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Tripartite genetic subdivisions in the ornate shrew (*Sorex ornatus*)

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Abstract

We examined cytochrome *b* sequence variation in 251 ornate shrews (*Sorex ornatus*) from 20 localities distributed throughout their geographical range. Additionally, vagrant (*S. vagrans*) and montane (*S. monticolus*) shrews from four localities were used as outgroups. We found 24 haplotypes in ornate shrews from California (USA) and Baja California (Mexico) that differed by 1–31 substitutions in 392 bp of mitochondrial DNA (mtDNA) sequence. In a subset of individuals, we sequenced 699 bp of cytochrome *b* to better resolve the phylogeographic relationships of populations. The ornate shrew is phylogeographically structured into three haplotype clades representing southern, central and northern localities. Analysis of allozyme variation reveals a similar pattern of variation. Several other small California vertebrates have a similar tripartite pattern of genetic subdivision. We suggest that topographic barriers and expansion and contraction of wetland habitats in the central valley during Pleistocene glacial cycles account for these patterns of genetic variation. Remarkably, the northern ornate shrew clade is phylogenetically clustered with another species of shrew suggesting that it may be a unique lowland form of the vagrant shrew that evolved in parallel to their southern California counterparts.

Keywords: allozymes, conservation, cytochrome *b*, mitochondrial DNA, phylogeography, Soricidae

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Introduction

The ornate shrew (*Sorex ornatus*) is a rare species restricted to coastal marshes and riparian communities of California, from 39° N latitude southward discontinuously to the tip of Baja California (Mexico). Nine subspecies are currently recognized; two of them historically had wide distributions while the other seven are found in small patches along coastal marshes, some inland valleys, and montane meadows. A relictual population exists in the Sierra de la Laguna at the tip of Baja California, as well as an insular population on Santa Catalina Island (Fig. 1; Owen & Hoffmann 1983).

The presence of physical and ecological barriers between populations may lead to their genetic isolation. Over time, the phylogeny of DNA sequences from isolated populations will become reciprocally monophyletic such that a phylogenetic tree of these sequences will show divisions corresponding to the location of topographic barriers (Avise 1992). Due to their small size, semifossorial habits and high degree of habitat specialization, populations of ornate shrews are predicted to be highly isolated. Ornate shrews are limited to patchily distributed palustrine or salt marsh habitats (Owen & Hoffman 1983). These habitats have increasingly discontinuous distributions towards southern California and Baja California. In addition, the shrew's high basal metabolic rates require them to feed often as their energy stores are continuously threatened by exhaustion (McNab 1991), further restricting long distance dispersal. Ornate shrews rarely live more than 12–16 months and populations cycle annually. Summer populations are composed of old adults and young of the year; by autumn most old adults have died and populations consist mainly of young of the year that replace the parents (Rudd 1953; Newman 1976). These ecological and physiological characteristics put severe constraints on the ability of ornate

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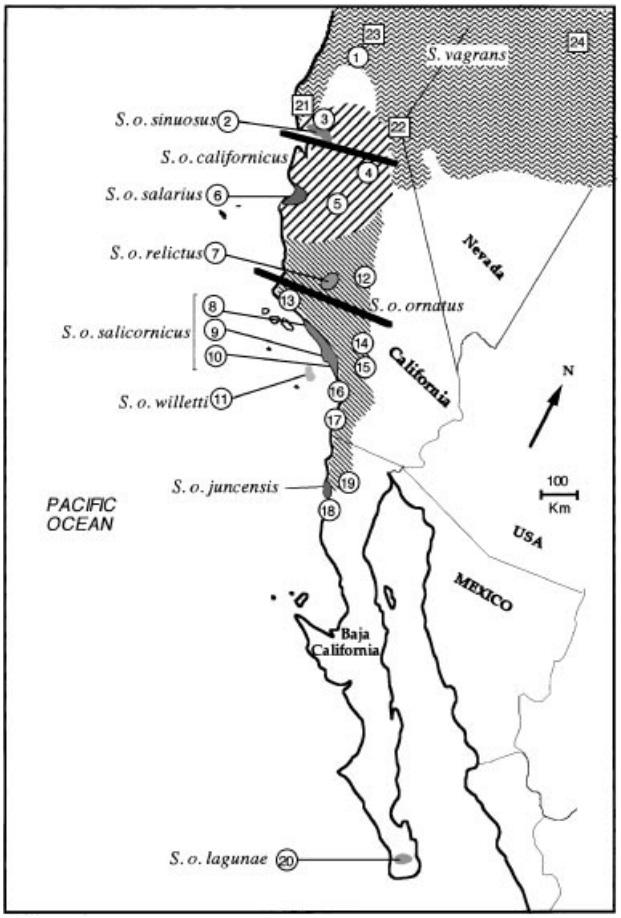

Fig. 1 Location of the 24 sampled shrew populations in the South-western US and North-western Mexico (Table 1). Circles indicate sampling localities for *Sorex ornatus*, squares are sampling localities for *S. vagrans* and/or *S. monticolus*. The distribution of the nine subspecies of ornate shrew (*S. ornatus*) are indicated (adapted from Owen & Hoffman 1983). The distribution of *S. vagrans* in Nevada and California (adapted from Hall 1981) is also shown. Genetic discontinuities are indicated by transverse thick lines (see text).

shrews to occupy inhospitable habitats over long periods of time and decrease their need for long distance dispersal. Consequently, genetic variation in the ornate shrew is expected to be geographically partitioned.

The systematics of ornate shrews and related forms has been poorly studied. Subspecies definitions in the ornate shrew often are based on the body size and pelage colouration of only one or two specimens (e.g. Owen & Hoffmann 1983). However, ornate shrews show a great degree of variation in size and pelage colouration and some populations exhibit different degrees of melanism (i.e. *S. o. sinuosus*, *S. o. salarius* and *S. o. relictus*) so subspecies definitions may not be reliable. Further, size and pelage colouration have been shown to be an ecophenotypically plastic character in small mammals (Patton & Brylski 1987). More recently, a morphometric study of 560 ornate shrew skulls from throughout the species' range showed low levels of geographical differentiation and no clear-cut differentiation between populations or subspecies was observed (J. E. Maldonado *et al*. in preparation).

In this paper, we measure genetic variation within and between populations of ornate shrews using mitochondrial DNA (mtDNA) sequences and allozyme electrophoresis. We quantified variation in cytochrome *b*, known to have moderate rates of evolution, over a wide range of mammalian taxa with relatively recent divergence times (Irwin *et al*. 1991; Smith & Patton 1993; Baker *et al*. 1994; Mouchaty *et al*. 1995). We complement the mtDNA data with a survey of nuclear encoded protein variation in 30 putative loci. We find a genetic uniformity within shrew populations that contrasts with remarkably high levels of divergence in mitochondrial sequence and allozymes between populations. Shrew populations are clustered into three distinct geographically defined clades that are similar to those found in other taxa and suggest common causes of genetic divergence in California vertebrates.

Materials and methods

Sample localities

Sampling sites in California and Baja California include topographically and ecologically diverse locales, including coastal marshes, enclosed basins and valleys, and mountain ranges (Fig. 1). The type localities of six of seven restricted range subspecies [*Sorex ornatus sinuosus*, *S. o. salarius*, *S. o. relictus*, *S. o. salicornicus* (three localities), *S. o. willetti*, *S. o. lagunae*] were sampled (Fig. 1 and Table 1). For *S. o. juncensis*, the habitat in the type locality was highly degraded and the subspecies could be extinct (Maldonado 1999). Instead, two neighbouring populations of *S. o. ornatus* were sampled (populations 18 and 19 in Fig. 1). Three populations from the northern widely distributed subspecies (*S. o. californicus*) and eight populations from the most widely distributed southern subspecies (*S. o. ornatus*) were also sampled. Finally, samples collected in northern California, around Shasta Lake (population 1 in Fig. 1), outside the range recognized for *S. ornatus*, were included. This population was identified as *S. ornatus* by its external morphology, and was different from *S. vagrans* living at higher altitudes in the same area. Two other shrew species from the genus *Sorex* that live in California, the montane shrew, *S. monticolus*, and the vagrant shrew, *S. vagrans*, were also sampled. These species were previously determined to be closely related to ornate shrews based on morphological (Findley 1955; Junge & Hoffmann 1981; Carraway 1990) and allozyme studies (George 1988) and occur at higher elevations (Williams 1991). The only

Table 1 Subspecies, sampling locality and sample size used in the mtDNA and allozyme analyses for *Sorex ornatus*, *S. vagrans* and *S. monticolus*. Locality codes correspond to localities in Fig. 1

Local. code	Subspecies	Locality	County	State	mtDNA (*n*)	Allozyme (*n*)
	Ornate shrews					
1	*Sorex ornatus (unamed)*	Dye Creek Ranch	Tehema	California	7	—
2	*S. o. sinuosus*	Grizzly Island	Solano	California	8	8
3	*S. o. californicus*	Rush Ranch	Solano	California	6	8
4	*S. o. californicus*	El Portal, Sierra Nevada	Mariposa	California	9	9
5	*S. o. californicus*	Los Banos Wildlife Area	Merced	California	14	14
6	*S. o. salarius*	Mouth of the Salinas River	Monterey	California	16	16
7	*S. o. relictus*	Kern Lake Preserve	Kern	California	17	10
8	*S. o. salicornicus*	Point Mugu	Ventura	California	3	—
9	*S. o. salicornicus*	Rancho Palos Verdes	Los Angeles	California	3	3
10	*S. o. salicornicus*	Bolsa Chica State Beach	Orange	California	1	—
11	*S. o. willetti*	Catalina Island	Los Angeles	California	3	—
12	*S. o. ornatus*	Kern River, Sierra Nevada	Kern	California	14	—
13	*S. o. ornatus*	Vandenburg Air Force Base	Santa Barbara	California	13	12
14	*S. o. ornatus*	Bluff lake, San Bernardino Mts.	San Bernardino	California	15	10
15	*S. o. ornatus*	James Reserve, San Jacinto Mts.	Riverside	California	3	—
16	*S. o. ornatus*	Camp Pendleton U.S.M.C.	San Diego	California	12	—
17	*S. o. ornatus*	Torrey Pines State Reserve	San Diego	California	36	—
18	*S. o. ornatus*	Mouth of El Rosario River	—	Baja California	5	5
19	*S. o. ornatus*	San Pedro Mts.	—	Baja California	10	—
20	*S. o. lagunae*	Sierra de la Laguna Mts.	—	Baja California	11	11
	Wandering shrews					
21	*S. v. vagrans*	Bodega Bay	Sonoma	California	21	13
22	*S. v. vagrans*	Sweetwater Mts.	Mono	California	2	—
23	*S. v. vagrans*	Shasta Mt.	Shasta	California	2	—
24	*S. v. vagrans*	Jarbridge Mts.	Elko	Nevada	1	—
	Montane Shrews					
22	*S. m. monticolus*	Sweetwater Mts	Mono	California	1	—
23	*S. m. monticolus*	Shasta Mt	Shasta	California	1	—

localities where vagrant and ornate shrews have been found together are in the Sierra Nevada and in marshes around the San Francisco Bay area. Samples from montane shrews from two different localities and vagrant shrews from four different localities, including areas of sympatry, were included in the study (see Table 1 and Fig. 1) and were used as outgroups in the phylogenetic analysis.

DNA extraction, polymerase chain reaction amplification and sequencing

Tissue samples (50 mg or 100 µL) were transferred to 1.7 mL eppendorf tubes containing 500 µL of 1× TNE pH 8.0. Genomic DNA was isolated by proteinase K digestion, followed by extraction with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and resuspended in TE pH 8.0 to yield a final concentration of about 1 µg/µL (Sambrook *et al*. 1989). Four universal primers (H15149, Kocher *et al*. 1989; L14724, Meyer & Wilson 1990; H15915 and L15513, Irwin *et al*. 1991) were used to amplify 425 bp and 402 bp of the mitochondrial cytochrome *b* gene. Each polymerase chain reaction (PCR) reaction mixture contained approximately 100 ng of genomic DNA, 25 pmoles of each primer and 1 mM dNTP mix in a reaction buffer including 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), and 2.5 U of *Taq* DNA polymerase in a total volume of 50 µL. Forty cycles of amplification were run in a programmable Perkin-Elmer Cetus DNA thermal cycler model 480 as follows: denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. The double-stranded products were separated in a 2% Nusieve (FMC Corporation, Rockland, MD) agarose gel in TAE buffer and stained with ethidium bromide. The appropriate band was excised, then purified using a Geneclean Kit (BIO 101).

Direct sequencing of double-stranded DNA was carried out using modifications of DMSO-based protocols (Green *et al*. 1989; Winship 1989) and a Sequenase Version 2.0 kit (US Biochemicals) labelling nucleotides with ^{35}S. The sequencing reaction products were separated by electrophoresis in a 6% polyacrylamide gel for 3 h at 55 W in a Stratagene Base Ace Sequencing apparatus. The

gels were then dried and exposed to autoradiographic film (Kodak Biomax) for 1–3 days. Sequence films were scored on an IBI gel reader, and entered into the MacVector computer program (IBI-Kodak). Sequences of both strands were obtained for 392 bp within the left domain of the cytochrome *b*. An additional 307 bp fragment (amplified with the primers L15513 and H15915) was sequenced from both strands of the right domain of the cytochrome *b* for representative taxa from each clade to provide better support for putative groupings of shrew haplotypes. Sequences were aligned first by eye, then by using Clustal V (Higgins & Sharp 1989), and rechecked by eye. Sequence data have been submitted to GenBank (accession numbers: AF300652–AF300699).

After we sequenced a representative sample of 1–5 individuals from each locality for the 392 bp segment of cytochrome *b*, we typed the additional samples from each population by single-stranded conformation polymorphism analysis (SSCP, Lessa & Applebaum 1993; Girman 1996). SSCP is a very sensitive method for detecting mutations in short DNA segments and was utilized to screen shrew samples from each population to determine the number of different haplotypes. However, because SSCP is most sensitive if fragments are less than 300 bp, an internal primer was designed at position L14841 (Kocher *et al*. 1989) and used in conjunction with the universal primer H15149. These primers amplify a 308-bp fragment, including the most variable area determined from the sequencing of the entire 392 bp fragment. Primers were end-labelled with [γ^{32}P]-ATP in a 25-μL polynucleotide kinase reaction. The end-labelled primers were then included in a PCR reaction identical to that above and the products were run in a nondenaturing gel. Haplotype standards representing each cytochrome *b* region sequence found in the initial survey were included on every gel. We typed a total of 232 individuals. New haplotypes identified using SSCP analyses were sequenced as explained above.

Sequence analysis

The cytochrome *b* sequence data from the two regions totalled 699 bp and were analysed using three phylogenetic methods: maximum parsimony, maximum likelihood, and neighbour-joining. To determine the most parsimonious tree, we used unweighted maximum parsimony with the branch-and-bound option of PAUP version 3.1.1 (Swofford 1993). Confidence in estimated relationships was determined using 1000 bootstrap pseudoreplicates (Felsenstein 1985). Maximum-likelihood trees were constructed using the PHYLIP program version 3.2 (Felsenstein 1989). For these analyses we used the empirically determined frequencies of nucleotides and an average transition/transversion ratio determined by pairwise comparisons of all taxa. Finally, the genetic distance between haplotypes was estimated by the Kimura 2-parameter model (Kimura 1981) and used to calculate a neighbour-joining tree (Saitou & Nei 1987).

We used AMOVA (analysis of molecular variance) to deduce the significance of geographical divisions among local and regional population groupings (Excoffier *et al*. 1992). AMOVA is a hierarchical approach analogous to analysis of variance (ANOVA) in which haplotype distances compared at various hierarchical levels are used as *F*-statistic analogs, designated as ϕ statistics. Gene flow within and among regions was approximated as $N_F m_F$, the number of female migrants between population per generation, and was estimated using the expression $F_{ST} = 1/(1 + 2N_F m_F)$ where N_F is the female effective population size and m_F is the female migration rate (Slatkin 1987, 1993; Baker *et al*. 1994). We used pairwise estimates of ϕ_{ST} as surrogates for F_{ST} among regional groupings of populations (e.g. Stanley *et al*. 1996). Following Slatkin (1993), we assessed differentiation by distance by plotting pairwise genetic distance values against geographical distance. The significance of the association between the two distance matrices was determined with a Mantel permutation test (Mantel 1967). A significant association between genetic distance and geographical distance indicates genetic structuring in populations and suggests that dispersal of individuals is limited (Slatkin 1993). We used the Arlequin 1.1 (Schneider *et al*. 1997) program to perform AMOVA as well as Fisher's exact test of population differentiation as described in Raymond & Rousset (1995). The DNAsp program (version 2.93, Rozas & Rozas 1997) was used to calculate the likelihood ratio test of linkage disequilibrium (Hill & Robertson 1968) and Tajima's test of selective neutrality (Tajima 1989).

Allozyme electrophoresis

A subset of the samples used for mtDNA analysis was analysed for allozyme variation (Table 1). Only populations for which we could obtain permits to sample more than five animals and extract heart, liver and kidney samples were studied. Extracted samples were stored at −70 °C until processed. Tissue preparation, horizontal starch-gel electrophoresis, and biochemical stain procedures modified from techniques described by Selander *et al*. (1971) and George (1988) were used to type samples (Appendix 1). Thirty presumptive loci were scored (George 1988; Collins & George 1990). Allelic frequencies, direct-count estimates of mean heterozygosity (H), and percentage of polymorphic loci (P) were calculated for each population using BIOSYS-1 (Swofford & Selander 1981). Each locus was tested to determine conformance with Hardy–Weinberg expectations. Cluster analyses were performed using Rogers' distance (Rogers 1972) and the Wagner procedure (Farris

1970). Wright's F_{ST} was calculated and used to estimate the number of migrants among populations using the expression, for diploid data, $F_{ST} = 1/(1 + 4Nm)$ (Wright 1969, 1978). Finally, we tested for gametic disequilibrium between all pairs of allozyme loci and between allozyme loci and mitochondrial haplotypes using a likelihood ratio test with an empirical distribution of disequilibrium values determined by permutation (Slatkin & Excoffier 1996). The Arlequin program version 1.1 was used for these calculations.

To assess the likelihood of finding one of the observed genotypes in each population or clade grouping we used an assignment test (Paetkau *et al*. 1995; Waser & Strobeck 1998). This approach calculates the likelihood of finding an individual genotype in a defined population grouping and assigns it to the population with the highest likelihood. The degree of differentiation between regions can be characterized by the percentage of correct assignments (Paetkau *et al*. 1995). Finally, the proportion of shared alleles between pairs of individuals was used to build a distance matrix. We used the distance $d_{ij} = -\ln(P_{ij})$, where P_{ij} is the proportion of shared alleles between individuals i and j. This distance matrix was calculated using the program MICROSAT (http://human.stanford.edu/microsat) and was used to construct a neighbour-joining tree of individuals.

Results

Sequence variation

We found 24 different haplotypes in ornate shrews. Except for three population groupings (Grizzly Island with Rush Ranch; James Reserve with San Bernardino, Los Banos with Salinas and Kern River), all populations had unique haplotypes (Table 2). In addition, the *Sorex vagrans* samples that were obtained from four different localities (Bodega Bay, Sweetwater Mountains, Mount Shasta, and Jarbridge Mountains) had seven different haplotypes and two *S. monticolus* from different localities (Sweetwater Mountains and Mount Shasta) had different haplotypes.

The cytochrome *b* gene of ornate shrews is one of the most polymorphic in mammals (Irwin *et al*. 1991; Smith & Patton 1993; Lara *et al*. 1996; Lessa & Cook 1998). In ornate shrews, we found 36 variable sites in the 392 bp cytochrome *b* fragment, 33 of which were transitions and three were transversions. Sixty-two positions varied across the 392 bp cytochrome *b* fragment in comparisons of ornate shrews to *S. vagrans* and *S. monticolus*. Fifty-seven of these were phylogenetically informative, 50 were transitions and seven were transversions. A total of 99 substitutions were observed over all pairwise comparisons; 14 occurred at first codon positions, seven at second positions, and 78 at third positions. Additionally, we sequenced 307 bp from the left domain of the cytochrome *b* gene sequenced in 16 shrews, including representatives of ornate shrews from each sequence clade defined in preliminary trees based on shorter sequences (Fig. 2a). In the combined 699 bp fragment, 109 variable sites were observed that distinguished sequences of the ornate, vagrant and montane shrews, 69 of which were phylogenetically informative. Of 139 substitutions observed in pairwise comparisons of sequences, 126 were transitions and 13 were transversions. Consequently, the transition to transversion ratio was 10.3. Twenty-three changes occurred at first codon positions, seven at second positions, and 109 at third positions.

Considering the complete 699 bp sequence, the mean divergence between the montane shrew sequence and two vagrant shrew sequences was 9.5% (9.5–9.6%) and between the montane shrew and 12 ornate shrew haplotypes it was 9.9% (SD = 0.6; range: 9.0–10.8%, $n = 12$). The divergence between vagrant and ornate shrew haplotypes was 5.3% (SD = 1.8, range: 1.2–7.3, $n = 24$), significantly lower than the divergence observed between montane and ornate shrews (Mann–Whitney's U = 288, $P < 0.001$). The range of the divergence observed between vagrant and ornate shrew haplotypes is almost identical to that observed between ornate sequences (range: 0.3–7.2%), however, the mean divergence is lower between ornate sequences (3.0%, SD = 2.0, U = 341, $P < 0.001$). Consequently, montane shrews have cytochrome *b* sequences highly divergent from those in ornate and vagrant shrews, whereas the latter two species are genetically similar.

Phylogeography of haplotypes

We found three distinct geographical clades in phylogenetic trees based on the 392 bp fragment in all samples and 699 bp sequences based on a reduced subset of 16 individuals (Fig. 2). These clades generally were supported in more than 80%, and 90%, of bootstrapped trees based on short and long sequences, respectively (Fig. 2). Within each clade, the topology varies among tree building methods, few nodes were supported in more than 50% of the bootstrap iterations and no support was evident for the subspecies that are distributed in several of the sampled populations (see, for example, the lack of support for a common origin of haplotypes ORNPTM, ORNSPV and ORNBCH, the haplotypes observed in populations attributed to *S. o. salicornicus*). However, in the northern clade, *S. vagrans* from higher elevations (Mount Shasta, Sweetwater Mountains and Jarbridge Mountains) formed a well-supported group (94% and 97% bootstrap values in the parsimony and neighbour-joining trees, respectively). Comparison of the 232 amino acids translated from the complete 699 bp sequence between the three *ornatus* clades shows that four amino acid changes were unambiguous: position 16, Asn-Ser (diagnoses the central clade); position

Table 2 *Sorex ornatus* (ORN), *S. vagrans* (VAG) and *S. monticolus* (MON) haplotype distribution at the 24 sampling localities in California, Baja California and Nevada

	Sorex ornatus																				*S. mont.*			*S. vagrans*		
Haplotype	1 Dye Creek	2 Grizzly Island	3 Rush Ranch	4 Sierra Nevada	5 Los Banos	6 Salinas	7 Kern Lake	8 Pt Mugu	9 Palos Verdes	10 Bolsa Chica	11 Catalina	12 Kern River	13 Sta Barbara	14 San Bernardino	15 James Reserve	16 Pendleton	17 San Diego	18 Rosario	19 San Pedro	20 Laguna	21 Bodega	22 Sweetwater Mts.	23 Shasta Mt.	24 Jarbridge Mts.	22 Sweetwater Mts.	23 Shasta Mt.	Total
ORNDYE-A	2																									2
ORNDYE-B	2																									2
ORNDYE-C	1																									1
ORNDYE-D	2																									2
ORNSGI-A		3	1																							4
ORNSGI-B		4	3																							7
ORNSGI-C		1	2																							3
ORNSIN-A				8																						8
ORNSIN-B				1																						1
ORNSAL					14	16						12														42
ORNSKL							17																			17
ORNPTM								3																		3
ORNSPV									3																	3
ORNBCH										1																1
ORNSCI											3															3
ORNKRP												2														2
ORNSBR													13													13
ORNSBM-B														7												7
ORNSBM-A														8	3											11
ORNPEN																12										12
ORNSND																	36									36
ORNROS																		5								5
ORNSSP																			10							10
ORNSLA																				11						11
VAGBOD-A																					12					12
VAGBOD-B																					7					7
VAGBOD-C																					1					1
VAGBOD-D																					1					1
VAGSWE																						2				2
VAGSHA																							2			2
VAGJAM																								1		1
MONSWE																									1	1
MONSHA																										1	1
TOTAL	7	8	6	9	14	16	17	3	3	1	3	14	13	15	3	12	36	5	10	11	21	2	2	1	1	1	234

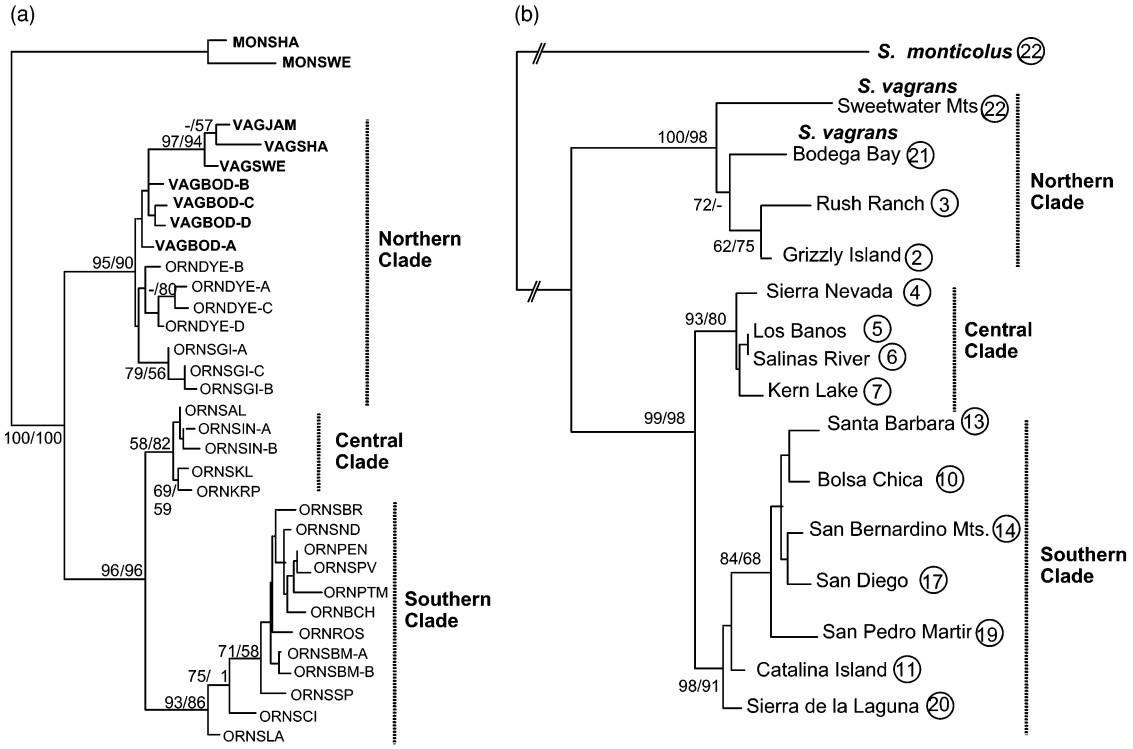

Fig. 2 Phylogenetic trees showing relationships between haplotypes found in ornate shrews form northern, central, southern California and Baja California. (a) Neighbour-joining (NJ) tree using Kimura 2-parameter sequence divergence values based on 392 bp of cytochrome *b* sequence. Codes for haplotypes correspond to those in Table 2. In bold are haplotypes corresponding to *Sorex vagrans* (VAG) and *S. monticolus* (MON). (b) NJ tree based on 699 bp of cytochrome *b* sequence. The numbers by the sequence indicate the population number in Fig. 1 and Table 1. Similar topologies were obtained using parsimony (MP) and maximum likelihood analysis in both cases. Percentage of support in 1000 bootstrap NJ (numerator) and MP (denominator) replicates is indicated by the node when it is over 50%.

39, Leu-Val (diagnoses the northern clade); position 53, Ileu-Met (diagnoses the central and southern clades); and position 147, Glu-Lys (diagnoses Bodega and Grizzly Island clade).

The northern clade includes haplotypes from populations north of the San Francisco Bay area: *S. vagrans* from Bodega Bay, Sweetwater Mountains, Shasta Mountain, and from the Jarbridge Mountains, in Elko, Nevada, *S. o. sinuosus* from Grizzly Island and *S. o. californicus* from surrounding mainland marshes in Rush Ranch. Ornate shrews from Dye Creek, although outside the recognized species range, are also grouped in the northern clade. The central clade contains haplotypes from the coastal, valley and Sierra Nevada populations north of the Tehachapi Mountains in central California and south of the San Francisco Bay area. The central clade includes individuals attributable to *S. o. californicus*, *S. o. salarius*, *S. o. relictus* and *S. o. ornatus*. The southern clade contains haplotypes from coastal populations in Santa Barbara, Ventura, Los Angeles, Orange and San Diego counties, as well as the San Bernardino Mountains, the San Jacinto Mountains, and El Rosario marsh and the San Pedro Martir Mountains in Baja California. Sequences from Santa Catalina Island and the relictual Sierra de la Laguna populations are the most basal in the southern clade. This clade includes representatives of the subspecies *S. o. salicornicus*, *S. o. willetti*, *S. o. ornatus* and *S. o. lagunae*.

Divergence within and among populations

The occurrence of unique haplotypes in most localities suggests that genetic subdivision is a common characteristic of ornate shrews throughout most of their range. In 14 of the 20 populations of ornate shrews only one 392 bp cytochrome *b* sequence was found (Table 2). The remaining six populations had 2–4 different haplotypes. The nucleotide diversity (π, Nei 1987) within populations was higher in the northern populations, where it ranged from 0.003 to 0.015, whereas in the central and southern populations, the Sierra Nevada, Kern River and San Bernardino populations had values of 0.001 and the rest had values of zero (Table 3). Overall, the northern populations had significantly higher ($P < 0.05$) nucleotide diversity ($\pi = 0.012 \pm 0.0011$) than the southern populations ($\pi = 0.005 \pm 0.0004$), which in turn had significantly higher values than the central populations ($\pi = 0.002 \pm 0.0002$). The average sequence

Table 3 Sequence statistics for shrew population for a 392-bp fragment of the cytochrome *b* sequence. Diagonal numbers in bold italics: Nei's (1987) nucleotide diversity within populations. Lower left, average sequence divergence between populations (underlined, populations not significantly differentiated; Fisher's exact test, $P > 0.05$). Upper right, number of female ($N_F m_F$) migrants per generation based on ϕ_{ST}. Sweetwater and Bodega are two populations of *Sorex vagrans*

	Sweetwater *S. vagrans*	Bodega *S. vagrans*	Dye Creek	Rush Ranch	Grizzly Island	Salinas	Kern Lake	Sierra Nevada	Los Banos	Kern River	Santa Barbara	San Bernardino	James Reserve	S. Catalina Island	Pt. Mugu	Palos Verdes	Pendleton	San Diego	San Pedro	Rosario	Laguna
Sweetwater	***0.001***	0.170	0.398	0.043	0.058	0.000	0.000	0.009	0.000	0.005	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Bodega	0.020	***0.005***	0.514	0.241	0.255	0.034	0.032	0.049	0.036	0.039	0.031	0.035	0.048	0.050	0.044	0.042	0.029	0.017	0.030	0.040	0.043
Dye Creek	0.024	0.009	***0.015***	0.665	0.610	0.046	0.041	0.081	0.051	0.056	0.042	0.047	0.102	0.107	0.093	0.089	0.041	0.017	0.044	0.073	0.060
Rush Ranch	0.027	0.014	0.013	***0.003***	inf	0.006	0.006	0.018	0.007	0.012	0.005	0.013	0.013	0.017	0.012	0.012	0.005	0.002	0.007	0.010	0.008
Grizzly Island	0.026	0.013	0.012	0.001	***0.003***	0.009	0.009	0.021	0.010	0.015	0.008	0.015	0.018	0.022	0.016	0.016	0.008	0.003	0.010	0.013	0.012
Salinas	0.068	0.052	0.054	0.055	0.055	***0.000***	0.000	0.098	inf	5.015	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Kern Lake	0.071	0.053	0.054	0.061	0.060	0.003	***0.000***	0.041	0.000	0.058	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Sierra Nevada	0.065	0.050	0.054	0.051	0.050	0.002	0.010	***0.001***	0.108	0.210	0.006	0.019	0.014	0.018	0.011	0.011	0.006	0.003	0.007	0.010	0.009
Los Banos	0.068	0.052	0.054	0.055	0.055	0.000	0.003	0.002	***0.000***	6.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Kern River	0.068	0.052	0.054	0.056	0.055	0.000	0.003	0.003	0.000	***0.001***	0.005	0.017	0.010	0.010	0.007	0.008	0.005	0.003	0.006	0.007	0.007
Santa Barbara	0.086	0.066	0.068	0.079	0.078	0.037	0.038	0.043	0.037	0.037	***0.000***	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
San Bernardino	0.085	0.066	0.069	0.080	0.078	0.038	0.039	0.044	0.038	0.038	0.009	***0.001***	1.875	0.038	0.073	0.106	0.066	0.060	0.050	0.169	0.021
James Reserve	0.084	0.065	0.068	0.079	0.077	0.037	0.038	0.043	0.037	0.037	0.011	0.001	***0.000***	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
S. Catalina Is.	0.081	0.062	0.065	0.059	0.058	0.034	0.036	0.030	0.034	0.034	0.019	0.017	0.019	***0.000***	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pt. Mugu	0.091	0.073	0.080	0.080	0.080	0.049	0.055	0.049	0.049	0.050	0.016	0.014	0.016	0.025	***0.000***	0.000	0.000	0.000	0.000	0.000	0.000
Palos Verdes	0.093	0.076	0.083	0.083	0.083	0.046	0.052	0.046	0.046	0.047	0.013	0.012	0.013	0.022	0.008	***0.000***	0.000	0.000	0.000	0.000	0.000
Pendleton	0.092	0.072	0.075	0.085	0.084	0.046	0.046	0.048	0.046	0.046	0.008	0.006	0.008	0.022	0.011	0.003	***0.000***	0.000	0.000	0.000	0.000
San Diego	0.091	0.071	0.075	0.084	0.083	0.043	0.046	0.048	0.043	0.043	0.008	0.006	0.008	0.019	0.011	0.008	0.003	***0.000***	0.000	0.000	0.000
San Pedro	0.097	0.077	0.081	0.075	0.074	0.040	0.043	0.043	0.040	0.041	0.014	0.009	0.011	0.014	0.017	0.014	0.014	0.011	***0.000***	0.000	0.000
Rosario	0.089	0.071	0.074	0.081	0.080	0.040	0.037	0.042	0.040	0.040	0.008	0.007	0.008	0.022	0.014	0.011	0.011	0.008	0.011	***0.000***	0.000
Laguna	0.069	0.051	0.055	0.056	0.055	0.028	0.031	0.031	0.028	0.029	0.020	0.021	0.023	0.014	0.023	0.026	0.025	0.023	0.023	0.022	***0.000***

inf, infinite.

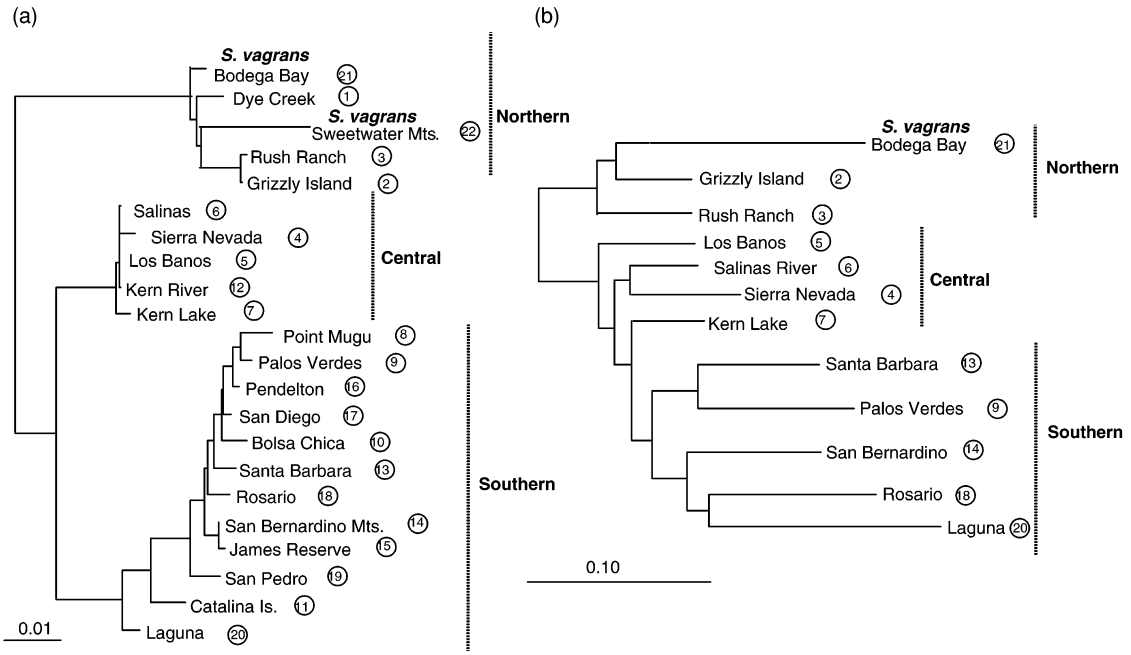

Fig. 3 (a) Unrooted neighbour-joining tree based on average sequence divergence (392 bp of cytochrome *b* sequence) between populations. (b) Distance Wagner tree based on allozymes using Rogers' (1972) distances. The length of the tree is 0.678 and the cophenetic correlation coefficient is 0.865. The numbers by the sequence indicate the population number in Fig. 1 and Table 1.

divergence between different populations of *S. ornatus* ranged from 0.1% to 8.5% (Table 3). The maximum is a large value compared with that found in other mammal species (Taberlet *et al*. 1998). Average sequence divergence between regions ranged from 4.11% ± 0.90 (southern vs. central) to 7.59 ± 0.15 (northern vs. southern).

An unrooted neighbour-joining tree based on genetic distance between populations (Nei 1972; Fig. 3a) shows a clear pattern of differentiation between the three geographical clades, with long branches separating the groups and short-terminal branches for each population within the clades. The populations from the central region show low degrees of differentiation. The results of Fisher's exact test of population differentiation (Table 3), as well as the pairwise computations of ϕ_{ST} using AMOVA, indicate that populations are significantly differentiated relative to a random collection of haplotypes. The only exceptions are populations that share haplotypes: Grizzly Island and Rush Ranch ($\phi_{ST} = 0.12$, $P = 0.732$); Salinas and Los Banos ($\phi_{ST} = 0.00$, $P = 0.990$); Salinas and Kern River ($\phi_{ST} = 0.09$, $P = 0.500$); Los Banos and Kern River ($\phi_{ST} = 0.08$, $P = 0.426$); and San Bernardino Mts. and James Reserve ($\phi_{ST} = 0.28$, $P = 0.099$). Therefore, our results suggest that in the past these populations were part of the same interbreeding population. With the exception of the five population pairs discussed above, pairwise values of *Nm* based on ϕ_{ST} are extremely low (*Nm* = 0.000–0.665; Table 3). Estimates of gene flow between populations from the three main clades found in the phylogenetic analysis are also low and range from 0.05 (central vs. southern) to 0.07 (northern vs. central) female migrants per generation. No haplotypes are shared between regions.

Overall, the degree of differentiation observed between localities increases with geographical distance (Fig. 4a; Mantel's test based on 10 000 permutations $P < 0.001$, $r = 0.49$). However, although there is a significant correlation, the portion of the total variance explained by the regression is small ($r^2 = 0.24$). The clustered distribution of different regional and interregion comparisons indicates that the differentiation between regions is more important than geographical distance to explain the genetic differences between populations. When each clade is examined separately, only the southern clade follows a predictable relationship with geographical distance (Mantel's test, $r = 0.52$, $P = 0.05$) suggesting that differentiation by distance is most apparent in the southern clade. Also in this case, however, the regression only explains a small part of the variance ($r^2 = 0.25$).

Relationship of S. ornatus *with* S. vagrans

As discussed above, ornate shrew haplotypes from Grizzly Island (*S. o. sinuosus*) and Rush Ranch (*S. o. californicus*) formed a monophyletic group supported in 79% of neighbour-joining bootstrapped trees (Fig. 2a). Populations from these two localities are not significantly differentiated

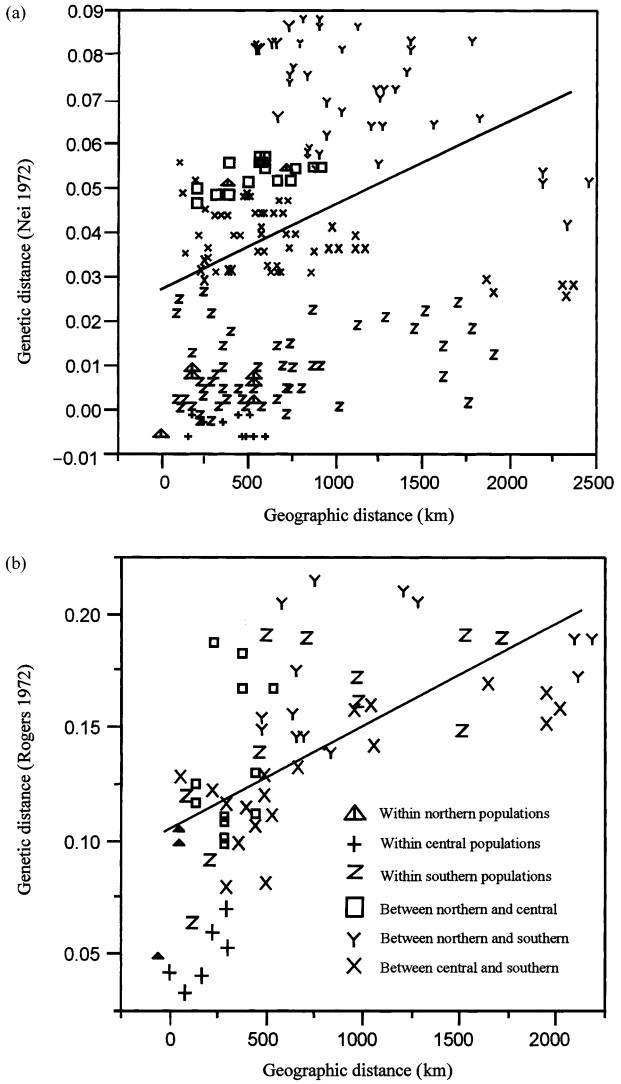

Fig. 4 Scatterplots showing the relationship between geographical distance and genetic distance between populations. (a) Results based on genetic distances (Nei 1972) derived from mtDNA analysis of 22 populations. Regression statistics: $y = -0.039 + 0.013(x)$, $r^2 = 0.24$. (b) Results based on genetic distances (Rogers 1972) derived from allozyme analysis of 11 populations. Regression statistics: $y = -0.023 + 0.026(x)$, $r^2 = 0.44$.

(Table 3) and have all haplotypes in common, implying that they are part of the same interbreeding population. Ornate shrew haplotypes from Grizzly Island, Rush Ranch and Dye Creek localities are grouped with those attributable to *S. vagrans* from Bodega Bay, Sweetwater Mountains, Mt. Shasta, and Jarbridge Mountains (Elko Co., Nevada) to form the northern clade of haplotypes (Fig. 2, Fig. 3). *S. vagrans* haplotypes from higher elevations formed a monophyletic group, which was supported in 94% and 97% of bootstrapped trees in maximum parsimony and neighbour-joining trees (Fig. 2), and differed by 2 (0.6%) to 4 (1.2%) substitutions from each other and 6 (1.5%) to 11 (3.6%) from haplotypes in the other populations in the northern clade. Consequently, the ornate shrew from northern California is a paraphyletic group that includes haplotypes from a related species.

Divergence time inferred from sequence data

The cytochrome *b* sequence data can be used to estimate divergence time of the three major ornate shrew clades when corrected for ancestral polymorphism within species (Nei 1987; Avise & Walker 1998; but see Hillis *et al*. 1996). The net sequence divergence between southern and central clades is 4.2%, and assuming a mutation rate of 2% per million years (Myr) (Wilson *et al*. 1985; the divergence rate would thus be about 4% per Myr if the sequences are far from saturation), the two clades diverged about 1.1 million years ago (Ma). Similarly, the southern and northern clades have a net sequence divergence of 4.9%, which implies a divergence time of approximately 1.2 Ma. The net sequence divergence between *S. monticolus* and the ingroup species is 6.5%, implying a divergence time of 1.6 Myr. Saturation was not evident in saturation plots of genetic distances vs. number of substitutions, and Tajima's test of selective neutrality (Kimura 1983) showed no significant rate differences ($P > 0.10$) among and within clades. Finally, the mean divergence between sequences within clades is 1.7%, 0.6% and 1.3% for northern, central and southern clades, respectively. These values correspond to divergence times of 0.425, 0.150 and 0.325 Ma. The lower mean divergence in the central clade suggests a more recent radiation of haplotypes.

Allozymic variation

Of the 30 loci examined, 10 were fixed for a single allele and 20 were polyallelic in at least one population (Appendix 1). Mean heterozygosity (H) within populations ranged from 0.069 to 0.142 (Table 4) (mean across all samples, 0.105) and falls within the values reported for soricids (Tolliver *et al*. 1985). On average, heterozygosity was significantly higher in northern populations (0.115 ± 0.040) than in central populations (0.094 ± 0.003) (Chi-square test; $P < 0.05$), but did not differ significantly from the southern populations (0.109 ± 0.01). Polymorphism within populations varied from 24.14 to 51.72% and was highest in northern populations (mean 42.53 ± 13.05) and lowest in the southern populations (mean 33.79 ± 9.25), although not statistically significant (Chi-square test; $P = 0.59$). Overall, no linkage disequilibrium was detected (maximum-likelihood ratio test with Bonferroni correction for multiple comparisons, $P > 0.05$).

Geographic patterns of variation are evident for a few loci. For example, the Est-2 allele 'a' occurs at high

Table 4 Allelic frequencies, mean heterozygosity (*H*), and per cent polymorphism (%*P*), for 20 polymorphic loci scored across 11 populations of *Sorex ornatus* and one population of *S. vagrans*. Sample size in parenthesis (*n*)

Locus		Bodega *S. vagrans*	Grizzly	Rush Ranch	Sierra Nevada	Salinas	Los Banos	Kern Lake	Sta Barbara	San Bernardino	Palos Verdes	Rosario	Laguna
		(13)	*(8)*	*(8)*	*(9)*	*(16)*	*(14)*	*(10)*	*(12)*	*(10)*	*(3)*	*(8)*	*(11)*
Est-1	**a**	0.65	0.56	0.38*	0.33	0.22*	0.39	0.35	0.00	0.00	0.00	0.00	0.00*
	b	0.35	0.44	0.50	0.67	0.78	0.61	0.65	1.00	1.00	1.00	1.00	0.32
	c	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.68
Est-2	**a**	0.15	0.06	0.19*	0.11	0.06	0.07	0.10	0.08	0.15	0.00	0.00	0.00*
	b	0.85	0.94	0.81	0.89	0.94	0.93	0.90	0.92	0.85	1.00	1.00	0.18
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.55
	d	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27
sMdh-A	**a**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	0.46*
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.55
Mdhp-A	**a**	0.00	0.00	0.00	0.00	0.00*	0.00	0.00	0.21*	0.30	0.50	0.00	0.00
	b	1.00	1.00	1.00	1.00	0.88	1.00	1.00	0.79	0.70	0.50	1.00	1.00
	c	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Iddh-A	**a**	0.96	0.94	0.94	0.89	0.94	0.93	0.95	0.96	0.95	1.00	1.00	1.00
	b	0.04	0.06	0.06	0.11	0.06	0.07	0.05	0.04	0.05	0.00	0.00	0.00
Ak-A	**a**	1.00	1.00	1.00	0.83	0.69	0.93	0.85	0.04	0.90	0.17	1.00	0.86
	b	0.00	0.00	0.00	0.17	0.31	0.07	0.15	0.96	0.10	0.83	0.00	0.14
Pep-D	**a**	0.00	0.00	0.00	0.22	0.16	0.21*	0.05	0.00	0.00	0.00	0.00	0.00
	b	1.00	0.69	0.94	0.78	0.84	0.79	0.95	0.83	0.80	1.00	1.00	1.00
	c	0.00	0.31	0.06	0.00	0.00	0.00	0.00	0.17	0.20	0.00	0.00	0.00
sSod-A	**a**	0.00	0.25	0.25	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	1.00	0.75	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
G6pdh-A	**a**	0.00	0.00	0.00	0.00	0.00*	0.00	0.00	0.08	0.05	0.00	0.00	0.14
	b	0.62	0.63	0.59	0.00	0.22	0.14	0.05	0.46	0.55	0.50	0.00	0.59
	c	0.39	0.38	0.27	1.00	0.78	0.86	0.95	0.46	0.40	0.50	1.00	0.27
Pgm-1	**a**	0.15	0.38	0.19	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.32
	b	0.85	0.63	0.81	1.00	1.00	1.00	0.90	1.00	1.00	1.00	1.00	0.68
Pgm-2	**a**	0.00	0.00	0.25	0.00	0.00	0.00	0.10*	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	0.75	1.00	1.00	1.00	0.90	1.00	1.00	1.00	1.00	1.00
6Pgdh-1	**a**	0.96	0.06	0.19*	0.06	0.00	0.00	0.00	0.00	0.50	0.00	0.40	0.41
	b	0.04	0.88	0.81	0.94	0.97	0.96	1.00	0.88	0.50	0.83	0.60	0.59
	c	0.00	0.06	0.00	0.00	0.03	0.04	0.00	0.13	0.00	0.17	0.00	0.00
sIcdh-A	**a**	0.00	0.06	0.04	0.00	0.06*	0.00	0.00	0.04	0.00	0.00	0.40*	0.00
	b	1.00	0.94	0.96	1.00	0.94	1.00	1.00	0.96	1.00	1.00	0.00	1.00
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.00
sAta-A	**a**	0.00	0.00	0.00	0.00	0.09*	0.14	0.35*	0.00	0.00	0.00	0.00	0.00
	b	1.00	1.00	1.00	1.00	0.91	0.86	0.65	1.00	1.00	1.00	1.00	1.00
mAta-A	**a**	1.00	0.94	0.93	1.00	1.00	0.93	1.00	0.92	1.00	1.00	1.00	1.00
	b	0.00	0.06	0.07	0.00	0.00	0.07	0.00	0.08	0.00	0.00	0.00	0.00
Pnp-1	**a**	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.08	0.00	0.17	0.00	0.00
	b	0.08	0.25	0.19	0.22	0.25	0.36	0.30	0.17	0.50	0.00	0.20	0.46
	c	0.69	0.75	0.81	0.78	0.63	0.64	0.70	0.75	0.50	0.83	0.40	0.18
	d	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.32
	e	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05
G3pdh-2	**a**	0.00	0.06	0.08	0.11	0.13	0.14	0.00	0.13*	0.00	0.00	0.20	0.32
	b	1.00	0.38	0.36	0.44	0.44	0.36	0.75	0.63	0.95	0.50	0.80	0.68
	c	0.00	0.56	0.56	0.44	0.44	0.50	0.25	0.25	0.05	0.50	0.00	0.00
Ada-1	**a**	0.77*	0.56	0.19	0.22	0.16	0.00	0.10	0.33	0.35*	0.00	0.10*	0.18
	b	0.23	0.31	0.69	0.78	0.84	0.96	0.55	0.67	0.30	1.00	0.30	0.82
	c	0.00	0.13	0.13	0.00	0.00	0.04	0.35	0.00	0.35	0.00	0.60	0.00
Xdh-A	**a**	0.00	0.00	0.00	0.00	0.13*	0.04	0.00	0.33*	0.15	0.17	0.00	0.05
	b	1.00	1.00	1.00	1.00	0.88	0.96	1.00	0.67	0.65	0.83	1.00	0.96
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00
Gpi-A	**a**	0.00	0.06	0.10	0.00	0.00	0.07*	0.10*	0.00	0.00	0.00	0.10	0.00
	b	1.00	0.94	0.90	1.00	1.00	0.93	0.90	1.00	1.00	1.00	0.90	1.00
	H	**0.069**	**0.142**	**0.134**	**0.096**	**0.097**	**0.094**	**0.090**	**0.092**	**0.121**	**0.103**	**0.108**	**0.122**
	%P	**27.59**	**48.28**	**51.72**	**31.03**	**48.28**	**37.93**	**44.83**	**44.83**	**37.93**	**24.14**	**24.14**	**37.93**

*Allelic frequencies do not conform with expectations from Hardy–Weinberg equilibrium.

Table 5 Matrix of genetic statistics based on allozyme data. Lower left, matrix of F_{ST} values between populations (underlined, populations that are not significantly differentiated, $P > 0.05$). Upper right: number of migrants per generation (Nm)

	Bodega *Sorex vagrans*	Grizzly Island	Rush Ranch	Sierra Nevada	Salinas	Los Banos	Kern Lake	Sta Barbara	San Bernardino	Palos Verdes	Rosario	Laguna
Bodega	—	0.567	0.650	0.235	0.272	0.233	0.267	0.220	0.401	0.168	0.197	0.297
Grizzly Island	0.271	—	22.666	0.786	0.827	0.758	0.795	0.508	0.721	0.525	0.384	0.509
Rush Ranch	0.276	0.010	—	1.005	1.100	0.107	0.988	0.572	0.793	0.728	0.434	0.629
Sierra Nevada	0.514	0.241	0.198	—	155.00	inf	4.555	0.809	0.798	0.810	0.461	0.502
Salinas	0.478	0.232	0.183	0.001	—	16.710	3.795	1.598	1.069	2.507	0.555	0.584
Los Banos	0.518	0.248	0.189	−0.003	0.014	—	3.838	0.677	0.718	0.817	0.440	0.516
Kern Lake	0.482	0.239	0.202	0.052	0.062	0.061	—	0.745	1.037	0.765	0.629	0.535
Sta Barbara	0.531	0.329	0.304	0.236	0.135	0.269	0.251	—	0.916	inf	0.357	0.447
San Bernardino	0.384	0.257	0.239	0.238	0.189	0.258	0.194	0.214	—	0.839	0.654	0.684
Palos Verdes	0.598	0.322	0.255	0.236	0.091	0.234	0.246	−0.009	0.229	—	0.306	0.473
Rosario	0.559	0.394	0.365	0.351	0.310	0.362	0.284	0.411	0.276	0.449	—	0.470
Laguna	0.456	0.329	0.284	0.332	0.300	0.326	0.317	0.358	0.267	0.345	0.347	—

inf, infinite.

frequencies in northern populations, is rare in central populations and is absent from southern populations. Allele 'b' for sSod-A occurs at high frequencies in northern populations and is absent in central and southern populations. For Pep-D, allele 'a' occurs at low frequencies only in central populations. Unique alleles were detected in four populations: in Laguna, Est-2 alleles 'c' and 'd', and Pnp-1 allele 'e'; in Rosario, sIcdh-A allele 'c'; in Salinas, Mdhp-A allele 'c'; and in San Bernardino, Xdh-A allele 'c'. In the latter two populations, the unique alleles were rare (Table 4).

The Wagner method tree (Fig. 3b) has a topology similar to that of UPGMA and neighbour-joining trees (not shown). There is a strong north to south pattern of clustering similar to that found with the mitochondrial sequence data. The Wagner method tree shows a northern cluster that includes populations from Bodega, Grizzly Island and Rush Ranch and a southern cluster which includes the populations from southern California and Baja California. The central populations from Salinas, Los Banos, Sierra Nevada and Kern Lake are sister to the southern clade. Some of the highest genetic divergence values are found in comparisons with the Laguna and Rosario populations as both have unusual allele frequencies at four loci (Est-1, Est-2, sMdh-A and Pnp-1; Table 4).

Population differentiation using allozymes

F-statistics averaged across all loci and all populations sampled were $F_{IS} = 0.163$, $F_{IT} = 0.430$ and $F_{ST} = 0.319$. Consequently, 32% of the total genetic variation found in these populations may be ascribed to among-population variation. With six exceptions, pairwise computations of F_{ST} using AMOVA indicate that populations are significantly differentiated relative to a random collection of genotypes (Table 5). The exceptions are for Grizzly Island and Rush Ranch, Kern Lake and Sierra Nevada, Salinas and Los Banos, Salinas and Sierra Nevada, Los Banos and Sierra Nevada, and Palos Verdes and Santa Barbara. Some pairs of populations significantly differentiated with mitochondrial sequences were not differentiated with allozymes. Grizzly Island and Rush Ranch and Salinas and Los Banos are also not significantly differentiated in the mtDNA analysis. The other groupings that were not significantly differentiated in the mtDNA analysis were not tested (see Materials and methods). The nuclear data suggest that none of the sampled central California populations are significantly differentiated from one another and suggest that in the past they were part of the same interbreeding population.

Pairwise values of Nm based on F_{ST} for the populations that are significantly differentiated range from 0.168 to 3.838 (Table 5) and are higher than estimates for mtDNA data. This result is expected because of the clonal and maternal inheritance of mtDNA, higher levels of differentiation and low levels of gene flow are expected for mitochondrial vs. nuclear markers (Hartl & Clark 1990). Estimates of gene flow between populations from the three main groupings found in the phylogenetic analysis range from 0.135 (central vs. southern) to 0.339 (northern vs. central) migrants per generation. The degree of differentiation observed between localities follows a predictable relationship with geographical distance (Fig. 4b; Mantel test based on 10 000 permutations $P < 0.001$; with a significant correlation $r = 0.663$, $r^2 = 0.440$) suggesting that limited dispersal and distance explain the level of genetic differentiation existing between shrew populations (Slatkin 1993). A visual examination of Fig. 4(b) shows

that the overall trend towards increased genetic distance with geographical distance is only clear for populations separated by less than 750–1000 km. After this point no obvious trend is observed. As with the mtDNA data, when each clade is examined separately, only the southern group follows a predictable relationship with geographical distance ($r = 0.778$; Mantel's test $P = 0.05$). However, the small sample size of the central and northern populations limits the statistical power of permutation tests (Mantel 1967). Finally, Roger's genetic distance between populations based on allozyme data is significantly correlated with the corresponding complete cytochrome *b* sequence divergence values (Mantel's test, 10 000 permutations, $r = 0.566$, $P = 0.004$).

An assignment test shows that 93% (27/29) of the northern shrews are correctly identified as belonging to the northern populations. Two individuals from Grizzly Island and Rush Ranch are incorrectly assigned to the southern region. A total of 92% (45/49) of the shrews from the central valley are correctly assigned, and four individuals are misassigned (one from Sierra Nevada and three from Salinas) to the southern populations. Finally, only 83% (34/41) of shrews from the southern populations are correctly identified, one individual from San Bernardino is assigned to the northern group of populations and six (five from Santa Barbara and one from Palos Verdes) are assigned to the central group. No shrew was wrongly cross-assigned between northern and central populations.

A tree of individuals based on allele sharing distance shows that in general the three population clades are well differentiated (Fig. 5). However, central and southern individuals are mixed in a portion of the tree. Some individuals from the central region, especially from Santa Barbara and Palos Verdes (northernmost of the southern populations) are very similar to individuals from the central populations. Overall, shrews from southern populations form a less cohesive group, and several groups of shrews from different populations are apparent (for example, shrews from Laguna, Rosario and San Bernardino, Fig. 5).

Divergence time inferred from allozyme data

Utilizing a protein molecular clock (Nei 1971; Sarich 1977; Wright 1983) and accounting for the combination of 'fast' and 'slow' evolving loci (Sarich 1977), a genetic distance unit of one (D = 1), is approximately equivalent to 20.5 million years (George 1988). Thus, using the average genetic distances from allozyme data (D = 0.046) we estimate central and southern populations diverged 950 000 years ago and northern and southern + central populations (D = 0.073) diverged 1.5 Ma. These values are similar to estimates of 1.1 and 1.2 million years, respectively, based on cytochrome *b* sequence divergence values.

Discussion

Three well-defined geographical population groupings were discovered in trees based on mtDNA sequences and allozymes. Several populations from different groupings were genetically divergent but geographically in close proximity. The clearest example occurs in the central populations where the Salinas population is approximately 300 km away from the Kern River and the Los Banos populations but the average Nei's allozyme genetic distance between Salinas and the latter two populations is only 0.009 and 0.002, respectively, and they share the same cytochrome *b* haplotypes. In contrast, the distance between Salinas and Grizzly Island, and Rush Ranch is at least 150 km less, but their average allozyme distance and sequence divergence values are more than 10 times higher (0.059 and 0.054, respectively). Similarly, the San Bernardino Mountains and Santa Barbara populations are 268 km apart and have a Nei's allozyme genetic distance of 0.098 and sequence divergence of 0.009, whereas the Kern Lake and Santa Barbara populations are separated by only a third of the distance (95 km) but have a Nei's distance value of 0.130, one and a half times higher and sequence divergence of 0.038, four times higher.

The divergence in mitochondrial sequence and allozyme frequencies suggests that the populations were fragmented beginning in the early Pleistocene, approximately 1.2–1.5 Ma. However, no striking geographical barriers presently separate the populations comprising the three clades: geographical barriers among populations within each clade are no less important than those between populations from different clades. Consequently, the divergence among clades likely reflects past topographic or climatic barriers that existed in California during the Pleistocene. Specifically, Quaternary cold periods that occurred 5–10 times throughout this period may have periodically isolated shrews. During these periods, wetland habitat available to shrews was more limited and fragmented (see below). As suggested by low rates of gene flow, shrews are poor dispersers and the imprint of past events may be long retained in present day populations.

Plio-Pleistocene history of California

Approximately 5 Ma the orogeny of the Sierra Nevada and Coast Ranges was initiated and large areas in central and southern California were covered by sea (Wahrhaftig & Birman 1965). Throughout much of the period that followed, the San Joaquin Valley was a wide seaway rather than the present-day continental river valley (Fig. 6). Mesic habitats suitable for shrews were restricted to the margin of this seaway. Two significant barriers to dispersal emerged across the central valley during the Plio-Pleistocene (Fig. 6). The most profound barrier likely developed at the point

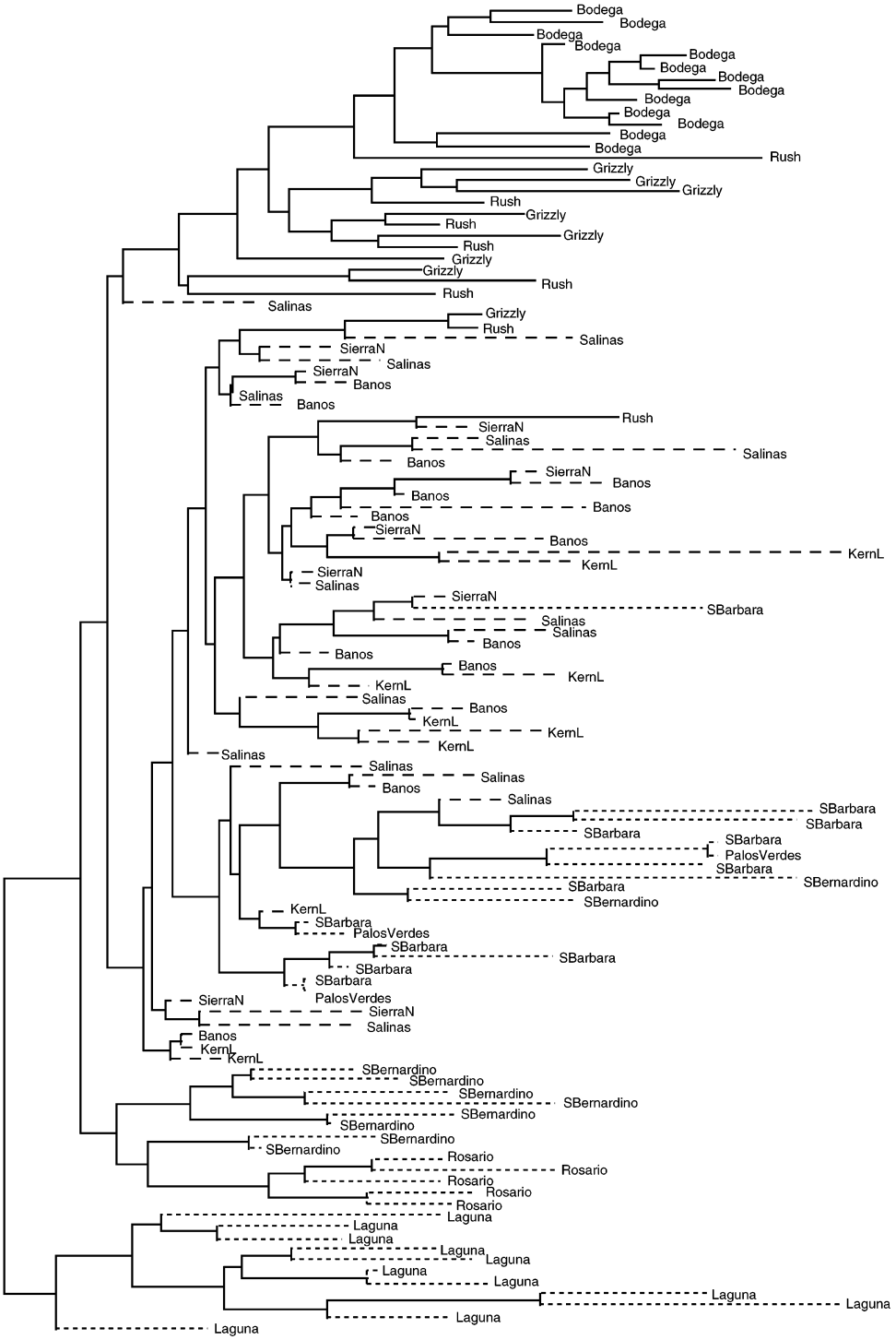

Fig. 5 Unrooted neighbour-joining tree based on the proportion of shared alleles between shrew allozyme genotypes. Shrews from northern populations are indicated with continuous lines, from central populations with dashed lines and from southern populations with dotted lines.

where the present day Central Valley drains into the Pacific Ocean near Monterey Bay. For various periods during the Pliocene, precursor drainages of the present-day Central Valley entered the Pacific Ocean in the vicinity of the present-day Monterey Bay where the largest marine canyon (of Grand Canyon scale) on the Pacific coast of north America is found (Yanev 1980). Beginning approximately 10 Ma and for most of the Plio-Pleistocene,

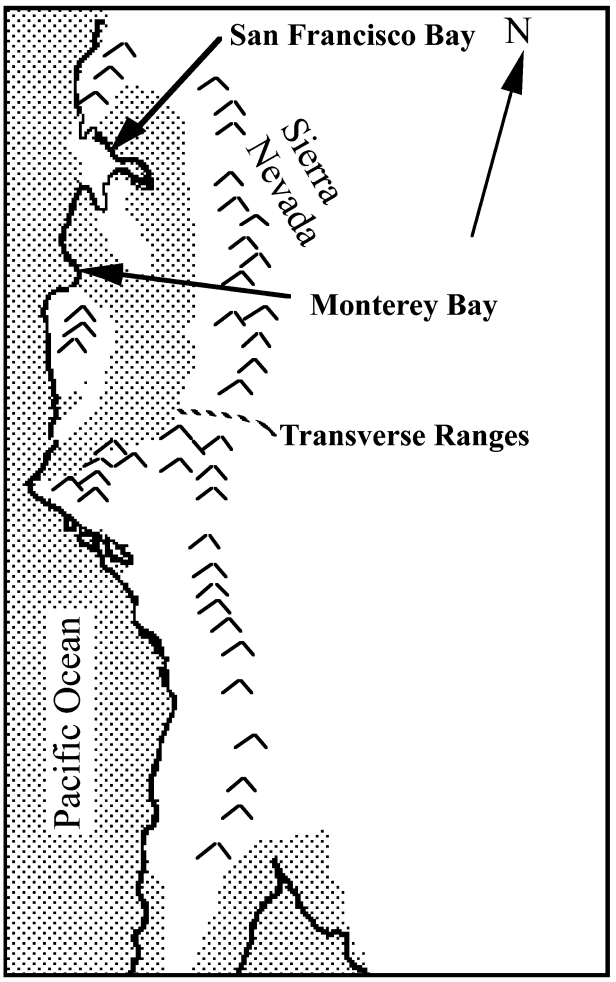

Fig. 6 Hypothetical palaeogeography of California in the Plio-Pleistocene. Stippled areas represent present areas covered by the ocean 5 million years before present. Map modified from Yanev (1980).

this area was a vast embayment that likely prevented dispersal for small vertebrates (Peabody & Savage 1958). Species transitions across the bay today are found in several species of mammals (Ingles 1965; Hall 1981; Jameson & Peeters 1988) and other vertebrate groups including salamanders (Yanev 1980; Tan & Wake 1995; Wake 1997). Another area that may have restricted the movement of shrews is the Transverse Ranges, which developed during the mid-Pliocene at the southern terminus of the San Joaquin Valley. Although today ornate shrews may be found along the Transverse Ranges, this area defines a sharp floral boundary between central and southern California by preventing northern storms from reaching southern areas. In the past, mesic habitat was unlikely to be continuous across the transverse range, whereas elsewhere habitats were joined during wet glacial periods (Yanev 1980).

We suggest that the development of these two topographic barriers is the cause of the divisions between the three clades of ornate shrew. The appearance of these barriers approximately 2 Ma agrees with estimates based on the molecular clock. However, the more recent split of the southern and central clades indicates the transverse ridge was an isolating barrier later than the emergence of the Monterey delta. Other vertebrate groups show similar geographical partitioning. Notably, in the plethodontid salamander *Ensatina*, the blotched subspecies occurring in the Sierra Nevada is divided into two distinct central and southern clades (Wake 1997). A northern clade existing to the north of Monterey Bay coincides approximately with the Northern clade of the ornate shrew. Other species with similar partitions include the California Newt (*Taricha torosa*; Tan & Wake 1995), the plethodontid *Batrachoseps* (Yanev 1980), the California mouse, *Peromyscus californicus* (Smith 1978), and the pocket gopher, *Thomomys bottae* (Patton & Smith 1994). More recently, a phylogeographic study of the California mountain kingsnake, *Lampropeltis zonata*, showed similar patterns of genetic structuring (Rodríguez-Robles *et al*. 1999).

Phylogeography and regional population expansion

The genetic distances between populations in the southern clade based on allozymes and mtDNA were significantly correlated with geographical distance, and geographical units defined by phylogenetic analysis correspond to geographical proximity. In addition, the diversity within clades varies. The northern clade has the highest nucleotide diversity within populations and the largest sequence divergence between populations (Table 3). The central clade is limited in sequence and allozyme diversity and the southern clade is intermediate. The differences in allozyme diversity and sequence coalescence between southern, central and northern populations suggests a more recent expansion of the central populations. The Pleistocene of California was characterized by periods of high rainfall and cool conditions during glacial maxima alternating with warm dry periods during interglacials. During the cool wet periods, mesic habitats for the ornate shrew expanded, whereas during interglacial dry periods, such as today, habitat for the ornate shrew was limited and severely fragmented (Fig. 1). In the southern clade, the expansion may have occurred from southern refugia as two of the southernmost populations are most basal in phylogenetic trees (La Laguna and Catalina Island; Fig. 2). Within the southern clade, there is a correspondence between genetic divergence and geographical distance as might be predicted if the expansion followed a south to north axis and thereafter populations experienced low rates of gene flow. Alternatively, the two refugial populations may simply be the outliers from an ancestral population

that was widespread in Baja and southern California. As geological processes changed the climate and moved Catalina Island to the north relative to the Cape region of Baja California, they also isolated these populations. The mainland populations of southern and northern Baja California subsequently evolved and differentiated, as we find them today.

Previous evolutionary hypotheses concerning the radiation of shrews in general (Findley 1955; Carraway 1990) have drawn on the conventional wisdom that Pleistocene climatic cycles precipitated a large portion of speciation events between extant sister taxa. A typical Pleistocene speciation scenario would suggest that a widespread ancestral population became isolated into separate glacial refugia where allopatric divergence leading to speciation was initiated (Hewitt 1996). Recently, this paradigm was challenged for avian species because 70% of bird species had genetic distance suggesting divergence over 5 Ma (Klicka & Zink 1997). However, many intraspecific divergences do date to Pleistocene events (Avise & Walker 1998) suggesting Ice Ages are important causes of differentiation. The tripartite division of ornate shrew populations dates to the early Pleistocene and does not reflect isolation in recent Ice Age refugia. In contrast, past patterns of genetic divergence within clades appear to be erased by population contractions during interglacials and re-established during glacial period expansions. In fact, a similar pattern of highly divergent clades, each with a recent coalescence, was found in a survey of European species (Taberlet *et al*. 1998). This suggests the Ice Age effects may have more pronounced impact on regional within clade diversity than on speciation.

The evolutionary origin of the ornate shrew

The northern clade is the most basal in mitochondrial and allozyme trees, suggesting that the northern populations of ornate and vagrant shrews are ancestral. The oldest known Pliocene *Sorex* from the Nearctic was found in the early Blancan Fox Canyon (Kansas) and Hagerman faunas (Idaho) approximately 3.5 Ma. The first fossil precursor of *Sorex vagrans*, *S. hoyi* and *S. fumeus* appears in the late Kansan (Cudahy fauna of Kansas) about 700 000 years ago (Kurtén & Anderson 1980), much more recently than suggested by the molecular data (1.3–1.6 Ma). However, the climatic conditions and boreal floral assemblage present in South-west during the Plio-Pleistocene were unfavourable to fossil deposition (Dietling 1968) and the fossil record of *Sorex* in North America is scanty (Kurtén 1967; Repenning 1967; Kurtén & Anderson 1980; Lundelius *et al*. 1983; Carraway 1990). Thus, the fossil data may substantially underestimate divergence time in shrews. In addition, palaeontologists have not attempted to distinguish between *S. monticolus* and *S. vagrans* when identifying fossil remains. Thus, it is not unreasonable to suggests that ancestors of this group of *Sorex* occurred within boreal floral assemblages present in the western US before the Pleistocene (Dietling 1968; Carraway 1990). Our results suggest that much older fossils of *S. ornatus*, *S. vagrans* and *S. monticolus* may be found.

Relationship of S. ornatus, S. vagrans *and* S. monticolus

S. monticolus, *S. vagrans* and *S. ornatus* are part of a group of closely related taxa included in the subgenus *Otisorex*. Distinguishing between these taxa has been difficult where the ranges of species overlap (George & Smith 1991). In the past, *S. monticolus* and *S. vagrans* have been variously identified as one or multiple species by different authors (e.g. Findley 1955; Brown 1974; Hennings & Hoffmann 1977; van Zyll de Jong 1982; George 1988; Carraway 1990). The results of our mtDNA analysis suggest that *S. monticolus* diverged from *S. vagrans* and *S. ornatus* approximately 1.6 Ma and should be considered a different species. A problematic finding concerns the inclusion of sequences from *S. ornatus sinuosus* and *S. o. californicus* in the northern clade that is otherwise composed of sequences from *S. vagrans* (Fig. 2), making *S. ornatus* a paraphyletic group. Four conceivable causes could give rise to the clustering of *S. vagrans* and ornate shrew sequences. First, hybridization may have occurred between the two species in northern California where their ranges meet. Mitochondrial haplotypes observed in vagrant shrews could have originated, in fact, in ornate shrews, or vice versa. Past morphological studies of shrews from the San Pablo Bay (in the San Francisco Bay area) showed that they were intermediate in external measurements and colour between *S. v. vagrans* and *S. o. sinuosus* (Rudd 1955a; Brown & Rudd 1981). However, northern populations do not show significant levels of gametic and cytonuclear disequilibrium that would be expected in hybridizing populations. Levels of disequilibrium are less than 1% of all pairwise comparisons between loci in all 22 populations of ornate shrew. Thus, if hybridization occurred it must be ancient because no disequilibrium can be detected. To further assess the possibility of hybridization, we sequenced a specimen of *S. vagrans* from the Jarbridge Mountains near Elko Nevada and the border with Idaho, approximately 400 miles from the presumed contact zone of the two species. *S. vagrans* has a distribution above 2000 meters in the eastern Slope of the Sierra Nevada mountains (Hennings & Hoffmann 1977), ranging from northern California to the northern Great basin and the Columbia Plateau (Fig. 1). In contrast, the ornate shrew is thought to be a lowland form restricted to California and Baja California (Owen & Hoffman 1983). *S. ornatus* is also the only shrew species occupying the blue oak/digger pine forest at the lowest elevations of the western slope of

the Sierra Nevada below 1200 m (Williams 1991). The *S. vagrans* haplotype from Nevada was classified within the northern clade of ornate shrew sequences in Fig. 2 and with the other haplotypes of *S. vagrans*. A hybrid zone of this extent is unlikely for a species of such limited mobility (Owen & Hoffmann 1983). Therefore, hybridization is an unlikely cause of the paraphyletic pattern. Additionally, all individuals with *S. ornatus* morphology in northern California have cytochrome *b* sequences similar to *S. vagrans*.

A second explanation is that lineage sorting is incomplete in ornate shrews such that long ancestral lineages are retained in the northern populations (Avise 1989). However, the *S. vagrans* haplotypes in the northern clade are not found in any other clades and are recently diverged from sequences in the northern clade. These findings contradict the expectation of incomplete lineage sorting.

A third explanation is that *S. vagrans* and *S. ornatus* are simply ecotypes of the same species. However, this is not consistent with the large sequence divergence between clades. The mean sequence divergence between the complete 699 bp sequences in the northern clade and those in the southern clade is 7.0% (4.2% between southern and central clades). In a phylogenetic study of cytochrome *b* sequences (1011 bp in length) across *Sorex* shrews throughout the world, Fumagalli *et al*. (1999) observed intraspecific distances ranging from 0 to 5.2%. Only in one case did they observe an instance of an exceptionally large divergence, up to 9.2%, in two well-differentiated subspecies of *S. arcticus*. The large divergence that we observed separating the northern clade from the central and southern clades suggest that they may correspond to different species.

Finally, populations in the northern clade may be misclassified as ornate shrews. In fact, ornate shrews are remarkably uniform phenotypically and cannot be easily distinguished from *S. vagrans* in northern California (Rudd 1955b; Brown 1971; Junge & Hoffmann 1981). Consequently, we conclude that the northern ornate shrews may represent a lowland form of vagrant shrews with a morphology resembling the ornate shrews, not closely related to ornate shrews from south of San Francisco Bay. Presumably the northern California ornate shrews are derived from *S. vagrans* that have a high altitude distribution in the Sierra Nevada mountains, whereas the southern and central clade evolved independently from a southern California ancestor.

Units for conservation

The ornate shrew is one of the most threatened small mammals in southern (Williams 1986) and Baja California (Elliot 1903; Huey 1964; Woloszyn *et al*. 1985; Maldonado 1999). It is thought to be threatened primarily due to destruction of wetlands and riparian habitats, and four subspecies (*S. o. salicornicus*, *relictus*, *willetti* and *sinuosus*) are listed as California Mammal Species of Special Concern. In Baja California, our field surveys also revealed that the few remaining populations are small and fragmented and have a high probability of extinction (Maldonado 1999). Ornate shrews may once have had a continuous distribution along the marshlands of Tulare Basin in the San Joaquin Valley (Grinnell 1932), but this habitat is now greatly fragmented due to cultivation and the recent disappearance of lakes and sloughs. Similarly, the loss of habitat due to development along the California coast may have caused the extirpation of numerous ornate shrew populations (Williams 1986).

Our results suggest that the southern and central populations of the ornate shrew form two distinct clades that have long and separate ancestry. Therefore, each is an evolutionary significant unit because they are reciprocally monophyletic and divergent in allele frequencies (Moritz 1995). Therefore, they should be managed as separate units for conservation. Moreover, within each region, gene flow is low among populations and nearly all have different mtDNA sequences and are differentiated with regard to allozyme allele frequencies (Tables 3–5). Therefore, these populations should be considered management units as they will respond independently to environmental changes (Moritz 1995). Once populations disappear they are unlikely to be recolonized from elsewhere. In addition, populations on Santa Catalina Island and the relictual population in Baja California are genetically the most distinct populations in the southern clade and are also the most endangered. Special attention needs to be placed on their conservation. Collaborative efforts between U.S. and Mexican federal governmental agencies are urgently needed to preserve coastal marshes, wetlands, and riparian habitats and to protect the few remaining populations of ornate shrews in southern and Baja California.

Finally, the populations north of San Francisco Bay may define a group having an independent origination from *S. vagrans* ancestors. Therefore, the conservation status of the northern populations may need to be re-evaluated in light of these newly discovered systematic affinities. In particular, efforts should be concentrated on elucidating the phylogenetic relationships of the threatened Salt Marsh Wandering shrew (*S. v. halicoetes*) which inhabits the salt marsh ecosystem of the San Francisco Bay area and is distinguished from other shrews in the area based on dubious phenotypic characteristics (i.e. dark pelage colouration and distribution). *S. o. sinuosus* was also originally considered a separate species because it was endemic to tide marshes and had a melanistic pelage (Rudd 1955b), but our results show this taxon is not differentiated from other northern populations. Past morphological studies of shrews from this area have been problematic and controversial (Rudd 1955a; Brown & Rudd 1981). The problems with the systematics of this

group of shrews have led Ingles (1965) and Hall (1981) to suggest that a future reviser might arrange both *sinuosus* and *ornatus* as subspecies of *S. vagrans*. Our results suggest that northern populations of the ornate shrew may be a unique lowland form of the vagrant shrew that has converged independently on the morphology of southern and central California *S. ornatus*.

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This study is part of the PhD research of Jesús Maldonado, who was directed by Dr Robert K. Wayne. Jesús Maldonado is interested in a variety of questions in evolutionary biology that can be answered with molecular tools. His primary interests are the evolutionary genetics and conservation biology of mammals. Carles Vilà works on the population and conservation genetics of mammals. Most of the laboratory analyses and all the data analyses were done in the Conservation Genetics laboratory of Dr Robert K. Wayne at UCLA.

Appendix 1

Enzyme systems examined and electrophoretic conditions employed. Buffer systems modified from Selander *et al*. (1971). Abbreviations: I, Continuous tris citrate II (pH 8.0; 8 h, 100 ma); II, Poulik (pH 8.7; 6 h, 250v); III, Lithium hydroxide (pH 8.2; 7 h, 350 v); IV, Continuous tris-citrate I (pH 7.5; 5 h, 75 ma)

Enzyme	Enzyme Commission Number	Locus	Number of alleles resolved	Tissue source	Electrophoretic conditions
6-phosphogluconate dehydrogenase	1.1.1.44	6-Pgdh-1	3	liver	I
Adelynate kynase	2.7.4.3	Ak-A	2	liver	I
Adenosine deaminase	3.5.4.4	Ada-1	3	liver	I
Alcohol dehydrogenase	1.1.1.1	Adh-1	1	liver	I
Aspartate transaminase	2.6.1.1	mAta-A	2	liver	I
Aspartate transaminase	2.6.1.1	sAta-A	2	liver	I
Esterase	3.1.1.1	Est-1	3	liver	I
Esterase	3.1.1.1	Est-2	4	heart and kidney	I
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6pdh-A	3	heart and kidney	II
Glucose-6-phosphate isomerase	5.3.1.9	Gpi-A	2	heart and kidney	III
Glutamate dehydrogenase	1.4.1.3	Gtdh-1	1	heart and kidney	II
Glycerol-3-phosphate	1.1.1.8	G3pdh-1	1	liver	I
Glycerol-3-phosphate	1.1.1.8	G3pdh-2	3	liver	I
Isocitrate dehydrogenase	1.1.1.42	mIcdh-A	1	heart and kidney	IV
Isocitrate dehydrogenase	1.1.1.42	sIcdh-A	3	heart and kidney	IV
Lactate dehydrogenase	1.1.1.27	Ldh-A	1	heart and kidney	IV
Lactate dehydrogenase	1.1.1.27	Ldh-B	1	heart and kidney	IV
L-Iditol dehydrogenase	1.1.1.14	Iddh-A	2	heart and kidney	I
Malate dehydrogenase (NADP +)	1.1.1.40	Mdhp-A	3	heart and kidney	I
Malate dehydrogenase	1.1.1.37	sMdh-A	2	heart and kidney	I
Malic dehydrogenase	1.1.1.37	mMdh-A	1	heart and kidney	I
Peptidase-glycyl-glycine	3.4.13.18	Pep-A	1	heart and kidney	III
Peptidase-leucine-glycine-glycine	3.4.14.1	Pep-B	1	heart and kidney	III
Peptidase-phenylalanine-proline	3.4.13.9	Pep-D	3	heart and kidney	III
Phosphoglucomutase	5.4.2.2	Pgm-1	2	heart and kidney	IV
Phosphoglucomutase	5.4.2.2	Pgm-2	2	heart and kidney	II
Purine nucleoside phosphorylase	2.4.2.1	Pnp-1	5	liver	I
Superoxide dismutase	1.15.1.1	sSod-A	2	heart and kidney	II
Xanthine dehydrogenase	1.1.1.204	Xdh-A	3	liver	II
Albumin	Non-enzymatic protein	ALB	1	heart and kidney	III