ORIGINAL ARTICLE

Species formation and geographical range evolution in a genus of Central American cloud forest salamanders (*Dendrotriton*)

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ABSTRACT

Aim Montane Central America offers an ideal system for testing geographical hypotheses of species diversification. We examined how the complex geological history of Nuclear Central America has shaped the diversification of a genus of cloud-forest-inhabiting salamanders (*Dendrotriton*). We applied parametric models of geographical range evolution to determine the predominant mode of species formation within the genus and to test existing hypotheses of geographical species formation in the region.

Location Montane cloud forests of Nuclear Central America.

Methods We estimated a species tree for *Dendrotriton* using a multi-locus DNA sequence data set and several coalescent methods, and performed molecular dating for divergence events within the genus. We then applied the species-tree estimate to a likelihood-based time-stratified model of geographical range evolution, based on current species distributions and available geological information for Central America.

Results Species trees from all methods contain two groups, one corresponding to species from the Sierra de los Cuchumatanes and the other containing all remaining species. In most cases, species formation within the genus involved an even division of the geographical range of the ancestral species between descendant species. The ancestor of extant *Dendrotriton* species was estimated to have occurred in either the Sierra de los Cuchumatanes or the Sierra Madre de Chiapas, and both of these areas appear to have been important for diversification within the genus. The single species found in the Quaternary-age Guatemalan volcanic cordillera dispersed to the volcanoes from an older highland area.

Main conclusions Models of geographical range evolution, when combined with robust species-tree estimates, provide insight into the historical biogeography of taxa not available from phylogenies or distributional data alone. Vicariant species formation, rather than peripatric or gradient speciation, appears to have been the dominant process of diversification, with most divergence events occurring within or between ancient highland areas. The apparent dispersal of *Dendrotriton* to the Quaternary-age volcanoes raises the possibility that the rich salamander community there is composed of species that dispersed from geologically older areas. The Motagua Valley appears not to have been as important in vicariant species formation within *Dendrotriton* as it is within other groups.

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INTRODUCTION

The high diversity of tropical species compared with that of the temperate zone has long been of interest to biogeographers, evolutionary biologists and systematists (reviewed in Mittelbach *et al.*, 2007), but the undescribed diversity and incomplete sampling available for many groups of tropical organisms can hinder the testing of biogeographical hypotheses. Mesoamerica, as the link between North and South America, has received much attention from biogeographers as an area of both faunal interchange (Webb, 2006; Weir *et al.*, 2009) and autochthonous diversification (Wake & Lynch, 1976; Savage, 1982; Campbell, 1999). Many studies have examined Central American biogeography at a broad scale (Savage, 1982; Savage & Wake, 2001; Crawford & Smith, 2005), while fewer have focused on biogeographical patterns within its distinct subregions. Nuclear Central America, defined as the region between the Isthmus of Tehuantepec and the Nicaraguan Depression (Schuchert, 1935), is topographically and biologically diverse, but has received comparatively little attention as a distinct biogeographical unit (but see Stuart, 1943a, 1957; Savage, 1982; Halffter, 1987; Campbell, 1999).

Nuclear Central America has a complex geological history, owing to its position at the interface of three major tectonic plates (Marshall, 2007). The high diversity of many taxa in the region led to its recognition as part of the Mesoamerican biodiversity hotspot (Myers *et al.*, 2000). The highlands of the region include ancient, non-volcanic uplifted ranges such as the Sierra de los Cuchumatanes, as well as volcanoes of Tertiary and Quaternary age along the Pacific versant of the south-east to north-west-sloping Guatemalan Plateau produced by the subduction of the Cocos Plate (Marshall, 2007). Many of these highland areas contain montane cloud forests and are currently separated by deep valleys with subhumid vegetation or intervening lowland habitat (Stuart, 1954), but these cloud forest patches were more connected in the past (MacVean & Schuster, 1981; Wake & Lynch, 1982). The discrete nature of the highland areas in the region, coupled with their differing ages and geological origins, makes Nuclear Central America an ideal system for testing hypotheses of species formation in a geographical context.

New parametric methods of biogeographical reconstructions (Ree *et al.*, 2005; Ree & Smith, 2008a; Sanmartín *et al.*, 2008), coupled with advances in species-tree estimation (Edwards *et al.*, 2007; Heled & Drummond, 2009; Kubatko *et al.*, 2009), allow hypotheses of evolutionary history and biogeography to be tested with greater rigour than in the past. Parametric biogeographical methods represent a major advance over nonparametric analyses. This is especially true of cladistic analyses focused on determining relationships between areas using taxon cladograms (Platnick & Nelson, 1978; Brooks, 1981, 1990), which rely on vicariance as the primary explanation of common patterns. In particular, the dispersal–extinction–cladogenesis (DEC) model (Ree *et al.*, 2005; Ree & Smith, 2008a), which models dispersal and extinction along branches of a phylogeny and estimates ranges of ancestral and descendant species at each node, can be used to test hypotheses of areas of origin of clades, geographical species formation scenarios, and dispersal routes, using all available geological and historical information for a region. We apply this method, along with several methods for species-tree estimation, to a clade of salamanders endemic to Nuclear Central America, for which we have complete species-level sampling, to examine patterns of diversification and biogeography within the region.

Salamanders assigned to species of *Dendrotriton*, and the superficially similar members of *Cryptotriton* and *Nototriton*, were long included in the genus *Chiropterotriton*, until the revisionary study of Wake & Elias (1983) restricted *Chiropterotriton* to Mexico west of the Isthmus of Tehuantepec. Molecular phylogenetic analyses with limited taxonomic sampling from these genera have shown that *Dendrotriton*, *Nototriton* and *Cryptotriton* are not a monophyletic group, and are not closely related to *Chiropterotriton* (Wiens *et al.*, 2007). The diminutive body size of these genera, along with a relatively conserved external morphology that has been hypothesized to be the result of specialization for life in arboreal (particularly bromeliad) microhabitats (Wake, 1987), made systematics of the group difficult. Wake & Elias (1983) erected the genus *Dendrotriton* for the five then-recognized species, using a combination of external morphological features and osteological characters of the skull and limbs.

No molecular phylogenetic analysis has included more than two species of *Dendrotriton*, and relationships among the species of the genus, including the three described or discovered since publication of previous phylogenetic estimates for the genus (Lynch & Wake, 1975; Collins-Rainboth & Buth, 1990), remain largely unknown. We here present a new phylogenetic hypothesis for the genus based on analyses of multi-locus DNA sequence data for all known species, and use this phylogeny to reconstruct the biogeographical history of the genus.

MATERIALS AND METHODS

Study system, sample collection and DNA sequencing

Dendrotriton megarhinus (Rabb, 1960) and *D. xolocalcae* (Taylor, 1941) are known from small areas of cloud forest above 2050 m elevation in the Sierra Madre de Chiapas, Mexico (Fig. 1). *Dendrotriton bromeliacius* (Schmidt, 1936) is known only from the slopes of Volcán Tajumulco (1900–2700 m elevation) (Wake & Lynch, 1976), and *D. rabbi* (Lynch & Wake, 1975) only from the Montañas de Cuilco (2100–3000 m elevation) (Lynch & Wake, 1975), both in northwestern Guatemala. *Dendrotriton cuchumatanus* (Lynch & Wake, 1975) is known from high-elevation (2860 m) oak forest in the Sierra de los Cuchumatanes, an uplifted limestone plateau of Mesozoic sediments (Marshall, 2007) and Atlantic drainage in north-western Guatemala. Two recently described species, *D. chujorum* and *D. kekchiorum* (Campbell *et al.*, 2010), both previously referred to *D. rabbi* (Elias, 1984), are known from high elevations (> 2000 m) in various parts of the
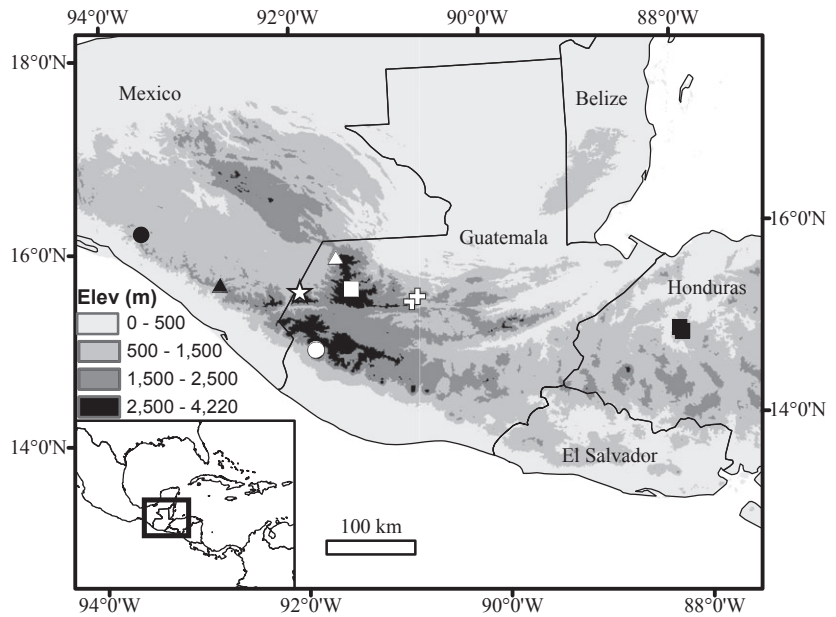
Figure 1 Distribution of species of *Dendrotriton* in Nuclear Central America. Black circle, *D. megarhinus*; black triangle, *D. xolocalcae*; white star, *D. rabbi*; white circle, *D. bromeliacius*; white triangle, *D. chujorum*; white square, *D. cuchumatanus*; cross, *D. kekchiorum*; black square, *D. sanctibarbarus*. Localities represent the entire known range of these species. Cloud forest habitat generally occurs above 1500 m elevation. North American Albers Equal-Area Conic projection used.

Sierra de los Cuchumatanes. *Dendrotriton sanctibarbarus* (McCranie & Wilson, 1997; Wake, 1998) is known only from montane forests of the isolated Montaña de Santa Barbara (1829–2744 m elevation) in Honduras (McCranie & Wilson, 1997), nearly 300 km south-east of its geographically closest congener. All species of *Dendrotriton* except *D. cuchumatanus* are primarily or exclusively bromeliad inhabitants (Taylor, 1941; Rabb, 1960; Lynch & Wake, 1975; Wake & Lynch, 1976; McCranie & Wilson, 1997). Specimens of all species except *D. cuchumatanus* and *D. kekchiorum* were collected between 2005 and 2009, and liver tissue was stored in liquid nitrogen. Existing frozen or ethanol-preserved tissues from museum collections were used for *D. cuchumatanus* and *D. kekchiorum*. Our sampling includes nearly all known localities for the genus *Dendrotriton*, and two to three individuals per species (Table 1) when multiple tissues were available. Only a single tissue sample was available for *D. rabbi* and *D. kekchiorum*, despite repeated attempts to collect additional samples.

Genomic DNA was extracted using either a Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA) or a guanidine thiocyanate extraction protocol (available from S.M.R. upon request). Portions of five genes (two mitochondrial and three nuclear) were sequenced: large subunit ribosomal RNA (16S, 520 bp), cytochrome *b* (cyt *b*, 798 bp), proopiomelanocortin (POMC, 484 bp) (Vieites *et al.*, 2007), recombination-activating gene 1 (RAG1, 749 bp) (San Mauro *et al.*, 2004), and solute carrier family 8 member 3 (SLC8A3, 789 bp) (Roelants *et al.*, 2007). Primer sequences and polymerase chain reaction (PCR) conditions are provided in Appendix S1 in the Supporting Information. PCR products were purified with 1 μL of 1:5 diluted ExoSAP-IT (USB Corporation, Cleveland, OH), cycle-sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730 DNA Analyzer automated sequencer. Sequences were edited using Sequen-

cher 4.7 (GeneCodes, Ann Arbor, MI, USA). GenBank accession numbers for all sequences are given in Table 1.

Phylogenetic analyses

Sequences were aligned using MUSCLE 3.6 (Edgar, 2004). Alignment length and number of variable sites for each gene are given in Appendix S2. Individual gene trees were constructed using both maximum likelihood (ML) and Bayesian phylogenetic analyses. Mitochondrial (mt) genes 16S and cyt *b* were analysed as a single locus for all phylogenetic analyses, with separate partitions for 16S, and for cyt *b* codon positions 1, 2 and 3. Nuclear genes were not partitioned because of the relatively low number of variable sites per locus. The program MRMODELTEST 2.2 (Nylander, 2004) was used to select substitution models for phylogenetic analyses. The following models were chosen: 16S–GTR + G; cyt *b* codon position 1–SYM + G; cyt *b* codon position 2–HKY + I; cyt *b* codon position 3–GTR + G; combined mtDNA–GTR + I + G; POMC–HKY; RAG1–K80 + I; SLC8A3–GTR + I. The likelihood ratio test in the program TOPALI 2.5 (Milne *et al.*, 2009) was used to test for intralocus recombination, which was detected only for SLC8A3. The largest non-recombining segment (730 bp) of SLC8A3 was used for all subsequent phylogenetic analyses.

Both ML and Bayesian phylogenetic analyses were performed to estimate gene trees for individual loci, as well as for a concatenated data set of all loci partitioned by gene (and by codon position for cyt *b*). ML analyses were performed using the program RAxML 7.0.4 (Stamatakis, 2006) with the GTR + I + G (mtDNA, SLC8A3, all data combined) or GTR + G (POMC, RAG1) substitution model. RAxML implements only GTR models, and substitution models for ML analyses were chosen to match those chosen by MRMODELTEST as closely as possible. One thousand bootstrap replicates were

Table 1 Locality information and GenBank accession numbers for *Dendrotriton* and outgroups used in the phylogenetic analyses.

Species	Specimen	Locality	16S	cyt *b*	POMC	RAG1	SLC8A3
D. bromeliacius	MVZ 263579, 263580	Guatemala: San Marcos: above El Rincon	JN560058, JN560059	JN559990, JN559991	JN560007, JN560008	JN560024, JN560025	JN560041, JN560042
D. chujorum	MVZ 265354, 265355	Guatemala: Huehuetenango: 19.1 km NW San Mateo Ixtatán	JN560060, JN560061	JN559992, JN559993	JN560009, JN560010	JN560026, JN560027	JN560043, JN560044
D. cuchumatanus	MVZ 231360, 231361	Guatemala: Huehuetenango: Montaña Madron, Sierra de los Cuchumatanes	JN560062, JN560063	JN559994, JN559995	JN560011, JN560012	JN560028, JN560029	JN560045, JN560046
D. kekchiorum	UTA-A 51086	Guatemala: Quiché: Road from El Chimel to San Pablo el Baldio	JN560064	JN559996	JN560013	JN560030	JN560047
D. megarhinus	MVZ 264179, 264181	Mexico: Chiapas: Cerro Tres Picos	JN560066, JN560065	JN559998, JN559997	JN560015, JN560014	JN560032, JN560031	JN560049, JN560048
D. rabbi	MVZ 263587	Guatemala: Huehuetenango: La Cumbre, Montañas de Cuilco	JN560067	JN559999	JN560016	JN560033	JN560050
D. sanctibarbarus	MVZ 263842, 263843, 263850	Honduras: Santa Barbara: San Luis de los Planes	JN560068, JN560069, JN560070	JN560000, JN560002, JN560001	JN560017, JN560019, JN560018	JN560034, JN560036, JN560035	JN560051, JN560053, JN560052
D. xolocalcae	ECOSUR AMA2489, MVZ 264219	Mexico: Chiapas: Campamento El Triunfo	JN560071, JN560072	JN560003, JN560004	JN560020, JN560021	JN560037, JN560038	JN560054, JN560055
Nyctanolis pernix	MVZ 263972	Guatemala: San José Maxbal, Huehuetenango	JN560074	JN560006	JN560023	JN560040	JN560057
Oedipina nica	MVZ 263774	Nicaragua: Jinotega: El Gobiado, Datanlí	JN560073	JN560005	JN560022	JN560039	JN560056

Gene abbreviations: 16S, large subunit ribosomal RNA; cyt *b*, cytochrome *b*; POMC, proopiomelanocortin; RAG1, recombination-activating gene 1; SLC8A3, solute carrier family 8 member 3. Museum codes: MVZ, Museum of Vertebrate Zoology, Berkeley, CA; UTA, University of Texas at Arlington, TX; ECOSUR, El Colegio de la Frontera Sur, San Cristobal de las Casas, Chiapas, Mexico.

conducted for each ML analysis in order to assess nodal support. Bayesian phylogenetic analyses were run for 50 million generations and sampled every 1000 generations, with four chains (one cold, three heated). Two separate runs were conducted for each analysis, and the cumulative, slide and compare plots in the program AWTY (Nylander *et al.*, 2008) were used to assess convergence after discarding the first 10,000 samples as burn-in. A sample of *Nyctanolis pernix*, the sister taxon of *Dendrotriton* in a previous molecular phylogenetic analysis (Wiens *et al.*, 2007), was also included in the data set. *Oedipina nica* was used as the outgroup for all phylogenetic analyses, based on the sister-group relationship of the *Dendrotriton–Nyctanolis* and *Oedipina–Nototriton–Bradytriton* clades from Wiens *et al.* (2007).

Species-tree estimation

Although the topology of each individual gene tree depends on the structure of the species tree, the stochastic nature of the coalescent means that the topology of gene trees may not match that of the species tree (Maddison, 1997; Rosenberg & Nordborg, 2002). The mismatch between gene trees and species trees can result from several factors, including introgression through hybridization (Doyle, 1992) and retention of ancestral polymorphism (deep coalescence) (Avise *et al.*, 1983; Pamilo & Nei, 1988; Maddison, 1997). Methods have been developed to estimate species trees while accounting for both hybridization and incomplete lineage sorting (Kubatko, 2009); this scenario, however, seems unlikely in *Dendrotriton*, given that all known species are allopatric and there are no known cases of hybridization in the genus. Retention of ancestral polymorphism can cause incongruence between gene and species trees, especially when internal branches are short or population sizes are large (Degnan & Rosenberg, 2005). Concatenation of multiple loci into a single data matrix (Rokas *et al.*, 2003) has commonly been used to infer species trees, but this approach fails to account for coalescent stochasticity and can produce misleading results (Kubatko & Degnan, 2007). Coalescent-based approaches that take advantage of the information available from multiple, independent loci have recently been developed (Liu, 2008; Heled & Drummond, 2009; Kubatko *et al.*, 2009). In addition, the species tree can be estimated with fewer loci under a coalescent approach than are required when using concatenation (Edwards *et al.*, 2007). In this study, we used several methods to infer the underlying species trees based on multi-locus sequence data. All species trees were estimated based on genotypic data to facilitate combining haploid mtDNA and diploid nuclear loci.

The program BEST 2.3.1 (Bayesian estimation of species trees; Liu, 2008) uses a hierarchical Bayesian Markov chain Monte Carlo (MCMC) approach to estimate a species tree (Edwards *et al.*, 2007; Liu & Pearl, 2007). BEST first uses an approximate prior distribution of gene trees to estimate the posterior probability of each gene tree given the sequence data within the program MRBAYES (Huelsenbeck & Ronquist,

2001). A second step uses a coalescent model to estimate the posterior probability of a species tree given each gene tree. Finally, samples are combined across loci to estimate the posterior distribution of the species tree, and importance sampling is used to correct for the use of the approximate gene tree prior in the first step (Liu, 2008). The MCMC analysis was run for 250 million generations with two separate runs of two chains each, using limits of 0.003–3 for the theta prior and 0.05–4 for the gene mutation rate prior based on results from preliminary BEST runs. The first 100,000 samples were discarded as burn-in, and the standard deviation of split frequencies was used to assess convergence. The program TRACER 1.5 (Rambaut & Drummond, 2007) was used to examine effective sample size (ESS) values for analysis parameters. In addition, multiple paired runs were conducted, and the resulting topologies compared to ensure that the analysis had converged on the correct species tree topology.

The program *BEAST 1.5.4 (Heled & Drummond, 2009) is an extension of the program BEAST (Drummond & Rambaut, 2007), and uses a multispecies coalescent model to estimate the posterior distribution of the species-tree topology, species divergence times and population sizes using a Bayesian MCMC approach. Multiple samples per species are necessary for estimation of population sizes, and the use of only a single individual per species may affect the estimated timing of species divergences as well (Heled & Drummond, 2009). We included at least two individuals for all species for which multiple tissue samples were available. The MCMC analysis was run for 1×10^9 generations, with sampling every 10,000 generations and the first 10,000 samples discarded as burn-in. Substitution models chosen by MRMODELTEST 2.2 (listed above) were used for each locus and a Yule prior was used for the species tree.

The program STEM 2.0 (Kubatko *et al.*, 2009) uses a ML approach to estimate species trees from multiple gene trees under a coalescent model. Unlike BEST and *BEAST, STEM requires gene trees as input files, and loci used in the analysis must obey the molecular clock. The program BEAST 1.5.4 was used to estimate gene trees. Each gene tree was estimated using a MCMC of 1×10^9 generations, sampled every 10,000 generations, with substitution models chosen by MRMODELTEST 2.2 (given above) and a strict clock model. In addition to gene trees, STEM requires an estimate of the rate of evolution of each locus as well as an overall theta value (Kubatko *et al.*, 2009). Relative rates of evolution were estimated by calculating the average GTR divergence from the outgroup (*Oedipina nica*) using the program PAUP* 4.0 (Swofford, 2003), and a theta value of 0.01 was used.

Divergence dating

The lack of any tropical bolitoglossine salamanders in the fossil record makes dating divergences based on molecular sequence data difficult. We attempted to use fossil calibration points from temperate salamander genera to make a rough estimate of divergence times within the genus. Wiens (2007) used

penalized likelihood (PL) analysis (Sanderson, 2002) of a RAG1 data set with 11 fossil calibration points to estimate divergence dates within the Plethodontidae. We conducted a BEAST analysis using the same set of calibration points and RAG1 sequences used by Wiens (2007), with the addition of the RAG1 sequences for *Dendrotriton*, *N. pernix* and *O. nica*. Following Wiens (2007), we fixed the root age of the tree to 210 Ma. All other calibration points were given uniform priors from the minimum age of the split to 210 Ma. Sequence data were partitioned by codon position, with a GTR + G substitution model used for each partition, and the uncorrelated relaxed lognormal clock method (Drummond *et al.*, 2006) was used. The analysis was run for 50 million generations, sampled every 1000 generations, and the first 5000 samples were discarded as burn-in. This method was used only to estimate the time to the most recent common ancestor (TMRCA) of *Dendrotriton*, given that the relationships in the RAG1 gene tree between species may not reflect the actual pattern of species divergence within the genus.

Geographical range evolution

Recent developments in historical biogeography that model the evolution of geographical ranges on a phylogenetic tree allow hypotheses about ancestral ranges and species range evolution to be tested with greater rigour than in the past (Ree & Sanmartín, 2009). The dispersal–extinction–cladogenesis (DEC) model (Ree *et al.*, 2005; Ree & Smith, 2008a) implemented in the program LAGRANGE 2.0 (Ree & Smith, 2008b) uses a ML approach analogous to models of character evolution to estimate the ancestral ranges of species along a phylogeny. The model requires that the set of areas that species can inhabit be defined in advance, and uses a matrix of areas inhabited by each species and a phylogeny as input parameters. At each node, species formation results in one descendant species inhabiting a single area and the other descendant species inheriting either the remainder or the entirety of the ancestral range. Along branches, range evolution can occur by dispersal to new areas or by extinction within an area of the range, with probabilities of dispersal and extinction estimated as part of the model (Ree & Smith, 2008a).

The species tree from the BEST analysis was used in all DEC analyses. Five areas were defined for the DEC analysis based on their status as geological units and separation from other mountainous areas by intervening lowland habitat: Sierra Madre de Chiapas (inhabited by *D. megarhinus* and *D. xolocalcae*), Montañas de Cuilco (containing *D. rabbi*), Sierra de los Cuchumatanes (inhabited by *D. cuchumatanus*, *D. chujorum* and *D. kekchiorum*), Guatemalan volcanic cordillera (inhabited by *D. bromeliacius*), and the Chortís highlands of Honduras (inhabited by *D. sanctibarbarus*). The Sierra Madre de Chiapas, Montañas de Cuilco and the Sierra de los Cuchumatanes are mountainous areas of the Mayan highlands geomorphological province and consist of ancient (Mesozoic) rock (Marshall, 2007). They are separated from each other by the deep valleys of the Cuilco and Selegua rivers, both

tributaries of the Río Grijalva and part of the Central American subhumid corridor (Stuart, 1954). The Guatemalan volcanic cordillera consists of Quaternary-age (< 2.6 Ma) volcanoes and is part of the Chortís volcanic front province, while the Chortís highlands of western Honduras consist of a dissected plateau of ancient bedrock that experienced significant uplift in the Miocene and Pliocene (between 10 and 3.8 Ma; Rogers *et al.*, 2002; Marshall, 2007).

The recent origin of the Guatemalan volcanic cordillera presumably precludes its inclusion in any ancestral species ranges before the Quaternary. For this reason, a time-stratified DEC model was constructed, in which the volcanic cordillera was excluded from the ancestral range of the root of *Dendrotriton*, and dispersal to or from this area was set to zero before the beginning of the Quaternary (2.6 Ma). The Motagua–Polochic fault system and associated valleys, which divide the Mayan highlands from the Chortís highlands (Fig. 2), are recognized as an important biogeographical barrier for many montane species (Stuart, 1954; Castoe *et al.*, 2009). While the Sierra de los Cuchumatanes is geographically closer to the Chortís highlands of Honduras where *D. sanctibarbarus* is found, a direct dispersal route between the two areas crosses the Motagua–Polochic fault system. Dispersal of a cloud-forest-dependent salamander such as *Dendrotriton* across the dry lowland Motagua Valley seems improbable, although it may have occurred in other cloud forest salamanders (*Cryptotriton* and *Nototriton*) that are found both north and south of the Motagua Valley. Lynch & Wake (1975) considered a trans-Motagua dispersal route for *Cryptotriton* to be unlikely, although palaeobotanical evidence from sites on the south side of the Motagua Valley suggests that the region may have been less arid during the Mio-Pliocene (Graham, 1998). Both *Cryptotriton* and *Nototriton* are found in eastern Guatemala north of the Motagua Valley, while *Dendrotriton* is not. A more probable dispersal route based on the current distribution of *Dendrotriton* species appears to be the Tertiary-age volcanic highlands of the Guatemalan plateau and southeastern Guatemala, included in the Western Rifted Highlands of the Chortís block (Marshall, 2007). All three Mayan highland areas in the DEC analysis are essentially contiguous with the Guatemalan plateau (Fig. 2), so no dispersal constraints were placed between the three Mayan highland areas and the Chortís highlands prior to 2.6 Ma. Although dispersal through the Guatemalan plateau and Tertiary highlands seems more likely based on the ecological requirements of *Dendrotriton*, the model does not explicitly prohibit dispersal across the Motagua Valley, given that only areas connected by dispersal routes are specified, rather than the actual dispersal routes themselves. During the Quaternary, the Guatemalan volcanic cordillera became available for colonization. Given that this cordillera presents a more direct dispersal route to the Chortís highlands of Honduras, two models were run: one in which all dispersal to and from the Chortís highlands passed through the Guatemelan volcanic cordillera, and a second in which dispersal was allowed between all areas during the Quaternary. The analysis was first run with a root age for
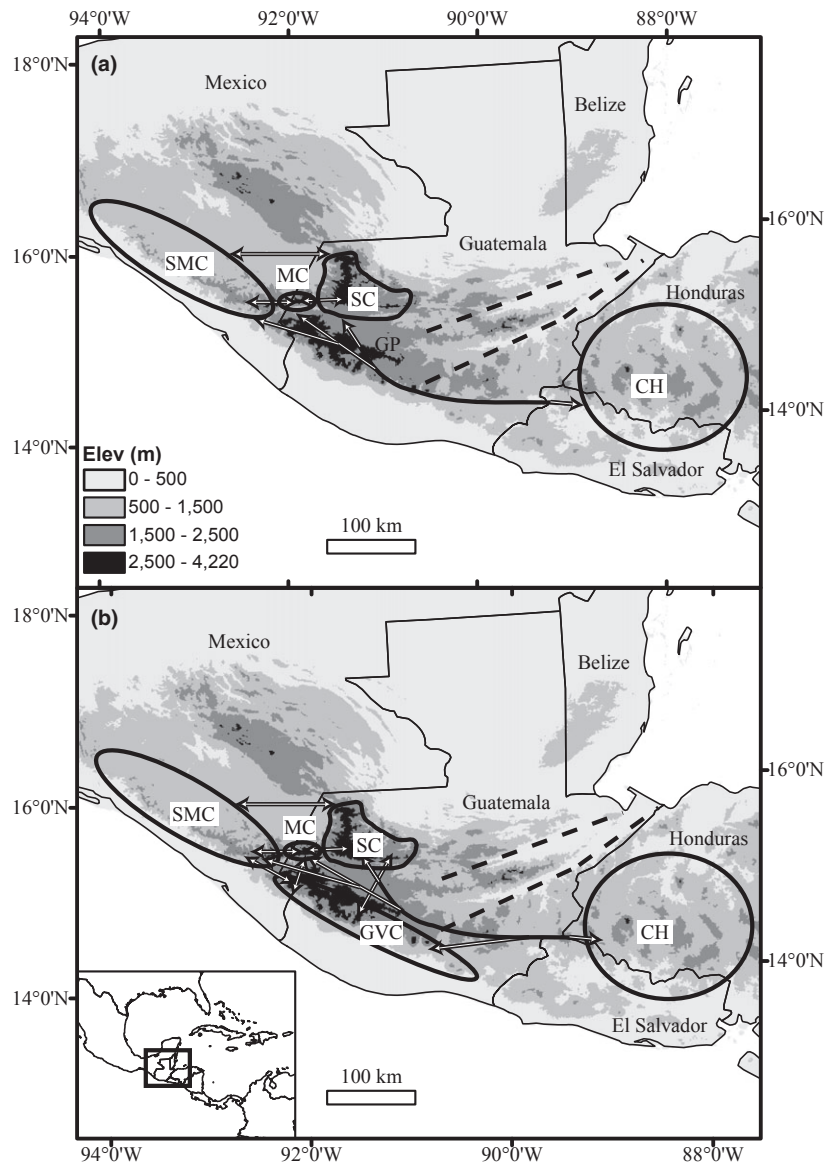
Figure 2 Areas and dispersal routes used in dispersal–extinction–cladogenesis (DEC) analysis of the geographical range evolution of *Dendrotriton* in Nuclear Central America. (a) Available areas and dispersal routes during the Tertiary (> 2.6 Ma), before formation of the Guatemalan volcanic cordillera. (b) Areas and dispersal routes during the Quaternary. Areas: SMC, Sierra Madre de Chiapas; MC, Montañas de Cuilco; SC, Sierra de los Cuchumatanes; CH, Chortís highlands; GVC, Guatemalan volcanic cordillera. Arrows indicate allowed dispersal between areas. Dotted lines represent the Motagua–Polochic fault zone. The Guatemalan plateau (GP) represents a possible highland dispersal route before the uplift of the GVC. North American Albers Equal-Area Conic projection used.

Dendrotriton of 16 Ma (see divergence dating results), but was repeated with root ages of 5, 10 and 25 Ma to reflect the large uncertainty in molecular dates. Ancestral species were allowed to have ranges consisting of a maximum of two areas, allowing only slightly broader distributions than are observed at present.

RESULTS

Species-tree estimates

The species tree estimated by BEST contains two major clades (Fig. 3). The first clade [posterior probability (PP) = 1.0] contains the three species from the Sierra de los Cuchumatanes; of these, *D. kekchiorum* and *D. cuchumatanus* are sister species (PP = 0.57). The other clade (PP = 1.0) contains all remaining species, with *D. sanctibarbarus* as sister to the species from Chiapas and north-western Guatemala (PP = 1.0).

Dendrotriton bromeliacius and *D. rabbi* are estimated to be sister taxa (PP = 1.0), and *D. xolocalcae* is estimated to be the sister taxon to both these two species (PP = 0.92; Fig. 3). The *BEAST tree has the same topology as the BEST species tree. Within the Cuchumatanes clade (PP = 1.0), the sister species of *D. cuchumatanus* is estimated to be *D. kekchiorum* (PP = 0.98). The other major clade (PP = 0.87) contains a subclade (PP = 0.98) of *D. bromeliacius*, *D. megarhinus*, *D. rabbi* and *D. xolocalcae*. As in the BEST results, *D. bromeliacius* and *D. rabbi* are sister species (PP = 1.0), with *D. xolocalcae* as their sister taxon. The ML estimate of the species tree (ln*L* = −1483.10) from STEM differs from the *BEAST tree in placing *D. megarhinus* as the sister species of *D. xolocalcae*, rather than as sister to (*D. bromeliacius* + *D. rabbi*); STEM also differs by placing *D. sanctibarbarus* as the sister to the Cuchumatanes clade (Fig. 3). The full search results from STEM, however, include 14 trees, the consensus of which conflicts with the *BEAST results only in resolving
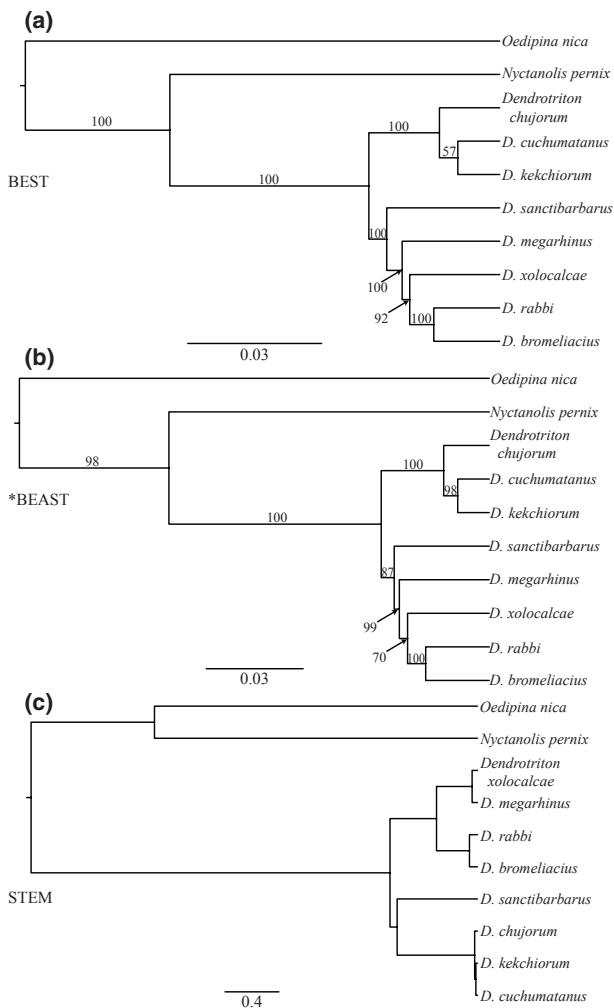
(a)

BEST

(b)

*BEAST

(c)

STEM

Figure 3 Multi-locus species-tree estimates for *Dendrotriton* from Nuclear Central America from (a) BEST, (b) *BEAST and (c) STEM. Numbers on branches represent posterior probabilities of clades (multiplied by 100). Branch lengths are measured in the number of substitutions per site.

D. megarhinus and *D. xolocalcae* as sister taxa. Species-tree estimates closely resemble, but are not identical to, the mtDNA gene-tree estimate (Fig. 4a): two major clades are present in both, and *D. sanctibarbarus* is sister to the remaining species in the larger clade. Results from both *BEAST and BEST, as well as analyses of individual gene trees, strongly support the monophyly of *Dendrotriton* (Figs 3 & 4). The topology of the tree from both ML and Bayesian analyses of the concatenated data set of all loci (results not shown) matches that of the mtDNA gene tree; this result is not surprising, given that the mtDNA data have many more variable sites than the nuclear data (Appendix S2).

Divergence dating estimates

The BEAST analysis of RAG1 sequence data estimated the TMRCA of *Dendrotriton* to be 16.4 ± 0.1 Ma, and the divergence between *Dendrotriton* and (*Nyctanolis* + *Oedipina*)

to be 57.6 ± 0.37 Ma. Our estimate of the timing of divergence between *Batrachoseps* and *Bolitoglossa* (77.8 ± 0.44 Ma) differs from that of Wiens (2007) (48.5 Ma), but closely matches that of Vieites *et al.* (2007), who used a multi-locus data set and multiple fossil calibration points to estimate the divergence between *Bolitoglossa* and *Batrachoseps* at 73 ± 11 Ma. Given the discrepancy between the age of the TMRCA of *Dendrotriton* and *Oedipina* from Wiens (2007) (roughly 37 Ma) and that from our study (57.6 Ma), we conducted all analyses of geographical range evolution using a number of ages of *Dendrotriton* (5, 10, 16 and 25 Ma) in order to reduce the dependence of the results on any single divergence date estimate.

Geographical range evolution

The DEC model results obtained using a root age of 16 Ma with dispersal between all areas during the Quaternary suggest that the Sierra Madre de Chiapas and the Sierra de los Cuchumatanes formed the ancestral range of *Dendrotriton*. The three most likely range reconstructions for the root node (Fig. 5) contain only these two areas, and, of the seven reconstructions within 2 log-likelihood units (2ln*L*) of the maximum for this node, all contain either the Sierra de los Cuchumatanes or the Sierra Madre de Chiapas within the ancestral range and only three contain a different area (Chortís highlands of Honduras). Both internal nodes in the clade containing *D. cuchumatanus*, *D. chujorum* and *D. kekchiorum* are reconstructed unambiguously with the Sierra de los Cuchumatanes as the range of the ancestor and both descendant species (Fig. 5). Under the four most likely of the seven root-node reconstructions (total relative probability of 0.61), this would imply that an ancestor of the Cuchumatanes clade (with a range limited to the Sierra de los Cuchumatanes in three of the four reconstructions) underwent divergence, resulting in two descendant species, one of which subsequently diverged into two species within the Cuchumatanes. Under the three other root-node range reconstructions within 2ln*L* of the maximum for this node (total relative probability = 0.15), the ancestor of the Cuchumatanes clade originated either in the Sierra Madre de Chiapas or in the Chortís highlands, implying a subsequent dispersal to the Cuchumatanes. The most likely reconstruction for the ancestral node of the other major clade in the phylogeny (that excluding the Cuchumatanes species) probably involved species formation within the Sierra Madre de Chiapas: three of the four reconstructions within 2ln*L* of the maximum involved either the Sierra Madre de Chiapas or the Chortís highlands (the fourth involved species formation within the Chortís highlands). The current range of *D. sanctibarbarus* within the Chortís highlands would imply dispersal from the Sierra Madre de Chiapas subsequent to this species formation event under the most likely reconstruction, while three of the other reconstructions involved species formation from an ancestral range including the Chortís highlands. The range of the ancestor of *D. megarhinus*, *D. xolocalcae*, *D. rabbi* and *D. bromeliacius* was unambiguously reconstructed as
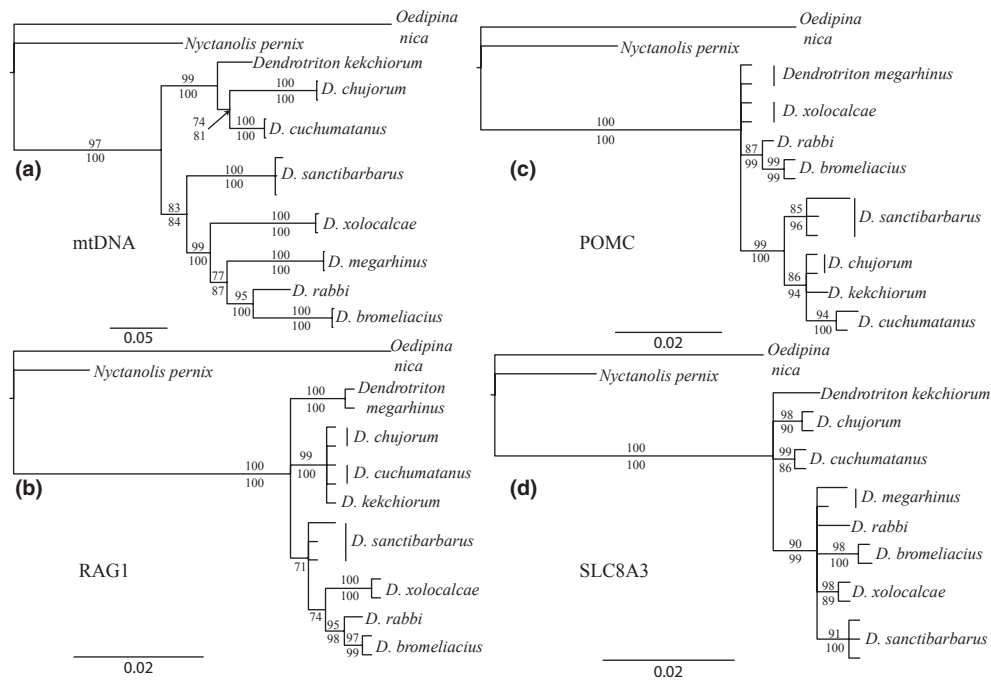
Figure 4 Gene trees of *Dendrotriton* species from Nuclear Central America from Bayesian analyses of (a) combined mtDNA (16S + cytochrome *b*); (b) recombination-activating gene 1, RAG1; (c) proopiomelanocortin, POMC; and (d) solute carrier family 8 member 3, SLC8A3. Numbers above branches are bootstrap support values from maximum likelihood analyses, and numbers below branches are posterior probabilities (multiplied by 100) from Bayesian analyses. Support values below 50 are not shown. Branch lengths are measured in the number of substitutions per site.

containing only the Sierra Madre de Chiapas, with the divergence between *D. megarhinus* and the other three species occurring in the Sierra Madre de Chiapas (Fig. 5). Similarly, the divergence of *D. xolocalcae* (from *D. rabbi* + *D. bromeliacius*) occurred within the Sierra Madre de Chiapas. Finally, the divergence between *D. rabbi* and *D. bromeliacius* occurred either within the Sierra Madre de Chiapas (most likely reconstruction), or within the Montañas de Cuilco, or between the Sierra Madre and the Montañas de Cuilco, and from an ancestor distributed either in the Sierra Madre, or in the Montañas de Cuilco, or in both (Fig. 5). Given that none of the ancestral range reconstructions for *D. bromeliacius* includes the Guatemalan volcanic cordillera, this result implies a dispersal event for *D. bromeliacius* to the volcanic cordillera subsequent to its origin in another area.

These results were robust to the variation in root age of *Dendrotriton* to ages between 10 and 25 Ma, and results were similar overall with a root age of 5 Ma. When the root age of *Dendrotriton* was set to 5 Ma, all ancestral reconstructions of the root node contained the Sierra de los Cuchumatanes, but two of the four most likely reconstructions did not contain the Sierra Madre de Chiapas, and one contained the Montañas de Cuilco. Furthermore, the range of the ancestor of the non-Cuchumatanes species excluding *D. sanctibarbarus* included the Montañas de Cuilco in one of the two most likely reconstructions, in contrast to when older root ages were used. Although the modelled unavailability of the Guatemalan volcanic cordillera to colonization prior to 2.6 Ma affects the

inclusion of this area in any range reconstruction, this result is robust to changes in the root age of the tree, including a root age of only 5 Ma. The model restricting dispersal to/from the Chortís highlands to occur only through the Guatemalan volcanic cordillera during the Quaternary produced the same most likely scenarios, in addition to a number of other scenarios of low relative probability for four nodes. The results for the two models were nearly identical when a root age of 10–25 Ma was used, and differences between models do not affect conclusions about range evolution within the genus in any substantive way.

DISCUSSION

Lungless salamanders (family Plethodontidae) are an exception to the generalization that diversity arises in the tropics and spreads to temperate regions (Wiens & Donoghue, 2004; Jablonski *et al.*, 2006; Wiens, 2007). One plethodontid clade (Bolitoglossini) differs from all other salamanders [nine other families and eight other plethodontid clades, all north-temperate in distribution (Wake, 1970; Duellman, 1999)] in that it entered the tropics relatively late in the evolutionary history of salamanders (Wiens, 2007) and underwent a radiation represented by nearly 300 species, 45% of all salamander species diversity (AmphibiaWeb, 2011). Investigations into the geography of species formation in tropical salamanders provide insights into the factors that have promoted high tropical species diversity in a group of
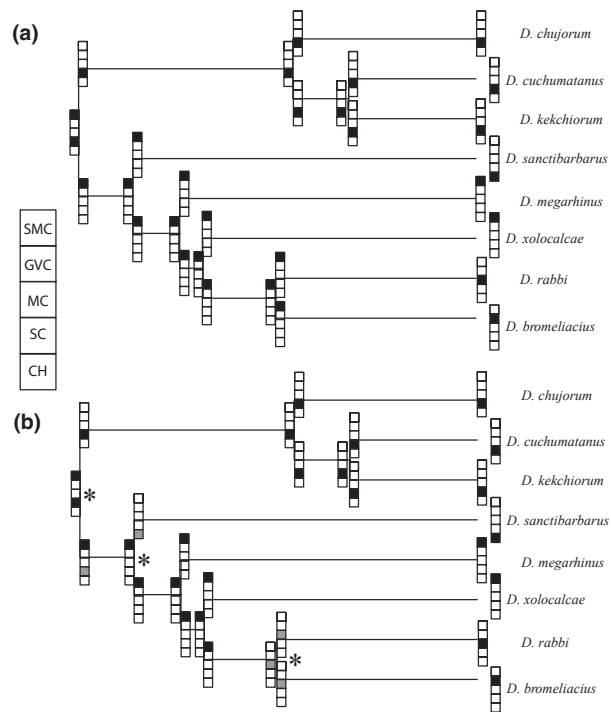
Figure 5 Maximum likelihood estimates of geographical range evolution for *Dendrotriton* from Nuclear Central America from Lagrange. (a) Most likely range reconstruction at each node, from the model with dispersal allowed between all areas in the Quaternary and a root age of 16 Ma. Black squares indicate geographical areas estimated as part of a species' range, with an ancestor's range on the left side of each node and the ranges of descendant species on the right side. (b) Second most likely reconstruction at each node. Inhabited areas not shared with the most likely range reconstruction are shown in grey. Asterisks indicate three nodes with additional range reconstructions within 2 log-likelihood units of maximum. Areas: SMC, Sierra Madre de Chiapas; MC, Montañas de Cuilco; SC, Sierra de los Cuchumatanes; CH, Chortís highlands; GVC, Guatemalan volcanic cordillera.

temperate origin, thus shedding light on the origins of latitudinal species diversity gradients (reviewed in Mittelbach *et al.*, 2007) in general. This is particularly true for bolitoglossine salamanders, given that most, like many tropical species, are habitat specialists with small geographical distributions (Wake & Lynch, 1976; Wake, 1987; Stevens, 1989). Our robust phylogenetic hypothesis for the genus provides the foundation for testing hypotheses of the geography of species formation in the geographically complex region of Nuclear Central America.

The species-tree estimates from our analyses of multi-locus sequence data are consistent in placing all species from the Sierra de los Cuchumatanes in one clade and all non-Cuchumatanes species in another clade. Results from the two Bayesian methods also correspond in placing *D. sanctibarbarus* as the most basal species in the non-Cuchumatanes clade, and in finding that *D. bromeliacius* and *D. rabbi* are sister species. The consensus tree from the stem search for all trees of high

likelihood only conflicts with the Bayesian results in placing *D. megarhinus* and *D. xolocalcae* as sister taxa.

Our species-tree estimates conflict with all previous morphological phylogenetic hypotheses for *Dendrotriton*, although previous estimates included only five of the eight currently recognized species. Lynch & Wake (1975) formulated the first phylogenetic hypothesis for the genus (then the *bromeliacius* group of *Chiropterotriton*) based on 11 morphological characteristics, and were able to separate all species in the genus based on morphology. Using Wagner parsimony and weighted invariant step strategy (WISS) methods to infer relationships, they hypothesized three different topologies, depending on whether or not two osteological characters were included. They concluded that relationships within the group remained uncertain and that molecular analyses probably offered the best prospect for clarifying them in the future. Collins-Rainboth & Buth (1990) recoded and reanalysed Lynch and Wake's data set using parsimony to produce a different topology. Wilkinson (1997) showed that differences in phylogenetic hypotheses between the two studies were largely the result of different methods of character coding and weighting, and randomization tests indicated that the morphological data set contained only weak phylogenetic signal.

In all three hypotheses of Lynch & Wake (1975) and that of Collins-Rainboth & Buth (1990), *D. cuchumatanus* is included in a clade with non-Cuchumatanes species, while in our species-tree estimates *D. cuchumatanus* and the other two species from the Sierra de los Cuchumatanes always form a clade separate from those in other areas. The closest previous phylogenetic hypothesis to our estimate was that of Lynch & Wake (1975) from Wagner parsimony and WISS analyses based on characters excluding osteological features, which conflicts with our *beast species tree only in placing *D. xolocalcae* as the sister species of *D. cuchumatanus*, rather than of the other three non-Cuchumatanes taxa.

Interestingly, the most likely range inheritance scenario for all nodes involved even division of the ancestral range into two single-area descendant ranges, rather than one descendant species inheriting an entire two-area range and the other inheriting a single area. The unambiguous reconstruction of divergence events within a single ancestral area (such as those within the Sierra de los Cuchumatanes) does not necessarily imply that such a divergence was the result of sympatric speciation (Ree *et al.*, 2005), because the areas used in the analysis (particularly the Sierra de los Cuchumatanes and the Sierra Madre de Chiapas) are large and topographically complex and could easily accommodate multiple, allopatric populations of an ancestral species. Thus, species formation in the group appears to be primarily allopatric, rather than through the isolation and divergence of peripheral populations (which would result in uneven range inheritance). Furthermore, *D. bromeliacius*, a member of the cloud forest community at one of the most diverse salamander sites in the Neotropics on Volcán Tajumulco (Wake & Lynch, 1976; Wake *et al.*, 1992), appears to have dispersed to the volcanic cordillera from the Sierra Madre of Chiapas or the Montañas

de Cuilco, rather than having originated *in situ* in the volcanic cordillera.

Given that even a narrowly endemic species to the volcanic cordillera such as *D. bromeliacius* appears to have originated elsewhere, this result supports the hypothesis that this rich community of tropical salamanders (several of which are endemic to a small region of Guatemala and Mexico) may have been assembled from species that originated in other, older highland areas (Wake & Lynch, 1982). Wake & Lynch (1982) hypothesized a Miocene origin of the *Bolitoglossa lincolni–franklini* species complex, which has a similar distribution to *Dendrotriton* in Guatemala and Mexico, in the ancient highlands of western Guatemala and Chiapas. Under their historical scenario, *B. franklini* colonized the Guatemalan volcanic cordillera from the Sierra Madre de Chiapas in the Pleistocene, and diverged morphologically on the Pleistocene volcanoes. The authors hypothesized that *B. lincolni* also colonized the Guatemalan volcanic cordillera during the Pleistocene from the Montañas de Cuilco. Under this scenario, another component of the volcanic cordillera salamander fauna originated in an older highland area and subsequently dispersed to the volcanic cordillera. Elias (1984) hypothesized that repeated fragmentation and reconnection of small highland areas on the volcanoes resulting from climatic fluctuations may have generated the high species diversity on Volcán Tajumulco, one of the Pleistocene volcanoes in the Guatemalan volcanic cordillera. Additional phylogenetic data from populations of other salamanders distributed in the volcanic cordillera (particularly *Pseudoeurycea* and *Bolitoglossa*, which have multispecies assemblages on the slopes of the volcano) will be critical to distinguish between these hypotheses.

The Sierra de los Cuchumatanes appears to have been an important area for the diversification of *Dendrotriton*, because of its probable inclusion within the ancestral range of the genus and because of subsequent divergence and species formation within the mountain range. This is consistent with the idea that the range has been biogeographically important for many taxa (Stuart, 1943a,b; Elias, 1984). The fact that *N. pernix*, the closest relative of *Dendrotriton* (Wiens *et al.*, 2007), is also found in the Sierra de los Cuchumatanes supports the inclusion of this area in the ancestral range of *Dendrotriton*. Stuart (1943a,b) hypothesized that the herpetofauna of the Sierra de los Cuchumatanes had strong affinities with that of the Guatemalan plateau, while Elias (1984) showed that the Sierra de los Cuchumatanes had several species in common with both the Montañas de Cuilco and the volcanic cordillera, but a deeper evolutionary history than either of the latter areas. Elias (1984) treated the salamander fauna of the Montañas de Cuilco and that of the Sierra de los Cuchumatanes as closely related, but our results show a closer relationship between species of *Dendrotriton* in the Montañas de Cuilco and the volcanic cordillera. While the Montañas de Cuilco have received comparatively little attention from biologists, they may form an important link between the ancient uplifted mountains of Nuclear Central America and the Pacific volcanoes, as hypothesized by Wake & Lynch (1982).

The importance of the Sierra de los Cuchumatanes in the biogeographical history of *Dendrotriton* results, at least in part, from the two recently described species from this mountain range (Campbell *et al.*, 2010). These species were previously considered to be part of *D. rabbi* (Elias, 1984); the inclusion of only two species in the Cuchumatanes, one of which was shared with the Montañas de Cuilco, would probably have produced a substantially different scenario of range evolution. This result highlights not only the importance of complete taxonomic sampling in phylogenetic estimates, but also the impact of undiscovered species or populations on biogeographical estimates.

The results of phylogenetic analyses of montane pitvipers (Castoe *et al.*, 2009) and multiple snake lineages (Daza *et al.*, 2010) show the Motagua Valley to have been an important biogeographical barrier, causing near-simultaneous divergence between and species formation of taxa on either side. Although the distribution of pitviper species in Castoe *et al.*'s (2009) study only generally approximates that of *Dendrotriton*, their phylogenetic estimates for the genera *Bothriechis* and *Cerrophidion* show species of *Bothriechis* and lineages of *Cerrophidion godmani* distributed in the Guatemalan volcanic cordillera and the Sierra de los Cuchumatanes to be more closely related to each other than to the corresponding taxa in the Chortís highlands. This result differs from our results, although the trans-Motagua Valley divergence between *D. sanctibarbarus* and the other non-Cuchumatanes *Dendrotriton* is the most basal split in its clade. The most likely range reconstruction for *Dendrotriton* postulates a dispersal event, rather than vicariance, from the Mayan highlands to the Chortís highlands, although fragmentation of an ancestral range of this clade containing the Chortís highlands is included within the set of likely range reconstructions. Unfortunately, the lack of appropriate calibration points for tropical salamanders prevents a direct comparison of divergence dates between *Dendrotriton* species and those available from time-calibrated phylogenies of Middle American snakes (Castoe *et al.*, 2009; Daza *et al.*, 2010). The inclusion of other montane salamander groups (*Cryptotriton*, *Nototriton*, *Bolitoglossa* subgenus *Magnadigita*) with trans-Motaguan distributions in future biogeographical analyses will clarify the importance of the Motagua Valley to divergence and species formation.

Our results show the utility of including reconstructions of ancestral ranges, in addition to present patterns of distribution, for testing the importance of biogeographical barriers in species formation. Major geological and topographical features, such as the division between the Sierra de los Cuchumatanes and other highland areas of north-western Guatemala, appear to have been important in promoting species formation within *Dendrotriton*. Other examples of species formation in the genus occurred within individual montane regions, however, suggesting that large-scale geological divisions or climatic features are not the only factors that cause divergence of these salamanders. Ancestral range reconstructions of species of *Dendrotriton* also demonstrate that areas of high species diversity, such as the Guatemalan volcanic cordillera,

do not necessarily correspond to areas of origin of the taxa currently found there, underscoring the importance of biogeographical history for understanding current patterns of species diversity. Our results also illustrate the dynamic history of species formation and range evolution over relatively small spatial scales within Nuclear Central America that could be missed when examining species diversification at a broader regional scale. Both phylogeographical studies of widely distributed upland species and systematic studies of groups such as *Dendrotriton* distributed in several montane areas in the region will serve to clarify the predominant biogeographical patterns within the Nuclear Central America region.

Species formation in *Dendrotriton* has been vicariant in nature, with no evidence of peripatric, parapatric or sympatric divergence. The diversification we have reported has taken place within, rather than between, individual montane areas, indicating that divergence occurs across very small spatial scales. Given the narrow elevational ranges that are typical of tropical organisms (Janzen, 1967; Ghalambor *et al.*, 2006), these results resemble expectations from a species pump mechanism operating within older montane blocks with complex topography (Savage, 2002), with subsequent dispersal to geologically younger areas. Such a mechanism operating within (rather than across) small montane areas, coupled with occasional dispersal between montane areas, could explain the build-up of species diversity within tropical regions, particularly for organisms of low vagility. Montane species pumps have been proposed for birds at a larger spatial scale in other tropical regions (Fjeldså & Rahbek, 2006; Sedano & Burns, 2010). The fragmentary nature of cloud forest habitat (Luna-Vega *et al.*, 1999) and the narrow range of climatic conditions under which it occurs (Stadtmüller, 1987) suggest that population vicariance caused by past climatic changes is an important mechanism behind the high endemism of cloud forest taxa. In addition to illustrating the generality of the biogeographical patterns seen for these salamanders, comparison with other taxa possessing greater dispersal abilities, such as birds, would allow the generality of such a species pump mechanism to be tested within montane areas.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Polymerase chain reaction conditions and primer sequences for loci sequenced in this study.

Appendix S2 Alignment length, number of variable/parsimony-informative sites, and gaps for loci used in phylogenetic analyses of *Dendrotriton*.

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BIOSKETCH

The authors share an interest in the diversification, species formation and conservation of Neotropical amphibians, particularly salamanders in Mexico and Central America. They are also interested in using amphibians as a model system for understanding the historical biogeography of Mesoamerica.

Author contributions: S.M.R. and D.B.W. designed the research; S.M.R., T.J.P., G.P-O, A.M-A. and C.R.V-A. collected the data; S.M.R. analysed the data; S.M.R., D.B.W. and G.P-O led the writing.

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