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Spatial and Temporal Drivers of Phenotypic Diversity in Polymorphic Snakes

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ABSTRACT: Color polymorphism in natural populations presents an ideal opportunity to study the evolutionary drivers of phenotypic diversity. Systems with striking spatial, temporal, and qualitative variation in color can be leveraged to study the mechanisms promoting the distribution of different types of variation in nature. We used the highly polymorphic ground snake (*Sonora semiannulata*), a putative coral snake mimic with both cryptic and conspicuous morphs, to compare patterns of neutral genetic variation and variation over space and time in color polymorphism to investigate the mechanistic drivers of phenotypic variation across scales. We found that strong selection promotes color polymorphism across spatial and temporal scales, with morph frequencies differing markedly between juvenile and adult age classes within a single population, oscillating over time within multiple populations, and varying drastically over the landscape despite minimal population genetic structure. However, we found no evidence that conspicuousness of morphs was related to which color pattern was favored by selection or to any geographic factors, including sympatry with coral snakes. We suggest that complex patterns of phenotypic variation in polymorphic systems may be a fundamental outcome of the conspicuousness of morphs and that explicit tests of temporal and geographic variation are critical to the interpretation of conspicuousness and mimicry.

Keywords: color polymorphism, frequency-dependent selection, geographic variation, mimicry, conspicuous coloration, *Sonora semiannulata*.

Introduction

Understanding how evolutionary processes drive phenotypic diversity is central to the study of evolutionary biology. A powerful way to study the origins and maintenance of phenotypic diversity is to examine systems with

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striking variability in phenotype, such as color polymorphism (the cooccurrence of multiple discrete color patterns within a single population). This sympatric phenotypic variation is especially attractive for understanding drivers of diversity because it creates a natural experiment in which to test how and why different color morphs can respond differently to the same biotic and abiotic environment. The mechanisms driving color polymorphism are of interest because the tendency of random genetic drift to fix neutral alleles over time suggests that the persistence of such variation must be explained by additional evolutionary processes (Fisher 1930; Ford 1930; Haldane 1930; Epling and Dobzhansky 1942). Both selective and neutral processes can promote polymorphism, and many important theoretical (e.g., Mather 1955; Van Valen 1960; Kelly and Wade 2000; Joron and Iwasa 2005) and empirical (e.g., Wright 1942; Dobzhansky 1947; Endler 1980; Gillespie and Oxford 1998) studies have debated the relative importance of these mechanisms in broad patterns of phenotypic evolution. However, color polymorphism can provide greater insight into how and why phenotypes vary over time and space beyond simply understanding simultaneous persistence of two morphotypes (Hoffman and Blouin 2000; Roulin 2004; Gray and McKinnon 2006).

Systems with color polymorphism can be described in terms of several distinct axes of variation. One type involves the amount of variation in color morphs. Some polymorphisms are striking because of the sheer number of color morphs (more than 10, dubbed "exuberant" polymorphisms; Franks and Oxford 2009; Croucher et al. 2011), while others are notable for the drastic differences in the color patterns among the morphs themselves (sometimes even erroneously designated as separate species; e.g., lepidopterans [Ford 1955], orthopterans [Rowell 1972], *Eclectus* parrots [Forshaw 1978], and snakes [Cox et al. 2012]). A second axis addresses the nature of variation, as morphs can vary remarkably in conspicuousness. Spe-

cies can display multiple cryptic morphs (e.g., snails [Lamotte 1959; Clarke 1960], water snakes [King and Lawson 1995], felids [Eizirik et al. 2003], and leopard frogs [Hoffman et al. 2006]), multiple aposematic or brightly colored morphs (e.g., Gouldian finches [Brush and Seifried 1968], various coral snake mimics [Brodie and Brodie 2004], and poison frogs [Noonan and Comeault 2009]), or even both brightly colored and cryptic morphs (e.g., ground snakes [Frost and Devender 1979], redback salamanders [Brodie and Brodie 1980], and poison frogs [Wang and Shaffer 2008]). Even though the distinction between cryptic and conspicuous color is not always straightforward and will vary depending on the sensory capability of the receivers and on background substrate characteristics, this categorization can still be helpful in the general sense (Bond 2007). A third type concerns the scale of variation, which can be across both spatial (within-population vs. among-population variation) and temporal (transient vs. stable polymorphism) dimensions. Variation in polymorphism across scales is especially important for identifying how processes generating variation may interact to produce phenotypic evolution over time and space. Notably, very different mechanistic processes can result in the same standing distribution of variation within and among populations, and understanding the drivers of this variation is fundamental to linking evolutionary processes at different spatial and temporal scales. Therefore, systems with considerable diversity along different axes can be leveraged to study not only the factors promoting the general phenomenon of polymorphism but more broadly when and why we find specific types of variation in nature.

The ground snake (*Sonora semiannulata*) is a dramatically polymorphic species, with very dissimilar sympatric color patterns that vary in conspicuousness and across geography. Although the majority of snakes use cryptic, muted color patterns for camouflage (Jackson et al. 1976), *S. semiannulata* has four strikingly distinct morphs that can all be categorized as variations of red and black pigmentation: individuals can be (1) red striped dorsally, (2) boldly banded with crosswise black rings or saddles, (3) simultaneously red striped and black banded, or (4) uniform gray or brown with no distinctive red or black markings of any kind (fig. 1). These color morphs do not vary between sexes or change ontogenetically, as the full complement of four morphs is present in both juveniles and adults of both sexes, and captive animals show no color change over time (Ernst and Ernst 2003). Individual populations can display the full spectrum of variation from a single morph up to all four morphs, in any combination. Many biologists interpret the bright "red- and black-ringed" color pattern as an aposematic signal in venomous coral snakes that is then deceitfully mimicked by harmless snakes, such as ground snakes (Batesian mimicry; Bates

1862; Dunn 1954; Jackson et al. 1976; Greene and McDiarmid 1981; Pfennig et al. 2001; Brodie and Brodie 2004), while others suggest that this color pattern is a convergent form produced by a single protective advantage through disruptive patterning (Brattstrom 1955; Hecht and Marien 1956; Gehlbach 1972; Grobman 1978). Regardless of these differing perspectives, ground snakes clearly have sharply contrasting color morphs that are both cryptic (uniformly brown) and brightly conspicuous (red and black), with red being of particular importance to predators with color vision (especially birds; Smith 1975; Jackson et al. 1976; Brodie 1993).

In this study, we used color polymorphism in ground snakes to examine the mechanisms driving variation in color pattern within the context of two research goals. The primary goal of our study was to compare variation within and among populations and over time to test whether similar or different evolutionary mechanisms promote polymorphism across temporal and spatial scales. A secondary goal of this study was to assess spatial patterns of morph presence by conspicuousness. As a putative mimic found both sympatrically and allopatrically to venomous coral snakes, *S. semiannulata* offers the opportunity to test a basic prediction of mimicry that (*a*) distribution of and (*b*) strength of selection on cryptic and conspicuous color patterns in mimetic species should vary as a function of geographic overlap with venomous model species. Most research in coral snake mimicry has studied selection on color pattern from the perspective of the predator by measuring avian response to synthetic replicas of snakes, providing important evidence that black and red color patterns are preferentially avoided by predators (Brodie 1993; Brodie and Janzen 1995; Pfennig et al. 2001; Kikuchi and Pfennig 2010*a*, 2010*b*). However, the color variation in ground snakes offers the opportunity to expand research focus to the real metric of interest: the phenotypic response (i.e., shifts in morph frequencies) of free-living snakes to potential selective agents in natural populations.

To assess how evolutionary processes act on a large geographic scale to drive color variation within and among populations of *S. semiannulata*, we first characterized the extent and variability of this polymorphism by examining ~2,500 living snakes and museum specimens from across the ground snake's geographic range. Then we tested among potential drivers of phenotypic variation by using two data types that yield different but complementary ways of assessing the relative role of neutral and selective processes in generating phenotypic diversity at different scales. First, we used population genetic data to compare genetic and phenotypic variation within and among populations (the locus comparison approach), specifically testing for the effects of population bottlenecks, gene flow, population structure, and natural selection on current variation in

Figure 1: The four sympatric color morphs of the ground snake (*Sonora semiannulata*). Clockwise from bottom left: striped morph (S) with red dorsal stripe (*bottom left*), banded morph (B) with black crosswise bands (*top left*), uniform morph (U) lacking any distinctive markings (*top right*), and mimetic morph (M) with both the red stripe and black bands (*bottom right*).

color pattern. Second, we analyzed extensive demographic data on (*a*) geographic location, (*b*) age cohorts, and (*c*) temporal changes in morph frequency within and among populations (the population dynamics approach) to test for the effects of isolation by distance, sympatry with coral snakes, selection on juveniles, and negative frequency dependence (rare morph advantage, a common mechanism for maintaining polymorphism; Gray and McKinnon 2006) on color variation. In combination, these two approaches lend insight into the mechanisms driving variation in cryptic and conspicuous phenotypes over space and time.

Methods

Study System

Ground snakes are small, insectivorous snakes that are distributed from southeastern Missouri and northern mainland Mexico to Baja California and southern Idaho and Oregon. Population density is not constant across this range (Ernst and Ernst 2003), as populations in the Great Plains are found in much greater abundance than ground snakes from elsewhere in their geographic range (C. L. Cox and A. R. Davis Rabosky, personal observation). There are three other species in the genus *Sonora* that are restricted in range to central and western Mexico (*S. aemula*, *S. michoacanensis*, and *S. mutabilis*). Notably, all three of these species also display striking color polymorphism of uniform red, bicolored (red and black), and tricolored (red, black, and white or yellow) morphs, and they are all considered coral snake mimics (Brodie and Brodie 2004; Cox et al. 2012).

While bright pigmentation and color polymorphism in other systems is often associated with sexual signaling, color variation in ground snakes is unlikely to be under sexual selection. First, the density and characteristics of

photoreceptors in snake retinas suggest that color vision is absent or limited in most snakes (Walls 1942; reviewed in Sillman et al. 1999), presumably due to the fossorial origin of the snake clade within lizards (Walls 1940; Vidal and Hedges 2004). Additionally, sexual dichromatism, which would be expected when color variation is driven by sexual selection, is often subtle or absent in snakes (Shine and Madsen 1994). Even in snake species with color polymorphism, the same color morphs are usually seen in both sexes, and there is usually little or no variation in morph frequencies between males and females (including in ground snakes; Kassing 1960). Although sexual selection in snakes likely exerts strong pressure on chemical cues (Mason et al. 1989) and body size (Shine 1994), coloration appears to be far more important for sexual communication in lizards than it is in snakes.

Although the genetic inheritance of color pattern has not been tested specifically in ground snakes, pigmentation synthesis pathways are known to be highly conserved across vertebrates (Bagnara et al. 1979; Kondo and Shirota 2009; Kanehisa et al. 2012). As in amphibians and fish, black (melanins) and red (pteridines) pigments in snakes are synthesized inside cellular organelles within two different chromatophores (melanophores and erythrophores, respectively) from different dermal tissue layers (Bagnara 1983). Common-garden breeding experiments (Bechtel and Bechtel 1962, 1978; Bechtel 1978) have shown that red and black coloration in other colubrid snakes is highly heritable and genetically controlled, likely by separate, unlinked loci with simple Mendelian inheritance. For the following analyses, we thus scored color pattern in one of two ways, either by morph (as uniform gray or brown [U], red striped [S], black banded [B], or red striped and black banded [M; mimetic]) or as two separate, unlinked traits by presence or absence of black bands or red stripes.

Locus Comparison

Population Genetic Data Collection and Screening. For the locus comparison approach, we generated a population genetic data set assessing the genetic structure of 224 ground snakes from 11 different populations limited to the Great Plains region (see table A1; tables A1–A5 are available online). We focused on this restricted area because populations are often highly polymorphic for dorsal color pattern and snakes are abundant enough for population genetic sampling. Snakes were collected between 2001 and 2010 by turning rocks in appropriate habitat across their geographic range. We preserved muscle, liver, and skin tissue in lysis buffer or 95% ethanol. Although some specimens were sampled for tissues and released, most specimens were fixed in 10% formalin and fluid-preserved in 70% ethanol. Specimens were deposited in

the Amphibian and Reptile Diversity Research Center at the University of Texas at Arlington and the Sternberg Museum of Natural History at Fort Hays State University.

We compared neutral genetic information to phenotypic (color pattern) information to study the evolutionary processes influencing color polymorphism (Gillespie and Oxford 1998; Andres et al. 2000; Hoffman et al. 2006; Abbot et al. 2008; Croucher et al. 2011). We used amplified fragment length polymorphisms (AFLPs) to determine the neutral genetic structure of ground snakes, following standard methods (Vos et al. 1995) with slightly modified primers (see table A2). We objectively scored AFLPs using the script AFLPscore (Whitlock et al. 2008) and processed AFLP loci for analysis using AFLPdat (Ehrich 2006) in R (ver. 2.14.0; R Development Core Team 2008). We then used two different Bayesian-based genome scan approaches to screen for AFLP loci under selection. First, we used Mcheza (Antao and Beaumont 2011), which implements the DFdist backend program (Beaumont and Balding 2004), to identify candidates for selection. Briefly, this approach uses hierarchical Bayesian methods with Markov chain Monte Carlo to identify loci with outlier F_{ST} values. We followed suggested parameter settings (Antao and Beaumont 2011) and estimated outliers using 100,000 generations and a conservative false discovery rate (FDR = 0.001). Second, we used the program BayeScan (Foll and Gaggiotti 2008) to identify candidate loci. This program uses a Bayesian approach with reversible-jump Markov chain Monte Carlo to identify outlier loci and differs from Mcheza (Antao and Beaumont 2011) in implementing more realistic population models that may be more robust to false positives (Foll and Gaggiotti 2008). We used suggested parameter settings (Foll and Gaggiotti 2008) and estimated outliers using 100,000 generations and a conservative false discovery rate (FDR = 0.001). We then removed loci under selection to obtain a neutral genetic data set of 106 AFLP loci (Cox and Davis Rabosky 2013). For analyses of color pattern, we treated black banding and red striping as separate dominant markers, similar to AFLP loci (i.e., Croucher et al. 2011). To assess neutral population genetic structure, we visualized population subdivision by performing a principal coordinates analysis using the packages "ape" and "vegan" in R.

Genetic Bottlenecks and Gene Flow. The role of genetic bottlenecks or other demographic processes in generating geographic variation in color pattern morphs was assessed by comparing genetic variability and color pattern variability within populations. We tabulated the number of color pattern morphs in each population and used Genalex (Peakall and Smouse 2006) to calculate Shannon's *I* and Nei's unbiased genetic variation statistics for both AFLPs and color pattern. AFLP and color pattern variation were

compared using Pearson's *R* (significance determined Bonferroni probabilities) and Spearman's rank correlation (Spearman's rank with significance assessed by resampling without replacement; 1,000 pseudoreplicates) on both untransformed and log-transformed data using the program Systat (Systat Software). If population bottlenecks or similar demographic processes are responsible for variation in the frequencies of color pattern morphs between populations, we predict that color pattern variation will increase with neutral genetic variation within populations. Therefore, we interpret a positive correlation to be consistent with population bottlenecks, and no correlation or a negative correlation to be consistent with selection.

We also tested for the association between gene flow and variation in color pattern morphs among populations. Pairwise Nei's unbiased genetic distance and binary genetic distance (a kind of simple Euclidean distance) was calculated in Genalex (Peakall and Smouse 2006) for each population pair. The AFLP and color pattern distance matrix for each distance type was then compared using simple and partial (with geographic distance as a covariate) Mantel tests, implemented in zt (Bonnet and van de Peer 2002). If gene flow is responsible for geographic variation in color pattern morphs, then the color pattern distance between populations should increase with neutral genetic (AFLP) distance. We interpret a positive correlation to be consistent with an important role for gene flow, and no correlation or a negative correlation to be consistent with selection.

Population Structure and Nature of Selection. The importance of neutral processes (genetic drift and local gene flow) in causing geographic variation in color pattern morphs was studied by comparing population subdivision for neutral genetic markers (AFLP) and color pattern (Gillespie and Oxford 1998; Andres et al. 2000; Hoffman et al. 2006; Abbot et al. 2008; Croucher et al. 2011). Similar levels of population structure for both neutral genetic markers and color pattern markers are consistent with neutral processes promoting variation in color morphs among populations. However, a mismatch between neutral and genetic markers implicates selection, and the nature of this mismatch can explain how selection is shaping color polymorphism among populations. High differentiation of neutral markers among populations relative to the phenotypic traits suggests that selection is driving populations toward similar color morph compositions. Low differentiation of neutral markers relative to the phenotypic traits suggests that selection is driving populations toward different color morph compositions (Gillespie and Oxford 1998; Runemark et al. 2010).

We compared patterns of population segregation between AFLP and color pattern loci using the statistic F_{ST}, which increases with increasing population subdivision. We used Genalex (Peakall and Smouse 2006) to calculate θ (F_{ST}, denoted as Φ_{PT} [Peakall and Smouse 2006]) for both types of loci and generated 95% confidence intervals by bootstrapping our data set with 9,999 replicates. We also compared AFLP and color pattern using genome-scan methods, which identify outlier loci on a per-locus basis (see "Population Genetic Data Collection and Screening"). For both methods, we consider $F_{ST\_color} = F_{ST\_AFLP}$ to be indicative of no selection, $F_{ST\_color} < F_{ST\_AFLP}$ to be indicative of balancing selection, and $F_{ST\_color} > F_{ST\_AFLP}$ to be indicative of diversifying selection (Gillespie and Oxford 1998; Abbot et al. 2008).

Population Dynamics

Demographic Data Collection and Screening. For the population dynamics approach, we augmented the data set described above by collecting demographic and morphological data from an additional 2,380 fluid-preserved *Sonora semiannulata* specimens from 12 institutional collections (see "Acknowledgments"), 60 observations from the North American Field Herping Association HERP database (accessed May 25, 2012), and 71 individuals sampled across the western United States. For the specimens from the HERP database, we only used entries with (*a*) reliable locality information and (*b*) one or more clear color photographs of the dorsal coloration of each snake for us to score for phenotype. For each museum or live-caught specimen, we measured snout-vent length and scored the color pattern as uniform (U), red striped (S), black banded (B), or mimetic red and black (M). Each individual was also photographed for (*a*) dorsum, (*b*) venter, and (*c*) close-up of dorsal color pattern, and these photographs were sometimes reexamined for quality control purposes to ensure correct color pattern scoring. Although some pigmentation (especially red) fades with the preservation process, even relatively old museum specimens were easy to score, as both stripes and bands are readily visible against the remaining background body pigment (see fig. A1; figs. A1 and A2 are available online). In the rare case of a snake with a subtle or unclear phenotype, the specimen was independently scored by at least two people, and any disagreement between the phenotype scores resulted in exclusion from further analysis.

Population Filtering. To obtain an accurate assessment of how many morphs are present within populations across the geographic range, we filtered this data set to contain only individuals from populations that met either of two criteria: (1) any population with individuals of all four morphs present, irrespective of total number of individuals sampled (minimum $N = 4$), or (2) for populations with putatively fewer than four morphs, any population for

which we had at least 23 individuals sampled. Using simple binomial probabilities, this sample size corresponds to a 90% probability of detecting a rare morph that is present at 10% frequency in a population dominated by a common morph, and many populations had sample sizes far above this threshold. We included three exceptions to this sample size criterion for populations (all western) that were bolstered by other published data (Sierra County, NM; Ricards 1961) or personal communications about unpublished data (Washoe County, NV, L. Diller and the Humboldt State University Wildlife Field Course; Inyo County, CA, M. Mulks). We viewed this threshold and our decision to include the few exceptions as striking the most appropriate balance between (*a*) conservatism in accurately depicting the number of morphs present in each population and (*b*) not overzealously rejecting the best available data in favor of no data, especially in the low-density western part of the range. Thus, our final data set contained a total of 1,760 snakes representing 39 populations from across the United States and northern Mexico.

For US specimens, we used political county boundaries as proxies for delineating populations, which was an efficient way to include specimens with only county-specific locality information and to generate latitude/longitude data for the many museum specimens that were not georeferenced. The only exception was for the Maricopa County, Arizona, population, which we conservatively restricted to the Phoenix greater metropolitan area. For the two Mexican populations, we used state (Coahuila) or geographic (the Baja peninsula) boundaries. The effect of county/state population area on detection of color morphs was tested (see "Geography and Isolation by Distance"). For each population, we assigned the latitude and longitude of each county as its midpoint (see table A3) and obtained the area of each county using metrics from the US Census Bureau (2011) and Mexican states from Wikipedia (2012). We then used published records (Conant and Collins 1998; Dixon 2000; Ernst and Ernst 2003; Stebbins 2003) to assign each population to sympatry or allopatry with venomous coral snakes (coral snake ranges are shown in light gray in fig. 2).

Geography and Isolation by Distance. We used this demographic data set to test for a relationship between geographic factors and (*a*) color polymorphism or (*b*) the presence of the red and black mimetic morph. We used parametric (Pearson's correlation with Bonferroni probabilities) and nonparametric (Spearman's rank with significance assessed by resampling without replacement; 1,000 pseudoreplicates) methods in Systat to test for a relationship between the number of morphs or the proportion of mimetic morphs and latitude, longitude, or area (km²) of each population census area. Exclusion of the

two positive outliers for area (the two Mexican populations) from the appropriate tests did not affect our results, so we report results from our total data set. Binomial logistic regression was used to evaluate the relationship between (*a*) the presence of bands, stripes, or a mimetic morph and (*b*) latitude or longitude among populations. We used the χ^2 test and Fisher's exact test in a contingency analysis to test for the role of sympatry with coral snakes on the type of color pattern morphs in each population. Finally, we tested for the effects of isolation by distance on the morph composition of populations using Mantel tests. We assessed morph distance between populations using Euclidean distance calculated from either morph data or the presence or absence of bands. We then used Mantel tests (significance assessed using 10,000 simulations) in zt (Bonnet and van de Peer 2002) to compare each type of color pattern distance matrix with the geographic distance matrix for all population pairs. For all tests, data were transformed as necessary to meet the assumptions of the statistical approach.

Age Class Variation. To test for a difference in color morph frequencies between juveniles and adults, we analyzed 203 preserved specimens collected between 1952 and 1962 from the greater Phoenix metropolitan area in Maricopa County, Arizona, which was the only polymorphic population for which we had a robust sample size within a time period small enough to reasonably approximate a single generation. We considered specimens juveniles if they had a snout-vent length of less than 230 mm (after Kassing 1960). First, we tested whether the frequency of the four morphs varied between juveniles and adults using Fisher's exact test in R. To conservatively rule out potential bias due to the better preservation of black than red pigment in museum specimens, we then repeated the analysis of snakes coded only for the presence or absence of black bands (collapsing individuals scored M or B into a banded category and those scored S or U into an unbanded category). As there is no ontogenetic color change known in *Sonora* (note that fig. 1 shows four juveniles), differences in morph frequencies between juveniles and adults can reasonably be interpreted as the result of variation in morph survivorship. To further ensure that differences in morph frequency between juveniles and adults were not a result of biases in the physical collection of the two age classes either (1) over the 10-year time interval or (2) across the metropolitan area, we also used χ^2 analysis to test whether the number of adults and juveniles varied between the first 5 years ($N_A = 41$, $N_J = 53$) and the second 5 years ($N_A = 37$, $N_J = 71$) or between the northwest ($N_A = 39$, $N_J = 63$) and southeast ($N_A = 39$, $N_J = 61$) areas of the Phoenix Valley following a natural separation in the spatial data.
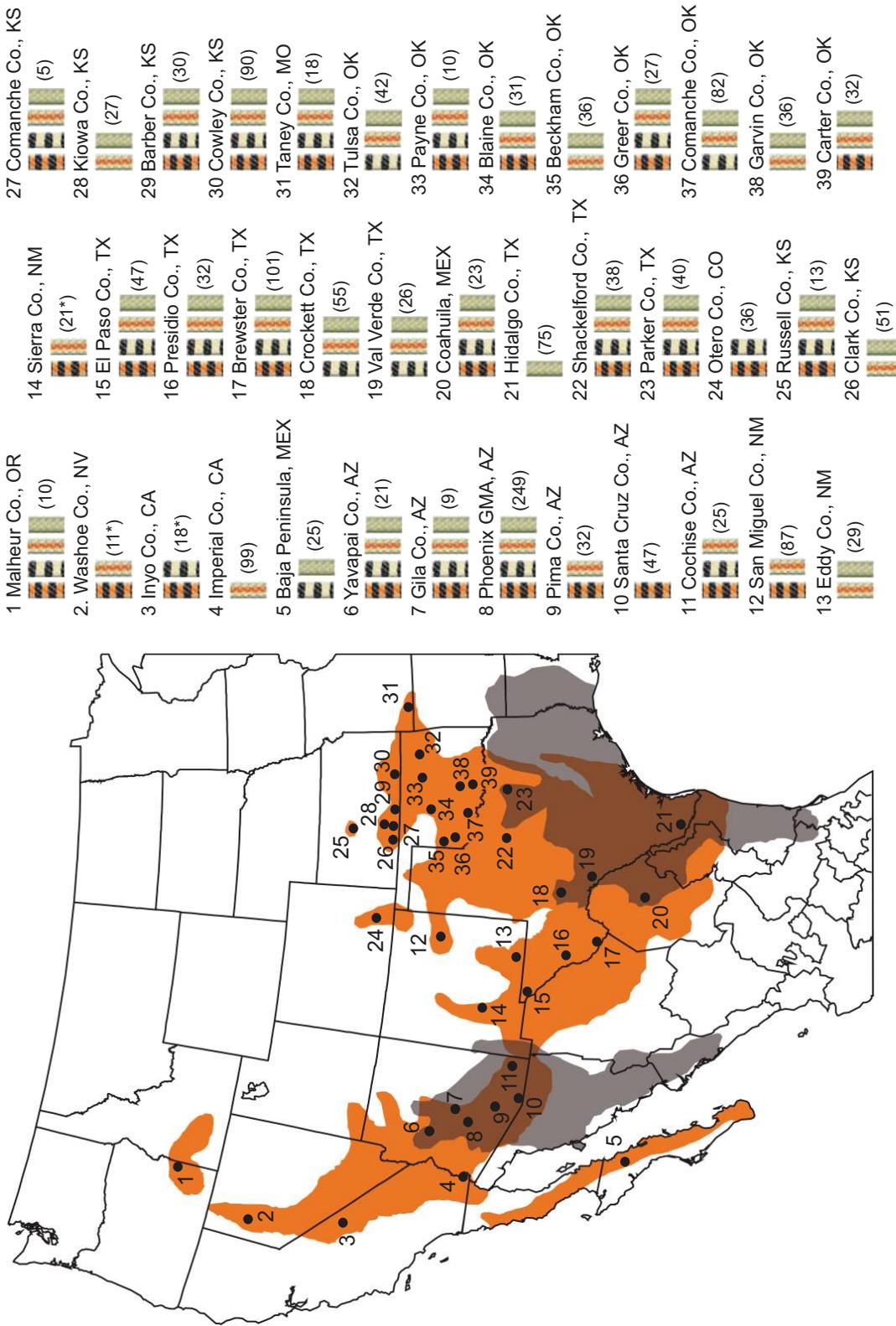
Figure 2: Distribution of color morphs across the geographic range (orange on map) of *Sonora semiannulata*. Vertical patterned bars indicate the presence of morphs (but not morph frequencies) in each population, with total sample size indicated parenthetically. Geographic distribution of two coral snake species are shaded in gray (west, Sonoran coral snake [*Micruroides euryxanthus*]; east, Texas coral snake [*Micrurus tener*]), and sympatry with ground snakes is shown with brown. Note that the black and red morph is found throughout the geographic range of *Sonora*, even in distant allopatry to coral snakes. Dorsal color pattern images are courtesy of http://www.reptilesofaz.org.

Temporal Variation. To test for change in morph frequency over time, we used only populations that had (*a*) more than 30 specimens over a time span of at least 40 years and (*b*) all four color morphs ($N = 5$ populations). For each of these populations, we used a multinomial logistic regression from the "mlogit" package in R to estimate the occurrence probabilities for each morph over that population's sampling time interval. To then test specifically for a rare-morph advantage (negative frequency-dependent selection), we analyzed the prediction that the frequency of the rarest morph in a population should increase over time by using the occurrence probabilities generated by the multinomial regression as a proxy for the true morph frequencies within a population.

First, for each population we determined (*a*) which morph was the rarest morph at time = 0 and (*b*) the magnitude and direction of change in occurrence probability for this morph. We then created a test statistic that summed the total change in occurrence probability for the rarest morph across all five populations with the equation

$$z = \sum_{i=1}^{5} (X_{i,t} - X_{i,0}),$$

where z is the test statistic, t is the final time point, and X represents the instantaneous occurrence probabilities for whichever morph is the rarest at the beginning of the time series for each population. Note that the "rarest morph" at the beginning of the time series in any specific population can be any one of the four morphs, and which morph is rarest can and does vary across populations. Although the comparison is specifically between the last time point and the first time point for the rarest morph, this measure in the observed data set was also mathematically equivalent to the difference between the maximum and minimum probability values for that morph.

To generate a null distribution of changes in rare morph frequency over time, we ran 10,000 simulations in which we first shuffled (without replacement) the order of individuals caught within each of the five populations while retaining the same capture histograms as the original collection data (e.g., if eight individuals were caught in 1952 within population 1, then only eight individuals were randomly drawn from the available pool for the 1952 time bin for that population's simulated data). It should be noted that this resampling scheme essentially creates block subsampling sets and therefore effectively avoids the autocorrelation concerns of some simulated time-series analyses (Politis 2003). Next, we fit the multinomial model to each population of randomized data and again calculated the test statistic as above for the five populations in each iteration of the simulation. Finally, we compared our observed z to the null distribution of test statistics generated

from the simulated data with the expectation that under negative frequency dependence rare morphs should have a greater positive change in frequency than expected by random chance.

Results

Extent and Variability of Polymorphism

We found incredible variation in color pattern among ground snake populations, with populations displaying the full set of possible variation, from a single morph to all four morphs (fig. 2). This variation often occurred on a very small spatial scale, with some neighboring populations displaying very different morph complements (fig. 3*A*). Most populations had multiple morphs, with almost half ($N = 17$; fig. 2) containing all four morphs, and only three populations had a single morph. These monomorphic populations were found fixed for all morphs except for banded (although a population with only black-banded individuals has been suggested in the literature using a limited number of specimens in the Grand Canyon, AZ [Stickel 1938]). Beyond simple presence and absence of color patterns, populations also varied greatly in the frequency of morphs in a population (see table A3). Some polymorphic populations were dominated by one or two morphs, while others contained equal proportions of each morph. Additionally, the red and black morph was distributed widely across the entire range of *Sonora semiannulata*, including areas distantly allopatric to coral snakes (fig. 2).

Locus Comparison

Population Bottlenecks and Gene Flow. Populations were very different in color pattern morph frequencies (fig. 3*A*). In contrast, individuals from all populations clustered together genetically, with no clearly geographically separated genetic clusters (fig. 3*B*), demonstrating a striking mismatch between genetic and phenotypic variation across populations.

We did not detect any evidence that genetic bottlenecks were responsible for variation in morphs among populations (fig. 4*A*). There was no relationship (all $P > .1$) between AFLP and color pattern variation within populations (Nei's unbiased heterozygosity, Shannon's I, number of color pattern morphs) on both untransformed and log-transformed data sets using both Pearson's and Spearman's rank correlation methods (all $P > .05$; see table A4).

Similarly, we did not find evidence that gene flow is responsible for variation in morphs among populations (fig. 4*B*). There was no significant relationship (all $P > .2$) be-
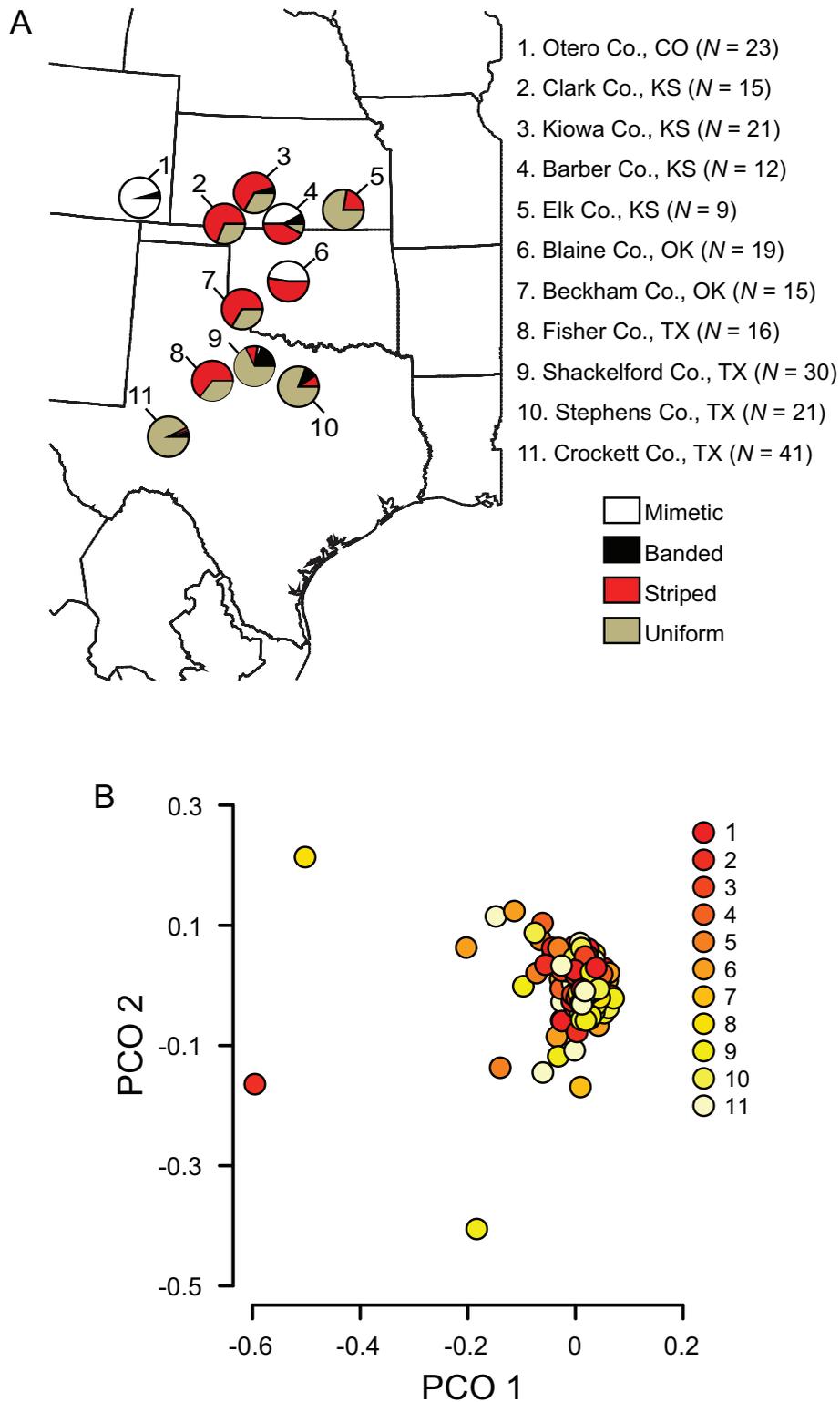
Figure 3: *A,* Populations from the Great Plains, United States, used for the locus comparison analysis. Pie charts represent the relative frequencies of each morph in each population. *B,* Principal coordinates analysis (PCO) of amplified fragment length polymorphism data for populations in *A.* Note the similar genetic clustering of all populations, despite large differences in morph frequencies.
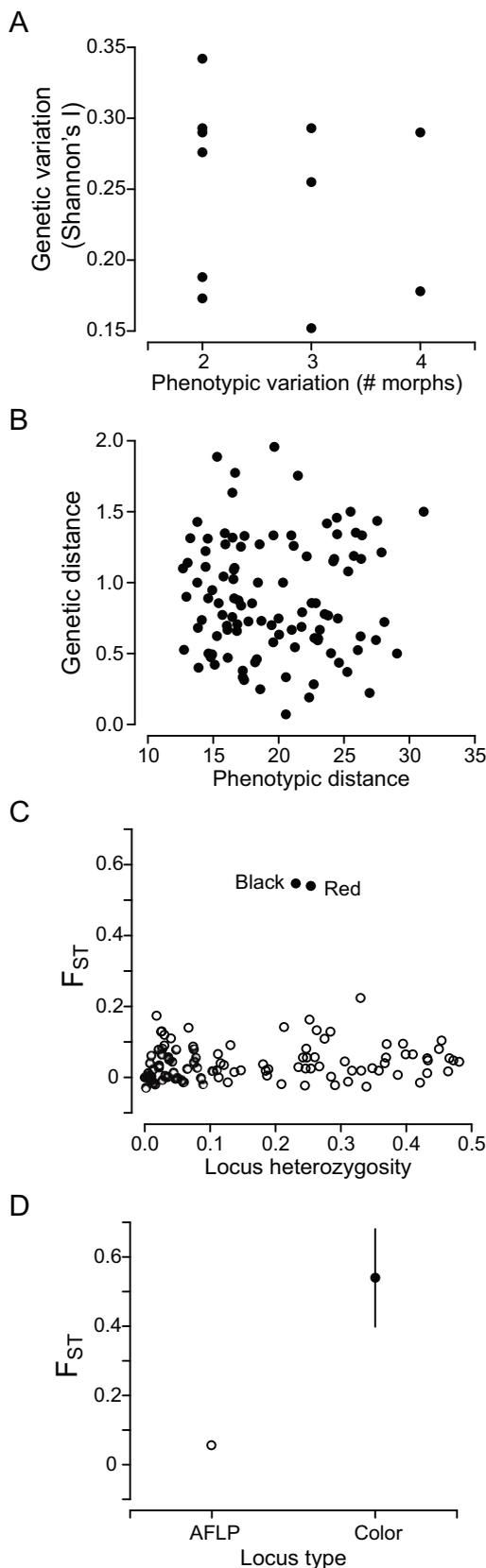
tween AFLP and color pattern distance between populations (Nei's unbiased genetic distance, Euclidean distance), even when controlling for geographic distance using simple and partial Mantel tests (see table A4).

Population Structure and Type of Selection. We did not find a significant role for genetic drift in creating the variation in color morphs among populations. Populations were very closely related genetically but were very different in morph composition (fig. 4C, 4D). Using two types of genome scans of AFLP and color pattern data (fig. 4C) and F_{ST} comparisons (fig. 4D), we found that the F_{ST} for color pattern was much higher than that for AFLPs. Thus, populations are highly subdivided with regard to color pattern but are very similar genetically. This mismatch indicates that selection is driving populations toward different color-morph compositions.

Population Dynamics

Geography and Isolation by Distance. We found no significant relationship (all $P > .05$) between the number of morphs or the proportion of mimetic morphs and latitude, longitude, or area (geographic size) of each population. There was also no significant relationship (all $P > .05$) between the presence of bands, stripes, or a mimetic morph and latitude or longitude within populations. Similarly, we did not find a significant relationship (all $P > .05$) between sympatry with coral snakes and the presence of any morph. There was also no significant relationship ($P = .22$) between geographic distance and color pattern distance calculated from the presence or absence of bands. However, we did find a significant positive correlation ($P = .018$) between geographic distance and color pattern distance calculated from the presence and absence of each morph between populations. See table A5 for a summary of all statistical tests.

Figure 4: *A*, Genetic versus phenotypic variation. There was no relationship between color pattern and genetic variation using multiple statistics and tests (see table A4). *B*, Genetic versus phenotypic distance between populations. Note that while we graphically present nonindependent pairwise distances here, we used the statistical approach of simple and partial Mantel tests to analyze these data. We did not detect a relationship between genetic and color pattern distance between populations using multiple statistics and statistical tests (see table A4). *C*, F_{ST} versus locus heterozygosity for all amplified fragment length polymorphism (AFLP) loci. The black and red color patterns were the only loci identified as significant outliers using multiple approaches. *D*, F_{ST} for AFLP loci and color pattern loci with 95% confidence intervals (the confidence interval for AFLP F_{ST} is smaller than the symbol). Note that F_{ST} for color pattern was significantly greater than that for all other marker categories.
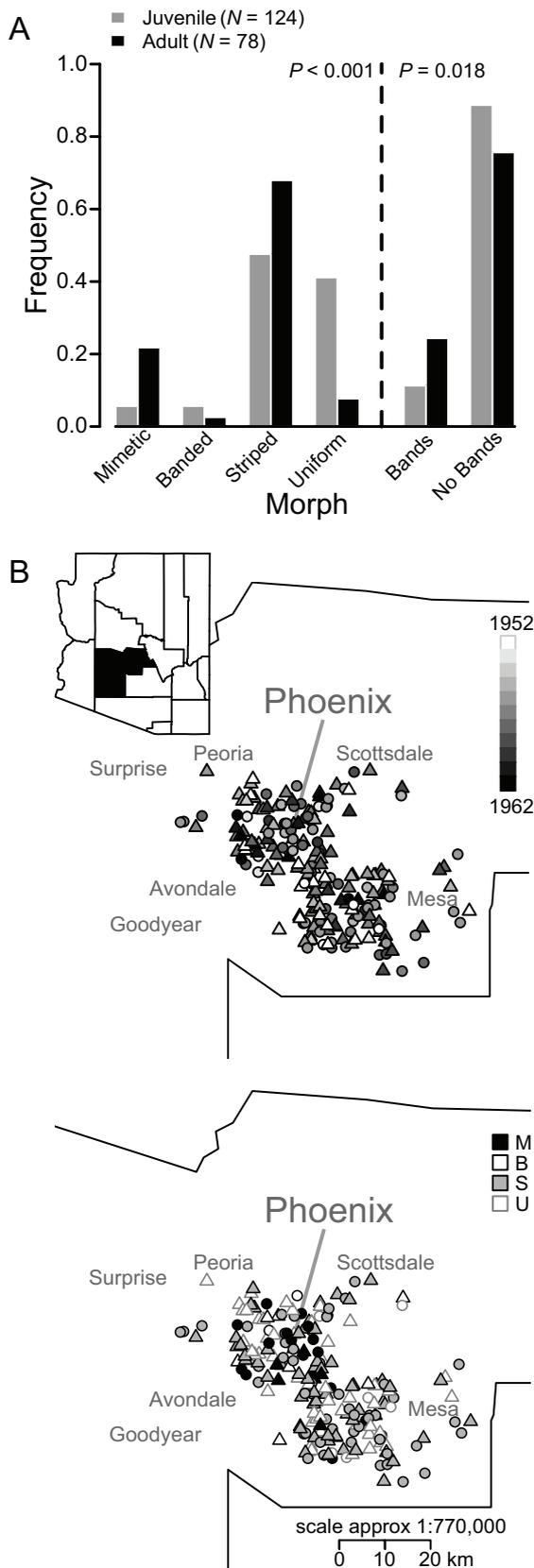
Age Class Variation. We found a dramatic difference in morph frequency between adults and juveniles in the Phoenix population (Fisher's exact test, $P < .001$). We found that there were far fewer uniform adults than juveniles and a corresponding greater frequency of mimetic and striped adults (fig. 5A, *left*). Even when considering only the presence and absence of black banding (see "Methods"), we still found significantly more (Fisher's exact test, $P = .018$) adults with bands compared to juveniles (fig. 5A, *right*). We did not detect any spatial ($\chi^2 = 0.0011$, df $= 1$, $P = .974$) or temporal ($\chi^2 = 1.4829$, df $= 1$, $P = .223$) bias in the distribution of adult and juvenile age classes across the greater Phoenix metropolitan area (fig. 5B, 5C), suggesting that these morph differences among age classes do not result from inadvertent analysis of a mixture of independent subpopulations (analogous to a Wahlund effect; Hartl and Clark 2007).

Temporal Variation. We found that the occurrence probabilities for each morph (a proxy for true morph frequency within a population) generally varied over time (fig. 6A–6E), although only two populations had a significant effect of time on occurrence probabilities (multinomial logistic regression, $P = .042$ for Maricopa County, AZ, and marginally significant effect at $P = .067$ for Presidio County, TX). Although these populations varied in the relative rank of morph frequencies and their patterns of change over time, we found that across populations the morphs that were the rarest at the beginning of each time interval increased in frequency over time. By comparing these observed values to randomized data simulations, we found that the rarest morphs clearly showed a greater increase over time than expected from simulated distributions ($P = .026$; fig. 6F). Although patchy sampling precluded robust statistical analysis of the remaining 24 populations with two to four morphs, we did qualitatively observe that morph presence/absence in these additional populations also suggests general stability in overall morph composition over time, despite similar shorter-term fluctuations in the relative morph frequencies themselves (fig. A2).

Figure 5: *A*, Frequency of color morphs (*left*) and black banding (*right*) among juvenile and adult *Sonora semiannulata* in the greater metropolitan area of Phoenix, Arizona. Note the dramatic difference in morph and band frequencies between juveniles and adults. *B*, Temporal (*top*) and morph (*bottom*) distribution of adults (circles) and juveniles (triangles) in the greater Phoenix metropolitan area (points have been randomly jittered around town midpoints to allow display of overlapping values). Inset map shows the position of Maricopa County within the state of Arizona. Note the homogeneous spatial and temporal distribution of size classes and color patterns in the area. M = mimetic, B = banded, S = striped, U = uniform.
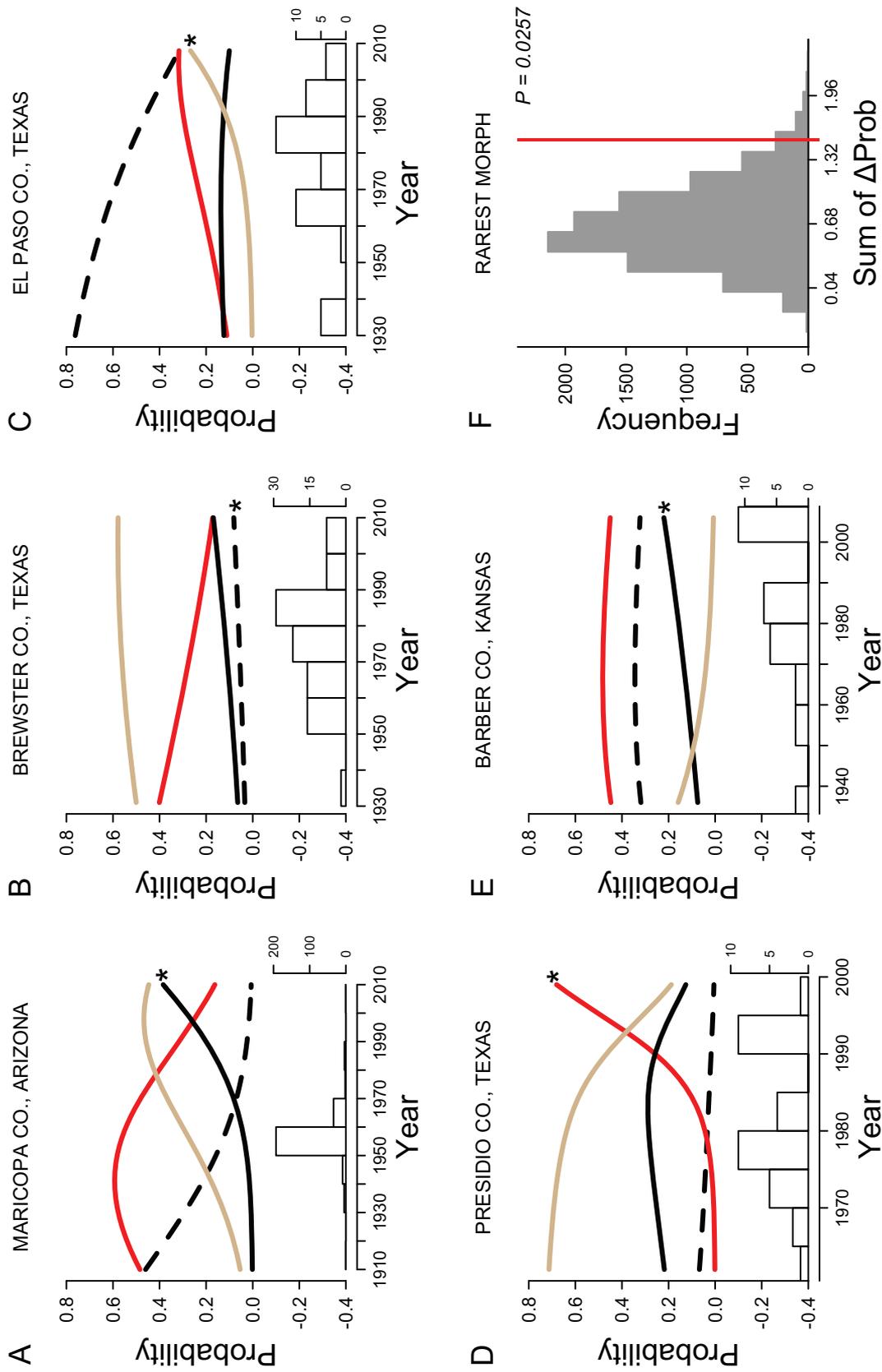
Figure 6: *A–E*, Predicted occurrence probabilities from multinomial logistic regression for striped (red), banded (solid black), uniform (tan), and mimetic (dashed) morphs over time for five populations. Rarest morphs at time = 0 are indicated with an asterisk. Histograms in each panel show the distribution of collected individuals over each population's time span, but note that histogram axis scales vary by population. *F*, Gray histogram depicting the frequency distribution of simulated test statistics, summing the change in probability for rarest morphs over time. Our observed value is indicated by the red line.

Discussion

Our study provides clear empirical data showing that strong selection promotes color polymorphism in ground snakes at multiple scales and from multiple lines of evidence. However, we found no evidence that conspicuousness of morphs was related either to geographic factors or to which color pattern was favored by selection. This research has important implications for understanding how evolutionary processes promote phenotypic variation at multiple scales, how and why certain types of polymorphism generate different patterns of geographic variation, and why explicit tests of temporal and geographic variation are critical to the interpretation of conspicuousness and mimicry.

Interpreting Patterns of Selection

Although the primary goal of this study was to discriminate among selective and neutral forces that govern the dynamics of polymorphism, we can further leverage our population genetic and demographic data sets to gain insight into the nature of selection and its mechanism of action on this polymorphism. However, many different terms have been used to describe spatial patterns of phenotypic variation and the selective forces that produce those patterns (balancing selection, divergent selection, diversifying selection, etc.); these terms have different meanings in different contexts, and they may describe both patterns of variation and the selective forces that generate variation. We chose to focus here on describing the outcome of selection: similarity or dissimilarity in phenotype across spatial and temporal scales. Our interpretation of mechanisms driving variation is centered on considering the combination of evolutionary forces that could plausibly generate our observed pattern of color polymorphism in ground snakes.

Mechanisms Driving Phenotypic Variation

An informative way to assess drivers of phenotypic diversity in *Sonora* is to compare the results from our locus comparison and population dynamics studies. Prior to the population genetic results of this study, the pattern of extraordinary variation in color within ground snakes could reasonably have been the result of neutral among-population genetic processes, especially in the context of a putative mimetic species now distributed widely beyond the range of its venomous model species. Surprisingly, we found no relationship between phenotypic and genetic variation within and among populations and a much greater F_{ST} for color pattern than neutral genetic markers in our locus comparison study, suggesting that strong selection drives color variation within and among populations. Although the evidence for selection is strong, the limitation of the locus-comparison approach is that it is only an indirect measure of selection. However, our population dynamics data set also suggests strong selection, showing clear differences in morph frequencies between age classes for the Phoenix population. We also found that morph frequencies within populations varied temporally, with the rarest morph tending to become more common over time in the classic signature of negative frequency dependence. Despite being a prime candidate for influential effects of population history, structure, and gene flow on the distribution of color morphs, the only neutral process driving color variation that we could detect in *Sonora* using different data sets and analytical approaches was a weak effect of isolation by distance on the presence or absence of morphs (but not black bands).

In fact, the difference in morph frequencies between age classes in the Phoenix population and the mismatch between the F_{ST} values for AFLP loci and color pattern are both quite dramatic. Although the long-term persistence of color patterns (e.g., uniform) experiencing very low survivorship to the adult stage in Phoenix may superficially seem counterintuitive, we offer two simple explanations for this pattern that may well work in concert. First, negative frequency-dependent selection can quickly reverse the direction of selection, and we note this apparent reversal as the uniform morph increases in frequency over time in the Phoenix population (fig. 6A). As a contributing factor during periods of negative selection, the persistence of the uniform morph within a population may also be prolonged through recessive null alleles carried by any of the other three color morphs, given the likely model of simple Mendelian inheritance of red and black pigmentation (see "Study System"). In our locus comparison study, although we found that the F_{ST} for color pattern was much greater than that for AFLPs (0.54 compared to 0.056), this is still close to the range of values (0.008–0.437 for color pattern, 0.01–0.14 for neutral markers) obtained in similar studies (Gillespie and Oxford 1998; Andres et al. 2000; Hoffman et al. 2006; Jorgensen et al. 2006; Abbot et al. 2008; Croucher et al. 2011). Thus, we consider our finding of such strong selection using multiple types of data to simply underscore the strength of the patterns that we report and represent a rare contribution from snakes to the more general observation that variation in color pattern is rarely driven by neutral processes.

One of the main goals of our research was to assess whether the processes generating variation within populations were the same as those driving among-population variation. Again, the key in this assessment is to compare the predicted patterns of divergence or similarity in phenotype expected under different selective mechanisms. We

suggest that there are two selective mechanisms that, working together, could simultaneously account for the discordance between genetic and phenotypic markers, the age-class differences in morph frequencies, and temporal variation in morph frequency: negative frequency dependence and spatially and temporally heterogeneous selection.

Negative frequency-dependent selection can create contrasting patterns of phenotypic variation at different scales. This type of selection should increase variation within populations by promoting polymorphism but increase similarity among populations by maintaining the presence of all color morphs in all populations. However, stochastic temporal asynchrony among populations should generate variation in their instantaneous position within the cycle and drive discrepancies in color morph frequencies among populations (Joron and Mallet 1998; Gray and McKinnon 2006; Sinervo et al. 2007). Our finding that the identity of the rare morph varied greatly among populations but showed similar increases in frequencies over time is consistent with this prediction of frequency dependence. The ecological agents of this rare-morph advantage of ground snakes could include dietary wariness of their predators (Thomas et al. 2003; Franks and Oxford 2009) or potential Batesian mimicry of coral snakes (Mallet and Joron 1999). We note that ground snakes are preyed on by various birds (Coulombe 1971; Kochert et al. 1979; Steenhof 1983) that both exhibit dietary wariness (Thomas et al. 2004) and are considered important drivers of coral snake mimicry systems (Greene and McDiarmid 1981; Brodie 1993). Although frequency dependence should maintain polymorphism widely, we found that more than half of the populations had fewer than four morphs. However, the number of morphs did not depend on the amount of genetic variation within a population or genetic distance between populations, so we suggest that processes other than population bottlenecks and gene flow must be invoked to explain this result.

Likewise, spatial and temporal heterogeneity in the type, strength, or direction of selection could generate a similar pattern of divergence across populations (Levins 1962, 1964), driving even geographically proximal populations toward different numbers of color morphs. However, such heterogeneity would not predict a consistent advantage to the rarest morphs within populations, which is what we observed in this study. Thus, neither negative frequency-dependent selection nor spatial and temporal heterogeneity alone are sufficient to explain the distribution of color morphs in ground snakes. However, the combination of these two selective forces is a reasonable primary mechanism driving phenotypic diversity in *Sonora* and could generate our observed pattern of extreme variation across spatial and temporal scales.

The pattern of color polymorphism within and among populations in ground snakes can be considered in the context of a geographic mosaic (Thompson 1994; Thompson 2005) that unites within- and among-population dynamics across a landscape. This theory predicts geographic and temporal variation in selection, which would produce phenotypic variation among populations (Brodie et al. 2002; Thompson 2005; Pfennig and Mullen 2010). Our results provide an empirical example of dramatic color polymorphism in a vertebrate that fits the theoretical expectations of a geographic mosaic.

Predicting Variation: Comparison to Geographic Variation in Other Polymorphic Species

Although it was initially surprising that we found no geographic effects on the distribution of morphs across the range of *Sonora*, comparison of our results to other polymorphic systems yields interesting insight into why number and conspicuousness of morphs may vary. While the maintenance of multiple morphs within single populations is fairly well studied (Gillespie and Oxford 1998; Nachman et al. 2003; Pryke and Griffith 2006; Rosenblum 2006; Abbot et al. 2008), less is known about when and why we should expect certain types of phenotypic variation and not others (mostly via theoretical models; Forsman et al. 2008; Franks and Oxford 2009, 2011). However, our comparisons of empirical data suggest that systems with only cryptic morphs (often a dark/light or patterned/patternless polymorphism) tend to have (1) fewer morphs and, more importantly, (2) a clinal pattern of geographic variation in which morphs are regionally common (especially in birds [Galeotti et al. 2003]; also in rock rattlesnakes [Vincent 1982; Farallo and Forstner 2012], spittlebugs [Stewart and Lees 1987], beach mice [Hoekstra et al. 2006], iguanid and teiid lizards [Rosenblum 2006], and skinks [Chapple et al. 2008]). In contrast, species with bright color morphs (under either sexual or natural selection) tend to (1) have a greater number of distinct color types (Franks and Oxford 2009) and (2) show no effect of geography, with either rampant variation in the composition of the polymorphism distributed across the species' entire range (this study; also ant-mimicking spiders [Ceccarelli and Crozier 2007], poison frogs [Rudh et al. 2007], *Papilio* butterflies [Clark and Vogler 2009], click beetles [Feder and Velez 2009], side-blotched lizards [Corl et al. 2010], killifishes [Dorn et al. 2011], and toads [Bonansea and Vaira 2012]) or the widespread maintenance of all morphs across all populations (*Theridion* spiders [Gillespie and Oxford 1998; Croucher et al. 2011], Gouldian finches [Gilby and Pryke 2009], and damselflies [Andres et al. 2000; Gosden et al. 2011]). There are exceptions to this pattern (e.g., grasshoppers [Rowell 1972] and frogs [Hoffman and Blouin

2000]), and as noted previously conspicuousness is not always easy to categorize (Bond 2007). However, these strikingly different geographic patterns suggest that the mechanisms generating variation in systems with multiple cryptic morphs may be quite different from those incorporating any conspicuous types. Specifically, geographic variation in cryptic morphs may be driven more by coarse-grained, relatively stable spatial heterogeneity, while geographic variation in species with conspicuous morphs is more likely to either be driven by unstable, fine-grained heterogeneity or be more influenced by pure frequency dependence rather than by exogenous spatial factors. Robust empirical data on spatial and temporal patterns of polymorphism can be difficult to obtain, especially for vertebrates, but they are essential for interpreting drivers of phenotypic variation.

Conspicuousness, Polymorphism, and Mimicry

We did not find evidence that coral snake sympatry influenced the presence or prevalence of the red and black (mimetic) phenotype in *Sonora*. Indeed, all four morphs were found over the entire geographic range of ground snakes, even in such distant allopatry to coral snakes (for which they were initially mistaken; Erwin 1925) as the Snake River drainage in the Pacific Northwest. These results may suggest that *Sonora semiannulata* is not part of a mimicry system, but then the widespread persistence of a conspicuous color morph in the presence of such strong selection is difficult to explain. Alternatively, *S. semiannulata* may be part of a mimicry system, but the benefits of red and black coloration extend beyond the geographic overlap with coral snakes (e.g., due to innate avoidance or to wide-ranging or migrating avian predators; Waldbauer 1988; Pfennig and Mullen 2010). If this is the case, then the high frequencies of other morphs across their geographic range remain enigmatic.

While our results can neither confirm nor reject the role of coral snake mimicry in the evolutionary dynamics of color pattern in ground snakes, they do suggest more complex selective factors than simple sympatry and allopatry with venomous species of similar color patterns. However, this complexity should be unsurprising considering the prevalence of color polymorphism in well-characterized mimicry systems, both within models and mimics (e.g., butterflies [Nijhout 2003], coral snakes and their mimics [Brodie and Brodie 2004], ant-mimicking spiders [Ceccarelli and Crozier 2007; Nelson 2010], and poison frogs [Darst and Cummings 2006; Wang and Shaffer 2008]). Because high levels of spatial and temporal variation in selection regimes are common within mimicry systems (Waldbauer 1988; Joron and Mallet 1998; Mallet and Joron 1999; Gilbert 2003), complex patterns of phenotypic var-

iation may be a fundamental outcome of mimetic interactions across taxa. A key way to test such ideas is to collect extensive geographic and temporal phenotypic data in systems with enough morphological variation to allow meaningful inferences about evolutionary processes. Our study suggests that despite the deceptively simple logic behind how selection drives color polymorphism, there may be surprising complexity in the forces that determine the distribution of phenotypes over space and time.

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