus* genome size data, the genome size of *P. serratus* was estimated to be 21.58 pg, consistent with the empirical median value of 21.01 pg. Genome sizes for extant species estimated from empirical data sets including and omitting *P. serratus* were highly similar (Table 3).

Discussion

Salamanders of the genus *Plethodon* appear to have undergone multiple contractions and expansions of genome size. Consistent with previous studies (Sessions and Larson 1987; Kraaijeveld 2010), our results suggest a genome size contraction preceding or concurrent with diversification of the eastern species. Some researchers (Kraaijeveld 2010; Herrick and Sclavi 2014) have used this result as an example of an evolutionary radiation following a reduction in genome size. However, Kozak et al. (2006) found that only the *P. glutinosus* group underwent a rapid radiation that produced a significantly higher number of extant lineages than expected. Our ACR analysis reconstructed a genome size expansion along the lineage leading to the *P.*

glutinosus group, suggesting that an expansion, rather than a reduction, in genome size is associated with the only radiation within *Plethodon*. On a broader phylogenetic scale, the family Plethodontidae has some of the smallest genome sizes of all salamanders and also the highest number of species, suggesting a negative correlation between genome size and speciation rate (Herrick and Sclavi 2014). However, our data of the genus *Plethodon* suggest a more complex relationship between genome size and speciation at this finer scale.

The three major lineages within the eastern *Plethodon* appear to be undergoing independent evolution of genome size, with one lineage showing a further reduction (*P. cinereus* group), one lineage showing an expansion (*P. glutinosus* group), and one lineage showing no change (*P. wehrlei* + *P. welleri* group). Within each group, there is little variation in genome size. The causes of genome size variation in salamanders are not yet well understood. However, various hypotheses have been proposed and explored, including life history constraints (Gregory 2002) and developmental constraints (Sessions and Larson 1987; Jockusch 1997). In addition, it has been proposed that smaller genome size is associated with younger lineages in salamanders, both among families and among genera within a family (Herrick and Sclavi 2014). Our results did not show this pattern for clades within the genus *Plethodon*. Species in the *P. cinereus* group have smaller genome sizes than species in the *P. glutinosus* group, yet the *P. glutinosus* group is younger (Martin et al. 2015). Further exploration of the potential factors underlying genome size variation in *Plethodon* is beyond the scope of this study.

We also highlight the need for a broad examination of genome size in *Plethodon* through collection of additional empirical genome size data. Of the 55 recognized species of *Plethodon*, empirical data are now currently available for only 15 species, or 28%. Furthermore, of the available data, only two data points were obtained in the past 15 years: one *P. cinereus* estimate

(Mueller et al. 2008) and *P. serratus* (this study). All other *Plethodon* genome size data were collected between 1968-1998, using several different methods. While any substantial deviation from the estimated values in our study would be unexpected, it is difficult to assess the biological significance of the variation in genome sizes among the eastern *Plethodon* groups without more complete information about intra-group variation among species. In addition, our empirical data for *P. serratus* show that even within a single population of a species, genome size estimates can vary (19.23 – 24.21 pg). A promising approach to collecting not only genome size data but also information about genome content is shotgun sequencing alongside chromosome capture and sequencing, as was recently used to estimate the genome size of *Ambystoma mexicanum* and explore the nature of repetitive elements of the genome (Keinath et al. 2015).

Full evaluation of the evolution of genome size in *Plethodon* requires a larger data set consisting of appropriate multilocus sequence data and genome size data for multiple individuals of each *Plethodon* species – with both genetic and genome size data for each individual. Our results are consistent with previous studies and also consistent with our expectations based on phylogeny – i.e., *Plethodon* species show clear genome size differentiation by clade – but the omission of within-species variation may mask other patterns or miss important outliers. For example, Gregory (2002) notes that while most *Desmognathus* undergo metamorphosis from aquatic larvae and possess the smallest genomes of salamanders, the one *Desmognathus* species now known to be direct developing (*D. aeneus*) has an unknown genome size. Hypothetically, if genome size is indeed associated with developmental cycle, the ACR method would fail to recognize *D. aeneus* as an outlier within its clade of small-genome *Desmognathus*. Further, inferences drawn from ACR analysis are based on the assumption that a Brownian motion model is a reasonable fit for genome size evolution. A Brownian motion model might not be

appropriate if, for example, genome size is under strong selection pressure (Elliot and Mooers 2014).

Our results reveal variation in the direction of genome size evolution among lineages within *Plethodon*. In particular, the genome size expansion at the base of the *P. glutinosus* group suggests that caution is needed when ascribing a particular pattern of evolution to higher-level clades. We also highlight the interdependence between studies of genome size evolution and the fields of phylogeography and population genetics. The new genome size estimates presented here will facilitate much-needed phylogeographic and population genetic studies of this system, and new information on patterns of genome size evolution in *Plethodon* will contribute to exploration of factors underlying large genomes.

The most species rich plethodontid genus, *Plethodon*, is also highly threatened. Of the 44 species that have been assessed and ranked by the IUCN Red List (excluding "Data Deficient" species), 20 are ranked at least Near-Threatened (IUCN 2016). In addition, many species have very small geographic distributions, restricted to one or a few mountain peaks. Future research on the evolution of genome size in plethodontids will enhance our understanding of the associations between genome size and other aspects of a salamander's biology – including potential adaptive significance of genome size.

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References

AmphibiaWeb. 2016. AmphibiaWeb: information on amphibian biology and conservation. Berkeley, CA. Available from http://amphibiaweb.org [accessed 18 July 2016].

Bachmann, K. 1970. Feulgen slope determinations of urodele nuclear DNA amounts. Histochemie **22**(4): 289–293. doi:10.1007/BF00277456.

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Sayers, E.W. 2013. GenBank. Nucleic Acids Res. **41**(212): D36–42. doi:10.1093/nar/gks1195.

Bonett, R.M., Chippindale, P.T., Moler, P.E., Van Devender, R.W., and Wake, D.B. 2009. Evolution of gigantism in amphiumid salamanders. PLoS One **4**(5): e5615. doi:10.1371/journal.pone.0005615.

Elliot, M.G., and Mooers, A.O. 2014. Inferring ancestral states without assuming neutrality or gradualism using a stable model of continuous character evolution. BMC Evol. Biol. **14**: 226.

Fisher-Reid, M.C., and Wiens, J.J. 2011. What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades. BMC Evol. Biol. **11**: 300. doi:10.1186/1471-2148-11-300.

Gregory, T.R. 2002. Genome size and developmental complexity. Genetica **115**(1): 131–146. doi:10.1023/A:1016032400147.

Gregory, T.R. 2016. Animal Genome Size Database. Available from http://genomesize.com [accessed 23 May 2016].

Hally, M.K., Rasch, E.M., Mainwaring, H.R., and Bruce, R.C. 1986. Cytophotometric evidence of variation in genome size of desmognathine salamanders. Histochemistry **85**(3): 185–192. doi:10.1007/BF00494802.

Hardie, D.C., Gregory, T.R., and Hebert, P.D.N. 2002. From pixels to picograms: a beginners' guide to genome quantification by Feulgen Image Analysis Densitometry. J. Histochem. Cytochem. **50**(6): 735–749. doi:10.1177/002215540205000601.

Herrick, J., and Sclavi, B. 2014. A new look at genome size, evolutionary duration and genetic variation in salamanders. Comptes Rendus - Palevol **13**(7): 611–621. doi:10.1016/j.crpv.2014.06.002.

Highton, R. 1995. Speciation in eastern North American salamanders of the genus *Plethodon*. Annu. Rev. Ecol. Syst. **26**(1995): 579–600.

Highton, R., Hastings, A.P., Palmer, C., Watts, R., Hass, C.A., Culver, M., and Arnold, S.J. 2012. Concurrent speciation in the eastern woodland salamanders (Genus *Plethodon*): DNA sequences of the complete albumin nuclear and partial mitochondrial 12s genes. Mol. Phylogenet. Evol. **63**(2): 278–290. doi:10.1016/j.ympev.2011.12.018.

Highton, R., and Larson, A. 1979. The genetic relationships of the salamanders of the genus *Plethodon*. Syst. Biol. **28**(4): 579–599. doi:10.2307/sysbio/28.4.579.

Hodges, E., Rooks, M., Xuan, Z., Bhattacharjee, A., Benjamin Gordon, D., Brizuela, L., Richard McCombie, W., and Hannon, G.J. 2009. Hybrid selection of discrete genomic intervals on custom-designed microarrays for massively parallel sequencing. Nat. Protoc. **4**(6): 960–974. doi:10.1038/nprot.2009.68.

IUCN. 2016. IUCN Red List of Threatened Species. Available from http://iucnredlist.org [accessed 19 July 2016].

Jockusch, E.L. 1997. An evolutionary correlate of genome size change in plethodontid salamanders. Proc. R. Soc. B **264**(1381): 597–604. doi:10.1098/rspb.1997.0085.

Kleinath, M.C., Timoshevskiy, V.A., Timoshevskaya, N.Y., Tsonis, P.A., Voss, S.R., and Smith, J.J. 2015. Initial characterization of the large genome of the salamander *Ambystoma mexicanum* using shotgun and laser capture chromosome sequencing. Sci. Rep. **5**: 16413.

Kozak, K.H., Weisrock, D.W., and Larson, A. 2006. Rapid lineage accumulation in a non-adaptive radiation: phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: *Plethodon*). Proc. R. Soc. B **273**(1586): 539–46. doi:10.1098/rspb.2005.3326.

Kraaijeveld, K. 2010. Genome size and species diversification. Evol. Biol. **37**(4): 227–233. doi:10.1007/s11692-010-9093-4.

Leon, P.E., and Kezer, J. 1978. Localization of 5S RNA genes on chromosomes of plethodontid salamanders. Chromosoma **65**: 213–230.

Licht, L.E., and Lowcock, L.A. 1991. Genome size and metabolic rate in salamanders. Comp. Biochem. Physiol. **100**(1): 83–92. doi:10.1016/0305-0491(91)90089-V.

Martin, S.D., Shepard, D.B., Steffen, M.A., Phillips, J.G., and Bonett, R.M. 2015. Biogeography and colonization history of plethodontid salamanders from the Interior Highlands of eastern North America. J. Biogeogr.: 410–422. doi:10.1111/jbi.12625.

McCartney-Melstad, E., Mount, G.G., and Shaffer, H.B. 2016. Exon capture optimization in

large-genome amphibians. Mol. Ecol. Resour. doi:10.1111/1755-0998.12538.

Mizuno, S., and Macgregor, H.C. 1974. Chromosomes, DNA sequences, and evolution in salamanders of the genus *Plethodon*. Chromosoma **48**(3): 239–296. doi:10.1007/BF00326507.

Mueller, R.L., Gregory, T.R., Gregory, S.M., Hsieh, A., and Boore, J.L. 2008. Genome size, cell size, and the evolution of enucleated erythrocytes in attenuate salamanders. Zoology **111**(3): 218–30. doi:10.1016/j.zool.2007.07.010.

Mueller, R.L., Macey, J.R., Jaekel, M., Wake, D.B., and Boore, J.L. 2004. Morphological homoplasy, life history evolution, and historical biogeography of plethodontid salamanders inferred from complete mitochondrial genomes. Proc. Natl. Acad. Sci. **101**(38): 13820–13825. doi:10.1073/pnas.0405785101.

National Research Council. 2011. Guide for the Care and Use of Laboratory Animals. National Academies Press, Washington, D.C. doi:10.17226/12910.

Newman, C.E., and Austin, C.C. 2015. Thriving in the cold: glacial expansion and post-glacial contraction of a temperate terrestrial salamander (*Plethodon serratus*). PLoS One **10**: e0130131. doi:10.1371/journal.pone.0130131.

Newman, C.E., and Austin, C.C. In press. Sequence capture and next-generation sequencing of ultraconserved elements in a large-genome salamander. Mol. Ecol. doi:10.1111/mec.13909.

Olmo, E. 1973. Quantitative variations in the nuclear DNA and phylogenesis of the Amphibia. Caryologia **25**(April 2015): 43–68. doi:10.1080/00087114.1973.10796525.

Olmo, E. 1974. Further data on the genome size in the urodeles. Boll. Di Zool. **41**(1): 29–33.

doi:10.1080/11250007409430082.

Pelletier, T.A., Crisafulli, C., Wagner, S., Zellmer, A.J., and Carstens, B.C. 2015. Historical species distribution models predict species limits in western *Plethodon* salamanders. Syst. Biol. **64**(6): 909–925. doi:10.1093/sysbio/syu090.

R Core Team. 2015. R: A Language and Environment for Statistical Computing. Vienna, Austria.

Rambaut, A., and Drummond, A.J. 2007. Tracer v.1.4.

Revell, L.J. 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. **3**(2): 217–223. doi:10.1111/j.2041-210X.2011.00169.x.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., and Huelsenbeck, J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**(3): 539–42. doi:10.1093/sysbio/sys029.

Sessions, S.K. 2008. Evolutionary cytogenetics in salamanders. Chromosom. Res. **16**: 183–201. doi:10.1007/s10577-007-1205-3.

Sessions, S.K., and Larson, A. 1987. Developmental correlates of genome size in plethodontid salamanders and their implications for genome evolution. Evolution. **41**(6): 1239–1251.

Sun, C., Shepard, D.B., Chong, R.A., López Arriaza, J., Hall, K., Castoe, T.A., Feschotte, C., Pollock, D.D., and Mueller, R.L. 2012. LTR retrotransposons contribute to genomic gigantism in plethodontid salamanders. Genome Biol. Evol. **4**(2): 168–83. doi:10.1093/gbe/evr139.

Vieites, D.R., Min, M.-S., and Wake, D.B. 2007. Rapid diversification and dispersal during periods of global warming by plethodontid salamanders. Proc. Natl. Acad. Sci. **104**(50): 19903–7. doi:10.1073/pnas.0705056104.

Wake, D.B. 2009. What salamanders have taught us about evolution. Annu. Rev. Ecol. Evol. Syst. **40**: 333–352. doi:http://dx.doi.org/10.1146/annurev.ecolsys.39.110707.173552.

Wiens, J.J., Engstrom, T.N., and Chippindale, P.T. 2006. Rapid diversification, incomplete isolation, and the "speciation clock" in North American salamanders (Genus *Plethodon*): testing the hybrid swarm hypothesis of rapid radiation. Evolution. **60**(12): 2585–603.

Table 1. Genome size estimates for *P. serratus* as determined by Feulgen image analysis densitometry.

Sample ID	IOD*	Genome size (pg)	Magnification
Plethodon serratus			
LSUMZ H-21468a	5644.15	22.14	63x
LSUMZ H-21468b	6170.92	24.21	63x
LSUMZ H-21469	5007.92	19.64	63x
LSUMZ H-21470	5371.06	21.07	63x
LSUMZ H-21471	5048.16	19.80	63x
LSUMZ H-21472	4902.28	19.23	63x
Ambystoma jeffersonianum	7342.36	28.80	63x
Oncorhynchus mykiss	2787.56	2.33	100x
Gallus domesticus	1495.93	1.25	100x

* Integrated optical density (IOD)

Table 2. Genome size for ancestral nodes, estimated by ACR.

Node	Clade Name	Genome size (pg)
1	Plethodontinae	32.63
2	-	32.49
3	*Desmognathus*	17.65
4	*Plethodon*	33.82
5	Western *Plethodon*	38.71
6	Eastern *Plethodon*	25.71
7	*P. cinereus* group	22.22
8	-	25.99
9	*P. wehrlei* + *P. welleri* group	24.96
10	*P. glutinosus* group + *P. websteri*	26.77
11	*P. glutinosus* group	29.07
12	-	28.74
13	-	29.84

Note: Node numbers correspond to Fig. 2.

Table 3. Empirical and estimated genome sizes for all species included in the full phylogeny.

Species	Genome size (pg)
Eastern *Plethodon*: *P. cinereus* group	
P. cinereus	**22.64**
P. electromorphus	21.25
P. hoffmani	**21.40**
P. hubrichti	21.67
P. nettingi	21.51
P. richmondi	**20.65**
P. serratus	**21.01**
P. shenandoah	**18.20**
P. virginia	21.42
Eastern *Plethodon*: *P. wehrlei* + *P. welleri* group	
P. angusticlavius	24.14
P. dorsalis	23.50
P. punctatus	24.37
P. ventralis	23.50
P. wehrlei	**24.20**
P. welleri	**22.60**
Eastern *Plethodon*: *P. glutinosus* group	
P. albagula	29.40
P. amplus	28.32
P. aureolus	28.43
P. caddoensis	30.82
P. chattahoochee	28.32
P. cheoah	28.32
P. chlorobryonis	28.32
P. cylindraceus	28.32

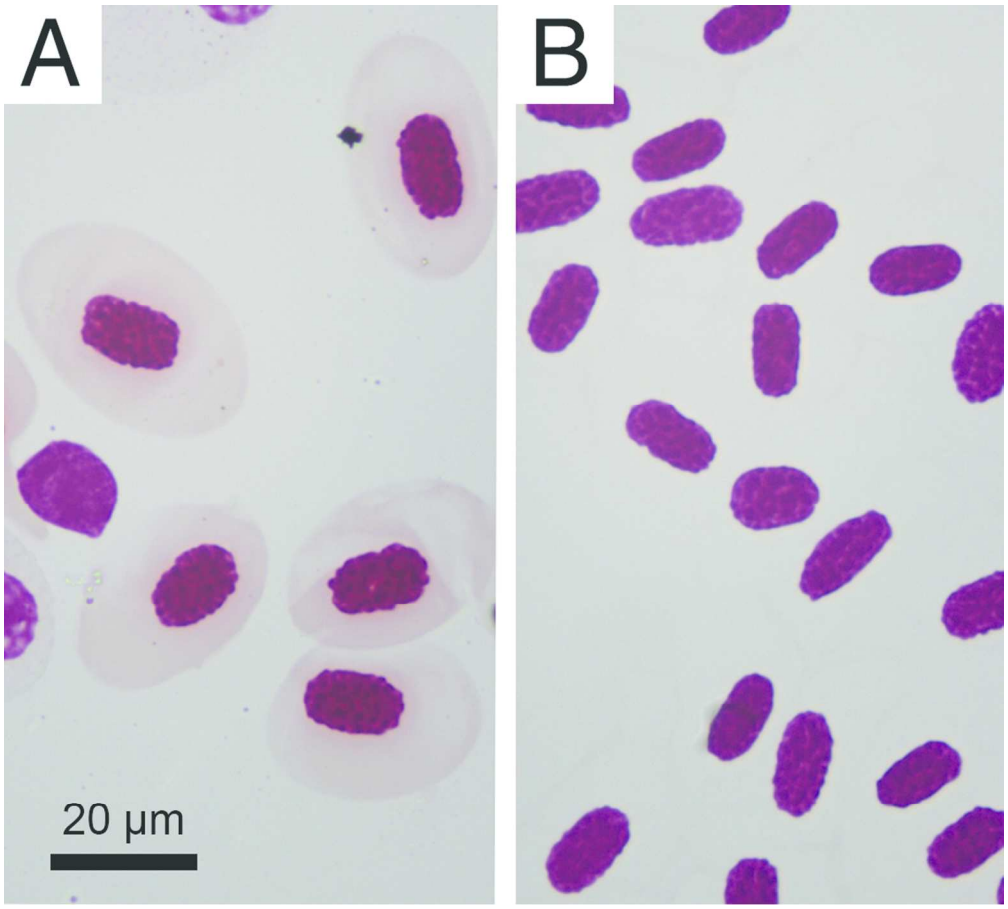
P. fourchensis	32.09
P. glutinosus	**28.00**
P. grobmani	29.40
P. jordani	**27.80**
P. kentucki	28.92
P. kiamichi	29.40
P. kisatchie	29.40
P. longicrus	28.62
P. meridianus	28.02
P. metcalfi	28.02
P. mississippi	29.40
P. montanus	28.02
P. ocmulgee	29.40
P. oconaluftee	28.32
P. ouachitae	**33.70**
P. petraeus	28.74
P. savannah	29.40
P. sequoyah	29.40
P. shermani	28.32
P. teyahalee	28.32
P. variolatus	28.32
P. websteri	26.77
P. yonahlossee	**30.75**
Western *Plethodon*	
P. elongatus	**33.63**
P. idahoensis	67.04
P. vandykei	**69.30**
P. vehiculum	**38.05**

Other Plethodontidae	
Eurycea bislineata	**26.92**
Aneides lugubris	**42.77**
Ensatina eschscholtzii	**38.87**
Desmognathus quadramaculatus	**17.19**
Desmognathus fuscus	**16.17**

Figure Legends

Fig. 1. Photomicrographs of Feulgen-stained erythrocyte nuclei from the salamanders A) *Ambystoma jeffersonianum* (1C = 28.8pg), and B) *Plethodon serratus* (1C = 21.0pg). Images taken under 63x magnification, scale bar = 20µm.

Fig. 2. ML reconstruction of extant and ancestral genome sizes in Plethodontidae. Phylogeny: Bayesian majority-rule consensus tree with all 55 species. For taxon names, red + asterisk: species with empirical genome size data, black: genome size estimated by ACR. Node labels correspond to Table 2. Inset photograph of LSUMZ 98343 (credit: CCA) from Kisatchie National Forest, Louisiana.



Photomicrographs of Feulgen-stained erythrocyte nuclei from the salamanders A) *Ambystoma jeffersonianum* (1C = 28.8pg), and B) *Plethodon serratus* (1C = 21.0pg). Images taken under 63x magnification, scale bar = 20µm.
Fig. 1
95x86mm (300 x 300 DPI)

Plethodon serratus

P. ouachitae *
P. fourchensis
P. caddoensis
P. kentucki
P. glutinosus *
P. sequoyah
P. mississippi
P. savannah
P. ocmulgee
P. kisatchie
P. kiamichi
P. grobmani
P. albagula
P. yonahlossee *
P. petraeus
P. longicrus
P. aureolus
P. chattahoochee
P. shermani
P. cheoah
P. variolatus
P. amplus
P. cylindraceus
P. chlorobryonis
P. oconaluftee
P. teyahalee
P. montanus
P. metcalfi
P. meridianus
P. jordani *
P. websteri
P. wehrlei *
P. punctatus
P. welleri *
P. ventralis
P. dorsalis
P. angusticlavius
P. hubrichti
P. richmondi *
P. electromorphus
P. shenandoah *
P. cinereus *
P. serratus *
P. nettingi
P. virginia
P. hoffmani *
P. vehiculum *
P. elongatus *
P. vandykei *
P. idahoensis
D. quadramaculatus *
D. fuscus *
A. lugubris *
Ensatina eschscholtzii *
Eurycea bislineata *

13
11
12
10
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4
1
5
3
2

P. glutinosus group
P. wehrlei + *P. welleri* group
P. cinereus group
Eastern *Plethodon*
Western *Plethodon*

15.06 Genome Size (pg) 69.3

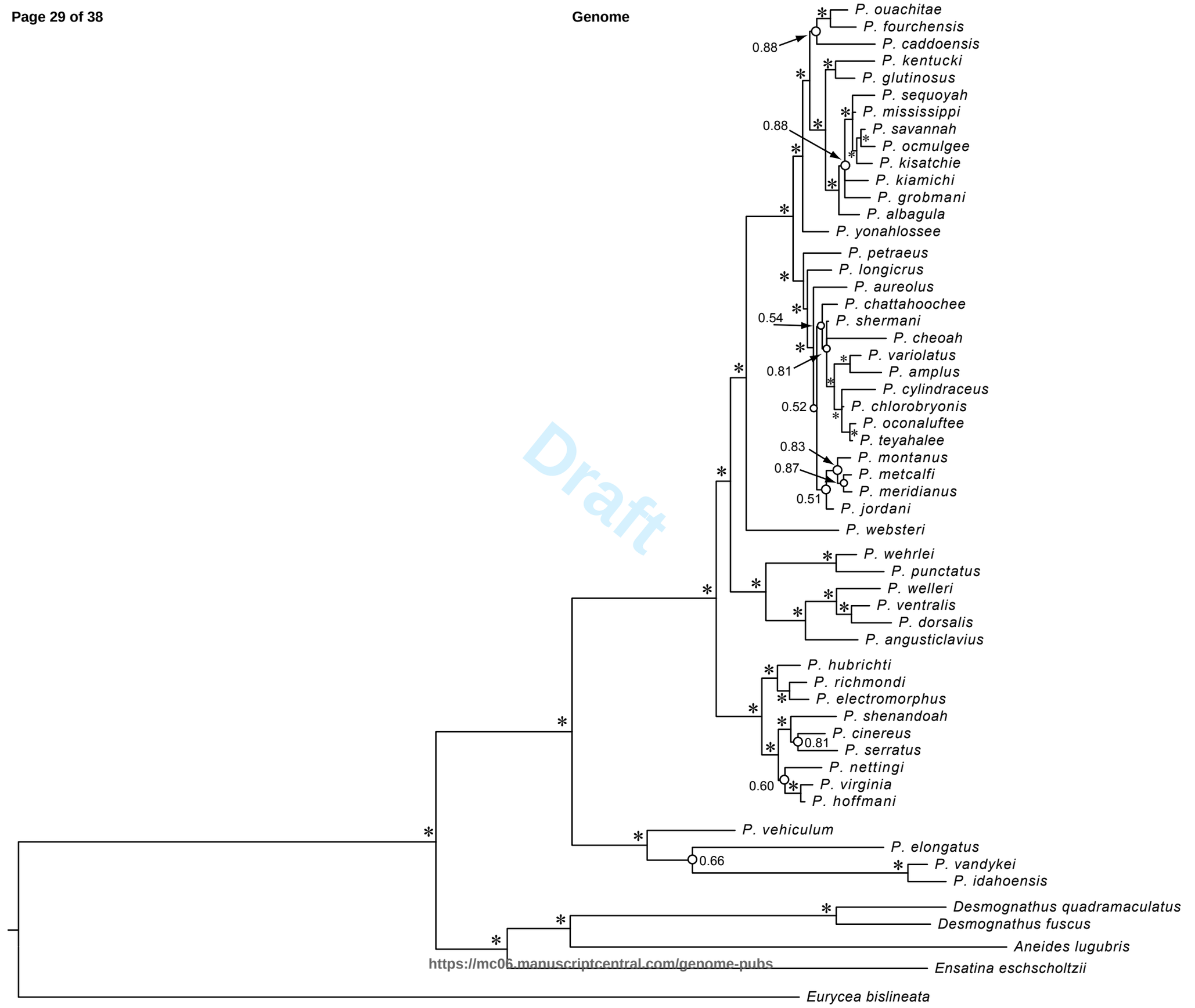
Online Supplementary Material, Figure Legends

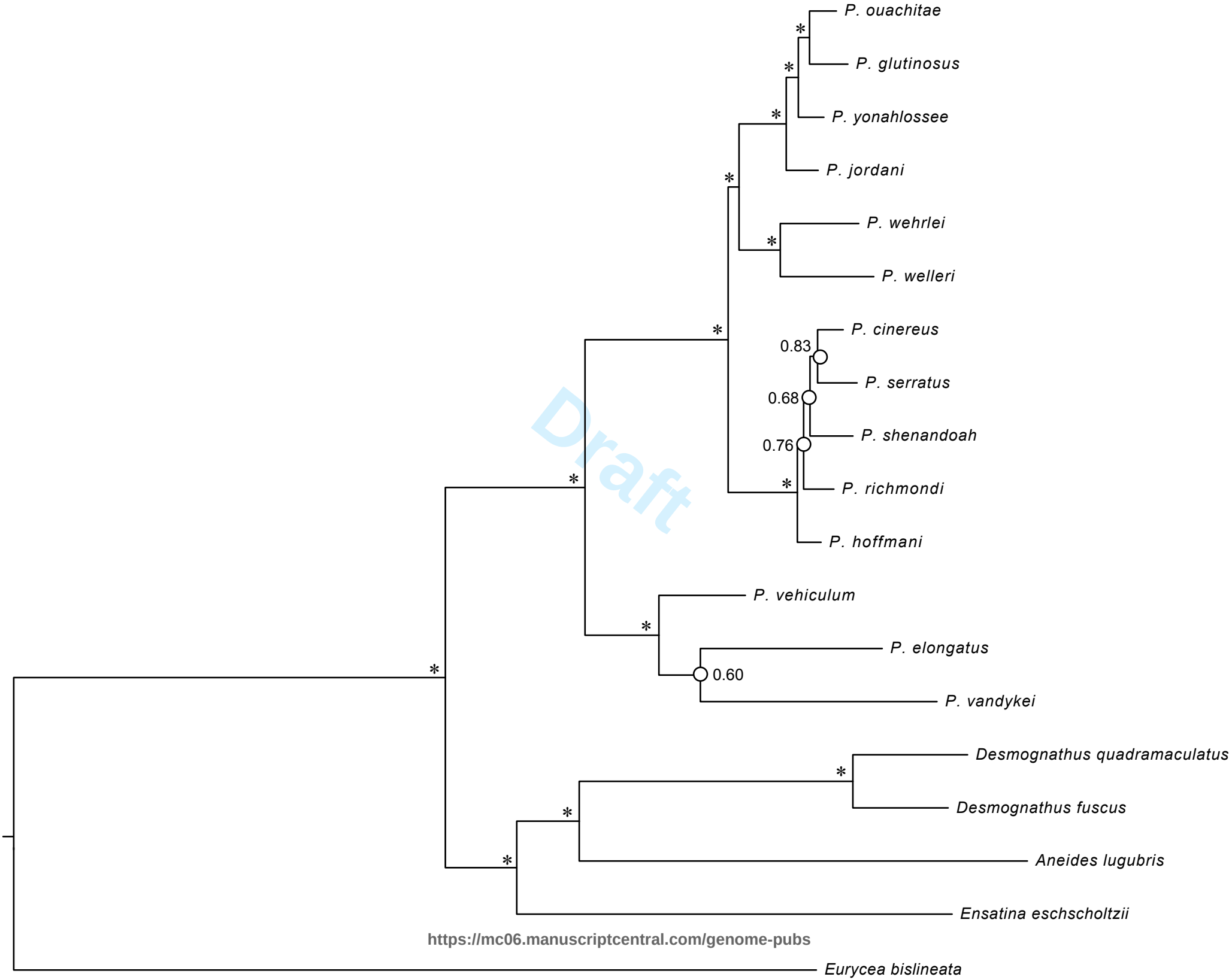
Fig. S1. Bayesian majority-rule consensus tree with all 55 species. Nodes supported by Bayesian posterior probability ≥ 0.9 are indicated by asterisk. Otherwise, posterior probability is noted.

Fig. S2. Bayesian majority-rule consensus tree with only species with empirical genome size data. Nodes supported by Bayesian posterior probability ≥ 0.9 are indicated by asterisk. Otherwise, posterior probability is noted.

Fig. S3. Node numbers on Bayesian majority-rule consensus tree with all 55 species. Node numbers correspond to Table S3.

Fig. S4. Node numbers on Bayesian majority-rule consensus tree with only species with empirical genome size data. Node numbers correspond to Table S3.


P. ouachitae
P. fourchensis
P. caddoensis
0.88
P. kentucki
P. glutinosus
P. sequoyah
P. mississippi
0.88
P. savannah
P. ocmulgee
P. kisatchie
P. kiamichi
P. grobmani
P. albagula
P. yonahlossee
P. petraeus
P. longicrus
P. aureolus
P. chattahoochee
0.54
P. shermani
P. cheoah
P. variolatus
0.81
P. amplus
P. cylindraceus
0.52
P. chlorobryonis
P. oconaluftee
P. teyahalee
0.83
P. montanus
0.87
P. metcalfi
P. meridianus
0.51
P. jordani
P. websteri
P. wehrlei
P. punctatus
P. welleri
P. ventralis
P. dorsalis
P. angusticlavius
P. hubrichti
P. richmondi
P. electromorphus
P. shenandoah
P. cinereus
0.81
P. serratus
P. nettingi
0.60
P. virginia
P. hoffmani
P. vehiculum
P. elongatus
0.66
P. vandykei
P. idahoensis
Desmognathus quadramaculatus
Desmognathus fuscus
Aneides lugubris
Ensatina eschscholtzii
Eurycea bislineata


P. ouachitae
P. glutinosus
P. yonahlossee
P. jordani
P. wehrlei
P. welleri
P. cinereus
0.83
P. serratus
0.68
P. shenandoah
0.76
P. richmondi
P. hoffmani
P. vehiculum
P. elongatus
0.60
P. vandykei
Desmognathus quadramaculatus
Desmognathus fuscus
Aneides lugubris
Ensatina eschscholtzii
Eurycea bislineata


P. ouachitae
P. fourchensis
P. caddoensis
P. kentucki
P. glutinosus
P. sequoyah
P. mississippi
P. savannah
P. ocmulgee
P. kisatchie
P. kiamichi
P. grobmani
P. albagula
P. yonahlossee
P. petraeus
P. longicrus
P. aureolus
P. chattahoochee
P. shermani
P. cheoah
P. variolatus
P. amplus
P. cylindraceus
P. chlorobryonis
P. oconaluftee
P. teyahalee
P. montanus
P. metcalfi
P. meridianus
P. jordani
P. websteri
P. wehrlei
P. punctatus
P. welleri
P. ventralis
P. dorsalis
P. angusticlavius
P. hubrichti
P. richmondi
P. electromorphus
P. shenandoah
P. cinereus
P. serratus
P. nettingi
P. virginia
P. hoffmani
P. vehiculum
P. elongatus
P. vandykei
P. idahoensis
Desmognathus quadramaculatus
Desmognathus fuscus
Aneides lugubris
Ensatina eschscholtzii
Eurycea bislineata

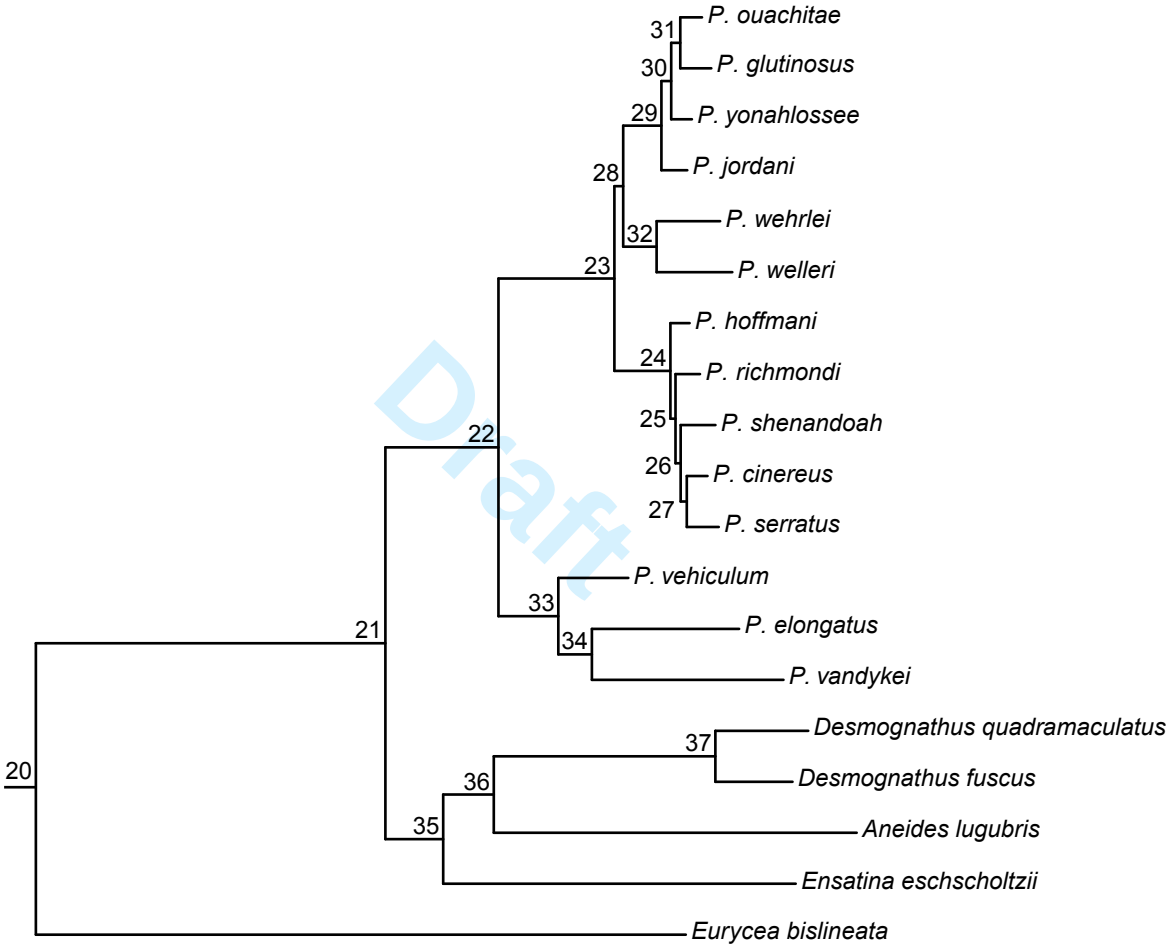

P. ouachitae
P. glutinosus
P. yonahlossee
P. jordani
P. wehrlei
P. welleri
P. hoffmani
P. richmondi
P. shenandoah
P. cinereus
P. serratus
P. vehiculum
P. elongatus
P. vandykei
Desmognathus quadramaculatus
Desmognathus fuscus
Aneides lugubris
Ensatina eschscholtzii
Eurycea bislineata
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Table S1. C-values of species included in analyses.

Species	C-value (pg) (All)	C-value (pg) (Species median)
Eurycea bislineata	20.75	24.50
	24.50	
	35.50	
Aneides lugubris	35.68	42.90
	42.80	
	43.00	
	49.60	
Ensatina eschscholtzii	31.00	41.39
	35.30	
	41.27	
	41.50	
	42.00	
	42.17	
Desmognathus quadramaculatus	14.50	15.06
	15.06	
	22.00	
Desmognathus fuscus	15.00	15.13
	15.00	
	15.13	
	17.70	
	18.00	
Plethodon cinereus	20.00	22.64
	21.40	
	22.30	
	22.50	
	22.64	
	23.00	
	23.08	
	26.13	
	26.20	
Plethodon elongatus	30.60	33.63
	33.63	
	33.63	
	33.80	
Plethodon glutinosus	22.50	28.00
	25.50	
	27.09	
	28.00	
	28.54	
	28.54	

	43.00	
Plethodon hoffmani	21.40	21.40
Plethodon jordani	23.10	27.80
	27.80	
	36.00	
Plethodon ouachitae	33.70	33.70
Plethodon richmondi	20.40	20.65
	20.90	
Plethodon serratus	22.14	20.43
	24.21	
	19.64	
	21.07	
	19.80	
	19.23	
Plethodon shenandoah	18.20	18.20
Plethodon vandykei	69.30	69.30
Plethodon vehiculum	35.60	38.05
	36.80	
	39.30	
	40.04	
Plethodon wehrlei	20.30	24.20
	28.10	
Plethodon welleri	22.60	22.60
Plethodon yonahlossee	25.40	30.75
	36.10	

Table S2. Genetic data used in the phylogenetic analysis. Data sources by accession number prefix: JN (Fisher-Reid & Wiens, 2011), EU (Vieites et al., 2007), FJ (Bonett et al., 2009), KR (Martin et al., 2015), DQ and AY (Wiens et al., 2006).

Species	BDNF	GAPD	ILF3	Mlc2a	POMC	RAG-1	RHO
P. albagula	-	JN798216	JN798264	JN798307	-	DQ995008	JN798363
P. amplus	-	JN798217	JN798265	JN798308	-	DQ995010	JN798364
P. angusticlavius	-	JN798218	-	-	-	DQ995011	-
P. aureolus	-	JN798219	JN798266	JN798309	-	DQ995012	JN798365
P. caddoensis	-	JN798221	JN798268	JN798311	-	DQ995013	JN798367
P. chattahoochee	-	JN798222	JN798269	JN798312	-	DQ995014	JN798368
P. cheoah	-	JN798223	JN798270	JN798313	-	DQ995015	JN798369
P. chlorobryonis	-	JN798224	JN798271	JN798314	-	DQ995016	JN798370
P. cinereus	-	JN798228	-	JN798317	FJ951365	DQ995021	JN798374
P. cylindraceus	-	JN798229	JN798272	JN798318	-	DQ995022	JN798375
P. dorsalis	-	-	JN798273	JN798319	-	DQ995023	JN798376
P. electromorphus	-	JN798230	-	JN798320	-	DQ995025	JN798377
P. elongatus	EU275882	JN798231	-	-	EU275836	AY650120	-
P. fourchensis	EU275884	JN798232	JN798274	JN798321	EU275838	DQ995026	-
P. glutinosus	-	JN798234	-	JN798324	-	DQ995027	JN798379
P. grobmani	-	JN798236	JN798276	JN798325	-	DQ995028	JN798381
P. hoffmani	EU275883	JN798238	-	JN798327	EU275837	DQ995029	JN798383
P. hubrichti	-	JN798239	-	JN798328	-	DQ995030	JN798384
P. idahoensis	-	-	-	JN798329	-	DQ995031	-
P. jordani	EU275881	-	JN798278	JN798330	EU275835	DQ995032	JN798385
P. kentucki	-	JN798240	JN798279	JN798331	-	DQ995033	JN798386
P. kiamichi	-	JN798241	JN798280	JN798332	-	DQ995034	JN798387
P. kisatchie	-	JN798242	JN798281	JN798333	-	DQ995035	JN798388
P. longicrus	-	JN798243	JN798282	JN798334	-	DQ995037	JN798389
P. meridianus	-	JN798244	JN798283	JN798335	-	DQ995038	JN798390
P. metcalfi	-	JN798245	JN798284	-	-	DQ995039	-
P. mississippi	-	JN798246	JN798285	-	-	-	JN798391
P. montanus	-	JN798247	JN798286	JN798336	-	DQ995043	JN798392

P. nettingi	-	JN798248	-	-	-	DQ995045	JN798393
P. ocmulgee	-	JN798250	JN798288	JN798338	-	DQ995048	JN798395
P. oconaluftee	-	JN798249	JN798287	JN798337	-	DQ995046	JN798394
P. ouachitae	EU275877	JN798251	JN798289	-	EU275831	AY691704	JN798396
P. petraeus	-	JN798252	JN798290	JN798339	-	DQ995049	JN798397
P. punctatus	-	JN798253	JN798291	-	-	DQ995050	JN798398
P. richmondi	-	JN798254	-	JN798340	-	DQ995053	JN798399
P. savannah	-	JN798255	JN798292	JN798341	-	DQ995055	JN798400
P. sequoyah	-	-	JN798293	JN798342	-	DQ995056	JN798401
P. serratus	EU275876	JN798256	-	JN798343	EU275830	DQ995057	JN798402
P. shenandoah	-	JN798257	-	JN798344	-	DQ995062	JN798403
P. shermani	-	JN798259	JN798294	JN798346	-	DQ995065	JN798405
P. teyahalee	EU275880	-	JN798295	JN798347	EU275834	DQ995068	JN798406
P. vandykei	EU275879	JN798260	-	-	EU275833	AY691715	-
P. variolatus	-	-	JN798296	JN798348	-	DQ995070	JN798407
P. vehiculum	-	-	-	JN798349	-	AY691716	JN798408
P. ventralis	-	JN798261	JN798297	JN798350	-	DQ995071	JN798409
P. virginia	-	-	JN798298	JN798351	-	DQ995072	JN798410
P. websteri	-	-	JN798299	JN798352	-	DQ995073	JN798411
P. wehrlei	-	-	JN798300	JN798353	-	DQ995075	JN798412
P. welleri	-	JN798262	JN798301	-	-	AY691717	JN798413
P. yonahlossee	EU275878	JN798263	JN798302	JN798354	EU275832	DQ995077	JN798414
Eurycea bislineata	EU275861	-	-	-	EU275815	AY691706	JN798360
Aneides lugubris	EU275893	-	-	-	EU275847	AY650118	JN798356
Ensatina eschscholtzii	EU275862	-	-	-	EU275816	EU275785	JN798361
Desmognathus quadramaculatus	-	-	-	-	KR732359	AY650117	-
Desmognathus fuscus	EU275858	-	-	-	EU275812	EU275781	-

Table S3. C-values estimated by ACR for all nodes on the full and known-only phylogenies. Node numbers correspond to Figs. S1, S2.

Node (all, known)	Clade Name	C-value (pg) (All)	C-value (pg) (Only known)
56, 20	(Root)	29.80	29.88
57, 21	Plethodontinae	32.63	32.77
58, 22	*Plethodon*	33.82	34.09
59, 23	Eastern *Plethodon*	25.71	26.37
60, 24	*P. cinereus* group	22.22	21.65
61		21.54	
62, 26		21.10	21.06
63, 27		21.29	21.30
64		21.51	
65		21.42	
66		21.67	
67		21.25	
68, 28		25.99	26.52
69	*P. glutinosus* group + *P. websteri*	26.77	
70, 29	*P. glutinosus* group	29.07	28.90
71, 30		29.84	29.93
72, 31		30.16	30.52
73		30.82	
74		32.09	
75		29.40	
76		29.40	
77		29.40	
78		29.40	
79		29.40	
80		29.40	
81		28.92	
82		28.74	
83		28.62	
84		28.43	
85		28.32	
86		28.02	
87		28.02	
88		28.02	
89		28.32	
90		28.32	
91		28.32	
92		28.32	

93		28.32	
94		28.32	
95, 32	*P. welleri* & *P. wehrlei* groups	24.96	25.02
96		24.14	
97		23.50	
98		23.50	
99		24.37	
100, 33	Western *Plethodon*	38.71	38.77
101, 34		41.99	41.75
102		67.04	
103, 35		32.49	32.57
104, 36		31.03	31.14
105, 37	*Desmognathus*	17.65	17.66
-, 25			21.29