

Extreme population subdivision throughout a continuous range: phylogeography of *Batrachoseps attenuatus* (Caudata: Plethodontidae) in western North America

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Abstract

Low-vagility species with deep evolutionary histories are key to our understanding of the biogeographical history of geologically complex areas, such as the west coast of North America. We present a detailed study of the phylogeography of the salamander *Batrachoseps attenuatus* (Caudata: Plethodontidae) using sequences of the mitochondrial gene *cob* from 178 individuals sampled from throughout the species' range. Sequences of three other mitochondrial genes (*16S*, *cox1*, *nad4*) and a nuclear gene (*RAG-1*) were used to investigate the deeper evolutionary history of the species. We found high levels of genetic diversity and deep divergences within a mostly continuous distribution, with five genetically well-differentiated and geographically structured mitochondrial DNA clades. Significant association between geographical and genetic distances within these clades suggests demographic stability, whereas F_u 's F_s tests suggest demographic expansions in three of them. Mantel tests identify two biogeographical barriers, the San Andreas Fault and the Sacramento–San Joaquin Delta, as important in the diversification of lineages. The timing of the main splitting events between intraspecific lineages was estimated by applying relaxed molecular clock methods combining several mutation rates and a fossil calibration. The earliest splitting events are old (Pliocene/Miocene), with more recent (Pleistocene) subdivisions in some clades. Disjunct populations distributed along the western foothills of the Sierra Nevada colonized this area relatively recently from a single refugium east of San Francisco Bay. The combination of fine-scale, comprehensive sampling with phylogenetic, historical demographic and hypothesis-based tests allowed delineation of a complex biogeographical scenario with general implications for the study of codistributed taxa.

Keywords: *Batrachoseps attenuatus*, mtDNA, phylogeography, *RAG-1*, Sacramento–San Joaquin Delta, San Andreas Fault

Received 22 May 2007; revision received 10 July 2007; accepted 1 August 2007

Introduction

The study of patterns of genetic structure in natural populations has increased dramatically since the late 1960s, when the first studies based on allozyme data sets were published (Avice 1974; Larson *et al.* 1984). These studies were followed by the generalized use of mitochondrial DNA (mtDNA) and, more recently, of nuclear introns and microsatellites in resolving patterns of intraspecific genetic diversification in different taxa across their geographical

ranges (Hare 2001; Zhang & Hewitt 2003). Large amounts of data are now available, setting the stage for comparative studies and, ultimately, for the emergence of new disciplines, such as comparative phylogeography (Bermingham & Moritz 1998; Avice 2000). The aim of comparative phylogeography is to infer the existence of common biogeographical events through the concordance of independent gene trees from a variety of taxa in a shared geographical background. Comparative studies have been used, for instance, to identify major historical events affecting a variety of organisms (Schneider *et al.* 1998; Riddle *et al.* 2002), to infer the location of refugial areas during cold periods in the

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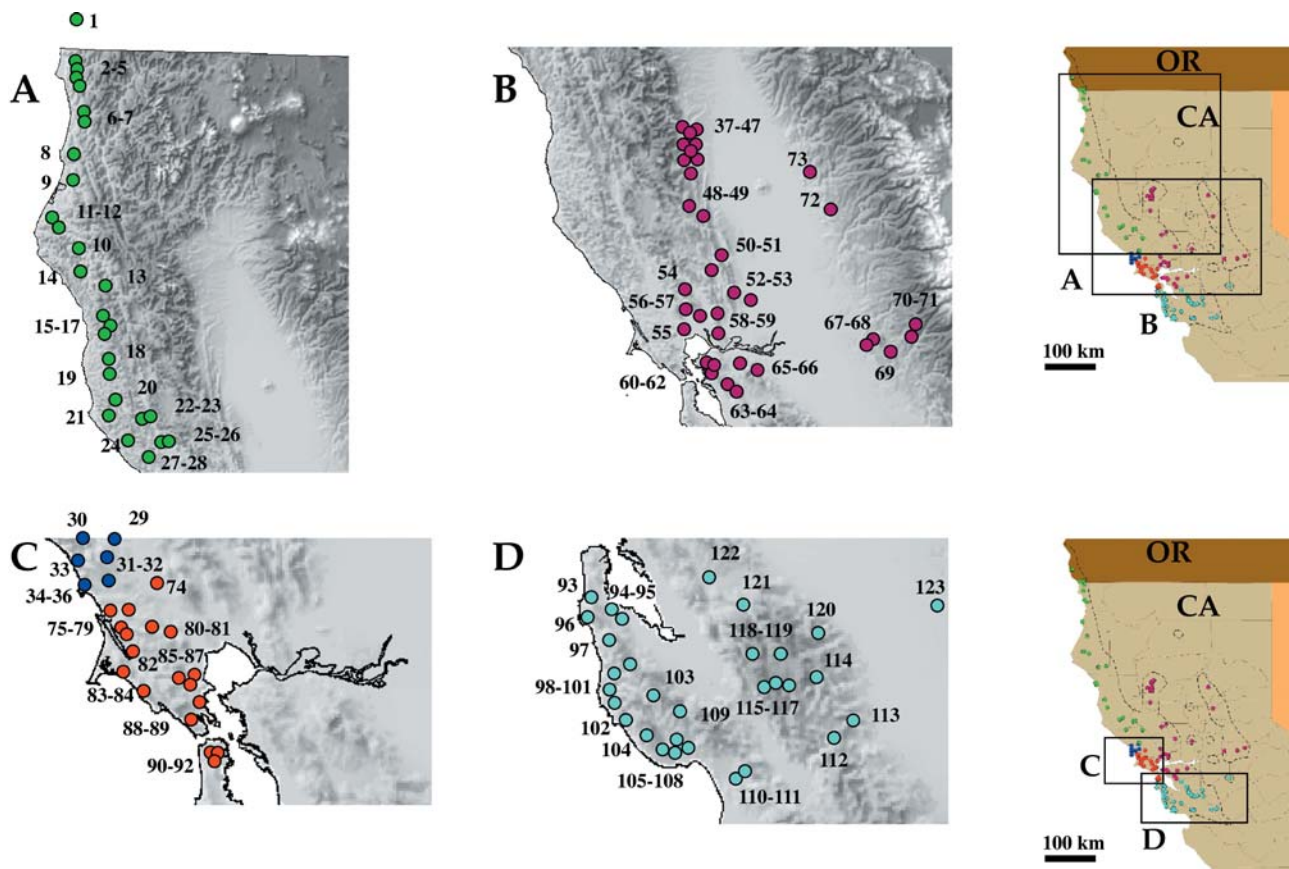


Fig. 1 Sampling localities and distribution of the main clades identified within *Batrachoseps attenuatus*. ID numbers as in the Appendix. Shading indicates topographic relief. (A) Northern clade (green); (B) Eastern clade (purple); (C) Bodega (dark blue) and Southern_North (red) clades; and (D) Southern_South (light blue) clade. Maps on the right show the complete range of the species (dashed line), sampling localities coded as in A–D, and the focus of each inset. Note the discontinuous distribution of the species in the Great Central Valley. OR, Oregon; CA, California.

Pleistocene as well as potential recolonization routes during warmer periods (Hewitt 1999), and to inform management policies concerned with the maintenance of evolutionary patterns and processes (Moritz *et al.* 2001).

The last decade has produced an increasing number of publications focused on phylogeographic patterns in western North America (Brunsfield *et al.* 2001; Calsbeek *et al.* 2003; Swenson & Howard 2005). The complex geological history of this region makes it challenging for comparative studies, and despite the identification of some general patterns (references above; Feldman & Spicer 2006; Rissler *et al.* 2006), many studies have also uncovered significant differences between taxa, suggesting considerable variation associated with historical factors in shaping present-day communities. Thus, species with similar distributions may have experienced very different evolutionary histories (Wake 2006).

Salamanders in the genus *Batrachoseps* provide an excellent system for testing the influence of biogeographical barriers for several reasons. First, they constitute an old clade, which probably has been native to western North

America for over 40 million years (Myr) (Yanev 1980; Wake 2006). Second, they are extremely sedentary, with home ranges among the smallest described within vertebrates (typically < 10 m, Hendrickson 1954). Third, while some species present restricted distributions, making them valuable for delimitation of areas of endemism, other species have relatively large distributions, providing opportunities to test biogeographical scenarios at broader geographical scales. Such is the case of *Batrachoseps attenuatus* (Eschscholtz 1883), which ranges from southwestern Oregon to central California, north of Monterey Bay (Stebbins 2003). The distribution is mostly continuous along the coast; there are also several isolated populations in the Great Central Valley, and a disjunct group of populations distributed along the western Sierra Nevada foothills (Fig. 1). This species is the sister taxon to a clade comprised of the 15 other species in the *Batrachoseps (sensu stricto)* clade (Jockusch & Wake 2002), and Late Miocene fossil remains attributed to *Batrachoseps* have been reported from east of San Francisco Bay (Clark 1985), suggesting a long presence of the genus in this area.

Previous studies based on limited geographical sampling have uncovered complex patterns of morphological (Yanev 1978; Jockusch 1997) and molecular (Yanev 1978; Jockusch & Wake 2002) variation in this species, emphasizing the need for more detailed studies.

Because of the characteristics cited above, in particular the deep evolutionary history and extremely low vagility of the species, we expected patterns of mtDNA variation in *B. attenuatus* to closely reflect the complex geological history of the geographical region they inhabit. Our main aim in this study is to reconstruct the evolutionary history of populations of *B. attenuatus*, including delineation of well-supported intraspecific lineages, and dating of the main splitting events. Based on the inferred history, we propose testable biogeographical hypotheses explaining evolutionary diversification in this species and discuss their implications for our knowledge of common phylogeographic patterns in the study region (central and northern California).

Materials and methods

Sampling design

The distribution of the species is mainly continuous, except in the Great Central Valley, which is inhospitable for terrestrial salamanders (Fig. 1). Three different data sets were used in the analyses. First, 178 individuals of *Batrachoseps attenuatus* from 123 localities were included in the analyses of the mitochondrial cytochrome *b* (*cob*) data set, used for phylogeographic and historical demographic analyses. Additionally, three other mitochondrial genes [16S ribosomal RNA (*16S*), cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 4 (*nad4*)] were sequenced in a subset of 47 individuals representing the main clades identified in the *cob* data set to resolve phylogenetic relationships between them (Fig. 2, Appendix). Finally, a fragment of a nuclear gene (*RAG-1*) was sequenced in a different subset of 31 individuals to test for geographical congruence between mtDNA and nuclear DNA haplotypes (GenBank Accession nos EU020135–EU020165).

PCR amplification and sequencing

Total genomic DNA was extracted from ethanol-preserved or frozen tissues using standard protocols (Sambrook *et al.* 1989) or QIAGEN DNeasy extraction kits. Polymerase chain reaction (PCR) was used to amplify ~800 base pairs (bp) of *cob*, ~500 bp of *16S*, ~800 bp of *cox1* and ~900 bp of *nad4* using the primers MVZ15 and MVZ16 for *cob* (Moritz *et al.* 1992), 16Sar and 16Sbr for *16S* (Palumbi 1996) and clade-specific primers for *cox1* and *nad4* designed for this study (available from I.M.S. on request) under standard conditions. A fragment of *RAG-1* was amplified using primers Amp-RAG1 F and Amp-RAG1 R1 (San Mauro *et al.* 2004).

Templates were cleaned using EXOSAP-IT. Sequencing reactions were performed for both strands and reaction products were separated on an ABI PRISM 3730 DNA sequencer.

Phylogenetic analyses

For the phylogenetic analyses, we chose as outgroups the *Batrachoseps* species *B. gabrieli*, *B. nigrirostris*, *B. diabolicus*, *B. gavilanensis* and *B. wrighti* (vouchers: MVZ 222959, DBW 6459, IMSZ386, IMSZ381 and MVZ 224903, GenBank Accession nos EU011248–EU011263). The sequences of *B. wrighti* were obtained from GenBank (Accession no. NC006333).

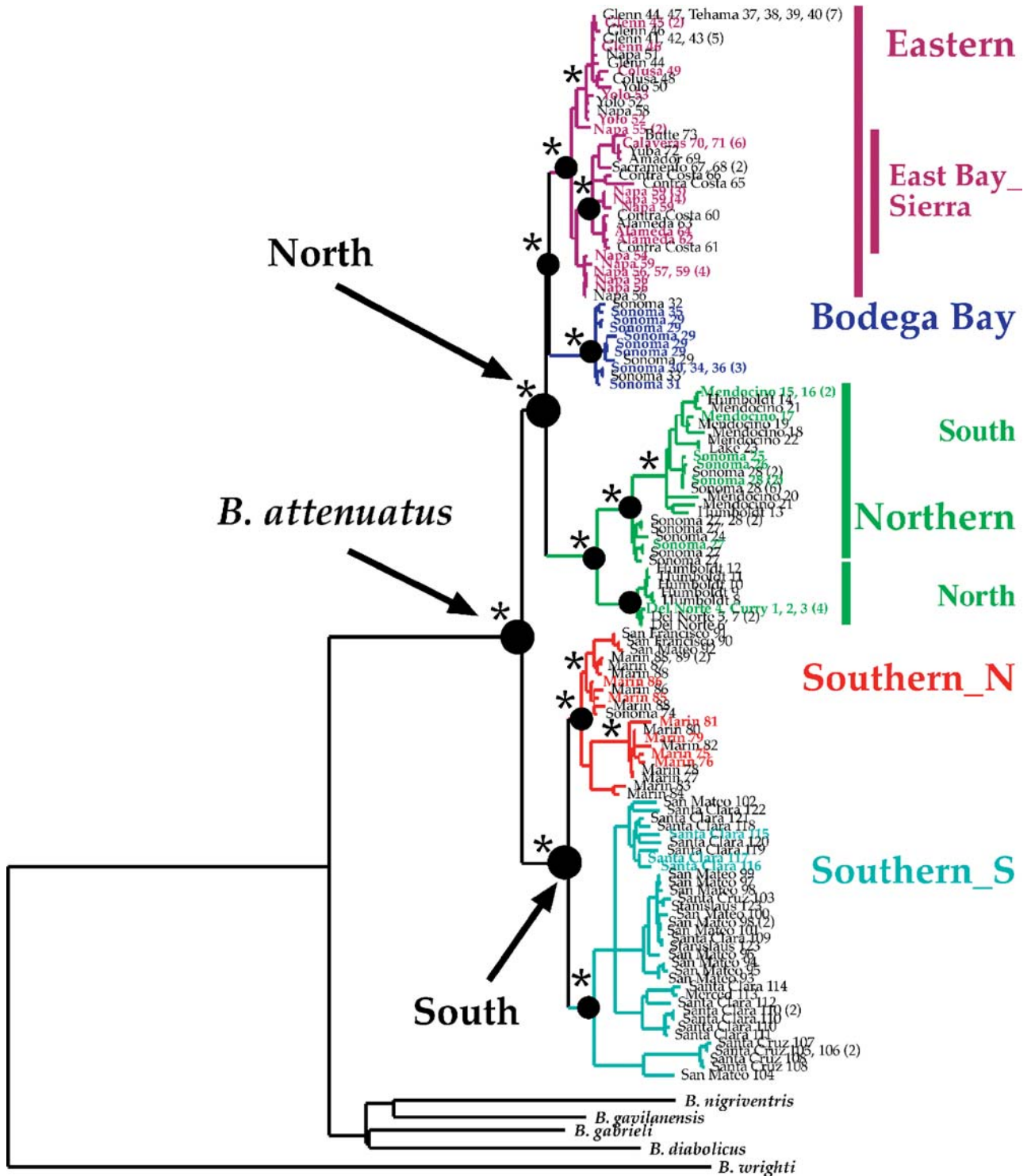
Sequences were aligned by eye. Alignment of 16S sequences required insertion of five gaps within the ingroup. Sequences of ingroup samples were deposited in GenBank (see Appendix). For all data sets, we conducted maximum parsimony (MP) analyses with PAUP 4.0b10* (Swofford 2002). We performed heuristic searches (tree-bisection–reconnection branch swapping, MULTREES option in effect, 10 random stepwise additions of taxa), bootstrapping the matrix 100 times to assess branch support. To reduce computation time, a maximum of 100 000 trees was saved at each bootstrap replicate.

We also performed phylogenetic analyses based on Bayesian inference using MRBAYES version 3.1 (Ronquist & Huelsenbeck 2003). We determined the best-fitting substitution model for the nucleotide matrix through the Akaike information criterion (AIC) implemented in MODELTEST 3.7 (Posada & Crandall 1998). We tested different partition strategies for each data set (unpartitioned, partition by gene, partition by gene and codon) and selected the best partitioning strategy by comparing Bayes factors (Kass & Raftery 1995). Default settings were used for the prior distribution in the Bayesian analyses. We ran Metropolis-coupled Markov chain Monte Carlo analyses with four chains for 20 million generations, sampling every 1000 generations. We discarded 5000 trees as burn-in, after checking for stationarity and convergence of the chains with the software TRACER 1.3 (Rambaut & Drummond 2004).

Phylogeographic and historical demographic analyses

All the analyses reported in this section were performed on the *cob* data set. Estimates of mean uncorrected genetic distances within and between the main mtDNA clades within *B. attenuatus* identified by the previous analyses were calculated with MEGA 3.1 (500 bootstrap replicates, Kumar *et al.* 2004).

We explicitly tested the plausibility of an isolation-by-distance (IBD) scenario in the different lineages of *B. attenuatus*. We performed Mantel tests (Mantel 1967) to analyse the relationships between genetic and geographical distances



— 0.01 substitutions/site

Fig. 2 Phylogram from BEAST showing relationships between 133 *cob* haplotypes of *Batrachoseps attenuatus* plus five outgroups. Taxa are labelled by county and haplotype number from the Appendix and numbers in parentheses represent haplotype frequencies when > 1. Asterisks indicate ingroup branches supported by bootstrap values > 70% in maximum parsimony analyses and posterior probabilities > 95% in Bayesian analyses of the combined (16S + *cob* + *cox1* + *nad4*) data set. Samples in colours indicate those used in the combined data set. Also indicated are groups for which TMRCA were estimated (black dots, see text for details).

in the main clades recovered in the phylogenetic analyses. The geographical distance matrix between samples (km) was generated with the program GEODIS 2.4 (Posada *et al.* 2000). The genetic distance matrix (uncorrected distance) was generated in MEGA 3.1. Mantel tests were carried out with the software package XLSTAT (Addinsoft, 1000 permutations).

We used partial Mantel tests as implemented in XLSTAT to examine whether the splits separating the main clades of *B. attenuatus* could be a result of the overall isolation-by-distance pattern in the data set, or whether additional factors were needed to account for any of the splits. In each case, we defined a binary matrix in which samples were coded as belonging to the same clade (0) or different clades (1), and tested for a significant correlation between this binary matrix and genetic distance, taking into account the effects of geographical distance.

Finally, in order to explore the demographic histories of the main mtDNA lineages within *B. attenuatus*, mismatch analyses of pairwise mtDNA differences (Slatkin & Hudson 1991) within each clade were performed with ARLEQUIN version 2000 (Schneider *et al.* 2000). This analysis compares the frequency distribution of pairwise differences between haplotypes with that expected under a model of population expansion. The frequency distribution is usually unimodal for lineages that have undergone recent population expansions and multimodal for lineages whose populations are either subdivided or in equilibrium. We also used Fu's F_S test (Fu 1997) as implemented in DNASP version 4.10.7 (Rozas *et al.* 2003; 10 000 replicates) to test for population growth in the main clades identified in previous analyses.

Tests of biogeographical hypotheses

Based on a comparative analysis of 75 phylogeographic lineages (Rissler *et al.* 2006), we considered two a priori major potential biogeographical barriers for *B. attenuatus*: (i) the Great Central Valley, which has played a significant role in shaping genetic variability in other taxa, including *Batrachoseps* (Yanev 1978) (see also Feldman & Spicer 2006; Starrett & Hedin 2007); and (ii) the Sacramento–San Joaquin Delta, a more recent barrier running east–west and including the San Francisco Bay, which has been associated with intraspecific differentiation in some taxa (Matocq 2002; Richmond & Reeder 2002). Additionally, we also considered as a potential barrier the San Andreas Fault, which runs northwest to southeast. It separates the Bodega Head and Point Reyes Peninsulas from the adjacent mainland and then runs through the San Francisco Peninsula. Although this region was not identified in comparative analyses (Rissler *et al.* 2006), it has been hypothesized to have caused ancient lineage splitting events in both *Batrachoseps* and the salamander genus *Ensatina* (Wake 1997, 2006; Jockusch & Wake 2002). We used Bayesian topology tests to estimate the posterior probabilities that

samples separated by these hypothesized barriers formed reciprocally monophyletic groups in analyses of the combined mtDNA data set. We used tree filters in PAUP to assess the proportion of trees in our sample that are consistent with each of these three hypotheses.

We also used partial Mantel tests (Smouse *et al.* 1986) to test whether biogeographical barriers and genetic distances between samples were correlated in the *cob* data set, taking into account the relationship between genetic and geographical distances. For each biogeographical barrier and clade of interest, a binary matrix was created which coded sample pairs as 0 if they were not separated by the barrier and 1 if they were. Partial Mantel tests were implemented in XLSTAT, and significance was judged using 1000 resamplings.

Divergence time estimates

We used a Bayesian coalescent approach to estimate the time to the most recent common ancestor (TMRCA) in subclades identified in the combined mtDNA data set. Divergence times and their credibility intervals were estimated by using a relaxed clock model with branch rates drawn from an uncorrelated lognormal distribution (Drummond *et al.* 2006), as implemented in BEAST 1.3 (Drummond & Rambaut 2005). Two different mutation rates were chosen (0.01 mutations/site/Myr and 0.004 mutations/site/Myr) to span the full range of estimates of the rate of evolution of the *cob* gene reported by Mueller (2006). Based on the fossil record of the genus (Clark 1985), we constrained the TMRCA of all *B. attenuatus* haplotypes to be at least 5.2 Myr old. For each mutation rate, we used a GTR + G model of sequence evolution and a Bayesian skyline coalescent model of population size with 10 groups. Analyses were run for 10 million generations, sampling every 1000. We checked for convergence and stability of the parameters and determined burn-in using TRACER 1.3 (Rambaut & Drummond 2004).

Results

Phylogenetic analyses

We analysed 677 base pairs of *cob* for 178 ingroup individuals and five outgroup samples. Of 677 characters, 392 were constant and 232 were parsimony-informative (441 constant and 188 informative when outgroups were excluded). We found 138 haplotypes among the 183 sequences. Haplotypes in *Batrachoseps attenuatus* clustered in five geographically well-structured clades (Figs 1 and 2), with high genetic distances separating lineages (up to 9.5%, Table 1). We refer to the three northernmost clades as Northern (distributed along a coastal strip from southern Oregon to Sonoma County in Central California), Eastern

Table 1 Mean values of uncorrected genetic distances within (diagonal) and between clades in *Batrachoseps attenuatus* (below diagonal), including standard deviations. Mean within-group genetic distances are mean divergences across the main split within each clade

	Northern	Eastern	Bodega	Southern_North	Southern_South
Northern	5.1 ± 0.7%				
Eastern	7.4 ± 0.7%	3.4 ± 0.6%			
Bodega	6.5 ± 0.7%	5.5 ± 0.7%	1.3 ± 0.3%		
Southern_North	8.4 ± 0.9%	7.3 ± 0.8%	6.6 ± 0.8%	3.9 ± 0.5%	
Southern_South	9.5 ± 1.0%	8.8 ± 0.9%	7.9 ± 0.9%	5.9 ± 0.6%	5.3 ± 0.7%

(eastern San Francisco Bay region, Central Valley, western slopes of the northern Sierra Nevada and eastern slopes of the North Coast Ranges in northern California), and Bodega (restricted to the vicinity of Bodega Bay, in coastal northern California). More southern populations form a southern clade that is in turn subdivided into North (distributed mainly north of the San Francisco Bay, but also on the San Francisco Peninsula) and South (distributed south of the San Francisco Bay) clades. In general, results of the MP and Bayesian analyses of the *cob* data set strongly supported the monophyly of each of these clades (MP bootstrap > 70%, Bayesian posterior probabilities (BPP) > 0.95), with the exception of the Southern_North clade, but no significant support was obtained for relationships between clades (except for the association between the two Southern clades, results not shown). Figure 1 depicts the geographical range of each clade in detail.

Geographical structuring is also pronounced within many of these clades. For example, the Northern clade is divided into a subclade comprising populations from the northernmost part of the distribution in southern Oregon and northern California (Northern_North) and a second clade including the remaining samples (Northern_South), again subdivided into two subclades meeting near the Russian River in Sonoma County. Within the Eastern clade, one subclade (East Bay_Sierra) includes all haplotypes from the western slopes of the Sierra Nevada and from south of the Sacramento–San Joaquin Delta in the eastern San Francisco Bay region, together with some of the haplotypes from population 59 in Napa County, the geographically closest population north of the Sacramento River Delta.

To resolve relationships among the three major clades in the north (Northern, Eastern and Bodega), we collected additional mtDNA sequence data from 42 ingroup samples representing all five clades plus outgroups (Fig. 2). The combined alignment included 495 characters of 16S, 677 bp of *cob*, 762 bp of *cox1* and 937 bp of *nad4*. For *nad4*, outgroup sequences were 270 bp shorter because a different set of primers was used due to cross-amplification problems with the primers designed for *B. attenuatus*. Five positions in the 16S data set were excluded from the analyses due to ambiguities in their alignment. Of the remaining 2866

characters, 1883 were constant and 707 were parsimony-informative (2237 and 487 excluding the outgroups). The best partitioning strategy was by gene and codon (10 partitions; harmonic mean of log-likelihoods: -15371.21); the resulting tree was fully consistent with the *cob* tree, and supported the monophyly of the Southern_North clade, and a sister group relationship between the Bodega and Eastern clades. The basal split in *B. attenuatus* divides the species into North and South clades. The North clade includes the Northern, Bodega and Eastern clades, whereas the South clade includes the Southern_North and the Southern_South clades. All clades and sister group relationships are supported by BPP values above 0.95 (Fig. 2). MP analyses also supported the same relationships.

The RAG-1 data set included sequences of 26 ingroup samples representing all five clades within *B. attenuatus* and the outgroups. The alignment comprised 819 characters (753 constant, 20 informative; 800 constant, eight informative in the ingroup). We found 21 haplotypes in 26 samples of *B. attenuatus*, with maximum pairwise sequence divergence (uncorrected distances) of 1.2%. However, genetic variation was not correlated with geography or mtDNA clades. Genetic distances to outgroups ranged from 1.1 to 1.8% with species within *Batrachoseps sensu stricto* and from 3.9 to 4.5% with *Batrachoseps wrighti*, a representative of the subgenus *Plethopsis* (Jockusch & Wake 2002).

Phylogeographic and historical demographic analyses

Mean values of uncorrected genetic distances for the *cob* data set within and between clades are presented in Table 1. Within-clade genetic distances across the basal split ranged from 1.3 (Bodega) to 5.3% (Southern_South), whereas between-clade values ranged from 5.5 (Bodega-Eastern clades) to 9.5% (Northern-Southern_South clades). The mean genetic distance between *B. attenuatus* haplotypes and outgroups was 15.9% (standard error: 1.0%) with respect to other species in *Batrachoseps sensu stricto* and 21.8% with respect to *B. (Plethopsis) wrighti* (standard error: 1.4%). Values of gene diversity were very high, ranging from 0.96 to 0.99 (Table 2), and in four of five clades, the number of haplotypes exceeded the number of localities sampled.

	Gene diversity	Haplotypes	Individuals	Localities
Northern	0.97	30	43	29
Eastern	0.96	36	62	38
Bodega	0.96	11	13	8
Southern_North	0.99	21	22	19
Southern_South	0.99	35	38	31

Table 2 Gene diversity for the five major mtDNA clades identified within *Batrachoseps attenuatus*. The number of haplotypes found, and individuals and localities sampled, is indicated for each clade

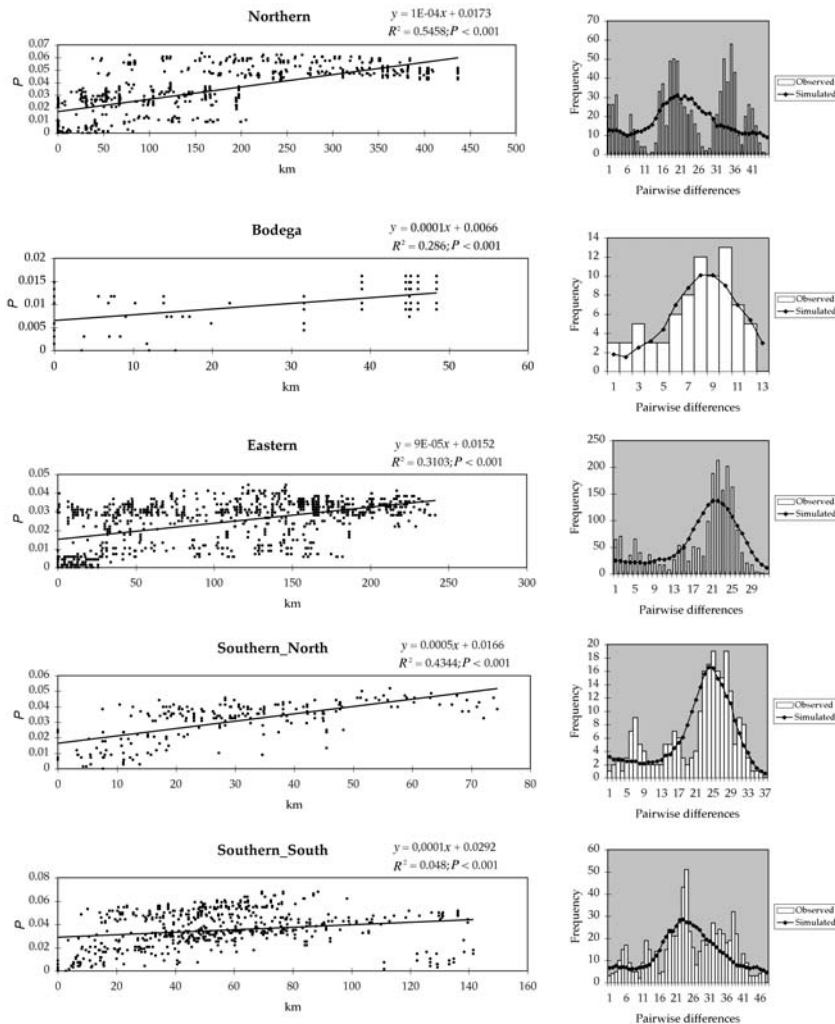


Fig. 3 Isolation by distance (IBD) plots (uncorrected genetic distances –p– vs. geographical distances, in kilometres) and mismatch distributions (observed vs. simulated values under a null model of exponential population growth) in each of the main clades within *Batrachoseps attenuatus*. IBD plots include regression equations, correlation coefficients and *P*-values.

All clades tested, including the geographically restricted Bodega clade, exhibited a significant relationship between geographical and genetic distances ($P < 0.001$ in all cases, Fig. 3). We also tested for IBD across major clades; all of these relationships were also significant ($P < 0.001$), but genetic divergence between clades was greater than expected on the basis of geographical distance alone (partial correlations between binary clade and genetic distance matrices, $P < 0.001$). Mismatch distributions appeared multimodal for most clades (Fig. 3); however, they were not significantly different from those generated under the null

model of population expansion in any of the clades studied. Fu's F_S test was significant in the Bodega ($F_S = -3.406$, $P = 0.046$), Southern_North ($F_S = -6.079$, $P = 0.008$) and Southern_South ($F_S = -12.173$, $P = 0.001$) clades, indicating population expansion, but not in the Northern ($F_S = -3.104$, $P = 0.178$) or Eastern ($F_S = -5.001$, $P = 0.121$) clades.

Tests of biogeographical hypotheses

All three of the biogeographical barriers were spanned by major, strongly supported clades within *B. attenuatus*,

Table 3 Results of partial Mantel tests testing effects of hypothesized biogeographical barriers. For each comparison, the partial correlation between the genetic distance matrix and the barrier or geographical distance matrix, taking into account the other, is shown

Hypothesized barrier Clade	Mantel r (barrier)	P -value	Mantel r (geographical distance)	P -value
Central Valley				
Eastern	-0.205	< 0.001	0.545	< 0.001
San Andreas Fault				
Southern_North	0.332	< 0.001	0.690	< 0.001
Southern_South	0.189	< 0.001	0.144	< 0.001
Southern_South (excluding population 123)	0.094	< 0.05	0.441	< 0.001
Bodega	-0.171	NS	0.551	< 0.001
South	0.180	< 0.001	0.632	< 0.001
Sacramento–San Joaquin Delta				
Eastern (excluding Sierran)	0.211	< 0.001	0.490	< 0.001
Southern_North	-0.073	NS	0.586	< 0.001
All (excluding Sierran)	0.392	< 0.001	0.303	< 0.001

NS, not significant.

resulting in posterior probabilities < 5% for each of the alternative topologies tested. The hypothesis of a coastal vs. inland fragmentation was rejected by the monophyly of the Eastern clade, which includes samples from both sides of the Great Central Valley (Fig. 1). The hypothesis of fragmentation associated with the San Andreas Fault was rejected by the monophyly of the North and South clades, both of which span the fault. A sister group relationship between the Southern_North and the Southern_South clades (both mostly distributed west of the San Andreas Fault, but in different land masses associated with its tectonic evolution) was strongly supported, however, suggesting a role of the San Andreas fault in genetic differentiation within the South clade in *B. attenuatus*. Finally, the hypothesis of genetic fragmentation associated with the formation of the Sacramento–San Joaquin Delta was rejected by the monophyly of the South clade and by the monophyly of the Eastern and Southern_North clades, each of which includes samples from both north and south of the Delta. In the latter two cases, however, most genetic variation is found north of the Sacramento Delta, suggesting recent colonization of areas south of the Delta by these clades.

Partial Mantel tests revealed significant effects of all three biogeographical barriers, although not in all clades. These tests showed that genetic distance is positively correlated with separation by the San Andreas Fault in the Southern_North and Southern_South but not Bodega clades, and with separation by the Sacramento Delta in the Eastern clade and whole data set but not in the Southern_North clade (Table 3). A negative correlation was observed between genetic distance and separation by the Central Valley in the Eastern clade. The correlations between genetic and geographical distances remained sig-

nificant when the presence of hypothesized biogeographical barriers were controlled for, indicating the barrier alone is not sufficient to account for the observed genetic structure. In two cases, the geographical barrier explained more of the observed genetic structure, whereas in the remaining seven cases geographical distance was more important (Table 3).

Divergence time estimates

Based on the clades recovered in the phylogenetic analyses, estimates of TMRCA were calculated for the following groups of haplotypes: the five main clades, the northern and southern subclades within the Northern clade (Northern_North and Northern_South), and the subclade including all East San Francisco Bay and Sierran samples within the Eastern clade (East Bay_Sierra, Fig. 2). To estimate the timing of deeper divergences within *B. attenuatus*, we estimated the TMRCA of the North and South clades. The results obtained for the two different substitution rates tested are shown in Table 4. Coalescence within all of the major clades except Bodega occurs in the Miocene [6.1–9.1 million years ago (Ma) assuming the slower calibration rate or Pliocene (2.5–3.7 Ma) assuming the higher rate, while coalescence within the Bodega clade is substantially younger (Pliocene or Pleistocene) (Table 4).

Discussion

*Intraspecific diversification in *Batrachoseps attenuatus**

We found a remarkable amount of genetic variability within *Batrachoseps attenuatus*. First, we identified five divergent mtDNA lineages, with average pairwise distances between

Table 4 Divergence time estimates [in million years (Myr)] in selected clades within *Batrachoseps attenuatus* (see text). Cladogenetic events were dated according to a Bayesian coalescent approach. We present mean values and 95% confidence intervals (CI) for estimates of time to the most recent common ancestor (TMRCA) for each clade. Divergence times were calculated under a relaxed clock model assuming two different mutation rates: 0.01 mutation/site/Myr (= 2% pairwise sequence divergence) and 0.004 mutations/site/Myr (= 0.8% pairwise sequence divergence)

Lineage	0.8%		2%	
	Mean	95% CI	Mean	95% CI
Northern	9.03	6.45–11.61	3.65	2.73–4.80
Northern_North	2.18	1.17–3.60	0.88	0.43–1.36
Northern_South	5.17	3.74–6.76	2.13	1.57–2.70
Eastern	6.13	4.58–7.79	2.49	1.79–3.21
East Bay_Sierra	2.63	1.42–3.89	1.10	0.64–1.65
Bodega	2.15	1.21–3.48	0.88	0.48–1.41
Southern_North	7.67	5.06–10.26	2.97	2.05–4.00
Southern_South	9.14	6.91–11.44	3.61	2.66–4.54
North	14.44	10.81–17.98	5.77	4.34–7.22
South	13.08	9.88–16.50	5.16	3.85–6.58
<i>B. attenuatus</i>	19.28	15.27–24.11	7.63	5.87–9.42

clades ranging from 5.5 to 9.5%, well above typical values reported for intraspecific comparisons in studies of other species of salamanders using the same mtDNA marker (Mead *et al.* 2001; Mahoney 2004; Carstens *et al.* 2005; but see Wake 2006). Also remarkable is the variation uncovered within each of these lineages. Values of gene diversity are extremely high, above 0.96 in all cases, and the number of segregating sites (*s*) between haplotypes within clades is also high (Northern = 89, Eastern = 89, Bodega = 22, Southern_North = 83, Southern_South = 129). There are also very few cases of haplotypes that are found in more than a single locality (only 11 out of 133 haplotypes in the ingroup). Moreover, in six of these cases, distances between populations sharing haplotypes do not exceed 10 km. The most geographically widespread haplotypes are those found in populations 37–40, 44 and 47 (Tehama and Glenn counties, maximum distance: 25 km), populations 5 and 7 (Del Norte County, California, distance: 27 km), and populations 1–4 (Del Norte County, California, and Curry County, Oregon, maximum distance: 35 km). These populations are the northernmost in the Eastern and Northern clades, suggesting *B. attenuatus* has recently colonized these areas. It is therefore surprising that we did not find evidence for population expansion in these two clades according to Fu's F_S test.

Fu's F_S test detects a significant excess of singleton mutations using information from the haplotype distribution, and is one of the most powerful tests for detecting population growth according to Ramos-Onsins & Rozas (2002). Apart from potential sampling issues, the inability to detect population expansion in this case may result from an intrinsic problem related to the F_S statistic, which presents atypical statistical power drops with increasing sample size or

number of segregating sites (Ramos-Onsins & Rozas 2002). Other statistics (Tajima's D , Ramos-Onsins and Rozas' R_2 , Fu and Li's F and D) also failed to detect signatures of demographic expansion (data not shown).

Diversity is also high at the intrapopulation level; in the few cases where we sequenced five or more individuals from the same locality, we almost always found several (between four and six) different, closely related haplotypes. The exception is population 71 (West Point, Calaveras County), where the five sampled individuals shared one haplotype. This is in accordance with the inferred recent origin of populations in the western foothills of the Sierra Nevada (see below). These values of genetic variability are exceptional in the literature and caution against over-interpretation of the observed patterns, especially at the lowest geographical scale, where even finer-scale sampling will be required to refine hypotheses about genetic diversification within clades (Cabe *et al.* 2007). In general, the observed levels of variability suggest the persistence of large populations through geological time without significant bottlenecks and perhaps sustained, continuous population growth, as inferred from results of Fu's F_S test. Isolation-by-distance plots, Mantel tests and mismatch distributions are consistent with a general scenario of vicariant splitting of the five main lineages and subsequent long-term demographic stability or moderate growth in the resulting clades, with an overall equilibrium between the opposing forces of genetic drift and gene flow.

With regard to taxonomy, one possible interpretation of the observed patterns, especially given the apparent allopatry or parapatry of the identified clades, would be that *B. attenuatus* is actually a complex of several cryptic species. Clearly, consideration of the taxonomic implications of our

results requires additional data, in particular on variation in nuclear markers. Yanev (1978) carried out an extensive study on allozyme variation (19 loci) in several species of *Batrachoseps*. This study included 13 populations of *B. attenuatus*, which we infer belong to three of the five clades described here (Southern_South, Northern and Eastern). Reported values of Nei's genetic distance (Nei 1972) in Yanev (1978) were 0.1 (mean between all *B. attenuatus* populations) and 0.2 between grouped 'Coast Ranges' (Southern_South and Northern clades, which are distantly related, in our study) and 'Sierra Nevada' populations (Eastern clade in our study). The latter have a fixed, exclusive allele at the MDH-2 locus, and present the lowest values of observed heterozygosity, suggesting possible bottleneck effects during the colonization of the Sierra Nevada foothills. Our RAG-1 data set also does not identify clades corresponding to the five mtDNA clades, and thus does not provide evidence for cryptic speciation. Incomplete lineage sorting, which is likely given the low mutation rate of this marker and the inferred large historical effective population sizes in this species, or alternatively, nuclear gene flow between different mtDNA clades, could explain this pattern. Further studies should analyse interactions in potential mitochondrial contact zones with more variable markers, like microsatellites and nuclear introns (studies in progress).

Comparative phylogeographic hypotheses

Comparative analyses of genetic vs. geographical distances (Calsbeek *et al.* 2003) and of the geography of lineage breaks (Rissler *et al.* 2006) in California herpetofauna have suggested two major barriers in the study region, the Great Central Valley and the Sacramento–San Joaquin Delta. In *Batrachoseps*, genetic breaks between major lineages do not coincide with these hypothesized biogeographical barriers. Nevertheless, we were able to detect an impact of these barriers on genetic divergence.

Many amphibian and reptile species are continuously distributed in northern California, with ranges that extend down the north Coast Ranges and foothills of the Sierra Nevada, with a gap in the Central Valley (Stebbins 2003). Despite this continuous distribution, most species show deep genetic breaks across the Central Valley (Rissler *et al.* 2006). By contrast, *B. attenuatus* has a disjunct distribution, with no connection across the mountains of northern California. However, we found that populations on opposite sides of this present-day barrier are actually more genetically similar than would be predicted, given their geographical distances. This, in combination with the placement of these populations nested within the Eastern clade, suggests that they originated recently by dispersal across the Central Valley. The persistence of isolated populations in patches of suitable habitat within the Central Valley further supports this interpretation. A 'Transvalley leak' pattern such as

this one was first described in the salamander *Ensatina eschscholtzii xanthoptica* (Wake 1997), and has also been documented in *Aneides lugubris* (Lapointe & Rissler, 2005). While a close genetic connection in species disjunctly distributed across the Central Valley and deep genetic breaks in species whose range is continuous may seem paradoxical at first, it may indicate that colonization of the Sierra Nevada during the Pleistocene was more likely to succeed in species that did not encounter close relatives.

One population of the Southern_South clade (Riverbank, no. 123) was also found well into the Central Valley (Fig. 1). While it is possible that this population resulted from a Pleistocene range expansion of the Southern_South clade, an alternative explanation is that it resulted from a human-mediated introduction. This alternative is suggested by the phylogenetic placement of these samples, nested within the coastal, rather than the geographically closer interior, haplotypes (Fig. 2). These Riverbank samples are responsible for the much lower correlation between genetic and geographical distance in the Southern_South clade than in other clades (Fig. 3); exclusion of these samples leads to a correlation coefficient of 0.545 ($P < 0.001$), suggesting a strong pattern of isolation by distance.

Our analyses of hypothesized biogeographical barriers suggest that the Sacramento–San Joaquin Delta has functioned at two levels in structuring genetic diversity within *B. attenuatus*. In the species as a whole as well as in the Eastern clade, there is a positive correlation between separation by the Delta and genetic distance, accounting for the effects of geographical distance. By contrast, separation by the Delta was not correlated with genetic distance in the Southern_North clade, which is found on both sides of the mouth of San Francisco Bay and has a demographic signature of recent range expansion (this opening is recent, and these samples may have been connected as recently as 15 000 years ago, Atwater 1979).

We also investigated the role of the San Andreas Fault in shaping patterns of genetic divergence in *B. attenuatus*. Plate tectonics has been suggested to explain the disjunct distribution of species in the *Batrachoseps pacificus* group (Wake 2006). Such an effect might remain detectable over long periods of time because of the very high geographical structuring of mtDNA in *Batrachoseps*. In two of the three clades spanning the San Andreas Fault, populations located on opposite sides of the Fault were more divergent than expected given their geographical separation. This is consistent with the hypothesized role of the Fault as a historical biogeographical barrier. An alternative interpretation is that the Fault zone serves as a current barrier to gene flow. Because *B. attenuatus* is continuously distributed across the Fault zone, and habitat appears suitable, we believe that a historical role is the more likely explanation for increased divergence between populations spanning the Fault in the Southern_North and Southern_South clades. The third

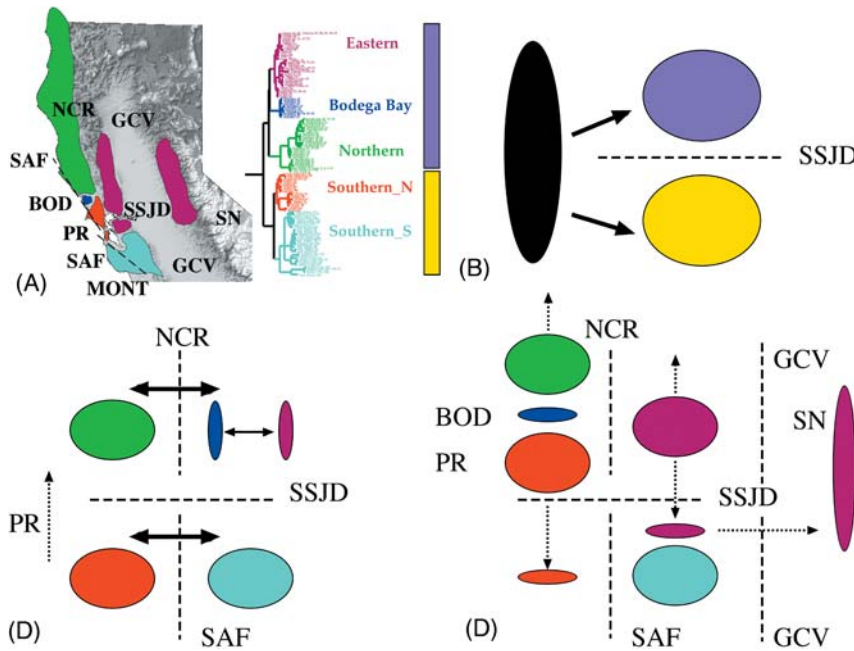


Fig. 4 Biogeographical reconstruction for *Batrachoseps attenuatus*. (A) Map of California, showing the main physiographic features discussed in the text, the distribution of each clade (colours), and our preferred phylogenetic hypothesis; (B) the first split produces the North (the ancestor of the Northern, Bodega and Eastern clades) and South (the ancestor of the Southern_South and Southern_North clades) clades; (C) later, each of these clades is in turn fragmented. The Northern and Eastern clades are separated by the North Coast Ranges (NCR), and the geographical origin of the Bodega clade is uncertain (see text). The Southern_North clade is isolated in the Point Reyes Peninsula (PR), which subsequently drifts north of the Sacramento-San Joaquin Delta (SSJD); (D) Colonization of areas south of the SSJD by the Southern_North and Eastern clades and subsequent colonization of the Sierra Nevada (SN) by the latter, across the Great Central Valley (GCV). Northward dispersal events are also shown for the Northern and Eastern clades. Not shown is dispersal of the Bodega and Southern_North clades across the San Andreas Fault (SAF). Colours as in Figs 1 and 2. Solid arrows indicate phylogenetic splits whereas dashed arrows represent dispersal events. BOD, Bodega Head Peninsula; MONT, Monterey Embayment.

clade spanning the Fault, the Bodega clade, had very low sequence divergence overall, and a negative, but nonsignificant, correlation between the Fault and genetic distance matrices when geographical distance was accounted for. Together, this evidence suggests that the Fault zone is not an inherent barrier to dispersal in the Bodega clade.

Biogeography

The complex geological history of California and its associated biota, rich in endemism, has long inspired studies aimed at unravelling the roles of historical factors in species formation, especially after range-wide allozyme and mtDNA data sets for many species became available (Yanev 1978, 1980; Wake 1997; Matocq 2002; Kuchta & Tan 2005). Based on our preferred gene tree, five well-supported clades are recovered within *B. attenuatus*. The distribution of these clades falls mainly into two very different geological substrates, separated by the San Andreas Fault: the Salinian block and the Franciscan complex (Blake 1984). The former includes the Bodega and Point Reyes Peninsulas as well as part of the southern San Francisco Peninsula and Santa Cruz mountains, currently inhabited by the Bodega,

Southern_North and Southern_South clades, respectively (Fig. 4). Rocks associated with the Franciscan formation include the Diablo Range in the East San Francisco Bay and the Coastal Ranges north and south of the Bay, inhabited by the Northern and Eastern clades. Based on palaeogeographical reconstructions, the tests of biogeographical barriers and the estimated divergence times, we propose a biogeographical scenario accounting for the observed patterns (Fig. 4).

Because *B. attenuatus* is the sister taxon to all other species in the *Batrachoseps (sensu stricto)* clade (Jockusch & Wake 2002), it seems reasonable to assume an ancestor for all extant mtDNA clades in *B. attenuatus* north of the Monterey Embayment (Fig. 4). This Monterey area constituted the main drainage for an interior sea occupying most of the Great Central Valley until around 600 000 years ago (Dupré 1990) and has been associated with important genetic breaks in a variety of taxa (see for example Jockusch & Wake 2002). The timing of the split between *B. attenuatus* and the other species of *Batrachoseps* cannot be precisely determined from our data set, but (taking into account the uncertainties associated to our time estimates) a minimum age of around 7.6 Ma for the common ancestor of all *B.*

attenuatus haplotypes can be assumed (Table 4); by that time, the ancestor of all five mtDNA clades would have been widespread in the areas north of the Monterey Embayment (Fig. 4).

The Point Reyes Peninsula, part of the Salinian block, has been displaced around 180 km northwest along the San Andreas Fault in the past 60 Myr to reach its present position north of the San Francisco Bay, with 70–90 km of displacement in the last 6–8 Myr (Burnham 2005). At the end of Miocene, most of the Peninsula mass was under the sea, but in the last 5 Myr most of the area has been above sea level and subject to erosion during its northward migration (Galloway 1977). Based on our results, we hypothesize that Point Reyes was colonized by *Batrachoseps* before it drifted north; the isolation of this landmass after contact with the mainland south of its present position would explain both the genetic distinctiveness of the Southern_North clade and also its evolutionary affinities with southern populations (the Southern_South clade) (Fig. 4C). If our hypothesis is correct, we predict that finer-scale studies will uncover the highest values of genetic diversity and ancestral haplotypes in the Point Reyes Peninsula, and a pattern of successive colonizations moving east into Marin County and south into the northern San Francisco Peninsula. A similar biogeographical pattern has been described in the spider *Atypoides riversi* (Ramírez & Chi 2004). We anticipate that future studies in other taxa will find similar relationships between populations north and south of the San Francisco Bay region, particularly in taxa with low vagility. The present distribution of the Bodega clade spans the San Andreas Fault and could be explained either as the result of a recent population expansion into areas west of the Fault or as the result of the Bodega ancestor having moved long ago into the Bodega Head Peninsula and later drifted north to its present location, subsequently expanding inland to the east (Fig. 4C). In the latter case, separation along the San Andreas Fault could have contributed to the isolation of lineages. The fact that three of the main clades identified in our study (Northern, Bodega, and Southern_North) are geographically close to each other near the mouth of the Russian river suggests changes in the flow pattern in this basin may have constituted temporary barriers to dispersal at different time periods, but further sampling is needed in this region to test this hypothesis. Rissler *et al.* (2006) found genetic breaks in the vicinity of the Russian River in four of five amphibian species examined, but not in any reptiles, suggesting that it is an amphibian-specific barrier.

With respect to the Northern and Eastern clades, both are old, with ancestral haplotypes dating back to around a minimum of 5.7 Ma. Thus an old vicariant event would have isolated the ancestors of these clades from the ancestors of the Southern clades (Fig. 4B). It is unclear which event caused this, although a plausible hypothesis would be the presence of marine seaways north of present-day

San Francisco Bay (Dupré 1990; Sims 1993). Later, the final stages in the orogeny of the northern Coast Ranges, associated with extensive volcanism, might have isolated the ancestors of the Northern (on the coastal side) and the Eastern (in the interior) clades on opposite slopes (Fig. 4C). In both clades, there are more recently derived subclades (Northern_North and East Bay_Sierra), both probably less than 2.2 Myr old (Fig. 2, Table 4), suggesting recent colonization of areas either previously affected by glaciations (Northern clade) or isolated by geographical barriers (Eastern clade). Most variation within the Eastern clade is north of the Sacramento–San Joaquin Delta, suggesting that the East Bay south of the Delta was colonized relatively recently, constituting the advancing front from which populations in the Sierra foothills were eventually founded (Fig. 4D). The ability to colonize large, distant areas in relatively short time periods by such an extremely sedentary species is in principle surprising but could be explained by the persistence of large, stable populations through time and the presence of favourable habitats through which dispersal was possible.

Our study uncovered extremely high levels of genetic variability within a morphologically uniform, continuously distributed salamander species. The combination of fine-scale, comprehensive sampling with phylogenetic, historical demographic and hypothesis-based tests allowed delineation of a complex biogeographical scenario, with general implications for the study of codistributed taxa. No single a priori hypothesis based on biogeographical patterns observed in other taxa fully explained the complex pattern of genetic diversification observed. However, the combination of analyses providing relevant information at different timescales allowed identification of the relative contributions of different putative barriers to dispersal. The patterns revealed are unique in providing unprecedented detail about biogeographical patterns in California, adding a new layer of resolution for future comparative studies, and will prove useful in further delineating patterns of microendemism in the study region, highlighting the relevance of common, widespread species for conservation planning.

Acknowledgements

D. Buckley, C. Cicero (MVZ Tissue Collection), T. Devitt, C. Grande, M. Koo and J. Vindum (California Academy of Sciences Tissue Collection), R. Mueller and T. Papenfuss helped in collecting samples or provided tissues. C. Grande provided assistance with primer design. R. Pereira and two anonymous reviewers made useful suggestions that improved the manuscript. The California Department of Fish and Game provided collecting permits. This study was funded by the National Science Foundation (EF-0334939, DEB-0543446) and the MVZ (Alexander and Mertens Funds). I.M.S. was supported by a postdoctoral fellowship from the Spanish Ministerio de Educación y Ciencia (Ref. EX2004-0921).

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Appendix

Sampling localities, population identifiers (ID, used in Fig. 1), sample sizes, voucher numbers, haplotypes numbers (used in Fig. 2) and GenBank Accession numbers for the sequences obtained for this study. Abbreviations: Hwy, Highway; Rd, Road; mi, miles; ca, circa; km, kilometres; Blvd, Boulevard

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos			
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>
1	Northern	N bank Pistol River Rd, 3.5 mi E junction Hwy 101, Curry County, Oregon	42.27800	-124.34930	1	MVZ 157301	66	EU011264	EU011449	EU011491	EU011533
2	Northern	Winchuck River Rd at Winchuck Campground, Curry County, Oregon	42.01722	-124.10694	1	MVZ 230761	66	EU011265			
3	Northern	N bank Winchuck Rd, 1.8 mi E junction Hwy 101, Curry County, Oregon	42.01110	-124.18700	1	MVZ 157310	66	EU011266			
4	Northern	Rowdy Creek Rd, 4.0 mi E (by road) Hwy 101 at Smith River, Del Norte County, California	41.94325	-124.10184	1	MVZ 172625	66	EU011267			
5	Northern	J. Smith State Park, 0.6 mi down Walker Rd, Del Norte County, California	41.81708	-124.11069	1	MVZ 151641	59	EU011268			
6	Northern	Wilson Creek Rd, 0.8 mi NE Hwy 101, Del Norte County, California	41.61492	-124.09109	1	MVZ 168712	71	EU011269			
7	Northern	3.5 mi N Klamath along Hunter Creek Rd, 0.4 mi E junction Hwy 101, Del Norte County, California	41.57725	-124.03788	1	MVZ 151634	59	EU011270			
8	Northern	S of Big Lagoon, Humboldt Co, California	41.14085	-124.13232	1	MVZ 151726	61	EU011271			
9	Northern	3.0 mi E of Bayside, just E of Arcata-Eureka on Jacoby Creek Rd, Humboldt County, California	40.82511	-124.03714	1	MVZ 151672	69	EU011272			
10	Northern	Just N Scotia on Hwy 101, Humboldt Co, California	40.48862	-124.09778	1	MVZ 151793	62	EU011273			
11	Northern	Rio Dell, Monument Drive, ca. 1 mi W junction Hwy 101, Humboldt County, California	40.48747	-124.12053	1	MVZ 151788	70	EU011274			
12	Northern	Pepperwood, Humboldt County, California	40.44591	-123.99074	1	MVZ 151771	72	EU011275			
13	Northern	0.5 mi S of Alderpoint, Humboldt County, California	40.17149	-123.60624	1	MVZ 151671	60	EU011276			
14	Northern	N of Garberville, Humboldt County, California	40.14514	-123.77982	1	MVZ 151761	63	EU011277			
15	Northern	2 mi S Usal, Mendocino County, California	39.80638	-123.84329	1	MVZ 152104	77	EU011278	EU011447	EU011489	EU011531
16	Northern	2 mi N Rockport, Mendocino County, California	39.76651	-123.81255	1	MVZ 152101	77	EU011279			
17	Northern	Hwy 1, 2.1 mi N Rockport, Mendocino County, California	39.76434	-123.82438	1	MVZ 152096	76	EU011280	EU011446	EU011488	EU011530
18	Northern	7.5 mi E Fort Bragg on Hwy 20, Mendocino County, California	39.44334	-123.64649	1	MVZ 152036	75	EU011281			
19	Northern	Comptche Airport Rd, Little River, 1 mi E junction Hwy 1, Mendocino County, California	39.26653	-123.59501	1	MVZ 152042	64	EU011282			
20	Northern	Crest of Boonville-Ukiah Rd [Hwy 253], Mendocino County, California	39.05073	-123.24645	1	MVZ 152030	74	EU011283			
21	Northern	Approximately 4 mi E Boonville along Hwy 253 toward Ukiah, Mendocino County, California	39.00868	-123.31965	2	MVZ 151997 MVZ 152018	68 73	EU011284 EU011285			

Appendix Continued

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos				
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>	
22	Northern	Hopland–Lakeport Road, 6.3 mi from Hopland, Mendocino County, California	38.96840	–123.02800	1	IMSZ 56	50	EU011286				
23	Northern	Hopland–Lakeport Road, 10.6 mi from Hopland, Lake County, California	38.98031	–122.97134	1	IMSZ 57	51	EU011287				
24	Northern	18.8 mi W (by air) Geyserville on Stewarts Point Rd, Sonoma County, California	38.79003	–123.18188	1	MVZ 152941	67	EU011288				
25	Northern	Geysers Road, 2.4 mi from junction with Geysers Resort Rd, Sonoma County, California	38.77990	–122.81565	1	IMSZ 51	48	EU011289	EU011462	EU011504	EU011546	
26	Northern	Geysers Road, 1.7 mi from junction with Geysers Resort Rd, Sonoma County, California	38.78793	–122.82512	1	IMSZ 52	49	EU011290	EU011463	EU011505	EU011547	
27	Northern	0.3 mi N Wohler Rd, bridge over Russian River, on W side of River, Sonoma County, California	38.50806	–122.88556	5	DBW 6370	56	EU011291	EU011473	EU011515	EU011557	
						DBW 6371	57	EU011292				
						DBW 6372	55	EU011293				
						DBW 6374	58	EU011294				
						MVZ 221033	65	EU011295				
28	Northern	0.2 mi N Wohler Rd bridge over Russian River, E side River, Sonoma County, California	38.50722	–122.88222	11	DBW 6351	52	EU011296				
						DBW 6352	53	EU011297	EU011468	EU011510	EU011552	
						DBW 6353	52	EU011298				
						DBW 6354	54	EU011299				
						DBW 6355	53	EU011300				
						DBW 6356	52	EU011301				
						DBW 6357	54	EU011302				
						DBW 6358	55	EU011303				
						DBW 6360	52	EU011304				
						DBW 6361	52	EU011305				
29	Bodega	Martinelli Road, Gruntal Ranch, Sonoma County, California	38.28574	–122.54405	6	MVZ 221030	52	EU011306				
						DBW 6362	5	EU011307	EU011469	EU011511	EU011553	
						DBW 6363	6	EU011308				
						DBW 6364	7	EU011309	EU011470	EU011512	EU011554	
						DBW 6365	8	EU011310				
						DBW 6366	9	EU011311	EU011471	EU011513	EU011555	
30	Bodega	Across from Duncan's Mills on S side Russian River, 1.2 mi W Moscow Rd on Freeze Out Rd, Sonoma County, California	38.44358	–123.06223	1	DBW 6369	10	EU011312	EU011472	EU011514	EU011556	
						MVZ 152917	2	EU011313				
31	Bodega	Barnett Valley Rd, 1 mi E Burnside Rd, Sonoma County, California	38.36650	–122.89199	1	MVZ 235986	11	EU011314	EU011451	EU011493	EU011535	
32	Bodega	Estero Lane, Sonoma County, California	38.33353	–122.98660	1	IMSZ 32	1	EU011315				
33	Bodega	Coleman Valley Road, Sonoma County, California	38.36811	–123.06215	1	IMSZ 47	4	EU011316				

Appendix Continued

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos			
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>
34	Bodega	Bodega Head, near junction of East Shore with Bay Flat, Sonoma County, California	38.33548	-123.05101	1	IMSZ 43	2	EU011317	EU011460	EU011502	EU011544
35	Bodega	Marina on Bodega Head, near parking lot, Sonoma County, California	38.24346	-123.05710	1	IMSZ 40	3	EU011318	EU011459	EU011501	EU011543
36	Bodega	Bodega Head, Bodega Bay, Sonoma County, California	38.30523	-123.06125	1	IMSZ 38	2	EU011319	EU011458	EU011500	EU011542
37	Eastern	Thomes–Newville Reservoir site, 0.5 mi S Williams Butte, Tehama County, California	39.86237	-122.57774	1	MVZ 158894	33	EU011320			
38	Eastern	Mendocino National Forest, Forest Rd M4, 3.8 mi E (by road) of County Rd 58 (Newville Rd), Tehama County, California	39.82758	-122.62806	1	CAS 223702	33	EU011321			
39	Eastern	Mendocino National Forest, Round Valley Rd (Forest Rd M4), 3.7 mi W (by road) of Newville Rd, Tehama County, California	39.82622	-122.63086	1	CAS 202566	33	EU011322			
40	Eastern	Mendocino National Forest, Round Valley Rd (Forest Rd M4), 4.1 mi W (by road) of Newville Rd, Tehama County, California	39.82200	-122.62919	1	CAS 202569	33	EU011323			
41	Eastern	Salt Creek, 4.8 km W Newville, 0.4 km E Mendocino National Forest Boundary, 0.6 km S Glenn–Tehama County line, Glenn County, California	39.79278	-122.61565	2	MVZ 158445 MVZ 158448	32	EU011324 EU011325			
42	Eastern	Mendocino National Forest, Forest Rd M9 (Hull Rd), 6.1 mi E (by road) of County Rd 309, Glenn County, California	39.71398	-122.61691	1	CAS 223705	32	EU011326			
43	Eastern	Mendocino National Forest, Grindstone Rd, 3.9 mi N (by road) of Hwy 162, Glenn County, California	39.69697	-122.71313	2	CAS 202564 CAS 202565	32	EU011327 EU011328			
44	Eastern	Mendocino National Forest, Old Hwy 162, ca 0.7 mi W (by road) of Hwy 162 (eastern boundary of Mendocino National Forest), Glenn County, California	39.65877	-122.60394	2	CAS 202552 CAS 202553	33 41	EU011329 EU011330			
45	Eastern	Mendocino National Forest, Old Hwy 162, ca 0.6 mi W (by road) of Hwy 162 (eastern boundary of Mendocino National Forest), Glenn County, California	39.65686	-122.60480	2	CAS 202555 CAS 202556	42	EU011331 EU011332	EU011442	EU011484	EU011526
46	Eastern	Mendocino National Forest, Sanhedrin Rd, 9.0 mi E (by road) of County Rd 309, Glenn County, California	39.63713	-122.65004	2	CAS 223711 CAS 223712	46 47	EU011333 EU011334	EU011445	EU011487	EU011529
47	Eastern	Mendocino National Forest, Sanhedrin Rd, 5.5 mi E (by road) of County Rd 309, Glenn County, California	39.63197	-122.60766	2	CAS 223709 CAS 223710	33	EU011335 EU011336	EU011444	EU011486	EU011528
48	Eastern	Mendocino National Forest, 0.1 mi N (by road) of North Fork Campground, Colusa County, California	39.38033	-122.64961	1	CAS 202597	44	EU011337			
49	Eastern	Mendocino National Forest, North Fork Campground, Colusa County, California	39.37947	-122.64913	1	CAS 202580	43	EU011338	EU011443	EU011485	EU011527
50	Eastern	Cache Creek Natural Area, Yolo County, California	38.90780	-122.30916	1	TJD 0184	12	EU011339			
51	Eastern	Lake Berryessa, 12 mi N along Knoxville Creek [tributary of Etnicnera Creek], Napa County, California	38.85712	-122.40395	1	MVZ 152253	36	EU011340			

Appendix Continued

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos																																													
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>																																										
52	Eastern	8 mi NW Winters on Hwy 128, Yolo County (=Fremont County), California	38.57851	-122.05978	2	MVZ 152809 MVZ 152818	28 34	EU011341 EU011342	EU011448	EU011490	EU011532																																										
53	Eastern	Along Putah Creek, 0.75 mi E Old Davis Rd, Yolo County (=Fremont County), California	38.53846	-121.74591	1	MVZ 222689	38	EU011343	EU011450	EU011492	EU011534																																										
54	Eastern	Archer Taylor Preserve, Napa County, California	38.36097	-122.42565	1	TJD 0192	13	EU011344	EU011453	EU011495	EU011537																																										
55	Eastern	Carneros Highway (Hwy 12), Cherry Hill, Napa County, California	38.18565	-122.44202	2	DBW 6397	25	EU011345																																													
56	Eastern	Carneros Highway (Hwy 12), 0.4 mi to Sonoma County line, Napa County, California	38.25408	-122.37260	4	DBW 6398	26	EU011346	EU011481	EU011523	EU011565																																										
						DBW 6390	23	EU011347	EU011479	EU011521	EU011563																																										
						DBW 6391	24	EU011348																																													
						DBW 6393	25	EU011349	EU011480	EU011522	EU011654																																										
57	Eastern	Carneros Highway (Hwy 12), junction with Old Sonoma Road, Napa County, California	38.25570	-122.34267	2	DBW 6394	21	EU011350																																													
						DBW 6388	21	EU011351																																													
58	Eastern	Soscol Creek Road, Napa County, California	38.24068	-122.26724	1	DBW 6385	22	EU011353																																													
59	Eastern	American Canyon, Oat Hill, Napa County, California	38.18038	-122.26131	10	DBW 6375	17	EU011354	EU011474	EU011516	EU011558																																										
						DBW 6376	18	EU011355	EU011475	EU011517	EU011559																																										
						DBW 6377	17	EU011356																																													
						DBW 6378	17	EU011357																																													
						DBW 6379	19	EU011358	EU011476	EU011518	EU011560																																										
						DBW 6380	20	EU011359	EU011477	EU011519	EU011561																																										
						DBW 6381	21	EU011360	EU011478	EU011520	EU011562																																										
						DBW 6382	19	EU011361																																													
60	Eastern	Point Richmond at Brickyard Cove, Richmond, Contra Costa County, California	37.91100	-122.38370	1	DBW 6383	19	EU011362																																													
						DBW 6384	19	EU011363																																													
						MVZ 244077	39	EU011364																																													
						61	Eastern	El Cerrito, Baron's Residence, Contra Costa County, California	37.90988	-122.30141	1	MVZ 151380	27	EU011365																																							
												62	Eastern	Vine Street, between Henry and Shattuck, Berkeley, Alameda County, California	37.87983	-122.27040	1	IMSZ 50	14	EU011366	EU011461	EU011503	EU011545																														
																		63	Eastern	Anthony Chabot Regional Park, near Bort Meadow Staging Area parking, Alameda County, California	37.77757	-122.12639	1	IMSZ 69	15	EU011367																											
																								64	Eastern	Willow Park Public Golf Course, Alameda County, California	37.72436	-122.07938	1	IMSZ 78	16	EU011368	EU011464	EU011506	EU011548																		
																														65	Eastern	Walnut Creek, Bruce Baigami's back yard, Contra Costa County, California	37.90099	-122.06186	1	MVZ 151627	40	EU011369															
																																				66	Eastern	4 mi E Clayton on Clayton Rd, Contra Costa County, California	37.93891	-121.85075	1	MVZ 151354	37	EU011370									
																																										67	Eastern	Howard Ranch (1), Sacramento County, California	38.32667	-121.03216	1	RLM 97	35	EU011371			

Appendix Continued

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos				
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>	
68	Eastern	Howard Ranch (2), Sacramento County, California	38.32600	-121.03699	1	RLM 103	36	EU011372				
69	Eastern	Jackson, just off Hwy 49, Amador County, California	38.33832	-120.75365	1	ELJ 1134	29	EU011373				
70	Eastern	End of Higdon Rd, 3.2 mi (by air) W of West Point, Calaveras County, California	38.39865	-120.58844	1	MVZ 237183	30	EU011374	EU011452	EU011494	EU011536	
71	Eastern	3.2 mi W of West Point, Calaveras County, California	38.39971	-120.58731	5	MVZ 205354	30	EU011375				
						MVZ 205355		EU011376				
						MVZ 205357		EU011377				
						MVZ 205358		EU011378				
						MVZ 205359		EU011379				
72	Eastern	University of California Sierra Foothill Range Field Station, E Brown's Valley, near dam on Engelbright Reservoir, Yuba County, California	39.25000	-121.28333	1	MVZ 211859	31	EU011380				
73	Eastern	Plumas National Forest, road along W side of S tributary to Jack Creek, Butte County, California	39.71480	-121.38402	1	CAS 205581	45	EU011381				
74	Southern (N)	0.8 mi S Blank Rd on Peterson Rd, 6675 Peterson Rd, Sonoma County, California	38.33529	-122.76050	1	MVZ 235950	127	EU011382				
75	Southern (N)	Tomales Community Park, Marin County, California	38.24535	-122.90568	1	IMSZ 22	118	EU011383	EU011456	EU011498	EU011540	
76	Southern (N)	Valley Ford Franklin School Rd, Marin County, California	38.25230	-122.94346	1	IMSZ 26	119	EU011384	EU011457	EU011499	EU011541	
77	Southern (N)	Highway 1, Marshall-Bodega Bay, 2.9 mi N from junction with Marshall-Petaluma Rd, Marin County, California	38.19603	-122.91961	1	IMSZ 20	117	EU011385	EU011455	EU011497	EU011539	
78	Southern (N)	Highway 1, Marshall-Bodega Bay, near junction with Marshall-Petaluma Rd, Marin County, California	38.16440	-122.89108	1	IMSZ 8	116	EU011386				
79	Southern (N)	Petaluma-Marshall Rd (1), Marin County, California	38.15181	-122.85796	1	IMSZ 6	115	EU011387				
80	Southern (N)	Petaluma-Marshall Rd (2), Marin County, California	38.16890	-122.79725	1	IMSZ 5	114	EU011388				
81	Southern (N)	Petaluma-Marshall Rd, near bridge over Salmon Creek, Marin County, California	38.16538	-122.73661	1	IMSZ 3	113	EU011389	EU011454	EU011496	EU011538	
82	Southern (N)	Along Hwy 1 at E margin Tomales Bay, Marin County, California	38.10470	-122.83840	1	MVZ 237275	130	EU011390				
83	Southern (N)	Along Coast Trail, 0.3 mi S American Youth Hostel, Point Reyes, Marin County, California	38.04004	-122.86519	1	MVZ 235954	128	EU011391				
84	Southern (N)	N end Glenn Trail, Point Reyes, Marin County, California	37.99533	-122.79594	1	MVZ 225931	129	EU011392				
85	Southern (N)	Indian Valley Golf Course, Stafford Lake, Marin County, California	38.10886	-122.64001	1	DBW 6415	123	EU011393	EU011483	EU011525	EU011567	
86	Southern (N)	Day Island Wildlife Area, Black Point, Marin County, California	38.10003	-122.50017	2	DBW 6400	121	EU011394	EU011482	EU011524	EU011567	
						DBW 6401	122	EU011395				
87	Southern (N)	Lucas Valley Rd, 3 mi from Bridge Point, Marin County, California	38.04290	-122.61650	1	DBW 6420	124	EU011396				
88	Southern (N)	Gerstle Park and adjoining land, San Rafael, Marin County, California	37.96549	-122.53557	3	MVZ 224437	125	EU011397				
						MVZ 224438	126	EU011398				
						MVZ 224446	120	EU011399				

Appendix Continued

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos			
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>
89	Southern (N)	near Mount Tamalpais State Park, Marin County, California	37.91875	-122.60031	1	IMSZ 115	120	EU011400			
90	Southern (N)	San Francisco, ca. 80 meters S of intersection of Ortega St. and 14th Avenue, in rocky outcrop 15 meters E of 14th Avenue, San Francisco County, California	37.75200	-122.47100	1	CAS 208078	133	EU011401			
91	Southern (N)	San Francisco, Lake Merced at S end of Harding Park Golf Course, ca. 100 meters W of intersection of Lake Merced Blvd and Higuera Street, San Francisco County, California	37.72097	-122.49326	1	CAS 200601	132	EU011402			
92	Southern (N)	Daly City, lot at junction of Mission Blvd and Alemany Blvd, San Mateo County, California	37.71006	-122.45612	1	MVZ 152457	131	EU011403			
93	Southern (S)	Pacifica, Sharp Park, field between Golf Course and Mori Point, San Mateo County, California	37.62147	-122.49233	1	MVZ 152533	92	EU011404			
94	Southern (S)	Hillsborough, San Mateo County, California	37.56895	-122.37665	1	MVZ 152476	88	EU011405			
95	Southern (S)	0.7 mi W El Camino Real, Crystal Spring Rd at Cunningham Way, San Mateo County, California	37.55555	-122.33540	1	MVZ 167301	89	EU011406			
96	Southern (S)	Moss Beach, San Mateo County, California	37.52764	-122.51226	1	MVZ 152523	105	EU011407			
97	Southern (S)	ca. 6.5 km ENE (by air) Half Moon Bay at Hwy 35, 5.6 mi N junction Tunitas Creek Rd near Hwy 92, San Mateo County, California	37.48459	-122.35827	1	MVZ 152472	104	EU011408			
98	Southern (S)	Purisima Creek Redwoods, San Mateo County, California	37.43088	-122.31418	3	IMSZ 122 IMSZ 123 IMSZ 124	86 86 87	EU011409 EU011410 EU011411			
99	Southern (S)	Tunitas, 3.2 mi E junction of Hwy 1 and 6.7 mi SW junction of Hwy 35 on Tunitas Creek Rd, San Mateo County, California	37.38281	-122.37250	1	MVZ 152571	108	EU011412			
100	Southern (S)	SE San Gregorio, 1.4 mi E Stage Rd on Pomponio Rd, San Mateo County, California	37.30423	-122.35534	1	MVZ 220569	106	EU011413			
101	Southern (S)	Along Pescadero Rd, 0.7 mi N Warr Rd and 0.4 mi S Jones Gulch Rd, which is 3.4 mi S Alpine Rd, San Mateo County, California	37.29167	-122.28056	1	MVZ 235966	91	EU011414			
102	Southern (S)	Approximately 8.5 km SSE Pescadero at Gazos Creek, 2.9 mi E Hwy 1 on Gazos Creek Rd, San Mateo County, California	37.18576	-122.33153	1	MVZ 152553	107	EU011415			
103	Southern (S)	Hwy 9, 7 mi N (by road) Boulder Creek, Santa Cruz County, California	37.21263	-122.14532	1	MVZ 158199	109	EU011416			
104	Southern (S)	At junction of New Years Creek Rd and Hwy 1, 0.9 mi N of Santa Cruz county line on Hwy 1, San Mateo County, California	37.11689	-122.30281	1	MVZ 205373	90	EU011417			
105	Southern (S)	0.7 mi WNW Empire Grade on Pine Flat Rd, Santa Cruz County, California	37.07910	-122.14065	1	MVZ 220533	111	EU011418			

Appendix *Continued*

ID	Clade	Locality (USA)	Latitude	Longitude	Sample size	Voucher nos	Haplotype nos	GenBank Accession nos				
								<i>cob</i>	16S	<i>cox</i>	<i>nad4</i>	
106	Southern (S)	Martin Road, 1.3 mi NE (by road) junction with Pine Flat Road, Santa Cruz County, California	37.05468	-122.13819	1	MVZ 218030	111	EU011419				
107	Southern (S)	Smith Grade, 0.6 mi SW Empire Grade, 2.5 mi SW (by air) Felton, Santa Cruz County, California	37.02374	-122.10433	1	MVZ 167514	110	EU011420				
108	Southern (S)	1.1 mi NE Hwy 1 on Bonny Doon Rd, Santa Cruz County, California	37.01485	-122.17350	2	MVZ 220530	112	EU011421				
						MVZ 220531	93	EU011422				
109	Southern (S)	Soda Springs Rd, 0.4 mi E Alma Bridge Rd, Santa Clara County, California	37.18333	-121.97500	1	MVZ 230762	94	EU011423				
110	Southern (S)	Summit Rd, 2.9 mi NW Mount Madonna Rd, Santa Clara County, California	37.04611	-121.75194	4	MVZ 230741	96	EU011424				
						MVZ 230742	95	EU011425				
						MVZ 230743	97	EU011426				
						MVZ 230744	96	EU011427				
111	Southern (S)	Redwood Retreat Rd, 2.4 mi W junction Watsonville Rd, Santa Clara County, California	37.03139	-121.69666	1	MVZ 230740	98	EU011428				
112	Southern (S)	Hagerman Canyon, Santa Clara County, California	37.03720	-121.30820	1	ELJ 1075	100	EU011429				
113	Southern (S)	Dinosaur Point Rd above San Luis Reservoir, Merced County, California	37.06667	-121.19333	1	MVZ 235987	101	EU011430				
114	Southern (S)	San Antonio Valley Rd, Santa Clara County, California	37.33279	-121.49629	1	IMSZ 111	85	EU011431				
115	Southern (S)	San Antonio Valley Rd, 2.8 mi from Hamilton Peak, Santa Clara County, California	37.35001	-121.60992	1	IMSZ 109	84	EU011432	EU011467	EU011509	EU011551	
116	Southern (S)	Highway 130, Santa Clara County, California	37.33166	-121.68648	1	IMSZ 104	83	EU011433	EU011466	EU011508	EU011550	EU011550
117	Southern (S)	Highway 130, 4.8 mi towards Hamilton Peak from San Jose, Santa Clara County, California	37.35974	-121.75417	1	IMSZ 102	82	EU011434	EU011465	EU011507	EU011549	EU011549
118	Southern (S)	Sierra Rd, 2.4 mi from Piedmont, Santa Clara County, California	37.41352	-121.82008	1	IMSZ 97	81	EU011435				
119	Southern (S)	Calaveras Valley, 0.8 mi from Felter Rd, Santa Clara County, California	37.44562	-121.81522	1	IMSZ 94	80	EU011436				
120	Southern (S)	Approximately 23 mi E (by air) Milpitas at junction of San Antonio Valley Rd and Mine Rd, 1.3 mi S Alameda county line, Santa Clara County, California	37.46111	-121.51667	1	MVZ 152670	99	EU011437				
121	Southern (S)	Calaveras Rd, 0.4 mi from Welch Creek, Santa Clara County, California	37.54029	-121.85612	1	IMSZ 91	79	EU011438				
122	Southern (S)	Palomares Rd, 5.1 mi from junction with Hwy 84, Santa Clara County, California	37.64921	-121.97622	1	IMSZ 80	78	EU011439				
123	Southern (S)	S bank of Stanislaus River 0.3 mi W junction of Claus Rd and Hwy 108, Riverbank, Stanislaus County, California	37.73770	-120.92560	2	MVZ 230638	102	EU011440				
						MVZ 230639	103	EU011441				
					Total:		178	133				