Genome Size Diversification in Central American Bolitoglossine Salamanders (Caudata; Plethodontidae)

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Genome sizes (expressed as C-values, or haploid genome sizes) of six species of Honduran plethodontid salamanders (one species of *Nototriton* and five of *Bolitoglossa*) vary greatly. *Nototriton* has a moderate-sized genome (29.2 pg) relative to other species of salamanders. Genome sizes in the species of *Bolitoglossa* span a range of 24 pg (~23.4 gigabases) of DNA and include the largest genomes (83.7 pg) reported for the genus and for the family Plethodontidae. A phylogenetic analysis indicates that genome evolution in this group of salamanders featured mainly large increases in the mass of nuclear DNA. We propose that these evolutionary changes in genome size reflect random drift in small, isolated populations in the highlands of Central America.

SALAMANDERS show more variation in genome size than any other vertebrate order (Gregory, 2005). Mean C-values in salamanders range from 13.8 picograms (pg) to over 120 pg of DNA (Gregory, 2016). This genome size variation does not involve polyploidy, and instead is driven by transposable elements (TEs) and other non-coding DNA sequences (such as repetitive sequences) spread across the karyotype, affecting chromosome size but not number or shape between species (Mizuno and Macgregor, 1974; Orgel and Crick, 1980; Sessions, 2008; Sun et al., 2012).

The lungless salamander family Plethodontidae is a highly diverse group that comprises approximately two-thirds of all species of salamanders (Shen et al., 2016; AmphibiaWeb, 2018). The published genome size range for plethodontid species (13.8–76.2 pg) encompasses nearly the entire range of genome sizes in the order Caudata (Sessions, 2008; Newman et al., 2016), with genomes at the high end that are exceeded only by species of the salamander families Amphiumidae and Proteidae, which include the largest genomes of any tetrapod (Gregory, 2016). Despite being the most species-rich and genomically diverse family, there is relatively poor genome size sampling across the genera of Plethodontidae. In particular, little is known about genome size variation among the most speciose group of plethodontids, the Neotropical bolitoglossines. Most of what we know about this group of 309 species comes from a single study of 41 species (Sessions and Kezer, 1991; AmphibiaWeb, 2018). C-values for these species range from 20.8 pg in *Parvimolge townsendi* to 68.9 pg in *Bolitoglossa pesrubra* (Sessions and Kezer, 1991). Within the most species-rich Neotropical genus, *Bolitoglossa*, genome sizes range from 42.3 to 68.9 pg (Sessions and Kezer, 1991), a difference in amount of DNA equivalent in mass to eight entire human genomes.

A growing body of evidence indicates that genome size has measurable phenotypic correlates from the cell level to the whole organismal level (Sessions and Larson, 1987; Roth et al., 1994; Gregory, 2005). Both nuclear volume and overall cell size are positively correlated with genome size, and the combination of large cell and small body size can lead to developmental constraints that have probably played an important role in the morphological evolution of plethodontid salamanders (Sessions, 1985; Sessions and Larson, 1987; Hanken and Wake, 1993; Roth et al., 1994, 1997; Parra-Olea et al., 2004; Sessions, 2008). Thus, genome size evolution in salamanders potentially reflects the accumulation of TEs and non-coding DNA at the molecular level with relaxed selective constraints at the organismal level (Gregory, 2005; Sessions, 2008; Sun and Mueller, 2014).

A full understanding of the biological significance of these relationships will depend on a thorough understanding of the variation in genome size in various groups, especially among the diverse Neotropical species. Genome size data offer a novel approach to the study of patterns of gene flow and speciation, as well as the identification of cryptic species. Here, we present new genome size data on Central American species of *Bolitoglossa* and *Nototriton* from Honduras and examine genome size diversity in these species within an explicitly phylogenetic context. Our data include the largest genome sizes yet reported for plethodontid salamanders and reveal intraspecific differences in genome size that may underlie biogeographic isolation and possible speciation events.

MATERIALS AND METHODS

Taxon sampling.—We measured genome sizes of six Central American bolitoglossine salamander species endemic to Honduras, including *Nototriton picucha* and five species of the genus *Bolitoglossa*. These included four members of the subgenus *Magnadigita*: *Bolitoglossa celaque* ($n = 3$), *B. diaphora* ($n = 2$), *B. heiroreias* ($n = 1$), *B. porrasorum* ($n = 2$), and one

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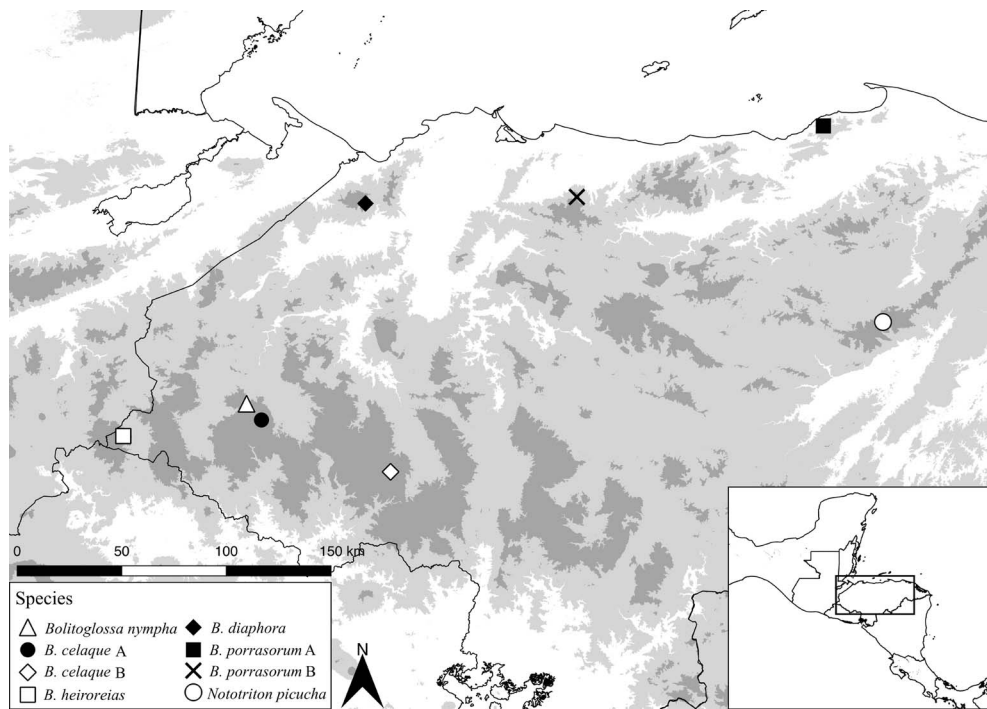
member of the subgenus *Nanotriton*: *B. nympha* ($n = 1$). Museum vouchers can be found in the Material Examined. All of these species have restricted ranges in montane regions in Honduras. *Nototriton picucha* occurs in the Sierra de Agalta of eastern Honduras at elevations of 1,890 to 1,930 m above sea level (Townsend et al., 2011). *Bolitoglossa celaque* is known from the Montaña de Celaque, the Sierra de Opalaca, and the Sierra Lenca in southwestern Honduras, at elevations of between 1,900 and 2,620 m (McCranie and Wilson, 2002). *Bolitoglossa diaphora* is known only from the Sierra de Omoa of northwestern Honduras at altitudes between 1,470 and 2,200 m (McCranie and Wilson, 2002). *Bolitoglossa heiroreias* is known only from the vicinity of Cerro Montecristo and Cerro Miramundo in the Trifinio International Park of El Salvador, Guatemala, and Honduras, and from Volcán Quetzaltepeque in Guatemala, at elevations of 1,800 to 2,300 m (Greenbaum, 2004). *Bolitoglossa porrasorum* occurs in the environs of Pico Pijol and Montaña Macuzal in the Sierra de Sulaco, Pico Bonito, and Cerro Corre Viento in the central Cordillera Nombre de Dios, and the environs of Texíguat in the western Cordillera Nombre de Dios, all in north-central Honduras, at elevations between 980 and 1,920 m (McCranie and Wilson, 2002; Townsend and Wilson, 2016). *Bolitoglossa nympha* occurs in western Honduras and is also found in eastern Guatemala (Campbell et al., 2010). Our samples include two allopatric populations each of *B. celaque* from Cerro Celaque (*B. celaque* A) and San Pedro La Loma (*B. celaque* B) and *B. porrasorum* from Corre Viento (*B. porrasorum* A) and Texíguat (*B. porrasorum* B; Fig. 1); the two populations of *B. porrasorum* have previously been shown to represent two deeply divergent lineages based on analyses of COI barcodes (Townsend and Wilson, 2016).

Genome size estimation.—Specimens were euthanized in 0.1% tricaine methanesulfonate solution (MS-222 buffered with 0.2% sodium bicarbonate, pH 7) and fixed in 10% buffered formalin for a minimum of 24 hours, then rinsed in water overnight, and stored in 70% ethanol. Blood cells were extracted from fixed specimens by making a small incision

under the gular fold, clipping the heart, aspirating blood cells with a pipet, and then dropping the blood cells onto glass slides, following the method of Sessions and Larson (1987).

The nuclei of blood cells were stained with Schiff's reagent using the Feulgen method (Sessions and Larson, 1987; Sessions, 1996). Slides were hydrated for 3 min in distilled water, then hydrolyzed in 5 N HCl for 20 min at room temperature, rinsed briefly in distilled water three times, and placed in a Coplin jar filled with Schiff's reagent (Humason, 1972) and stained for 90 min at room temperature. Next, we soaked the slides in 0.5% sodium metabisulfite solution, three changes, 5 min each and rinsed them three times with distilled water. We then dehydrated the slides in an alcohol series (70%, 95%, 100%, 1 min each) followed by air drying, and mounted them in immersion oil and covered them with a glass coverslip.

Stained slides were examined using an Axioskop 2 MOT (Carl Zeiss) microscope. Images were taken with a color CCD digital camera (Sony DXC-950P), and integrated optical densities of individual nuclei were measured via KS400 (Carl Zeiss Vision) software as described by Vilhar and Dermastia (2002). Measurement images were shading-corrected and densitometrically calibrated using a set of neutral-density filters. For the C-values, 15–55 nuclei of RBCs were measured per individual, depending on the quality of the cells (i.e., avoiding atypically shaped nuclei). We used *Bolitoglossa pesrubra* from the Cerro de la Muerte in the Talamanca mountains of Costa Rica as our standard based on the mean of published C-values: 65.2 pg (Sessions and Kezer, 1991; Vinogradov, 1998). Nuclear and cell areas of fixed, Feulgen-stained RBCs ($n = 20$) were calculated using Leica Application Suite (v. 4.0) from images taken at magnification 100× with a Zeiss Option Axioskop microscope and Leica DFC 290 HD digital camera. Our estimated C-values were assessed by comparing with a regression analysis of nuclear area vs. genome size using nuclear areas calculated from stained RBC nuclei and published data for a variety of taxa representing a wide range of C-values (Gregory, 2016). Although red blood cell (RBC) nuclei are diploid somatic cells, we have followed

Table 1. Species and samples used in the phylogenetic analysis.

		GenBank accession number
Species	Locality	*16S*
Bolitoglossa (Magnadigita) celaque A	Honduras: Lempira	MN429138
B. (Magnadigita) celaque B	Honduras: Intibucá	MN116215
B. (Magnadigita) cuchumatana	Guatemala: El Quiché	GU725454
B. (Magnadigita) diaphora	Honduras: Cortés	GU725447
B. (Magnadigita) dunni	Honduras: Cortés	GU725446
B. (Magnadigita) engelhardti	Guatemala: San Marcos	GU725448
B. (Magnadigita) flavimembris	Mexico: Chiapas	GU725449
B. (Magnadigita) heiroreias	Guatemala: Chiquimula	MN116218
B. (Magnadigita) helmrichi	Guatemala: Zacapa	GU725450
B. (Magnadigita) lincolni	Guatemala: San Marcos	AY526148
B. (Magnadigita) morio	Guatemala: Chimaltenango	KJ175098
B. (Nanotriton) nympha	Honduras: Santa Barbara	KC288003
B. (Nanotriton) occidentalis	Mexico: Chiapas	KC287912
B. (Eladinea) pesrubra	Costa Rica: San Jose	EU448104
B. (Magnadigita) porrasorum A	Honduras: Colón	MN116217
B. (Magnadigita) porrasorum B	Honduras: Atlántida	MN116216
B. (Magnadigita) rostrata	Guatemala: Totonicapán	KJ175099
B. (Nanotriton) rufescens	Guatemala: Alta Verapaz	KC287937

convention and have expressed genome size as C-value, the mass of DNA in a haploid genome expressed as picograms (pg), where 1 pg ≈ 1 billion base pairs (0.978 Gb) of DNA.

DNA extraction, PCR, and sequencing.—Cellular DNA was extracted from muscle tissue preserved in SED buffer using a Qiagen PureGene DNA Isolation Kit (Qiagen, Valencia, CA) following the kit protocol. Fragments of 16S were amplified using the 16Sar-L/16Sbr-H primer pairs (Palumbi et al., 1991). PCR reactions were 20 μL in total volume, containing ~25 μng of DNA template, 4 μL 5X PCR buffer, 1.2 μL MgCl$_2$ (25 mM), 0.09 μL dNTPs (10 mM), 0.8 μL of each primer (10 μM), 0.2 μL GoTaq Flexi polymerase (Promega, Madison, WI), and 11.91 μL H$_2$O. Amplification protocol was as follows: initial denaturation for 3 minutes at 94°C, 35 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 45 seconds, with a final elongation at 72°C for 5 minutes. PCR products were verified using electrophoresis on a 1.5% agarose, ethidium bromide-stained gel. Unincorporated nucleotides were removed from PCR products using 1 μL of ExoSAP-IT (USB, Santa Clara, CA) per 10 μL of PCR product, which was followed by cycle sequencing of both complementary strands using the BigDye Terminator 3.1 Cycle Sequencing kit, followed by spin column filtration through Sephadex before electrophoresing on an ABI 3130xl (Applied Biosystems, Inc).

Phylogenetic analysis.—We constructed a phylogeny of *Bolitoglossa* including the two subgenera presented in this study: *Magnadigita* and *Nanotriton*. DNA sequences for the 16S ribosomal RNA gene were generated for *B. heiroreias* and two representatives from two separate populations each of *B. celaque* and *B. porrasorum*. Additional 16S data for species in *Eladinea*, *Magnadigita*, and *Nanotriton* with known genome sizes, including those presented in this study, were obtained through NCBI (https://www.ncbi.nlm.nih.gov/genbank; Table 1).

The 16S dataset included 18 individuals representing 16 taxa, which were aligned using MUSCLE in MEGA v7 with default parameters and trimmed to 528 bp (Edgar, 2004; Kumar et al., 2016). We used an HKY+G+I nucleotide

substitution model for 16S based on PartitionFinder 2 (Lanfear et al., 2016) and constructed a Bayesian-inference (BI) phylogeny using BEAST v2.4.5 (Bouckaert et al., 2014) with a coalescent constant population model and constraining monophyly among the *Eladinea* + *Magnadigita* subgenera as resolved by Rovito et al. (2015). The BI analysis was run with four chains for 10^6 generations, sampling every 1,000. Our results were assessed for stationarity with Tracer v1.6.0 (Rambaut et al., 2014), and trees were sampled using TreeAnnotator with 10% burnin (Bouckaert et al., 2014).

In order to estimate the magnitude and direction of genome size evolution in these species, a maximum likelihood ancestral character reconstruction was used to estimate genome sizes at each node in the phylogeny using the package phytools (Revell, 2012) in R v.3.4.2 (R Core Team, 2016).

RESULTS

As expected from published C-values from other members of the genus *Nototriton* (Sessions and Kezer, 1991), the smallest C-value was measured in *N. picucha* (29.2 pg), with correspondingly small nuclear areas (47.2 μm^2; Table 2; Fig. 2). C-values measured for species of the genus *Bolitoglossa* ranged from 59.7 pg for *B. nympha* to 83.7 pg for *B. porrasorum* A, the largest genome size yet reported for the genus *Bolitoglossa* and for the family Plethodontidae, and one of the largest genomes among salamanders (Table 2; Sessions, 2008; Gregory, 2016). A regression analysis of nuclear area vs. genome size showed a strong overall correlation (Fig. 2; R^2 = 0.91). However, this relationship is weaker only among species of *Bolitoglossa* (R^2 = 0.67). Overall RBC surface area appears also to be positively correlated with genome size and nuclear area (Table 2). Like other members of the subgenus *Nanotriton* (Villolobos et al., 1988), *B. nympha* has partially enucleated RBCs (approximately 60%).

The individuals sampled from each of the two species, *B. celaque* A and *B. diaphora,* show very similar C-values that differ by less than 1 pg in each case (Table 2). The population referred to as *B. celaque* A, however, has a genome size that is nearly 10 pg larger than that of the geographically separate

Table 2. Species sampled, the number of specimens (N), the total number of measured cells (n), estimated C-values, RBC nuclear area (NA), and cell areas (CA) of *Bolitoglossa* salamanders and *Nototriton picucha* analyzed in this study (standard deviations in parentheses). We used *B. subpalmata* (C-value = 65.2 from previously published values; Sessions and Kezer, 1991; Gregory, 2016) as our standard. ND: no data.

Species	N	n	C-value (pg)	NA (μm^2)	CA (μm^2)
Nototriton picucha	1	55	29.2 (2.25)	47.1 (3.17)	216.9 (27.3)
Bolitoglossa (Eladinea) subpalmata	1	31	65.2 (5.58)	105.8 (9.1)	647.5 (117.2)
B. (Magnadigita) celaque A	1	30	81.5 (7.84)	125.6 (11.1)	521.3 (82.1)
B. (Magnadigita) celaque A	1	34	82.3 (10.5)	ND	ND
B. (Magnadigita) celaque B	1	15	72.4 (3.72)	109.2 (7.56)	588.4 (121.7)
B. (Magnadigita) diaphora	1	47	79.3 (7.80)	125.1 (6.35)	597.0 (115.9)
B. (Magnadigita) diaphora	1	29	79.5 (6.77)	ND	ND
B. (Magnadigita) heiroreias	1	32	69.8 (8.01)	119.6 (14.4)	508.9 (114.2)
B. (Magnadigita) porrasorum A	1	31	83.7 (6.87)	153.6 (19.4)	ND
B. (Magnadigita) porrasorum B	1	52	81.6 (7.29)	119.0 (11.2)	491.3 (81.9)
B. (Nanotriton) nympha	1	43	59.7 (4.77)	105.0 (7.88)	361.7 (73.9)

population referred to as *B. celaque* B. In contrast, specimens from two populations of *B. porrasorum*, A and B, differ from each other by only 2.1 pg (Table 2).

Our ancestral character reconstruction of genome size suggests that evolutionary change in genome size in these species included both large increases and decreases in C-values (Fig. 3). The ancestral value for *Bolitoglossa* was estimated to be 56.4 pg, with a decrease at the base of the *Nanotriton* clade and an increase leading to *Eladinea* + *Magnadigita* clade. *Magnadigita* has the most dynamic variation in genome size, with two major shifts early in this subgenus. One clade (*B. cuchumatana, B. engelhardti, B. helmrichi, B. rostrata*) underwent a large decrease in genome size and little diversification thereafter. Genome size appears to have increased slowly across the rest of *Magnadigita*, with rapid changes in both directions towards the tips.
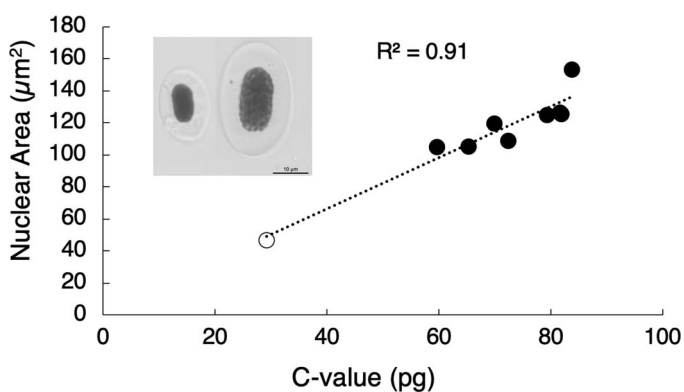
DISCUSSION

Previously published data on species of plethodontid salamanders show that they have an enormous range of genome sizes (13.8–76.2 pg; $\bar{x} = 36.4$; Gregory, 2016), with the Neotropical species (tribe: Bolitoglossini; Wake, 2012) having genome sizes averaging in the upper range of the family (20.8–68.9 pg; $\bar{x} = 43.4$; Sessions and Kezer, 1991; Gregory, 2016). The results of this study extend the known genome size variation for the genera *Nototriton* and *Bolitoglossa*, as well as the family Plethodontidae. The largest

Fig. 2. Erythrocyte (RBC) nuclear area vs. C-values of the specimens of bolitoglossine salamanders examined in this study. Dotted line shows the "best fit" line. The single specimen of *Nototriton* is indicated by the open circle. Inset: Feulgen-stained RBC of *Nototriton picucha* (left) and *Bolitoglossa porrasorum* B (right) at the same magnification.
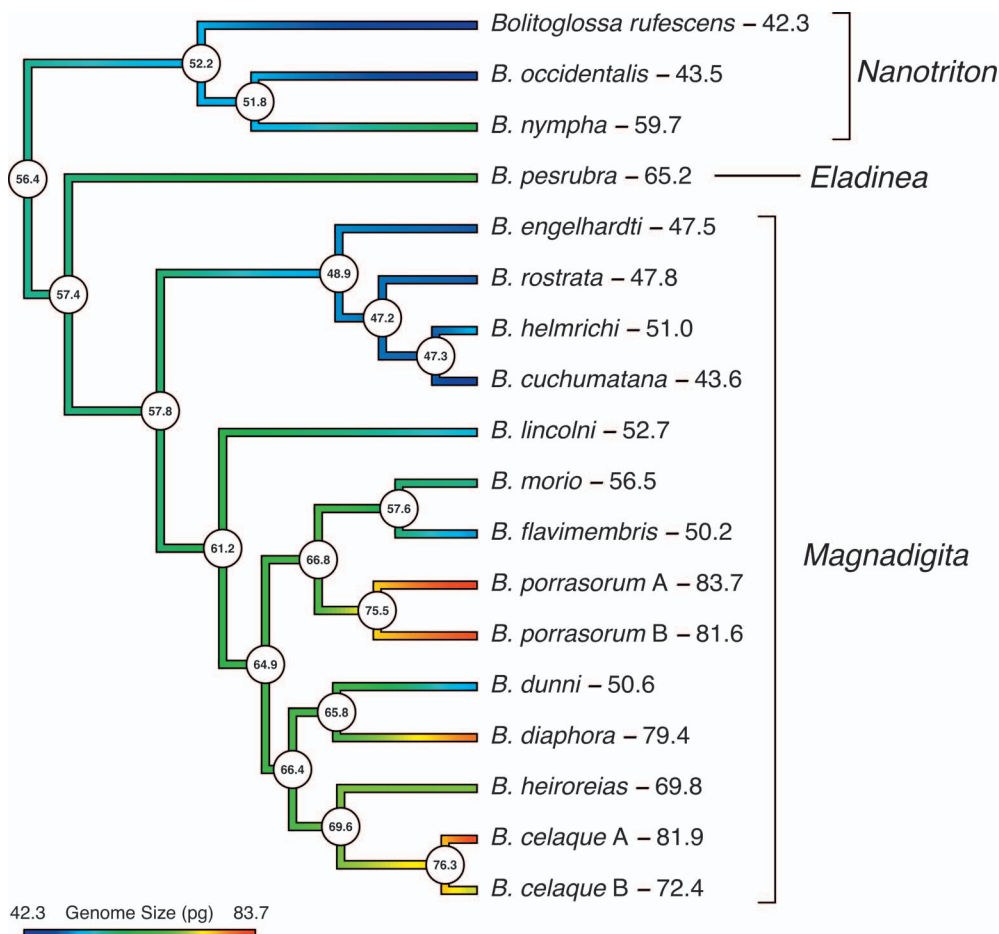
previously reported genome sizes in *Bolitoglossa* and Plethodontidae were 68.9 pg (*B. subpalmata*) and 76.2 pg (*Hydromantes italicus*), respectively (Sessions and Kezer, 1991; Sessions, 2008). With the exception of *B. nympha*, all of the species of *Bolitoglossa* presented in this study exceed the largest known genome size previously known in the genus, and with a C-value of 83.7 pg, *B. porrasorum* A now holds the record as the largest reported genome size in the genus *Bolitoglossa* and in the family Plethodontidae, and has one of the largest genomes among salamanders (Sessions, 2008; Gregory, 2016).

The phenotypic consequence of large shifts in genome size is a topic of on-going investigation (Gregory, 2005). Genome size is correlated with important developmental parameters, including cell size, cell cycle time, and rates of growth and differentiation (Sessions and Larson, 1987; Gregory, 2001, 2002; Sessions, 2008), as well as tissue and organ complexity (Roth et al., 1993, 1994, 1997; Gregory, 2002; Mueller, 2015), that may play a role in heterochrony (e.g., paedomorphosis; Alberch and Alberch, 1981; Sessions and Larson, 1987; Gregory, 2002; Jaekel and Wake, 2007; Sessions, 2008). It is possible that these phenotypic correlates, such as slower rates of development in organisms with large genomes, may be maladaptive consequences of "run-away" genome expansion via TE insertions and other non-coding sequences (Orgel and Crick, 1980; Sessions, 2008; Sun and Mueller, 2014). The possible role that these phenotypic correlates have played in the morphological evolution of bolitoglossine salamanders should be investigated further.

An unexpected result of our study is the discovery that *B. nympha*, a member of the subgenus *Nanotriton*, has about 60% enucleated RBCs, a trait that has evolved independently in different lineages of miniaturized plethodontid salamanders with relatively large genomes and cells (Villolobos et al., 1988; Mueller et al., 2008). The estimated genome size (59.7 pg) is larger than that of other members of this subgenus, *B. occidentalis* (43.5 pg) and *B. rufescens* (42.3 pg). Because both *B. occidentalis* and *B. rufescens* also have enucleated RBCs (Villolobos et al., 1988), as does a newly described Mexican species of *Nanotriton* (*B. chinanteca*; Rovito et al., 2012; S. Rovito pers. comm.), the presence of enucleated RBCs is a synapomorphy for the subgenus *Nanotriton*. Further investigation is required to understand the relationship between RBC enucleation, miniaturization, and cell size in plethodontid salamanders, and to identify the mechanism and possible adaptive significance of this phenotype (Villolobos et al., 1988; Mueller et al., 2008).

Fig. 3. Ancestral state reconstruction of genome size across the *Eladinea*, *Magnadigita*, and *Nanotriton* subgenera of *Bolitoglossa*. Node estimates for genome size are denoted at each node in the circles. Subgenera (*Eladinea*, *Magnadigita*, and *Nanotriton*) are identified using the taxonomy of Parra-Olea et al. (2004) and Rovito et al. (2015). See Data Accessibility for tree file.

Only relatively limited research has been done on intraspecific variation in genome size in salamanders (Litvinchuk et al., 2004), including plethodontid salamanders (Sessions and Larson, 1987; Licht and Lowcock, 1991; Mueller et al., 2008). However, these studies found that there is little intraspecific variation in genome size within species and populations. In this study, we were able to examine two specimens each of *B. celaque* A and *B. diaphora* from the same populations. Our results from these small sample sizes reveal modest intraspecific variation (1.0–3.0%) in genome size within a given population. We found that differences in genome size between geographically isolated populations can be either relatively small (~2.5%), as in *B. porrasorum* A vs. B, or relatively large (~13.0%), as in *B. celaque* A vs. B. Interestingly, the populations of *B. porrasorum* with relatively small differences in genome sizes are thought to represent distinct species based on 16S and COI mtDNA data (Townsend and Wilson, 2016). On the other hand, genetic distance between *B. celaque* A and *B. celaque* B is relatively low, yet these populations differ by about 10 pg in genome size, suggesting that *B. celaque* A and *B. celaque* B are genetically isolated from each other (Itgen, 2016). These data show that evolutionary changes in genome size can be independent of divergence in sequence structure of functional genes. These results highlight the need for better sampling within species across geographic ranges as it is unknown how much variation in genome size can exist in a single species.

Some research suggests that genetic drift dynamics alone cannot explain genome size evolution (Whitney and Garland, 2010), including large genomes in salamanders (Mohlhenrich and Mueller, 2016). However, TE-driven genome size changes could be a major factor in genetic divergence among geographically highly fragmented populations of salamanders. The "mutational hazard hypothesis" proposes that increased genome size via non-coding DNA is driven by the degree of genetic drift (Lynch and Conery, 2003). Therefore, it is more likely for non-coding DNA (i.e., TE insertion) to become fixed in species with small effective population sizes (N_e), resulting in gradual, or even rapid, increases in genome size. The heterogeneous landscape of the Neotropics in particular has resulted in high rates of genetic fragmentation and speciation for bolitoglossine salamanders, which is thought to be primarily driven by high ecological variation across narrow elevational gradients (Wake and Lynch, 1976; Rovito, 2017). In bolitoglossine salamanders, this relationship has generated a pattern of highly specialized species with limited distribution, and individual species (including ones used in this study) are often found isolated on a single mountain peak. Future work incorporating population genetics and distribution may provide insight into whether variation in N_e and genetic drift pressure is correlated with the diversification in genome size in plethodontid salamanders.

In summary, genome size evolution in plethodontid salamanders appears to be highly dynamic, involving large increases as well as decreases (Sessions and Larson, 1987; Sessions and Kezer, 1991; Sessions, 2008). The Neotropical genera show the widest range of genome sizes in the family, and our results confirm that the most variation in absolute mass of DNA is shown in the diverse genus *Bolitoglossa*, despite the relatively limited sampling within the genus (Sessions and Kezer, 1991; Sessions, 2008). We have added at

least five additional species and can now report that genome size variation within the genus *Bolitoglossa* ranges from 42 pg to 84 pg, a range of 42 pg, a difference equivalent to about 14 whole human genomes. Among the species examined in this study, genome size diversity appears to reflect genetic drift in small, isolated populations living in the extremely mountainous regions of Central America, especially in the mountains of northwestern Honduras (Townsend, 2014).

MATERIAL EXAMINED

Bolitoglossa celaque: (3) CM 170509–10, Lempira; CM 1705011, Intibucá.

Bolitoglossa diaphora: (2) CM 170506–7.

Bolitoglossa heiroreias: (1) CM 170508.

Bolitoglossa nympha: (1) CM 163310.

Bolitoglossa porrasorum: (2) CM 170504, Colón; CM 170505, Atlántida.

Nototriton picucha: (1) CM 163327.

DATA ACCESSIBILITY

Supplemental material is available at https://www.copeiajournal.org/ch-18-156.

ACKNOWLEDGMENTS

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