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A multilocus perspective on the speciation history of a North American aridland toad (*Anaxyrus punctatus*)

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ABSTRACT

Interpretations of phylogeographic patterns can change when analyses shift from single gene-tree to multilocus coalescent analyses. Using multilocus coalescent approaches, a species tree and divergence times can be estimated from a set of gene trees while accounting for gene-tree stochasticity. We utilized the conceptual strengths of a multilocus coalescent approach coupled with complete range-wide sampling to examine the speciation history of a broadly distributed, North American warm-desert toad, *Anaxyrus punctatus*. Phylogenetic analyses provided strong support for three major lineages within *A. punctatus*. Each lineage broadly corresponded to one of three desert regions. Early speciation in *A. punctatus* appeared linked to late Miocene–Pliocene development of the Baja California peninsula. This event was likely followed by a Pleistocene divergence associated with the separation of the Chihuahuan and Sonoran Deserts. Our multilocus coalescent-based reconstruction provides an informative contrast to previous single gene-tree estimates of the evolutionary history of *A. punctatus*.

1. Introduction

Phylogenetic power from the accumulated signal of many gene trees has been long recognized (Avise and Ball, 1990; Avise, 2000), yet only recently have robust methods for reconstructing evolutionary history from these gene trees been developed (see Edwards, 2009). Among these methods are approaches using multispecies coalescent theory designed to model gene-tree stochasticity that can mislead single gene-tree estimates of evolutionary history (Liu and Pearl, 2007; Liu et al., 2008; Heled and Drummond, 2010). Gene trees are 'embedded' inside a species tree by following the stochastic coalescent process back in time along each branch to a most recent common ancestor (Rannala and Yang, 2003; Heled and Drummond, 2010). Using this approach, a species-tree topology, divergence times, and ancestral population sizes can be estimated from a set of gene trees while accounting for gene-tree stochasticity caused by heterogeneous processes such as incomplete lineage sorting.

Multilocus species-tree reconstructions using coalescent theory represent a powerful approach to estimating biogeographically informative demographic parameters (Hickerson et al., 2010). Instead of using gene trees directly to infer demographic history, coalescent methods use genealogies as a transitional parameter to obtain estimates of phylogeographic parameters (e.g., ancestral population sizes, divergence times, and migration rates) given the stochastic timing of coalescent events (Hey and Machado, 2003; Wakeley, 2008; Hickerson et al., 2010). Because phylogeography is rooted in understanding causal relationships between geography, species distributions, and the mechanisms driving speciation (Avise et al., 1987), robust estimates of these parameters are important. As evidenced in recent studies, interpretations of phylogeographic patterns can change when analyses shift from a single gene tree to multilocus coalescent analyses (e.g., Gifford and Larson, 2008; Galbreath et al., 2010; Kubatko et al., 2011).

The red-spotted toad (*Anaxyrus punctatus*, also known as *Bufo punctatus*) is a widespread denizen of the warm deserts of North America (Fig. 1). Because of its broad distribution, *A. punctatus* has been used as a model organism to explore hypothesized vicariant events associated with the early formation of North American deserts (Riddle et al., 2000; Jaeger et al., 2005; Pyron and Burbrink, 2010). Three mitochondrial DNA (mtDNA) haplotype clades of *A. punctatus*, corresponding to the general boundaries of three warm desert regions (Chihuahuan Desert, Sonoran Desert, and Baja California peninsula), diverged during the late Neogene about 5 million years ago (Jaeger et al., 2005). This divergence was attributed to two vicariant events: the early development of the Baja California peninsula, and orogeny associated with secondary uplifting of the Sierra Madre Occidental. In the previous studies

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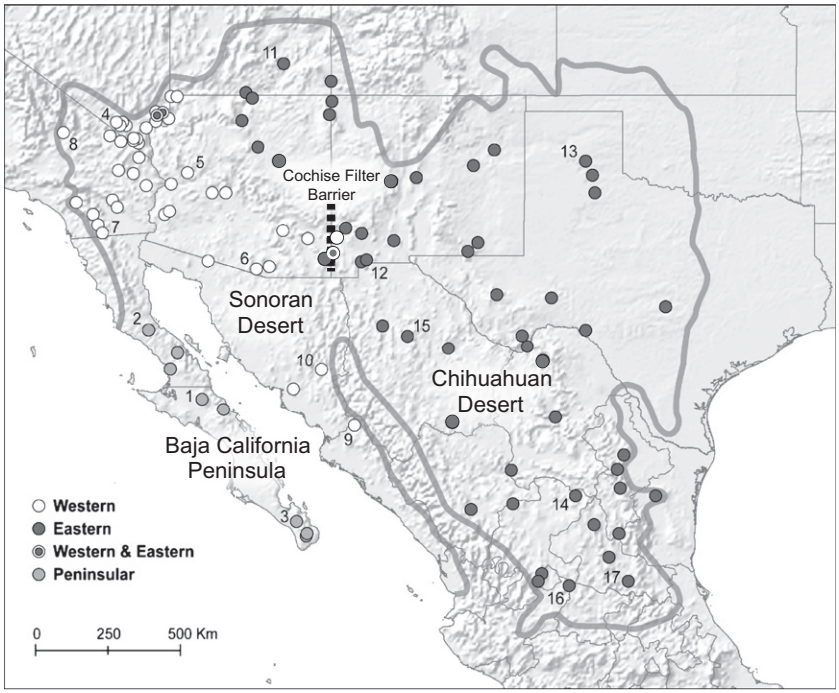

Fig. 1. Map of sampling localities for *Anaxyrus punctatus* in relationship to topography and political boundaries. Shaded dots indicate occurrence at sampling localities of the Eastern (dark grey), Western (white), and Peninsular (light grey) lineages diagnosed using molecular markers. Numbers reference sites from which samples were selected for multilocus analyses (see Table 1). Heavy grey line indicates approximate distribution of *A. punctatus* following published accounts (Korky, 1999; Stebbins, 2003) and our sampling. The general locations of important biogeographic regions discussed in the text are noted.

of *A. punctatus*, however, geographic sampling across central Mexico was lacking, and phylogeographic patterns in co-distributed taxa across this region (Neiswenter and Riddle, 2010; Bryson et al., 2011) indicate the possibility that additional geographic structure may be present in this toad.

Phylogeographic breaks across a variety of co-distributed taxa have been found to be temporally and spatially concordant with those inferred for *A. punctatus* (e.g., Riddle et al., 2000; Jaeger et al., 2005; Leavitt et al., 2007; Bryson et al., 2010, 2011). These interpretations of shared histories among the taxa were based on inferences from gene trees. Explicitly accounting for the inherent stochasticity associated with the gene-tree coalescence, however, might yield different phylogeographic interpretations. For example, coalescent tests of vicariance for 12 species distributed across the Baja California peninsula reveal two separate divergence events, whereas previous studies revealed only one (Leaché et al., 2007).

In this study, we utilize the conceptual strengths of a multilocus coalescent species tree coupled with complete range-wide sampling to expand earlier phylogeographic studies of *A. punctatus*. We first reconstruct a mtDNA gene tree to determine the maternal lineages of new samples of *A. punctatus* from mainland Mexico. We then estimate a species tree and divergence times from our multilocus dataset. We compare our multilocus reconstructions of the evolutionary history of *A. punctatus* with previous hypotheses for this species and co-distributed North American desert species, and propose several potential explanations for the phylogeographic patterns of *A. punctatus*.

2. Methods and materials

2.1. Taxon sampling and DNA sequencing

To complete range-wide sampling of *A. punctatus*, we obtained tissues from 22 individuals from 22 localities across the Sonoran and Chihuahuan Deserts and Central Mexican Plateau of Mexico (Fig. 1; Appendix A). These samples were added to the dataset of 192 samples from 82 locations generated in a previous study by Jaeger et al. (2005). We used *Anaxyrus nelsoni* and *Incilius occidentalis* as outgroups (Pauly et al., 2004). Total genomic DNA was extracted from liver or toe clips using the QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following manufacturer's recommendations.

For phylogenetic assessment of our new Mexican samples, we sequenced a 666-bp fragment of cytochrome *b* (cyt-*b*) used in the previous study (Jaeger et al., 2005). For species-tree analysis, we sequenced exemplars ($n = 17$; Table 1) representing mtDNA clades for an additional four genes: a segment of the mitochondrial ribosomal RNA 16S (16S, 852 bp), the nuclear intron beta-crystallin (cryba, 341 bp), and the nuclear exons proopiomelanocortin (POMC, 593 bp) and rhodopsin 1 (Rho1, 315 bp). These genes have previously been used to reconstruct bufonid (Pramuk, 2006; Maciel et al., 2010; Thomé et al., 2010) and other anuran (Bryson et al., 2010) phylogenetic relationships. Primer sequences are given in Jaeger et al. (2005; cyt-*b*), Pramuk (2006; 16S), Dolman and Philips (2004; cryba), Wiens et al. (2005; POMC), and Bossuyt and Milinkovitch (2000; Rho1). We generated sequence data following methods described in Bryson et al. (2010), with annealing temperatures for amplifications at 55 °C for cyt-*b* and Rho1, and 57 °C for16S, cryba, and POMC. We edited and manually aligned the forward and reverse sequences for each individual using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). For cryba data, which contained numerous indels, an additional sequence alignment was performed with MAFFT v6 using default settings and the G-INS-i algorithm (Katoh et al., 2002; Katoh and Toh, 2008). We identified heterozygous sites in nuclear segments when two different nucleotides were present at the same position in electropherograms of both strands, with the weakest peak reaching at least 50% of the strongest signal. We computationally determined the gametic phase of the variants using PHASE 2.1.1 (Stephens and Donnelly,