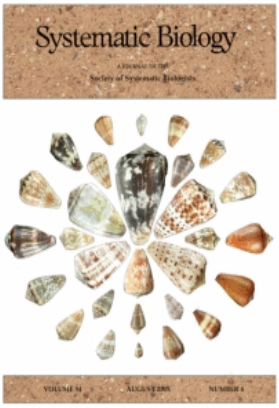

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Adding More Ecology into Species Delimitation: Ecological Niche Models and Phylogeography Help Define Cryptic Species in the Black Salamander (**Aneides flavipunctatus**)

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Adding More Ecology into Species Delimitation: Ecological Niche Models and Phylogeography Help Define Cryptic Species in the Black Salamander (*Aneides flavipunctatus*)

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Abstract.— Being able to efficiently and accurately delimit species is one of the most basic and important aspects of systematics because species are the fundamental unit of analysis in biogeography, ecology, and conservation. We present a rationale and approach for combining ecological niche modeling, spatially explicit analyses of environmental data, and phylogenetics in species delimitation, and we use our methodology in an empirical example focusing on *Aneides flavipunctatus*, the black salamander (Caudata: Plethodontidae), in California. We assess the relationships between genetic, environmental, and geographic distance among populations. We use 11 climatic variables and point locality data from public databases to create ecological niche models. The suitability of potential contact zones between parapatric lineages is also assessed using the data from ecological niche modeling. Phylogenetic analyses of portions of the mitochondrial genome reveal morphologically cryptic mitochondrial lineages in this species. In addition, we find that patterns of genetic divergence are strongly associated with divergence in the ecological niche. Our work demonstrates the ease and utility of using spatial analyses of environmental data and phylogenetics in species delimitation, especially for groups displaying fine-scaled endemism and cryptic species. [*Aneides*; California; Maxent; niche modeling; salamanders; species delimitation.]

Integrating genetic and ecological approaches in the study of mechanisms driving geographic distributions of organisms is becoming more common (e.g., Hugall et al., 2002; Johnson and Cicero, 2002; Graham et al., 2004a; Lapointe and Rissler, 2005). This is partly due to the ever-increasing amount and accuracy of natural history collections' (NHCs) point-locality data, accessibility of public databases housing fine-scaled climate data, and new modeling techniques (Graham et al., 2004b; Hijmans et al., 2005; Elith et al., 2006). The use of such data in environmental niche modeling (Soberón and Peterson, 2005) is proving to be a powerful approach to understanding how abiotic factors (e.g., temperature, precipitation, and seasonality) impact the geographic limits of lineages and species (Graham et al., 2004b; Wiens and Graham, 2005).

New spatially explicit methods that combine bioclimatic information and presence-absence locality information from natural history databases are useful for understanding the ecological processes driving biodiversity patterns (Graham et al., 2004b; Elith et al., 2006). For example, environmental niche modeling uses georeferenced data from specimen records in combination with environmental data layers, usually in a GIS framework, using diverse algorithms (see Elith et al., 2006, for details on alternative algorithms), to identify areas of predicted presence on a map (Soberón and Peterson, 2005). This correlative approach identifies areas that are ecologically similar to regions where the point locality information was used to build the models. These predicted regions of occurrence represent the ecological niche, and they are presumed to contain the suite of environmental conditions necessary to maintain a viable population (Grinnell, 1917, 1924; Hutchinson, 1957; Graham et al., 2004b). Whether this predicted region is a true spatial representation of the fundamental or realized niche has been a point of controversy (Kearney and Porter, 2004; Soberón and Peterson, 2005; Araújo and Guisan, 2006), but in general, the "fundamental niche" has been defined as the environmental space where fitness is greater than or equal to one, in the absence of range-limiting biotic interactions and dispersal barriers (Hutchinson, 1957; see Chase and Leibold, 2003, for a detailed review of the niche concept). Because correlative models take presence data from natural history databases, and thus implicitly incorporate biotic interactions that are dependent on the abiotic variables, researchers have argued that ecological niche modeling more correctly identifies the "realized niche" (Kearney and Porter, 2004; Soberón and Peterson, 2005; Araújo and Guisan, 2006; Kearney, 2006).

Results from this type of correlative ecological niche modeling have provided insight into a variety of questions relevant to conservation and evolutionary biology. These include (1) the importance of niche conservatism to speciation (e.g., Peterson et al., 1999; Kozak and Wiens, 2006); (2) the geographic spread of invasive species (e.g., Peterson, 2003; Peterson and Robins, 2003); (3) distributions of undiscovered species (Raxworthy et al., 2003); (4) pinpointing regions of high endemicity and conservation value for genetic lineages (Rissler et al., 2006); (5) inferences of historical and future distributions (Hugall et al., 2002; Peterson et al., 2002; Thomas et al., 2004; Araújo et al., 2006; Hijmans and Graham, 2006); and (6) historical biogeography (Wiens et al., 2006). One area where niche modeling has not been used extensively is phylogeography (but see Hugall et al., 2002), although there is potential for niche modeling to provide insight into the abiotic factors affecting the geographic limits of genetic lineages and to shed light on whether speciation or genetic variation is associated with divergence in the ecological niche (Graham et al., 2004b; Kidd and Ritchie, 2006).

Historical biogeography and comparative phylogeography, in part, seek to explain patterns of geographic congruence in phylogenetic breaks across multiple taxa. The processes driving lineage divergence, speciation,

and the buildup of biodiversity are many and include geographical, historical, and environmental factors that favor isolation and the cessation of gene flow between populations (Wiens, 2004). Recent work on modes of speciation has examined geographic, genetic, and climatic patterns across sister species in the hope of inferring the mechanisms driving differentiation (e.g., Kozak and Wiens, 2006). This approach has been somewhat controversial because it assumes that present-day distributions can provide information on the ancestral species at the time of speciation. Perhaps more importantly, and more relevant for the work in this paper, combining niche modeling and genetic analyses can provide some insight into the role of ecological divergence in speciation, regardless of the particular geographic mode of speciation (i.e., parapatric, sympatric, allopatric).

In addition, an understanding of the mechanisms driving divergence can also help in species delimitation. By combining information on geographic distributions, ecological niche models based on environmental data, and genetic information from multiple loci, researchers can make strong inferences when diagnosing species (Wiens and Graham, 2005). For example, two genetically unresolved lineages, with unique ecological niches, that are geographically distributed either parapatrically or allopatrically warrant further study to determine if the intervening region at the contact zone contains suitable habitat. If there is no suitable habitat in the contact zone, then gene flow between the lineages may be impeded, and this would support the conclusion that the two lineages are distinct, even with limited genetic divergence. However, if those two lineages had similar ecological niches with no biogeographic barrier separating their distributions and high-quality habitat for one or both at the contact zone, then it is less likely that the two lineages are distinct with no gene flow. Of course, the extent of gene flow in a suitable contact zone is still dependent on the level of reproductive isolation among lineages, but this information is often difficult to obtain. Therefore, an understanding of geographic distributions, environmental niche dimensions, and genetic patterns may help in species delimitation.

Species delimitation can be especially difficult for organisms displaying cryptic morphological characters and fine-scaled endemism patterns. Most species have been diagnosed using morphological data, but the use of genetic data has led to the discovery of many unique, morphologically cryptic lineages (Sites and Marshall, 2003, 2004). Recent analyses have demonstrated high concordance in patterns of global terrestrial endemism across taxa (Brooks et al., 2006), but it is unclear whether or not conservation measures based on classically defined species adequately reflect evolutionary lineages (Agapow et al., 2004; Rissler et al., 2006). In addition, true biodiversity hotspots can be missed if taxonomic lists do not adequately reflect nature (Köhler et al., 2005). Therefore, it is important to develop methods that can be used efficiently and easily to aid in species delimitation.

In this paper we use the salamander species *Aneides flavipuncatatus* to demonstrate a general approach for combining ecological niche modeling and phylogenetics in species delimitation. Additional goals are to (1) examine phylogeographic patterns in *A. flavipunctatus*, a species for which there have been no previous mitochondrial analyses; (2) develop ecological niche models using georeferenced museum locality data and environmental data layers; and (3) assess whether genetic variation across lineages is associated with divergence in the ecological niche.

General Approach

Our view of species follows the evolutionary species concept (ESC) and general lineage species concept (GLC) (Wiley, 1978; de Queiroz, 1998), and our goal is to recognize historically distinct evolutionary lineages that are likely to remain distinct (e.g., Wake, 2006). We present a general approach to combining spatially-explicit environmental data with phylogenetic information in Figure 1 for aiding species delimitation. In general, one needs to (1) define the potential phylogenetic lineages that may deserve species' status; (2) determine the extent of ecological divergence for these lineages; and (3) assess whether the contact zones between lineages contain suitable habitat that may impede or facilitate gene flow.

Combining independent sets of data (ecological and genetic) can provide a more robust view of the independence of evolutionary lineages. Our approach focuses on adding information on ecological divergence because this is often an important step in the process of speciation (Mayr, 1947; Van Valen, 1976; Andersson, 1990; Streelman and Danley, 2003; Funk et al., 2006), and the information is available from public databases (Graham et al., 2004b; Hijmans et al., 2005; Elith et al., 2006). Information on the ecological niche can be especially important when other data (e.g., multiple genetic loci or behavioral) are lacking or insufficient to determine whether the lineages in question are truly distinct. Therefore, we consider those phylogenetic lineages that are also separated in ecological space as distinct evolutionary lineages that are likely to remain distinct; i.e., species.

Materials and Methods

Species of Interest

Aneides flavipunctatus is endemic to the Californian Floristic Province and is distributed north of San Francisco Bay along the California coast and northeast into Shasta County, with an isolated and southern disjunct population around Santa Cruz, north of Monterey Bay. The range also extends northward into the Applegate Valley of extreme southern Oregon (Blackburn et al., 2001).

Tissue samples from frozen (−80°C) collections at the Museum of Vertebrate Zoology supplemented live specimen collections. We analyzed 42 samples from 18 localities of *A. flavipunctatus*, and almost all individuals were geocoded (latitude and longitude) or had detailed locality information associated with the specimen (Table 1). Sampling covered the majority of the species' range.

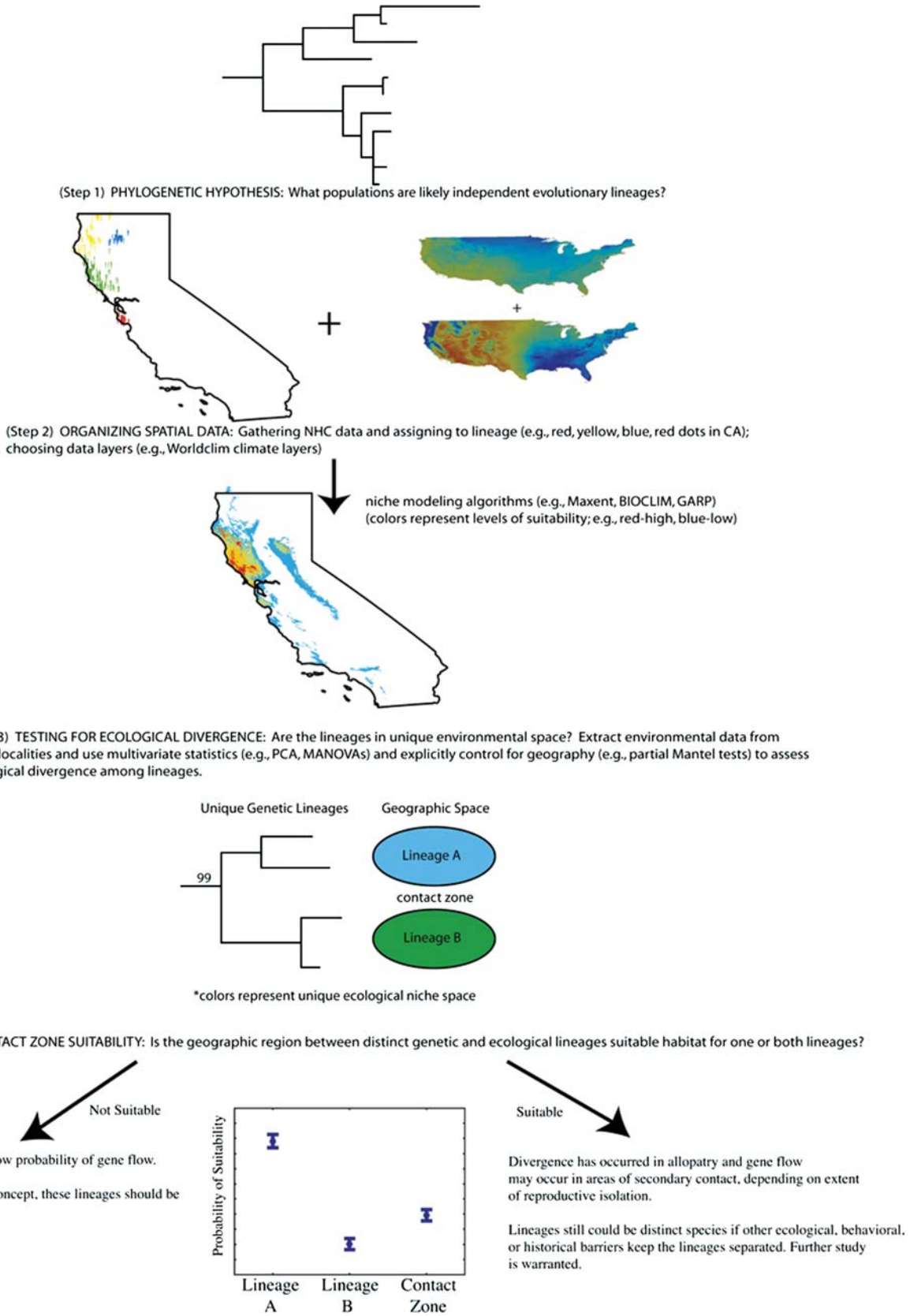

FIGURE 1. General schematic of the steps for using spatially explicit ecological data and phylogenetic information to aid in species delimitation.

TABLE 1. Locality information for samples used in genetic analysis. Tree codes correspond to Figure 3, and map codes correspond to Figure 4.

Museum number	County/parish	Lat.	Long.	Genbank (ND4)	Genbank (12S)	Tree code	Map code
MVZ231255	Santa Clara	37.260000	−122.10000	AY274658	AY274749	AF1	A
MVZ133028	Santa Clara	37.260000	−122.10000	AY274664	AY274755	AF2	A
MVZ98923	Santa Clara	37.260000	−122.10000	AY274661	AY274752	AF3	A
MVZ231248	Santa Clara	37.260000	−122.10000	AY274660	AY274751	AF4	A
MVZ231253	Santa Clara	37.260000	−122.10000	AY274663	AY274754	AF5	A
MVZ98922	Santa Clara	37.260000	−122.10000	AY274659	AY274750	AF6	A
MVZ219972	Shasta	40.864992	−122.03990	AY274654	AY274745	AF7	B
MVZ219971	Shasta	40.864992	−122.03990	AY274653	AY274744	AF8	B
MVZ221019	Shasta	40.911416	−122.20719	AY274649	AY274740	AF9	C
S-273 (MVZ)	Shasta	40.873257	−122.25932	AY274650	AY274741	AF10	D
MVZ133024	Shasta	40.873257	−122.25932	AY274651	AY274742	AF11	D
MVZ133025	Shasta	40.873257	−122.25932	AY274652	AY274743	AF12	D
MVZ231256	Shasta	40.873257	−122.25932	AY274656	AY274747	AF13	D
MVZ145014	Shasta	40.753579	−122.45930	AY274655	AY274746	AF14	E
MVZ145015	Shasta	40.753579	−122.45930	AY274657	AY274748	AF15	E
MVZ221016	Humboldt	40.848142	−123.92435	AY274647	AY274738	AF16	F
MVZ221018	Humboldt	40.846271	−123.89568	AY274646	AY274737	AF17	G
MVZ219974	Siskiyou	41.433626	−123.50524	AY274648	AY274739	AF18	H
MVZ219975	Siskiyou	41.433626	−123.50524	AY274643	AY274734	AF19	H
MVZ219973	Siskiyou	41.433626	−123.50524	AY274644	AY274735	AF20	H
MVZ217462	Trinity	40.881667	−123.55030	AY274645	AY274736	AF21	I
S-10760 (MVZ)	—	—	—	AY274634	AY274725	AF22	—
MVZ222663	Colusa	38.970826	−122.33890	AY274636	AY274727	AF23	J
MVZ222664	Colusa	38.970826	−122.33890	AY274635	AY274726	AF24	J
MVZ219969	Mendocino	39.847792	−123.70807	AY274642	AY274733	AF25	K
MVZ219970	Mendocino	39.847792	−123.70807	AY274640	AY274731	AF26	K
MVZ98924	Mendocino	39.742679	−123.79365	AY274641	AY274732	AF27	L
MVZ137133	Mendocino	39.249793	−123.11974	AY274624	AY274715	AF28	M
MVZ137134	Mendocino	39.249793	−123.11974	AY274625	AY274716	AF29	M
MVZ222519	Mendocino	39.335830	−123.20667	AY274665	AY274756	AF30	N
TJ-96	Mendocino	.	.	AY274626	AY274717	AF31	—
MVZ219977	Sonoma	38.589298	−122.92093	AY274623	AY274714	AF32	O
MVZ219978	Sonoma	38.589298	−122.92093	AY274637	AY274728	AF33	O
MVZ219979	Sonoma	38.589298	−122.92093	AY274638	AY274729	AF34	O
MVZ133023	Sonoma	38.693173	−123.02314	AY274639	AY274730	AF35	P
MVZ158442	Mendocino	39.065654	−123.43955	AY274627	AY274718	AF36	Q
MVZ158443	Mendocino	39.065654	−123.43955	AY274628	AY274719	AF37	Q
MVZ219966	Mendocino	39.065481	−123.43576	AY274630	AY274721	AF38	Q
MVZ219968	Mendocino	39.066308	−123.43576	AY274633	AY274724	AF39	Q
DBW5967 (MVZ)	—	—	—	AY274632	AY274723	AF40	—
MVZ222518	—	—	—	AY274629	AY274720	AF41	—
MVZ219967	Mendocino	39.180043	−123.69507	AY274631	AY274722	AF42	R

DNA Extraction and Sequencing

Extraction of DNA was done using the DNeasy tissue kit protocol (Qiagen, Valencia, CA). We sequenced portions of the ND4 and 12S regions in the mitochondrial genome. The valine transfer RNA region of the 12S gene was amplified using primers valB and valG (Rissler and Taylor, 2003). The ND4 region was amplified with primers designed for *A. flavipunctatus*: NADH-f (flav) (5′-GGGTATGGTATTATTCGAATT-3′) and NADH-r (flav) (5′-TGGGGCAGATAATTAGCAGT-3′). All PCR reactions were carried out in a volume of 25 μL containing 5 to 50 ng of genomic DNA, 2.5 μL PCR buffer, 0.5 μL of dNTPs (10 mM of each dNTP), 2.5 μL of 100 mM primer, 0.2 μL Taq, and water. Double-stranded PCR products were cleaned with a QIAquick PCR purification kit (Qiagen) or ExoSAP-IT (USB, Cleveland, OH) and labeled with fluorescent-dye labels through a cycle-sequencing reaction following standard protocols (Applied Biosystems, Perkin-Elmer). Cycle-sequencing products were cleaned with Sephadex columns and sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems). All samples were sequenced in both directions and then combined in Sequencher (version 4.5). We used ClustalW in MacVector (version 8.0) for sequence alignments. Alignments were further corrected manually, and there was no difficulty in aligning sequences in hypothesized loop regions of the 12S gene. The two mitochondrial genes were appended and treated as a single locus.

Genetic Analyses

We used maximum likelihood (ML; PAUP*4.0b10 [Swofford, 2000] and Garli v.0.951 (Zwickl, 2006]) and Bayesian inference (MrBayes 3.01 [Huelsenbeck and Ronquist, 2001]) to infer the phylogeny. The model of evolution was chosen by ModelTest 3.04 (Posada and Crandall, 1998) using the AIC values, and model parameters were used in ML analyses. ML analyses were conducted using a heuristic search with the chosen model, TBR branch swapping, and 10 random-addition replicates. Nodal support was assessed using bootstrap analysis with 100 replicates for ML and Bayesian posterior

probabilities (PP) for Bayesian analysis. For the Bayesian analyses, we used a flat Dirichlet probability density for the GTR rate matrix. Four incrementally heated Markov chains were run for 5.0 million generations, and trees were sampled every 5000 generations. The log-likelihood values of the chains fluctuated within a stable range of 0.008, and we plotted the ln-likelihood values against generation time to assess stationarity (data not shown). We allowed a burn-in of 2.5 million generations, and the remaining trees were used for measuring posterior probabilities of nodes. Data files have been uploaded to TreeBASE.org as accession number SN3580.

We defined lineages as those groups of populations that were monophyletic with good support values (>95% ML bootstrap and 1.0 Bayesian PP) and were distributed in distinct geographic space; other methods could have been used (e.g., Templeton, 2001; Wiens and Penkrot, 2002; Pons et al., 2006). Some potential lineages were not considered as potential species in our analyses because of low sample sizes and/or support (e.g., AF22-27, AF28-31; Table 1), but with additional sampling, other lineages/species within our "Central" lineage could become evident.

We used DnaSP 3.52 (Rozas and Rozas, 1999) to examine nucleotide diversity and other population genetic parameters within and across lineages. PAUP*4.0b10 was used to calculate maximum-likelihood corrected distances for comparisons of percent-corrected divergence across lineages.

Bioclimatic Modeling

Ecological niche models for *A. flavipunctatus* and its phylogeographic lineages were created using Maxent version 2.0 (Phillips et al., 2006). Maxent creates species distributional models by combining presence only data with ecological layers using a statistical approach known as maximum entropy. The maximum entropy approach estimates a species' environmental niche by finding a probability distribution that is based on a distribution of maximum entropy (with reference to a set of environmental variables). The principle of maximum entropy is useful for analyzing the available information to determine a unique probability distribution that assumes the least biased distribution encoding the given information is that which maximizes the information entropy. This method is equivalent to finding the maximum-likelihood distribution of a species (Phillips et al., 2004). Although Maxent is a new approach in species distributional modeling, it seems to perform better than other established methods like BIOCLIM, GARP, or DOMAIN (Phillips et al., 2004, 2006; Elith et al., 2006).

Maxent was run using point locality information from specimens in natural history museum collections summarized in two data portals, HerpNET (www.herpnet.org) and GBIF (www.gbif.org), combined with climatic layers downloaded from the WorldClim database (Hijmans et al., 2005). The WorldClim bioclimatic variables are biologically relevant temperature and precipitation layers (Hijmans et al., 2005). The WorldClim climate layers were created by interpolating observed climate from climate stations around the world, using a thin-plate smoothing spline set to a resolution of approximately 1 km, over the 30-year period from 1960 to 1990 (Hijmans et al., 2005).

To ensure that we did not overparameterize our niche models with redundant climatic information, we conducted a series of correlation tests intended to remove redundant variables. We extracted the environmental information from 50,000 randomly generated points from across the United States. Correlation matrices were then generated for all 19 variables within each of the two general climatic categories: temperature and precipitation. We used a Pearson correlation coefficient of 0.75 to identify highly correlated variables (Rissler et al., 2006). For pairs that were highly correlated we chose the variable that we considered more biologically meaningful and easier to interpret. Eleven variables were chosen and used in all subsequent analyses. These included BIO1 = annual mean temperature; BIO2 = mean diurnal range in temperature; BIO3 = isothermality (monthly/annual temperature range); BIO7 = annual range in temperature; BIO8 = mean temperature of wettest quarter of the year; BIO9 = mean temperature of driest quarter of the year; BIO15 = precipitation of seasonality (coefficient of variation); BIO16 = precipitation of wettest quarter of the year; BIO17 = precipitation of driest quarter of the year; BIO18 = precipitation of warmest quarter of the year; BIO19 = precipitation of coldest quarter of the year. Finally, each climate layer was entered into Maxent as an ASCII raster grid.

Models for *A. flavipunctatus* were created using a total of 502 specimen localities, and the unsampled populations were split into four classes as defined by the phylogeographic analyses to create lineage models. Similar methods were used in Rissler et al. (2006). All runs were set with a convergence threshold of 1.0E–5 with 1000 iterations, and the regularization value was set to 1.0E–4. The resultant ASCII file was placed into raster format using ArcGIS 9.1 and viewed. The predicted potential distribution (ecological niche) of each lineage was displayed in four distinct categories using the natural breaks method of classification (Jenks' method) in ArcGIS 9.1. This method assigns the data to classes using an iterative algorithm that minimizes the variance within classes and maximizes variance between classes.

Spatial Statistical Analyses.—We conducted principal components analyses (PCA) using each lineage with the values extracted for each climate layer to examine the overall levels of divergence in the ecological niche in STATISTICA v.6.0. We then used multivariate analyses of variance (MANOVA) with PCA axis scores as dependent variables and lineage as the fixed factors to determine whether separation in the ecological niche was statistically significant. Post hoc tests (e.g., Tukey's HSD) were used to understand which lineages differed, and we plotted the least-squares means (ls-means) to visually compare the differences in environmental variables across lineages.

It is also important to establish whether the associations are strictly due to spatial autocorrelation, and to do

this, the geographic location of specimens must be statistically controlled for in multivariate analyses. We used partial Mantel tests to examine the relationships between genetic and environmental distances while controlling for geographic distance. Significant, positive partial correlations support the conclusion that genetic divergence is congruent with climatic gradients (e.g., Kozak and Wiens, 2006). Statistical significance was assessed by 999 permutations in the R-package v. 4.0 (Casgrain and Legendre, 2001). In these tests, the genetic distance matrices were based on the maximum-likelihood distances, and the environmental distances were based on Euclidean distances of the factor scores from the PCA of the 11 climatic variables at each locality. Only individuals that had genetic, geographic, and environmental information were used in the partial Mantel tests (n = 39).

Contact Zone Analyses.—To determine whether the areas of potential geographic contact between parapatric lineages (contact zones) were broadly suitable for one or both lineages, we compared the probability of occurrence for each lineage in their respective ranges to those within the contact zone. If the contact zones are broadly suitable for both lineages, this suggests that environmental boundaries are not strong and gene flow could occur in the region, depending on the extent of reproductive isolation. On the other hand, if the two lineages are found in unique environmental space and separated by an inhospitable contact zone, then dispersal across this unsuitable region into an environmental space that may be outside of the physiological tolerance limits of the organisms may be unlikely. In fact, Kozak and Wiens (2006) suggest that even if lineages occupy the same environmental niche space, an unsuitable environment in the contact zone would still prevent gene flow.

We compared the contact zones of the following parapatric lineages: Northwest-Shasta and Northwest-Central. The ecological niche modeling technique (Maxent) provides probability of occurrence values for each grid cell (Phillips et al., 2006). Therefore, we extracted those probabilities for each historic collection locality from their respective ecological niche models. To assess contact zone suitability, we created 1,000 random points within a minimum convex polygon that spanned the contact zone (Fig. 2). Maxent probability values were then extracted from each lineage's ecological niche model within the contact zone polygon for those random points. Probability values for each lineage and contact zone were compared using ANOVAs in STATISTICA v.6.0. Low probability of occurrence values for one or both lineages in their contact zone is considered evidence that gene flow is unlikely between the parapatric lineages.

RESULTS

Gene Genealogies

Sequence lengths for the valine transfer region of the 12S gene averaged around 600 bp, and the ND4 region averaged around 730 bp. All sequences have been deposited in Genbank (Table 1). There were 296 parsimony-informative characters. The chosen model of evolution was the K81+Γ with base frequencies A = 0.3332, C = 0.2179, G = 0.1379, and T = 0.3110; substitution model A-C = 1.0000, A-G = 3.4425, A-T = 1.2944, C-G = 1.2944, C-T = 3.4425, and G-T = 1.000; and gamma distribution shape parameter equal to 0.6363.

All methods of phylogenetic inference identified the same lineages within *A. flavipunctatus*; therefore, we only present the ML topology (Fig. 3). This tree suggests that *A. flavipuncatus* can be divided into four lineages (Southern Disjunct, Shasta, Northwest, and Central). The ML tree was rooted with the Southern Disjunct lineage because it clearly is a monophyletic and geographically bounded unit that is the sister taxon of other lineages within *A. flavipunctatus* based on unpublished data (Rissler, unpublished) that includes multiple outgroups (including other species within the genus *Aneides* and other species within the families Plethodontidae, Ambystomatidae, and Salamandridae). The Shasta and Northwest California lineages are also distinct (Figs. 3, 4). The remaining samples include individuals from the central coastal region of California (Central lineage, Fig. 3, 4). High genetic diversity that is not geographically structured characterizes this lineage, yet there is a deeply diverged lineage found within Mendocino County (AF28–31; Fig. 3).

The four main lineages within *A. flavipuncatus* are quite distinct (Tables 2, 3). For example, there is an average of 66 nucleotide differences between the Southern Disjunct lineage and the Shasta lineage (Table 3). Even within a lineage, there is high haplotype diversity (Table 2). The Northwest lineage (Siskiyou, Humboldt, and Trinity counties) also has high haplotype diversity and number of nucleotide differences, despite a much smaller sample size. The Shasta lineage has the lowest number of nucleotide differences between individuals (Table 2), but it differs from each of the other lineages by an average of more than 29 nucleotide sites (Table 3). The Southern Disjunct lineage has both high haplotype and nucleotide diversity despite a very isolated geographic distribution.

Bioclimatic Modeling and Spatial Statistical Analyses

For the lineages within *A. flavipunctatus*, the ecological niche models had little overprediction, meaning the models did not predict broadly outside of the modeled populations, except for some common overprediction into the Sierra Nevada Mountains (Fig. 5). However, the Southern Disjunct lineage that was sampled only around Santa Clara County (Fig. 4, A on map), predicted an optimal range that extended north into the Bay Area. The PCA indicated that the phylogenetic lineages were predominately distributed in unique environmental space, with the Central and Northwest lineages showing the most overlap (Fig. 6). The Southern Disjunct lineage showed no overlap with any lineage, and the Shasta lineage had very little variation across the first two factor scores. The PCA scores differed significantly between the lineages (Wilks' lambda = 0.204; $F_{6,960} = 194.350$; $P < 0.000$), and post hoc tests revealed both factor 1 and factor

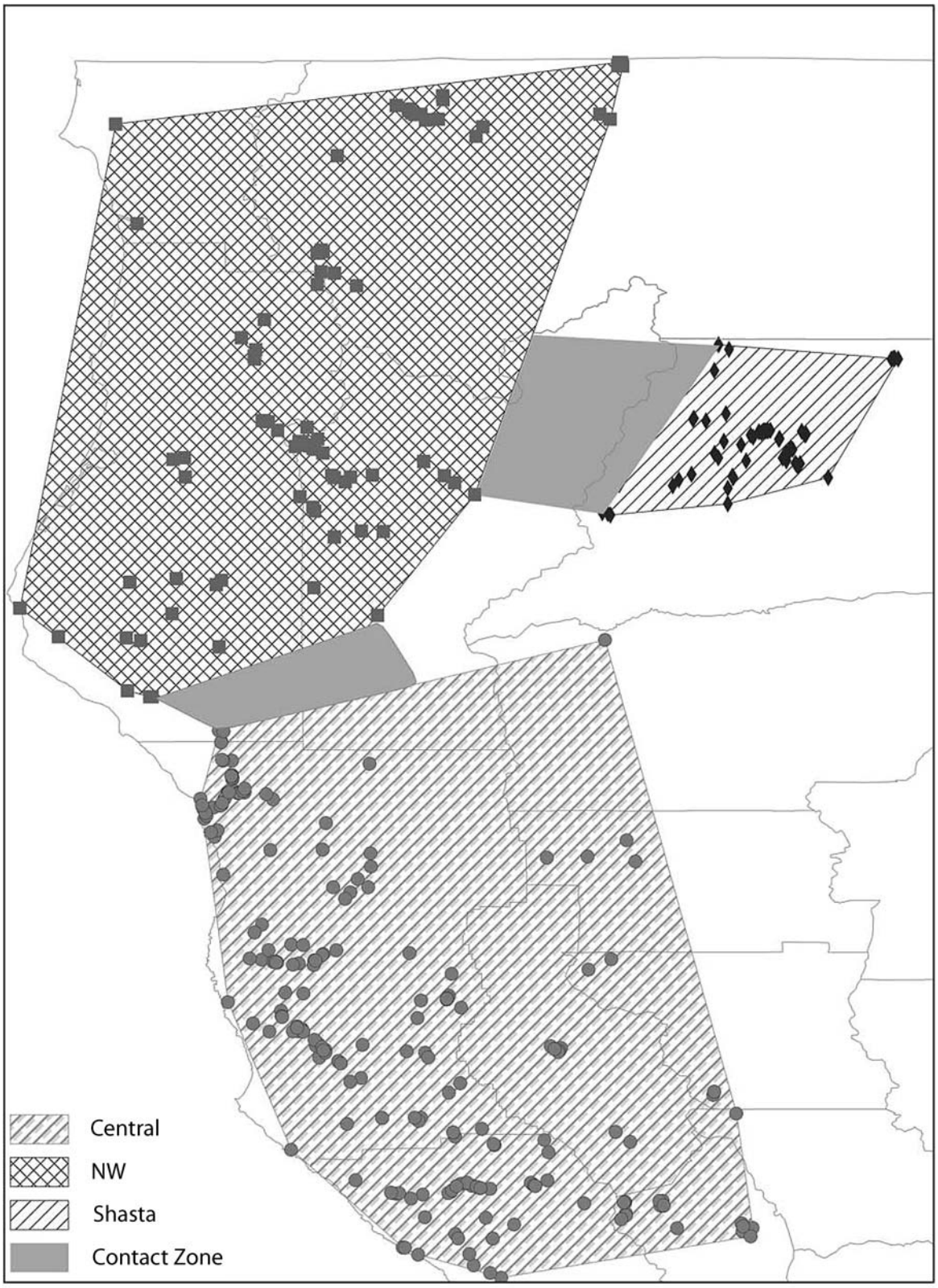

FIGURE 2. Minimum convex polygons around three parapatric lineages (Central, Northwest (NW), and Shasta) and the contact zones between the lineages shown in solid grey. Natural history collection (NHC) point localities are also shown for each lineage. Maxent probability values were extracted from the NHC points and 1,000 random points within each contact zone to compare the suitability of that region for each lineage (see text for detail).

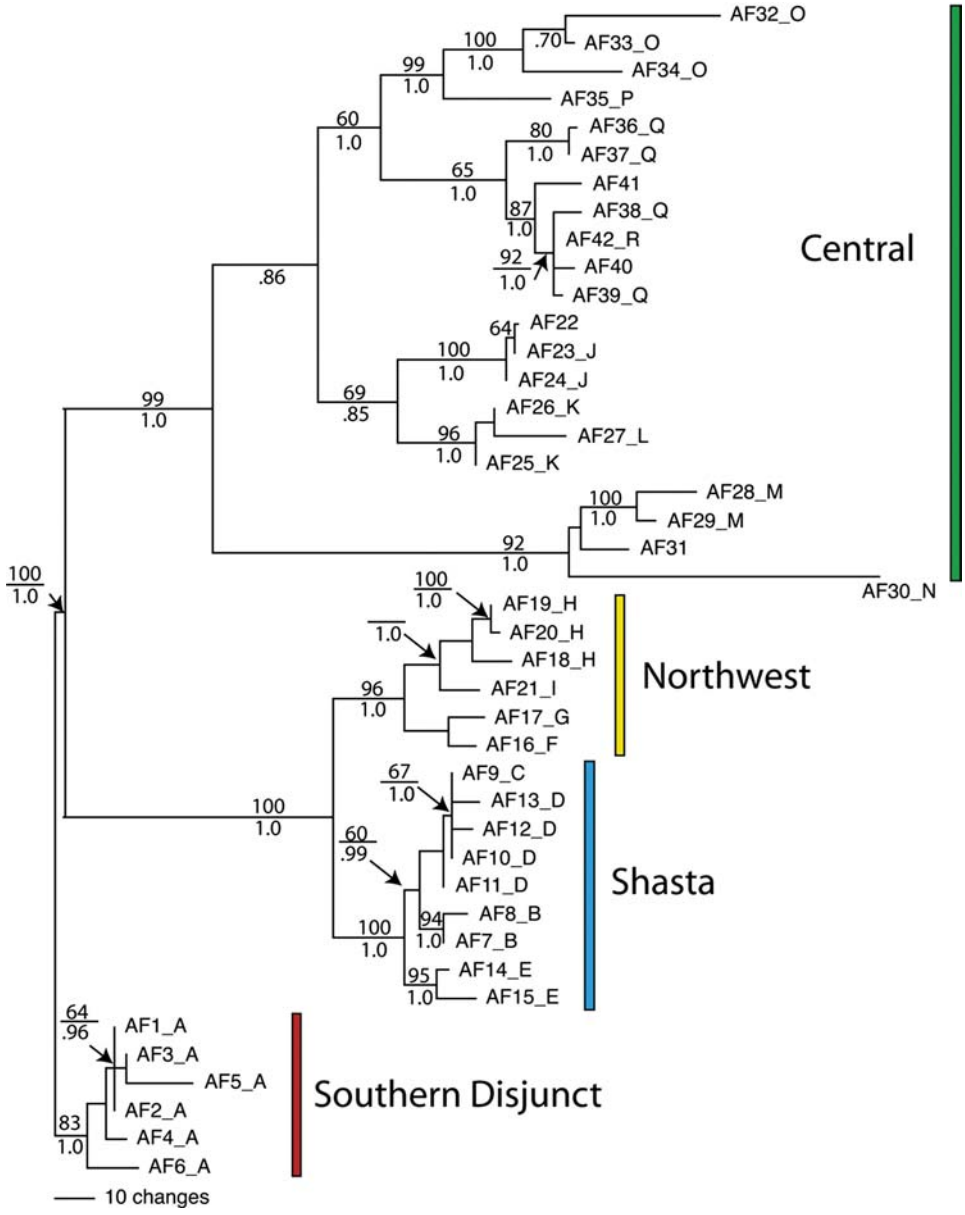

FIGURE 3. Phylogram resulting from a maximum likelihood (ML) analysis of the ND4 and 12S regions of the mitochondrial DNA. Numbers at nodes represent bootstraps from a ML analysis (above) and Bayesian posterior probabilites (PP; below). Bootstraps and PP are only placed on major nodes with at least 60% support. Localities are designated by the letter following the underscore for those with lat/long information, see Table 1 and Figure 4.

2 were significant. Six of the 11 climatic variables differed significantly across all lineages at $P < 0.000$ (Fig. 7).

The partial Mantel test that examined the relationship of environmental distance and genetic distance, while controlling for geographic distance, was significant ($r = 0.331$; $P = 0.002$). To determine if the Southern Disjunct lineage was driving that significance, we tested the relationship using only the other three lineages and found that the significant relationship held ($r = 0.155$; $P = 0.005$). However, when only the Shasta and Northwest lineages were included in the analysis, the relationship between environmental distance and genetic distance was not significant ($r = -0.008$; $P = 0.509$).

Contact Zone Analyses

For the first major contact zone (Northwest and Shasta), Maxent probabilities of all NHC locality points under the Northwest ecological niche model (Fig. 5) averaged around 78% for the Northwest points, less than 50% for the Shasta points, and about 34% for the 1000 random points in the contact zone (Figs. 2, 8a). In this comparison

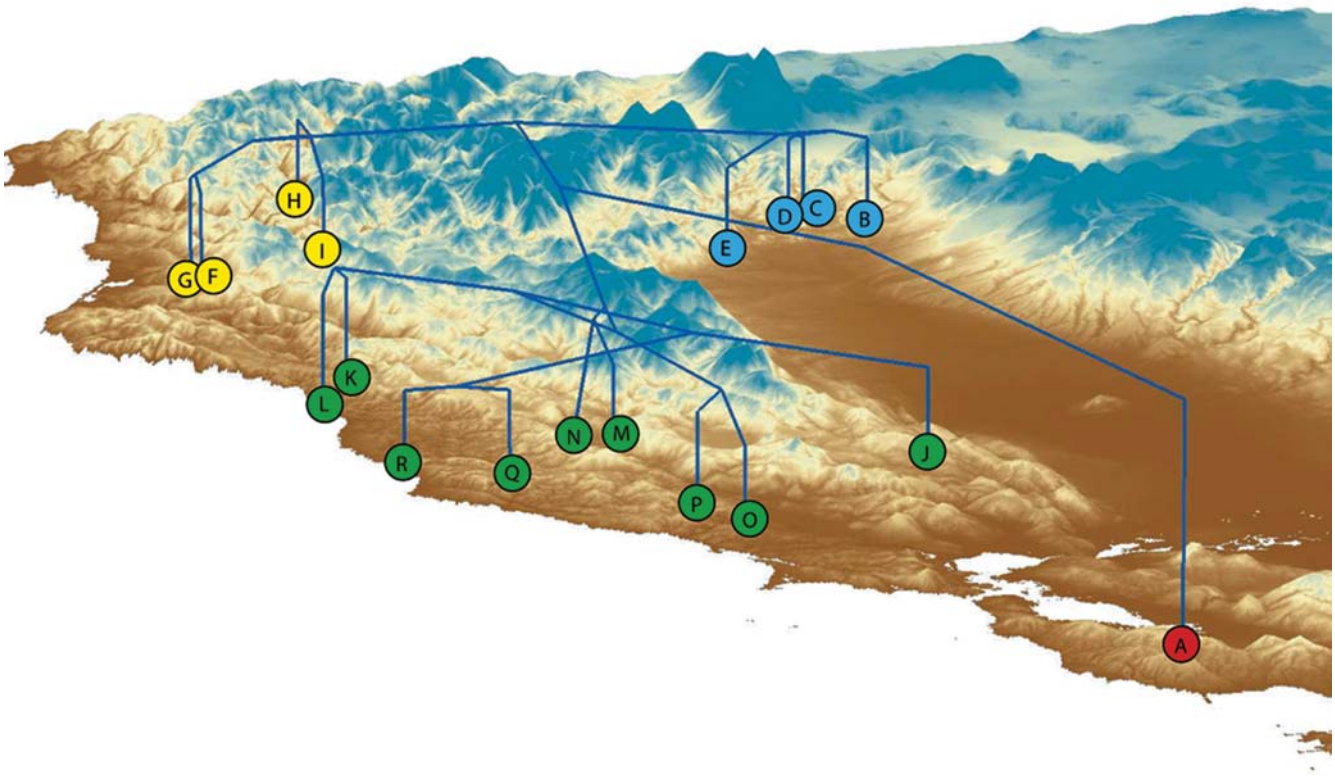

FIGURE 4. Two-dimensional spatial representation of the genetic information using GeoPhyloBuilder 1.0 (Kidd and Ritchie, 2006) and viewed using ArcGIS 9.1. Letters and colors correspond to Figure 3.

the contact zone was even less suitable for the Northwest lineage than the parapatric sister lineage's range. The overall ANOVA was significant ($F_{2,269} = 125.202$; $P < 0.001$), and all pairwise comparisons were significantly different from each other. The same comparison but using the Shasta ecological niche model (Fig. 5) averaged around 88% for the Shasta points, about 20% for the Northwest points, and about 38% for the random points in the contact zone (Figs. 2, 8b). The overall ANOVA was significant ($F_{2,269} = 274.130$; $P < 0.001$), and all pairwise comparisons were significant. Therefore, for the Shasta lineage, the contact zone was not suitable, nor was the range of the parapatric sister lineage.

For the second major contact zone (Central versus Northwest), Maxent probabilities of all NHC locality points under the Central ecological niche model (Fig. 5) averaged around 70%, about 25% for the Northwest points, and about 45% for the random points in the contact zone (Figs. 2, 8c). The contact zone in this case is relatively unsuitable at 45% for the Central lineage, and the range of the Northwest lineage is not suitable for the Central lineage. The overall ANOVA was significant ($F_{2,458} = 188.860$; $P < 0.001$), and all pairwise comparisons were significant. Under the Northwest ecological niche model (Fig. 5), Northwest points averaged around 78%, Central points less than 25%, but points in the contact zone averaged around 72%. Although the overall ANOVA was significant ($F_{2,\ 458} = 205.514$, $P < 0.001$), the important comparison of the contact zone points and the Northwest points did not differ (Figs. 2, 8d; $P = 0.3245$). Therefore, the contact zone is suitable for the Northwest lineage.

TABLE 2. Genetic diversity measures for all lineages as determined by the phylogenetic analyses (Fig. 3).

Group	n	Haplotype diversity (+SD)	*P* (+SD)	q	k
Aneides flavipunctatus	42	0.976 (0.012)	0.057 (0.004)	0.078	24.060
Southern Disjunct	6	0.524 (0.209)	0.033 (0.022)	0.047	20.667
Shasta	9	0.833 (0.127)	0.010 (0.003)	0.013	8.611
Northwest	6	1.000 (0.096)	0.020 (0.003)	0.019	22.067
Central	21	0.974 (0.022)	0.044 (0.005)	0.051	25.450

p = Nucleotide diversity; q = number of mutations per site; k = average number of nucleotide differences.

TABLE 3. Genetic diversity among lineages. Upper diagonal is % corrected divergence using the K81 + G model of evolution. Lower diagonal is the average number of nucleotide differences (k; Tajima 1983) of the raw data between phylogeographic lineages of *Aneides flavipunctatus* (as defined in the text).

	Shasta	Disjunct	Northern	Central
Shasta	0.00	9.36	4.47	9.46
Disjunct	66.15	0.00	8.61	9.10
Northern	29.46	59.39	0.00	9.48
Central	40.92	38.91	44.07	0.00

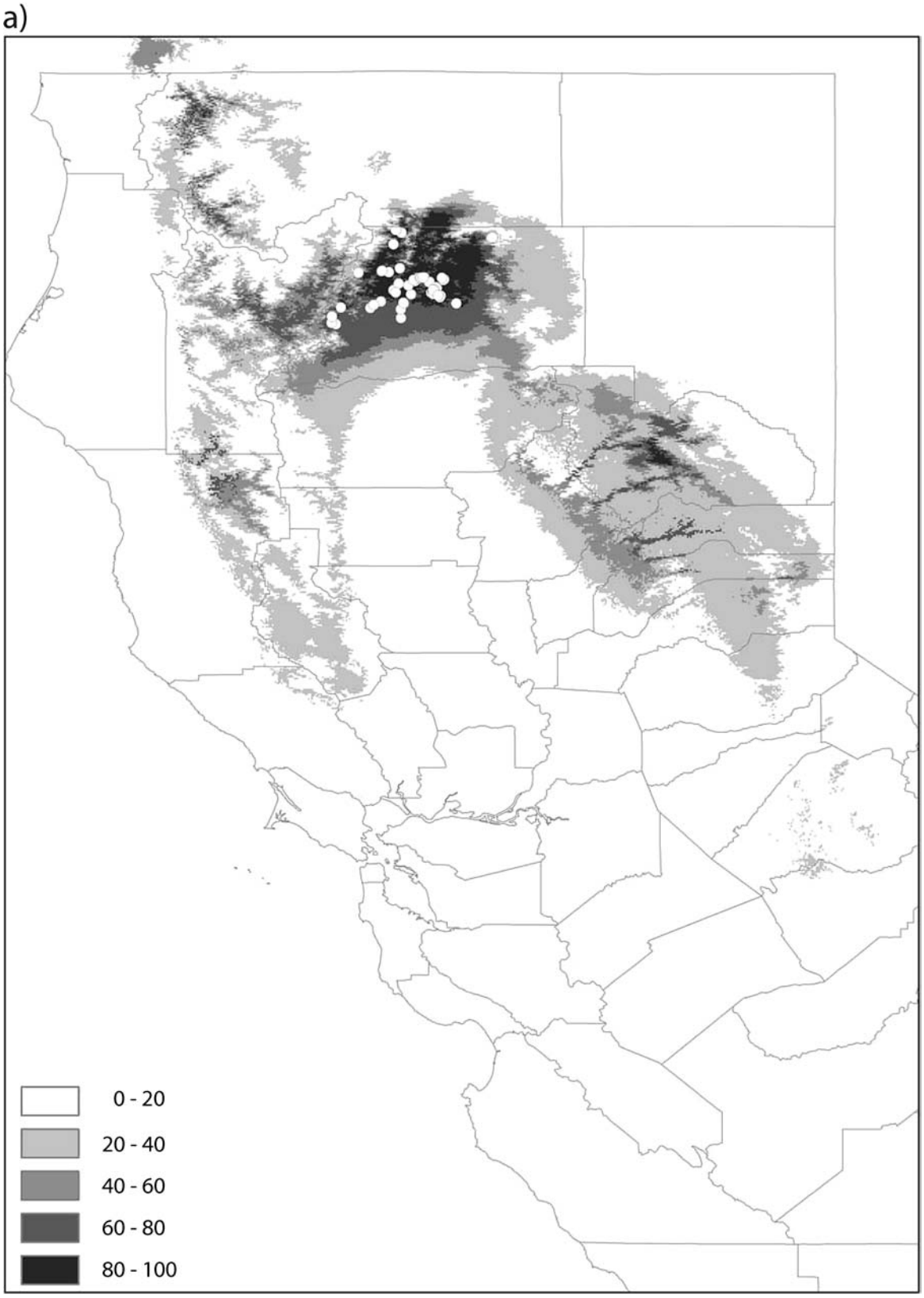

FIGURE 5. Ecological niche models for lineages in *Aneides flavipunctatus* using 11 WorldClim data layers. Locality points are given in white circles, and Maxent probability value levels are listed in a graded series of grey to black. (a) Shasta lineage; (b) Northwest lineage; (c) Central lineage; (d) Southern Disjunct lineage. *(Continued)*

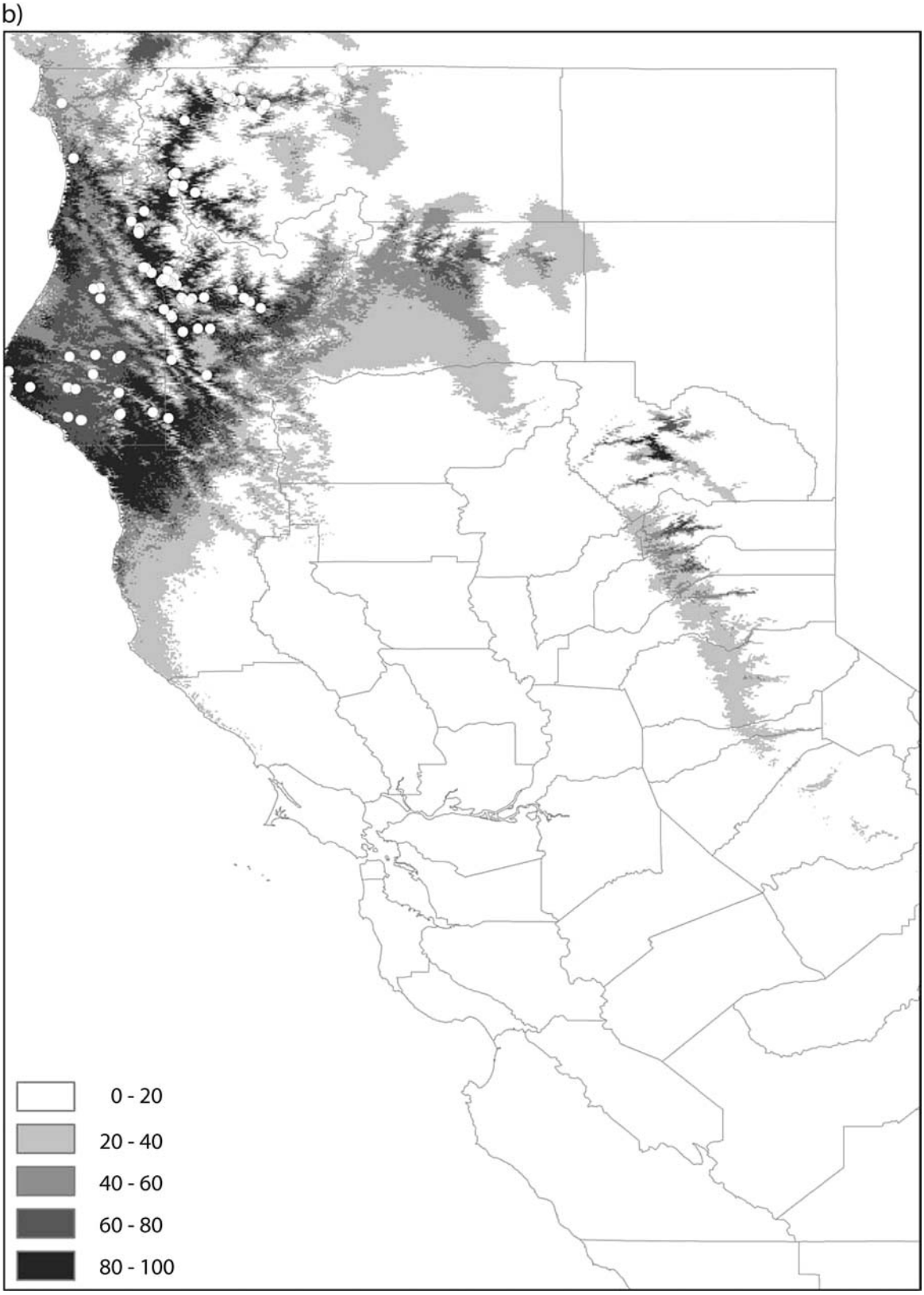

FIGURE 5. (Continued).

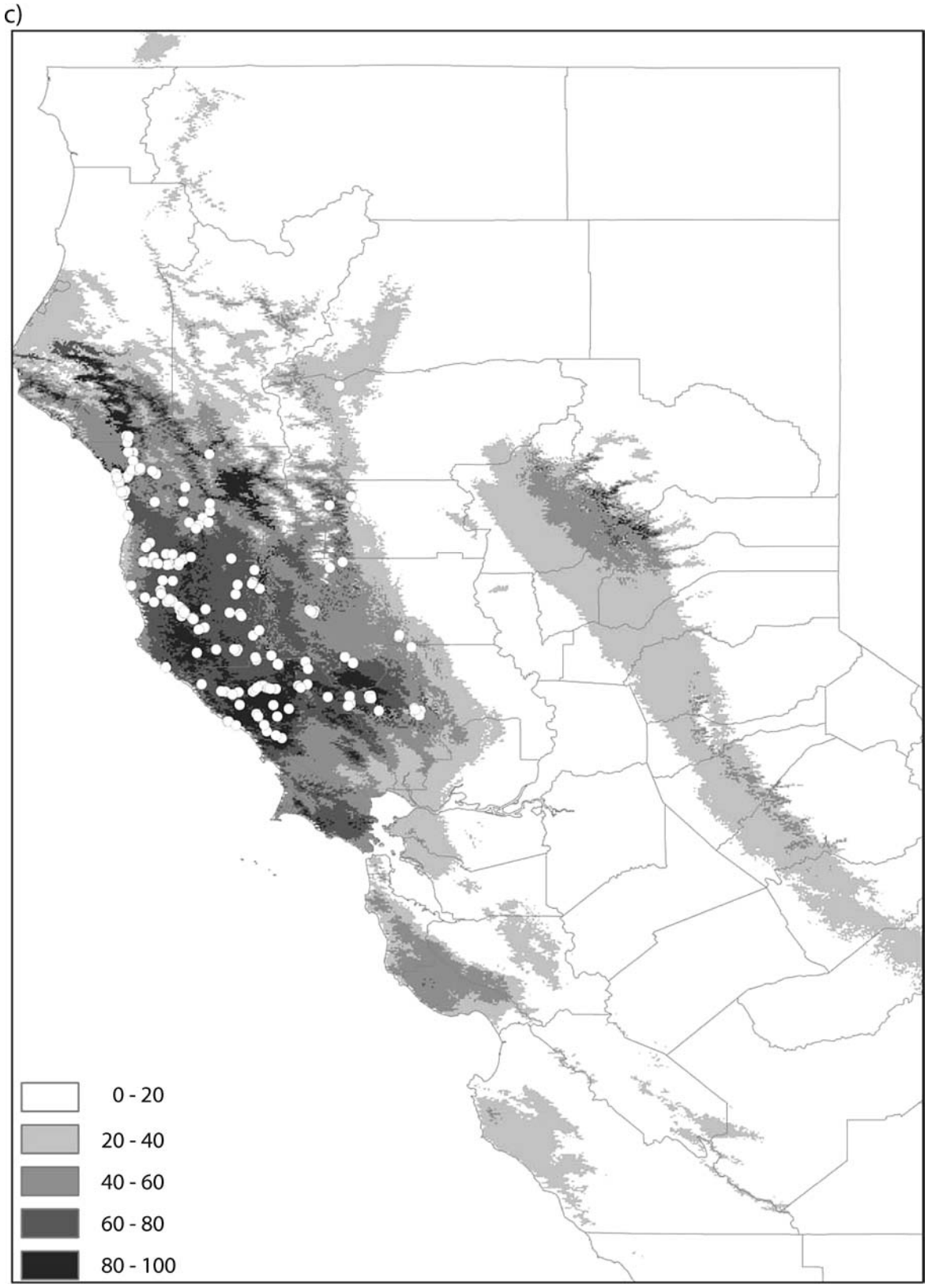

FIGURE 5. (Continued).

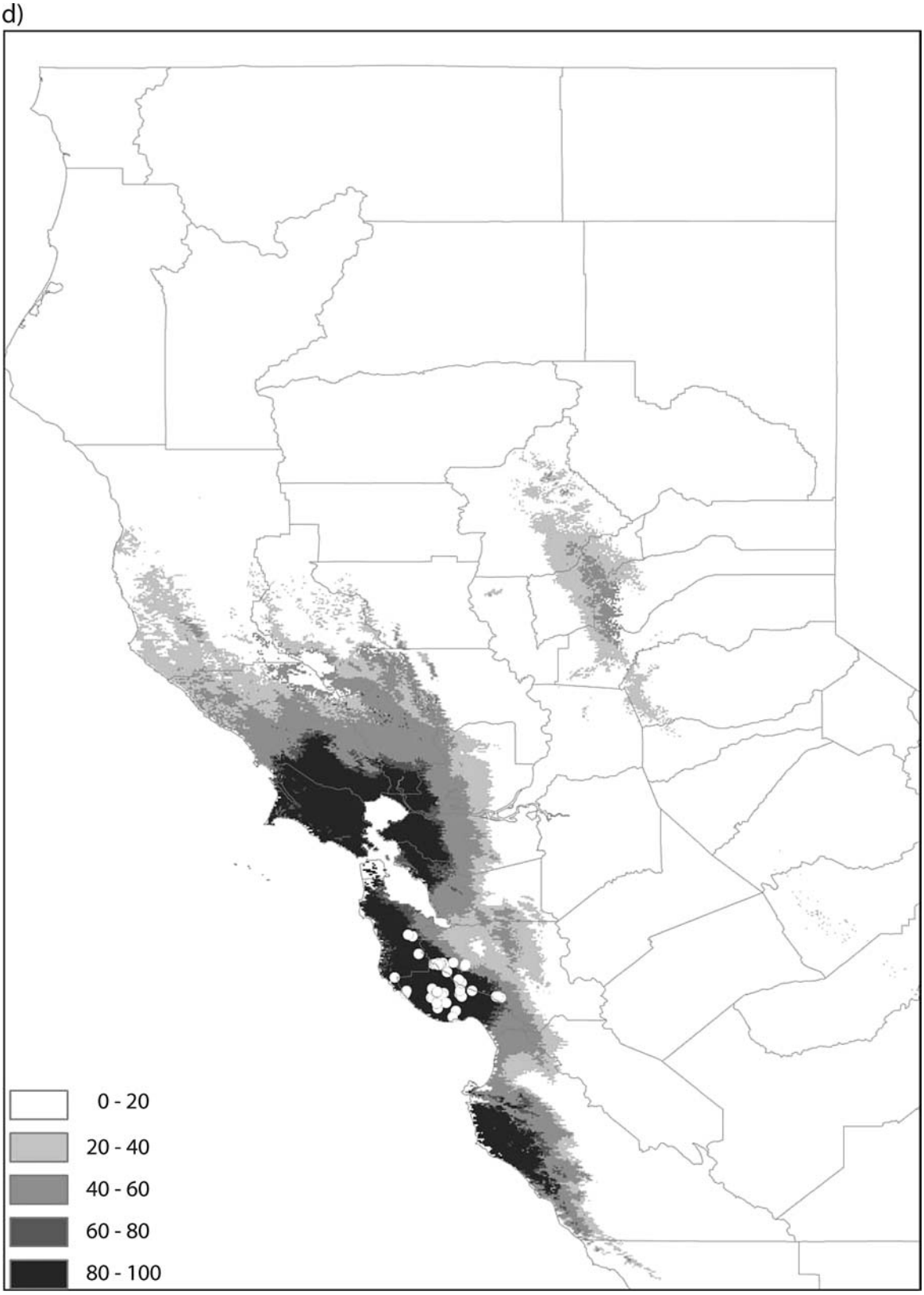

FIGURE 5. (Continued).

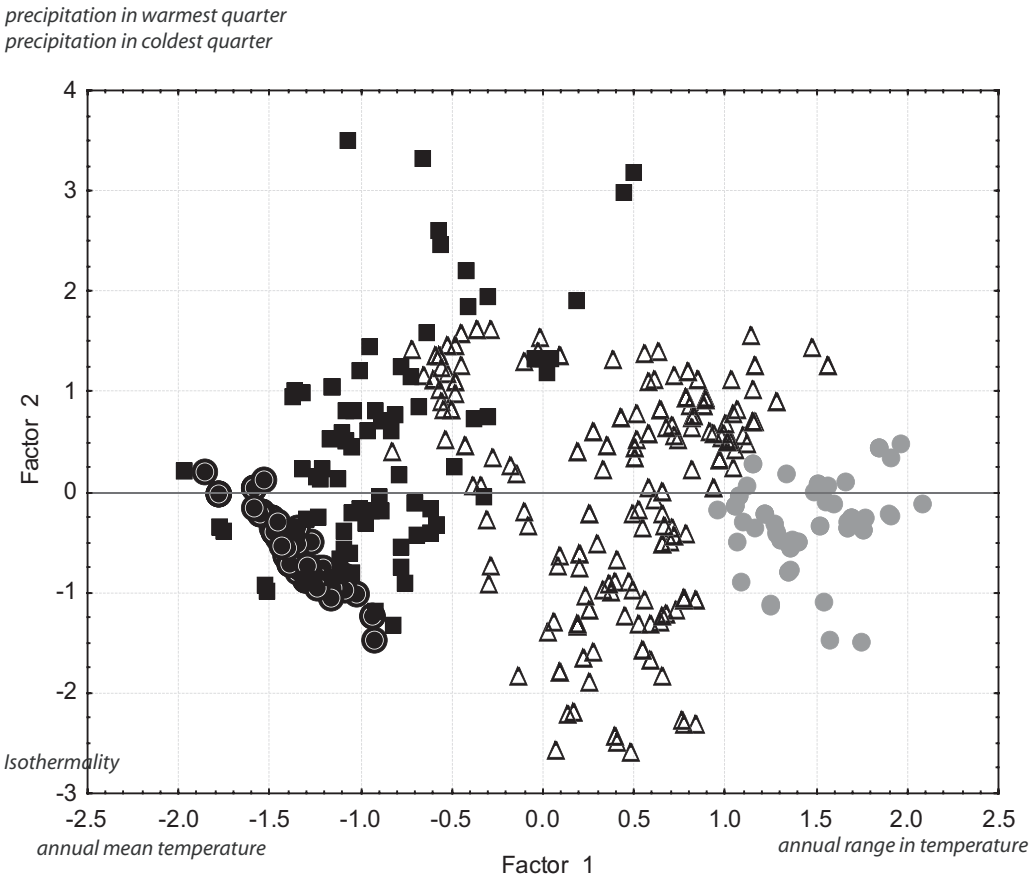

FIGURE 6. Principal components analysis (PCA) of lineages in *Aneides flavipunctatus*. The *x*-axis explains 46.76% of the variation, and the *y*-axis explains 29.33%. Total variation explained by the first two principal components is 76.09%. ■Northwest; ●Shasta; ●Southern Disjunct; △Central lineage.

DISCUSSION

Implications for Species Delimitation in Aneides flavipunctatus

Our analyses of the spatial data (PCA, MANOVA, partial Mantel) all support the conclusion that genetic diversity is associated with significant divergence in the ecological niche of *A. flavipunctatus*. The extent of the divergence across lineages varies and is a result of isolation that can be geographic or environmental. For example, in the case of the Shasta lineage, the population is not necessarily geographically isolated (as is the Southern Disjunct lineage) but it is environmentally isolated. The potential contact zone region between the Northwest and Shasta lineages is unlikely to support viable populations of either lineage, because the Maxent probability values are well under 40% in that region under both lineage niche models. This was found despite the nonsignificant association between environmental and genetic distances (controlling for geographic distance) in the partial Mantel test, suggesting that the two environments for the Shasta and Northwest lineages are not that different but these sister lineages are isolated by intervening, unsuitable habitat. However, to confirm this, more thorough sampling should be conducted in this potential contact zone. We acknowledge that there could be very limited intervening suitable habitat in this region, but to date (2007), no records of *A. flavipuntatus* are known from this potential contact zone.

The Central lineage is genetically quite distinct from the Shasta, Northwest, and Southern Disjunct lineages. However, the contact zone between the Northwest lineage and Central lineage is broadly suitable for the Northwest lineage, but, interestingly, not for the Central lineage. Despite suitability, the extent of gene flow in this region is entirely dependent on the level of reproductive isolation between these lineages, and we have no evidence of reproductive isolation at the current time. More thorough sampling should be conducted across the Central lineage's range to determine whether additional lineages exist and to refine the location of potential contact zones.

Currently, *A. flavipunctatus* is considered a distinct species, and sometimes two subspecies are recognized: *A. f. flavipunctatus* (speckled black salamander) and

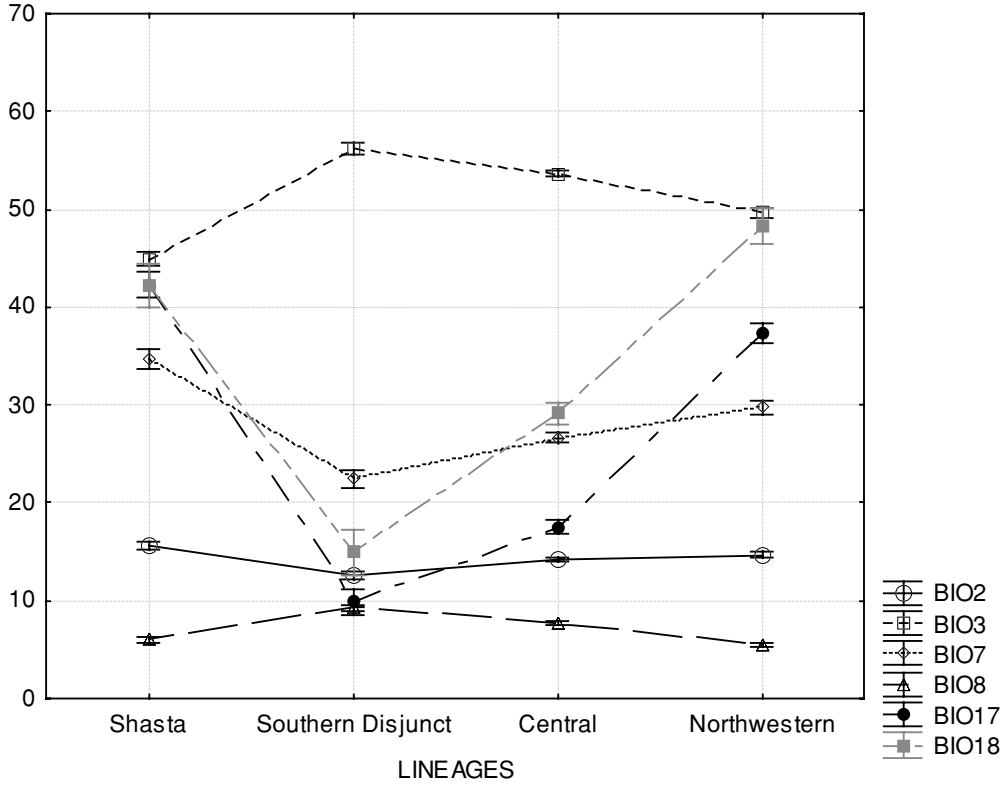

FIGURE 7. Ls-means plot of each of the environmental variables that varied significantly across all lineages. BIO2 = mean diurnal temperature range (mean of monthly [max temperature − min temperature]); BIO3 = isothermality; BIO7 = annual range temperature; BIO8 = mean temperature of wettest quarter of year; BIO17 = precipitation in the driest quarter of the year; BIO18 = precipitation in the warmest quarter of the year (temperature in Celcius; precipitation in mm). See Hijmans et al., 2005, for additional details on layers.

A. f. niger (Santa Cruz black salamander; Myers and Maslin, 1948). Petranka (1998) follows Lynch's (1981) recommendations to not recognize subspecies based on the conclusion that morphological and color variation is predominately clinal and ontogenetic (Lynch, 1981). *Aneides f. niger* corresponds to our Southern Disjunct lineage, and it is completely geographically isolated. However, other lineages within the species are not geographically isolated, and ecological niche modeling can aid in our assessment of species status, especially when morphological delineations are not clear-cut. Although morphological variation is somewhat high in this species (i.e., in degree of brassy pigmentation and diameter of white dorsal iridophores), there is no evidence that this is genetically or racially based (Lynch, 1974, 1981). Allozyme analyses have also indicated that both the Shasta lineage and Southern Disjunct lineage are distinct (Larson, 1980). Highton (2000) reanalyzed Larson's (1980) data and found that there were three groups within *A. flavipunctatus* (Southern Disjunct, Shasta, and remaining populations) that differed from each other by Nei $D > 0.15$. However, both Larson (1980) and Lynch (1974, 1981) concluded that there was insufficient evidence to elevate these populations to species status, although Highton (2000) disagreed. Interestingly, early ecological and morphological studies by Lowe (1950) in a University of California Los Angeles unpublished doctoral thesis separated *A. flavipunctatus* into five subspecies that roughly correspond to the genetic lineages we found. Names are available for three of these: flavipunctatus Strauch 1870 (most similar to our Central lineage), niger Myers and Maslin 1948 (our Southern Disjunct lineage), and iëcanus Cope 1883 (our Shasta lineage). Should subsequent work validate species status for all five clusters, new names will be required for the Northwest and AF28–31 lineages. Lowe's evidence was almost completely ecological and based on the separation of humid, coniferous forest habitat along the coastal populations and the drier, more openly vegetated interior populations; populations along the coast had reduced levels of white spotting, although later analyses have shown morphological variation in *A. flavipuncatatus* to be largely due to ontogenetic variation (Lynch, 1981).

Based on our mtDNA analyses and ecological modeling, along with the corroboration of allozyme data, we believe that the Shasta and Southern Disjunct lineages should clearly be elevated to species status, and the southern extent of the Northwest lineage should be determined before that lineage is elevated to species

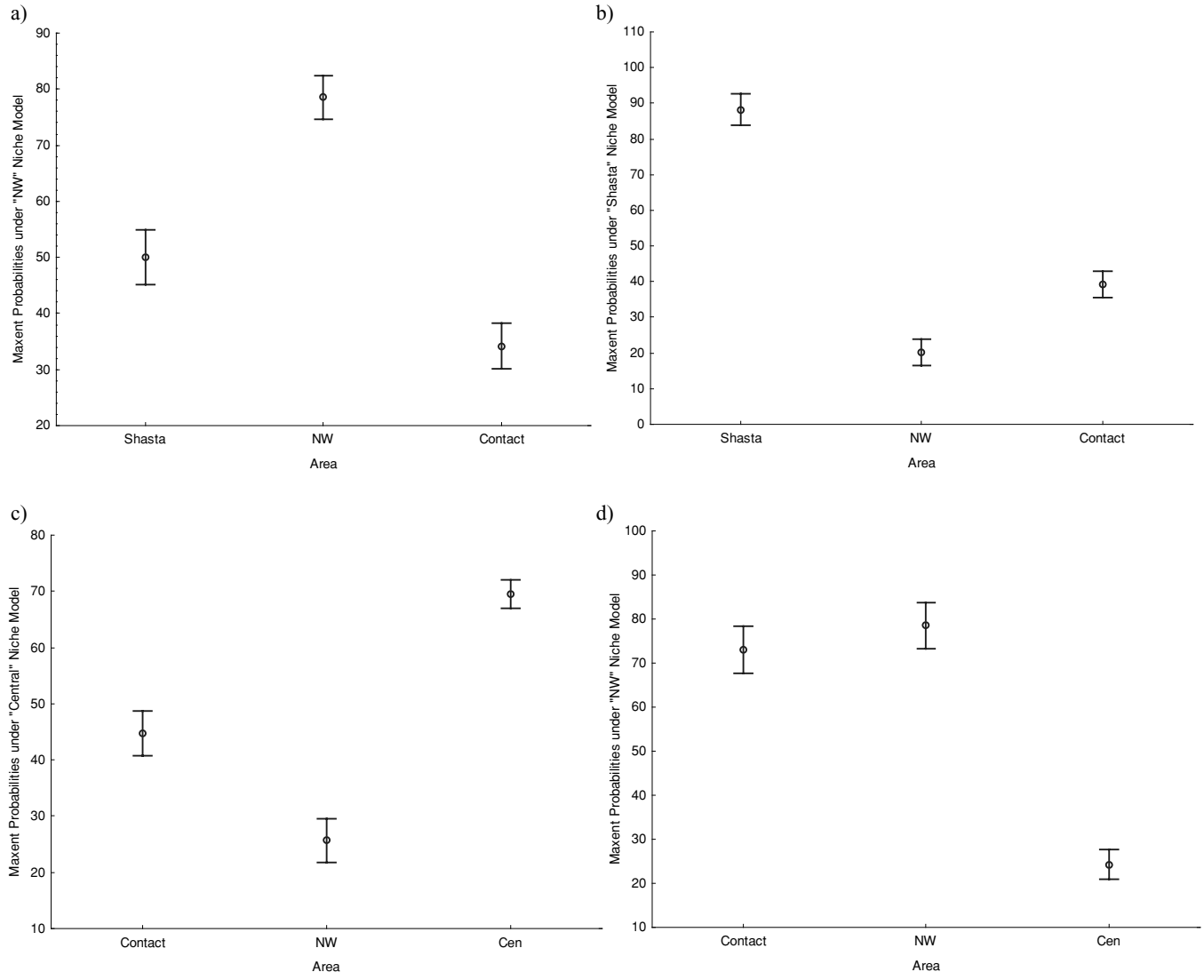

FIGURE 8. Contact zone analyses. Means with vertical bars denoting 95% confidence intervals of the Maxent probability values under unique ecological niche models for each lineage (separate figures) in their respective range (see Fig. 2) and in the contact zone. For example, in (a), the Northwest (NW) ecological niche model was run, and the Maxent probability values for all NHC point localities in the NW minimum convex polygon averaged around 78%. The values from the Shasta NHC point localities under the NW ecological niche model averaged around 50%. The contact zone had a very low probability of suitability at about 34%. For (a) to (c), all comparisons have contact zones with low suitabilities. However, suitability is high for the Central and NW contact zone (d), suggesting that the NW lineage could move south into the Central lineage's range. (a) Contact zone analysis for NW and Shasta region under the NW ecological niche model. (b) Contact zone analysis for NW and Shasta region under the Shasta ecological niche model. (c) Contact zone analysis for the Central and NW region under the Central ecological niche model. (d) Contact zone analysis for the Central and NW region under the NW ecological niche model.

status. We have taxonomic publications in progress, and we suggest additional geographic and genetic sampling should be conducted to refine the limits of populations within the Central lineage, especially around Mendocino County (e.g., AF28–31, Fig. 3). There appears to be little morphological divergence across these species, and in general we suggest that genetic analyses combined with ecological data can be far stronger evidence for delimiting species than morphological data due to the ubiquitous nature of morphological homoplasy from convergence, parallelism, and reversals (e.g., Wake, 1991; Mueller et al., 2004).

Adding Information on the Ecological Niche to Studies of Speciation and Species Delimitation

We believe that natural selection through abiotic pressures can be a major driver of divergence and ultimately speciation, and the analyses of ecological divergence in relation to phylogenetic diversity can shed insight into biodiversity patterns and the potential processes driving those patterns. A recent analysis of over 500 comparisons across plant, invertebrate, and vertebrate taxa found a highly consistent and significant association between ecological divergence (including habitat divergence) and reproductive isolation (Funk

et al., 2006). Funk et al. (2006) concluded that speciation is an "... inherently ecological process," and this has been supported by theoretical and experimental work (Mayr, 1947; Dobzhansky, 1951; Rice and Salt, 1990; Rice and Hostert, 1993; Filchak et al., 2000; Schluter, 2000; Rundle and Nosil, 2005; Dettman et al., 2007). Another recent review (Streelman and Danley, 2003) focused on the stages of vertebrate evolutionary radiation and concluded that habitat was the initial axis of divergence in many adaptive radiations, followed by divergence in morphology and communication. Therefore, we suggest that quantifying divergence in the ecological niche should be an important component of current phylogeographic studies and could be useful for species delimitation (e.g., Wiens and Graham, 2005).

In species delimitation it is important to distinguish between the primary species concept (entities believed to be species) and the secondary species concept (operational methods for the discovery of those entities; Sites and Marshall, 2003, 2004). As mentioned previously, our view of species follows the evolutionary species concept (ESC) and general lineage species concept (GLC; Wiley, 1978; de Queiroz, 1998) with the goal of recognizing historically distinct evolutionary lineages that are likely to remain distinct (e.g., Wake, 2006). The methodologies used to distinguish those lineages are likely to vary across systems because of the plethora of evolutionary processes operating within and among populations at varying spatiotemporal scales (Harrison, 1998; reviewed in Sites and Marshall, 2003, 2004). Methods are generally grouped into tree-based and non–tree-based methods, but none of the currently recognized operational criteria for delimiting species considers spatially explicit environmental (climatic) information on the niche (e.g., Sites and Marshall, 2003, 2004; Wiens and Graham, 2005; Pons et al., 2006). We argue that data on the ecological niche can be highly informative, especially in combination with more traditional phylogenetic data (e.g., mtDNA), because it can provide a surrogate for physiological adaptations as well as a direct view of the abiotic variables likely impacting divergence. Our approach, therefore, focuses on the distinctness of lineages in historical (genetic) and ecological space, rather than giving primacy to gene flow.

SUMMARY

Amphibians, as well as many other taxa, commonly exhibit conservative morphological evolution that masks significant genetic diversity (e.g., Highton, 1995; Bickford et al., 2007). This hampers our understanding of the patterns of biodiversity and the processes that drive those patterns, and because amphibians are one of the most threatened groups of organisms on the planet (Stuart et al., 2004), a limited understanding of their biodiversity patterns can significantly affect conservation assessments.

The California Floristic Province is one of 34 world hotspots of biological diversity (Mittermeier et al., 2005), and it is a region with high endemism and concordant phylogeographic breaks that are often congruent with environmental gradients (Calsbeek et al., 2003; Lapointe and Rissler, 2005; Rissler et al., 2006). Temperature and precipitation regimes are especially likely to impact the distributions of amphibians because of their permeable skin and general physiology (Duellman and Trueb, 1986; Buckley and Jetz, 2007). When populations become isolated geographically, unique environmental conditions can drive divergence (e.g., Dobzhansky, 1940; Orr and Smith, 1998; Schluter, 2001; Funk et al., 2006). Within the range of *A. flavipunctatus*, environmental gradients are steeper in an east-west direction (Rissler et al., 2006), and morphological and genetic data support this (Lynch, 1974, 1981; Larson, 1980). These strong gradients in combination with the generalized phenomenon of niche conservatism (see Wiens, 2004) likely provided multiple opportunities for isolation and subsequent divergence in the early evolutionary history of *A. flavipunctatus*. Whether or not reproductive isolation is complete across lineages/species in *A. flavipunctatus* is unknown at the current time, but plethodontid salamanders can show reproductive isolation even in the absence of observable morphological or ecological changes (Highton, 1979).

We suggest that future phylogeographic and biogeographic analyses will be more explicitly concerned with the spatial and geographic components of genetic variation and speciation than is currently evident (Kidd and Richie, 2006). We believe ecological niche modeling of climatic variables has shown promise in helping to elucidate the abiotic processes impacting evolutionary lineages, but the technique is not a panacea for inadequate sampling, nor can one definitively conclude that the climatic variables that statistically differentiate two lineages are the reason for their genetic divergence. We do, however, suggest that because amphibians are physiologically constrained to particular environmental conditions (Feder and Burggren, 1992), climate is likely to play an especially important role in driving global and local biodiversity patterns. In fact, Buckley and Jetz (2007) analyzed the distributions of 5,634 of the known 6,200 amphibian species (www.amphibiaweb.org; accessed 30 August 2007) and concluded that while history plays an important role in broad scale richness patterns, environment is by far the stronger predictor of species richness within geographic realms. Future research is important to elucidate the causal relationships between environmental variables, ecological divergence in phenotypic traits, and speciation. For example, for species showing divergence in the ecological niche, further physiological and behavioral comparisons should be made across lineages. These types of analyses will become increasingly important for predictions of range distributions and extinctions during climate change, especially for species that are physiologically constrained to environmental conditions (e.g., Pounds et al., 1999; Lips et al., 2003; Buckley and Jetz, 2007).

In summary, we have demonstrated the ease and utility of combining ecological niche models, spatially explicit analyses of environmental data, and phylogeographic information to help define cryptic species.

Especially for organisms displaying fine-scaled endemism and cryptic diversity, these methods should provide an objective and rigorous tool to aid in investigations of biogeography and species delimitation.

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