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Phylogeography and lineage-specific patterns of genetic diversity and molecular evolution in a group of North American skinks

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Geography influences the evolutionary trajectory of species by mediating opportunities for hybridization, gene flow, demographic shifts and adaptation. We sought to understand how geography and introgression can generate species-specific patterns of genetic diversity by examining phylogeographical relationships in the North American skink species *Plestiodon multivirgatus* and *P. tetragrammus* (Squamata: Scincidae). Using a multilocus dataset (three mitochondrial genes, four nuclear genes; a total of 3455 bp) we discovered mito-nuclear discordance, consistent with mtDNA introgression. We further tested for evidence of species-wide mtDNA introgression by using comparisons of genetic diversity, selection tests and extended Bayesian skyline analyses. Our findings suggest that *P. multivirgatus* acquired its mitochondrial genome from *P. tetragrammus* after their initial divergence. This putative species-wide mitochondrial capture was further evidenced by statistically indistinguishable substitution rates between mtDNA and nDNA in *P. multivirgatus*. This rate discrepancy was observed in *P. multivirgatus* but not *P. tetragrammus*, which has important implications for studies that combine mtDNA and nDNA sequences when inferring time since divergence between taxa. Our findings suggest that by facilitating opportunities for interspecific introgression, geography can alter the course of molecular evolution between recently diverged lineages. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: Edwards Plateau – lizard – mitochondrial introgression – mito-nuclear discordance – phylogeography – *Plestiodon*.

INTRODUCTION

The geographical features of a landscape can play an important role in structuring patterns of diversification in terrestrial organisms (Bermingham & Moritz, 1998; Arbogast & Kenagy, 2001; Swenson & Howard, 2005). For example, landscapes that lack significant barriers to biotic interchange can facilitate high rates of gene flow among populations, effectively limiting opportunities for diversification (Hewitt, 2001; Manel *et al.*, 2003). In contrast, geographical features can be barriers to migration, limiting gene flow and promoting divergence between lineages (Hewitt, 2001; Manel *et al.*, 2003). Following speciation, the landscape may further influence evolutionary trajectories by enabling or preventing interspecific gene flow between sister species (Hewitt, 2001; Bryson *et al.*, 2010). Thus, landscapes can impact genetic diversity at multiple evolutionary tiers by generating opportunities for demographic shifts, adaptation and hybridization (Saetre *et al.*, 2001; Donnelly *et al.*, 2004; Toews & Brelsford, 2012).

Geography can have multiple impacts on the molecular evolution of recently diverged lineages. First, limited gene flow at the geographical range edge can lead to lower genetic diversity at the geographical range periphery (Blows & Hoffmann, 2005;

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Trumbo *et al.*, 2013). This can be exacerbated during a rapid geographical range expansion, where allelic variants can increase in frequency and move with the new range front whether they are deleterious, neutral or advantageous (Excoffier, Foll & Petit, 2009), and can lead to the stochastic depletion of genetic variation at the geographical range front (Makowsky, Chesser & Rissler, 2009; Mattoccia *et al.*, 2011; Streicher *et al.*, 2012). Second, rapid adaptive evolution and selective sweeps during geographical expansion can also lead to the loss of genetic diversity (Galtier & Duret, 2007; Irwin, Rubtsov & Panov, 2009). Finally, mitochondrial introgression in the geographical contact zones between recently divergent lineages can create mitonuclear discordance and molecular evolution dynamics (Jiggins, 2003; Bachtrog *et al.*, 2006; Gompert *et al.*, 2008; Wiens, Kuczynski & Stephens, 2010; Toews & Brelsford, 2012; Boratynski *et al.*, 2014; Jones & Searle, 2015). Introgression can also lead to the uncoupling of mtDNA and nDNA substitution rates because of divergent evolutionary history in different geographical areas and even lead to an increase in the mutation rate in the mitochondria as it adapts to the new host nuclear genome (Linnen & Farrell, 2007; McGuire *et al.*, 2007; Bryson *et al.*, 2010). Examining variation in molecular evolution among geographically delimited lineages can help explain how geography and evolutionary relationships can shape intraspecific patterns of genetic diversity.

We studied phylogeography and molecular evolution of taxa in south-western North America, focusing on Texas and northern Mexico. One of the most important geographical features in this landscape is the Balcones Escarpment of the Edwards Plateau, which is located in central Texas (see Fig. 1) and can act as both an important barrier to gene flow and as a foster of endemism (e.g. cotton rats: Andersen & Light, 2012; snakes: Castoe, Spencer & Parkinson, 2007; salamanders: Chippindale *et al.*, 2000; fish: Edwards, Garrett & Allan, 2004; pocket mice: Neiswenter & Riddle, 2010; cave crickets: Taylor *et al.*, 2007). This geographical feature provides an excellent opportunity to characterize patterns of

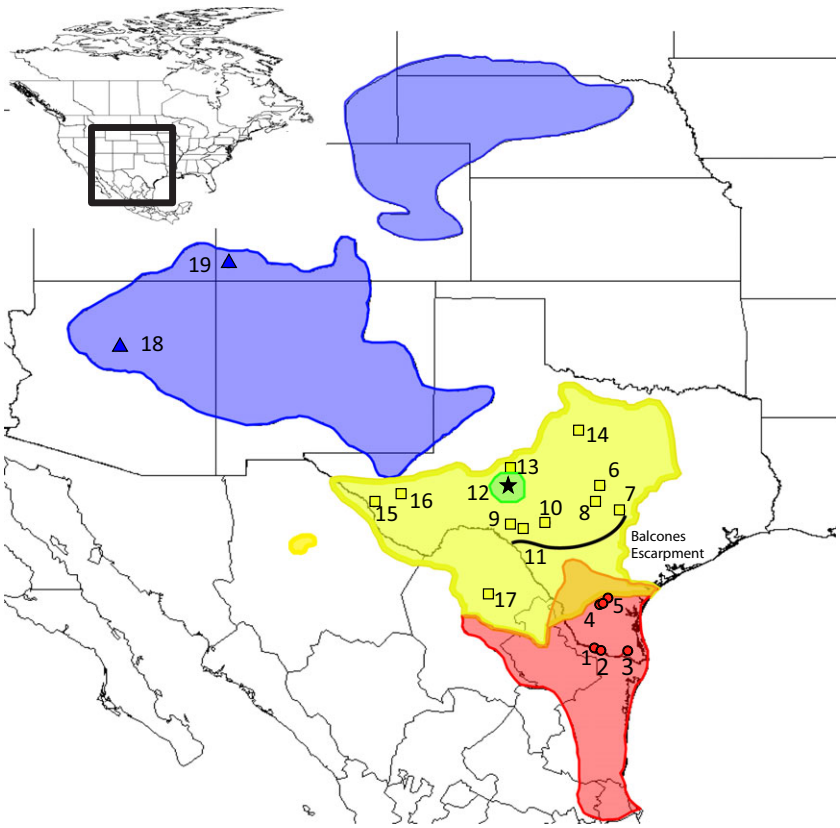

Figure 1. Geographical distribution of *P. t. tetragrammus* (red), *P. t. brevilineatus* (yellow) and *P. multivirgatus* (blue) based on Dixon (2013), IUCN Redlist (2007) and Lieb (1985). Green indicates range overlap between *P. t. brevilineatus* and *P. multivirgatus*. Orange indicates overlap between *P. t. tetragrammus* and *P. t. brevilineatus*. Symbols and numbers indicate collection localities used in the study. Circles: *P. t. tetragrammus*; squares: *P. t. brevilineatus*; triangles: *P. multivirgatus*. Star indicates locality where both *P. t. brevilineatus* and *P. multivirgatus* were sampled together.

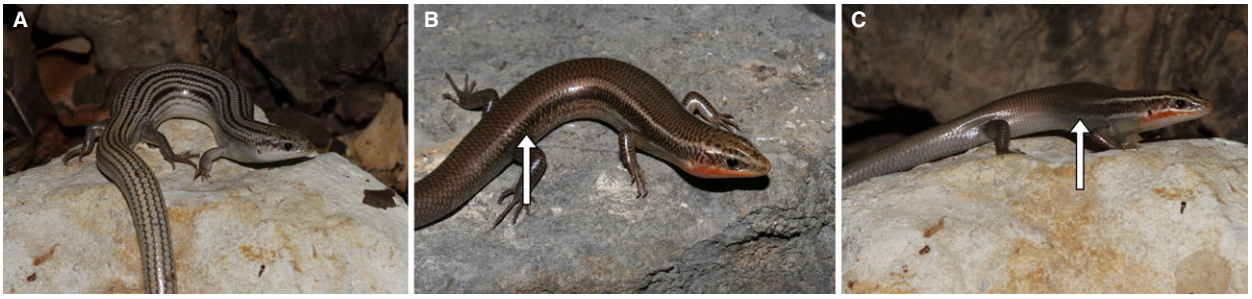

Figure 2. External morphology of *P. multivirgatus* (A), *P. t. tetragrammus* (B) and *P. t. brevilineatus* (C). Arrows indicate the termination of the lateral light and dark stripes in *P. t. tetragrammus* and *P. t. brevilineatus*, which is the character used to distinguish these two subspecies.

molecular evolution in lineages separated by a potential barrier to gene flow.

Our research focuses on the four-lined skink, *Plestiodon tetragrammus* Baird, 1859, and the many-lined skink, *Plestiodon multivirgatus* Hallowell, 1857, which are sister taxa (Brandley *et al.*, 2012) and are distributed in south-western North America. The many-lined skink is endemic to the United States (but see unconfirmed Mexican record, Smith & Taylor, 1950) and is found in Arizona, Colorado, New Mexico, Nebraska, South Dakota, Utah, Wyoming and Texas. A disjunct population of *P. multivirgatus* occurs in west-central Texas (Fig. 1). Two subspecies of the many lined skink, the northern many-lined skink (*P. m. multivirgatus*) and the variable skink (*P. m. epipleurotus*), have been named and some authors have suggested that these be recognized as separate species (Hammerson, 1999), although this is not widely accepted (Crother, 2012). The four-lined skink occurs in the United States and Mexico and is currently separated into two geographically distinctive subspecies (Lieb, 1985). The four-lined skink, *P. t. tetragrammus*, is found in Texas south of the Balcones Escarpment down the Mexican coast to northern Veracruz. The short-lined skink, *P. t. brevilineatus* Cope (1880), is distributed north and west of the Balcones Escarpment of central Texas into northern Mexico, and both western and northern Texas. Beyond geographical distribution, these two subspecies can be distinguished based on external morphology (Fig. 2), as the lateral stripes terminate at the forelimb in *P. t. brevilineatus* and extend to the hindlimb in *P. t. tetragrammus* (Lieb, 1985).

We analysed how genetic divergence of populations across the geographical landscape impacts patterns of molecular evolution among populations of *P. multivirgatus* and *P. tetragrammus*. While the utility of subspecies in evolutionary biology is debatable (Burbrink, Lawson & Slowinski, 2000; Phillimore & Owens, 2006), and genetically distinct subspecies are often elevated to species (Burbrink, 2001; Streicher *et al.*, 2012), we treated *P. t. brevilineatus* and *P. t. tetragrammus* as potentially distinct lineages given their parapatric distributions and distinct morphologies. Using nuclear and mitochondrial loci, we reconstructed phylogenetic relationships of both subspecies of *P. tetragrammus* and southern populations of *P. multivirgatus* and analysed levels of genetic variation and substitution rates in each species. We hypothesized that geographical distribution would shape patterns of genetic diversity and molecular evolution among lineages, and that any introgression between lineages would decouple mitochondrial and nuclear evolution among lineages.

METHODS

Taxon and tissue collection

Skinks were collected by turning rocks and cover objects in appropriate habitat across their geographical range from 2008 to 2013 (Fig. 1). We preserved muscle, liver or skin tissue in lysis buffer, 95% ethanol or an RNA-preserving buffer. Specimens were deposited in the University of Texas Arlington Amphibian and Reptile Diversity Research Center. We obtained additional tissues from the Texas Museum of Natural History (TNHC collection) at the University of Texas Austin (Table 1). We used a hierarchical outgroup strategy to root our analyses, with multiple other *Plestiodon* species (*Plestiodon japonicus*, *P. septentrionalis*, *P. fasciatus*, *P. anthracinus*, *P. obsoletus* and *P. inexpectatus*), a more distantly related skink (*Scincella lateralis*) and a lizard species from another lineage (family Gerrhosauridae; *Gerrhosaurus major*) as the most distant outgroup.

DNA isolation, PCR and sequencing

DNA was isolated using Qiagen DNeasy (Qiagen) kits following standard protocols. We amplified and sequenced three mitochondrial genes, totalling 1579 bp: a fragment of NADH dehydrogenase subunit 1 and flanking tRNAs (481 bp), 16S ribosomal RNA

Table 1. Samples used in this study with locality information and GenBank accession numbers

Locale	Voucher	Taxon	State	County	c-mos	BDNF	SNCAIP	PRLR	ND1	12S	16S
12	CLC750	PTB	TX	Crockett	KT326054	KT326031	KT326130	KT326097	KT325937	KT325979	KT326000
12	CLC751	PTB	TX	Crockett	KT326063	KT326032	–	KT326098	KT325938	KT325980	KT326001
17	JAC26325	PTB	MX:COAH	–	KT326064	KT326033	KT326131	KT326099	KT325939	KT325982	KT326002
17	JAC26395	PTB	MX:COAH	–	KT326065	KT326034	KT326132	KT326100	KT325940	KT325983	KT326003
14	JWS 057	PTB	TX	Stephens	KT326066	KT326035	KT326133	KT326101	KT325941	KT325984	–
9	TNHC61293	PTB	TX	ValVerde	KT326075	KT326044	KT326139	KT326110	KT325950	KT325963	KT326012
9	TNHC61360	PTB	TX	ValVerde	KT326076	KT326045	KT326140	KT326111	KT325951	KT325964	KT326013
11	TNHC53241	PTB	TX	Real	KT326073	KT326042	KT326138	KT326108	KT325948	KT325961	KT326010
6	TNHC53240	PTB	TX	SanSaba	KT326072	KT326041	KT326137	KT326107	KT325947	–	KT326009
8	TNHC65483	PTB	TX	Travis	KT326077	KT326046	KT326141	KT326112	KT325952	KT325965	KT326014
10	TNHC64025	PTB	TX	Edwards	KT326082	KT326050	KT326146	KT326117	KT325956	KT325970	KT326019
15	TNHC67003	PTB	TX	Presidio	–	KT326047	KT326142	KT326113	KT325953	KT325966	KT326015
15	TNHC67348	PTB	TX	Presidio	KT326078	KT326048	KT326143	KT326114	–	KT325967	KT326016
15	TNHCGDC14027	PTB	TX	Presidio	KT326081	KT326051	KT326147	KT326118	KT325957	KT325959	KT326020
7	TNHC60585	PTB	TX	Llano	KT326074	KT326043	KT326121	KT326109	KT325949	KT325962	KT326011
13	TNHC84900	PTB	TX	Sterling	KT326079	–	KT326144	KT326115	KT325954	KT325968	KT326017
16	TJL643	PTB	TX	Jeff Davis	KT326071	KT326040	KT326136	KT326106	KT325946	KT325960	KT326008
1	CLC372	PTT	TX	Hidalgo	KT326055	KT326021	KT326120	KT326084	KT325927	KT325981	KT325989
4	CLC625	PTT	TX	Duval	KT326056	KT326022	–	KT326085	KT325928	KT325971	KT325991
5	CLC651	PTT	TX	Duval	KT326057	KT326023	KT326122	KT326086	KT325929	KT325972	KT325992
5	CLC652	PTT	TX	Duval	KT326058	KT326024	KT326123	KT326087	KT325930	KT325973	KT325993
5	CLC695	PTT	TX	Duval	KT326052	KT326025	KT326124	KT326088	KT325931	KT325974	KT325994
2	JWS236	PTT	TX	Starr	KT326067	KT326036	KT326134	KT326102	KT325942	KT325985	KT326004
2	JWS238	PTT	TX	Starr	KT326068	KT326037	–	KT326103	KT325943	KT325986	KT326005
3	JWS244	PTT	TX	Cameron	KT326069	KT326038	–	KT326104	KT325944	KT325987	KT326006
3	JWS245	PTT	TX	Cameron	KT326070	KT326039	KT326135	KT326105	KT325945	KT325988	KT326007
12	CLC745	PM	TX	Crockett	KT326059	KT326026	KT326125	KT326089	KT325932	KT325975	KT325995
12	CLC746	PM	TX	Crockett	KT326053	KT326027	KT326126	KT326090(1)	KT325933	KT325976	KT325996
12	CLC747	PM	TX	Crockett	KT326060	KT326028	KT326127	KT326092(3)	KT325934	KT325977	KT325997
12	CLC748	PM	TX	Crockett	KT326061	KT326029	KT326128	KT326094(5)	KT325935	–	KT325998
12	CLC749	PM	TX	Crockett	KT326062	KT326030	KT326129	KT326096	KT325936	KT325978	KT325999
19	ADL274	PM	CO	Montezuma	–	HM160645.1	HM161309.1	HM160932.1	HM160836.1	–	–
18	TNHCFS05485	PM	AZ	Coconino	KT326080	KT326049	KT326145	KT326116	KT325955	KT325969	KT326018
	CLC298	*Plestiodon obsoletus*	OK	Beckham	–	–	KT326119	KT326083	KT325926	KT325958	KT325990
		P. fasciatus			AY217869.1	HM160622.1	HM161286.1	HM160909.1	AY315600.1	AY315505.1	AY315554.1
		P. septentrionalis			–	HM160655.1	HM161319.1	HM160942.1	HM160847.1	AY308363.1	AY308214.1
		P. inexpectatus			AY217888.1	HM160626.1	HM161290.1	HM160913.1	HM160817.1	AY308353.1	AY308204.1
		P. anthracinus			–	HM160596.1	HM161260.1	HM160883.1	HM160787.1	JF497943.1	JF498071.1
		P. japonicus			–	HM160627.1	HM161291.1	HM160914.1	HM160818.1	EU202993.1	EU203034.1

Table 1. *Continued*

Locale	Voucher	Taxon	State	County	c-mos	BDNF	SNCAIP	PRLR	ND1	12S	16S
		Scincella lateralis			AY217857.1	HM160667.1	HM161331.1	HM160954.1	AY649213.1	AY649131.1	AY649172.1
		Gerrhosaurus major			EU366459.1	HM160588.1	HM161254.1	HM160876.1	HM160779.1	AY167345.1	AY167362.1

Unless noted otherwise all samples from the United States. Numbers respond to geographic locations in Fig. 1.
TNHC, Texas natural history collection; CLC, Christian L. Cox field series; JWS, Jeffrey W. Streicher field series; TX, Texas, CO, Colorado; AZ, Arizona; MX, Mexico; COAH, Coahuila; PM, *Plestiodon multivirgatus*, PTB, *P. tetragrammus brevilineatus*; PTT, *P. t. tetragrammus*.

gene (462 bp) and 12S ribosomal RNA gene (634 bp). We amplified and sequenced four nuclear genes, totalling 1876 bp: synuclein alpha interacting protein (*SNCAIP*, 410 bp), prolactin-like receptor (*PRLR*, 564 bp), brain-derived neurotropin factor (*BDNF*, 547 bp) and oocyte maturation factor (*c-mos*, 352 bp). Primer information is given in Table 2. All mitochondrial loci were amplified with an initial 2 min of denaturation at 95 °C followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 50 °C and 1 min extension at 72 °C. Following these cycles, we performed a final 10 min extension at 72 °C. Nuclear loci were amplified with a touchdown procedure using an initial 2 min denaturation step at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 56–50°C and 1 min at 72 °C. Following these cycles, we used a final extension of 10 min at 72 °C. We used gel electrophoresis in 1% agarose to test amplification, and we cleaned PCR products for sequencing by treatment with the ExoSAP-IT kit (United States Biochemical). We used the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) for sequencing reactions with the sequenced products precipitated using an ethanol/sodium acetate/EDTA method and rehydrated in formamide (Hi-Di). Each sample was analysed on an ABI PRISM 3100xl Genetic Analyzer in the Genomics Core Facility at the University of Texas Arlington. We edited and assembled sequences using Sequencher (Genes Code Corp.). We obtained sequences for outgroup taxa from GenBank. Sequences were aligned using the ClustalW algorithm (Larkin *et al.*, 2007) implemented in MEGA v5.2 (Tamura *et al.*, 2011), using default parameters, visually inspected and trimmed, then concatenated by eye. Concatenated datasets were then partitioned by gene and codon position using sequences from Brandley *et al.* (2012). Individuals that were missing complete sequence data for any genes were removed from the analysis.

Phylogenetic reconstruction

We used MEGA v5.2 (Tamura *et al.*, 2011) to perform model testing on each gene partition. The GTR+I+G model ranked in the top three models for each partition based on Akaike information criterion (AIC) and Bayesian information criterion (BIC) scores, so we chose this model for the subsequent analyses. We used MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) with default priors to perform Bayesian tree searches. We ran multiple tree searches with varying priors and partition schemes without major changes to topology. We ran Markov chain Monte Carlo (MCMC) searches for 10 000 000 generations, sampling every 500 generations, using three heated chains and one cold chain and checked stationarity using parameter outputs in

Table 2. Primer information and sources of the initial primer sequence

Gene	Primer name	Sequence (5′–3′)	Source
12S	tPHE	AAAGCACRGCACTGAAGATGC	Wiens and Reeder (1997)
	12e	GTRCGCTTACCMTGTTACGACT	Wiens and Reeder (1997)
16S	16aR2	CCCGMCTGTTTACCAAAAACA	Schmitz *et al.* (2005)
	16d	CTCCGGTCTGAACTCAGATCACGT	Reeder (1995)
ND1	INTF3	ATAATRTGRTTYATYTCNACNCTAGCAGA	Leache and Reeder (2002)
	tMET	TCGGGGTATGGGCCCRARAGCTT	Brandley *et al.* (2005)
c-mos	FU-F	TTTGGTTCKGTCTACAAGGCTAC	Gamble *et al.* (2008)
	FU-R	AGGGAACATCCAAAGTCTCCAAT	Gamble *et al.* (2008)
BDNF	BDNF-F	GACCATCCTTTTCCTKACTATGGTTATTTCATACTT	Brandley *et al.* (2011)
	BDNF-R	CTATCTTCCCCTTTTAATGGTCAGTGTACAAAC	Brandley *et al.* (2011)
PRLR	PRLR_f1	GACARYGARGACCAGCAACTRATGCC	Brandley *et al.* (2011)
	PRLR_r3	GACYTTGTGRACTTCYACRTAATCCAT	Brandley *et al.* (2011)
SNCAIP	SNCAIP_f10	CGCCAGYTGYTGGGRAARGAWAT	Brandley *et al.* (2011)
	SNCAIP_r13	GGWGAYTTGAGDGCACTCTTRGGRCT	Brandley *et al.* (2011)

Mitochondrial genes: 12S, 12S ribosomal RNA; 16S, 16S ribosomal RNA; *ND1*, NADH dehydrogenase 1. Nuclear genes: *c-mos*, oocyte maturation factor; *BDNF*, brain derived neurotropin factor; *PRLR*, prolactin-like receptor; *SNCAIP*, synuclein alpha interacting protein.

MrBayes (Huelsenbeck & Ronquist, 2001) and the program TRACER v. 1.4 (Drummond & Rambaut, 2007). For this and all subsequent analyses we discarded 25% of the trees as burnin. For maximum-parsimony (MP) analysis we used MEGA v5.2 (Tamura *et al.*, 2011) for nuclear and mitochondrial alignments, with 10 000 bootstrap replicates for nodal support. We used RAxML v7.0.3 (Stamatakis, 2006) to conduct maximum-likelihood (ML) analysis using 100 ML tree searches and 10 000 bootstrap replicates on the best scoring topology to obtain nodal support values. These processes were completed separately for each concatenated mitochondrial and nuclear dataset. We then used *BEAST (Heled & Drummond, 2010) to perform coalescent species tree analysis on the combined mitochondrial and nuclear datasets using the same priors and MCMC criteria as the Bayesian tree searches. We then used DensiTree v2.1.10 (Bouckaert, 2010) to examine variation in the topologies obtained from the coalescent analysis. We used FigTree v1.3.1 (Rambaut, 2007) for tree visualization and manipulation.

Genetic diversity and testing for evidence of species-wide mitochondrial capture in sister taxa

We implemented a series of tests to compare patterns of nuclear and mitochondrial genetic variation. These tests were designed to identify mitochondrial and nuclear variation consistent with mitochondrial introgression and assume that *P. multivirgatus* and *P. tetragrammus* are sister taxa. We used the program MEGA v5.2 to generate average within-group distance measures (uncorrected 'p') and SYSTAT v11 (Systat Software) to conduct several non-parametric Mann–Whitney *U* tests (Mann & Whitney, 1947) of genetic diversity. We used all available sequences for each locus to calculate uncorrected 'p' distances.

First, we tested for deviation from a well-documented pattern of molecular evolution in animals: mtDNA evolves more rapidly than nDNA (Brown, George & Wilson, 1979; Eytan & Hellberg, 2010; Willett, 2012). To test this expectation, we compared mtDNA and nDNA genetic variation within *P. multivirgatus* and *P. tetragrammus*. If we failed to reject the null hypothesis of similar levels of intraspecific mtDNA and nDNA diversity we interpreted the test as evidence consistent with species-wide mtDNA introgression. Second, because sister taxa have been evolving for approximately the same amount of time, we expect that neutral (or nearly neutral) mutations will fix at similar rates. We tested this null expectation by comparing nDNA and mtDNA diversity between *P. mulitvirgatus* and *P. tetragrammus*. If this test rejected the null hypothesis of similar diversity levels between sister taxa for mtDNA but not for nDNA, we interpreted the test as consistent with species-wide mtDNA introgression. In this scenario we identified the taxon with lower levels of mtDNA diversity as the likely recipient of introgressed mtDNAs.

Testing for population size and neutral molecular evolution

Our genetic diversity comparisons of sister taxa assumed neutrally evolving loci and similar population sizes through time. To test these assumptions in

P. multivirgatus and *P. tetragrammus* we performed codon-based selection tests using the proportion of nonsynonymous subtitutions, d_N, and synonymous subtitutions, d_S (d_N/d_S) using MEGA v5.2. We examined translated amino acid sequences with both intra- and interspecific datasets (Table 4). Specifically, we used a Z-statistic to test the null hypothesis of strict neutrality ($d_N = d_S$) against a model of positive selection ($d_N > d_S$). We used 1000 bootstrap pseudoreplicates to generate Z-statistics and *P*-values in all tests.

We used loci that displayed d_N/d_S ratios consistent with neutral or purifying selection to perform Bayesian skyline analysis (Drummond *et al.*, 2005) and extended Bayesian skyline analysis (Heled & Drummond, 2008). We performed both skyline analyses using BEAST v1.7.5 (Drummond *et al.*, 2012) and restricted our analyses to nDNA loci given that, if present, species-wide mtDNA introgression may mislead our estimations of population demography. Our justification for these analyses was that, under a model of coalescence, demographic shifts should result in predictable patterns of nucleotide sequence divergence (Heled & Drummond, 2008). Based on simulations, the best strategies for estimating historical demography are achieved by sampling widely throughout the range of a species (Ho & Shapiro, 2011; Heller, Chikhi & Siegismund, 2013). Our sampling spanned large segments of the range of both species with samples of *P. multivirgatus* that originated from Arizona and Texas ($N = 6$) and samples of *P. tetragrammus* that originated from throughout Texas and Mexico ($N = 16$). A potential issue with our sampling was the small number of individuals of *P. multivirgatus*. We decided to estimate historical demographics from these samples because simulation studies have shown that the error rate of extended Bayesian skyline analysis is much more sensitive to the number of loci than individuals (Heled & Drummond, 2008). Specifically, error rates from simulations run on 3–20 individuals with constant population sizes all performed with < 20% relative error. Nonetheless, we acknowledge that demographic estimates we present for *P. multivirgatus* should be interpreted more cautiously than those we obtained for *P. tetragrammus*. In our extended Bayesian skyline analysis we focused on the population size change parameter (demographic.populationSizeChanges) to assess if variation was consistent with a constant population size through time. We ran tree searches for 10–50 million generations sampling every 1000 generations until the effective sample size of all parameters exceeded 200. Given the relatively conserved nature of nDNA sequences used in this study, we used a simple model of nucleotide evolution (HKY) and reduced the number of groups to 5 in Bayesian skyline analyses to avoid excessive parameterization. For the remaining parameters we used default settings in all BEAST v1.7.5 runs and summarized log files in TRACER v1.4 (Rambaut & Drummond, 2007).

RESULTS

Phylogenetic relationships

Although phylogenetic trees derived from mitochondrial and nuclear loci differed, we found identical topologies and similar branch lengths with all methods of phylogenetic reconstruction within each marker type, so we present phylogenetic relationships from Bayesian analysis with nodal support from Bayesian analyses (posterior probabilities) and ML and MP (bootstrap proportion). We found that *P. tetragrammus* and *P. multivirgatus* form a monophyletic group that is sister to a clade of *P. anthracinus*, *P. fasciatus* and *P. septentrionalis*, which is broadly consistent with larger phylogenetic studies of skinks (Brandley *et al.*, 2012).

Using phylogenetic reconstruction based on mitochondrial loci, we found that *P. t. tetragrammus* and *P. t. brevilineatus* were in separate, well-supported clades (Fig. 3). Moreover, there were two separate and well-supported clades within *P. t. brevilineatus* that corresponded to the eastern (northern and central Texas) and western (west Texas and the state of Coahuila) parts of their geographical range. Interestingly, *P. multivirgatus* mitochondrial haplotypes from across their geographical range (Texas–Arizona) were nested within *P. tetragrammus*, specifically *P. t. brevilineatus*.

Phylogenetic relationships based on nDNA between *P. multivirgatus*, *P. tetragrammus* and the other North American species were not well resolved. In contrast to the mitochondrial data, phylogenetic reconstructions using nuclear loci support a monophyletic *P. tetragrammus* (including *P. t. brevilineatus* and *P. t. tetragrammus*) while excluding *P. multivirgatus* (Fig. 3). Substructure within *P. tetragrammus* was neither consistent with subspecies designation nor geographically with clades that were resolved using mtDNA.

Coalescent species tree analysis with both mitochondrial and nuclear loci recovered *P. multivirgatus* and *P. tetragrammus* as monophyletic with strong statistical support (Fig. 4). Additionally, this analysis recovered *P. t. brevilineatus* and *P. t. tetragrammus* as reciprocally monophyletic and sister to *P. multivirgatus*. Although the sister relationship between *P. multivirgatus* and *P. tetragrammus* received limited statistical support (0.63; Fig. 4), it is topologically consistent with previous studies (Brandley

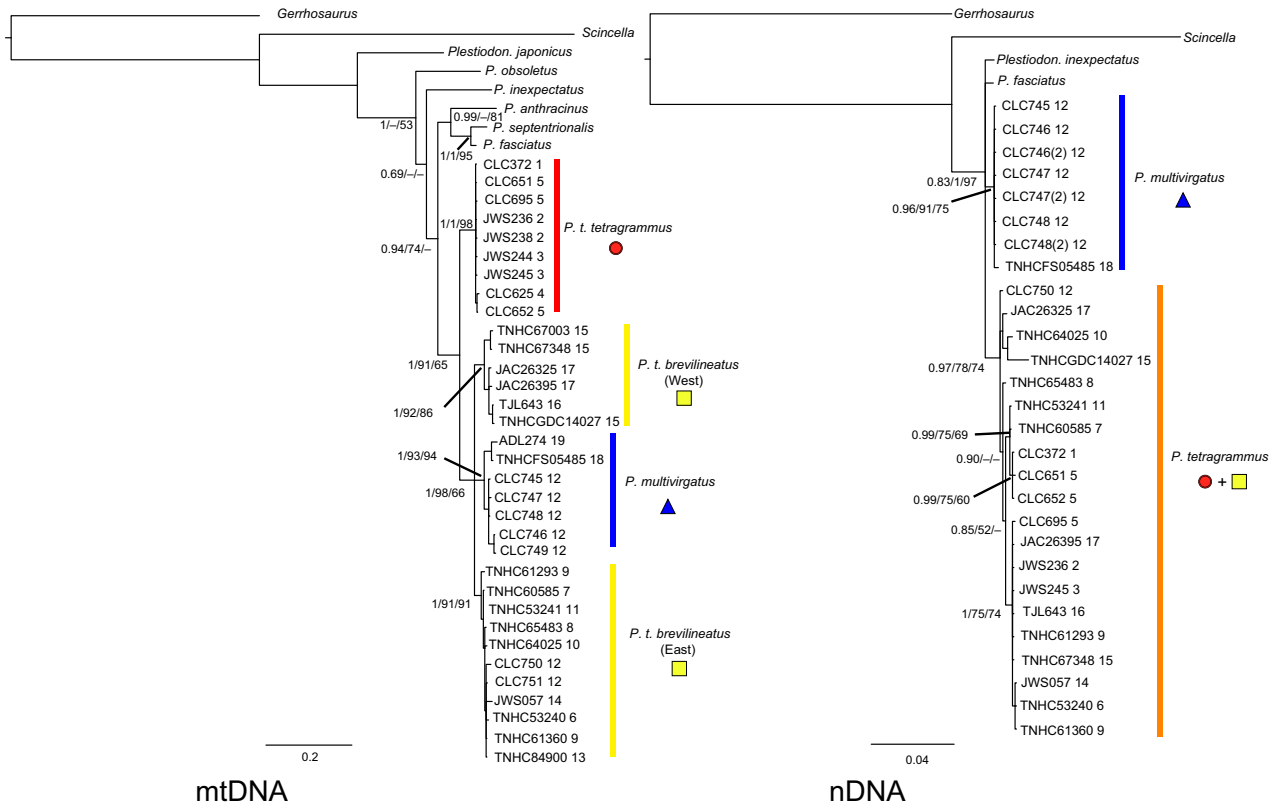

Figure 3. Phylogenetic reconstructions of relationships among specimens of *P. t. tetragrammus*, *P. t. brevilineatus* and *P. multivirgatus* based on mtDNA and nDNA. Trees are based on Bayesian analyses with nodal support values as Bayesian posterior probabilities, non-parametric bootstrap values using MP, and non-parametric bootstrap values using ML analysis, respectively. Symbols correspond to localities in Fig. 1.

et al., 2012). Our findings that mtDNA supports *P. multivirgatus* as nested within *P. t. brevilineatus*, and that this relationship is not supported by either phylogenetic analysis of concatenated nuclear loci or combined mitochondrial and nuclear loci in a coalescent framework, is consistent with mitochondrial introgression from *P. t. brevilineatus* into *P. multivirgatus* across its geographical range.

Mitochondrial and nuclear variation

We found that while *P. tetragrammus* had significantly different levels of mitochondrial and nuclear genetic variation (Mann–Whitney *U* test statistic = 9, $P = 0.046$), *P. multivirgatus* did not ($U = 7.5$, $P = 0.184$). This finding is consistent with a decoupling of mitochondrial and nuclear evolutionary rates in *P. multivirgatus* (Fig. 5).

Mann–Whitney *U* statistics indicated that levels of genetic variation of nuclear loci were not significantly different between *P. tetragrammus* and *P. multivirgatus* ($U = 3$, $P = 0.484$). This result matches our expectations for neutrally evolving sister taxa with similar population sizes. Mitochondrial levels of genetic variation, however, were significantly different ($U = 0$, $P = 0.050$), a result consistent with introgression. Although *P. t. brevilineatus* had higher levels of overall mitochondrial genetic variation than *P. multivirgatus* (Table 3), this difference was not statistically significant ($U = 3$, $P = 0.184$).

Evidence for neutral evolution and similar population sizes through time

All of our selection tests failed to reject the null model of neutral evolution (Table 4). Interestingly, the *SNCAIP* dataset of *P. multivirgatus* was the only test that received a *P*-value < 1. In these sequences a segregating single nucleotide polymorphism (SNP) produced the translation of glutamine (polar neutral side chain) in the Texas population and leucine (aliphatic hydrophobic side chain) in the Arizona population.

Bayesian skyline analysis revealed that collectively the four nDNA loci were more variable (inferred from the distribution of tree root heights) in

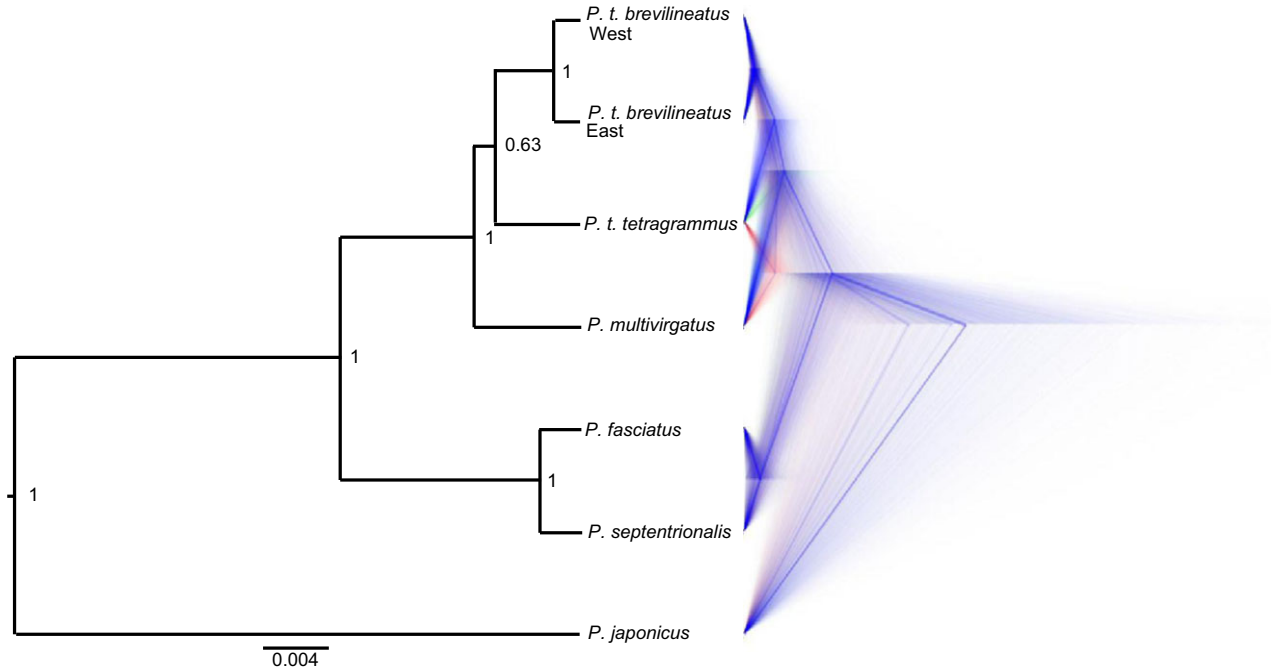

Figure 4. Topologies resulting from coalescent species tree analysis of combined mtDNA and nDNA data. Tree on left is a consensus topology with Bayesian posterior probabilities for nodal support. Tree on right is a DensiTree diagram of all trees recovered from coalescent analysis; the most frequently sampled topology is shown in blue with differing topologies shown in red and green revealing areas of uncertainty in the species tree topology.

P. tetragrammus than in *P. multivargatus* (Fig. 6A). The number of population size changes inferred by our extended Bayesian skyline analysis was most frequently estimated at 0 for *P. tetragrammus* and 1 for *P. multivirgatus* (Fig. 6B). However, 0 changes was the second-most frequently estimated state for *P. multivirgatus*. Thus, we cannot reject a history of constant population size for either species. A visual examination of the four nDNA alignments from *P. multivirgatus* revealed that the only polymorphisms were segregating sites between Arizona and Texas. In *PRLR*, there is a three-nucleotide indel (present in the Arizona population) and *BDNF* and *SNCAIP* each feature an SNP that differentiate the populations. These differences may account for the single demographic shift suggested by our extended Bayesian skyline plot analysis (Fig. 6B). Because nDNA sequences from *P. multivirgatus* featured less variation than *P. tetragrammus*, we scaled Bayesian skyline plots so that they were on the same timescale (Fig. 6C). As expected given intraspecific levels of nDNA sequence variation, median population size estimates from scaled Bayesian skyline plots suggest that *P. tetragrammus* has had larger population sizes through recent time than *P. multivirgatus*. However, the 95% HPD intervals for each species feature substantial overlap, which indicates that historical population sizes were probbaly similar. Collectively, selection tests and Bayesian skyline analyses produced evidence for (1) purifying or neutral selection on protein coding sequences and (2) similar population sizes through time.

DISCUSSION

Geographic patterns of genetic diversity

We found a well-supported phylogenetic break in the mtDNA dataset between individuals of *P. t. tetragrammus* and *P. t. brevilineatus*. This break is concordant with the Balcones Escarpment, suggesting this landscape feature may act as an obstacle to gene flow in *P. tetragrammus*. The impact of the Balcones Escarpment on lineage diversification has been found in snakes (Burbrink, 2002; Castoe *et al.*, 2007) and mammals (Riddle, 1995; Andersen & Light, 2012), but not anurans (Mulcahy & Mendelson, 2000; Lemmon *et al.*, 2007; Streicher *et al.*, 2012). Beyond geographical vicariance, it is also possible that these lineages have diverged ecologically. The Balcones Escarpment divides this region into a drier, xeric habitat to the north and west (the Edwards Plateau, Trans-Pecos desert and the mixed grass prairie of the Great Plains) and a more humid, mesic habitat to the south in the Tamaulipan thornscrub and coastal habitats (Smith & Buechner, 1947; Lieb,

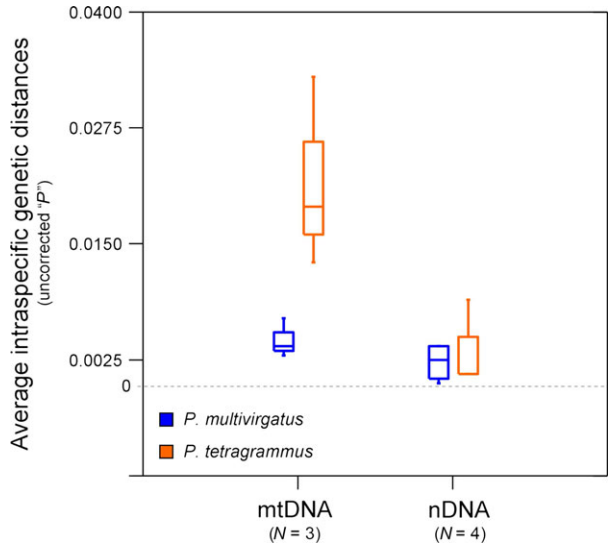

Figure 5. Average intraspecific genetic distances for three mitochondrial and four nuclear gene sequences from *Plestiodon multivigatus* and *P. tetragrammus*. Note that while *P. tetragrammus* possesses the expected pattern of mtDNA–nDNA variation for animals, *P. multivirgatus* has statistically indistinguishable levels of mtDNA–nDNA variation.

Table 4. Codon-based tests of positive selection for *Plestiodon multivirgatus* and *P. tetragrammus*

Test	Locus	*N*	Amino acids	*Z* statistic	*P*
multivirgatus	*ND1*	6	112	−2.254	1.000
multivirgatus	*BDNF*	6	182	−0.996	1.000
multivirgatus	*C-MOS*	6	117	0.000	1.000
multivirgatus	*PRLR*	6	157	−1.070	1.000
multivirgatus	*SNCAIP*	6	135	1.011	0.157
tetragrammus	*ND1*	25	112	−4.885	1.000
tetragrammus	*BDNF*	16	182	−1.005	1.000
tetragrammus	*C-MOS*	16	117	0.000	1.000
tetragrammus	*PRLR*	16	155	−1.007	1.000
tetragrammus	*SNCAIP*	16	137	−2.272	1.000
multivirgatus + *tetragrammus*	*ND1*	31	112	−5.837	1.000
multivirgatus + *tetragrammus*	*BDNF*	22	182	−1.577	1.000
multivirgatus + *tetragrammus*	*C-MOS*	22	117	−1.544	1.000
multivirgatus + *tetragrammus*	*PRLR*	22	155	−1.110	1.000
multivirgatus + *tetragrammus*	*SNCAIP*	22	137	−2.201	1.000

Each analysis tests the null hypothesis of strict neutrality ($d_N = d_S$) in favour of the alternative hypothesis of positive selection ($d_N > d_S$).

1985). Additionally, any ecological divergence could have also occurred during range expansion east into both habitat types (Riddle, 1995; Andersen & Light, 2012).

We also found two well-supported clades within *P. t. brevilineatus*, one limited to the Trans-Pecos and the other to the Edwards Plateau and Great Plains ecoregions. The herpetofaunal assemblage of the Trans-Pecos is quite distinct (Ward, Zimmerman & King, 1990), and the transition between the Trans-Pecos and the Great Plains corresponds to phylogeographical structure in some taxa (Jaeger, Riddle & Bradford, 2005; Pyron & Burbrink, 2009; Streicher *et al.*, 2014). Both deep (between *P. t. brevilineatus* and *P. t. tetragrammus*) and shallow (both clades of *P. t. brevilineatus*) divergences between lineages follow transitions between ecological regions, suggesting that ecological specialization may play an important role in driving the genetic diversity of this clade. In contrast to *P. tetragrammus*, we found low mitochondrial sequence divergence among populations of *P. multivirgatus*, albeit with limited sampling. Although separated by hundreds of kilometres and a well-described biogeographical boundary, the Cochise filter barrier (Castoe *et al.*, 2007; Pyron &

Table 3. Within-species genetic distances (uncorrected 'p-distances') for mitochondrial (mtDNA) and nuclear (nDNA) loci used in this study

Locus	Genome	*tetragrammus* (both subspecies)	*brevilineatus* only	*multivirgatus*
12S	mtDNA	0.019	0.012	0.003
16S	mtDNA	0.013	0.004	0.004
ND1	mtDNA	0.033	0.021	0.007
BDNF	nDNA	0.001	N/A	0.001
SNCAIP	nDNA	0.009	N/A	0.004
PRLR	nDNA	0.001	N/A	0.000
c-mos	nDNA	0.001	N/A	0.004

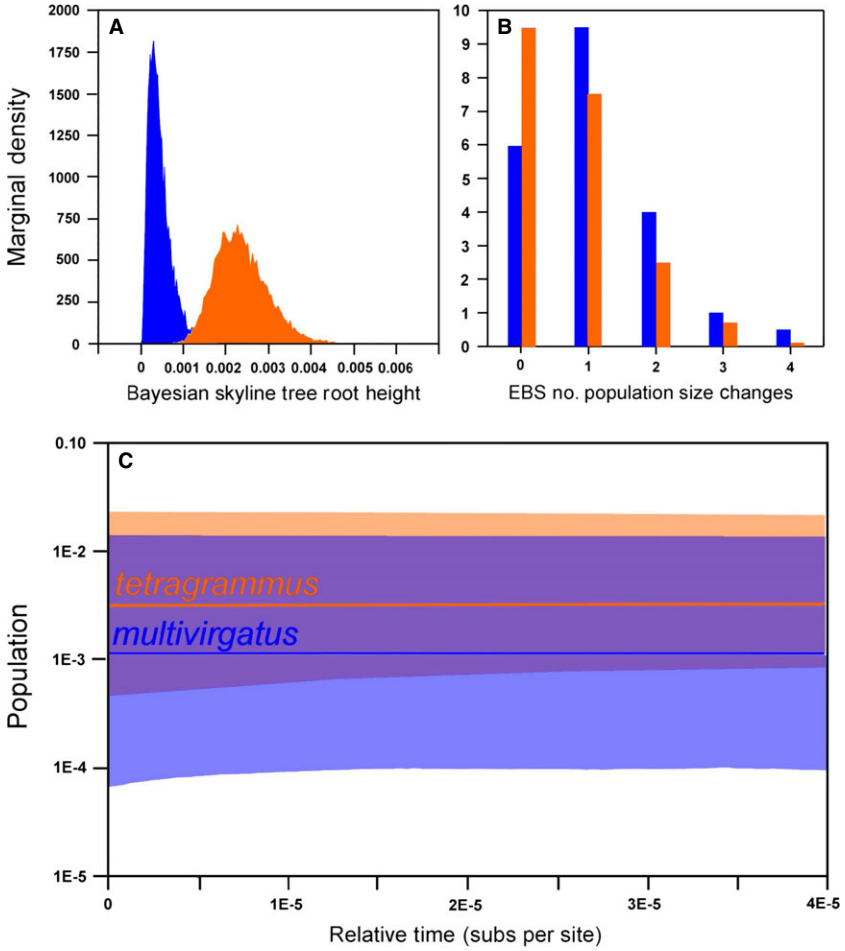

Figure 6. Results from extended Bayesian skyline (EBS) analyses of six *Plestiodon multivirgatus* and 16 *P. tetragrammus*: A, marginal densities of tree root height; B, marginal densities of the number of estimated population size changes; and C, skyline plots of population size over relative time in substitutions per site. Values for *P. multivirgatus* and *P. tetragrammus* are depicted in blue and orange, respectively.

Burbrink, 2009), samples of *P. multivirgatus* from Arizona were only 0.003–0.007% divergent in mtDNA sequence from those in Texas. However, our sampling included individuals only from *P. m. epipleurotus*, and additional samples from throughout the range of *P. multivirgatus* including *P. m. multivirgatus* will be necessary to ascertain phylogenetic relationships among populations of *P. multivirgatus*.

Signatures of introgression

Our phylogenetic trees support mitonuclear discordance, with mtDNA nesting *P. multivirgatus* within *P. t. brevilineatus*, and coalescent analysis of nDNA indicating reciprocal monophyly of both subspecies of *P. tetragrammus*, sister to a monophyletic *P. multivirgatus*. This pattern is consistent with the ancient introgression of *P. t. brevilineatus* mtDNA into *P. multivirgatus*. This interpretation suggests that introgression must have taken place after the diversification of *P. tetragrammus*, but before the two clades of *P. t. brevilineatus* diverged. Interestingly, we found *P. t. brevilineatus* mitochondrial haplotypes even in *P. multivirgatus* from Arizona, hundreds of kilometres from the range of *P. t. brevilineatus*. While introgression has been suggested as an explanation for mitonuclear discordance in lizards multiple times (Morando *et al.*, 2004; Leache & McGuire, 2006; McGuire *et al.*, 2007; Wiens *et al.*, 2010; Ng & Glor, 2011), these studies showed introgression only in narrow hybrid zones. Based on these studies we would expect to see identical *P. t. brevilineatus* haplotypes found within *P. multivirgatus* where the two are sympatric and less frequently away from the contact zone. In contrast, we found introgressed mitochondria throughout the range of *P. multivirgatus*, not just the individuals from the locality where they are sympatric with *P. t. brevilineatus* in Texas. There are two

possible explanations for this: (1) the introgression event took place prior to an expansion of *P. multivirgatus* into its current range or (2) a selective sweep of the introgressed mitochondria throughout the range of *P. multivirgatus*.

Our data do not currently allow us to favour either of these possibilities. Our finding that all loci are under purifying selection suggests that this haplotype is being maintained as might be expected following a selective sweep. Recent research has found evidence for a rapid range expansion of rattlesnakes (genus *Crotalus*) following the last glacial maxima (Castoe *et al.*, 2007; Schield *et al.*, 2015), and the lack of genetic variability in our data could follow that model. Neiswenter & Riddle (2010) suggest a pattern of radiation in pocket mice (genus *Perognathus*) where multiple groups were sympatric during glacial maxima, allowing for introgressive hybridization, and then follow the range expansion of arid grasslands at the conclusion of the glacial cycle. Our data are also consistent with *P. multivirgatus* following the expansion of arid grasslands after glacial maxima, while maintaining a small fraction of their range in sympatry with *P. t. brevilineatus*. The addition of more rapidly evolving nuclear markers (Toews & Brelsford, 2012) and ecological niche modelling of past distributions of *P. t. brevilineatus* and *P. multivirgatus* are needed to help identify which of these two scenarios is most likely.

Species-specific effects of introgression

We found significantly different patterns of mitochondrial diversity within *P. tetragrammus* and *P. multivirgatus*. Specifically, intraspecific mtDNA variation was much lower in *P. multivirgatus* than in *P. t. brevilineatus*, whereas intraspecific nDNA variation was similar between each species (Table 3, Fig. 5). Thus, the differences between mtDNA and nDNA variation in *P. multivirgatus* (but not *P. tetragrammus*; Fig. 5) run counter to expectations for animals (Brown *et al.*, 1979). One explanation for this pattern might be that our geographical sampling for *P. multivirgatus* was not as complete as for *P. tetragrammus* (Fig. 1). As such, we may have excluded a substantial proportion of extant mtDNA variation in *P. multivirgatus*. However, we note that we sampled individuals of *P. multivirgatus* from the US states of Arizona, Colorado and Texas, a geographical region that is similar in size to the entire range of *P. tetragrammus*.

We hypothesize that low mtDNA diversity in *P. multivirgatus* is related to the introgression and subsequent fixation of non-native mtDNAs, probably from an ancestral population of *P. t. brevilineatus*, based on the phylogenetic evidence (Fig. 3). Assuming this scenario, *P. multivirgatus* and *P. tetragrammus* have been impacted in very different ways by their historical interaction (Fig. 5). For example, mtDNA variation within *P. tetragrammus* has phylogeographical signal whereas mtDNAs from *P. multivirgatus* (despite having a larger range size) do not. This potential for decoupling of mitochondrial and nuclear variation in lineages that have experienced introgression is an important reminder that the evolutionary histories of mitochondrial and nuclear genomes can be uncoupled within an entire species (as we propose here for *P. multivirgatus*). If undetected, this phenomenon can lead to incorrect inferences of divergence time and historical demography when relying on datasets consisting mostly of mtDNA loci.

Taxonomic implications

We found that the individuals of *P. t. tetragrammus* and *P. t. brevilineatus* included in our study were reciprocally monophyletic (Fig. 3). However, because we did not sample individuals originating from central Texas (where these subspecies putatively overlap; Fig. 1), we refrain from suggesting taxonomic modification, in favour of testing these results using more extensive geographical and genetic sampling (Burbrink *et al.*, 2000; Sackett *et al.*, 2014). Regardless, our findings indicate the genetic distinctiveness of each subspecies, and further suggest complex dynamics of lineage diversification and introgression in this species complex.

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