Deep divergences and extensive phylogeographic structure in a clade of lowland tropical salamanders

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Abstract

Background: The complex geological history of Mesoamerica provides the opportunity to study the impact of multiple biogeographic barriers on population differentiation. We examine phylogeographic patterns in a clade of lowland salamanders (Bolitoglossa subgenus Nanotriton) using two mitochondrial genes and one nuclear gene. We use several phylogeographic analyses to infer the history of this clade and test hypotheses regarding the geographic origin of species and location of genetic breaks within species. We compare our results to those for other taxa to determine if historical events impacted different species in a similar manner.

Results: Deep genetic divergence between species indicates that they are relatively old, and two of the three widespread species show strong phylogeographic structure. Comparison of mtDNA and nuclear gene trees shows no evidence of hybridization or introgression between species. Isolated populations of Bolitoglossa rufescens from the Los Tuxtlas region constitute a separate lineage based on molecular data and morphology, and divergence between Los Tuxtlas and other areas appears to predate the arrival of B. rufescens in other areas west of the Isthmus of Tehuantepec. The Isthmus appears responsible for Pliocene vicariance within B. rufescens, as has been shown for other taxa. The Motagua-Polochic fault system does not appear to have caused population vicariance, unlike in other systems.

Conclusions: Species of Nanotriton have responded to some major geological events in the same manner as other taxa, particularly in the case of the Isthmus of Tehuantepec. The deep divergence of the Los Tuxtlas populations of B. rufescens from other populations highlights the contribution of this volcanic system to patterns of regional endemism, and morphological differences observed in the Los Tuxtlas populations suggests that they may represent an undescribed species of Bolitoglossa. The absence of phylogeographic structure in B. nympha, in contrast to other widespread species in the subgenus, may be due to historical forest contraction and more recent range expansion in the region. Phylogeographic data provide substantial insight into the evolutionary history of these morphologically similar species of salamanders, and contribute to our understanding of factors that have generated the high biodiversity of Mesoamerica.

Keywords: Salamander, Phylogeography, Mesoamerica, Isthmus of Tehuantepec, Biogeography, Bolitoglossa
Background
Phylogeography and molecular systematics have been of great utility for delimiting the species boundaries of morphologically cryptic taxa [1-3] and understanding the origins of diversity both at and below the species level [4-7]. Within taxa, high genetic diversity in a small region often indicates long-term historical persistence in the face of environmental change, while genetic breaks between populations suggest a more complex demographic history involving periods of isolation or restricted gene flow. Climatic barriers or geological features are often associated with such phylogeographic breaks and can be hypothesized to have caused population isolation. Given that allopatric divergence is hypothesized to be the prevailing geographic mode of speciation for most animal taxa [8], understanding which barriers have led to divergence within species should elucidate the impact of such barriers on species diversity over longer timescales. An improved understanding of both species boundaries and phylogeographic structure enables tests of hypotheses related to the geographic origin of clades, connecting their divergence to regional processes, and facilitates comparisons of patterns of lineage divergence across taxa.

Understanding the historical factors promoting population divergence and species formation is of particular interest in areas of high species diversity, such as humid tropical regions. Here we investigate how the complex geological history of southern Mexico and Nuclear Central America, the area between the Isthmus of Tehuantepec and the Nicaraguan Depression [9], relates to population divergence in a group of morphologically similar plethodontid salamanders. We interpret their divergence history in the light of patterns seen in other taxa in order to understand how regional biogeography may have influenced current patterns of species diversity and faunistic relationships between subregions.

Salamanders have often been shown to have historical signatures of geological or climatic changes in their geographic patterns of genetic variation [10-12], due to their short dispersal distances [13] and environmental sensitivity [14]. Most species of Neotropical salamanders, however, are characterized by small range sizes, and often are known from only a single mountain range [15-17], precluding studies of population differentiation at a regional scale. This tendency toward small range size could be a direct result of stronger population isolation in the tropics, leading to higher rates of allopatric speciation and thus producing many species with small geographic ranges [18,19]. The few wide-ranging tropical salamanders offer the chance to study species of low dispersal ability that are distributed across major geographic barriers, and allow us to understand the processes that may have been important in generating the high diversity seen in the tropical salamanders as a whole.

Our study focuses on species of Bolitoglossa subgenus Nanotriton, which has only four described species, yet is distributed throughout a wide area of Mesoamerica [20]. The species of this subgenus span multiple biogeographic boundaries in Mesoamerica, including the Isthmus of Tehuantepec, which appears to have restricted dispersal of mesic-adapted species [21-23], while acting as a corridor for arid-adapted species [24]. Species in the subgenus occur in multiple geological regions, including the eastern terminus of the Trans-Mexican Volcanic Belt (TMVB) and Los Tuxlas region in Veracruz, the Northern highlands of Oaxaca, the Sierra de los Chimalapas, Mexico and on both the Pacific and Caribbean sides of Chiapas, Mexico and Guatemala in Nuclear Central America (Figure 1). Bolitoglossa rufescens, the most widely distributed species in the subgenus, ranges across the Isthmus of Tehuantepec, making it one of only three salamander species to occur on both sides of this major barrier [25,26]. As presently known, B. rufescens also occurs on both sides of the Motagua-Polochic fault system, an important barrier for many other taxa in Central America [21,22].

Although species of Nanotriton generally occur only in mid- to low elevations, populations are known from up to 2000 m elevation and are not found in dry forest, savannah, or other low-elevation subhumid habitats. Many populations are currently associated with banana plantations and other anthropogenic habitats adjacent to forest, but salamanders can also be found in forested habitats. Because of these associations, species of Nanotriton, like higher elevation salamanders, could be used to test for effects of historical forest fragmentation or expansion, in addition to examining effects of geological barriers such as major mountain ranges or fault zones.

In at least two localities, two species of the subgenus are found in sympatry [27,28] and another instance of near-sympatry is known [29], despite overall morphological similarity of these species that might be expected to limit their co-occurrence [27]. The only obvious character separating two of the species (Bolitoglossa chinanteca and B. occidentalis) from the other two (B. nympha and B. rufescens) is the presence or absence of maxillary teeth, and even this character is variable within B. rufescens [29]. This group of salamanders exhibits little variation in morphology, possibly because of their generally paedomorphic state; development of features such as digits or skull bones that distinguish other species of salamanders is truncated, leading to a reduction or absence of these elements [30,31]. Because of this high similarity in external appearance, genetic markers provide one of the best tools to delimit these species’ geographical limits, an essential endeavor for understanding the biogeographic history of the group. The use of both mtDNA and nuclear markers also
allows us to test for a signature of introgression across species boundaries, with finer-scale sampling in possible areas of contact identified by analyses of allozymes [29].

In this study, we use both mitochondrial (mtDNA) and nuclear DNA (nDNA) to investigate phylogeographic structure within species of *Nanotriton* as well as phylogenetic relationships between described species. We compare patterns of mtDNA and nDNA to look for discordance that might indicate introgression across species boundaries, especially near previously identified possible contact zones, and to identify hybrids or signatures of past population admixture. We use a likelihood-based phylogeographic history estimation method (Phylomapper [32]) to test hypotheses of the geographic location of the origins of clades. In particular, we examine in which biogeographic region (southern Mexico vs. Central America) and in which mountain range or subregion species originated. We examine the impact of major biogeographic boundaries known from other taxa on the phylogeographic patterns within these salamander species, and compare patterns across these boundaries to those seen in other taxa.

**Results**

Phylogenetic analyses reveal deep divergences between species, as well as a high degree of phylogeographic structure within species. The mtDNA results (Figure 2) strongly support the monophyly of *Nanotriton* (likelihood bootstrap proportion [BS]=100, posterior probability [PP]=1.0), as well as that of all four members of the group: *Bolitoglossa occidentalis* (BS=99, PP=1.0), *B. chinanteca* (BS=100, PP=1.0), *B. nympha* (BS=100, PP=1.0) and *B. rufescens* (BS=100, PP=1.0). *B. occidentalis* and *B. chinanteca* are strongly supported as sister taxa (BS=99, PP=1.0), as are *B. nympha* and *B. rufescens* (BS=96, PP=0.99).
Within species, markedly different patterns of phylogeographic structure emerge. *Bolitoglossa chinanteca* shows little divergence in 16S sequence between the only known localities (GTR distance=0.004 substitutions/site). *Bolitoglossa occidentalis* shows several strongly supported lineages (BS>70, PP>95) corresponding to populations from 1) the Pacific coast of Chiapas and Guatemala, 2) a single population from the eastern side (Caribbean-draining) side of the Sierra Madre de Chiapas (Locality 35; Figure 1, Table 1), 3) a single population from Cerro Baúl in the isolated Sierra de los Chimalapas (Locality 22), and 4) various populations from other areas of northern and central Chiapas (Localities 23–34). Maximum GTR distances between samples of *B. occidentalis* are large (0.043

**Figure 2** Mitochondrial gene tree from RAxML analysis of 16S and cytb sequence data. Asterisks indicate branches with bootstrap proportions >70 for ML analysis and posterior propabilities >95 for Bayesian analysis. Numbers in parentheses after voucher numbers refer to all sampled localities (from Table 1) where haplotype was found. Geographic areas are shown for samples of *Bolitoglossa rufescens*. Abbreviations: Ch–Chiapas; TMVB–eastern terminus of Trans-Mexican Volcanic Belt; OAX–highlands of northern Oaxaca. Arrows indicate nodes whose daughter lineages are separated by the Isthmus of Tehuantepec.
Table 1 Populations of *Bolitoglossa* (*Nanotriton*) used in phylogenetic analyses

| Locality number | Species       | Country: State/Province | Locality                                      |
|-----------------|---------------|-------------------------|-----------------------------------------------|
| 1               | *B. chinanteca*, *B. rufescens* | Mexico: Oaxaca          | 9.2 km S of Valle Nacional on Hwy 175          |
| 2               | *B. chinanteca* | Mexico: Oaxaca          | Cocorales-Zacatepec highway, Sierra Mixte      |
| 3               | *B. rufescens*  | Mexico: Veracruz        | Cerro Chickahuatl, Cuautitlan                  |
| 4               | *B. rufescens*  | Mexico: Veracruz        | Fortín de las Flores                           |
| 5               | *B. rufescens*  | Mexico: Veracruz        | Coetzala, 8.9 km S of Amatlán                  |
| 6               | *B. rufescens*  | Mexico: Veracruz        | Playa Escondida, 30 km NNE Catemaco           |
| 7               | *B. rufescens*  | Mexico: Veracruz        | 9.2 km NE of Catemaco                          |
| 8               | *B. rufescens*  | Mexico: Veracruz        | Lake Catemaco, 2.5 km SE Coyame                |
| 9               | *B. rufescens*  | Mexico: Oaxaca          | 1.5 km SE of La Fortaleza                       |
| 10              | *B. rufescens*  | Mexico: Chiapas         | 10 km NW Oculapa, Ocozocuaúl                   |
| 11              | *B. rufescens*  | Mexico: Chiapas         | 15 km N Ocozocuaúl                             |
| 12              | *B. rufescens*  | Mexico: Chiapas         | 26.5 km N Ocozocuaúl                           |
| 13              | *B. rufescens*  | Mexico: Chiapas         | 12.4 km W Berriozabal                          |
| 14              | *B. rufescens*  | Guatemala: Huehuetenango| Siglo Veinte Ermit, Barillas                   |
| 15              | *B. rufescens*  | Guatemala: Huehuetenango| Las Victorias Chancolín, Barillas              |
| 16              | *B. rufescens*  | Guatemala: Huehuetenango| Palmiras de Chiblac, Barillas                  |
| 17              | *B. rufescens*  | Guatemala: Huehuetenango| El Valle, 4.5 km N of RN 9 at Aldea La Concepción|
| 18              | *B. rufescens*  | Guatemala: Alta Verapaz  | San Ramon, Barillas                            |
| 19              | *B. rufescens*  | Guatemala: Alta Verapaz  | western border of Parque Nacional Laguna Lachua|
| 20              | *B. rufescens*  | Guatemala: Alta Verapaz  | Parque Nacional Laguna Lachua                   |
| 21              | *B. rufescens*  | Guatemala: Alta Verapaz  | Finca Cuxmán, San Pedro Carchá                 |
| 22              | *B. occidentalis* | Mexico: Chiapas        | Cerro Baúl                                    |
| 23              | *B. occidentalis* | Mexico: Chiapas        | 11.4 km NW Berriozabal                         |
| 24              | *B. occidentalis* | Mexico: Chiapas        | Vista Hermosa, 7.5 km N Berriozabal            |
| 25              | *B. occidentalis* | Mexico: Chiapas        | Cuhumbác, 10.4 km N Berriozabal               |
| 26              | *B. occidentalis* | Mexico: Chiapas        | 11.2 km N San Fernando, Tuxtla Gutiérrez       |
| 27              | *B. occidentalis* | Mexico: Chiapas        | W San Fernando, 13 km N Tuxtla Gutiérrez       |
| 28              | *B. occidentalis* | Mexico: Chiapas        | 15.3 km ENE Copainalá                         |
| 29              | *B. occidentalis* | Mexico: Chiapas        | 9 km ENE Coapilla                             |
| 30              | *B. occidentalis* | Mexico: Chiapas        | 2.7 km W Pantepec                             |
| 31              | *B. occidentalis* | Mexico: Chiapas        | Puerto del Viento, Pueblo Nuevo Solistahuacán  |
| 32              | *B. occidentalis* | Mexico: Chiapas        | Julian Grijales, W of Pueblo Nuevo Solistahuacán|
| 33              | *B. occidentalis* | Mexico: Chiapas        | W of Rayón                                    |
| 34              | *B. occidentalis* | Mexico: Chiapas        | SE of Puerto Cate                             |
| 35              | *B. occidentalis* | Mexico: Chiapas        | Finca Prusia                                  |
| 36              | *B. occidentalis* | Mexico: Chiapas        | 14 km N Tapachula on road to Finca Nueva Alemania|
| 37              | *B. occidentalis* | Mexico: Chiapas        | 7.5 km N Cacahoaúán                           |
| 38              | *B. occidentalis* | Guatemala: San Marcos  | Finca Santa Julia                              |
| 39              | *B. occidentalis* | Guatemala: San Marcos  | 2 km S San Rafael Pie de la Cuesta             |
| 40              | *B. nympha*     | Guatemala: Huehuetenango| Chancolín                                     |
| 41              | *B. nympha*     | Guatemala: Alta Verapaz  | Finca Sabó, Purulhá                           |
| 42              | *B. nympha*     | Guatemala: Alta Verapaz  | Finca el Volcán, Serahú                       |
| 43              | *B. nympha*     | Belize: Toledo         | Blue Creek National Park                       |
| 44              | *B. nympha*     | Guatemala: Izabal       | Cerro Sanitín, Livingston                     |
| 45              | *B. nympha*     | Guatemala: Izabal       | Las Escobas, Cerro San Gil                    |
| 46              | *B. nympha*     | Guatemala: Zacapa       | Finca la Bendición, Pinalito                   |
| 47              | *B. nympha*     | Guatemala: Zacapa       | Finca las Granadillas, Pinalito                |
for 16S between localities 39 and 28, 0.10 for cytb between localities 22 and 37); GTR distances between individuals from all localities for each gene are given in Additional files 1, 2, 3; Tables S2, S3, S4. Our samples from the Sierra de los Chimalapas (locality 22; Figure 1) are from near the type locality of Bolitoglossa bilineata [33], synonymized with B. occidentalis by Wake and Brame [34]. Locality 22 is nested within B. occidentalis in our mtDNA gene tree.

Bolitoglossa rufescens comprises several well-supported, divergent lineages. Two groups of populations from the Los Tuxtlas region form a basal polytomy with all remaining populations. Samples from either side of the Isthmus of Tehuantepec are not reciprocally monophyletic; those from the eastern end of the TMVB in Veracruz and northern highlands of Oaxaca are in a clade with samples from Chiapas and the Sierra de los Chimalapas (Figure 2). Yet another clade of B. rufescens consists of samples from northwestern Guatemala.

Although B. nympha was described only from the type locality (locality 50) in Guatemala [35], Rovito et al. [27] hypothesized that populations from eastern Guatemala and western Honduras could be assigned to this species based on morphological data as well as allozyme results from Larson [29]. Campbell et al. [35] also stated that a specimen from Belize might be assignable to B. nympha based on a published cytb sequence. The mtDNA gene tree shows a deep divergence between populations formerly assigned to B. rufescens from Mexico and northwestern Guatemala and those from eastern Guatemala, Belize, and Honduras (Figures 1, 2). Given that the latter group of populations includes the type locality of B. nympha, we assign all these populations to that species, which is now known from a much broader geographic area than in the original description.

Results from phylogenetic analyses of the POMC data (Figure 3) mirror those from mtDNA in most respects. Bolitoglossa occidentalis and B. chinanteca are again placed in a clade that is the sister lineage of the other two species of Nanotriton, and B. chinanteca is monophyletic. The sample from nearest the type locality of B. bilineata is again nested within a group of other samples of B. occidentalis from Chiapas, in agreement with the mtDNA results, providing no support for the distinctiveness of this named taxon. The single sample of B. hartwegi included in the dataset fell within this clade, rather than outside Nanotriton; this relationship is strongly supported (BS=88, PP=0.99), suggesting that the relationship between the subgenera Nanotriton and Mayamandra should be further investigated using additional markers. Both B. rufescens and B. nympha are supported as monophyletic. Within B. rufescens, the initial divergence is once again resolved to be between populations from Los Tuxtlas and all other populations (Figure 3), and samples on either side of the Isthmus of Tehuantepec are not reciprocally monophyletic. A haplotype network constructed using the program TCS with the POMC data shows four separate networks and similar patterns to the phylogenetic tree results (Figure 4). The first corresponds to B. nympha and B. rufescens, which are separated by 8 mutational steps. The second network consists of samples of B. occidentalis and B. chinanteca, which are separated by 7 mutational steps, and the final two networks correspond to samples of B. hartwegi and B. mexicana (the outgroup in phylogenetic analyses). Bolitoglossa rufescens and B. occidentalis show both a higher diversity of haplotypes and more divergence between haplotypes compared to B. nympha. There was no discordance between the mitochondrial and POMC gene trees in terms of individuals being placed within clades corresponding to species; all samples from near contact zones fall into a clade corresponding to the same species in both the mtDNA and POMC gene trees. No individual has POMC haplotypes belonging to two different species, as we would expect if our dataset contained interspecific hybrids.

We are reluctant to undertake a molecular dating analysis of all divergences in the group due to a lack of appropriate fossil calibration points. Despite this, we estimated a chronogram with mtDNA data from all

| Table 1 Populations of Bolitoglossa (Nanotriton) used in phylogenetic analyses (Continued) |
|-----------------------------------------------|
|   | Species | Location                        | Distance from type locality |
| 48 | B. nympha | Guatemala: Zacapa | 5.2 km SE La Unión |
| 49 | B. nympha | Honduras: Copan | El Limón, Sierra del Espiritu Santo |
| 50 | B. nympha | Honduras: Copan | Santa Rosa de Copan |
| 51 | B. nympha | Guatemala: Izabal | Finca la Firmenza, Sierra Caral |
| 52 | B. nympha | Honduras: Santa Barbara | Montaña de Joconales |
| 53 | B. nympha | Honduras: Cortés | Santa Teresita |
| 54 | B. nympha | Honduras: Cortés | Aldea Buenos Aires |
| 55 | B. nympha | Honduras: Cortés | Sierra del Espiritu Santo, W San Pedro Sula |
| 56 | B. nympha | Honduras: Yoro | 11 km W of CA-5 at Hospital Cermeza, San Pedro Sula |
| 57 | B. nympha | Honduras: Yoro | 386 km NE Santa Rita |
samples used in our phylogenetic analyses in order to calculate the divergence time of populations of *B. rufescens* that span the Isthmus of Tehuantepec. We used two different published substitution rate estimates for *cytb* from a fossil-calibrated phylogenetic analysis of plethodontids [36] and a rate that has been used as a general vertebrate molecular clock [37] in order to compare the timing of this divergence to that seen in other species. Populations of *B. rufescens* separated by the Isthmus of Tehuantepec were estimated to have diverged 3.3
A high degree of phylogeographic structure characterizes both *Bolitoglossa rufescens* and *B. occidentalis*, as revealed both by the significant phylogeographic association test from Phylomapper and the presence of multiple divergent lineages present within both species.
**Bolitoglossa rufescens** is known from a number of localities on both sides of the Isthmus of Tehuantepec and in the isolated Sierra de los Tuxtlas, making it one of only two species of salamander (along with *B. platydactyla*) to be distributed in all three of these areas. Although the Phylomapper results reconstruct a Central American origin for the species, an ancestral location within either the highlands of northern Oaxaca or in Los Tuxtlas could not be rejected. Indeed, the null hypothesis of an ancestral location at the center of the species’ current range could also not be rejected. Our inability to distinguish between these hypotheses may be due to the fact that the three major areas where *B. rufescens* is found meet in approximately the center of its range, and its history appears to be relatively deep in both Los Tuxtlas and Nuclear Central America. Nucleotide diversity for 16S is low in the TMVB/northern Oaxacan highlands (\(\pi = 0.0040 \pm 0.0037\)) compared to both los Tuxtlas (\(\pi = 0.014 \pm 0.011\)) and Central America (\(\pi = 0.021 \pm 0.012\)), which points to an origin for the species outside of the TMVB/northern Oaxacan highlands. The fact that initial divergences within *B. rufescens* are between Los Tuxtlas and all other areas in both the mtDNA and POMC gene trees suggests that this area may have been the origin of the species, or minimally that *B. rufescens* has been present in Los Tuxtlas for a long period of time. Volcanism that created the Sierra de los Tuxtlas began around 7 Ma, and several of the major volcanoes were formed sometime between from 3–1 Ma [38]. Although no calibration points are available for an accurate molecular dating analysis, the deep divergence between the samples from Los Tuxtlas and the clade from the TMVB/Oaxacan highlands and Nuclear Central America for both mtDNA and POMC most likely coincided with the early formation of the Tuxtlas volcanic complex.

Multiple species of amphibians show either disjunct distributions (*Pseudoeurycea werleri* [17], *Pseudoeurycea nigromaculata* [39], *Anotheca spinosa* [40]) or sister species pairs (*Pseudoeurycea orchimelas* and *P. orchileucos* [41]; *Thorius pennatulus* and *T. narismagnus* [16,39]) between the eastern terminus of the TMVB of Veracruz or highlands of northern Oaxaca and Los Tuxtlas, while other sister species pairs are found on either side of the Isthmus of Tehuantepec in Los Tuxtlas and the Sierra de los Chimalapas [42]. The only other lowland *Bolitoglossa* in the area, *B. platydactyla*, is present at low elevations in the Sierra Madre Oriental, eastern terminus of the TMVB, northern Oaxaca, Los Tuxtlas, and in northern Chiapas. Campbell (1984) proposed a closer relationship between the herpetofauna of Los Tuxtlas and the Chimalapas than between Los Tuxtlas and the northern Oaxacan highlands, and observed that the lowland area between los Tuxtlas and other highland areas of southern Mexico must have contained forest in the past to allow for interchange between these areas. The lowland areas around Los Tuxtlas today, which include both savannah and wetlands, do not contain any known populations of *B. rufescens* that would indicate a more continuous distribution in the recent past between these volcanoes and other areas where *Nanotriton* are found, although more searches would be needed to confirm this. The high nucleotide diversity of Los Tuxtlas compared to the TMVB/northern Oaxacan highlands and the high genetic distance between Los Tuxtlas and all other
populations of *B. rufescens* follow the pattern of divergence and endemism seen in more highland amphibian and reptile species with similar distributions and indicate the Los Tuxtlas populations have been isolated from other highland areas of southern Mexico for a long time. Indeed, the *B. rufescens* from Los Tuxtlas are the only population of the species with maxillary teeth [29], a morphological character often used to delimit species of tropical salamanders [16,43]. The Los Tuxtlas populations may warrant description as an additional species endemic to the volcanic complex, and merit further study.

Mulcahy et al. [44] found that the Isthmus of Tehuantepec corresponded to a phylogeographic break in both *Incilius valliceps* and *Rhinella marina*. They dated this divergence to approximately 2–3 Ma in both species, and suggested that these data were consistent with the existence of a Pliocene seaway across the Isthmus that may have caused vicariance between populations on either side. Using divergence values recalculated separately for each gene, their data show levels of divergence across the Isthmus for both species that are higher those seen for *B. rufescens* for 16S (*R. marina*: mean 16S GTR distance=0.011, *I. valliceps*: 0.0088) but lower for cytb (*R. marina*: mean cytb GTR distance=0.026; *I. valliceps*=0.020); *B. rufescens* populations from the TMVB/northern Oaxacan highlands and those from Chiapas in Nuclear Central America have mean...
pairwise GTR distances of 0.0062 for 16S and 0.040 for cytb. The fact that the trans-Isthmus divergence values for *B. rufescens* are not consistently higher or lower suggests that *B. rufescens* may have been affected by this barrier in a similar manner as the toads. Although substitution rate estimates for this group of tropical salamanders are not available, using two substitution rates for cytb from the literature gives a divergence estimate of 1.9 or 3.2 Ma, roughly concordant with the timing of the Pliocene seaway; the second estimate uses a rate derived from a phylogenetic analysis of plethodontids [36], and may represent a better estimate of the divergence time. Samples of *B. rufescens* from north and south of the Isthmus are not reciprocally monophyletic for mtDNA; samples from the TMVB/northern Oaxacan highlands render those from Central American paraphyletic. This suggests that any phylogeographic structure caused by the breaks in forested habitat associated with formation of the Isthmus or by formation of a seaway across the Isthmus postdates earlier phylogeographic structure associated with geologic or climatic barriers within Central America. The fact that genetic structure within *B. rufescens* associated with the Isthmus is relatively shallow compared to that within Nuclear Central America is reflected in the Barrier analysis results, which showed that the Isthmus corresponds to the fourth most important barrier within the species, after barriers within Nuclear Central America and Los Tuxtlas. Similarly, the AMOVA results show that while significant genetic structure is present among populations within regions, genetic structure is not significant at the regional level, indicating that divisions between regions such as the Isthmus do not correspond to locations of the primary genetic divisions between the species.

In addition to its possible role as a barrier between the Oaxacan highlands and Central America, the Isthmus may have isolated populations in Los Tuxtlas from those in Central America. The much deeper divergence seen between Los Tuxtlas and all populations in Central America may have been partly driven or reinforced by the formation of the Isthmus in the late Miocene or early Pliocene [45]. At an even deeper timescale, *Bolitoglossa occidentalis* and *B. chinanteca* were found to be sister taxa and are separated by the Isthmus of Tehuantepec. Based on our rate calibrations, their divergence is much too old to have been caused by a Pliocene seaway and likely predates the event. Given this difference in timing of the divergence between the two species and the formation of the Isthmus, the fact that their ranges are currently separated by the Isthmus suggests that caution must be used when inferring a causal role for geographic barriers based solely on overlap with genetic breaks.

Despite its small range and its distribution entirely within the Mayan geological block [46] of Nuclear Central America, *B. occidentalis* displays a high level of genetic diversity for both mtDNA and POMC. The Phylomapper results show significant phylogeographic structure within the species and reject an origin in any of the three main clusters of populations within our sampling or from the center of its current distribution. These results provide strong support for an origin of *B. occidentalis* on the eastern side of the Sierra Madre de Chiapas, near one of our sampled populations (Locality 35). While increased sampling of populations on both sides of the Sierra Madre de Chiapas could potentially change this result, our analyses indicate that *B. occidentalis* arose in an area of Caribbean drainage. *Bolitoglossa rufescens* is entirely confined to Caribbean-draining areas of Mexico and Central America, while *B. occidentalis* is primarily found on the Pacific side of Central America [15] (Figure 1). If both species arose within the Caribbean drainage, some other factor besides isolation across the Caribbean-Pacific divide must have been responsible for their initial divergence. The Barrier results show that the primary division within the species is between populations in southeastern Chiapas and Guatemala and all others, and the AMOVA results also show significant structure between the different areas where *B. occidentalis* is found.

The limited divergence between populations and lack of strong phylogeographic structure within *Bolitoglossa nympha* stands in contrast to that seen in both *B. rufescens* and *B. occidentalis*. *Bolitoglossa nympha* ranges across a major geographic barrier, the Motagua-Polochic fault system [21,22], yet this fault and associated subhumid areas seem not to have caused vicariance or population isolation within *B. nympha*. *Bolitoglossa nympha* and *B. rufescens* may have been separated by the Motagua-Polochic fault in the past, with a subsequent expansion of *B. nympha* to the northwest of the fault, bringing the two species into secondary contact. Likewise, areas where the distributions of *B. nympha* and *B. rufescens* approach each other in both the Sierra de los Cuchumatanes (localities 14–18 and 40, Figure 1C) and the Sierra de Xucanéb (localities 21 and 41–42; Figure 1D) in Guatemala present no obvious geological or climatic barrier that could have either caused the divergence between the two species or maintained them in allopatry. The low haplotype diversity and lack of strong phylogeographic structure within *B. nympha* suggest that the species may have undergone a historical bottleneck or reduction in range. Modeling of the extent of wet forest habitat, with which species of *Nanotriton* are generally associated, in Central America at the Last Glacial Maximum predicted an absence of this habitat in northeastern Honduras and eastern Guatemala [47], covering most of the current range of *B. nympha*. While this area was previously hypothesized as a Pleistocene refugium for plant taxa [48], the absence of humid forest over most of the range of *B. nympha* east of the Motagua fault during the Pleistocene could
explain both the lack of strong phylogeographic structure within the species compared to *B. rufescens* and *B. occidentalis* and the lack of a phylogeographic break across the Motagua-Polochic fault.

**Conclusions**

Species of *Nanotriton* have a long history in Central America. The deepest divergences within and between species of *Nanotriton* do not appear to have been caused by restricted dispersal across biogeographic barriers identified for other taxa, such as the Isthmus of Tehuantepec and Motagua-Polochic fault system. While the Isthmus of Tehuantepec did cause vicariance of populations of *Bolitoglossa rufescens*, this divergence is shallow compared to others within both *B. rufescens* and *B. occidentalis*. The distinctiveness of *B. rufescens* from Los Tuxtlas appears to be the exception to this pattern, and reinforces the importance of Los Tuxtlas as a site of high endemism and biogeographic distinctiveness within southeastern Mexico. Aside from Los Tuxtlas, the highest intrapopulation divergence within both *B. occidentalis* and *B. rufescens* has taken place within the Mayan block of Nuclear Central America. This relatively strong phylogeographic structure over small spatial scales could result in the formation of separate species over longer timescales. The external morphological similarity of all species in the subgenus has hidden the high phylogeographic structure present within two of these species, and subsequent morphological examination of populations from Los Tuxtlas or *B. occidentalis* from Chiapas could show that additional independent lineages warrant recognition as distinct species. Despite their morphological similarity, we found no evidence of introgression at or near contact zones. Differences in levels of phylogeographic structure between *B. rufescens* and *B. nympha* may be related to regional differences in historical forest extent, which is hypothesized to have varied over time [47,48]. This phenomenon should be tested by examining patterns of genetic variation within other forest-inhabiting taxa with similar distributions to these two salamander species. The phylogeographic history of *Nanotriton* serves as a useful comparison to both higher elevation forest taxa [21,22,49] and lowland, arid-adapted species [24], and provides an important addition to our understanding of factors responsible for population divergence and speciation across a geologically complex landscape.

**Methods**

**Study system and sample collection**

Populations of all four species of *Nanotriton* were sampled throughout Mexico, Guatemala and Honduras during fieldwork from 2005 to 2011, as well as from the Museum of Vertebrate Zoology tissue collection. Populations of *B. rufescens* in eastern Guatemala and Honduras belong to a second, recently described species, *B. nympha* [35]; this species was described only from the type locality, but other populations from the region bear a morphological resemblance to the type series [27,29]. *Bolitoglossa occidentalis* occurs from extreme eastern Oaxaca to the Pacific coast of Guatemala; we examined a single individual reported to *B. occidentalis* from the Caribbean side of Honduras [50], and determined it to be a juvenile of another subgenus (most likely subgenus *Bolitoglossa*). When this record is excluded, nearly all portions of the known range of these species were sampled in our study. Permits for specimen collection and export were provided to GPO in Mexico by SEMARNAT, to CRVA in Guatemala by CONAP, and to SMR in Honduras by COHDEFOR.

We collected a total of 96 individuals from 57 localities (Additional file 4: Table S1, Figure 1). Most individuals were found by searching in the outer layers of the trunks of banana plants in plantations or coffee groves, but some were found in arboreal bromeliads, under cover objects, or at night on vegetation. Liver and/or tail tissue was collected and stored either in ethanol, liquid nitrogen, or RNA later buffer in the field, and subsequently transferred to ~80°C. Voucher specimens were deposited in the Museum of Vertebrate Zoology (MVZ) at the University of California, Berkeley, the Instituto de Biología, Universidad Nacional Autónoma de México (IBH), or the Museo de Historia Natural, Universidad de San Carlos, Guatemala (USAC).

**Sequencing and phylogenetic analysis**

We extracted DNA from liver or tail tissue using either DNeasy extraction kits (Qiagen, Valencia, CA, USA) or a guanidine thiocyanate extraction protocol. The guanidine thiocyanate protocol involves cell lysis at 55°C for 3–12 hours, protein precipitation using guanidine thiocyanate followed by centrifugation for 10 min, and DNA precipitation using 100% isopropanol followed by centrifugation for 10 min. We sequenced two mitochondrial genes, the large subunit ribosomal RNA gene (16S) using primers 16Sar and 16Sb [51] and cytochrome *b* (*cytb*) using primers MVZ15 and MVZ16 [52], as well as one nuclear gene, proopiomelanocortin (*POMC*) using primers POMC_DRV_F1 and POMC_DRV_R1 [53]. PCR amplification consisted of an initial denaturation step at 95°C for 2 min, followed by 38 cycles of denaturation at 95°C for 30 s, annealing at 48°C (*16S* and *cytb*) or 57°C (*POMC*) for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were purified using 1uL of EXOSAP-IT (USB Corp., Cleveland, OH, USA), cycle sequenced with BigDye3.1 terminator sequencing (Applied Biosystems, Foster City, CA, USA), purified using ethanol precipitation and run on an ABI-3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited using Sequencher.
(GeneCodes, Ann Arbor, MI, USA) or Geneious v 5.1.7 [54] GenBank accession numbers for all sequences are given in Additional file 4: Table S1.

Sequences were aligned with Muscle v3.6 [55] using default parameters. Alignment lengths were 522 base pairs (bp) for 16S, 772 bp for cytb, and 481 bp for POMC. Gametic phase of POMC sequences was determined computationally using PHASE [56], and haplotype determinations from the best pairs output were used. When an individual had two distinct haplotypes for POMC, both were used in gene tree reconstruction. We constructed gene trees separately for concatenated mitochondrial genes (16S+cytb) and POMC using both maximum likelihood (ML) and Bayesian analyses, and removed redundant haplotypes prior to phylogenetic analysis. For ML analyses, the program RAXML v7.0.4 [57] was used. Mitochondrial data were partitioned by gene, and cytb data were further partitioned by codon position. A GTR+G+I substitution model was used for all ML analyses, and 1000 bootstrap replicates were run to assess nodal support; models less complex than the GTR model are not implemented in RAxML. We estimated maximum likelihood (ML) and Bayesian consensus trees sampled from the posterior distribution by MrBayes 3.1.2 [58], with two runs and four chains (one cold, three heated) per run. MCMC analyses were run for 2 x 10^7 generations, sampled every 1000 generations, and the first 5000 samples were discarded as burn-in. Rate variation across partitions was permitted, and default priors were used for other parameters. We used the sliding window and compare plots in the program AWTY [59] to check for convergence in Bayesian analyses. A sequence of Bolitoglossa (Bolitoglossa) mexicana was used as the outgroup for all phylogenetic analysis, and a sequence of Bolitoglossa (Mayamandra) hartwegi was also included in the dataset, given that the subgenera Bolitoglossa and Mayamandra were found to be the closest relatives of Nanotriton in previous analyses of mtDNA [20]. Sequence alignments and Bayesian consensus trees are archived in TreeBase (Submission 13756; http://purOrg/phylo/treebase/phylo\study\TB2: S13756).

For Bayesian analyses, we tested several partitioning strategies for the mtDNA dataset: 1) all data as a single partition, 2) 16S and cytb as separate partitions (two partitions), and 3) 16S as one partition with cytb further partitioned by codon position (four partitions). For POMC, we compared results with all data as single partition and with each codon position as a separate partition. The program MrModeltest [60] was used to determine the most appropriate model of nucleotide substitution for each partition using the AIC. We determined the most appropriate partitioning strategy for each locus using Bayes factors [61]. Comparison of results of Bayesian analyses with different partitioning strategies supported the 4-partition strategy for mtDNA (2ln (Bayes factor) 4 vs. 2 partitions–466; 4 vs. 1 partition–624; 2 vs. 1 partition–158) and 3 partitions for the POMC data (2ln(Bayes factor) 3 vs. 1 partition–15). The following substitution models were used in the favored partitioning strategy: 16S, GTR+I+G; cytb codon positon 1, 2 – HKY+G; cytb codon position 3, POMC codon position 3 – GTR+G; POMC codon position 1 – F81; POMC codon position 2 – HKY+I. A haplotype network for the POMC data was constructed using TCS v 1.2.1 [62].

Detection of interspecific hybridization
No examples of hybridization are currently known for the subgenus Nanotriton, and these phylogeographic data are suitable for assessing whether these morphologically similar species come into contact and hybridize. In order to detect possible interspecific hybrids, larger numbers of salamanders were sequenced for zones where different species may contact each other in Chiapas, Mexico and Guatemala. Although B. chinanteca and B. rufescens are known to occur syntopically in Oaxaca, Mexico [27], only a few samples from this locality were available. In the area of Berriozabal, Chiapas, Mexico (localities 13, 23–25; Figure 1) and Chancolín, Guatemala (localities 14–18, 40; Figure 1), multiple individuals per locality were included in both 16S and POMC phylogenetic analyses. Not all these individuals were sequenced for cytb, since both mitochondrial genes are inherited as a single unit. If F1 hybrids were included in the sample, we would expect to see some individuals with distinct POMC haplotypes from two different species, given the degree of structure in the POMC gene tree (see Results). Additionally, for both F1 hybrids and some backcrosses, we would expect to see individuals with 16S haplotypes from one species and POMC haplotypes from a different species.

Phylogeographic analyses
In order to determine the geographic origin of the three species with larger distributions (all except B. chinanteca), we used Phylomapper v1 [32]. This program uses geographic coordinates of collection localities of samples included in a phylogeny, along with branch length information, to estimate the geographic location of the ancestor of a clade using a random walk model of migration. Because branches of zero length produce infinite dispersal distance estimates (Phylomapper manual), the number of identical haplotypes was first reduced by sampling only a single individual from each site or cluster of sites. Mitochondrial sequences (either 16S and cytb or 16S alone) were used to construct a mtDNA gene tree using MrBayes from this sample set, with the same analysis parameters as in the analysis of all mtDNA sequences. To deal with remaining redundant haplotypes, one tree from the 15,000 fully resolved trees sampled from the posterior distribution by MrBayes was selected for each run using the “random” option in Phylomapper. The geographic origin of each species was calculated separately, using 100 runs of Phylomapper. For
each analysis, rate smoothing was conducted for the clade of interest (the species whose geographic origin was being estimated), rather than for the entire *Nanotriton* clade.

Phylomapper was also used to test statistically for phylogeographic association, or a correlation between geographic distance and genetic distance, within a clade and for a difference between the estimated location of the ancestor of a clade and the geographic center of that clade. The Bayesian consensus tree was used for all hypothesis testing, rather than choosing a tree randomly from the posterior distribution, in order to conduct all tests on the same gene tree. Polytomies in the consensus tree were resolved arbitrarily by inserting branches of very short length (0.0001). Statistical testing was not done for *B. nympha* because resolving the large number of polytomies between closely related haplotypes significantly changed the likelihood compared to runs using randomly chosen trees from the posterior distribution. For the phylogeographic association test, geographic coordinates from sampling localities were assigned randomly across tips and 10 000 iterations were used to construct a null distribution of the scaled dispersal parameter ($\Psi$).

Several additional hypotheses related to the ancestral location of clades were also tested by fixing the location of the ancestor of the clade, optimizing the other parameters in the model and comparing the likelihood of the constrained model (location fixed) with the model with the ancestral location unconstrained using a likelihood ratio test with two degrees of freedom [32]. Within each region tested as an alternative ancestral location, the geographically closest population in our dataset to the estimated origin of the clade was used as the point locality for the alternative origin location. *Bolitoglossa rufescens* was tested for an origin in the northern Oaxacan highlands (locality 1), and for an origin in the Los Tuxtlas region of Veracruz (locality 8). *Bolitoglossa occidentalis* is confined to the Nuclear Central America region, but is distributed primarily in three areas within the region, and was tested for an ancestral location in each: north-central Chiapas (locality 24), the Pacific coast (locality 36), and the Sierra de los Chimalapas (locality 22).

For species with a significant result from the Phylomapper phylogeographic association test, we used the program Barrier v.2.2 [63] to infer the geographic location of barriers between samples with Monmonier's (1973) maximum difference algorithm [64]. Barrier identifies the spatial location of genetic breaks by dividing up geographic space using a Voronoi tessellation, creating a network of polygons each edge of which is equidistant to two sampling localities, and the centroids of these polygons (sample localities) are connected in a Delaunay triangulation. Monmonier's algorithm finds the edge of the triangulation with the maximum genetic distance, and traces a barrier along the edge of the Voronoi polygon perpendicular to this edge. It proceeds along adjacent edges until the edge of the triangulation or a previously defined barrier is reached [63]. We used PAUP [65] to calculate GTR distances between samples used in the Phylomapper analyses, and ran the analysis with four barriers for *B. rufescens* and three for *B. occidentalis* (which has a smaller geographic range). Larger numbers of barriers were tested, but began to finely subdivide geographically proximate populations.

We used Arlequin v3.5 [66] to calculate nucleotide diversity for clades within species with the Tamura-Nei (TN) [67] substitution model (the most parameterized model implemented in Arlequin) with a gamma correction, in order to compare levels of genetic diversity within subregions. We used the same mtDNA dataset in Phylomapper in order to remove multiple redundant haplotypes from sites near contact zones. The value of gamma from the substitution model chosen by MrModeltest2.2 was used for distance calculation. For *Bolitoglossa rufescens*, samples were grouped into those from the eastern terminus of the TMVB/northern highlands of Oaxaca, Mexico (localities 1–5), Central America (localities 7–13), and those from Los Tuxtlas (localities 6–8), and the first two populations were used to compare divergence levels across the Isthmus of Tehuantepec. We also calculated nucleotide diversity for each species as a whole. Finally, we calculated mean GTR distances between species, as well as between geographic groups of populations within species of *Nanotriton*, using PAUP [65]. We also recalculated GTR distances across the Isthmus for two toad species (*Rhinella marina* and *I. valliceps*) from Mulcahy et al. [44], separating their 16S and *cytb* data in order to allow comparison with our data. We also performed a hierarchical Analysis of Molecular Variance (AMOVA) [68] in Arlequin for *B. rufescens* and *B. occidentalis*, which showed significant phylogeographic structure in the Phylomapper analyses. Populations were divided into three regional groups for *B. rufescens*: Los Tuxtlas, Nuclear Central America, and the TMVB/Oaxaca Highlands, and the latter two groups were subdivided into populations from Chiapas, Guatemala, TMVB, and Oaxacan highlands. A hierarchical AMOVA was performed using 16S sequences, with 1000 permutations used to assess significance. For *B. occidentalis*, an AMOVA with four groups was performed: the Sierra de los Chimalapas (locality 22), populations from northern and central Chiapas (localities 23–34), the population from the Sierra Madre de Chiapas (locality 35), and populations from the Pacific coast of southeastern Chiapas and Guatemala (localities 36–39).

**Divergence dating**

No fossil calibration points are available for tropical salamanders, complicating the use of molecular data to infer
divergence times. In order to gain a rough estimate of the divergence time between populations of *B. rufescens* on either side of the Isthmus of Tehuantepec, we used two different substitution rate estimates for cytb in a BEAST analysis [69] of the mtDNA data. The first rate of 0.0062 substitutions/site/Myr per lineage was estimated using a variety of fossil calibration points on a mitochondrial phylogeny of the Plethodontidae [36], while the second, faster rate of 0.01 substitutions/site/Myr [37] has been used as a general vertebrate mtDNA clock. The BEAST analysis was done a separate GTR+G substitution model for gene. An uncorrelated lognormal relaxed clock model was used to estimate divergence dates. Analyses were run for 20 × 10⁹ generations, sampled every 1000 generations, and Tracer v1.6 [70] was used to summarize posterior distributions of divergence times.

### Additional files

- **Additional file 1: Table S2.** General time reversible (GTR) distances between 165 haplotypes used in phylogenetic analyses.
- **Additional file 2: Table S3.** General time reversible (GTR) distances between cytb haplotypes used in phylogenetic analyses.
- **Additional file 3: Table S4.** General time reversible (GTR) distances between POMC haplotypes used in phylogenetic analyses.
- **Additional file 4: Table S1.** Museum catalog numbers, locality information, geographic coordinates, and GenBank accession numbers for all tissues used in phylogenetic analyses.

### Competing interests

The authors declare that they have no competing interests.

### Authors’ contributions

SMR helped design study, conducted fieldwork, carried out the molecular genetic studies, performed phylogenetic analyses, and drafted the final manuscript. GPO and DBW helped design study, carried out fieldwork, and assisted with manuscript preparation. CRVA and RLR conducted fieldwork. Collecting permits were provided by CONAP for help in the field, J. R. McCranie for providing tissues, and L. Márquez for help in the laboratory. Collecting permits were provided by CONAP (Guatemala), SEMARNAT (Mexico), and COHDEFOR (Honduras). S.M.R was funded by a postdoctoral fellowship from UC MEXUS-CONACYT postdoctoral fellowship and a NSF Bionventory grant (DEB 1026396). The project was partially funded by PAPIIT-UNAM IN212111 to G. P.-O.

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